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Cooperative Effects of Light and Temperature on the Activity of Phosphoenolpyruvate Carboxylase from *Amaranthus paniculatus* L.[1]

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ABSTRACT

The phosphoenolpyruvate carboxylase of *Amaranthus paniculatus* shows *in vitro* optimum affinity ($S_{0.5}$) to phosphoenolpyruvate at a relatively high temperature (about 35°C); even in the presence of activators, it functions efficiently only above 25 to 27°C. At lower temperatures, a steep increase of activity with temperature is observed, due to the high activation energy for the catalyzed reaction. The same behavior *in vivo* could amplify the photoactivation of the enzyme to a large extent, since the night/day transition is soon followed by a considerable rise in leaf temperature.

Photoregulation of enzymic activities appears to be a prevalent phenomenon in photosynthetic cells (1, 2), assuring a smooth and efficient shifting from the respiratory to the autotrophic operational mode. In studying the modulation of enzymic activities by light, however, it is often vexing to find only a small effect, when physiological considerations indicate a larger one. This is clearly the case with PEPCase[3] of C_4 plants. Though photoactivated (7–9, 16) the observed light/dark activity ratios (1.5–3.5, depending on extraction and assay conditions) are not large enough to be considered of primary importance for the regulation of the C_4 sequence. Additional factors which may amplify the light effect (pH, substrate and activator levels) have been pinpointed (9, 15), but the picture of PEPCase regulation *in vivo* is still far from complete.

In studies of the properties of PEPCase, an unusually high dependence of activity on temperature was observed in some C_4 plants (*Portulaca oleracea* L., *Amaranthus paniculatus* L.). Since an environmental factor rarely acts in isolation, it became apparent that the temperature rise accompanying the night-day transition could bring about, at least in some C_4 plants, a strong amplification of the light activation of the enzyme. To establish the extent of the light-temperature cooperativity on the activity of PEPCase, a detailed study of the temperature relationship in PEPCase of *A. paniculatus* was undertaken.

MATERIALS AND METHODS

Amaranthus paniculatus L. was grown from seed both in a growth chamber (temperature, RH and light/dark cycles of 30/18°C, 40/70% and 13/11 h, respectively) and in the field; *Zea mays* L. was grown in the growth chamber as above and *Atriplex halimus* L. was collected from the field. Irradiance at plant level in the growth chamber was around 250 $\mu mol \cdot m^{-2} \cdot s^{-1}$ PAR given by a mixture of fluorescent and incandescent lamps. The plants in the field were under full sunlight most of the growth period and the prevailing air temperatures were 24 to 27°C during the day (maxima 25–36°C) and 20–25°C at night (minima 15–19°C).

For PEPCase extraction, 1 g of mature leaves was ground in a prechilled mortar with purified sea sand and 5 ml of extraction buffer containing 100 mM Tris-Cl (pH 7.8), 1 mM EDTA, 10 mM $MgCl_2$, glycerol (25% v/v), PVP(3% w/v), and approximately 100 mg of insoluble PVP. Glycerol was added for stabilization of the enzyme upon storage (8). The extract was centrifuged and the clear supernatant was desalted through a Sephadex G-25 column equilibrated with 100 mM Tris-Cl (pH 7.8) in 25% v/v glycerol. The volume of the desalted extract was adjusted to be double of that layered on the column. All above steps were carried out at 4°C.

Assays of PEPCase activity were run in 3 ml final volume containing 100 mM Hepes-KOH (pH 7.2 or 7.8), 1 mM $NaHCO_3$, 5 mM $MgCl_2$, 0.14 mM NADH, 4.5 units of malate dehydrogenase (pig heart, Sigma), and PEP as specified. The reaction was started with the addition of the desalted enzymic extract (0.05–0.2 ml, depending on assay temperature) and the rate was measured by the decrease in A at 340 nm (oxidation of NADH). Since the activity declined rapidly during the assay at low PEP concentrations, the true initial activities were calculated by extrapolation to zero time (5). The Hepes buffer was selected for the assay because of its low temperature coefficient; its pH at 100 mM was found practically constant within the 5 to 30°C temperature range. For temperatures above 30°C, the pH was measured and corrected in the cuvette, before the addition of the enzyme.

RESULTS AND DISCUSSION

Effects of Temperature on Kinetic Parameters of PEPCase. Kinetic analyses on the two forms of PEPCase (day- and night-form) from *A. paniculatus* leaves were conducted at pH 7.2 and 7.8 and at temperatures ranging from 16 to 45°C. Lower temperatures could not be used with this enzymic source, since the activity was too low for dependable measurements. Plants grown in the growth chamber or in the open were used separately in different experiments; however the same trends were observed with both enzymic sources.

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[2] From her Doctoral Thesis.

[3] Abbreviations: PEPCase, phosphoenolpyruvate carboxylase; PEP, phosphoenolpyruvate; E_a, activation energy; ΔT, day-night temperature difference.

Results of two representative experiments with both types of plants are shown in Tables I and II. As reported previously (9), both darkness and lower assay pH tend to increase the sigmoidicity of the rate curves. High temperatures seem also to increase this apparent allostericity toward PEP, though a similar effect was observed at 20°C (pH 7.8) in plants grown in the open. The catalytic efficiency ($V_{max}/S_{0.5}$) of the enzyme is strongly promoted at higher temperatures due to an increase in V_{max} with temperature and a decrease of $S_{0.5}$ up to 35°C. Results with plants grown in the open confirmed that the day-form of the enzyme has a slightly higher affinity for PEP and the temperature for optimum affinity to the substrate is around 35°C. for both forms (Fig. 1). This rather high temperature for optimum affinity to the substrate could be considered as strong biochemical evidence for adaptation of *A. paniculatus* to high growth temperatures. Ecological observations support the tenet that *A. paniculatus* is a thermophilic species. Under the Mediterranean climate of Greece, it grows vigorously only from June (seedling stage) to August (mature, seeding plants), when the mean day temperature range is 20 to 30°C (maxima at 25–35°C); under full sunlight, though, leaf temperatures should be considerably higher. In *A. halimus*, a lower temperature for optimum enzyme-substrate affinity was found (Fig. 1). This species is a C_4 perennial shrub, which in N. Peloponnese withstands the summer temperatures but makes new growth only in spring. A comparable optimum, at about 30°C, has been reported from *Atriplex vesicaria* (Fig. 9.23 in Ref. 13). An increase of K_m (PEP) above 30°C has also been found for PEPCase of *Kalanchoë daigremontiana*, a CAM plant (3) (Fig. 7 in Ref. 12). It is noteworthy, however, that a clear-cut temperature optimum for enzyme-substrate affinity is not evident in *K. daigremontiana*, as the lower temperatures do not affect the K_m (PEP). The probable physiological significance of that difference between CAM- and C_4-PEPCase is rather obvious, since the former functions at lower night temperatures, whereas the latter fixes CO_2 under higher temperatures during the day.

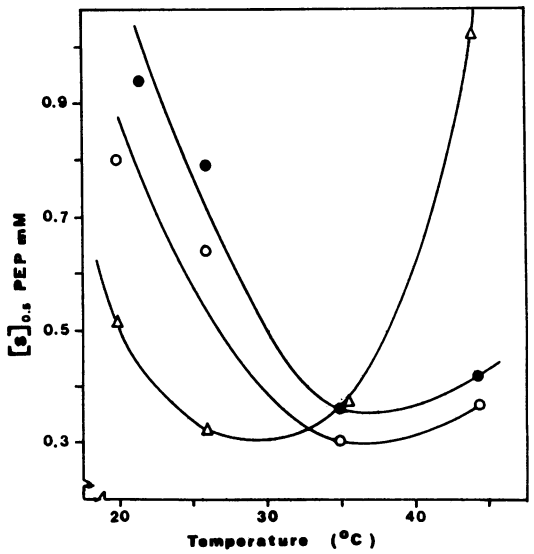

FIG. 1. Temperature dependence of $S_{0.5}$(PEP) in day- (○) and night-form (●) of PEPCase of *A. paniculatus* L. and day-form (△) of *A. halimus* L. Enzymic source: desalted leaf extracts from plants grown in the open. Activities assayed at pH 7.2.

Table I. *Kinetic Parameters of PEPCase (Day- and Night-Form) as Affected by Temperature and pH*

Enzymic source: desalted leaf extracts from *A. paniculatus* L. plants grown in the growth chamber; activities in mmol $CO_2 \cdot min^{-1} \cdot g^{-1}$ fresh wt; n = Hill coefficient.

pH	Day-Form					Night-Form				
	Temp.	$S_{0.5}$ (PEP)	V_{max}	n	$V_{max}/S_{0.5}$	Temp.	$S_{0.5}$ (PEP)	V_{max}	n	$V_{max}/S_{0.5}$
	°C	mM				°C	mM			
7.2	16	1.62	3.1	1.4	2	16	3.80	4.5	1.5	1
	26	0.83	12.0	1.4	14	26	0.92	8.5	2.0	9
	33	0.34	20.8	1.7	61	33	0.45	16.0	2.0	36
	42.5	0.32	38.5	1.8	120	45	0.52	33.0	2.3	63
7.8	16	1.10	6.1	1.0	5	16	1.60	5.2	1.0	3
	26	0.56	16.4	1.0	29	26	0.40	12.4	1.3	31
	33	0.33	24.0	1.0	73	32.9	0.30	18.5	1.6	62
	42	0.40	50.0	1.0	125	45	0.42	33.3	1.8	79

Table II. *Kinetic Parameters of PEPCase (Day- and Night-Form) as Affected by Temperature and pH*

Enzymic source: desalted leaf extracts from *A. paniculatus* L. plants grown in the field; activities in mmol $CO_2 \cdot min^{-1} \cdot g^{-1}$ fresh wt; n = Hill coefficient.

pH	Day-Form					Night-Form				
	Temp.	$S_{0.5}$ (PEP)	V_{max}	n	$V_{max}/S_{0.5}$	Temp.	$S_{0.5}$ (PEP)	V_{max}	n	$V_{max}/S_{0.5}$
	°C	mM				°C	mM			
7.2	20	0.80	4.0	1.7	5	21.5	0.94	4.4	2.5	5
	26	0.64	9.1	1.9	14	26	0.79	7.7	2.0	10
	35	0.30	16.6	2.1	55	35	0.36	15.8	2.4	44
	44.5	0.37	25.6	3.1	69	44.5	0.42	22.7	3.3	54
7.8	20	0.49	4.4	1.4	9	22	0.34	6.1	1.6	18
	26	0.40	10.0	1.0	25	26	0.34	8.5	1.3	25
	35	0.18	14.3	1.3	79	35	0.21	15.2	1.7	72
	44.5	0.37	24.4	2.6	66	44.5	0.42	18.2	2.5	43

Most characteristic of the PEPCase from *A. paniculatus* is the steep increase of activity as the temperature is raised up to 35°C (Figs. 2 and 3). The high E_a values calculated from the corresponding Arrhenius plots show that this enzyme *in vitro* is a rather inefficient catalyst at temperatures lower than 31 to 32°C at low and 26 to 27°C at high substrate (PEP) level. Few differences are noted when the activity is assayed at pH 7.8; discontinuities in the slopes are found at 26 to 27°C at both substrate levels, and the estimated E_a values are 15 to 20% lower (data not shown).

Arrhenius plots for the same reaction with enzymic preparations from corn (11, 18), *Eleusine indica* (14), and other C_4 grasses (10), either show a break at lower temperatures or the slope is uniform and modest throughout the range examined (usually 5–35°C). The discontinuity in the slope often appears at 10 to 12°C (10, 18) though breaking points at higher temperatures (17–20°C and 25°C) have also been found (11, 14). The reported E_a values at temperatures above the breaking point or in the uniform slopes are low (in the range of 6.6–18.3 kcal/mol), whereas they become much higher (11.6–49.7 kcal/mol) below the breaking point. Our results with PEPCase extracted from *A. halimus* and *Z. mays* (Table III) are fairly comparable to values reported in the literature, confirming that PEPCase from *A. paniculatus* is indeed exceptional.

Cooperativity of Light and Temperature on PEPCase Activity. The inefficiency of PEPCase as a catalyst at low temperatures may have a dramatic consequence on *in situ* activity, suppressing it to a very low level during the night and amplifying the photoactivation after the onset of light, as a gradual increase in leaf temperature follows the night/day transition. Depending on climate and transpirational rate, this day-night temperature difference (ΔT) may eventually become considerably higher than 10°C. The amplification of the light effect by temperature could be particularly important for plants possessing a PEPCase adapted to high temperature, as it is apparently the case with *A. paniculatus*. The potential for such an amplification is clearly shown in Figure 4. When photoactivation is assessed, as usual, at the same temperature, the effect of light on PEPCase of this species appears to be marginal. At 0.48 mM PEP, a day/night activity ratio of only 1.3 is obtained, when activities are assayed at 35°C. However, a temperature difference of 13.5°C between night and day amplifies the above ratio 24-fold to 31.3. Admittedly, this is an extreme case where catalytic inefficiency (high E_a) is combined with a rather high ΔT. Nevertheless, it can be calculated that even with an efficient PEPCase (*e.g.* E_a = 10.7 kcal/mol) and a moderate ΔT of 10°C (*e.g.* 25 and 15°C at day and night, respectively) the amplification of the light effect would be almost 2-fold; though small, such a temperature effect is not negligible, if the diurnal modulation of PEPCase activity is achieved through a multifactorial system of coarse and fine controls (9).

Temperature Response in the Presence of Activators. Both the efficiency of PEPCase at a given temperature range and the temperature of transition to a more efficient form are affected by the presence of glucose-6-P and 3-phosphoglycerate. The former is the best known activator of PEPCase (4, 6, 17, 19),

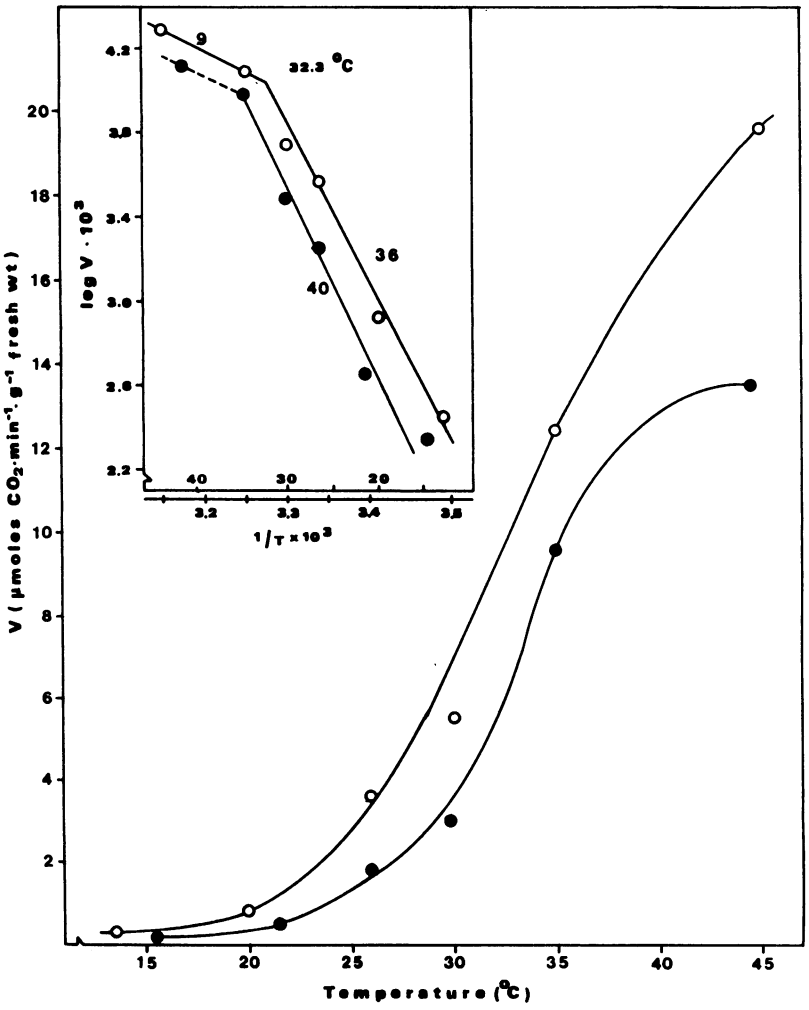

FIG. 2. Temperature effects, from a representative experiment, on PEPCase activity (○ = day-form; ● = night-form) assayed at 0.49 mM PEP and pH 7.2. Arrhenius plots of the same data are shown in the inset. The numbers by each line are the estimated E_a values in kcal/mol. Enzymic source: desalted leaf extract from *A. paniculatus* L. plants grown in the open.

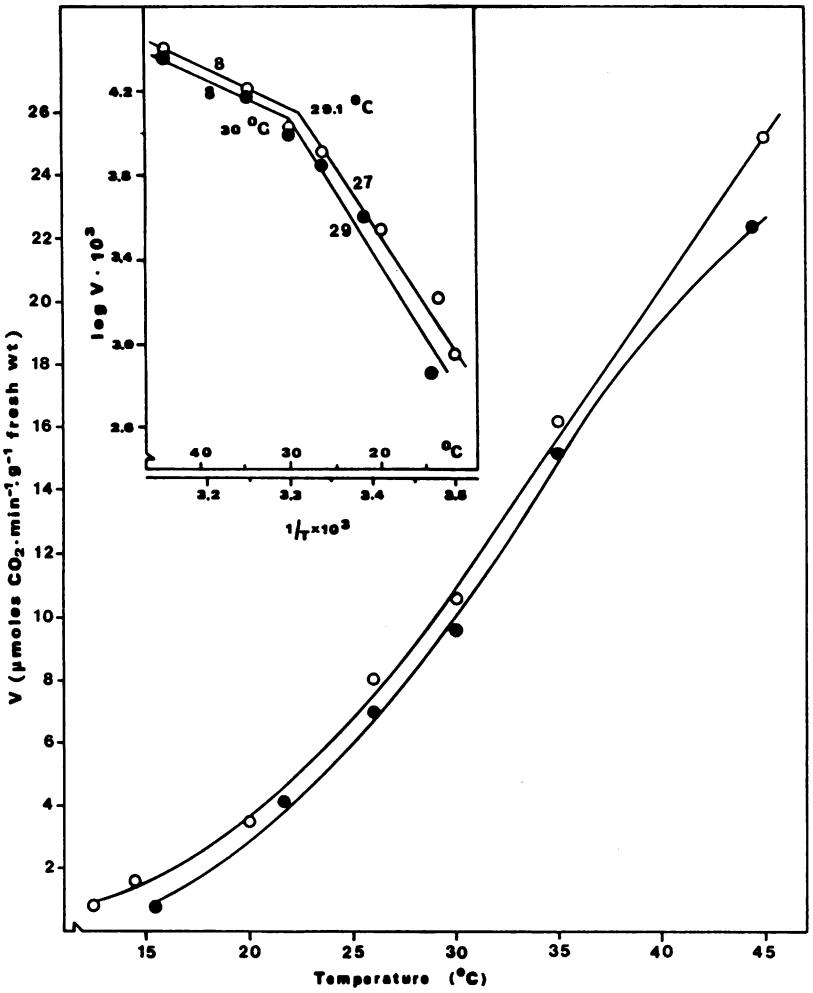

FIG. 3. Enzymic source and assay as in Figure 2 but at 2.43 mM PEP.

Table III. *Data from Arrhenius Plots of PEPCase Activity Extracted from A. halimus L. and Z. mays L. Leaves and Assayed at Two pH Values and PEP Levels*

pH	*A. halimus*			*Z. mays*		
	PEP	Break	E_a[a]	PEP	Break	E_a[a]
	mM	*°C*	*kcal/mol*	*mM*	*°C*	*kcal/mol*
7.2	0.49	no	17	0.24	14.5	10/20
	2.43	30.0	10/17	2.43	no	15
7.8	0.49	29.1	7/18	0.24	14.5	6/21
	2.43	29.6	8/17	2.43	no	17

[a] E_a upper/lower are for temperatures above/below the breaking point.

whereas the latter was recently shown to act as activator at low pH and PEP level (15). The effects of the above activators, at 3 PEP levels, on the performance of PEPCase (day- and night-form), within the temperature range 16–44°C, are given in Table IV.

When PEP is not saturating, the presence of activators lowers considerably the temperature of transition from the inefficient to the efficient form. This transition-point seems to be slightly higher in the night-form of PEPCase. Assuming that the enzyme behaves similarly *in vivo*, we may conclude that the PEPCase of *A. paniculatus*, at low PEP levels and in the presence of activators, is efficient only above 25 to 27°C during the day, whereas at night even higher temperatures would be necessary. Under field

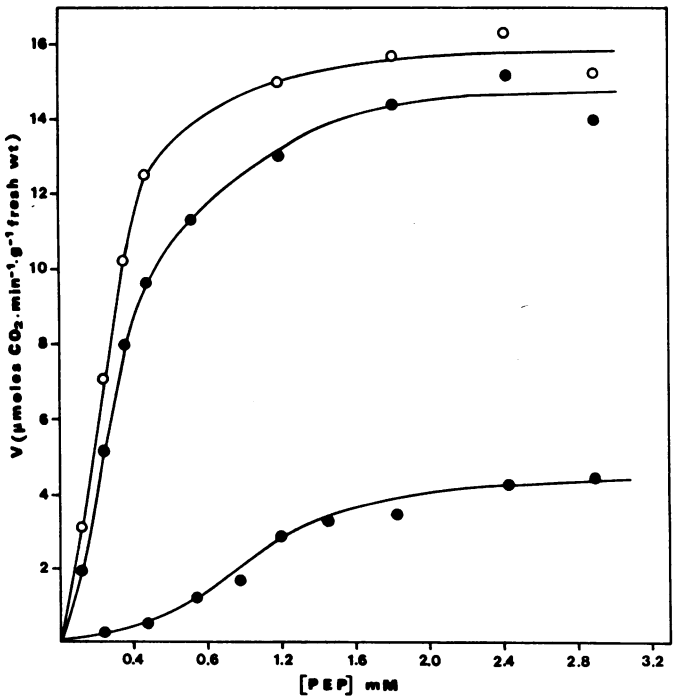

FIG. 4. Rate curve of the day-form (O) of PEPCase in 35°C as compared to the rate curves of the night form (●) in 35°C (upper curve) and 21.5°C (lower curve). Enzymic source and assay as in Figure 2.

Table IV. *Data from Arrhenius Plots of PEPCase Activity, Extracted from A. paniculatus Leaves (Day- and Night-Form) and Assayed at pH 7.2 in the Absence or Presence of Activators, at Three PEP Levels*

[PEP]	No Activator		3-PGA (4 mM)		G-6-P (2 mM)	
	Break	E_a[a]	Break	E_a[a]	Break	E_a[a]
mM	*°C*	*kcal/mol*	*°C*	*kcal/mol*	*°C*	*kcal/mol*
			Day-Form of PEPCase			
0.24	>34	ND[b]/35	27.7	12/20	26.8	12/36
0.49	32.3	9/36	25.0	12/38	26.7	11/32
2.43	29.1	8/27	25.5	10/28	26.0	11/20
			Night-Form of PEPCase			
0.24	>35	ND/32	28.7	11/42	33.3	13/29
0.49	>35	ND/40	30.0	7/37	31.4	9/33
2.43	30	8/29	27.3	10/30	28.7	9/25

[a] E_a upper/lower are for temperatures above/below the breaking point. [b] ND = not determined because of insufficient experimental data above the breaking point.

conditions, therefore, if the night temperature is much lower than 25°C—a reasonable postulate for temperate climates—the photoactivation of PEPCase would be greatly amplified by the day-night temperature difference.

CONCLUSIONS

On the basis of our results and the common assumption that the behavior of an enzyme *in vitro* represents faithfully the *in vivo* situation, it can be concluded that the properties of PEPCase in *A. paniculatus* are indicative of biochemical adaptation to elevated temperatures. Both the high temperature for optimum K_m (PEP) and the apparent catalytic inefficiency at temperatures lower than 25 to 27°C, even in the presence of activators, are strong evidence for such an adaptation.

Obviously, the high temperature requirement, in cooperation with other variables, could play a decisive role in the diurnal regulation of PEPCase activity; the amplification of its photoactivation is a typical case of such a cooperative action of two factors (light and temperature). It seems probable that even the commonly expected amplification (about 2-fold) of the photoactivation, due to the usual day-night temperature difference, could be significant. As suggested previously (9), additional factors, such as the concentrations of substrates and effectors and/or shifts in the pH, may be involved in a metabolic on-off switch for PEPCase *in vivo*.

It is unlikely that *A. paniculatus* is a rarity among C_4 species in having a PEPCase adapted to function at elevated temperatures. *Portulaca oleracea* L. possesses a similar PEPCase (E Selinioti, Y Manetas, NA Gavalas, unpublished data) and a survey among C_4 plants would undoubtedly reveal many more thermophilic species or even thermophilic ecotypes within species. C_4 dicots should be the main target, since an extensive screening of C_4 monocots (21 species) has already been made (10), without locating a thermophile comparable to *A. paniculatus*. In light of the results with PEPCase, studies of the temperature relationships of other photoregulated enzymes appear to be a fertile field for further investigation.

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