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Short Communication

Induction of PEP Carboxylase and Crassulacean Acid Metabolism by Gibberellic Acid in *Mesembryanthemum crystallinum*

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The induction of Crassulacean acid metabolism in *Mesembryanthemum crystallinum* was investigated in response to foliar application of gibberellic acid (GA). After 5 weeks of treatment, GA-treated plants showed 1.7- to almost a 4-fold increase of phosphoenolpyruvate carboxylase (PEPcase) activity with a concomitant increase in acid metabolism when compared to control plants. Immunoblot analysis indicated an increase in the PEPcase protein similar to that of salt treatment while Rubisco did not show a similar rise. The results indicate that exogenously applied GA accelerates plant developmental expression of PEPcase and Crassulacean acid metabolism in *M. crystallinum*.

Key words: Crassulacean acid metabolism — Development — Gibberellic acid — *Mesembryanthemum crystallinum* — Phosphoenolpyruvate carboxylase.

Abbreviations: GA, gibberellic acid; CAM, Crassulacean acid metabolism; LP, leaf pair; PEPcase, phosphoenolpyruvate carboxylase; Rubisco, ribulose biphosphate carboxylase.

The annual, *Mesembryanthemum crystallinum*, is a facultative CAM (Crassulacean acid metabolism) species, shifting from C₃ photosynthesis to CAM, as noted by an increasing amount of nocturnal CO₂ uptake, increases in the titratable diurnal acid fluctuation, and a shift of the carbon isotope composition from –26 to –16‰ during the course of the season (Winter and Troughton 1978, Bloom and Troughton 1979, Winter et al. 1978). During CAM induction in *M. crystallinum*, the primary enzymes, phosphoenolpyruvate carboxylase (PEPcase) and NADP-malic enzyme, of the CAM pathway are synthesized de novo (Foster et al. 1982). In addition, salt stress in *M. crystallinum* induces transcription of a CAM-specific isoenzyme of PEPcase that can be separated from its C₃ counterpart (Cushman et al. 1989).

Other factors, such as leaf aging and development have been implicated in the plant's ability to perform CAM in *M.*

crystallinum (Von Willert et al. 1985). There is a slow rise in PEPcase during development under unstressed conditions (Cushman et al. 1990). Three-week-old plants show little induction when salt stressed, but by 6 weeks of age, salt stressed plants are able to show a large induction of the PEPcase protein (Cushman et al. 1990). Hormonal control has also been implicated in the induction of CAM. Exogenous application of ABA increased diurnal acid fluctuations and PEPcase activity (Chu et al. 1990) and increased the expression of PEPcase mRNA (Cushman et al. 1989). This indicated that salinity might induce CAM by increasing the levels of ABA in the plant. In contrast, work by Dai et al. (1994) found three growth regulators (ABA, farnesol, a precursor of ABA, and benzylaminopurine) when fed through the roots were able to induce CAM in *M. crystallinum* comparable to that of salt stress. However, GA fed through the roots showed no observable CAM induction (Dai et al. 1994).

Research has also shown that leaf age and long day photoperiod may play a role in the expression of CAM in *M. crystallinum* (Cushman et al. 1990, Cheng and Edwards 1991). It is known that GA can substitute for a long day photoperiod requirement in a number of plant species (Taiz and Zeiger 1998). In this study we tested the hypothesis that CAM induction may be mediated through exogenous foliar applications of gibberellic acid by altering plant development. Our results show that exogenously applied GA increased the levels of PEPcase activity and the abundance of PEPcase protein in *M. crystallinum* with a concomitant increase in acid metabolism.

Mesembryanthemum crystallinum (Family: Aizoaceae) seeds were germinated on vermiculite moistened with Hoagland's solution. After 1–2 weeks, when the first leaf pair (LP) was visible, the seedlings were transferred into larger containers. The plants were grown in a growth chamber with a 10-h day/14-h night period with a day/night temperatures of 30/17°C, respectively. The plants were watered daily and once a week with half strength Hoagland's solution with micronutrients. Plants were grown under an irradiance of 300–400 μmol m⁻² s⁻¹ with a relative humidity of 50–60%. When the plants were 3 weeks old, they were split into two groups, a control

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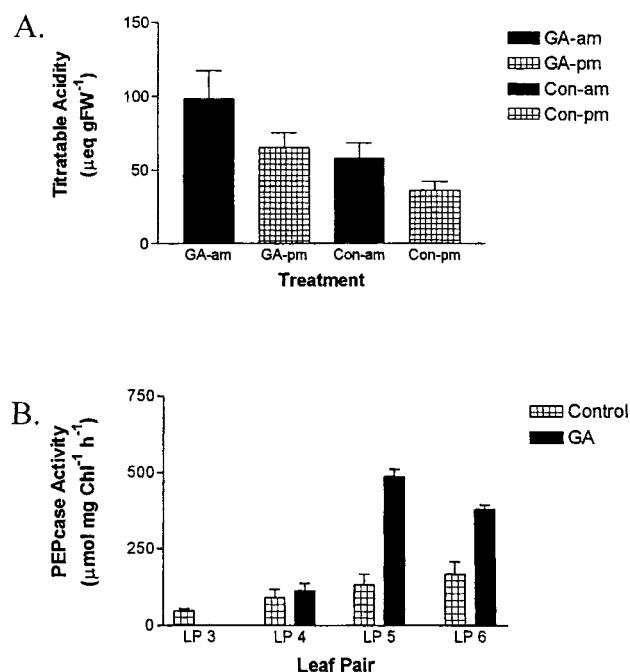


Fig. 1 Diurnal fluctuation of total titratable acids and PEPcase activity in *M. crystallinum* after 6 weeks of treatment. (A) Total titratable acidity (am and pm acid levels) of axillary leaves. Samples were taken at the beginning and end of the light period. (B) PEPcase activity in control and GA-treated plants. Leaf pairs (LP) were counted with LP 1 from the bottom of the plants. LP 3 for GA-treated plants had senesced when the assays were completed. Error bar represents 1 SE of the mean. Typical results from replicate experiments.

group which was sprayed every third day with a 1% Tween solution and gibberellic acid (GA₃, Sigma Chemical Co.) treated group, which was treated with 10⁻⁵ M GA and 1% Tween solution, and similarly sprayed every third day. Plants were sprayed 1 h before the end of the light period to wet the leaves, which depended on the size of the plant, with between 10 and 20 ml of solution. Plants at 3 weeks had two LPs with the third LP still developing. Portions of the control plants were irrigated with 450 mM NaCl for the last 5 d of the treatment period for CAM induction prior to collection. Samples for analysis were collected after 3–6 weeks of treatment as indicated in the figure legends. Plants at the end of treatment had six LPs and axillary stems.

Two leaves representing each LP were collected at beginning and end of the light period, and frozen (–20°C) until assayed. The leaves were weighed and ground in 20 ml double distilled H₂O. They were titrated with 0.01 M KOH to an endpoint of pH 7.0. The results are expressed in (µeq g FW)⁻¹.

Leaf samples for PEPcase activity were collected in duplicate in the late afternoon when levels of malate and potential for inhibition would be the lowest. Extraction of PEPcase was according to the method of Guralnick and Ting (1988). An

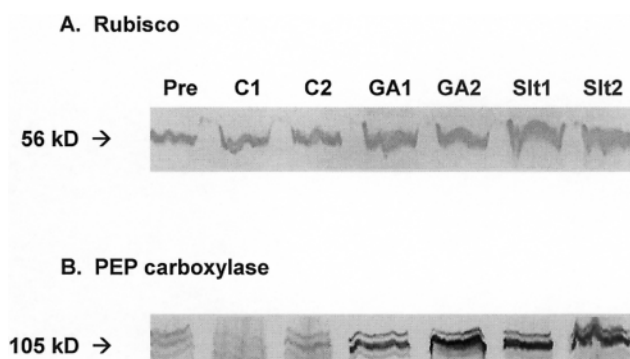


Fig. 2 Western immunoblots for *M. crystallinum* leaf Rubisco and PEPcase. Leaf samples were harvested at the end of a 6-week treatment period. Pairs of samples were collected consisting of LP 5 and axillary leaves. Salt-treated plants were control plants irrigated for 5 d prior to the end of experiment. Abbreviations: Pre=pretreatment (3-week-old plants prior to spraying); C=control; GA=gibberellic acid treated; Slt=salt treated; 1=LP 5; 2=axillary leaves.

aliquot of 1 ml was taken for chlorophyll determination as described by Guralnick and Ting (1988). The crude extract was used to assay enzyme activity spectrophotometrically by following the method of Guralnick and Ting (1988).

Approximately 1 g of tissue was collected for each sample and ground in 5 ml extraction buffer containing 0.7 M sucrose, 0.5 M Tris (pH 7.5), 30 mM HCl, 50 mM EDTA, 0.1 M KCl, 2% (v/v) β-mercaptoethanol and 5% (w/v) insoluble PVP, and 5 ml of water-saturated phenol. The protein was isolated following the procedure of Drincovich et al. (1998). This procedure avoids proteolysis of protein. For electrophoretic analysis of proteins, a SDS-PAGE system with a 7.5% to 15% acrylamide linear gradient was used. Twenty micrograms of protein per sample was loaded onto the gels for PEPcase immunoblots and 1 : 4 dilution of the sample was used for ribulose biphosphate carboxylase (Rubisco) large subunit immunoblot analysis. Immunoblots were done according to Drincovich et al. (1998).

Overall plants that were sprayed with GA showed earlier branching, flowering, and marked stem elongation when compared to control plants. Senescence of the lower leaves occurred earlier in the GA group. Marked stem elongation was in particular indicative that the foliar application of GA was entering into the leaf where as the Tween solution alone had no effect on the overall development of the control plants.

The degree of diurnal acid fluctuations increased in leaves of both the control and GA-treated plants over the course of the experiment (data not shown). The diurnal fluctuations in acidity in axillary leaves after 6 weeks of treatment did not show a statistically significant difference between the control and GA-treated plants (Fig. 1A). Leaf pair 5 and 6 also showed a 22 (µeq g FW)⁻¹ acid fluctuation similar in magnitude to the

axillary leaves. However, the GA treated plants accumulated approximately 1.7 times more titratable acid than the control plants. After 5 weeks of treatment the PEPcase activity was higher in the GA treated plants for both LP 5 and 6 (Fig. 1B). Repeats of the experiments showed similar increases in PEPcase activity in GA-treated plants, ranging from 1.7 times to almost a 4-fold increase over control plants (data not shown).

Immunoblot analysis was done for both PEPcase and the large subunit of Rubisco (Fig. 2). There were slight differences in Rubisco with the GA- and salt-treated plants showing a slightly higher abundance than that of control plants. PEPcase showed a different pattern. Samples taken before treatment at 3 weeks showed three weak bands consistent with those reported earlier (Dai et al. 1994). Control leaves harvested at the end of the experiment indicated a similar pattern in both LP 5 and the axillary leaves but the axillary leaves had more PEPcase protein (Fig. 2). GA-treated plants had a higher abundance of PEPcase protein in both LP 5 and the axillary leaves when compared to control plants, especially the second isoform, which increased the most. The pattern and abundance of PEPcase protein in GA-treated plants was comparable to that of salt-treated plants.

It is known that both stage of development and stress (salinity or drought) effects the induction of CAM in *M. crystallinum* (above). The results of the present study indicate that exogenously applied GA increased the levels of PEPcase activity and the abundance of PEPcase protein in *M. crystallinum*. The immunoblot pattern and abundance of PEPcase protein in GA-treated plants was comparable to the results obtained by Cushman et al. (1990). Also, there was increased PEPcase activity in younger leaves in the GA-treated plants when compared to the control plants. These results indicate a possible role of GA in the development of CAM in *M. crystallinum*. Other developmental effects of GA, such as earlier flowering, were also observed. Consistently, the acid levels in the GA-treated group were higher than the control group. As plants became older there was an increase in the acid fluctuation which is consistent with previous results. This was most likely due to the developmental status of the plant as the capacity for CAM increases with age of the plant (Chu et al. 1990, Cushman et al. 1990, Cheng and Edwards 1991).

These results with GA-treated plants are in contrast to those of Dai et al. (1994). Dai et al. (1994) fed GA through the roots of the plants in a nutrient solution and found there was no induction of the PEPcase protein. In this study, we applied GA to the leaves of the plants by spraying. There is little evidence thus far for root transport of GA (Coolbaugh 1985) unlike that for the transport of cytokinin from the root as proposed by Schmitt and Piepenbrock (1992).

However, results by Dai et al. (1994) with ABA, farnesol, and benzylaminopurine showed an induction of CAM activity within a week of application. The results in this study with GA show a slower time course of increased expression of PEPcase of 3–6 weeks under the short day photoperiod. GA may accel-

erate development of CAM in *M. crystallinum* by inducing earlier branching and flowering under short photoperiods. Cheng and Edwards (1991) found that longer photoperiods accelerated branching and flowering of *M. crystallinum* and development of CAM. Thus, exogenously applied GA may mimic the effect of long days in development.

In *M. crystallinum*, both long days and developmental status are important in the induction of CAM. The diurnal acid fluctuations found in this study were similar in magnitude to the fluctuations found by Cheng and Edwards (1991) when well-watered plants were grown under a long-day photoperiod. Cheng and Edwards (1991) also found an increase in PEPcase activity under the long-day photoperiod similar to results obtained in the present study under short-day photoperiods plus GA. The similarity of results of the GA treatment and long-day photoperiod without GA may indicate a role of GA in photoperiod-dependent processes. GA interactions with photoperiodic processes in plants has been well documented (Metzger 1995). It has been shown that GA can replace the long day requirement for floral induction in CAM plant, *Bryophyllum (Kalanchoe) daigremontianum* (Zeevaart and Lang 1962). Interestingly floral induction is correlated with CAM induction in *Kalanchoe* spp. (Queiroz and Brulfert 1982). These effects indicate gibberellin may be an important growth hormone in controlling development of CAM metabolism in some species (e.g. *M. crystallinum*) and having a role in the induction and development of CAM as the photoperiod increases. Future research is needed to determine how GA exerts its effect and whether it can promote the induction of CAM by its actions on promoter of genes for the key enzymes of the CAM signaling pathway via protein kinases (Cushman and Bohnert 1997). Also, it would be of interest to evaluate endogenous levels of GA during the development of leaves under different photoperiods and interactions with ABA in induction of CAM in facultative CAM plants under drought or salinity conditions.

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