Planta (1982) 154:326–331

Planta


# Photoperiodism and Crassulacean acid metabolism

## I. Immunological and kinetic evidences for different patterns of phosphoenolpyruvate carboxylase isoforms in photoperiodically inducible and non-inducible Crassulacean acid metabolism plants

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**Abstract.** Plants of *Kalanchoe blossfeldiana* v. Poelln. Tom Thumb and *Sedum morganianum* E. Walth. were grown under controlled photoperiodic conditions under either short or long days. Gaz exchange measurements confirmed that in *K. blossfeldiana* Crassulacean acid metabolism (CAM) was photoperiodically inducible and that *S. morganianum* performed CAM independently of photoperiod. With *K. blossfeldiana*, a comparison of catalytic and regulatory properties of phosphoenolpyruvate carboxylase (PEPC, EC 4.1.1.31) from short-day and long-day grown plants showed differences, but not with *S. morganianum*. Ouchterlony double diffusion tests and immunotitration experiments (using a *S. morganianum* PEPC antibody) established that CAM is induced in *K. blossfeldiana* – but not in *S. morganianum* – through the synthesis of a new PEPC isoform; this form shows an immunological behavior different from that prevailing under non-inductive conditions and can be considered as specific for CAM performance.

**Key words:** Crassulacean acid metabolism induction – *Kalanchoe* – Phosphoenolpyruvate carboxylase (isoforms) – Photoperiodism – *Sedum*.

### Introduction

Among plants which are capable of performing Crassulacean acid metabolism (CAM) (Kluge and Ting 1978), a number of species (Queiroz 1979) are known in which a convenient photoperiodic treatment, i.e., the application of a convenient ratio between diurnal lengths of day and night, enhances the CAM pathway or even induces it, as defined by Brulfert et al. (1976), which implies non-reversibility of the effect. *Kalanchoe blossfeldiana* "Tom Thumb" is the best investigated example of this behavior: Preceding work (Gregory et al. 1954; Queiroz 1974, 1979) with this plant established a clearcut dependence on short days which act as an inducing factor for CAM involving detection of photoperiod by phytochrome. On the other hand, in the majority of CAM plants, CAM is established independently of photoperiod but can be modulated to some extent by photoperiodic treatments (Kluge and Ting 1978).

The mechanism of CAM induction by photoperiod in plants, e.g., *Kalanchoe blossfeldiana*, largely includes photoperiodic control of the CAM key enzyme phosphoenolpyruvate carboxylase (PEPC):

a) Short-day treatment increases the capacity of PEPC (Queiroz and Morel 1974), following a kinetics similar (Brulfert et al. 1975) to that of the increase in dark $CO_2$ fixation and in amplitude of the diurnal malic acid rhythm, which are usually considered as criteria for CAM operation;

b) short-day treatment induces a new PEPC isoform distinguishable from the formerly present PEPC by its electrophoretic properties, elution profiles from ion-exchange chromatography, and different molecular weight (Brulfert et al. 1979).

The aim of the present study was to look for further evidence that the photoperiodic induction of CAM in *Kalanchoe blossfeldiana* is linked to changes in the PEPC isoform pattern through the synthesis of a protein functionally responsible for CAM operation and showing kinetic and regulatory properties different from those of the enzyme existing under long days.

For comparative reasons parallel experiments were carried out with *Sedum morganianum* which performs CAM independently of photoperiod. Antise-

*Abbreviations:* CAM = Crassulacean acid metabolism; PEP = phosphoenolpyruvate; PEPC = phosphoenolpyruvate carboxylase (EC 4.1.1.31)

0032-0935/82/0154/0326/$01.20

rum against PEPC from *Sedum morganianum* was already available (Müller et al. 1980), thus allowing immunological comparisons between the two plant species.

## Materials and methods

*Plant material. Kalanchoe blossfeldiana* Poelln. Tom Thumb (clone from Gif-sur-Yvette) and *Sedum morganianum* (clone from Botanical Garden Darmstadt) were obtained from cuttings and cultivated under standard conditions at the Phytotron of Gif-sur-Yvette. Growth conditions were as follows: long day, 16 h light/8 h darkness; short day, 9 h light/15 h darkness. The light intensity was 100 $Wm^{-2}$ at the level of the plants. The temperature during the day was 27° C for *K. blossfeldiana* and 22° C for *S. morganianum* and during the night 17° C for *Kalanchoe* and 12° C for *Sedum*.

*PEPC extraction and assay.* Leaves were harvested 1 h after the beginning of the light period. In *K. blossfeldiana,* the second pair of leaves from the apex was extracted. In *S. morganianum,* the enzyme was obtained from the first fully developed leaves (i.e., about the 10th leaves from the apex).

Fresh material was thoroughly ground in 2 vol tris(hydroxymethyl)-aminomethane (Tris)-HCl buffer (200 mM, pH 8.0) containing: $MgCl_2$ 3.5 mM, dithiothreitol (DTT) 0.3 mM, polyethylene glycol (MW 20,000) 2% fresh weight, and polyclar AT 5% fresh weight. The final pH after extraction was 7.4. Before being used in the assays, the extracts were centrifuged for 15 min at 100,000 *g*.

PEPC capacity, Km(PEP), PEPC inhibition by L-malate and activation by glucose-6-P were measured at pH 7.4 in extracts desalted through Sephadex G25 columns, according to Kluge et al. 1981. The estimation of molecular weights by polyacrylamide gel electrophoresis was done using the method of Brulfert et al. 1979.

*Immunological methods.* The anti-PEPC immunserum used was raised against PEPC from *S. morganianum* by immunization of a rabbit. The antigen purification procedure, immunization, preparation of the antiserum, and the criteria of antiserum specificity were described earlier (Müller et al. 1980). It is important to note that the immunological comparisons reported here were carried out with the same immunserum.

Double diffusion tests were carried out after Ouchterlony (1958). It has been checked that the antibodies obtained against PEPC from *S. morganianum* were monospecific for plant PEPCs (Müller et al. 1980).

For immunotitration of PEPC, increasing volumes of antiserum were mixed with aliquots of enzyme crude extract corresponding to similar leaf dry weight (10 mg) to afford a comparison between long-day and short-day treated plants; fractions were completed to a constant vol. of 0.9 ml by Tris-HCl buffer (50 mM, pH 7.4) and stored overnight at 4° C. After centrifugation (50,000 *g*, 45 min) the residual PEPC activity in the supernatant was measured. The immunoprecipitates obtained were washed with 0.2 ml distilled water, 0.2 ml 95% ethanol, dessicated, redissolved in 0.5 ml NaOH (0.1 M), heated 20 min at 100° C, and assayed for protein, according to Lowry et al. 1951. PEPC residual activity was expressed as % of enzyme activity in the control fraction containing no antiserum.

*Measurement of $CO_2$ exchange and malic acid content of the leaves.* $CO_2$ exchange was monitored by an infrared gas analyzer. Malate was assayed according to Hohorst (1970).

## Results

### *Effects of photoperiod on CAM parameters*

Figure 1 documents that, as far as performance of CAM is concerned, *Kalanchoe blossfeldiana* and *Sedum morganianum* responded differently to photoperiodic treatment. This evaluation is important in the context of comparative enzymological studies.

As predictable from earlier results (Queiroz 1965), short-day treatment induced CAM in *K. blossfeldiana,* as indicated by the nocturnal net $CO_2$ fixation and a clear diurnal malic acid rhythm (Fig. 1 A). These CAM criteria were practically lacking in the long-day grown *K. blossfeldiana* (Fig. 1 B), which therefore can be considered as non CAM-performing.

In contrast, *S. morganianum* showed net dark $CO_2$ fixation and diurnal malic acid rhythm under long- and short-day treatment (Fig. 1 C and 1 D). Hence, in this species occurrence of CAM is not controlled by photoperiodism. The only effect of the daily length of the light period which was detectable in *S. morganianum* during our experiments consisted in a substantial net $CO_2$ uptake by the long-day-treated plants during the second half of the light period (Fig. 1 D). This $CO_2$ uptake was lacking in the short-day-grown plants (Fig. 1 C), but inspection of curves C and D indicates that this lacking could result simply from an earlier onset of darkness and not from a basic difference in mechanism: In both photoperiods a net $CO_2$ production occurs during roughly 8–9 h of light.

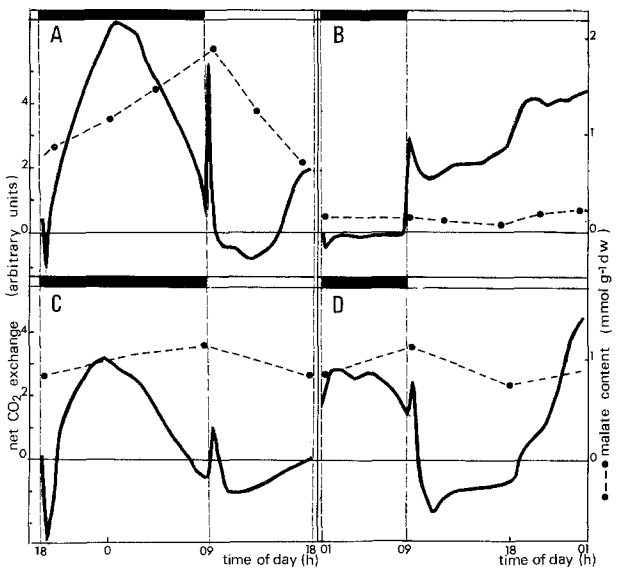


**Fig. 1 A–D.** Diurnal patterns of net $CO_2$ exchange and malic acid content in *Kalanchoe blossfeldiana* and *Sedum morganianum* under short-day and long-day treatments. *Kalanchoe:* **A,** short days; **B,** long days; *Sedum:* **C,** short days; **D,** long days. Black bars indicate duration of daily dark period

**Table 1.** Kinetic data and sensitivity to effectors of PEPC obtained from long-day (LD) or short-day (SD) grown *Kalanchoe blossfeldiana* and *Sedum morganianum* (extraction 1 hour after beginning of day; extracts desalted through Sephadex G25 columns); data obtained at pH 7.4 and 30° C. Activation by glucose-6-phosphate (13.3 mM) and inhibition by malate (6.6 mM) measured in presence of respectively non-saturating (1.25 mM) and saturating (7.5 mM) PEP concentration; results expressed as a % of initial activity

| | *Kalanchoe blossfeldiana* | | *Sedum morganianum* | |
|---|---|---|---|---|
| | LD | SD | LD | SD |
| PEPC capacity (mmol $h^{-1}g^{-1}$DW) | 0.2 | 6.4 | $0.97 \pm 0.4$ | $0.805 \pm 0.6$ |
| Km(PEP) (mM) | 1.4 | 2.8 | 5.2 | 5.2 |
| G-6-P activation (% of initial activity) | 160 | 380 | 240 | 300 |
| Malate inhibition (% of initial activity) | 60 | 35 | 36 | 27 |
| Molecular weight | 174,000 | 184,000 | 232,000 | |

*Effects of photoperiod on catalytical and regulatory properties of PEPC*

*PEPC capacity.* Table 1 shows that the different responses of CAM to photoperiod in *K. blossfeldiana* and *S. morganianum* coïncide with divergent catalytic and regulatory properties of PEPC. In *K. blossfeldiana,* as already described (Queiroz, 1968, 1969), there was a drastic increase in the capacity of PEPC (i.e., in the maximal extractable enzyme activity) when CAM was induced by short-day treatment. In contrast, there was no photoperiodic-dependent difference in PEPC capacity in *Sedum.*

*Affinity for PEP.* In *K. blossfeldiana,* results published elsewhere (Kluge et al. 1981; Brulfert and Queiroz 1982) established a), the existence of a diurnal rhythm in Km(PEP) for PEPC extracted from either long- or short-day-grown plants with a larger amplitude in the case of the plants under short days; b) no significant difference in the daily mean values of these rhythms. Results in Table 1 show that short-day treat-

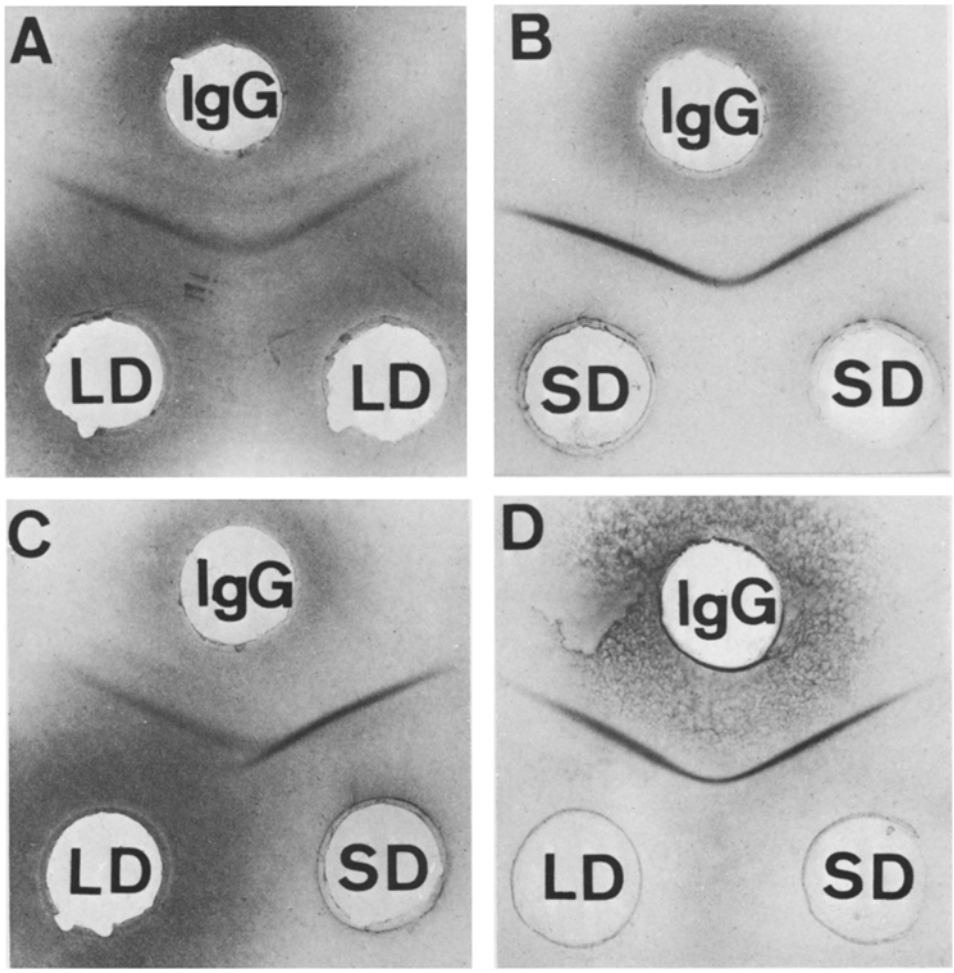


**Fig. 2A–D.** Immunological comparison of PEP carboxylases by Ouchterlony test. PEP carboxylases were obtained from *Kalanchoe blossfeldiana* and *Sedum morganianum* grown in long days (*LD*) or in short days (*SD*). The IgG were raised against PEP carboxylase of *Sedum morganianum.* **A**, *K. blossfeldiana* LD/LD; **B**, *K. blossfeldiana* SD/SD; **C**, *K. blossfeldiana* LD/SD; **D**, *S. morganianum* LD/SD

ment seems to lower the affinity of PEPC for PEP when enzyme extraction is performed 1 h after the beginning of light (10 h).

In fact a similar comparison during the night would have shown a remarkable inverse situation: PEPC affinity for PEP was significantly higher at 0.00 h in extracts from plants under short days [Km(PEP) = 0.9 mM under short days, 1.7 under long days], as could be expected for CAM efficiency (Brulfert and Queiroz 1982). In summary, different photoperiods resulted, with *Kalanchoe blossfeldiana,* in different Km(PEP) of PEPC, but not with *Sedum morganianum.*

*Activation by glucose-6-P and inhibition by malate.* The well-known effects of glucose-6-P and of malate on the activity of PEPC were utilized for further characterization of the effects of photoperiodism on the enzyme. Results reported in Table 1 show a significant difference in the sensitivity to glucose-6-P and to malate between the enzymes extracted from long- or short-day-treated *Kalanchoe blossfeldiana.* In *S. morganianum* only slight, non-significant differences could be noticed.

*Molecular weights.* As previously described (Brulfert et al. 1979), an electrophoretically and chromatographically distinguishable PEPC appears with CAM induction under short days in *K. blossfeldiana;* its molecular weight was shown to differ from that of the form prevailing under long days. In contrast, the PEPC obtained from long- and short-day-treated *S. morganianum* plants had an identical MW of 232,000 (Müller et al. 1982) and behaved identically in gel electrophoresis (Table 1).

### *Effects of photoperiod on immunological properties of PEPC*

*Double immunodiffusion tests.* Ouchterlony double diffusion tests revealed that not only PEPC from *S. morganianum,* i.e., the homologue antigen, was precipitated by the antiserum, but also PEPC obtained from *K. blossfeldiana.* However, there were significant differences in the enzymes extracted from the two plants (Fig. 2).

In *S. morganianum,* the precipitation lines of the long- and short-day-extracted PEPC fused completely, indicating immunological identity between the two enzymes (Fig. 2C). Contrastingly, in *K. blossfeldiana,* the precipitation lines obtained with long- and short-day-extracted PEPC did not fuse completely, but formed a spur (Fig. 2C), showing that the compared antigens share common antigenic determinants but are immunologically not completely identical. Hence, in *Kalanchoe,* not however in *Sedum,* photoperiodical treatment created differences in the immunological properties of PEPC.

*Immunotitration.* Results reported in Fig. 3A present immunotitration curves for PEPC extracted from *Kalanchoe blossfeldiana* grown under long and short days. Consideration of the equivalence points (i.e., the amount of antiserum required to precipitate all PEPC) or, for more accuracy, the 50% residual activity points shows that the amount of PEPC-protein per unit of leaf dry weight is much higher under short days than under long days. This is confirmed by the difference between the variations in the amounts of protein in the immunoprecipitates obtained from the two extracts.

On the other hand, it appears from the results that the amount of immunoprecipitates (i.e., the approximative index of the PEPC protein) increased 4.6 fold with short-day treatment, whereas the capacity of PEPC was enhanced 32.0 fold: These data indicate that beyond the amount of PEPC protein its specific activity is also increased by short days. However, because it is not yet known if the affinity of the proposed PEPC isoforms for the antibody is identical, this question can not be resolved without further experimentation.

In contrast to results with *Kalanchoe blossfeldiana,* the amounts of antiserum required to reach the 50%

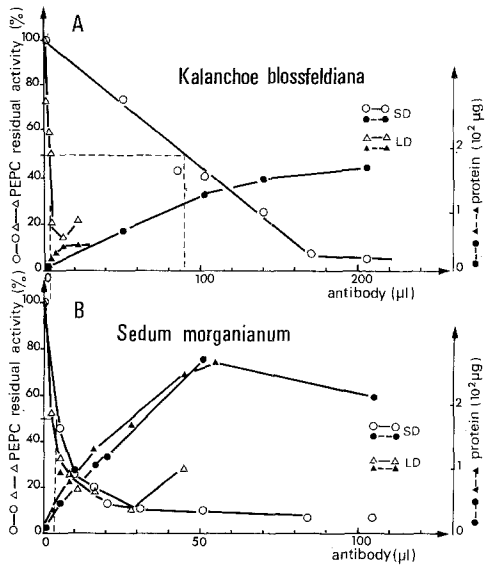


**Fig. 3A, B.** Immunotitration of PEP carboxylase from short-day- and long-day-grown *Kalanchoe blossfeldiana* (**A**) and *Sedum morganianum* (**B**). The antibody used was raised against PEPC from *Sedum morganianum.* PEPC residual activity remaining in the supernatant (open symbols) was expressed as % of PEPC activity in a control fraction where no antibody was added. Closed symbols indicate protein recovery in immunoprecipitate

residual activity were practically identical for the extracts from long- and short-day-treated *Sedum morganianum* (Fig. 3B). Increase in protein immunoprecipitate with increasing amounts of antiserum is similar for the two extracts.

## Discussion

The above results support the hypothesis that in *Kalanchoe blossfeldiana* photoperiodic regulation of PEP carboxylase is integrated in the mechanism of CAM induction. Until recently, evidence that induction of increased PEP carboxylase capacity by short days resulted from changes in the isozymic pattern was based mainly on electrophoretical and chromatographical studies (Brulfert et al. 1979). The present results provide more clear-cut evidence that the mechanism of photoperiodic induction of CAM by short days acts through the synthesis (see Fig. 3) of a protein which was only weakly present under long days: This is evidenced by the Ouchterlony double diffusion test which showed that the short-day dependent PEPC is immunologically not totally identical to the form prevailing under long days. This divergency is also supported by the differences in the kinetic and regulatory behavior of PEPC derived from either long- or short-day treatment.

In summary, short-day photoperiods produce a large increase in the PEPC capacity form already present in minor amounts in the tissues (examination of the Ouchterlony plates indicates trace amounts of this form in extracts from long-day-treated plants). Nevertheless, the effects of short days are so dramatic that they practically turn into a new qualitative situation, i.e., CAM effective operation, as compared to the non-CAM operation of plants under long days (see Fig. 1B). Therefore, we assume that the photoperiod-sensitive form is the form specific for CAM.

As the results show, significant photoperiodically determined changes of molecular, immunological, and regulatory properties were lacking in *Sedum morganianum*, i.e., in a plant where CAM is performed independently of the photoperiodic conditions. In this regard a fundamental difference between the enzymic response to short days in *Kalanchoe blossfeldiana* and the response to saline conditions in *Mesembryanthemum crystallinum* should be noted (v. Willert et al. 1976). In the latter case, the PEPC form correlated with growth in saline soil disappears when salt is removed. Therefore the pattern observed under salinity should not be considered as "induced" by these conditions. In contrast, a few short days (e.g., 7 or 10) were shown to result in intermediate levels of CAM in *Kalanchoe* which persisted after the plants were returned to long-day conditions.

Performance of CAM in the short-day-treated *Kalanchoe* is certainly not only due to the observed changes of regulatory PEPC properties but also, or perhaps even more, to the drastic enhancement of PEPC capacity. Certainly this latter factor is important for maintainance of a high rate of nocturnal $CO_2$ fixation, i.e., malic acid synthesis. The present studies revealed that an increase in the amount of PEPC protein and possibly an increase in specific activity of the enzyme contribute to the photoperiodically determined increase in PEPC capacity. This contrasts with the mechanism of diurnal oscillations of PEPC capacity performed by *Kalanchoe blossfeldiana* (Queiroz 1974, 1979), where solely changes in specific activity and not in the level of PEPC-protein are involved (unpublished results).

These investigations were supported by the Centre National de la Recherche Scientifique (Action Thématique Programmée Internationale n° 4927) and the Deutsche Forschungsgemeinschaft. The authors thank Mme D. Guerrier and Mr. S. Güçlü for technical assistance.

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Received 22 August 1981; accepted 20 January 1982