Environmental Control of Phosphoenolpyruvate Carboxylase Induction in Mature *Mesembryanthemum crystallinum* L.¹

Mechtild Piepenbrock and Jürgen M. Schmitt*

Institut für Pflanzenphysiologie und Mikrobiologie, Freie Universität, Königin Luise Strasse 12–16, W-1000 Berlin 33, Germany

**ABSTRACT**

*Mesembryanthemum crystallinum* L. plants shift the mode of carbon assimilation from C₃ to Crassulacean acid metabolism when stressed by high salinity. A prerequisite for Crassulacean acid metabolism induction is the synthesis of phosphoenolpyruvate carboxylase (PEPCase). A moderate increase in the abundance of PEPCase transcripts and activity is observed in 7-week-old, well-watered plants. This increase in PEPCase coincides in time with a decrease in the growth rate of the shoots. The steady-state level of PEPCase activity is uniform along the leaves of well-watered plants, as can be shown by comparing leaves of different age from individual 7-week-old plants. In contrast, the rate of induction in response to salt stress varies with the age of plants and to a lesser extent with the age of the leaves. Two-week-old seedlings induce PEPCase slowly under a moderate salt stress regimen, whereas older plants induce faster. When individual leaves from a seven-week-old plant are compared with respect to induction velocity, no clear-cut correlation with leaf age is apparent. The highest induction is observed in leaves from node five that are about 2 weeks old at the beginning of the experiment. PEPCase transcripts are readily down-regulated to minute levels when detached leaves are hydrated. The levels reached after 8 hours of rehydration are very similar, regardless of whether the leaves were cut from young or old plants or whether the plants were previously salt-stressed or well-watered. It is concluded that environmental rather than developmental factors are predominant in determining abundance of PEPCase activity and transcripts in leaves of mature *M. crystallinum* plants.

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Land plants have adapted to arid environments in numerous ways. In *Mesembryanthemum crystallinum*, water deficits, whether caused by low air humidity in combination with high light intensity (18) or by salinity (19), trigger a biochemical shift from C₃ to CAM. Plants operating in the CAM mode of photosynthesis open their stomates at night when evaporation demand is relatively low. This results in an improved water balance.

Several carbon-metabolizing enzymes increase in activity during the shift from C₃ to CAM (10). The induction of the key enzyme of CAM, PEPCase, has been characterized at the molecular level (1). During induction, a CAM-specific isoform of PEPCase (8) is synthesized de novo (9). Translation is directed by mRNA newly transcribed from one of two PEPCase isogenes (3). Rehydration of stressed plants (17) or detached leaves (14) leads to a rapid decrease in PEPCase mRNA abundance with a half-life in the range of hours.

It has been recognized that well-watered old plants induce PEPCase (2, 4) and switch to CAM under normal greenhouse conditions (18). Furthermore, it has been found that the competence for the induction of PEPCase activity under stress is acquired when plants are approximately 6 weeks of age but not before (12). It has been claimed (4) that CAM inducibility may be under the control of a developmental program that supposedly could have evolved as an adaptation to the natural environment in which young plants grow in the humid season whereas old plants have to cope with arid conditions (21). If age-dependent PEPCase induction in plants grown in soil of high water potential was dependent on an internal program, short-term changes in environmental conditions should have little influence on the expression of CAM-related genes. Therefore, we have investigated how the age of plants or leaf tissues, respectively, influences the level of PEPCase activity and *ppc* transcripts under various stress conditions as well as the capability to down-regulate PEPCase mRNA after rehydration.

**MATERIALS AND METHODS**

All methods have been described previously (14). Plant age is indicated in weeks after germination. When indicated in the figure legends, autoradiographic signals were quantified using a Cybertech CS1 eight bit video densitometer (kindly provided by Prof. B. Friedrich) in the 1D mode.

**Probes**

Inserts from pMcCAM7 (15) or pPPC1 (13) were used to probe for transcripts of PEPCase (*ppc*). These clones are specific for the stress-inducible isoform 1 of the enzyme (3). Insert from pMcss5 (5) was used to probe for *rbcS* transcripts coding for the small subunit of ribulose bisphosphate carboxylase/oxygenase.

**RESULTS**

When *M. crystallinum* plants are grown under well-watered conditions, PEPCase activity and transcripts increase in abundance around week 7 after germination (Fig. 1A, B). Thereafter, a higher steady-state level is maintained until flowers...
start to develop around week 9. The degree of induction in response to a short, 4-d salt treatment also varies with the age of the plants. Two-week-old seedlings with a shoot fresh weight of less than 1 g respond only weakly to a short 4-d stress treatment. The lack in responsiveness in young seedlings is, however, not absolute. As can be seen in Figure 2, a 5 fold induction in both PEPCase activity and ppc transcripts can be achieved after 6 d of stress in seedlings that were 2 weeks old at the beginning of stress treatment. Plants older than 2 weeks, weighing 1 g or more (Fig. 1E), respond to a short salt stress with a strong up-regulation of transcripts for PEPCase. The highest induction factor for transcripts over a low basic level is seen at an age from 4 to 5 weeks. At this age, side shoots start to develop. Five-week-old plants are still rather small, with a shoot fresh weight of about 10 g. In older, well-developed plants, transcript levels are about doubled in stressed as compared with unstressed leaves. Ten-week-old plants are in the process of setting flowers and salt treatment results in an approximate doubling of PEPCase activity but little increase in PEPCase mRNA. It is interesting to note that the time of the increase in PEPCase activity in unstressed plants correlates with a decrease in the growth rate of the shoots (Fig. 1A, E).

In contrast, levels of mRNA for the small subunit of ribulose bisphosphate carboxylase decline during growth of the plants with a sigmoidal curve in both stressed and unstressed plants. The largest concentration changes occur between weeks 5 and 7, when the levels of PEPCase and ppc transcripts increase in unstressed leaves. As expected, rbcS transcripts were somewhat lower in stressed as compared with unstressed leaves.

It should be kept in mind that induction responses in plants of different age have to be measured in different leaves because the life span of individual leaf layers is considerably shorter than that of the whole plant. The leaf layers that were harvested and pooled each week are therefore indicated in Fig. 1D.

Old and young leaves play different physiological roles. Old leaves, which have reached between 30 and 60% of their final length, generally serve as source leaves, whereas younger and therefore smaller leaves transport assimilates bidirectionally or they import exclusively (16). In *Mesembryanthemum*, any leaf pair is functional only for a fraction of the life span of the plants, usually not much longer than about 6 weeks (Fig. 1D, E), and eventually, most of the organic matter ends up in stems and reproductive organs. At a plant age of 4 to 6 weeks, branches arise from axillary buds, on which smaller leaves develop. When the side shoots develop, the cotyledons and primary leaves show symptoms of senescence and leaf pair 2 already loses turgor. A photograph of a 10-week-old plant has been published previously (19).

The increase in PEPCase activity in well-watered, mature plants could be caused by a developmental program in which newly made leaves express CAM constitutively, whereas leaves that had developed earlier would perform C₃ photosyn-

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**Figure 1.** PEPCase induction as a function of plant age. Plants of different ages were grown in 0.7-L pots and stressed for 4 d with 0.5 M NaCl in nutrient solution (C). Controls were irrigated with nutrient solution (E). PEPCase activity is given in μmol mg⁻¹ protein min⁻¹ (panel A). Steady-state levels of transcripts for PEPCase (ppc, panel B) and the small subunit of ribulose bisphosphate carboxylase (rbcS, panel C) are also shown. The leaves that have been included in the samples for extraction of RNA and protein are indicated by the stippled areas in panel D. For a schematic drawing of a 7-week-old plant with a leaf numbering scheme, compare Figure 3. Shoot fresh (Δ) and dry (□) weights are given in panel E.

**Figure 2.** Time course of PEPCase induction in seedlings. Two-week-old seedlings were stressed with 0.5 M NaCl in nutrient solution for the time indicated. Maximum PEPCase activity (□) was 0.41 μmol mg⁻¹ protein min⁻¹. Steady-state levels of transcripts for PEPCase (ppc, □) and the small subunit of ribulose bisphosphate carboxylase (rbcS, Δ) are given.
thesis unless the plants were stressed. Therefore, we were interested to determine whether the differences in PEPCase abundance and inducibility that are found during the life cycle of plants were caused by leaves in different stages of development. PEPCase activity is relatively uniform between leaves of widely different age, and younger leaves have somewhat less and not more PEPCase activity than older leaves (data not shown).

The time course of PEPCase induction in individual leaf pairs in response to stress is shown in Figure 3. The approximate life-span of individual, nonsenescent leaf pairs in such a 7-week-old plant is given in Fig. 1D. It can be seen that leaves induce with somewhat different rates when stressed. Very young leaves (compare Fig. 1) originating at the side stems (5.1 and 6.1) induce slowly, reaching only about half the transcript levels of the older leaves. Enzyme activities follow the transcripts with a lag phase of days. There is, however, no general correlation between leaf age and rate of induction. Leaf pair five, which starts to develop about 3 weeks after germination, induces with a higher rate than pairs 4 and 3, which are about 2 or 3 weeks older, respectively.

In a previous paper, we have shown that wilting, detached leaves are able to induce PEPCase mRNA within hours, much faster than intact plants under our standard stress conditions (14). We have compared expanded leaves from old (9 weeks of age) and young (5 weeks of age) plants for their capability to induce PEPCase transcripts within 8 h under wilting conditions. Plants were either well-watered or stressed with salt for 6 d before detaching the leaves. The degree of dehydration by evaporation is comparable between leaves of 5-week-old plants, irrespective of whether they were stressed previously. A similar water loss is observed in leaves derived from 9-week-old, unstressed plants. Leaves from 9-week-old, prestressed plants lose less water in comparison with leaves from unstressed plants. As can be seen in Figure 4, rapid ppc mRNA induction occurs in all wilted samples. Leaves from young and old plants strongly induce PEPCase transcripts within 8 h, irrespective of the fact that old, well-watered plants have a higher basic level of PEPCase activity as compared with young, well-watered plants (compare Fig. 1A). It is interesting to note that the increase in PEPCase transcripts is very similar between leaves derived from 5-week-old prestressed as compared with leaves derived from 9-week-old well-watered plants.

The transcripts present in old, well-watered plants are rapidly down-regulated when the leaves are hydrated. The level reached after only 8 h of hydration is comparable to the level in unstressed leaves from 5-week-old plants. In other words, the accumulation of PEPCase transcripts during maturation of well-watered plants is rapidly and fully reversible by hydration within a few hours.

Wilting of leaves is a very efficient way to increase PEPCase mRNA abundance. Until now, only leaves having lost 20% or more of their initial water content have been analyzed for PEPCase induction (14). We were interested to determine the threshold water stress leading to up-regulation of PEPCase mRNA. As little as 5% loss of the initial water content induces a strong increase in PEPCase mRNA abundance within 8 h (Fig. 5). This increase is near saturation, because wilting to 15 or 20% water loss gives similar induction.

**DISCUSSION**

Lowering the water potential in the rooting medium by adding solutes (usually NaCl) has been the standard procedure in many laboratories, including ours, to inflict the stress leading to the expression of CAM in *M. crystallinum*. Salting rather than wilting has been popular, presumably because the amount of salt added to the soil or hydroponic medium can be accurately quantified and reproduced. However, it must be kept in mind that dehydration by evaporation is different from dehydration caused by soil salinity. Because *Mesembryanthemum* adjusts osmotically by accumulating salt when stressed (19), it is obvious that abundance of NaCl in the rooting medium may facilitate osmotic adjustment. This may explain why detached leaves losing 5% water by evaporation strongly induce PEPCase transcripts (Fig. 5). More than 5%
diurnal changes in water content of leaves are found in intact well-watered *M. crystallinum* plants (7, 18). No concomitant diurnal fluctuations in PEPCase transcripts, however, are found in well-watered plants (R. Höfner, personal communication). Therefore, it is likely that a substance from the roots down-modulates PEPCase induction. Preliminary results indicate that exogenously applied cytokinin (100 μM benzyladenine sprayed on leaves) diminishes PEPCase induction under conditions of salt and drought stress and in well-watered old plants. This suggests a possible signal transduction chain, implying that a decrease in water and concomitant cytokinin movement from the roots may be measured by a cytokinin concentration sensor in the leaves and trigger induction. This hypothesis is currently under investigation in our laboratory. If this turns out to be correct, it follows that detached leaves that are cut off from water and cytokinin supply may be an excellent system to dissect the relative effects of these two factors on PEPCase induction.

CAM induction in *Mesembryanthemum* has been interpreted to be an adaptation to water deficit, resulting in water conservation and, therefore, in dehydration avoidance. CAM as measured by acid cycling, however, is expressed after a lag period of days, following the decrease of the water and solute potentials of leaves (7). Water conservation by CAM, therefore, has been considered to be a late, secondary response to salt stress, which supposedly must be preceded by other adaptive mechanisms like proline accumulation to enable survival of the plant under saline conditions (1).

Rapid induction of PEPCase transcripts (Figs. 4 and 5) and activity (14) under wilting rather than saline conditions, however, shows that leaves are able to rapidly react to water deficits inducing PEPCase synthesis within hours. Similar observations have been made in *Sedum telephium*. This species, CAM induction as measured by acid cycling can be observed 3 to 5 d after withholding water from whole plants, but within 20 h in the drying detached leaves (6).

In a previous paper, we have shown that induction of PEPCase protein occurred in both young and old leaves. It was observed that the age of the tissue source within the plant had no influence on the induction of PEPCase in response to salt stress (12). This clearly was an oversimplification. It is apparent that both the rate of induction of PEPCase transcripts and activity as well as their abundance after a 9-d stress treatment vary somewhat between leaves (Fig. 3). It should be noted that leaf pair 5, which shows the highest induction rate for PEPCase transcripts, is almost totally unshaded by other leaves.

![Diurnal changes in water content of leaves](image)

**Figure 4.** Abundance of transcripts for PEPCase (ppc) and ribulose biphosphate carboxylase (rbcS) in leaves from young and old plants as affected by salt stress, wilting, and rehydration. For 6 d before harvesting the leaves, plants were either stressed with 0.5 M salt solutions (prestressed) or well-watered. Three hours after the beginning of the light period, leaves were cut from node 3 of 5-week-old plants and from node 5 of 4-week-old plants. For an 8-h period, the detached leaves were either wilted (Wi) on the greenhouse bench or hydrated (Rh) by immersing the petioles in 1 mM CaCl2. Water content (RWC) as determined by weighing is given relative to the initial weight. Control leaves (Co) were left attached to the plants and were harvested at the end of the experiment from nodes 2 and 4 of 5-week-old plants and from node 4 from 4-week-old plants. Autoradiographic signals for *rbcS* and *ppc* mRNA from Northern hybridization detection were quantified using a Cybertech video densitometer.

![Steady-state transcript levels](image)

**Figure 5.** Steady-state transcript levels for PEPCase and the small subunit of ribulose biphosphate carboxylase (SSU) as a function of water loss of detached leaves. Leaves (node 5) derived from 2-week-old well-watered plants were cut in the morning, 4 h after the beginning of the light period. They were wilted on the greenhouse bench (25°C and 50% relative humidity) and were subsequently transferred into a humid, thermostated chamber in the light. Eight hours after cutting, all samples were weighed and then frozen in liquid nitrogen for later RNA extraction. Leaves from the same node (0% water loss) were harvested from the plant at the end of the experiments to serve as controls. Water loss is indicated in relation to the fresh weight immediately after cutting the leaves.
At a certain plant age, usually about week 7, induction also becomes apparent in well-watered plants (Fig. 1A, B). It should be kept in mind that we have used the term well-watered instead of unstressed because irrigation with too much nutrient solution may cause anoxia in the roots, which is known to induce CAM in *Mesembryanthemum* (20). The plant age at which a response of *ppc* transcripts to a short 4-d salt stress was observed varied between 2 and 4 weeks, i.e. the curves as seen in Fig. 1A and B were shifted along the time axis, as was the case with respect to PEPCase mRNA and activity increases in well-watered plants (compare Figs. 1 and 4). This may explain our previous observation (12) that young, 5-week-old plants are not competent to induce PEPCase enzyme in a 5-d stress period. The general sigmoidal shape of the induction curves, however, was similar between experiments. There is no apparent juvenile age that would not permit the induction of *ppc* transcripts in response to stress. Even 2-week-old seedlings with a shoot fresh weight of less than 1% of the mature plants are able to induce PEPCase transcripts to 400% of the unstressed level in a 6-d stress period (Fig. 2). One-week-old seedlings with a shoot weight of 25 mg are able to induce *ppc* transcripts after 8 h of wilting (data not shown). Freshly germinated seedlings weighing 6.5 mg (median) have not been tested.

When detached leaves are drought-stressed, both 5-week- and 9-week-old plants induce PEPCase transcripts rapidly. Drought-stressed leaves derived from a well-watered 9-week-old plant induce better than drought-stressed leaves derived from a well-watered 5-week-old plant. Based on the degree of previous induction, however, further induction by drought becomes very similar; the response of a wilting leaf from a 9-week-old plant can be mimicked in a leaf from a 5-week-old plant by prestressing with sodium chloride. The reverse process of hydration also leads to similar and extremely low transcript levels in leaves, regardless of plant age or pretreatment (Fig. 4). Our data would be consistent with the view that well-watered old plants differ from young plants with respect to PEPCase transcript induction kinetics under stress mainly by intensity and duration of previous water deficits. We propose that mild water deficits caused by diurnal transient decline in leaf water content (7, 18) are sufficient (Fig. 5) to lead to induction in well-watered plants (Fig. 1) and intensify the response to salt stress in intact plants (Fig. 1) or drought stress in detached leaves (Fig. 4).

In an ecological study, Winter and coworkers (21) have measured CAM induction in *M. crystallinum* under natural conditions at a site near Caesarea, Israel. *Mesembryanthemum* plants germinate in the humid winter season. When precipitation decreases in spring, plants gradually start to perform CAM. The decrease of soil water content in the observation period had a half-time of approximately 2 months and pronounced acid cycling was not observed before the plants were 12 weeks old. At that time, soil water content had decreased to about half its maximal value (21). A stepwise increase in PEPCase activity around week 7, which is observed in the climate chamber (Fig. 1A), has not been observed in nature (21). There is evidence that the synchronous and relatively uniform growth of *Mesembryanthemum* plants as observed in Caesarea may not be the only growth pattern. At a site on the island of Teneriffa (Cañon del Infierno), all stages of development from seedling to flowering plant could be observed side by side at the beginning of March 1991 (G. Meyer, personal communication). A predetermined, developmentally controlled increase in PEPCase would have no adaptive value for the plants at this site. On the contrary, plants switching to CAM too early in the season under still favorable water conditions would be expected to grow slower than their *C3* counterparts and would thus be at a disadvantage. A rigid differentiation program switching to CAM metabolism in anticipation of summer drought is not necessary for survival in the natural habitat because the perception of water deficit is highly sensitive and the response is very rapid (Fig. 5).

The increase in PEPCase activity in well-watered plants occurs in a stepwise manner within 1 week (Fig. 1A) rather than continuously over the lifetime of the plant (4). The time of the increase correlates with a decrease in growth rate (Fig. 1E). This decrease in growth is not caused by a programmed developmental switch, because plants in the natural habitat show no break in growth rate and grow much larger, reaching shoot weights of 1 kg or more (21). The decrease in growth is probably caused by limitations in water and/or nutrients imposed by the root system in the small pots used in this and previous work (12). Seven-week-old plants grown in 5.7 L pots under our conditions have a shoot fresh weight of about 400 g, compared with 70 g for plants grown in our standard 0.7 L pots (Fig. 1). Preliminary results indicate that PEPCase induction occurs later in well-watered plants if the plants are grown in larger pots.

CAM, as measured by acid cycling, has been induced in 9-week-old plants grown in the absence of added salt at 50% relative humidity and 16,000 lux. When plants were grown in half the light intensity and 95% relative humidity, *i.e.* conditions favoring water conservation, no acid cycling could be observed in plants of the same age (18).

PEPCase mRNA, once accumulated, can rapidly be degraded upon rehydration of detached leaves (14) or intact plants (17). PEPCase transcripts in leaves are down-regulated regardless of whether the original level had been reached by prior salt-stress or by stepwise accumulation without apparent stress in old, well-watered plants. The rapid and extensive down-regulation of *ppc* transcripts by hydration of leaves suggests that water status rather than developmental status is the predominant factor in PEPCase induction.

Taken together, present evidence indicates that CAM induction and the preceding increase of PEPCase activity that is observed in old, well-watered plants is regulated by environmental factors rather than by a genetically determined developmental program.

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**LITERATURE CITED**


