Journal of
Experimental
Botany
www.jxb.oxfordjournals.org

RESEARCH PAPER

Interplay of light and temperature during the *in planta* modulation of C$_4$ phosphoenolpyruvate carboxylase from the leaves of *Amaranthus hypochondriacus* L.: diurnal and seasonal effects manifested at molecular levels

Uday K. Avasthi[1], Katsura Izui[2] and Agepati S. Raghavendra[1],*

[1] Department of Plant Sciences, School of Life Sciences, University of Hyderabad, Hyderabad 500046, India
[2] Kinki University, Institute of Advanced Technology, 14-1 Minamiakasaka, Kainan, Wakayama, 642-0017, Japan

* To whom correspondence should be addressed. E-mail: as_raghavendra@yahoo.com

Abstract

The interactive effects of light and temperature on C$_4$ 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 C$_4$ 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: C$_4$ 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 C$_4$ 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 C$_4$ 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 C$_4$ 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 C$_4$ 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 C_4 PEPC in leaves of *A. hypochondriacus* are studied *in vivo/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 C_4 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 $MgCl_2$, 2 mM K_2HPO_4, 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 $MgCl_2$, 0.2 mM NADH, 2 U of NAD-MDH, 2.5 mM PEP, 10 mM $NaHCO_3$, 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 $NaHCO_3$ 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 diflouride 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 (RuBisCO 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 CTTAACCGGGTGTTCTGC-3′; RuBisCO 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 monthly/seasonal 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 Supplementay 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 C$_4$ 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
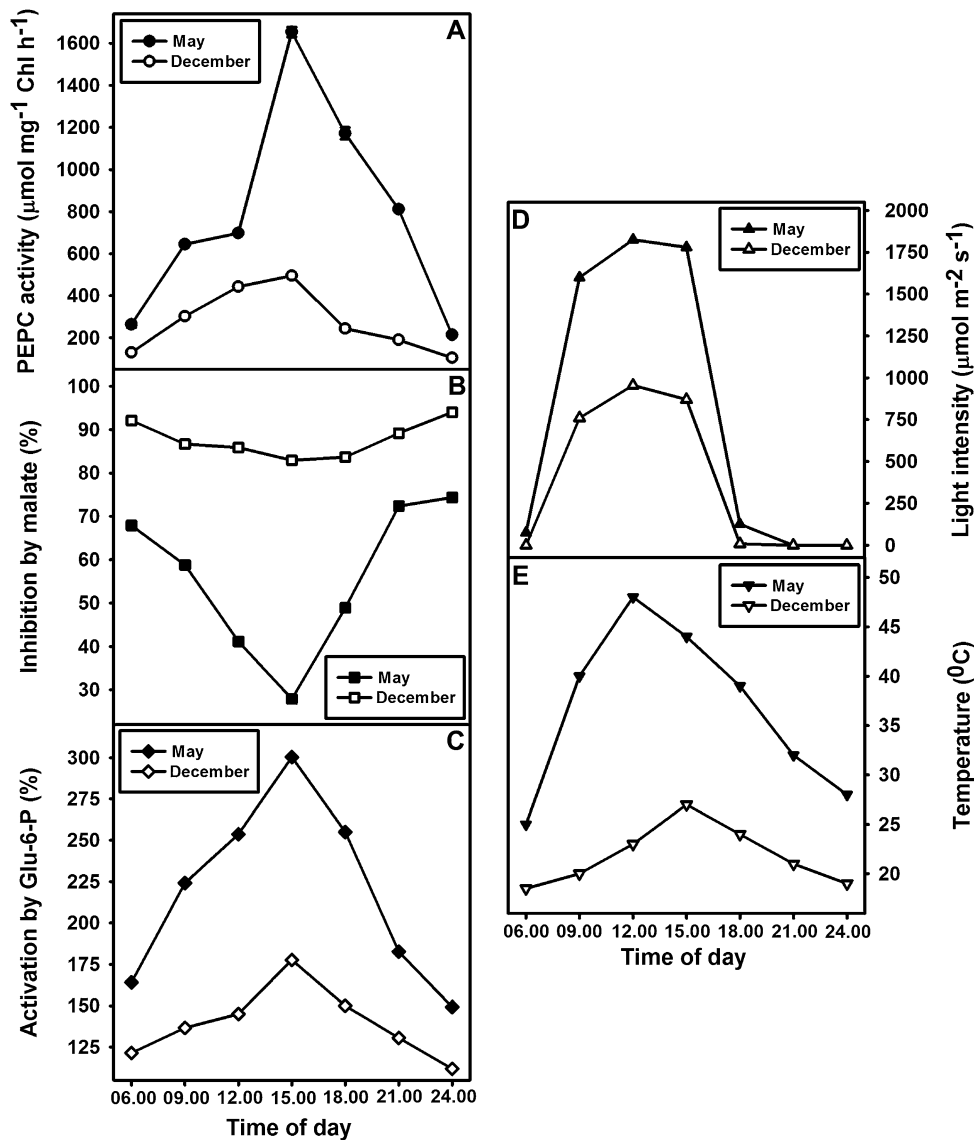
Fig. 1. Patterns of change in PEPC activity (A), malate sensitivity (B), activation by Glu-6-P (C), light intensity (D), and temperature (E) during a typical day in May and December 2007. Standard errors, if not visible, fall within the symbols.
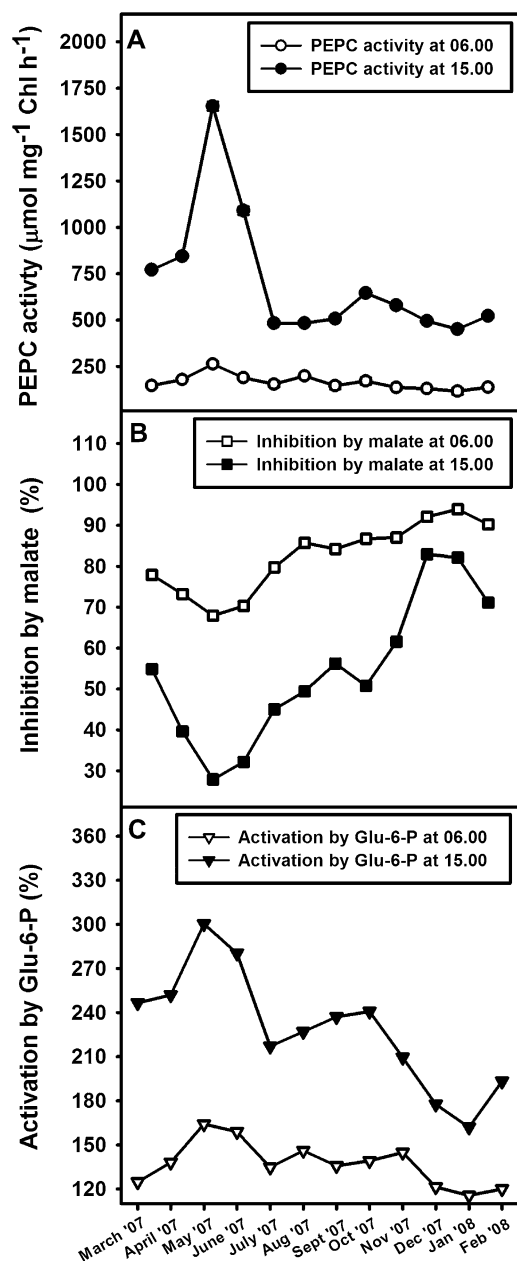
from 06.00 h up until 12.00 h and then decreased to reach a minimum at 24.00 h only in May. The protein levels remained almost constant throughout the day in December. The PEPC protein levels were also higher during May than in December (Fig. 4). The phosphorylation levels increased as the day progressed, 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,

Fig. 2. PEPC activity at 06.00 h and 15.00 h during a typical day of each month of the year (A). Sensitivity of PEPC to malate at 6.00 h and 15.00 h during a typical day of each month of the year (B). Activation of PEPC by Glu-6-P at 06.00 h and 15.00 h during a typical day of each month of the year (C). Standard errors, if not visible, fall within the symbols.

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*

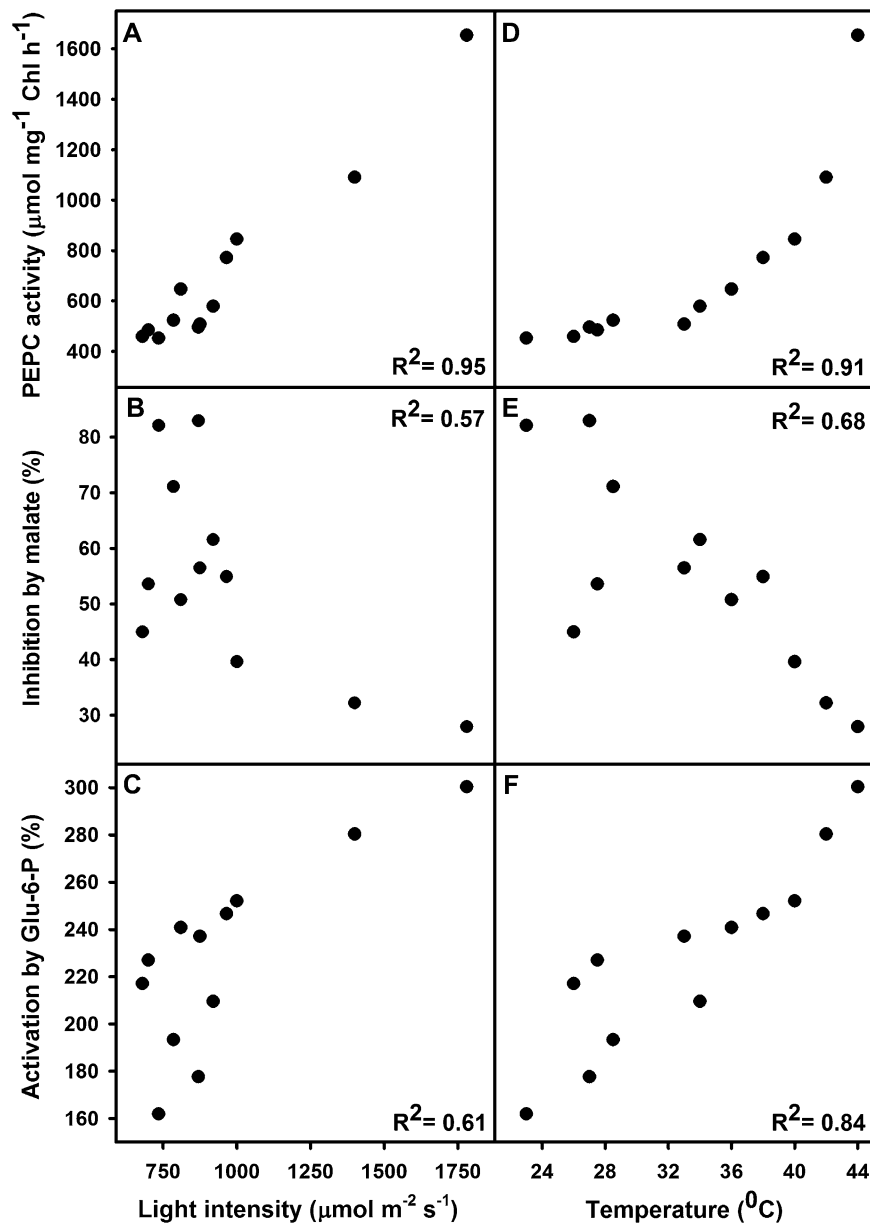| Property/parameter	Ranges during the day	
	May 2007	**December 2007**
PEPC activity (without effector) (μmol mg^{-1} Chl h^{-1})	214–1653[a]	85–495
Inhibition by malate (%)	74–28[a]	94–83
Activation by Glu-6-P (%)	150–300[b]	112–178
Light intensity (μmol m^{-2} s^{-1})	0–1825	0–955
Temperature (°C)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

[a] 2.5 mM PEP and 10 mM NaHCO$_3$.
[b] 0.5 mM PEP and 0.05 mM NaHCO$_3$.

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 C$_4$ 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 NaHCO$_3$ (subsaturating substrate concentrations) than at 2.5 mM PEP and 10 mM NaHCO$_3$ (saturating substrate concentrations). Illumination increases the affinity of PEPC for PEP or HCO$_3^-$ (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

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

Fig. 3. Scatter plots of PEPC activity (A), malate sensitivity (B) and activation by Glu-6-P (C) at 15.00 h versus the maximum light intensity during a typical day of the different months of the year. Scatter plots of PEPC activity (D), malate sensitivity (E) and activation by Glu-6-P (F) at 15.00 h versus the maximum temperature during a typical day of the different months of the year. The R^2 of the curve fit to each data set is indicated. Further details are described in Results.

Table 2. Correlation coefficients (using linear or exponential fits) of variations in light or temperature with the modulation of the activity and regulatory properties of C_4 PEPC in leaves of *A. hypochondriacus*

PEPC activity and inhibition by malate were determined at 2.5 mM PEP and activation by Glu-6-P at 0.5 mM PEP.

Parameter	PEPC activity (μmol mg^{-1} Chl h^{-1})	Inhibition by 1 mM malate (%)	Activation by 2 mM Glu-6-P (%)
Light (μmol m^{-2} s^{-1})	0.95	–0.57	0.61
Temperature (°C)	0.91	–0.68	0.84

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).
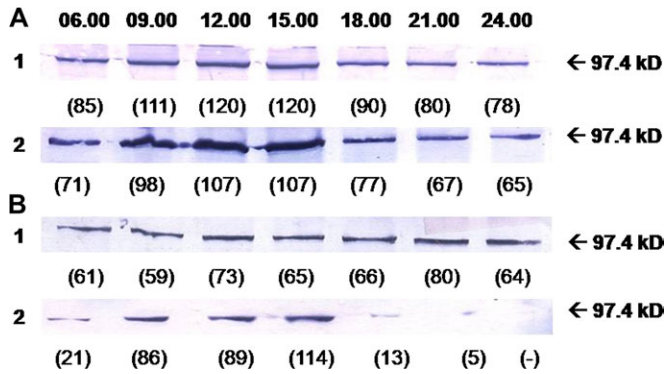
Fig. 4. Patterns of PEPC protein (1) and phosphorylation (2) levels during a typical day in May 2007 (A) and December 2007 (B). The values given in parentheses are the pixel intensities measured by using IMAGE software. The molecular weight of the bands detected is annotated in the figure.
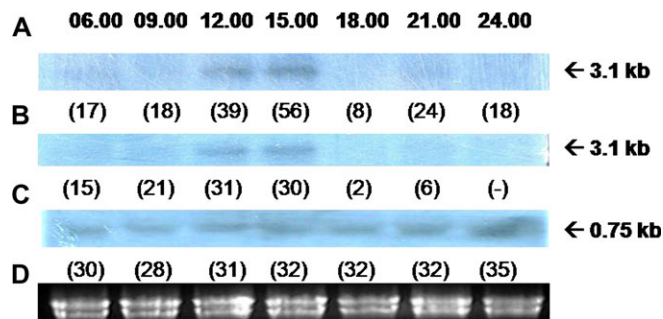
Fig. 5. Patterns of PEPC mRNA levels during a typical day in May 2007 (A) and December 2007 (B). Levels of RuBisCO ssu (C) and an equal loading gel (D) as controls are shown. The values given in parentheses are the pixel intensities measured by using IMAGE software. The molecular weight of the bands detected is annotated in the figure.

In the case of maize PEPC (pure recombinant enzyme) the V_{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 C$_4$ 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 C$_4$ 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 C$_4$ 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 for the *Ppc*1 gene (C$_4$-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 C$_4$ 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 protein 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*.
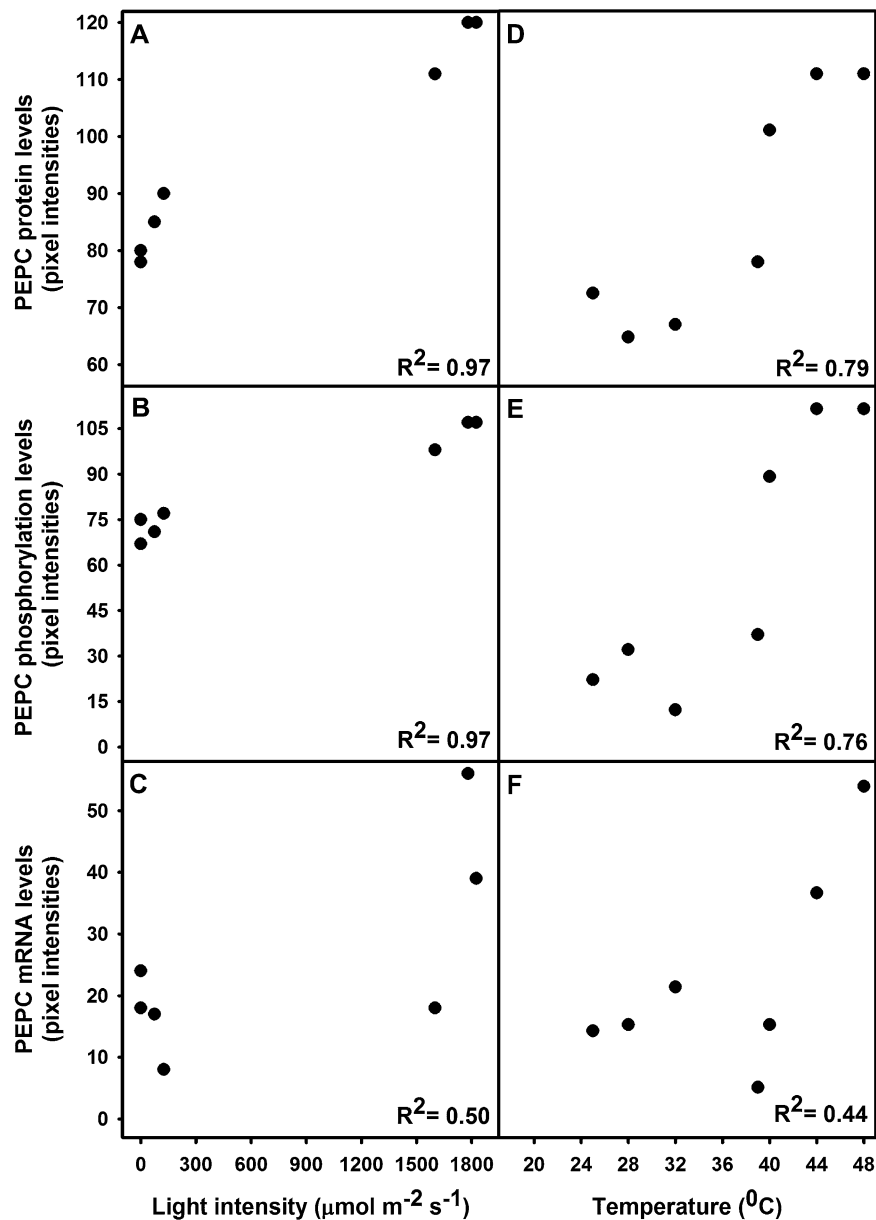
Fig. 6. Scatter plots of the PEPC protein levels (A), phosphorylation status (B) and mRNA levels (C) versus light intensity, at different times, during a typical day in May 2007. Scatter plots of the PEPC protein levels (D), phosphorylation status (E) and mRNA levels (F) versus temperature during a typical day in May 2007. The R^2 of the curve fit to each data set is indicated. Further details are described in Results.

Table 3. Correlation coefficients (using linear fits) of variation in light or temperature with the protein levels, phosphorylation status, and mRNA levels of PEPC in leaves of *A. hypochondriacus* during a typical day in May and December 2007

Parameter	Protein levels	PhosphoPEPC	mRNA levels
May 2007 (summer)			
Light (µmol m^{-2} s^{-1})	0.97	0.97	0.50
Temperature (°C)	0.79	0.76	0.44
December 2007 (winter)			
Light (µmol m^{-2} s^{-1})	0.27	0.52	0.75
Temperature (°C)	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 C$_4$ 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 C$_4$ photosynthesis. In a related study, the photosynthetic rates under high summer temperatures were observed to be higher in the C$_4$ subspecies of *Alloteropsis semialata* than in the C$_3$ 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 C$_4$ photosynthesis.

The activities of at least three more key enzymes—RuBisCO, pyruvate P$_i$ dikinase (PPDK), and NADP-malate dehydrogenase (NADP-MDH)—are subject to marked regulation in C$_4$ plants and are relevant to their C$_4$ photosynthesis in the field. Among these, the activities of PEPC, PPDK, and RuBisCO appeared to be highly correlated with rates of C$_4$ 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 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.

Acknowledgements

References

Arnon DI. 1949. Copper enzymes in isolated chloroplasts. Polyphenol oxidase in *Beta vulgaris*. *Plant Physiology* **24,** 1–15.

Avasthi UK, Raghavendra AS. 2008. Mutual stimulation of temperature and light effects on C$_4$ phosphoenolpyruvate carboxylase in leaf discs and leaves of *Amaranthus hypochondriacus*. *Journal of Plant Physiology* **165,** 1023–1032.

Bailey KJ, Gray JE, Walker RP, Leegood RC. 2007. Coordinate regulation of phosphoenolpyruvate carboxylase and phosphoenolpyruvate carboxykinase by light and CO$_2$ during C$_4$ photosynthesis. *Plant Physiology* **144,** 479–486.

Chinthapalli B, Murmu J, Raghavendra AS. 2003. Dramatic difference in the responses of phosphoenolpyruvate carboxylase to temperature in leaves of C$_3$ and C$_4$ plants. *Journal of Experimental Botany* **54,** 707–714.

Chomczynski P, Sacchi N. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. *Analytical Biochemistry* **162,** 156–159.

Crafts-Brandner SJ, Salvucci ME. 2002. Sensitivity of photosynthesis in a C$_4$ plant, maize, to heat stress. *Plant Physiology* **129,** 1773–1780.

Du YC, Nose A, Kondo A, Wasano K. 2000. Diurnal changes in photosynthesis in sugarcane leaves. I. Carbon dioxide exchange rate, photosynthetic enzyme activities and metabolite levels relating to the C$_4$ pathway and the Calvin cycle. *Plant Production Science* **3,** 3–8.

Fukuyama H, Hatch MD, Tamai T, Tsuchida H, Sudoh S, Furbank RT, Miyao M. 2003. Activity regulation and physiological impacts of maize C$_4$-specific phosphoenolpyruvate carboxylase overproduced in transgenic plants. *Photosynthesis Research* **77,** 227–239.

Furbank RT, Chitty JA, Jenkins CLD, Taylor WC, Trevanion SJ, von Caemmerer S, Ashton AR. 1997. Genetic manipulation of key photosynthetic enzymes in the C$_4$ plant *Flaveria bidentis*. *Australian Journal of Plant Physiology* **24,** 477–485.

Furumoto T, Izui K, Quinn V, Furbank RT, von Caemmerer S. 2007. Phosphorylation of phospho *enol*pyruvate carboxylase is not essential for high photosynthetic rates in the C$_4$ species *Flaveria bidentis*. *Plant Physiology* **144,** 1936–1945.

Grammatikopoulos G, Manetas Y. 1990. Diurnal changes in phosphoenolpyruvate carboxylase and pyruvate, orthophosphate dikinase properties in the natural environment: interplay of light and temperature in a C$_4$ thermophile. *Physiologia Plantarum* **80,** 593–597.

Hartwell J, Gill A, Nimmo GA, Wilkins MB, Jenkins GI, Nimmo HG. 1999. Phosphoenolpyruvate carboxylase kinase is a novel protein kinase regulated at the level of expression. *The Plant Journal* **20,** 333–342.

Hartwell J, Smith LH, Wilkins MB, Jenkins GI, Nimmo HG. 1996. Higher plant phosphoenolpyruvate carboxylase kinase is regulated at the level of translatable mRNA in response to light or a circadian rhythm. *The Plant Journal* **10,** 1071–1078.

Horst I, Offermann S, Dreesen B, Niessen M, Peterhansel C. 2009. Core promoter acetylation is not required for high transcription from the phosphoenolpyruvate carboxylase promoter in maize. *Epigenetics and Chromatin* **2,** 17.

Ibrahim DG, Gilbert ME, Ripley BS, Osborne CP. 2008. Seasonal differences in photosynthesis between the C_3 and C_4 subspecies of *Alloteropsis semialata* are offset by frost and drought. *Plant, Cell and Environment* **31,** 1036–1050.

Izui K, Matsumura H, Furumoto T, Kai Y. 2004. Phospho *enol*pyruvate carboxylase: a new era of structural biology. *Annual Review of Plant Biology* **55,** 69–84.

Kalt-Torres W, Kerr PS, Usuda H, Huber SC. 1987. Diurnal changes in maize leaf photosynthesis I. Carbon exchange rate, assimilate export rate, and enzyme activities. *Plant Physiology* **83,** 283–288.

Karabourniotis G, Manetas Y, Gavalas NA. 1983. Photoregulation of phosphoenolpyruvate carboxylase in *Salsola soda* L. and other C_4 plants. *Plant Physiology* **73,** 735–739.

Kurotani K, Hata S, Izui K. 1999. Light-regulated expression of the gene for C_4-form phospho *enol*pyruvate carboxylase in maize: destabilization of mRNA by okadaic acid. *Plant and Cell Physiology* **40,** 423–430.

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature* **227,** 680–685.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. 1951. Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry* **193,** 265–275.

Luo H, Song F, Zheng Z. 2005. Overexpression in transgenic tobacco reveals different roles for the rice homeodomain gene OsBIHD1 in biotic and abiotic stress responses. *Journal of Experimental Botany* **56,** 2673–2682.

Parvathi K, Bhagwat AS, Ueno Y, Izui K, Raghavendra AS. 2000. Illumination increases the affinity of phosphoenolpyruvate carboxylase to bicarbonate in leaves of a C_4 plant, *Amaranthus hypochondriacus*. *Plant and Cell Physiology* **41,** 905–910.

Pierre JN, Preito JL, Gadal P, Vidal J. 2004. *In situ* C_4 phospho *enol*pyruvate carboxylase activity and kinetic properties in isolated *Digitaria sanguinalis* mesophyll cells. *Photosynthesis Research* **79,** 349–355.

Rozen S, Skaletsky HJ. 2000. Primer3 on the WWW for general users and for biologist programmers. In: Krawetz S, Misener S, eds. *Bioinformatics methods and protocols, methods in molecular biology*. Totowa, NJ: Humana Press, 365–386.

Sage RF, Kubien DS. 2007. The temperature responses of C_3 and C_4 photosynthesis. *Plant, Cell and Environment* **30,** 1086–1106.

Samaras Y, Manetas Y, Gavalas NA. 1988. Effects of temperature and photosynthetic inhibitors on light activation of C_4 phosphoenolpyruvate carboxylase. *Photosynthesis Research* **16,** 223–242.

Selinioti E, Manetas Y, Gavalas NA. 1986. Cooperative effects of light and temperature on the activity of phosphoenolpyruvate

carboxylase from *Amaranthus paniculatus* L. *Plant Physiology* **82,** 518–522.

Solar Radiation Hand Book. 2008. Typical climatic data for selected radiation stations (The data covered: 1986–2000). A joint project of Solar Energy Centre, MNRE, Indian Meterological Department. http://mnes.nic.in/sec/srd-sec.pdf.

Takahashi-Terada A, Kotera M, Ohshima K, Furumoto T, Matsumura H, Kai Y, Izui K. 2005. Maize phosphoenolpyruvate carboxylase. Mutations at the putative binding site for glucose-6-phosphate caused desensitization and abolished responsiveness to regulatory phosphorylation. *Journal of Biological Chemistry* **280,** 11798–11806.

Taylor WC. 1989. Transcriptional regulation by a circadian rhythm. *The Plant Cell* **1,** 259–264.

Tazoe Y, Hanba YT, Furumoto T, Noguchi K, Terashima I. 2008. Relationships between quantum yield for CO_2 assimilation, activity of key enzymes and CO_2 leakiness in *Amaranthus cruentus*, a C_4 dicot, grown in high or low light. *Plant and Cell Physiology* **49,** 19–29.

Thomas M, Cretin C, Vidal J, Keryer E, Gadal P, Monsinger E. 1990. Light-regulation of phosphoenolpyruvate carboxylase mRNA in leaves of C_4 plants: evidence for phytochrome control on transcription during greening and for rhythmicity. *Plant Science* **69,** 65–78.

Towbin H, Staehlin T, Gordon J. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets. Procedure and some applications. *Proceedings of the National Academy of Sciences, USA* **76,** 4350–4354.

Ueno Y, Imanari E, Emura J, Kumagaye KY, Nakajima K, Inami K, Shiba T, Hata S, Izui K. 1998. Regulatory phosphorylation of maize C_4-form PEP carboxylase (PEPC): antibody detection system and purification of PEPC-protein kinase. In: Garab G, ed. *Photosynthesis: mechanisms and effects*, Vol. V. Dordrecht: Kluwer Academic Publishers, 3407–3410.

Ueno Y, Imanari E, Emura J, Kumagaye KY, Nakajima K, Inami K, Shiba T, Sakakibara H, Sugiyama T, Izui K. 2000. Immunological analysis of the phosphorylation state of maize C_4-form phosphoenolpyruvate carboxylase with specific antibodies raised against a synthetic phosphorylated peptide. *The Plant Journal* **21,** 17–26.

Wang D, Portis Jr. AR, Moose SP, Long SP. 2008. Cool C_4 photosynthesis: pyruvate pi dikinase expression and activity corresponds to the exceptional cold tolerance of carbon assimilation in Miscanthus× giganteus. *Plant Physiology* **148,** 557–567.

Weigend M, Hincha DK. 1992. Quaternary structure of phosphoenolpyruvate carboxylase from CAM-C_4- and C_3-plants—no evidence for diurnal changes in oligomeric state. *Journal of Plant Physiology* **140,** 653–660.