*Plant Physiology*, November 1999, Vol. 121, pp. 889–896, www.plantphysiol.org © 1999 American Society of Plant Physiologists

# Metabolite Control Overrides Circadian Regulation of Phospho*enol*pyruvate Carboxylase Kinase and $CO_2$ Fixation in Crassulacean Acid Metabolism[1]

**Anne M. Borland*, James Hartwell, Gareth I. Jenkins, Malcolm B. Wilkins, and Hugh G. Nimmo**

Department of Agricultural and Environmental Science, University of Newcastle, Newcastle upon Tyne NE1 7RU, United Kingdom (A.M.B.); and Plant Molecular Science Group, Institute of Biomedical and Life Sciences, University of Glasgow, Glasgow G12 8QQ, United Kingdom (J.H., G.I.J., M.B.W., H.G.N.)

**Phospho*enol*pyruvate carboxylase (PEPc) catalyzes the primary fixation of $CO_2$ in Crassulacean acid metabolism plants. Flux through the enzyme is regulated by reversible phosphorylation. PEPc kinase is controlled by changes in the level of its translatable mRNA in response to a circadian rhythm. The physiological significance of changes in the levels of PEPc-kinase-translatable mRNA and the involvement of metabolites in control of the kinase was investigated by subjecting *Kalanchoë daigremontiana* leaves to anaerobic conditions at night to modulate the magnitude of malate accumulation, or to a rise in temperature at night to increase the efflux of malate from vacuole to cytosol. Changes in $CO_2$ fixation and PEPc kinase activity reflected those in kinase mRNA. The highest rates of $CO_2$ fixation and levels of kinase mRNA were observed in leaves subjected to anaerobic treatment for the first half of the night and then transferred to ambient air. In leaves subjected to anaerobic treatment overnight and transferred to ambient air at the start of the day, PEPc-kinase-translatable mRNA and activity, the phosphorylation state of PEPc, and fixation of atmospheric $CO_2$ were significantly higher than those for control leaves for the first 3 h of the light period. A nighttime temperature increase from 19°C to 27°C led to a rapid reduction in kinase mRNA and activity; however, this was not observed in leaves in which malate accumulation had been prevented by anaerobic treatment. These data are consistent with the hypothesis that a high concentration of malate reduces both kinase mRNA and the accumulation of the kinase itself.**

In plants with Crassulacean acid metabolism (CAM), phospho*enol*pyruvate carboxylase (PEPc) (EC 4.1.1.31) catalyzes the nocturnal fixation of atmospheric $CO_2$ (as $HCO_3^-$) into oxaloacetate, which is subsequently reduced to malate and stored in the vacuole. During the day, the decarboxylation of malate released from the vacuole generates a high intercellular partial pressure of $CO_2$, which results in stomatal closure and the conservation of water. The fixation of this internally generated $CO_2$ by Rubisco continues behind closed stomata until malate decarboxylation nears completion and the $CO_2$ partial pressure drops. Stomata may subsequently re-open and atmospheric $CO_2$ can then be fixed directly via the Calvin cycle.

The temporal separation of these $C_4$ and $C_3$ carboxylation processes, which distinguishes CAM from $C_4$ photosynthesis, requires that the activity of PEPc be reduced during the day to curtail futile cycling of $CO_2$ from concurrent malate synthesis and breakdown. The day/night regulation of flux through PEPc is achieved by reversible phosphorylation that reduces the sensitivity of the enzyme to inhibition by L-malate with the phosphorylated, malate-insensitive (active) form of PEPc present at night (Nimmo et al., 1984, 1986). The phosphorylation state of PEPc is determined by the presence or absence of a specific $Ca^{2+}$-independent protein kinase termed PEPc kinase (Carter et al., 1991; Li and Chollet, 1994). Recently, Hartwell et al. (1996) used a novel approach in which the products of in vitro translation of leaf RNA were assayed directly for PEPc kinase activity to demonstrate that the activity of PEPc kinase reflects changes in the level of its translatable mRNA. Thus, levels of kinase mRNA were approximately 20-fold higher at night than during the daytime in leaves of the CAM plant *Kalanchoë* (*Bryophyllum*) *fedtschenkoi* (Hartwell et al., 1996).

While the levels of PEPc kinase mRNA in $C_3$ and $C_4$ plants appear to respond to photosynthesis and, thus, light-dark transitions, in CAM plants a circadian oscillator controls the levels of kinase activity and translatable mRNA under constant environmental conditions (Carter et al., 1991; Hartwell et al., 1996). This results in a circadian rhythm in the phosphorylation state of PEPc (Nimmo et al., 1987), which plays an important role in generating the endogenous rhythms of $CO_2$ exchange in CAM plants first described by Wilkins (1959). To date, the exact nature of the circadian oscillator in CAM is unknown, but recent observations indicate that the timing of PEPc activation/deactivation varies between different CAM species grown under identical environmental conditions (Borland and Griffiths, 1997).

Physiological manipulations of dark $CO_2$ uptake and malate accumulation have indicated that the storage capacity of the vacuole for malate plays a key role in determining the timing of the inactivation of PEPc (Winter and Tenhunen, 1982; Fischer and Kluge, 1984; Borland and Griffiths, 1997). Thus, in plants prevented from accumulating malate overnight in an atmosphere of $N_2$, flux through

[1] Financial support was provided by the Natural Environment Research Council and the Biological and Biotechnological Science Research Council, United Kingdom.

* Corresponding author; e-mail a.m.borland@ncl.ac.uk; fax 191–222–5228.

PEPc increases substantially at the start of the day in ambient air, and the inactivation of PEPc is delayed by 2 to 3 h (Borland and Griffiths, 1997). Observations that the circadian rhythms of phosphorylation of PEPc and $CO_2$ exchange can be disrupted and re-initiated by temperature changes have also pointed to a key role for the tonoplast in malate compartmentation, and for malate itself in the generation of the endogenous rhythm of PEPc activity (Wilkins, 1983; Carter et al., 1991; Grams et al., 1997). Malate inhibits PEPc kinase by binding to PEPc (Carter et al., 1991; Li and Chollet 1993, 1994), although it is not clear whether this effect is physiologically significant. Malate or other metabolites might also affect the phosphorylation of PEPc by acting at steps closer to the circadian oscillator. Such effects on the output from the oscillator could provide CAM plants with the flexibility to adjust C flux in response to changes in environmental conditions.

The aim of the present work was to study the relationship between leaf malate content, PEPc kinase activity, and levels of translatable kinase mRNA in intact plants of *Kalanchoë daigremontiana* Hamet et Perr. Physiological manipulations involving anaerobic treatments and temperature changes in the dark were used to modulate the magnitude of dark $CO_2$ uptake and malate accumulation. The results highlight the physiological importance of changes in translatable PEPc kinase mRNA in the CAM cycle and suggest that metabolites, most likely malate, affect the phosphorylation of PEPc at several levels.

## MATERIALS AND METHODS

### Plant Material and Growth Conditions

Plants of *Kalanchoë daigremontiana* Hamet et Perr., which were approximately 1 year old and growing in 10-cm-diameter pots, were acclimated in the growth chamber for 4 weeks prior to experimentation. All measurements were conducted on the fourth leaf pair from the growing tip.

The plants were acclimated in a growth chamber (Fitotron, Sanyo Gallenkamp, Leicester, UK) programmed to provide gradual changes in temperature, humidity, and photsynthetic photon flux density (PPFD) at the start and end of the photoperiod in an attempt to mimic conditions found naturally at dawn and dusk. From 8:30 AM until 12 PM, PPFD was increased to a maximum of 530 $\mu$mol $m^{-2}$ $s^{-1}$ at leaf height, the temperature was increased from 19°C to 27°C, and the relative humidity (RH) was decreased from 80% to 60% (the vapor pressure deficit was increased from 1.8–2.9 kPa). These conditions were maintained until 4 PM, when PPFD was decreased gradually until the lights were off at 7:30 PM, the temperature was decreased to 19°C, and the RH was increased to 80% (the vapor pressure deficit was 1.8 kPa). Over the 13-h dark period, the temperature (19°C) and RH (80%) remained constant.

### Manipulation of CAM

Previous studies on *K. daigremontiana* have indicated that exposure of the plants to $CO_2$-free air still permits the accumulation of malate (up to 25% of that observed in controls) through refixation of respiratory $CO_2$ by PEPc (A. Borland, unpublished data). Thus, in order to completely inhibit PEP carboxylation at night, individual leaves of intact plants were enclosed in an atmosphere of $N_2$ overnight, as described by Borland and Griffiths (1997), thereby preventing access to external $CO_2$ and inhibiting the release of internal (respiratory) sources of $CO_2$ (full $N_2$). Some leaves were enclosed in an atmosphere of $N_2$ for the first half of the dark period (until 2 PM) and then exposed to ambient air for the remainder (half $N_2$). Control leaves were exposed to the ambient atmosphere in the growth chamber.

A set of plants, half of which were maintained in ambient air (control), and half with leaves enclosed in an atmosphere of $N_2$ (half $N_2$), was subjected to an increase in temperature from 19°C to 27°C in the middle of the dark period (2:30–3 AM). The leaves enclosed in $N_2$ were subsequently exposed to ambient air from 3 AM onward, with the temperature maintained at 27°C and the RH at 70%.

### Gas Exchange Measurements

Rates of net $CO_2$ assimilation were measured continuously on the same leaf over 24 h. The leaf was enclosed in a porometer head that tracked the environmental conditions in the growth chamber with gas exchange parameters measured using an open infrared (IR) gas exchange system (H. Walz, GmbH Effeltrich, Germany) with a gas analyzer (Binos, H. Walz). Gas exchange parameters were calculated using DIAGAS software supplied by H. Walz. Each gas exchange curve presented is for a representative leaf from three replicate determinations.

### Malate Content

Discs were punched from three replicate leaves, subjected to the various treatments at intervals over the dark and light periods, and immediately plunged into hot (80°C) methanol (80%, v/v). The methanolic extracts were heated for 1 h at 70°C before being evaporated to dryness, taken up in 100 mM *N,N′*-bis(2-hydroxyethylglycine) (Bicine), pH 7.8, and the malate content determined enzymatically using malate dehydrogenase, as described by Hohorst (1965).

### PEPc and PEPc Kinase Assays

Leaf extracts were prepared and desalted as described by Hartwell et al. (1996). The activity of PEPc was assayed and its apparent $K_i$ for L-malate estimated as described by Nimmo et al. (1984). PEPc kinase activity in desalted extracts was assayed according to the method of Carter et al. (1991) using purified dephosphorylated PEPc from *Kalanchoë fedtschenkoi* as the substrate. Incubations were for 30 min at 30°C.

### Assay of PEPc-Kinase-Translatable mRNA

Following the method of Hartwell et al. (1996), RNA was isolated and translated in vitro using a rabbit reticulocyte lysate, and a sample of the translation products was as-

sayed for PEPc kinase activity. The PEPc was isolated by immunoprecipitation, resolved by SDS gel electrophoresis, and the incorporation of $^{32}P$ into PEPc was quantified by phosphor imaging. These values were corrected to take into account any differences in the efficiency of translation between the different samples, as estimated by the incorporation of [$^{35}S$]Met into protein (Hartwell et al., 1996). The values are therefore equivalent to data from northern blot analysis corrected for RNA loading. Control experiments in which [$\gamma$-$^{32}P$]ATP was omitted from the kinase assays showed that when *K. daigremontiana* RNA was used, small amounts of [$^{35}S$]Met were incorporated into immunoprecipitated PEPc from de novo synthesis of PEPc during the translations. This incorporation was less than the amount of $^{32}P$ incorporated into PEPc in controls using the products of translations with no added RNA. The background incorporation of $^{32}P$ was as a result of trace contamination of the PEPc substrate with PEPc kinase. However, this was <4% of the maximum labeling obtained with samples containing RNA. All experiments were repeated at least twice, with similar results, and the data presented are from representative individual experiments.

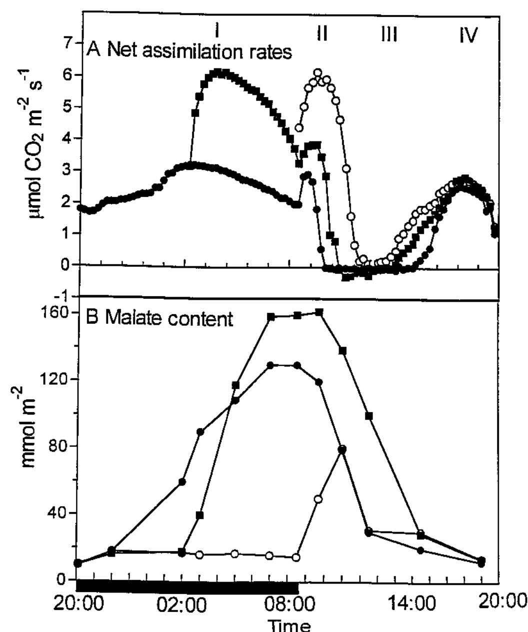


**Figure 1.** Rates of net $CO_2$ uptake and malate content in leaves exposed to anaerobic conditions for part or all of the dark period. A, Leaves were enclosed in an atmosphere of $N_2$ for the first half (half $N_2$) or entire duration (full $N_2$) of the 13-h dark period before transfer to ambient air. Rates of net $CO_2$ assimilation were measured. Control leaves were exposed to the ambient atmosphere in the growth chamber. Each gas exchange curve is representative of three replicate runs with SE <10%. B, Malate content was measured in leaves subjected to the above treatments with each point being the mean of three replicates with SE <10%. ●, Control leaves; ○, full-$N_2$ leaves; ■, half-$N_2$ leaves. The solid bar on the *x* axis represents the period of darkness.

## RESULTS

### Physiology of CAM and Manipulation by $N_2$

Figure 1A illustrates how the dark/light pattern of net $CO_2$ uptake, which may be dissected into four phases (Osmond, 1978), was modulated in response to anaerobic conditions that were imposed for part or all of the dark period. Inhibiting $CO_2$ uptake over the first half of the 13-h dark period by enclosing leaves in an atmosphere of $N_2$ for 7.5 h resulted in a substantial increase in rates of net $CO_2$ assimilation when darkened leaves were removed from $N_2$ and transferred to ambient air (half $N_2$) compared with control plants exposed to ambient air throughout the night. The malate content of the half-$N_2$-treated leaves increased rapidly when darkened leaves were transferred to ambient air (Fig. 1B). After only 3 h in ambient air, the malate content of half $N_2$ leaves was somewhat higher than that in control leaves that had accumulated malate over 9 h. At the end of the dark period, the malate content of the half-$N_2$ leaves was about 25% higher than that measured in control leaves.

At the start of the 11-h photoperiod, leaves that had been exposed to $N_2$ for the first half of the dark period (half $N_2$) showed a small increase in the magnitude and duration of phase II net $CO_2$ uptake compared with control plants (Fig. 1A). However, in leaves that had been enclosed in an atmosphere of $N_2$ for the entire duration of the dark period (full $N_2$), transfer to ambient air at the start of the photoperiod resulted in a substantial increase in the rates of net $CO_2$ assimilation over both control and half-$N_2$ leaves during phase II. Stomatal closure was delayed by about 2 h compared with controls, as judged by the time at which net $CO_2$ assimilation fell to zero (Fig. 1A). Moreover, after transfer to ambient air at the start of the photoperiod, the full-$N_2$ leaves accumulated about 60 mmol $m^{-2}$ malate over the first 2.5 h of the photoperiod. Thus, in these leaves PEPc was still active at a period during which net breakdown of malate occurred in control and half-$N_2$ leaves (Fig. 1B). Despite this accumulation of malate in full-$N_2$ leaves during the photoperiod, the malate content attained only about 50% of that measured in control leaves, and the majority of decarboxylation was accomplished within 2 h. Consequently, during phase III, stomata remained closed for only 2 h in full-$N_2$ leaves compared with 5 h in control leaves (Fig. 1A).

### PEPc Kinase Activity, Translatable mRNA, and Manipulation by $N_2$

Figure 2 shows the changes in PEPc kinase activity and the level of translatable mRNA for the kinase in control and full-$N_2$ leaves throughout the dark period. In control leaves, PEPc kinase activity increased over the first part of the dark period, reaching a plateau after 9.5 h in darkness (Fig. 2A). For leaves maintained in $N_2$ during the entire dark period (in which malate content remained low; Fig. 1B), PEPc kinase activity increased steadily over the course of the dark period and was substantially higher than that measured in control leaves at comparable stages throughout the night. However, Figure 2B indicates that the levels of translatable PEPc kinase mRNA in control and full-$N_2$

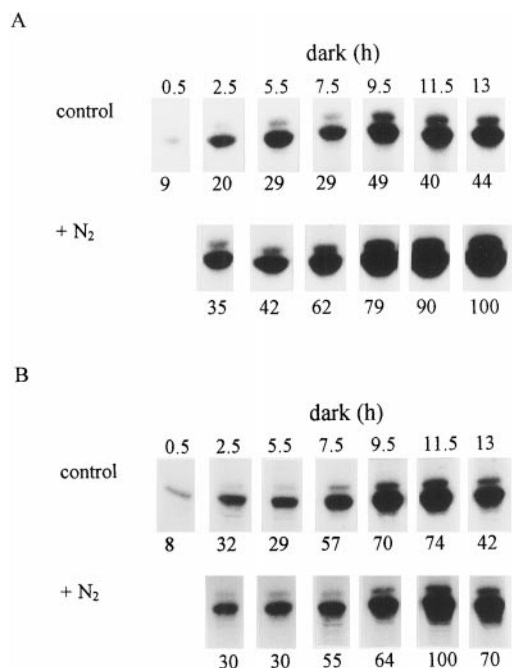


**Figure 2.** PEPc kinase activity and levels of translatable PEPc kinase mRNA under ambient and anaerobic conditions at night. Leaves were enclosed in an atmosphere of $N_2$ overnight to prevent malate accumulation or maintained in ambient air. Samples for PEPc kinase assays (A) and RNA isolation and measurement of PEPc kinase translatable mRNA (B) were taken simultaneously from the same leaves at intervals over the 13-h dark period. Shown are autoradiographs of the $^{32}$P-labeled PEPc bands following SDS-PAGE. The doublet of PEPc bands is caused by the presence in a ratio of about 10:1 of two related subunits in *K. fedtschenkoi* PEPc, both of which are phosphorylated by PEPc kinase (Carter et al., 1991). The relative intensity of the PEPc bands, shown below each track, was determined by phosphor imaging. The total incorporation of [$^{35}$S]Met into in vitro translation products using RNA isolated from control and $N_2$-treated leaves was similar (data not shown). The results are from duplicate experiments

leaves were similar for the first 9.5 h of the dark period. Subsequently, levels of translatable mRNA in leaves enclosed in $N_2$ were higher than those measured in control leaves.

The time course of changes in PEPc kinase activity and translatable mRNA (Fig. 2), together with changes in the apparent $K_i$ of PEPc for malate for control and full-$N_2$ leaves, are illustrated in Figure 3. Changes in the apparent $K_i$ for malate reflect the phosphorylation state of PEPc (Carter et al., 1991). In *K. daigremontiana*, the apparent $K_i$ in control leaves increased from 0.5 to 5.0 mM during the night, compared with the range of 0.3 to 3.0 mM seen with *K. fedtschenkoi* in earlier work (Nimmo et al., 1984). However, the apparent $K_i$ in full-$N_2$ leaves reached 8 mM, implying that the enzyme was not fully phosphorylated in control leaves. The changes in apparent $K_i$ closely followed changes in PEPc kinase activity. The increase in the apparent $K_i$ measured in leaves enclosed in $N_2$ compared with controls was reflected by an increased PEPc kinase activity in the full-$N_2$ leaves. In control leaves, the levels of kinase-translatable mRNA reached a plateau at 2 AM, whereas kinase activity and apparent $K_i$ achieved maximum values 2 h later. In full-$N_2$ leaves, a peak in kinase mRNA levels occurred at 6 AM. The levels of mRNA were substantially higher than those measured in control leaves at this time. In both control and full $N_2$ leaves, levels of translatable mRNA declined over the last part of the dark period.

Figure 3 also illustrates changes in apparent $K_i$ for malate, PEPc kinase activity, and levels of translatable mRNA that occurred when leaves exposed to $N_2$ for the first half of the dark period were subsequently transferred to ambient air for the remainder of the night. In these leaves, following transfer to ambient air, the apparent $K_i$ for malate and PEPc kinase activity were appreciably

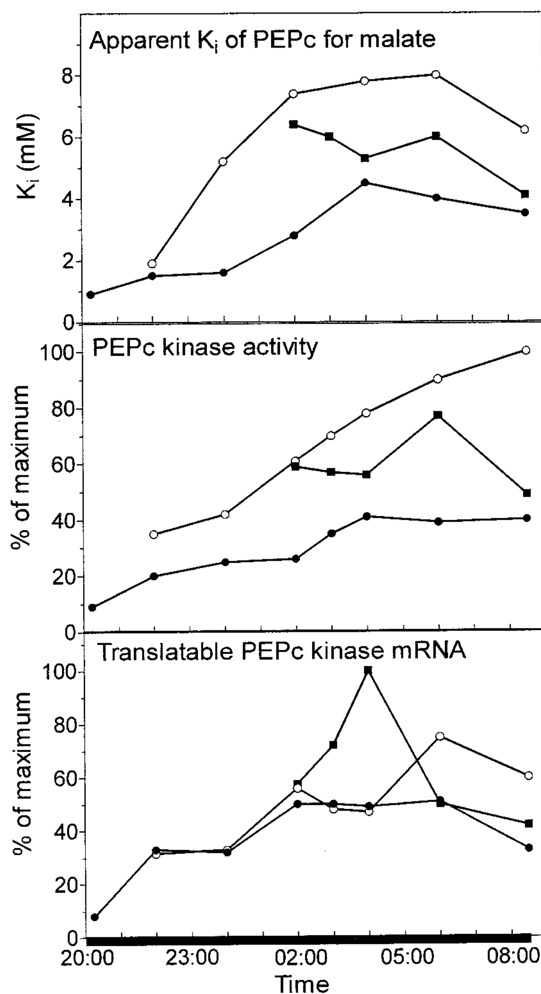


**Figure 3.** Apparent $K_i$ of PEPc for L-malate, PEPc kinase activity, and translatable kinase mRNA under ambient and anaerobic conditions at night. Control leaves (●) were kept in ambient air throughout. Full-$N_2$ leaves (○) were enclosed in an atmosphere of $N_2$ overnight to prevent malate accumulation. Half-$N_2$ leaves (■) were enclosed in an atmosphere of $N_2$ to prevent malate accumulation for the first half of the dark period before transfer to ambient air. Samples for PEPc and PEPc kinase assays and RNA isolation were taken simultaneously from the same leaves at intervals over the dark period. Kinase activity and translatable mRNA values are expressed as percentages of the maximum reached during the 13-h dark period. The results are from duplicate experiments.

higher than in the controls. In the 2 h following transfer of the half-$N_2$ leaves to ambient air, rates of net $CO_2$ uptake reached a maximum (Fig. 1A). Over this period, the level of kinase mRNA in the half-$N_2$ leaves rose significantly. By 6 AM, when malate content peaked (Fig. 1B), kinase mRNA had dropped to a level comparable to that measured in control leaves. The peak in kinase mRNA at 4 AM preceded the time when maximum PEPc kinase activity was reached in half-$N_2$ leaves at 6 AM. There was little change in apparent $K_i$ over this period.

Figure 4 compares the changes that occurred at the start of the photoperiod in control leaves with those in leaves maintained in $N_2$ throughout the dark period (full $N_2$) but transferred to ambient air at the start of the photoperiod. In control leaves, the rapid down-regulation of PEPc activity was shown by a decrease in the apparent $K_i$ of PEPc for L-malate and by the low level of PEPc kinase activity over the 1st h of the photoperiod as rates of net $CO_2$ assimilation fell to zero and malate was broken down (Fig. 1). In the same leaves, the low levels of kinase mRNA detected at the start of the photoperiod declined to essentially zero after 100 min in the light. In contrast, the apparent $K_i$ for malate,

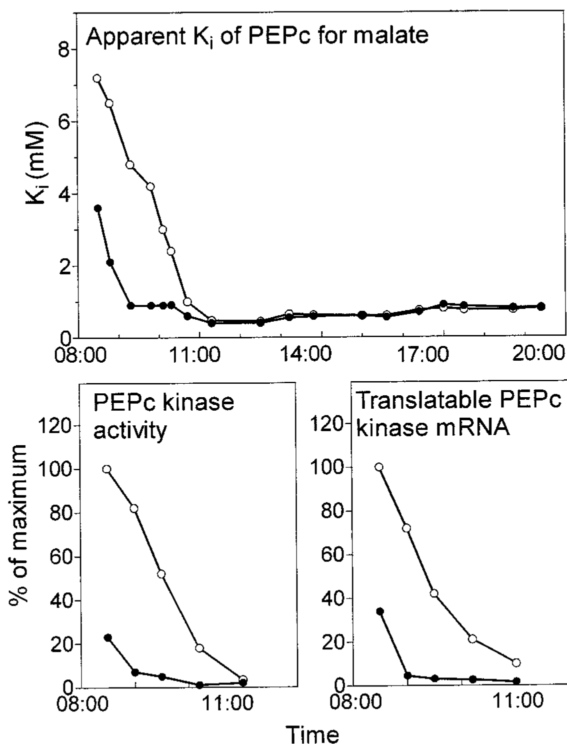


**Figure 4.** Changes in the apparent $K_i$ for L-malate, PEPc kinase activity, and translatable kinase mRNA at the start of the photoperiod after a night in ambient or anaerobic conditions. Leaves that had been maintained in an atmosphere of $N_2$ overnight to prevent malate accumulation were transferred to ambient air at the start of the photoperiod (○). Control leaves were maintained in ambient air (●). Samples for PEPc and PEPc kinase assays and RNA isolation were taken simultaneously from the same leaves at intervals over the light period. Kinase activity and translatable mRNA values are expressed as percentages of the maximum reached during the photoperiod. The results are from duplicate experiments.

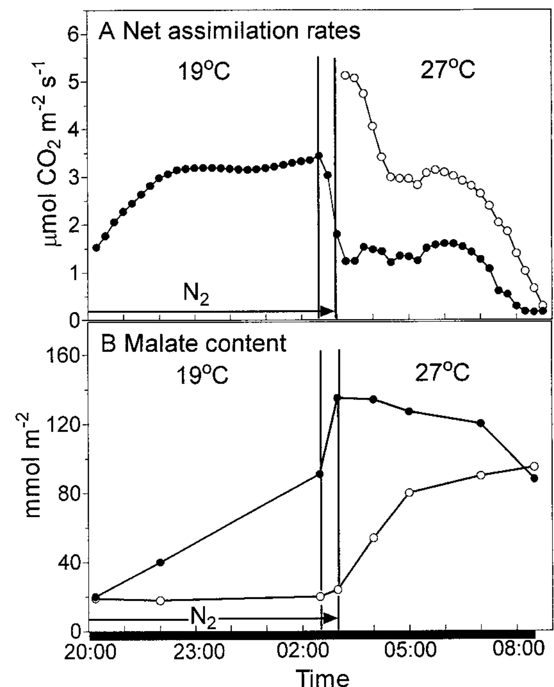


**Figure 5.** Modulation of net $CO_2$ assimilation rates and malate accumulation by a temperature increase at night. Control leaves (●) were exposed to ambient air. Half-$N_2$ leaves (○) were enclosed in an atmosphere of $N_2$ for the first half of the dark period to prevent malate accumulation. Leaves were subjected to an 8°C rise in temperature from 2:30 to 3 AM. The half-$N_2$ leaves were subsequently exposed to ambient air at 27°C for the duration of the dark period. A, Rates of net $CO_2$ uptake by leaves under the two treatments with each gas exchange curve representative of three replicate runs with SE <10%. B, Malate content was measured in leaves subjected to the above treatments, with each point the mean of three replicates with SE <10%. The solid bar on the *x* axis represents the period of darkness.

PEPc kinase activity, and kinase mRNA at the start of the photoperiod were substantially higher in leaves previously exposed to $N_2$ overnight than in control leaves, and remained high well into the photoperiod as net $CO_2$ uptake continued and malate was accumulated (Fig. 1).

### Physiological Aspects of Temperature Manipulations

It has been suggested that the disruption of the circadian oscillations of $CO_2$ exchange in CAM plants by high temperature may be a consequence of increased efflux of malate from the vacuole to the cytosol, the site of PEPc activity (Wilkins, 1983; Grams et al., 1997). Figure 5 illustrates the physiological consequences of exposing either control leaves or leaves prevented from accumulating malate over the first half of the dark period (half $N_2$) to an 8°C increase in temperature in the middle of the night (from 2:30–3 AM). In control leaves there was a rapid decline in the rate of net $CO_2$ assimilation as the temperature was increased from 19°C to 27°C (Fig. 5A). The sharp increase in malate content over the 30-min rise in temperature (Fig. 5B) may be attributed to an increase in refixation of respiratory $CO_2$ by PEPc. Overall, the maximum net

assimilation rate at 27°C was <50% of that measured at 19°C in control leaves. Despite the continued net uptake of $CO_2$, the malate content of the control leaves dropped slightly over the first few hours of exposure to the higher temperature, suggesting consumption of malate through increased rates of mitochondrial respiration. However, marked breakdown of malate was observed over the last hour of the dark period, when net $CO_2$ assimilation had virtually ceased. Rates of net $CO_2$ assimilation in leaves removed from $N_2$ immediately after the temperature had been increased to 27°C were approximately 5-fold higher than those measured in control leaves. However, net assimilation rates dropped sharply during the first 1.5 h at the higher temperature in $N_2$-treated leaves, reached a plateau for 3 h, and then decreased over the last hour of the dark period. Malate content in the $N_2$-treated leaves showed a marked increase over the first 2 h at the higher temperature and a more gradual increase over the remaining 3.5 h. In contrast to control leaves, the net breakdown of malate in $N_2$-treated leaves did not commence until the start of the photoperiod (data not shown).

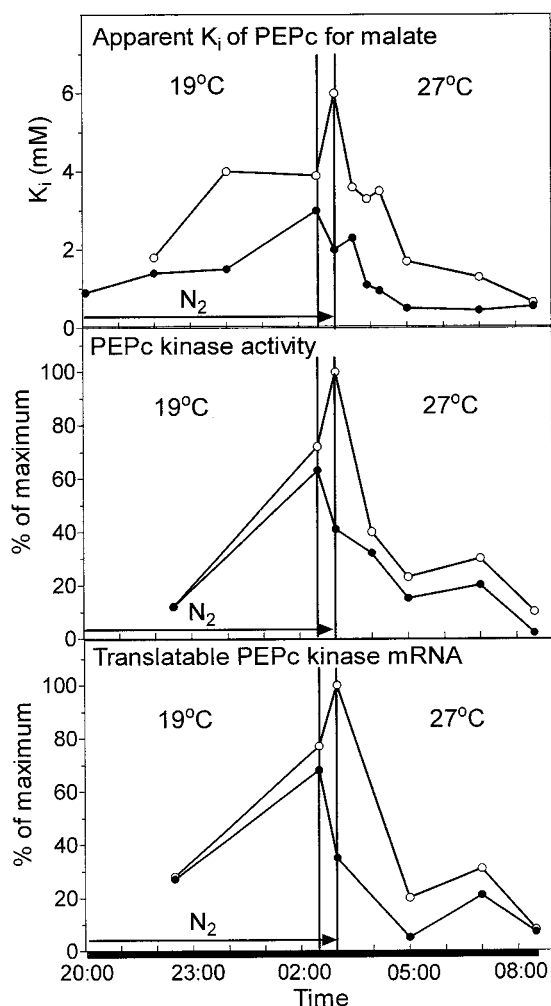


**Figure 6.** Modulation of the apparent $K_i$ for L-malate, PEPc kinase activity, and translatable kinase mRNA by an increase in temperature at night. Control leaves (●) were exposed to ambient air. Half-$N_2$ leaves (○) were enclosed in an atmosphere of $N_2$ for the first half of the dark period to prevent malate accumulation. All leaves were subjected to an 8°C rise in temperature from 2:30 to 3 AM. The half-$N_2$ leaves were subsequently exposed to ambient air at 27°C for the duration of the dark period. Samples for PEPc and PEPc kinase assays and RNA isolation were taken simultaneously from the same leaves at intervals over the dark period. Kinase activity and translatable mRNA values are expressed as percentages of the maximum reached in leaves during a normal dark period at 19°C. The results are from duplicate experiments.

### Modulation of PEPc Kinase Activity and Translatable mRNA by Temperature

Figure 6 indicates that an 8°C rise in temperature over 30 min resulted in a decrease in PEPc kinase activity and kinase-translatable mRNA and a slight decrease in apparent $K_i$ in control leaves. These parameters continued to fall over the following 70 min at 27°C. In contrast, in leaves prevented from accumulating malate over the first half of the dark period, an 8°C rise in temperature over 30 min resulted in an increase in apparent $K_i$, PEPc kinase activity, and kinase-translatable mRNA. However, transfer of the $N_2$-treated leaves to ambient air after the temperature rise resulted in a marked decrease in translatable PEPc kinase mRNA, kinase activity, and the apparent $K_i$ for L-malate over 70 min at the higher temperature as malate accumulated, presumably in the cytosol. Additional experiments in which the levels of PEPc kinase mRNA were measured at more frequent intervals after the temperature increase confirmed a steady decline in the levels of mRNA from 3 until 4:10 AM (data not shown). From 4:10 until 7 AM, the levels of kinase mRNA in half-$N_2$ leaves were maintained at 20% of maximum. This was mirrored by a maintenance of PEPc kinase activity and by the plateau in net $CO_2$ assimilation in half-$N_2$ leaves (Fig. 5A). For the latter part of the dark period, the apparent $K_i$ for L-malate and the levels of kinase activity and mRNA were somewhat higher in $N_2$-treated leaves, in which the malate content was low but rising compared with controls, in which the malate content was high but declining (Fig. 5B).

## DISCUSSION

In this work we have manipulated intact plants to affect the magnitude of dark $CO_2$ uptake and malate accumulation, and monitored the effects of these manipulations on the levels of PEPc kinase mRNA and activity. The results allow a number of conclusions about the control of PEPc kinase to be drawn. First, the data clearly demonstrate the physiological significance of PEPc phosphorylation, as shown by the close correlation between the activity in vitro of PEPc kinase, net $CO_2$ uptake by PEPc, and malate accumulation in vivo under ambient air and after transfer from anaerobic conditions to ambient air. For example, leaves prevented from accumulating malate overnight in an atmosphere of $N_2$ exhibited an extended period of $CO_2$ uptake by PEPc for 2 to 3 h at the start of the photoperiod under ambient air (Fig. 1) (Borland and Griffiths, 1997). Under these conditions, kinase activity remained detectable and PEPc remained phosphorylated (as judged by its

malate sensitivity) for several hours into the photoperiod (Fig. 4). In leaves moved from $N_2$ to ambient air midway through the dark period, malate accumulated significantly faster, PEPc kinase activity was higher, and PEPc was more highly phosphorylated than in control leaves. (Figs. 1 and 3). The data presented here support and extend those of Hartwell et al. (1996) on *B.* (*K.*) *fedtschenkoi* in showing that these physiologically significant changes in PEPc kinase activity reflect changes in the translatable mRNA for this protein. Moreover, recent work using northern analysis with a PEPc kinase cDNA has shown that there are very similar changes in the level of PEPc kinase transcripts (J. Hartwell, A.M. Borland, G.I. Jenkins, and H.G. Nimmo, unpublished data).

Previous work has demonstrated clearly that PEPc kinase mRNA and activity and the phosphorylation state of PEPc are under circadian control (Nimmo et al., 1987; Carter et al., 1991; Hartwell et al., 1996). These effects contribute to the well-established circadian control of $CO_2$ fixation in CAM plants (e.g. Wilkins, 1992). The influence of a circadian oscillator, rather than light/dark control, is illustrated by the fact that in *K. fedtschenkoi* in an 8-h photoperiod, both the increase and decrease in PEPc kinase mRNA and activity occur during the dark period (Hartwell et al., 1996). In the present work using *K. daigremontiana* in an 11-h photoperiod, the increase in PEPc kinase mRNA and activity also occured during the dark period (Figs. 2 and 3). The decline in PEPc kinase mRNA commences during the dark period, but the decline in kinase activity occurs only at the start of the light period.

The data in this paper allow a further conclusion to be drawn about the control of PEPc kinase. The circadian control of kinase mRNA and activity can be influenced by metabolic status, specifically by treatments that affect the content or compartmentation of malate. For example, in leaves that cannot accumulate malate, PEPc kinase activity is significantly higher than in control leaves, even though PEPc kinase mRNA levels are similar (Figs. 2 and 3). Although subjecting leaves to an anaerobic environment under $N_2$ could in itself affect mRNA abundance, the data shown here present a number of testable hypotheses. Thus, in leaves with a high malate content, translation of PEPc kinase mRNA is reduced, the rate of inactivation (possibly by turnover) of PEPc kinase is increased, or both. The mechanisms(s) responsible could involve sensing of malate itself or of another metabolite the level of which correlates with the total leaf malate content.

Another effect of the prevention of malate accumulation was observed in experiments in which the temperature was increased from 19°C to 27°C in the middle of the dark period. In control leaves, this increase in temperature was accompanied by a reduction in the level of PEPc kinase mRNA. In contrast, in leaves in which malate accumulation had been prevented, there was a marked increase in kinase mRNA as temperature increased (Fig. 6). Experiments conducted with *K. fedschenkoi* have indicated that low temperature (i.e. 4°C) stabilizes the levels of kinase mRNA and postpones de-phosphorylation (Hartwell et al., 1996).

The effect of increased temperature on circadian rhythms of $CO_2$ fixation has been ascribed to increased permeability of the tonoplast to malate and efflux of malate to the cytoplasm (Wilkins, 1983, 1992). There is direct experimental support for this hypothesis (Friemert et al., 1988). One possible explanation of our data is that PEPc-kinase-translatable mRNA is negatively regulated by cytosolic malate. However, it must be emphasized that no direct measurements of cytosolic malate have been made in CAM plants, and we have not been able to ascertain whether the temperature increase reduced PEPc-kinase-translatable mRNA through an increase in total malate (Fig. 5B), an increase in cytosolic malate, a lowering of cytosolic pH, or a change in another metabolite. Either transcription of the PEPc kinase gene or the stability of the kinase mRNA could be affected. Presumably, the relevant metabolite level in control leaves was insufficient to reduce the accumulation of PEPc kinase mRNA observed during the first 10 h of darkness (Fig. 3). PEPc kinase mRNA started to decline later in control leaves than in half-$N_2$ leaves (6 and 4 AM, respectively) (Fig. 3). Because the total leaf malate contents were actually similar at these times in the two treatments (Fig. 1B), there may be a threshold level of total malate in the dark (at about 120 mmol $m^{-2}$) above which malate is sufficient to reduce PEPc kinase mRNA. However, PEPc kinase mRNA starts to decline after 6 AM, even in leaves treated with full $N_2$ and unable to accumulate malate (Fig. 3), so at least part of the decline at this time may reflect circadian control.

Overall, the control of flux through PEPc is multilayered. Fine control is achieved by changes in cytosolic levels of pH and opposing metabolic effectors such as malate (negative) and Glc 6-P (positive), whereas the phosphorylation of PEPc represents a means for coarse control of flux through this enzyme. The timing of phosphorylation is set by a circadian oscillator. The data in this paper show that circadian control can be overridden by metabolite control, probably in various ways. Our data are consistent with the view that metabolites can affect PEPc kinase gene expression or mRNA stability, and perhaps the stability of the kinase itself. Such metabolite effects may influence entrainment of the circadian rhythm to environmental conditions that support photosynthetic plasticity and survival through temporarily optimizing $CO_2$ uptake. Identification of the factors responsible will require measurement of the amount and distribution of a number of key metabolites, including malate.

## ACKNOWLEDGMENT

A.M.B. is grateful to Professor H. Griffiths (Department of Agricultural and Environmental Sciences, University of Newcastle) for his continued interest in this work.

Received April 19, 1999; accepted August 2, 1999.

## LITERATURE CITED

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

**Carter PJ, Nimmo HG, Fewson CA, Wilkins MB** (1991) Circadian

rhythms in the activity of a plant protein kinase. EMBO J **10:** 2063–2068
**Fischer A, Kluge M** (1984) Studies on carbon flow in Crassulacean acid metabolism during the initial light period. Planta **160:** 121–128
**Friemert V, Heininger D, Kluge M, Zeigler H** (1988) Temperature effects on malic-acid efflux from the vacuoles and on the carboxylation pathways in Crassulacean acid metabolism plants. Planta **174:** 453–461
**Grams TEE, Borland AM, Roberts A, Griffiths H, Beck F, Lüttge U** (1997) On the mechanism of reinitiation of endogenous Crassulacean acid metabolism rhythm by temperature changes. Plant Physiol **113:** 1309–1317
**Hartwell J, Smith LH, Wilkins MB, Jenkins GI, Nimmo HG** (1996) Higher plant phospho*enol*pyruvate carboxylase kinase is regulated at the level of translatable mRNA in response to light or a circadian rhythm. Plant J **10:** 1071–1078
**Hohorst HJ** (1965) L-(−)Malate: determination with malic acid dehydrogenase and DPN. *In* HU Bergmeyer, ed, Methods of Enzymatic Analysis. Academic Press, London, pp 328–332
**Li B, Chollet R** (1993) Resolution and identification of $C_4$ phospho*enol*pyruvate-carboxylase protein-kinase polypeptides and their reversible light activation in maize leaves. Arch Biochem Biophys **307:** 416–419
**Li B, Chollet R** (1994) Salt induction and the partial purification/characterisation of phospho*enol*pyruvate carboxylase protein-serine kinase from an inducible Crassulacean acid metabolism (CAM) plant, *Mesembryanthemum crystallinum* L. Arch Biochem Biophys **314:** 247–254
**Nimmo GA, Nimmo HG, Fewson CA, Wilkins MB** (1984) Diurnal changes in the properties of phospho*enol*pyruvate carboxylase in *Bryophyllum* leaves: a possible covalent modification. FEBS Lett **178:** 199–203
**Nimmo GA, Nimmo HG, Hamilton ID, Fewson CA, Wilkins MB** (1986) Purification of the phosphorylated night form and dephosphorylated day form of phospho*enol*pyruvate 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 phospho*enol*pyruvate carboxylase from *Bryophyllum fedtschenkoi* leaves and in its sensitivity to inhibition by malate. Planta **170:** 408–415
**Osmond CB** (1978) Crassulacean acid metabolism: a curiosity in context. Annu Rev Plant Physiol **29:** 379–414
**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** (1983) The circadian rhythm of carbon-dioxide metabolism in *Bryophyllum*: the mechanism of phase-shift induction by thermal stimuli. Planta **157:** 471–480
**Wilkins MB** (1992) Circadian rhythms: their origin and control. New Phytol **121:** 347–375
**Winter K, Tenhunen JD** (1982) Light-stimulated burst of carbon dioxide uptake following nocturnal acidification in the Crassulacean acid metabolism plant *Kalanchoë daigremontiana*. Plant Physiol **70:** 1718–1722