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Communication

PEPCase Transcript Levels in *Mesembryanthemum crystallinum* Decline Rapidly upon Relief from Salt Stress[1]

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ABSTRACT

Mesembryanthemum crystallinum **plants respond to water stress by changing their pathway of carbon assimilation from C_3 to Crassulacean acid metabolism (CAM). Stressed plants are characterized by elevated levels of phosphoenolpyruvate carboxylase (PEPCase) mRNA, protein, and enzyme activity. We wanted to determine whether CAM is a reversible response to environmental conditions or a developmentally programmed adaptation that is irreversibly expressed once induced. Plants were osmotically stressed by irrigation with 500 millimolar NaCl for 12 days to elicit CAM. Salt was then thoroughly flushed from the soil and PEPCase protein and transcript levels were monitored. PEPCase mRNA levels dropped by 77% within 2.5 hours after salt removal. PEPCase activity and polypeptide levels declined more slowly, with a half-life of 2 to 3 days. These results show that PEPCase expression in *M. crystallinum* is a reversible response to stress that is regulated at the level of transcription or stability of the PEPCase mRNA.**

When subjected to water stress induced by drought or salinity, *Mesembryanthemum crystallinum* plants shift their pathway of carbon assimilation from C_3 metabolism to CAM (9). This change in metabolism involves *de novo* synthesis of PEPCase[2] (1, 2) and an increase in the level of translatable PEPCase mRNA (5). Increased PEPCase enzyme activity is evident 2 to 3 d after irrigation with 500 mM NaCl (5).

We are using CAM induction and the expression of PEPCase in *M. crystallinum* as a system to study changes in gene expression caused by environmental stress. It has not yet been shown whether salt stress affects gene expression reversibly. There is evidence that PEPCase expression is regulated in part by development. PEPCase activity does not increase during a 5 d irrigation with 500 mM NaCl in plants less than approximately 5 weeks of age (5). In addition, we have observed that PEPCase activity, protein, and transcript levels rise gradually with age in unstressed plants (our unpublished data). It seems possible, therefore, that plants might be developmentally programmed to express CAM enzymes and that stress acts to trigger the irreversible expression of this pathway. Von Willert *et al.* (8) have reported that removing NaCl from the soil of salt-stressed *M. crystallinum* leads to a decline in PEPCase activity. Here we correlate a drop in activity with changes in gene expression by demonstrating that relief from stress causes a rapid decrease in PEPCase mRNA followed by a slower decline in the amount of PEPCase protein.

MATERIALS AND METHODS

Plant Material. *M. crystallinum* plants were grown from seed as previously described (5). Plants were stressed when 6 weeks old by watering each day with nutrient solution containing 500 mM NaCl. Salt was removed from the soil (destress) after 12 d of NaCl treatment by flushing the soil with distilled water continuously for 2 h at the start of the light period. Plants were watered with nutrient solution immediately after salt removal and daily until harvest. Controls were irrigated with either nutrient solution or nutrient solution with 500 mM NaCl until harvest. Plant material was collected for RNA extraction immediately prior to NaCl removal and at 0.5, 2.5, 4.5, 8, and 32 h after destress. Plant material was harvested for protein extraction immediately before NaCl removal and 2 h before dark on d 1, 2, 3, 6, and 9 of destress. Each sample included material from three individual plants. All samples were frozen in liquid N_2 and stored at −70°C.

Preparation and Analysis of Protein and RNA Samples. RNA and soluble protein extractions, PEPCase activity assays, SDS-PAGE, and immunoblotting were performed as previously described (5).

Total RNA was slot-blotted onto nitrocellulose (7) following the protocol supplied by the manufacturer (Schleicher and Schuell, Keene, NH). Nitrocellulose filters were prehybridized at 42°C in 6× SSC containing 50% (v/v) formamide and 0.25% (w/v) nonfat dry milk (3). Filters were then hybridized overnight under prehybridization conditions with an [α-^{32}P]dCTP-labeled probe made from a cloned 950-bp portion of the 3′ coding region of a *M. crystallinum* PEPCase gene (pMcPEP1; 6). High stringency washes were carried out in 0.1× SSC/0.1% (w/v) SDS at 55°C, and results were visualized by autoradiography. Quantitation was performed with a GS 300 densitometer (Hoefer Scientific Instruments).

RESULTS

Total activity of PEPCase was followed for 9 days after destress (Fig. 1). PEPCase began to decline by the evening of d 2, 32 h after salt removal. After 3 days PEPCase activity had dropped

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[2] Abbreviations: PEPCase, phosphoenolpyruvate carboxylase (EC 4.1.1.31); SSC, 15 mM NaCl, 1.5 mM sodium citrate (pH 6.8).

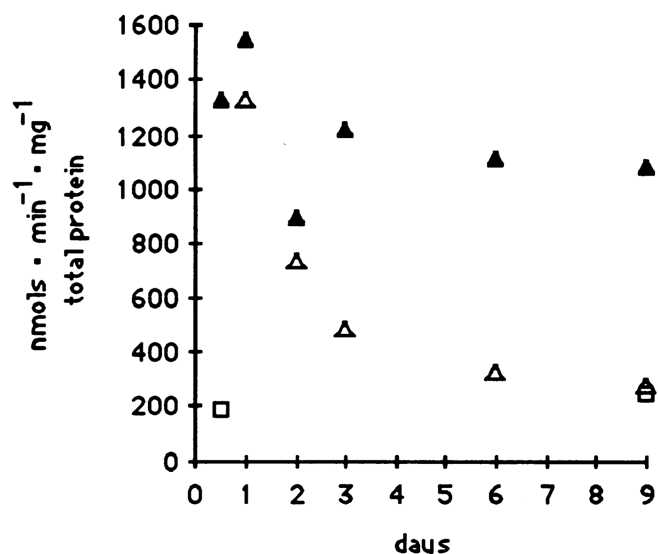

FIG. 1. Total PEPCase activity in samples from destressed plants and salt-stressed and unstressed controls. Total PEPCase activity was measured in soluble protein extracts from destressed (△), stressed (▲), and unstressed (□) plants. Plants were harvested 2 h before dark on the days indicated, with the exception of the controls harvested before destress on d 1 (data points at 0.5 d). Material from 3 plants was combined for protein extractions from each timepoint.

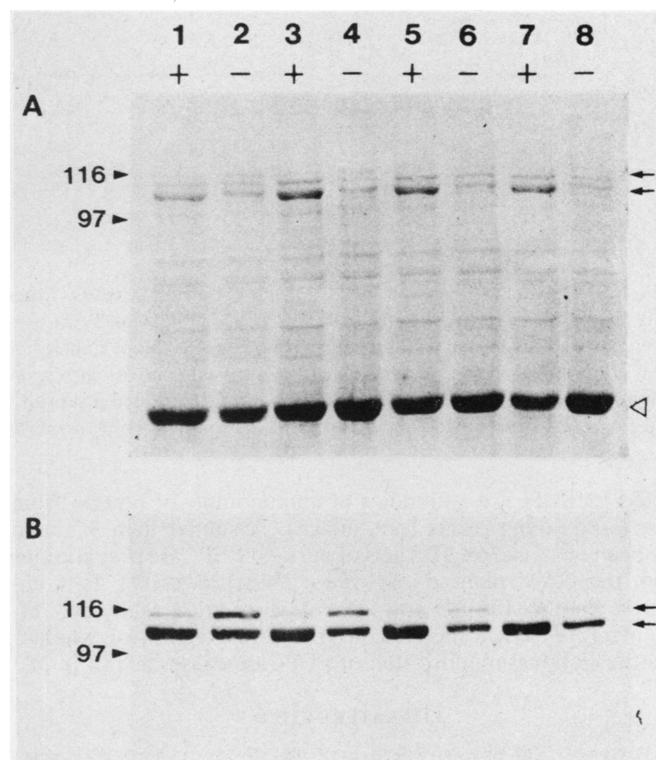

FIG. 2. Decline in the amount of PEPCase protein after destress. Equal amounts of total soluble protein from stressed (+) and destressed (−) plants were resolved by 10% SDS-PAGE and either stained (A) or immunoblotted with anti-PEPCase antibodies (B). Plants were harvested 2 h before dark on d 2 (lanes 1 and 2), d 3 (lanes 3 and 4), d 6 (lanes 5 and 6), and d 9 (lanes 7 and 8) of destress. Arrows indicate the position of the 105 and 115 kD PEPCase bands. The position of the large subunit of ribulose-1,5-bisphosphate carboxylase is indicated (∇).

to approximately 40% of the value of stressed controls. By d 9 PEPCase activity was 26% of that in stressed controls of the same age, and it was almost equal to the activity in unstressed plants harvested the same day.

The decline in PEPCase activity was accompanied by a decline in PEPCase protein levels (Fig. 2). Equal amounts of total soluble protein extracted from plants harvested 2, 3, 6, and 9 d after salt removal were separated by SDS-PAGE, and the gels were either stained (Fig. 2A), or immunoblotted with anti-PEPCase antibodies (Fig. 2B). A gradual decrease in the amount of PEPCase (approximately 105 kD) was evident in samples from destressed plants. An immunologically reactive band was also present at a position corresponding to a M_r of approximately 115 kD. The intensity of this band increased transiently after salt removal.

The most dramatic response to destress was the decline in PEPCase mRNA levels (Fig. 3). Equal amounts of total RNA from stressed and destressed plants were blotted and probed with a cDNA fragment containing 950 bp of the 3′ coding region of a PEPCase gene from *M. crystallinum*. Densitometry of the autoradiogram demonstrated that the amount of PEPCase mRNA declined by 77% within 2.5 h after destress (lane 3 *versus* lane 4) and by 84% by the evening of d 2, 32 h after salt removal (lane 7 *versus* lane 9).

Figure 3 also reveals that PEPCase mRNA levels in stressed plants fluctuated during the day. The transcript was least abundant in the morning (lanes S and 1) and it increased during the day (lanes 3, 5, and 7). This result has recently been confirmed and is being investigated in more detail (R Höfner, personal communication).

DISCUSSION

We have shown that PEPCase activity increases following irrigation with 500 mM NaCl only when plants are approximately 5 weeks of age or older (5). In addition, PEPCase activity, mRNA transcript, and protein levels appear to increase gradually with age in unstressed plants (our unpublished data). One interpretation of these observations is that the expression of CAM enzymes in *M. crystallinum* is governed at least in part by development. If this is the case, it might be expected that relief from stress would have little effect on PEPCase levels once the enzyme is induced. The data reported here, however, demonstrate that the induction of PEPCase can be reversed after the plants have shifted to CAM. When salt is removed from the soil of stressed plants there is a rapid decrease in the amount of mRNA for the enzyme followed by a slower decline in PEPCase protein and activity levels. It has been shown that PEPCase activity declines after salt removal in 9 week old plants that have been stressed for 5 weeks with 300 mM NaCl (8). In our experiments we correlated a decline in activity with a parallel decline in PEPCase protein and reduced amounts of PEPCase mRNA. Further work will be necessary to determine whether the decline in mRNA is due to transcriptional regulation or reduced mRNA stability after destress.

It should be noted that the response to salt stress varies between plants. In these experiments we analyzed 7.5 week old plants that had been stressed for 12 d. Generally, PEPCase activities in unstressed 7.5 week old plants vary from 110 to 190 nmol·min^{-1}·mg^{-1} total protein. In plants of the same age subjected to 12 d of stress with 500 mM NaCl, PEPCase activities range from 800 to 1600 nmol·min^{-1}·mg^{-1} total protein (Fig. 1; our unpublished data).

The immunoblot of protein extracts from destressed plants revealed the gradual decline of a PEPCase band of an apparent M_r of 105 kD (Fig. 2B). This band is the most prominent band in stressed plants. Another band (approximately 115 kD) increased transiently after destress. This polypeptide is also de

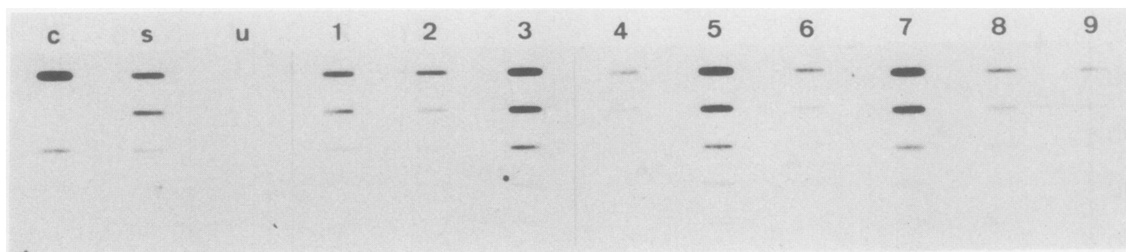

FIG. 3. Decline in PEPCase mRNA levels following destress. Equal amounts of total RNA from destressed plants and controls were blotted onto nitrocellulose and hybridized to a [α-^{32}P]dCTP-labeled PEPCase cDNA. Twofold serial dilutions (starting with 10 μg) of RNA from each timepoint were loaded in order of decreasing concentration. Lanes 2, 4, 6, 8, and 9 contain RNA from plants harvested 0.5, 2.5, 4.5, 8, and 32 h after salt removal, respectively. RNA from stressed controls corresponding to the first four time points is in lanes 1, 3, 5, and 7. Lanes S and U contain RNA from stressed and unstressed controls harvested just prior to salt removal. Lane C, Controls for RNA binding and probe hybridization: 10 μg of RNA from CAM (upper slot) and C_3 (lower slot) *M. crystallinum*.

tected by PEPCase antibodies in immunoblots of protein from unstressed young plants (not shown). A similar high M_r band has been observed on SDS gels of purified PEPCase preparations from the CAM plant *Bryophyllum fedtschenkoi* (4). It is unknown whether the 105 and 115 kD bands are actually two different proteins, *e.g.* derived from different genes, or whether posttranslational modification of PEPCase causes a shift in M_r.

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