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On the Mechanism of Reinitiation of Endogenous Crassulacean Acid Metabolism Rhythm by Temperature Changes[1]

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Under continuous light the endogenous Crassulacean acid metabolism (CAM) rhythm of *Kalanchoë daigremontiana* Hamet et Perrier de la Bâthie disappears at high (>29.0°C) or low (<8.0°C) temperatures. We investigated the reinitiation of rhythmicity when temperature was reduced from above the upper and increased from below the lower threshold level via measurements of (a) short-term changes in carbon-isotope discrimination to illustrate shifts between C_3 and C_4 carboxylation in vivo, and (b) the malate sensitivity of phospho*enol*pyruvate carboxylase (PEPC) in vitro. When the net CO_2-exchange rhythm disappears at both temperatures, the instantaneous discrimination indicates low PEPC activity. Leaf malate concentration and osmolarity attain high and low values at low and high temperatures, respectively. After small temperature increases or reductions from the low and high temperatures, respectively, the rhythm is reinitiated, with phases shifted by 180° relative to each other. This can be related to the contrasting low and high leaf malate concentrations due to direct inhibition of PEPC and possibly also of the phosphorylation of PEPC by malate. The experimental results were satisfactorily simulated by a mathematical CAM-cycle model, with temperature acting only on the passive efflux of malate from the vacuole. We stress the important role of the tonoplast in malate compartmentation and of malate itself for the reinitiation and generation of endogenous CAM rhythmicity.

CAM is an ecophysiological adaptation of plants to limited availability of water or CO_2 (Kluge and Ting, 1978). At night CO_2 is fixed via the C_4 carboxylation enzyme PEPC, with the product, malate, stored in the vacuole. During the day the decarboxylation of malate releases CO_2, which is refixed by the C_3 carboxylation enzyme Rubisco when the stomata are closed. CAM has been intensively studied, with approaches on various levels such as ecophysiology, membrane transport processes, molecular biology, and endogenous rhythmicity (Winter and Smith, 1996). Since the early work of Wilkins (1959, 1962) and Nuernbergk (1961), net CO_2 exchange of CAM plants is known to exhibit a circadian rhythm that persists in continuous darkness and CO_2-free air, as well as in continuous light and normal air. It was proposed that such rhythms of CO_2 output are directly attributable to changes in flux through PEPC (Warren and Wilkins, 1961).

More recent studies stress the role of the tonoplast in the circadian rhythmicity of CAM. The net CO_2-exchange rhythm of the obligate CAM plant *Kalanchoë daigremontiana* Hamet et Perrier de la Bâthie disappears at increased temperature (>29.0°C) and/or irradiance (>180 μmol m^{-2} s^{-1}) under continuous illumination and normal air (Buchanan-Bollig, 1984). In a more detailed study, Lüttge and Beck (1992) presented pronounced threshold values of the control parameters temperature and irradiance for the disappearance of the rhythm. Under continuous light, when temperature (or irradiance) is reduced from values above threshold (evoking arrhythmicity) to values below threshold, the circadian CO_2-exchange rhythm is reinitiated and set in phase (Wilkins, 1962; Anderson and Wilkins, 1989; Grams et al., 1996). The mechanism of this reinitiation is not yet understood, but Wilkins (1983) proposed that high temperature opens "gates" in the tonoplast, which also may be related to the effect of temperature on the state of order of isolated tonoplast vesicles of *K. daigremontiana* (Kluge et al., 1991). This could modulate the passive efflux of malate from the vacuole to the cytoplasm (Lüttge and Smith, 1984). Using this temperature-dependent behavior of the tonoplast to model the influence of temperature on the endogenous CAM rhythm, Grams et al. (1996) were able to reproduce the experimental net CO_2-exchange data, although simulated leaf malate concentrations did not fit with the observed values. Thus, as presented in this work, we made another effort to improve

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Abbreviations: Δ, instantaneous discrimination; mOsm, milliosmol; PEPC, PEP carboxylase.

the computer model of the CAM cycle with respect to leaf malate content.

The flux of carbon through PEPC is thought to be regulated via reversible phosphorylation mediated by a specific PEPC kinase that alters the sensitivity of PEPC to malate inhibition (Nimmo et al., 1987; Carter et al., 1991). The activity of the PEPC kinase is in turn regulated by an endogenous rhythm of protein synthesis and destruction (Carter et al., 1991, 1996). However, recent findings of rhythmic net CO_2-exchange rates without a concomitant PEPC phosphorylation/dephosphorylation rhythm under certain temperature regimes indicate that PEPC kinase is not a mandatory requirement for the endogenous CAM rhythm (Carter et al., 1995b). On the other hand, Carter et al. (1995a) reported temperature-dependent regulation of PEPC of *Kalanchoë fedtschenkoi* (syn. *Bryophyllum fedtschenkoi*). Thus, reinitiation of the CAM rhythm by temperature changes could also be due to direct effects of temperature on PEPC.

To clarify the role of both PEPC and the tonoplast in the reinitiation of the endogenous CAM rhythm in *K. daigremontiana*, we investigated PEPC activity by monitoring (a) the sensitivity of PEPC to malate inhibition in vitro, and (b) short-term changes in carbon-isotope discrimination during net CO_2 uptake, which demonstrate shifts between C_3 and C_4 carboxylation in vivo (Griffiths et al., 1990; Borland and Griffiths, 1996) at low and high temperatures, and after temperature increase and decrease under continuous light. If the state of order of the tonoplast affects malate efflux from the vacuole, a very high state of order (at low temperature) should restrict the malate efflux. To test this hypothesis we measured leaf malate content of *K. daigremontiana* at low temperature, which should inhibit malate efflux and thus endogenous CAM rhythmicity.

MATERIALS AND METHODS

Plants of *Kalanchoë daigremontiana* Hamet et Perrier de la Bâthie were grown under natural light conditions in a greenhouse from adventitious plantlets from plants of the collection of the Botanical Garden in Darmstadt, Germany. In winter, additional light was provided by fluorescent tubes (L58 W77 Fluora, Osram, Munich, Germany). Before the experiments plants with five to seven leaf pairs were transferred to a climate-controlled chamber and adapted for 2 to 3 weeks to day/night temperatures of about 25.0/15.0°C and RH of about 60/80%.

Gas-Exchange Measurements

Measurements were performed in a walk-in climate chamber using a minicuvette system (CMS 400, Walz, Effeltrich, Germany), as described elsewhere (Grams et al., 1996). One fully developed leaf was enclosed in a Plexiglas cuvette. For all investigations irradiance was kept constant for individual experiments at 90 to 110 μmol m^{-2} s^{-1}, whereas leaf temperature was changed between 5.0 and 30.0°C, depending on the experiment. Inside the cuvette RH was set to 70% and controlled by a humidity bypass control system (Walz). This system pumps air from the cuvette through a cold trap (KF18/2, Walz), which was set to a dew point 2°C lower than inside the cuvette. The dried air reenters the cuvette and, by different flow rates through the bypass system, compensates for increased RH due to leaf transpiration. Absolute and differential CO_2 concentrations were measured (BINOS 4P, Rosemount, Hanau, Germany) and gas-exchange parameters were calculated according to the equations of von Caemmerer and Farquhar (1981). The transpiration rate (E) of plants was calculated using the flow rate through the bypass system ($flow_{bp}$) and the water vapor partial pressures at the cold trap in the bypass (P_{Wbp}), at the entrance (P_{We}), and at the outlet of the cuvette (P_{Wo}), as follows:

$$E = \frac{1}{A} \cdot \left(flow_{bp} \frac{P_{Wo} - P_{Wbp}}{P_{cuv} - P_{Wbp}} + flow \frac{P_{Wo} - P_{We}}{P_{cuv} - P_{We}} \right) (\text{mmol m}^{-2}\ \text{s}^{-1}),$$

where A represents the single leaf area, *flow* the flow rate through the cuvette, and P_{cuv} the air pressure in the cuvette.

Measurement of Instantaneous Carbon-Isotope Discrimination

For these experiments a gas cylinder with compressed air was used to provide source air with a constant CO_2 concentration and isotopic signature. During critical times CO_2 from the airstream of the gas-exchange measurements (analytical and reference) was sampled according to the method of Griffiths et al. (1990). Air was drawn into the collection line using a rotary vacuum pump with the flow rate restricted to 250 mL/min^{-1} through a needle valve on the collection line. The decrease in pressure between the valve and pump allowed the collection of CO_2 over 15 min in a single cold trap of liquid N_2. The CO_2 was subsequently cryodistilled into a side arm using liquid N_2, and sealed to form a vial. The collected CO_2 was repurified by cryodistillation through a series of liquid N_2 and solid CO_2/acetone cold traps via oxidation and reduction columns to remove N_2O and H_2O. The recovered CO_2 was analyzed using an isotope-ratio mass spectrometer (Isospec 44; modified by CJS Sciences, Winsford, UK). Δ was calculated according to the method of Evans et al. (1986):

$$\Delta = \frac{\xi(\delta_o - \delta_e)}{1 + \delta_o - \xi(\delta_o - \delta_e)},$$

where $\xi = c_e/(c_e - c_o)$ and δ_e and δ_o are the isotope composition and c_e and c_o are the CO_2 concentration of air entering (e) and leaving (o) the cuvette, respectively.

The predicted Δ for C_3 plants was calculated according to the simple model of Farquhar et al. (1989):

$$\Delta_{pred.} = a + (b - a)\frac{p_i}{p_a},$$

where a represents the isotopic fractionation occurring due to diffusion of CO_2 in air (theoretical value, 4.4‰), b is the isotope effect of CO_2 fixation via Rubisco (27‰; Farquhar et al., 1989), and p_a and p_i are the ambient and intercellular partial pressures of CO_2, respectively, as measured during gas exchange.

Measurement of PEPC Activity

For these experiments plants were kept under the same conditions as the plants used for gas-exchange measurements in the same climate chamber. Accuracy of the temperature and RH inside the chamber was ±1°C and ±10%, respectively. Extracts were rapidly prepared from the fifth to sixth leaf pair following the method of Winter (1982). Extraction buffer, desalting of the crude extract using Sephadex G-25 medium, and the PEPC activity assay were performed according to the method of Nimmo et al. (1984). Care was taken to handle all steps quickly and at 4°C to avoid changes of the phosphorylation state of PEPC. Activity was measured with and without the addition of 2 mM malate (Kusumi et al., 1994). The reaction was initiated by the addition of extract.

Malate Content and Osmolarity

Samples were taken from the fifth to sixth leaf pair at critical stages of the experiments. Osmolarity of the leaf sap was measured (Osmomat 030, Gonotec, Berlin, Germany) and its malate content was determined using the method of Möllering (1974).

Model for the CAM Cycle

For simulations of the CAM rhythm at different temperatures under continuous illumination, a computer model first described by Nungesser et al. (1984) was used. This model is explained in detail by Lüttge and Beck (1992) and was subsequently improved for the use of simulation of the endogenous CAM rhythm (Lüttge and Beck, 1992; Grams et al., 1996). Further refinement of the model with respect to vacuolar malate concentration is presented in "Results."

Sinus Fit

Data of the reinitiated endogenous rhythm were fitted using the fit function of Sigma-Plot (Jandel Scientific, Erkrath, Germany) with the following equation:

$$f(x) = p_1 \cdot \sin(p_2 \cdot x + p_3) + p_4,$$

where p_1 represents the amplitude, p_3 the phase, and p_4 the mean value of the oscillator. The period length of the rhythm ($L = 2\Pi/p_2$) for one experiment was determined using the net CO_2-exchange data and was used for all other fits of the same experiment.

RESULTS

Temperature Reduction under Continuous Light

Four gas-exchange experiments with temperature reduction during the time of continuous light were performed. Temperature reductions from 30.0 to 22.1°C, from 30.0 to 23.0°C, and in two cases from 29.1 to 27.1°C were imposed. One representative experiment is shown in Figure 1. During the first 24 h (Fig. 1A) under the night/day cycle and a constant temperature of 30.0°C, *K. daigremontiana* showed all four phases of CAM as described by Osmond (1978).

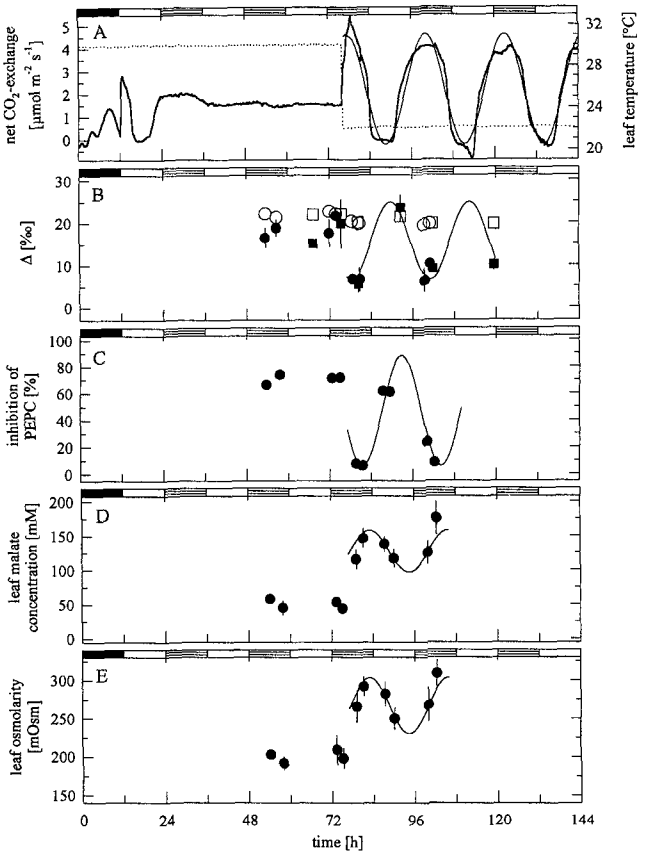

Figure 1. Net CO_2 exchange, $^{13}CO_2$ discrimination (Δ), inhibition of PEPC by 2 mM malate, leaf malate concentration, and leaf osmolarity of *K. daigremontiana* in an experiment with reduction of the temperature from 30.0 to 22.0°C during continuous light. A, Net CO_2 exchange (—) and leaf temperature (····) of one leaf under night/day conditions (0–24 h) and continuous light (24–144 h). B, Values of $^{13}CO_2$ discrimination (Δ) measured (●) for the same leaf as used in A and predicted for C_3 carboxylation (○). Data from a second experiment with temperature reduction from 30.0 to 23.0°C are given by the squares. C, Inhibition of PEPC by 2 mM malate. D, Leaf malate concentration. E, Leaf osmolarity. All measured data after temperature reduction were fitted using a sinus fit (—). Data of B to E are means (±SD), $n \geq 3$. Dark bars indicate nighttime, open bars daytime, and striped bars subjective night during time of continuous light.

Subsequently, under continuous illumination at otherwise unchanged conditions the phases of CAM were no longer evident and a net CO_2-exchange rate of about 1.7 µmol m^{-2} s^{-1} was established. After the temperature reduction from 30.0 to 22.1°C, the circadian rhythm was reinitiated, starting with increasing net CO_2-exchange rates, followed by oscillations between rates of 5.4 and −0.8 µmol m^{-2} s^{-1}. A period length of this rhythm of 22.7 h was obtained at 22.1°C using the sinus-fit function (Fig. 1A). The Δ of the leaf used for gas-exchange measurements in this and in a comparable experiment with a temperature reduction from 30.0 to 23.0°C is shown in Figure 1B. A third experiment with a temperature reduction from 29.1 to 27.1°C gave similar results. Before temperature reduction, Δ was arrhythmic at about 18‰ (closed symbols), a value only

slightly lower than that predicted for C_3 metabolism (open symbols), indicating low PEPC activity under these conditions. Plants kept in the same climate chamber under the same conditions as indicated in Figure 1A were used to determine inhibition of PEPC by 2 mM malate (Fig. 1C), leaf malate concentration, and leaf osmolarity (Fig. 1, D and E; representative for a total of three experiments). During the time of high temperature (i.e. 30.0 ± 1.0°C) and continuous illumination, PEPC activity was highly inhibited by 2 mM malate at a rather constant level of about 70 to 80% (Fig. 1C). At the same time, leaf malate concentration and osmolarity were maintained at a constant low level (about 50 mM and 200 mOsm, respectively; Fig. 1, D and E).

After temperature reduction, Δ dropped within a few hours to values of about 6‰, followed by a circadian oscillation between 25 and 5‰, which was shifted in phase by about 180° relative to the net CO_2-exchange rhythm. In contrast, the values of Δ predicted for C_3 carboxylation remained at a more or less constant level of about 20‰. This indicates rhythmic activity of PEPC in vivo. Inhibition of PEPC by 2 mM malate drastically changed in response to the temperature reduction: after 3 h at 22.0°C, inhibition was reduced to 5 to 10%, followed by an oscillation between 60 and 10%, which was well in phase with the observed rhythm of Δ (Fig. 1C). Moreover, after the temperature reduction, leaf malate content and osmolarity also showed endogenous oscillations. At their initiation they started with increasing values and were almost in phase with the reinitiated net CO_2-exchange rhythm (Fig. 1, D and E).

Low Temperature under Day/Night Cycle

Under a day/night cycle at 5.0°C, *K. daigremontiana* showed very low rates of nocturnal net CO_2 uptake. During the daytime more or less stable rates of about 1.2 μmol m^{-2} s^{-1} were reached (Fig. 2A). Thus, the typical CAM pattern of gas exchange as seen at 30.0°C in the first 24 h of Figure 1A was not expressed at 5.0°C (see also the first 24 h of Fig. 3A). Moreover, malic acid present in the morning hours (about 125 mM) was not decarboxylated and remained constant throughout the day (Fig. 2C). This was confirmed by the high and constant levels of osmolarity (about 300 mOsm) during the daytime, which were measured in the same leaf press sap samples that were used for the malate determination (Fig. 2D). The measured instantaneous discrimination of *K. daigremontiana* did not change significantly during the day, with values observed between 15 and 18‰ (Fig. 2B). Calculated values of instantaneous discrimination for C_3 carboxylation were somewhat higher, in the range of 21 to 23‰. This suggests low PEPC activity during the daytime (Fig. 2B).

Temperature Increase under Continuous Light

Four gas-exchange experiments with temperature increase during the time of continuous light were performed (from 5.0 to 15.0°C, 5.4 to 8.3°C, 5.6 to 10.5°C, and 7.0 to 15.0°C). Under continuous light at 7.0°C, plants showed an arrhythmic pattern of net CO_2 exchange at a level of about 2.5 μmol m^{-2} s^{-1}, similar to the behavior at 30.0°C (Fig. 3A). During this time of the experiment, instantaneous discrimination of the investigated leaf was in the range of 20 to 25‰, which was slightly below the value of about 26‰ predicted for C_3 carboxylation (Fig. 3B). After the temperature increase (from 7.0 to 15.0°C), plants showed an endogenous rhythm in net CO_2 exchange, with a period length of 16.6 h. In contrast to the experiment with temperature reduction, the reinitiation of the rhythm started with a decrease in net CO_2-exchange rate (compare Figs. 1A and 3A). Within the first 3 h after the temperature change, Δ increased to about 30‰ and decreased down to 13‰ within the following 12 h (Fig. 3B). The evaluation of the data by the sinus-fit function revealed a circadian

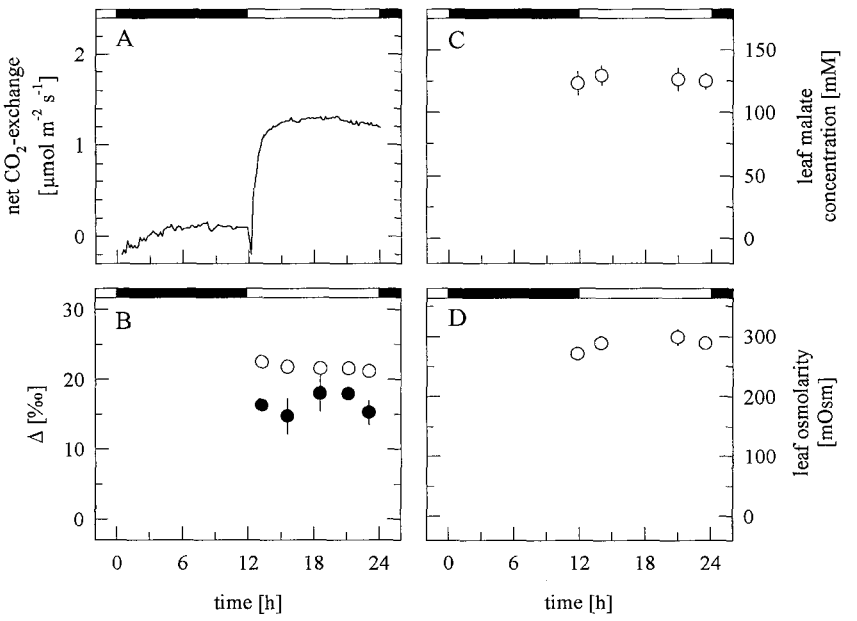

Figure 2. Net CO_2 exchange, $^{13}CO_2$ discrimination (Δ), leaf malate concentration, and leaf osmolarity of *K. daigremontiana* under night/day conditions at 5.0°C. A, Net CO_2 exchange (—) of one leaf. B, Values of $^{13}CO_2$ discrimination (Δ) of one leaf measured (●) and predicted for C_3 carboxylation (○). C, Leaf malate content. D, Leaf osmolarity. For details, see Figure 1.

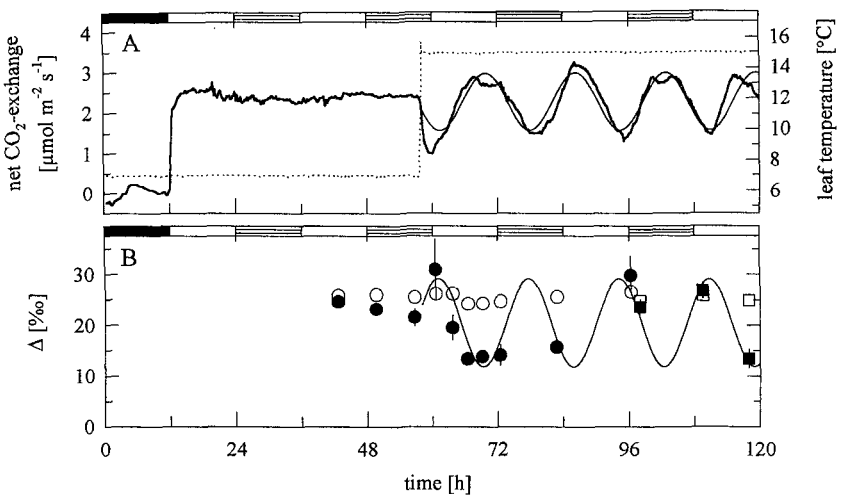

Figure 3. Net CO_2 exchange and $^{13}CO_2$ discrimination of one leaf of *K. daigremontiana* in an experiment with increase of temperature from 7.0 to 15.0°C during continuous light. A, Net CO_2 exchange (—) and leaf temperature (····) under night/day conditions (0–24 h) and continuous light (24–120 h). B, Values of $^{13}CO_2$ discrimination (Δ) measured (●) for the same leaf used in A and predicted for C_3 carboxylation (○). Data from a second experiment with increase of temperature from 5.0 to 15.0°C are given by the squares. For details, see Figure 1.

rhythm of Δ, which was shifted in phase by 180° in relation to the net CO_2-exchange rhythm. In contrast, Δ predicted for C_3 photosynthesis showed more or less stable values of about 25‰. The biggest differences between measured Δ and Δ predicted for C_3 carboxylation were found at the times of high net CO_2 uptake. This indicates rhythmic activity of PEPC, with high PEPC activity at times of high net CO_2-exchange rates.

Leaf malate concentrations and osmolarities for several days of continuous illumination and high or low temperatures are given in Table I. At low temperatures (i.e. 5.0 and 7.0°C) leaf malate contents attain relatively high levels, whereas at 30.0°C low malate concentrations were found in the leaves. These data are supported by the osmolarities measured in the same leaf press sap samples that were used for malate determinations, also showing high and low values at low and high leaf temperatures, respectively.

Model Simulations

Three CAM functional modes had been incorporated into the existing model, namely vacuolar "influx" and "efflux" of malate and "influx near the maximal malate storage capacity of the vacuole" (Grams et al., 1996). In the present work they were reduced to the two modes influx and efflux of malate into and from the vacuole, respectively. Below the lower temperature threshold value (i.e. 7.0°C) the model is set to the CAM mode influx, and, complementarily, above the upper temperature threshold value (i.e. 29.0°C) the model is set to the CAM mode efflux. If the temperature is in between the two temperature threshold values, the setting of the CAM mode depends on the vacuolar malate concentration. If the minimal vacuolar malate concentration is reached, the model switches to the mode influx, whereas it switches back to the mode efflux when the maximal malate concentration is attained. The possible modes and the switches of the CAM cycle model between the two modes are shown in Table II.

Table I. *Leaf malate concentration and osmolarity at low and high leaf temperatures in different experiments under continuous light*

Values are given ± SD with number of samples (*n*).

Leaf Temperature	Malate Concentration	Osmolarity
°C	*mM*	*mOsm*
5.0	159 ± 17 (*n* = 3)	288 ± 19 (*n* = 3)
5.0	114 ± 9 (*n* = 12)	311 ± 9 (*n* = 12)
7.0	148 ± 11 (*n* = 4)	259 ± 14 (*n* = 4)
30.0	55 ± 11 (*n* = 20)	178 ± 16 (*n* = 20)
30.0[a]	49 ± 10 (*n* = 15)	200 ± 14 (*n* = 15)

[a] Data taken from the experiment shown in Figure 1.

Figure 4 shows three model simulations of net CO_2 exchange and vacuolar malate concentrations starting with a night/day cycle at 20.0°C followed by different temperatures under continuous illumination of low light intensity. At a temperature of 20.0°C (between the upper and lower temperature threshold value) under the night/day cycle the model generated the characteristic features of CAM, with malate accumulation and breakdown during the night and day, respectively, and the typical gas-exchange pattern (first 24 h of Fig. 4, A–C). Under continuous illumination and unchanged temperature at 20.0°C, the model showed an endogenous circadian rhythm of net CO_2 exchange and vacuolar malate content (Fig. 4A). When the control parameter temperature was raised to 30.0°C under the conditions of continuous illumination (48–96 h of Fig. 4B), the model showed a strictly stable, medium net CO_2-exchange rate, and the vacuolar malate content reached a low concentration (minimal malate concentration). After a switch back to 20.0°C, the endogenous CAM rhythm was reinitiated, with a slightly lower period length starting with an increase of malate concentration and net CO_2-exchange rate (Fig. 4B). Under continuous light at low temperatures (5.0°C) the net CO_2 exchange of the model was also fixed to medium rates, but simulated malate content reached the maximal vacuolar malate concentration (48–96 h of Fig. 4C). After a switch back to 20.0°C the endogenous CAM rhythm reinitiated again, with a slightly lower period length. The rhythm reinitiated by a temperature increase from 5.0 to 20.0°C was shifted in phase by 180° as related to the rhythm reinitiated by temperature decrease from 30.0 to 20.0°C. The responses of the net CO_2-exchange rates and

Table II. *Mode-switch matrix of the CAM-cycle model.*

The matrix should be read in the following way: the model can shift from the CAM modes given on the left side (From) to the CAM modes shown at the top (To). The conditions for the shifts in CAM modes are indicated in the corresponding boxes.

	To Influx	To Efflux
From Influx	Temperature is below 7.0°C or Temperature is between 7.0 and 28.0°C and maximal vacuolar malate concentration is not yet reached	Temperature is above 28.0°C or Temperature is between 7.0 and 8.0°C and maximal vacuolar malate concentration is reached
From Efflux	Temperature is below 7.0°C or Temperature is between 7.0 and 28.0°C and minimal vacuolar malate concentration is reached	Temperature is above 28.0°C or Temperature is between 7.0 and 28.0°C and minimal vacuolar malate concentration is not yet reached

malate levels were also different with different directions in the two cases, i.e. in contrast to the reinitiation by temperature decrease the rhythm reinitiated by temperature increase started with a decrease of malate concentration and net CO_2-exchange rate (Fig. 4C).

DISCUSSION

Reinitiation of endogenous CAM rhythmicity in *Kalanchoë* species when temperature was reduced from above to below a threshold value of about 29.0°C has previously been reported in the literature (Wilkins, 1962; Anderson and Wilkins, 1989; Grams et al., 1996). The absolute value of this upper threshold of temperature depends on the growth temperature of the plants (Grams et al., 1995). In the present work we were able to reinitiate the rhythm by temperature decreases of only 2°C. This was also the smallest temperature difference tested and effective in plants adapted to high growth temperatures (Grams et al., 1995). After the temperature reduction the rhythm started with increasing net CO_2-exchange rate, which can be related to increasing PEPC activity (Fig. 1), since before the temperature reduction PEPC activity was low as indicated by high carbon-isotope discrimination. There was apparent rhythmicity of PEPC activity: at high net CO_2-exchange rates the plants showed low discrimination, and at low net CO_2-exchange rates high discrimination was observed (Fig. 1), confirming earlier data from $^{14}CO_2$-labeling experiments during the persisting net CO_2-exchange rhythm of *Kalanchoë tubiflora* under continuous light (Ritz and Kluge, 1987). At high temperature PEPC showed a constant and high

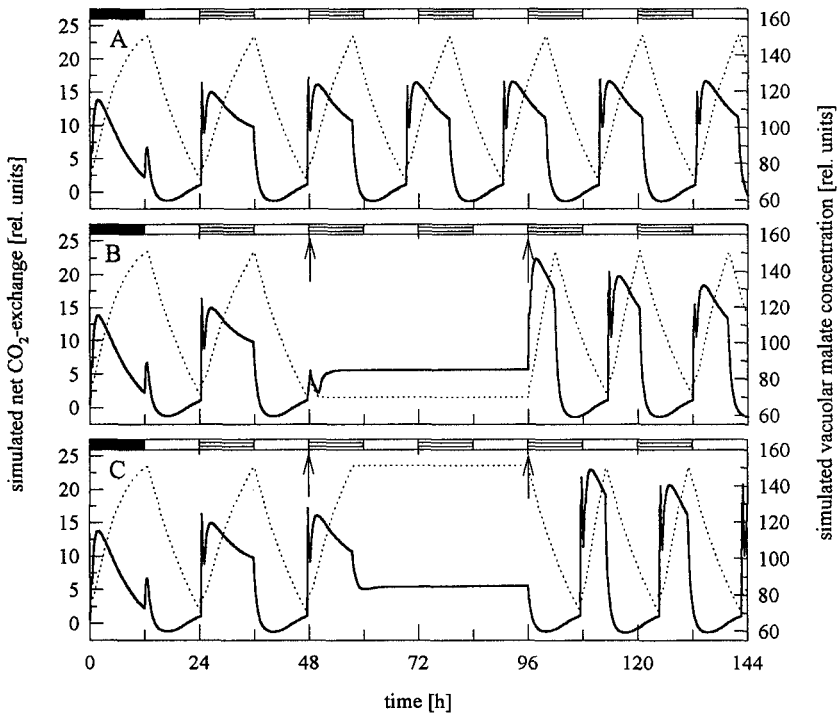

Figure 4. Simulated net CO_2 exchange (—) and vacuolar malate concentration (····). A, Simulation at a constant temperature of 20.0°C. B and C, Simulations in which temperatures changed between 48 and 96 h from 20.0 to 30.0 and to 5.0°C, respectively (arrows). Dark bars indicate nighttime, open bars daytime, and striped bars subjective night during time of continuous light.

level of inhibition by 2 mM malate. This indicates that the enzyme was present in the dephosphorylated inactive form (as also proposed for *K. fedtschenkoi* by Carter et al., 1995a). We show here for the first time to our knowledge that only a few hours after the temperature reduction, the inhibition of PEPC by malate is reduced to only 5%, indicating that PEPC is rapidly phosphorylated. Moreover, circadian phosphorylation/dephosphorylation of PEPC occurred, as indicated by changing levels from inhibition by malate, as has also been shown previously for *K. fedtschenkoi* and *Graptopetalum paraguayense* (Nimmo et al., 1987; Kusumi et al., 1994). The rhythmic conversion of the phosphorylation state of PEPC might be brought about by rhythmic activity of PEPC kinase (Carter et al., 1991) on a background of constant dephosphorylating phosphatase activity (Carter et al., 1990). Leaf malate content and osmolarity were low under conditions of high temperature. After temperature reduction both also showed endogenous rhythmicity starting with increasing values and 180° phase shifts to PEPC activity (Fig. 1) (see Grams, 1992; Grams et al., 1996).

At low temperature the night/day pattern of net CO_2 exchange in CAM species of *Kalanchoë* changes to a typical C_3 pattern (Kluge, 1968) (Figs. 2 and 3) and there is also no endogenous CAM rhythm in *K. daigremontiana* (Fig. 3) or in *K. fedtschenkoi* (Wilkins, 1962; Anderson and Wilkins, 1989) at low temperatures in continuous light. After the temperature increase (smallest tested and effective temperature difference was 2.9°C, i.e. from 5.4 to 8.3°C) the endogenous CAM rhythm was reinitiated with a 180° shift in phase compared with the rhythm reinitiated by temperature reduction. Again, rhythmic changes of PEPC activity were most likely (Fig. 3). Although rhythmicity of PEPC kinase activity plays an important role in the reinitiation and persistence of endogenous CAM rhythmicity, it seems not to be an obligatory requirement (Carter et al., 1995b). Therefore, reinitiation of endogenous CAM rhythmicity is probably not solely related to the phosphorylation status of PEPC. Furthermore, changes from arrhythmicity to rhythmicity of net CO_2 exchange and instantaneous discrimination are elicited by small temperature changes (e.g. 2.0 and 2.9°C in this work and 0.8°C in the work of Lüttge and Beck [1992]), which in themselves are unlikely to bring about substantial changes in gross activity of PEPC, which appears to be largely unaffected by changes in ambient temperature over a 20.0°C range (Carter at al., 1995b).

To assess the mechanism of reinitiation of endogenous CAM rhythmicity by temperature increase and decrease, one needs to understand why the rhythm is reinitiated at phases that differ by 180° under these two conditions. The beat oscillator of the rhythm appears to be fixed in its phase at low and high temperatures; the phases in which it is fixed are 180° apart from each other in the two cases. One factor found to be fixed in its phase in this way was leaf malate content, which is shown in Table I for *K. daigremontiana* and by Anderson and Wilkins (1989) for *K. fedtschenkoi*. These high and low malate concentrations, i.e. 115 to 160 mM at 5.0 to 7.0°C and 49 to 55 mM at 30.0°C, might be based on different states of order of the tonoplast at low and high temperatures. A low state of order of the tonoplast (expected at high temperatures) might affect active malate transport (Bettey and Smith, 1993; Ratajczak et al., 1994) or malate storage in the vacuole due to facilitated passive malate efflux (Lüttge and Smith, 1984; Kluge et al., 1991), inhibiting PEPC in the cytoplasm (Kluge et al., 1981; Nimmo et al., 1984, 1986). Moreover, since phosphorylation of PEPC is also inhibited by malate (Carter et al., 1991; Li and Chollet, 1994), PEPC must have been in the dephosphorylated state (Fig. 1C). After temperature reduction malate can be stored in the vacuole, PEPC becomes phosphorylated (decreasing inhibition by malate; Fig. 1D), and the rhythm is reinitiated with increasing net CO_2-exchange rates, increasing leaf malate content, and decreasing discrimination (Fig. 1).

On the other hand, at low temperatures and, thus, high state of order of the tonoplast, malate efflux seems to be inhibited (Fig. 2C). As a consequence vacuolar malate content rises to the maximal level and remains high. Carter et al. (1995a) found PEPC to be in the phosphorylated active form at low temperatures under continuous darkness. We measured high values of discrimination under continuous light, indicating low PEPC activity. This may have been brought about by the low temperatures, although the enzyme was in the phosphorylated active form. This situation was also found under the day/night cycle at 5.0°C (Fig. 2), when PEPC activity was low (indicated by high values for discrimination) and maximum vacuolar malate storage capacity was reached. This causes low nocturnal rates of net CO_2 exchange in an obligate CAM plant such as *K. daigremontiana* (Fig. 2A). Largely reduced nocturnal net CO_2 uptake due to low daytime temperatures were also reported by Kluge (1968) and Lange and Zuber (1980). The low PEPC activity at low temperatures (i.e. 5.0°C) might be due to inhibition by malate, which cannot be stored in the vacuole, or to reduced activity due to low temperatures (Carter et al., 1995a). After the temperature increase under continuous light, malate efflux from the vacuole begins, and malate in the cytoplasm highly inhibits PEPC and PEPC kinase. Thus, net CO_2 exchange rhythm is reinitiated with decreasing net CO_2-exchange rates, decreasing leaf malate content, and increasing discrimination due to malate efflux from the vacuole (Fig. 3). It should be noted that the restriction of nocturnal net CO_2 uptake under day/night cycles and the disappearance of endogenous CAM rhythmicity under continuous light are observed at the same temperature threshold value of about 8.0°C, below which Kluge et al. (1991) observed a permanent state of high order, indicating a rigid state of the tonoplast in isolated vesicles of *K. daigremontiana*.

In the CAM cycle model, the temperature-dependent state of order of the tonoplast was used as the only site of action of temperature (Grams et al., 1996). At low temperatures the CAM mode was set to the mode influx, allowing only malate influx until the maximal vacuolar malate concentration was reached. In contrast, at high temperatures the model was fixed to the mode efflux and, consequently, vacuolar malate concentration was lowered until the minimal vacuolar malate concentration was reached. At intermediate temperatures the mode depends on vacuolar

malate concentration (Table II) (see Lüttge and Beck, 1992) and changes of the state of order of the tonoplast might be brought about by free Ca^{2+} concentration changing with organic acid accumulation in the vacuole (Schomburg, 1994; Kluge and Schomburg, 1996). Thus, in agreement with the experimental observations in plants, the beat oscillator of the circadian rhythm in the model (malate concentration) is fixed by high and low temperatures in phases shifted by 180° to each other. Thus, simulation of the reinitiation of endogenous CAM rhythmicity in net CO_2 exchange and malate content was quite satisfactory (compare Fig. 4 with Figs. 1A and 3A).

It should be noted that the CAM cycle model in its present state does not include rhythmic changes of PEPC sensitivity toward malate. This is not needed for the simulation of the endogenous rhythm, although it is important for subtle reproduction of experimental observations of the shape of gas-exchange curves and metabolite pools (B. Blasius, unpublished data).

In conclusion, our data emphasize the important role of malate compartmentation by the tonoplast. Malate concentrations in the vacuole and cytoplasm and the regulatory effects of malate on PEPC activity and phosphorylation of PEPC are the key factors in the reinitiation and generation of endogenous CAM rhythmicity.

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LITERATURE CITED

Anderson CM, Wilkins MB (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

Bettey M, Smith JAC (1993) Dicarboxylate transport at the vacuolar membrane of the CAM plant *Kalanchoë daigremontiana*: sensitivity to protein-modifying and sulphydryl reagents. Biochim Biophys Acta **1152:** 270–279

Borland AM, Griffiths H (1996) Variations in the phases of Crassulacean acid metabolism and regulation of carboxylation patterns determined by carbon-isotope-discrimination techniques. *In* K Winter, JAC Smith, eds, Crassulacean Acid Metabolism. Springer-Verlag, Berlin, pp 230–246

Buchanan-Bollig IC (1984) Circadian rhythms in *Kalanchoë*: effects of irradiance and temperature on gas exchange and carbon metabolism. Planta **160:** 264–271

Carter PJ, Fewson CA, Nimmo GA, Nimmo HG, Wilkins MB (1996) Roles of circadian rhythms, light and temperature in the regulation of phosphoenolpyruvate carboxylase in Crassulacean acid metabolism. *In* K Winter, JAC Smith, eds, Crassulacean Acid Metabolism. Springer-Verlag, Berlin, pp 46–52

Carter PJ, Nimmo HG, Fewson CA, Wilkins MB (1990) *Bryophyllum fedtschenkoi* protein phosphatase type 2A can dephosphorylate phosphoenolpyruvate carboxylase. FEBS Lett **263:** 233–236

Carter PJ, Nimmo HG, Fewson CA, Wilkins MB (1991) Circadian rhythms in the activity of a plant protein kinase. EMBO J **10:** 2063–2068

Carter PJ, Wilkins MB, Nimmo HG, Fewson CA (1995a) Effects of temperature on the activity of phosphoenolpyruvate carboxylase and on the control of CO_2 fixation in *Bryophyllum fedtschenkoi*. Planta **196:** 375–380

Carter PJ, Wilkins MB, Nimmo HG, Fewson CA (1995b) The role of temperature in the regulation of the circadian rhythm of CO_2 fixation in *Bryophyllum fedtschenkoi*. Planta **196:** 381–386

Evans JR, Sharkey TD, Berry JA, Farquhar GD (1986) Carbon isotope discrimination measured concurrently with gas exchange to investigate CO_2 diffusion in leaves of higher plants. Aust J Plant Physiol **13:** 281–292

Farquhar GD, Ehleringer JR, Hubick KT (1989) Carbon isotope discrimination and photosynthesis. Annu Rev Plant Physiol Plant Mol Biol **40:** 503–537

Grams TEE (1992) Untersuchungen zum CAM-Rhythmus unter Dauerlichtbedingungen an *Kalanchoë daigremontiana*. Diplom Biologie thesis. Technische Hochschule, Darmstadt, Germany

Grams TEE, Beck F, Lüttge U (1996) Generation of rhythmic and arrhythmic behaviour of Crassulacean acid metabolism in *Kalanchoë daigremontiana* under continuous light by varying the irradiance or temperature: measurements *in vivo* and model simulations. Planta **198:** 110–117

Grams TEE, Kluge M, Lüttge U (1995) High temperature-adapted plants of *Kalanchoë daigremontiana* show changes in temperature dependence of the endogenous CAM rhythm. J Exp Bot **293:** 1927–1929

Griffiths H, Broadmeadow MSJ, Borland AM, Hetherington CS (1990) Short-term changes in carbon-isotope discrimination identify transitions between C_3 and C_4 carboxylation during Crassulacean acid metabolism. Planta **181:** 604–610

Kluge M (1968) Untersuchungen über den Gaswechsel von *Bryophyllum* während der Lichtperiode. Planta **80:** 359–377

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

Kluge M, Kliemchen A, Galla H-J (1991) Temperature effects on Crassulacean acid metabolism: EPR spectroscopic studies on the thermotropic phase behaviour of the tonoplast membranes of *Kalanchoë daigremontiana*. Bot Acta **104:** 353–360

Kluge M, Schomburg M (1996) The tonoplast as a target of temperature effects in Crassulacean acid metabolism. *In* K Winter, JAC Smith, eds, Crassulacean Acid Metabolism. Springer-Verlag, Berlin, pp 72–78

Kluge M, Ting IP (1978) Crassulacean Acid Metabolism: Analysis of an Ecological Adaptation. Springer-Verlag, Berlin

Kusumi K, Arata H, Iwasaki I, Nishimura M (1994) Regulation of PEP-carboxylase by biological clock in a CAM plant. Plant Cell Physiol **35:** 233–242

Lange OL, Zuber M (1980) Temperature dependence of CO_2 exchange in stem-succulent members of the Asclepiadaceae having Crassulacean acid metabolism. Flora **170:** 529–553

Li B, Chollet R (1994) Salt induction and the partial purification/characterisation of phosphoenolpyruvate carboxylase protein-serine kinase from an inducible Crassulacean-acid-metabolism (CAM) plant, *Mesembryanthemum crystallinum* L. Arch Biochem Biophys **314:** 247–254

Lüttge U, Beck F (1992) Endogenous rhythm and chaos in Crassulacean acid metabolism. Planta **188:** 28–38

Lüttge U, Smith AC (1984) Mechanism of passive malic-acid efflux from vacuoles of the CAM plant *Kalanchoë daigremontiana*. J Membr Biol **81:** 149–158

Möllering H (1974) L (-) Malat. *In* HU Bergmeyer, ed, Methoden der Enzymatischen Analyse, Vol 2. Verlag Chemie, Weinheim, Germany, pp 1636–1639

Nimmo GA, Nimmo HG, Fewson CA, Wilkins MB (1984) Diurnal changes in the properties of phosphoenolpyruvate carboxylase in *Bryophyllum* leaves: a possible covalent modification. FEBS Lett **179:** 199–203

Nimmo GA, Nimmo HG, Hamilton ID, Fewson CA, Wilkins MB (1986) Purification of the phosphorylated night form and dephosphorylated day form of phosphoenolpyruvate carboxylase from *Bryophyllum fedtschenkoi*. Biochem J **239:** 213–220

Nimmo GA, Wilkins MB, Fewson CA, Nimmo HG (1987) Persistent circadian rhythms in the phosphorylation state of phosphoenolpyruvate carboxylase from *Bryophyllum fedtschenkoi* leaves and its sensitivity to inhibition by malate. Planta **170:** 408–415

Nuernbergk EL (1961) Endogener Rhythmus und CO_2-Stoffwechsel bei Pflanzen mit diurnalem Säurerhythmus. Planta **56:** 28–70

Nungesser D, Kluge M, Tolle H, Oppelt W (1984) A dynamic computer model of the metabolic and regulatory processes in Crassulacean acid metabolism. Planta **162:** 204–214

Osmond CB (1978) Crassulacean acid metabolism: a curiosity in context.Annu Rev Plant Physiol **29:** 379–414

Ratajczak R, Kemna I, Lüttge U (1994) Characteristics, partial purification and reconstitution of the vacuolar malate transporter of the CAM plant *Kalanchoë daigremontiana* Hamet et Perrier. Planta **195:** 226–236

Ritz D, Kluge M (1987) Circadian rhythmicity of CAM in continuous light: coincidences between gas exchange parameters, $^{14}CO_2$ fixation patterns and PEP-carboxylase properties. J Plant Physiol **131:** 285–296

Schomburg M (1994) Untersuchungen über das thermotrophe Phasenverhalten des Tonoplasten bei der CAM-Pflanze *Kalanchoë daigremontiana*. PhD thesis. Technische Hochschule, Darmstadt, Germany

von Caemmerer S, Farquhar GD (1981) Some relationships between the biochemistry of photosynthesis and the gas exchange of leaves. Planta **153:** 376–387

Warren DM, Wilkins MB (1961) An endogenous rhythm in the rate of dark fixation of carbon dioxide in leaves of *Bryophyllum fedtschenkoi*. Nature **191:** 686–688

Wilkins MB (1959) An endogenous rhythm in the rate of dark fixation of carbon dioxide in leaves of *Bryophyllum*. II. The effects of light and darkness on the phase and period of the rhythm. J Exp Bot **10:** 377–390

Wilkins MB (1962) An endogenous rhythm in the rate of dark fixation of carbon dioxide in leaves of *Bryophyllum*. III. The effects of temperature changes on the phase and period of the rhythm. Proc R Soc Lond B Biol Sci **156:** 220–241

Wilkins MB (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 (1982) Properties of phosphoenolpyruvate carboxylase in rapidly prepared, desalted leaf extracts of the Crassulacean acid metabolism plant *Mesembryanthemum crystallinum* L. Planta **154:** 298–308

Winter K, Smith JAC (1996) Crassulacean Acid Metabolism: Biochemistry, Ecophysiology and Evolution. Springer-Verlag, Berlin