Interplay of light and temperature during the in planta modulation of C₄ phosphoenolpyruvate carboxylase from the leaves of Amaranthus hypochondriacus L.: diurnal and seasonal effects manifested at molecular levels

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Abstract

The interactive effects of light and temperature on C₄ phosphoenolpyruvate carboxylase (PEPC) were examined both in vivo and in situ using the leaves of Amaranthus hypochondriacus collected at different times during a day and in each month during the year. The maximum activity of PEPC, least inhibition by malate, and highest activation by glucose-6-phosphate were at 15.00 h during a typical day, in all the months. This peak was preceded by maximum ambient light but coincided with high temperature in the field. The highest magnitude in such responses was in the summer (e.g. May) and least in the winter (e.g. December). Light appeared to dominate in modulating the PEPC catalytic activity, whereas temperature had a strong influence on the regulatory properties, suggesting interesting molecular interactions. The molecular mechanisms involved in such interactive effects were determined by examining the PEPC protein/phosphorylation/mRNA levels. A marked diurnal rhythm could be seen in the PEPC protein levels and phosphorylation status during May (summer month). In contrast, only the phosphorylation status increased during the day in December (winter month). The mRNA peaks were not as strong as those of phosphorylation. Thus, the phosphorylation status and the protein levels of PEPC were crucial in modulating the daily and seasonal patterns in C₄ leaves in situ. This is the first detailed study on the diurnal as well as seasonal patterns in PEPC activity, its regulatory properties, protein levels, phosphorylation status, and mRNA levels, in relation to light and temperature intensities in the field.

Key words: C₄ PEPC, diurnal rhythm, Glu-6-P, in situ, interactive effects, light, malate, phosphorylation, seasonal rhythm, temperature.

Introduction

Phosphoenolpyruvate carboxylase (PEPC, EC 4.1.1.31) which catalyses the primary step of phosphoenolpyruvate (PEP) carboxylation in C₄ and CAM plants, is regulated by internal metabolites, its common inhibitor being malate and its activator glucose-6-phosphate (Glu-6-P). Despite being a cytosolic enzyme, the C₄ PEPC is modulated markedly by light as well as temperature. The individual effects of either temperature or light on the activity and regulatory properties of C₄ PEPC have been studied extensively (Chinthapalli et al., 2003; Izui et al., 2004; Pierre et al., 2004). However, there are only a few reports on the interactive influence of light and temperature on C₄ PEPC (Selinioti et al., 1986; Grammatikopoulos and Manetas, 1990). The interaction between light and temperature while modulating both the activity and regulatory properties of PEPC in leaf discs and leaves of Amaranthus...
hypochondriacus has recently been reported (Avasthi and Raghavendra, 2008). These earlier studies were carried out in vitro.

In the present study the interactive effects of light and temperature on C₄ PEPC in leaves of A. hypochondriacus are studied in situ. During the in vitro experiments, it was noticed that the activities of PEPC in leaves collected within 2 h of sunlight were higher during the summer than during the winter. This could be due to the natural variation in the amount of sunshine and/or temperature which are quite marked during different times of the day as well as during the year. The influence of the interactions between light and temperature on the daily and seasonal rhythms of PEPC in situ, in plants grown under field conditions, was therefore investigated. Diurnal variations in the activity of PEPC were noticed >29 years ago (Kalt-Torres et al., 1987), yet there have been only limited attempts to examine in detail the diurnal variation in the catalytic and regulatory properties of C₄ PEPC.

In general, the day form of PEPC was more active than the night form (Selinioti et al., 1986; Grammatikopoulos and Manetas, 1990). A few studies carried out on the PEPC protein/phosphorylation/mRNA levels have yielded either unclear or intriguing results. Experiments using gel exclusion chromatography had suggested that the reversible association/disassociation of PEPC subunits is not the mechanism that regulates the diurnal transitions in the kinetic properties of PEPC (Weigend and Hincha, 1992). Ueno et al. (1998) have reported that the changes in phosphorylation of PEPC did not follow any diurnal rhythm, unlike later reports by Ueno et al. (2000) and Fukuyama et al. (2003). However, Ueno et al. (2000) have also observed that the levels of PEPC protein remained constant throughout the day. The day–night changes in PEPC mRNA were either undetectable (Taylor, 1989) or followed only a circadian rhythm (Thomas et al., 1990; Hartwell et al., 1999). Some of these changes in mRNA were expected to be due to the destabilization of mRNA (Kurotani et al., 1999). As such, another aim of this study was to understand the mechanisms behind such a diurnal/seasonal regulation of PEPC at the molecular level by determining the PEPC protein/phosphorylation/mRNA levels at different times of the day.

The major aim of the present article is to examine critically whether there are any significant diurnal and seasonal variations in the properties of PEPC in the leaves of A. hypochondriacus, in situ. Our university campus, being located in a typical, subtropical environment, offers a good choice, with significant fluctuations in the intensity of incident light as well as temperature during a day and during the year. The characteristics of PEPC were assessed in extracts prepared from leaves collected from the field, at a given time of the day and in different months of the year. The physiological/biochemical properties of PEPC were studied, followed by the molecular analysis of protein levels, phosphorylation status, and the mRNA levels.

Materials and methods

Plant material and growth conditions

Plants of A. hypochondriacus (cv. AG-67) were raised from seeds. The plants were grown in earthenware pots, filled with soil supplemented with farmyard manure (in a ratio of 5:1). They were grown outdoors in the field, in the campus of the University of Hyderabad (latitude 17.41°N, longitude 78.47°E, and altitude 536 m) under a natural photoperiod and temperature regime. The average sunshine (h d⁻¹) and mean maximum temperature (at 15.00 h) during a typical 12 month period are shown in Supplementary Fig. S1 (available at JXB online). These data are the averages of the observations made during the period 1986–2000 obtained from the Solar Radiation Hand Book (2008).

Harvest of leaf tissue

The fully developed leaves from the first three whorls of different, 3- to 4-week-old plants were harvested. The leaf samples were cut into two portions, immediately frozen, and stored in liquid nitrogen. One portion was used for the isolation of total RNA and the other for the preparation of total protein extract for PEPC enzyme assays and western blots. The field air temperature at the time of harvest was measured using a thermometer and the maximum light intensity at leaf level was measured by using a quantum meter (Apogee Instruments Inc.). These experiments spanned the period of March 2007 to February 2008.

Extraction and assay of PEPC

The extraction and assay of PEPC were as already described (Parvathi et al., 2000; Chinthapalli et al., 2003). Leaf material frozen in liquid nitrogen was thawed, weighed, and 125 mg of it was quickly extracted using a chilled mortar and pestle with 1 ml of extraction medium [containing 100 mM TRIS-HCl (pH 7.3), 10 mM MgCl₂, 2 mM K₂HPO₄, 1 mM EDTA, 10% (v/v) glycerol, 10 mM β-mercaptoethanol, 10 mM NaF, 2 mM phenylmethylsulphonyl fluoride (PMSF), 10 mg ml⁻¹ chymostatin, and 2% (w/v) insoluble polyvinylpyrrolidone]. The homogenate was centrifuged at 15 000 g for 5 min and the supernatant was used as ‘crude extract’. A small aliquot was kept aside, prior to centrifugation, for chlorophyll estimation.

Maximum PEPC activity was assayed by coupling to NAD-malate dehydrogenase (NAD-MDH) and monitoring NADH oxidation at 340 nm in a Shimadzu 1601 UV-Visible spectrophotometer at a temperature of 30°C. The assay mixture (1 ml) contained 50 mM TRIS-HCl (pH 7.3), 5 mM MgCl₂, 0.2 mM NADH, 2 U of NAD-MDH, 2.5 mM PEP, 10 mM NaHCO₃, and leaf extract equivalent to 1 µg of chlorophyll. The sensitivity of PEPC to malate was checked by adding malate to make a final concentration of 1 mM in the assay mixture. Similarly, activation by Glu-6-P was checked by adding Glu-6-P to make a final concentration of 2 mM in the assay medium. During the studies on the sensitivity of PEPC to malate or Glu-6-P, 0.5 mM PEP and 0.05 mM NaHCO₃ were used. Each assay was done in triplicate for each sample.

Estimation of chlorophyll and protein

Chlorophyll was estimated by extraction with 80% (v/v) acetone (Arnon, 1949). Total protein content was estimated by the method of Lowry et al. (1951), by using bovine serum albumin (BSA) as a standard.

Western blot analysis of PEPC protein and phosphorylation state

Leaf extracts equivalent to 5 µg of total protein were subjected to 10% SDS-PAGE as per the principles of Laemmli (1970). The proteins were then transferred electrophoretically from the gel onto polyvinylidene difluoride membranes by employing the method of Towbin et al. (1979). Further details are described in
an earlier paper (Chinthapalli et al., 2003). For assessment of the phosphorylation state of PEPC, the blots were probed with antibody specific to phosphorylated PEPC at a dilution of 1:500 (provided by KI). Acquisition and analysis of the images of these blots were done by using the software IMAGE for windows from the NIH, USA (version 1.9.1).

**Extraction of total RNA and reverse transcription**

Total RNA was isolated by using the single-step guanidine isothiocyanate method (Chomczynski and Sacchi, 1987) with the TRI® reagent (Sigma) according to the manufacturer’s instructions. The RNA concentration was estimated spectrophotometrically and the quality was determined by 1.0% denatured agarose-formaldehyde gel electrophoresis.

About 5 µg of total RNA was reverse transcribed to cDNA using a SuperScript® first-strand synthesis kit (Invitrogen). PCR was then performed by using Platinum® Taq DNA polymerase High Fidelity (Invitrogen). The PEPC and ribulose-1,5-bisphosphate carboxylase oxygenase small subunit (RuBiCO ssu) gene-specific primers were designed using the software, Primer3 input [Whitehead Institute for Biomedical Research (Rozen and Skaletsky, 2000); version 0.4.0].

The sequences of the primers used were as follows: PEPC, forward 5’-TATGGCTAGTGGGAAAGGTG-3’, reverse 5’-TTG CTCAAACGGGTGTCTCTGC-3’; RuBiCO ssu, forward 5’-TGT AAGCAATGAAACTGAG-3’, reverse 5’-AATGGCTTCCTC- TATGCTTT-3’. The PCR products were separated on 1.0% agarose gels. After confirmation of amplification and determination of the amplicon size, the products were purified by using a QIAquick® gel extraction kit (Qiagen). All procedures used were according to kit protocols.

**Northern blot analysis of PEPC mRNA**

Northern blots were performed according to the method of Luo et al. (2005) with a few modifications. A 20 µg aliquot of RNA was separated through a 1.0% agarose-formaldehyde gel and transferred to a Hybond N+ nylon membrane (Amersham) by using the capillary method. After overnight transfer, the membrane was washed with 2× SSC and UV cross-linked. The DNA probe was radiolabelled by using the kit, HexaLabel® DNA (Fermentas). Pre-hybridization of the membrane was carried out at 42 °C for 45 min using ULTRAhyb® hybridization buffer (Ambion). Hybridization was carried out overnight for 15 h at 42 °C. The membrane was then washed twice with 2× SSC and 0.1% SDS for 10 min each followed by two washes with 0.1× SSC and 0.1% SDS for 15 min each at 42 °C and the radioactivity was reduced to 10–15 counts per second (cps). The membrane was exposed by using an X-ray film at –80 °C for 5 d. Ethidium bromide staining was done to observe the equal loading of total RNA in each lane.

**Replications and statistical analysis**

All assays were performed three times for each sample. The average values ±SE are presented. Statistical analysis of the data was done using the software SigmaPlot (version 10.0). Correlation coefficients were calculated and curve fitting of data sets was done by using Microsoft Office Excel, 2007.

**Results**

**Patterns of PEPC activity and regulatory properties during the day and during the year**

The monthlyseasonal patterns of PEPC were examined throughout a year, and the data obtained during a typical day in May 2007 (month of maximum sunshine hours and highest air temperature) was compared with a day in December 2007 (month of minimum sunshine hours and lowest air temperature). The times 06.00 h and 15.00 h were chosen to represent the times of the minimum and maximum light intensity and temperature. When examined at intervals of every 3 h from 06.00 h to 24.00 h during a day, a clear diurnal trend in the activity of PEPC was observed. On a typical day in May 2007, the activity of PEPC in the morning at 06.00 h was quite low, reached a maximum at 15.00 h, and then decreased by 24.00 h (Fig. 1A). A similar trend was also noticed during a typical day in December 2007. In both months, the extent of inhibition of PEPC by malate was quite high at 06.00 h, fell to a minimum at 15.00 h, and then rose again up until 24.00 h (Fig. 1B). In contrast, the activation of PEPC by Glu-6-P was lowest at 06.00 h, rose to a maximum at 15.00 h, and then decreased by 24.00 h (Fig. 1C). The field temperatures and light intensities at leaf level at the time of leaf sampling were noted. As the day progressed, the light intensity reached its maximum by 12.00 h and the temperature by 15.00 h, and then decreased by 24.00 h. This trend was consistent for the entire year. However, the temperature and light intensities were significantly higher during the summer (e.g. May) and least during the winter (e.g. December) (Fig. 1D, E). When examined at 06.00 h and 15.00 h during different months of the year, the activity of PEPC, decrease in inhibition by malate, and increase in activation by Glu-6-P were maximal in summer (e.g. May) and minimal in winter (e.g. December) (Fig. 2). The ranges of these variations are summarized in Table 1. The average values for daily sunshine and the maximum temperature (at 15.00 h) at Hyderabad for every month over the years 1986–2000 are shown in Supplemental Fig. S1 (available at JXB online) for comparison.

A further critical analysis of the data revealed that the peak in activity/decrease in malate sensitivity/increase in activation by Glu-6-P is always preceded by maximum light but coincided with high temperature, a trend which was consistent throughout the year (Fig. 1). The use of scatter plots was quite informative, indicating an apparently strong relationship between the modulation of PEPC activity and regulation by malate/Glu-6-P with light or temperature (Fig. 3). Curve fitting of the data sets suggested that the modulation of PEPC activity by light and modulation of activation by Glu-6-P by light or temperature fitted best into linear curves. On the other hand, the modulation of PEPC activity by temperature and modulation of malate sensitivity by light or temperature fitted best into exponential curves. The calculation of correlation coefficients (Table 2) conveyed that light had a greater influence on PEPC activity while temperature exerted a much greater effect on the regulatory properties of C4 PEPC.

**Western blot analysis of PEPC protein and phosphorylation levels**

When the changes in the PEPC protein and phosphorylation levels were followed during the day in a summer (May) or winter (December) month, the protein levels increased
from 06.00 h up until 12.00 h and then decreased to reach a minimum at 24.00 h only in May. The protein levels remained almost constant throughout the day in December. The PEPC protein levels were also higher during May than in December (Fig. 4). The phosphorylation levels increased as the day progressed, reached a maximum at ~15.00 h, and then decreased to reach a minimum at 24.00 h. These levels were much higher in May than in December. In May, PEPC remained phosphorylated even in the night, but during December PEPC was almost completely dephosphorylated in the night. The diurnal variation in phosphorylation was more prominent in December (Fig. 4).

Northern blot analysis of PEPC mRNA transcript levels

The PEPC mRNA levels reached a maximum by 12.00 h, remained stable up to 15.00 h, and then decreased by 24.00 h in May and December (Fig. 5). The PEPC mRNA levels were however, higher during May (pixel intensities in the range of 8–56) than during December (pixel intensities in the range of 2–31). For comparison, the mRNA levels of the small subunit of another photosynthetic enzyme, RuBisCO, remained almost constant throughout the day (pixel intensities in the range of 28–35).

Scatter plots suggested a stronger modulation of PEPC protein levels, phosphorylation status, and mRNA levels by light or temperature during May (Fig. 6) than during December (data not shown). All these scatter plots fitted best into linear curves. The calculation of correlation coefficients (Table 3) revealed that during May, light had a greater influence on PEPC protein levels while temperature exerted a much greater effect on both the PEPC protein and the phosphorylation levels. On the other hand, light has the greatest influence on the PEPC mRNA levels, and temperature has the greatest influence on the phosphorylation status during December.
Discussion

In an earlier study, a marked interaction between light and temperature while modulating the activity and regulatory properties of PEPC from leaf discs of *A. hypochondriacus* in *vitro* had been reported (Avasthi and Raghavendra, 2008). During the present extended studies on PEPC during different months of the year, it was noticed that the activity of PEPC in leaves during summer was somewhat higher, with much less inhibition by malate, than that in winter. These marked variations in PEPC appear to be related to light and temperature intensities in the field. A few previous studies indicated diurnal changes in only the PEPC phosphorylation status and mRNA levels (Hartwell et al., 1999; Fukuyama et al., 2003), but not in PEPC protein levels (Ueno et al., 2000). The present article describes efforts to use a combined approach, to study diurnal as well as seasonal variations in PEPC protein levels together with the phosphorylation status as well as mRNA levels in leaves, with reference to the variation in ambient light and temperature *in situ*.

In *A. paniculatus* L., another C₄ plant, a peak in PEPC activity was observed at 15.00 h and the photoactivation of PEPC was also observed to be higher when assayed at lower PEP concentrations (Grammatikopoulos and Manetas, 1990). In leaves of *Saccharum* sp., PEPC activity reached a maximum at 14.00 h (Du et al., 2000). In maize, PEPC activity showed a diurnal rhythm when assayed at sub-optimal pH and PEP concentrations (Kalt-Torres et al., 1987). In this study too, PEPC activity increased from 06.00 h to reach a peak at 15.00 h and then decreased. Further, there was a concomitant decrease in malate sensitivity and increase in activation by Glu-6-P which also reached a peak at 15.00 h (Fig. 1). The activity of PEPC during the light period was higher when assayed at 0.5 mM PEP and 0.05 mM NaHCO₃ (subsaturating substrate concentrations) than at 2.5 mM PEP and 10 mM NaHCO₃ (saturating substrate concentrations). Illumination increases the affinity of PEPC for PEP or HCO₃⁻ (Parvathi et al., 2000; Izui et al., 2004) which may enable the enzyme to function at higher rates even at low substrate concentrations during the day.

The present observations emphasized the strong correlation between the modulation of the activity and regulatory properties of PEPC with ambient light and temperature (Fig. 1). Selinioti et al. (1986) and Grammatikopoulos and Manetas (1990) suggested that PEPC activity could be higher during the daytime due to an interaction between light and temperature in the field. The rate and levels of photoactivation of PEPC were not only influenced by light intensity (Karabourniotis et al., 1983) but also by

![Fig. 2](image-url)

**Table 1.** The range of changes in PEPC activity, inhibition by malate, activation by Glu-6-P, light intensity, and temperature during a typical day in May and December 2007 in leaves of *A. hypochondriacus*.

| Property/parameter | Ranges during the day | May 2007 | December 2007 |
|--------------------|-----------------------|----------|---------------|
| PEPC activity (without effector) (µmol mg⁻¹ Chl h⁻¹) | 214–1653<sup>a</sup> | 85–495 |
| Inhibition by malate (%) | 74–28<sup>a</sup> | 94–83 |
| Activation by Glu-6-P (%) | 150–300<sup>b</sup> | 112–178 |
| Light intensity (µmol m⁻² s⁻¹) | 0–1825 | 0–955 |
| Temperature (°C) | 25–48 | 18.5–27 |

<sup>a</sup> 2.5 mM PEP and 10 mM NaHCO₃.
<sup>b</sup> 0.5 mM PEP and 0.05 mM NaHCO₃.
temperature (Samaras et al., 1988). During the study, light preceded and temperature coincided with the peak in PEPC activity/decrease in malate sensitivity/increase in activation by Glu-6-P, suggesting that phosphorylated PEPC is more responsive to modulation by warm temperature, and vice versa in situ. In a previous study, the modulation of C_4 PEPC in vitro by the synergistic effects of light and temperature in leaf discs of _A. hypochondriacus_ has been reported (Avasthi and Raghavendra, 2008).

Light modulated C_4 PEPC through phosphorylation (Pierre et al., 2004; Bailey et al., 2007; Tazoe et al., 2008), whereas temperature was proposed to cause conformational changes in the protein structure (Chinthapalli et al., 2003).
Regulation by light and temperature of C₄ PEPC

In the case of maize PEPC (pure recombinant enzyme) the \( V_{\text{max}} \) was not increased by the phosphorylation, but it had a dramatic effect on the kinetic and allosteric properties of the enzyme (Takahashi-Terada et al., 2005). The correlation coefficients presented in Table 2 suggested that light plays a dominant role in modulating the PEPC activity whereas temperature is quite effective in modulating the regulatory properties of C₄ PEPC. Curve fitting of the data (Fig. 3) suggested that (i) light shows a linear correlation with PEPC activity and activation by Glu-6-P but an exponential relationship with malate sensitivity; and (ii) temperature shared an exponential correlation with activity and malate sensitivity but a linear relationship with Glu-6-P activation. A complex interaction between light and temperature may be operating in vivo at the molecular level during the remarkable modulation of C₄ PEPC by the natural variations in both light and temperature under in situ conditions.

The reports on the occurrence of a diurnal rhythm in the PEPC mRNA/protein/phosphorylation levels in C₄ plants have not been clear. In Zea mays L., no diurnal changes in the transcription of the PEPC gene have been reported (Taylor, 1989). In Sorghum vulgare Pers., PEPC RNA was found to follow a rhythmic behaviour, being higher during the daytime, unlike PEPC protein (Thomas et al., 1990). Experiments using gel exclusion chromatography suggested that association/disassociation of PEPC subunits was not the mechanism that regulates the diurnal transitions in the kinetic properties of PEPC (Weigend and Hincha, 1992). Diurnal changes in the mRNA levels for the Ppc1 gene (C₄-form PEPC) have been observed, the levels being higher during the day (08.00–20.00 h) than during the night (Kurotani et al., 1999). Studies using western blot and/or dot blot analysis in Z. mays L. have shown the absence of a circadian rhythm in the phosphorylation of C₄ PEPC (Ueno et al., 1998). However, it has also been reported that the phosphorylation of PEPC started before dawn, reached a maximum by mid-day, decreased before sunset, and the dephosphorylation was almost complete by midnight (Ueno et al., 2000), and that although the levels of PEPC protein remained constant throughout the day, phosphorylation is higher during the daytime than during the night (Fukuyama et al., 2003). In the present study, during the months of May and December selected for further molecular studies, PEPC mRNA, protein, and phosphorylation were found to follow a similar pattern to the PEPC activity (Figs 4, 5). The mRNA levels reached their maximum by 12.00 h, remained stable up to 15.00 h, and then decreased. This observation corroborates those made earlier (Kurotani et al., 1999). The mRNA levels, however, may not be limiting PEPC. The protein levels usually peaked by noon and phosphorylation by 15.00 h. These observations, however, are in contrast to those made by Ueno et al. (2000).

The correlation coefficients presented in Table 3 confirmed that light or temperature had a much greater influence on the modulation of the PEPC protein levels, phosphorylation status, and mRNA levels during May (summer) than during December (winter). The different fits of the curves of the relationship between temperature and PEPC activity suggest that the mechanisms of modulation of PEPC by light and/or temperature are different. This aspect needs further detailed investigation.

The maximum PEPC activity, minimum malate sensitivity, and highest levels of activation by Glu-6-P at 15.00 h can be attributed to maximum phosphorylation levels at this point, which in turn could be attributed to higher photoactivation of PEPC at warm temperatures. The PEPC mRNA and phosphorylation levels were higher during May than December. In summer, for example May, PEPC was found to be phosphorylated even during the night; thus the higher PEPC activities during May can be attributed to higher phosphorylation levels. In winter, for example December, the diurnal variation in phosphorylation was prominent, suggesting that the diurnal variation in the properties of PEPC was mostly due to phosphorylation of protein. In both cases, PEPC was found to be phosphorylated by as early as 06.00 h. Hartwell et al. (1996) found that the mRNA levels of PEPC-protein kinase start increasing well before sunrise in leaves of Z. mays, which explains the present results obtained with A. hypochondriacus.
Thus it can be concluded that the phosphorylation status and not the PEPC mRNA/protein levels are more crucial for the daily and seasonal patterns in the activity and regulatory properties of PEPC. The results obtained in a previous study also suggest a major role for conformational changes affecting phosphorylation while modulating the effect of temperature on the light activation of C₄ PEPC in vitro (Avasthi and Raghavendra, 2008). The phosphorylation status of PEPC is modulated by the daily and seasonal changes in the light and temperature intensities to which the plants are unavoidably exposed. However, the diurnal cycling of PEPC gene transcription in *Z. mays* is believed to be under circadian control (Horst *et al.*, 2009).

Recent reports suggested that PEPC achieves maximum phosphorylation under high light intensities and that the

![Fig. 6](image-url) Scatter plots of the PEPC protein levels (A), phosphorylation status (B) and mRNA levels (C) versus light intensity, at different times, during a typical day in May 2007. Scatter plots of the PEPC protein levels (D), phosphorylation status (E) and mRNA levels (F) versus temperature during a typical day in May 2007. The $R^2$ of the curve fit to each data set is indicated. Further details are described in Results.

| Parameter | Protein levels | PhosphoPEPC | mRNA levels |
|-----------|---------------|--------------|-------------|
| May 2007 (summer) | 0.97 | 0.97 | 0.50 |
| Light (µmol m⁻² s⁻¹) | 0.79 | 0.76 | 0.44 |
| Temperature (°C) | 0.27 | 0.52 | 0.75 |
| December 2007 (winter) | 0.20 | 0.58 | 0.45 |

Thus it can be concluded that the phosphorylation status and not the PEPC mRNA/protein levels are more crucial for the daily and seasonal patterns in the activity and regulatory properties of PEPC. The results obtained in a previous study also suggest a major role for conformational changes affecting phosphorylation while modulating the effect of temperature on the light activation of C₄ PEPC in vitro (Avasthi and Raghavendra, 2008). The phosphorylation status of PEPC is modulated by the daily and seasonal changes in the light and temperature intensities to which the plants are unavoidably exposed. However, the diurnal cycling of PEPC gene transcription in *Z. mays* is believed to be under circadian control (Horst *et al.*, 2009).

Recent reports suggested that PEPC achieves maximum phosphorylation under high light intensities and that the
phosphorylation levels increased with increasing photon flux density (Bailey et al., 2007; Tazoe et al., 2008), corroborating the present inferences. However, Furumoto et al. (2007) have suggested that PEPC phosphorylation is essential only for relieving malate inhibition but not for efficient C₄ photosynthesis. In a related study, the photosynthetic rates under high summer temperatures were observed to be higher in the C₄ subspecies of Alloteropsis semialata than in the C₃ subspecies, a trend which was reversed at low winter temperatures (Ibrahim et al., 2008). The present results suggest that a combined effect of enhanced protein levels and high phosphorylation status (Fig. 4) help to achieve the maximum rate of PEPC activity. Obviously, the warm temperature and high light during the summer months are the most favourable for sustaining the very high rates of PEPC activity and subsequently may also affect the rates of C₄ photosynthesis.

The activities of at least three more key enzymes—RuBisCO, pyruvate P dikinase (PPDK), and NADP-malate dehydrogenase (NADP-MDH)—are subject to marked regulation in C₄ plants and are relevant to their C₄ photosynthesis in the field. Among these, the activities of PEPC, PPDK, and RuBisCO appeared to be highly correlated with rates of C₄ photosynthesis and appeared more rate limiting than those of NADP-MDH (Furbank et al., 1997). The recovery and maintenance of photosynthesis rates at cold temperatures in Miscanthus×giganteus appeared to be due to an increase in the levels of PPDK (Wang et al., 2008). The activities of RuBisCO activase and subsequently RuBisCO activation were rate limiting at cold temperatures (Crafts-Brandner and Salvucci, 2002; Sage and Kubien, 2007). As per the present observations, the multimode modulation of PEPC (through the modulation of protein, phosphorylation status, and mRNA levels) by the diurnal/seasonal variation in light as well as well temperature highlights the importance of PEPC and its regulation.

**Supplementary data**

Supplementary data are available at JXB online.

**Figure S1.** The patterns of average daily sunshine and the maximum daily temperature during different months of the year at Hyderabad, adapted from the Solar Radiation Hand Book (2008) data collected by the Indian Metrological Department. Average values for every month from the 1986 to 2000 are shown.

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