Plant Physiol. (1987) 84, 1270-1275
0032-0889/87/84/1270/06/$01.00/0

Salt Stress Increases the Level of Translatable mRNA for Phosphoenolpyruvate Carboxylase in *Mesembryanthemum crystallinum*[1]

Received for publication February 19, 1987 and in revised form April 28, 1987

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ABSTRACT

Mesembryanthemum crystallinum **responds to salt stress by switching from C_3 photosynthesis to Crassulacean acid metabolism (CAM). During this transition the activity of phosphoenolpyruvate carboxylase (PEPCase) increases in soluble protein extracts from leaf tissue. We monitored CAM induction in plants irrigated with 0.5 molar NaCl for 5 days during the fourth, fifth, and sixth week after germination. Our results indicate that the age of the plant influenced the response to salt stress. There was no increase in PEPCase protein or PEPCase enzyme activity when plants were irrigated with 0.5 molar NaCl during the fourth and fifth week after germination. However, PEPCase activity increased within 2 to 3 days when plants were salt stressed during the sixth week after germination. Immunoblot analysis with anti-PEPCase antibodies showed that PEPCase synthesis was induced in both expanded leaves and in newly developing axillary shoot tissue. The increase in PEPCase protein was paralleled by an increase in PEPCase mRNA as assayed by immunoprecipitation of PEPCase from the *in vitro* translation products of RNA from salt-stressed plants. These results demonstrate that salinity increased the level of PEPCase in leaf and shoot tissue via a stress-induced increase in the steady-state level of translatable mRNA for this enzyme.**

Little is known about the mechanisms which govern plant gene expression during periods of environmental stress. *Mesembryanthemum crystallinum* responds to salt stress, or other conditions which create water stress, by switching the primary path of CO_2 fixation from C_3 photosynthesis to CAM (22–24). CAM is a well-defined biochemical and physiological adaptation to water stress (for reviews see Refs. 18 and 20). The induction of CAM in response to salinity provides a unique opportunity to examine the molecular basis of stress perception and response in a higher plant species. We are investigating the sequence of events that lead from the initial stages of salt stress to the synthesis of polypeptides essential to the CAM photosynthetic pathway in *M. crystallinum*. As part of this work we have examined the effect of salt stress on the level of translatable mRNA for PEPCase,[3] a key enzyme in CAM.

PEPCase is a prominent high molecular mass (100 kD) soluble protein in plants assimilating carbon dioxide via the CAM pathway and is a useful indicator of CAM induction in *M. crystallinum*. PEPCase increased from approximately 1% of the total soluble protein in young plants to 10% of the soluble protein in mature salt-stressed leaves (27). On a shorter time scale, PEPCase activity in 8 week old plants increased 10-fold during a 7 d induction period (6). The increase in PEPCase activity was correlated with an increase in a 100 kD polypeptide tentatively identified as PEPCase (6). *In vivo* labeling and immune precipitation of newly synthesized PEPCase demonstrated that the enzyme was synthesized *de novo* in response to salt stress (13). Our objectives here were to determine (a) how soon after germination we could induce PEPCase synthesis in response to salt stress, (b) which plant tissue sources were most suitable for studying the induction process, and (c) if the induction of PEPCase synthesis during salt stress was due to increased levels of translatable mRNA for this enzyme.

MATERIALS AND METHODS

Plant Materials. *M. crystallinum* seed was planted in a potting soil mixture (Bacto potting soil/sand/vermiculite/peatmoss 3:2:2:1 v/v/v/v) and transplanted 10 d later into 1 L containers of the same soil mixture. Plants were thinned to one plant per pot and watered as needed with nutrient solution containing: 300 μM KNO_3, 200 μM $Ca(NO_3)_2$, 50 μM $(NH_4)H_2PO_4$, 50 μM $(NH_4)_2HPO_4$, 50 μM $MgSO_4$, 1 μM FeH_2-EDTA, 1.25 μM H_3BO_3, 0.01 μM $MnSO_4$, 0.1 μM $ZnSO_4$, 0.025 μM $CuSO_4$, and 0.025 μM MoO_3. Plants were grown until harvest in a growth chamber on a 12-h light (22°C)/12-h dark (18°C) cycle. Fluorescent and incandescent lighting provided a photon flux density of 200 $\mu mol/m^{-2} \cdot s^{-1}$.

NaCl Induction of PEPCase. All plants were grown under nonstressed conditions as described above prior to the start of each experiment. At the beginning of the fourth, fifth, and sixth week after germination plants were divided into groups which were watered each day for 5 d with either 100 ml of nutrient solution, or 100 ml of nutrient solution containing 0.5 M NaCl. All plant samples were taken 1 h before the end of the light period. Whole plants were rinsed briefly in water and frozen immediately in liquid N_2. Frozen plant tissue was ground in liquid N_2 using a mortar and pestle and stored at −70°C until extraction.

In another experiment unstressed plants and plants which had been watered with 0.5 M NaCl for 5 d during the sixth week after

[1] Supported by grants from the United States Department of Agriculture (85-CRCR-1-1677) to H. J. B., Deutsche Forschungsgemeinschaft and Deutscher Akademischer Austauschdienst to J. M. S. Travel was supported by NATO Collaborative Research grant RG230/84 to J. M. S. and H. J. B.

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[3] Abbreviations: PEPCase phosphoenolpyruvate carboxylase (EC 4.1.1.31); DEPC, diethylpyrocarbonate; Igs, immunoglobulins;

germination were separated into individual leaf and axillary shoot positions and frozen in liquid N_2. Leaf positions were numbered 1, 2, 3, etc., beginning with the first leaf pair above the cotyledons. Axillary shoots were designated 2A, 3A, etc., corresponding to their subtending leaf pair.

Soluble Protein Extraction. Frozen leaf tissue (0.1 g) was homogenized in 250 μl of TND buffer (90 mM Tris-HCl [pH 8.3] at 4°C, 90 mM NaCl, 9 mM DTT, 2 mM leupeptin) in a ground glass homogenizer. Extracts were clarified by centrifugation (15,000*g*, 10 min, 4°C). Soluble protein samples were prepared by removing the supernatants and adding glycerol to a final concentration of 10% (v/v) to the supernatants. The samples were frozen in liquid N_2 and stored at −70°C.

Determination of PEPCase Activity and Soluble Protein Concentration. Duplicate 50 μl aliquots of the soluble protein samples from unstressed and salt-stressed plants were assayed for PEPCase activity as described by Winter (25), except that the samples were not desalted before the assay. The concentration of soluble protein in each sample was determined in duplicate using the Bradford (3) assay with BSA as the standard.

PEPCase Purification. PEPCase was isolated from mature leaves of *Pennisetum americanum*, cv Senegal Bulk, as described by Hatch and Heldt (11) and HÖFNER *et al.* (13), using high performance anion exchange chromatography as a final step. The identity of the purified protein was confirmed by comparing the N-terminal sequence of several cyanogen bromide cleavage fragments with the deduced amino acid sequence of *Escherichia coli* PEPCase (7).

SDS-PAGE and Immunoblot Analysis. Samples were prepared for SDS-PAGE by mixing equal parts of the soluble protein samples with preheated SDS sample buffer (100°C) and boiling for 2 min. Proteins were resolved by SDS-PAGE using a discontinuous buffer system (17). Proteins were transferred electrophoretically onto nitrocellulose (4, 21). Nonspecific protein binding sites were blocked by incubating the nitrocellulose in NDM/TBS (5% w/v nonfat dry milk in 25 mM Tris-HCl [pH 7.3], 150 mM NaCl) for 1 h at room temperature (16). Immunoblots were prepared by incubating the nitrocellulose for 1 h at room temperature in NDM/TBS containing antibodies raised against *P. americanum* PEPCase. The nitrocellulose was washed in NDM/TBS (3 × 10 min) and incubated with horseradish peroxidase-linked goat anti-rabbit antibodies (Bio-Rad) for 1 h at room temperature. The nitrocellulose was then washed in NDM/TBS (3 × 10 min) and rinsed in distilled water. The protein-antibody complexes were visualized using a solution containing 3,3′-diaminobenzidine and cobalt (1).

RNA Preparation and *in Vitro* Translation. Frozen leaf tissue was ground in sterile TNE buffer (100 mM Tris-HCl [pH 8.0] at 25°C, 150 mM NaCl, 10 mM EDTA) containing 2 mM aurintricarboxylic acid (9) and filtered through Miracloth. SDS was added to the filtrate to 1% (w/v). The filtrate was mixed with an equal volume of phenol (500 g/200 ml water) containing 0.1% (w/v) 8-hydroxyquinoline. After 1 to 2 h at 4°C with occasional shaking the aqueous phase was removed and extracted twice with an equal volume of chloroform. Ammonium acetate was added to a final concentration of 0.3 M and nucleic acids were precipitated overnight at −20°C after the addition of an equal volume of isopropanol. The precipitates were pelleted and dissolved in sterile DEPC-treated water. Total RNA was recovered by two precipitations in the presence of 2 M LiCl. The RNA pellets were washed with 70% ethanol (v/v), dried under vacuum, dissolved in DEPC treated water, and again precipitated in the presence of 0.3 M ammonium acetate and an equal volume of isopropanol at −20°C.

Polyadenylated RNA [(A^+)RNA] was prepared from total RNA by oligo(dT)-chromatography (2). The A^+ RNA fractions were extracted once with phenol/chloroform (1:1, w/v) and twice with chloroform before precipitation in 0.3 M ammonium acetate and three volumes of ethanol at −20°C. Equal amounts of either total or (A^+)RNA from unstressed and salt-stressed plants were translated *in vitro* using a rabbit reticulocyte lysate system (Bethesda Research Laboratories). Optimal magnesium, potassium, and RNA concentrations were determined in preliminary experiments by following the incorporation of L-[^{35}S]methionine into TCA precipitable radioactivity. *In vitro* translations were terminated after 90 min by adding RNAse to a final concentration of 100 μg/ml and incubating the reactions at 30°C for 15 min. Duplicate 1 μl aliquots of the translation reaction mixtures were spotted onto Whatmann 3MM paper, incubated in 10% TCA (10 min, 4°C), in 5% TCA (2 × 5 min, 25°C), rinsed twice with ethanol and air dried. Radioactivity was determined by liquid scintillation counting.

Immunoprecipitations. Immunoprecipitations were preformed as previously described (5, 15). PEPCase Igs were prepared from serum by immunoaffinity chromatography using *P. americanum* PEPCase immobilized on an agarose gel support (Affigel-10, Bio-Rad). Total RNA from a 5-d salt-stressed plant was translated *in vitro* in the presence of L-[^{35}S]methionine. Aliquots (48 μl) of the translation mixture were heated to 100°C in the presence of 2% SDS (w/v) and diluted 10-fold with TNET buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, mM EDTA, 2% (v/v) Triton X-100, 20 mM L-methionine). The boiled and diluted translation mixtures were incubated with the affinity purified anti-PEPCase Igs (0.5, 1.0, 5.0 μg) at room temperature for 45 min before adding 2.5, 5.0, and 25 μl, respectively, of a 10% (w/v) *Staphylococcus aureus* cell suspension (Pansorbin-Calbiochem). After 15 min the *S. aureus* cells were pelleted through a 40% (w/v) sucrose pad. The pellets were washed twice in 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 0.1% (w/v) SDS, and rinsed once with 125 mM Tris-HCl (pH 6.8). The final pellets were resuspended in SDS sample buffer and boiled for 2 min.

RESULTS

Induction of PEPCase Activity by Salt Stress Is Influenced by Plant Age. Preliminary work with *M. crystallinum* showed that low levels of PEPCase activity were detectable in young unstressed plant material and that PEPCase activity increased gradually in older unstressed plants. Experiments were conducted to

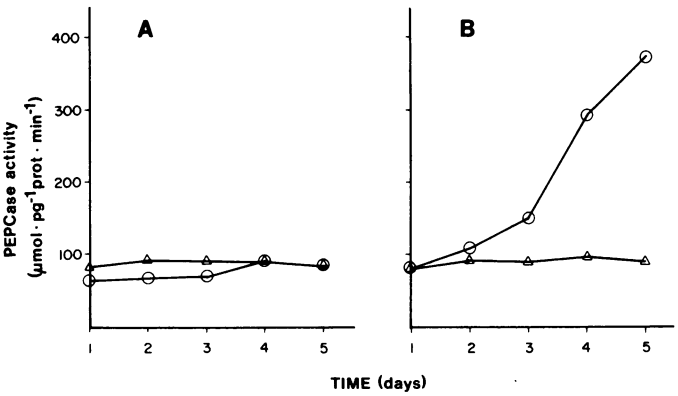

FIG. 1. Salt-stress induction of PEPCase activity in *M. crystallinum* during the fifth (A) and sixth (B) week after germination. Plants were watered with 100 ml of either nutrient solution (Δ) or nutrient solution containing 0.5 M NaCl (O) within 2 h after the beginning of each light period. Soluble protein extracts were prepared from whole plant samples taken 1 h before the end of the light period. The number of hours between the initial application of nutrient or NaCl solution and sample harvest is 8, 32, 56, 80, and 104 for d 1, 2, 3, 4, and 5, respectively. PEPCase activity in each sample was assayed in duplicate. Values shown are the means of two experiments.

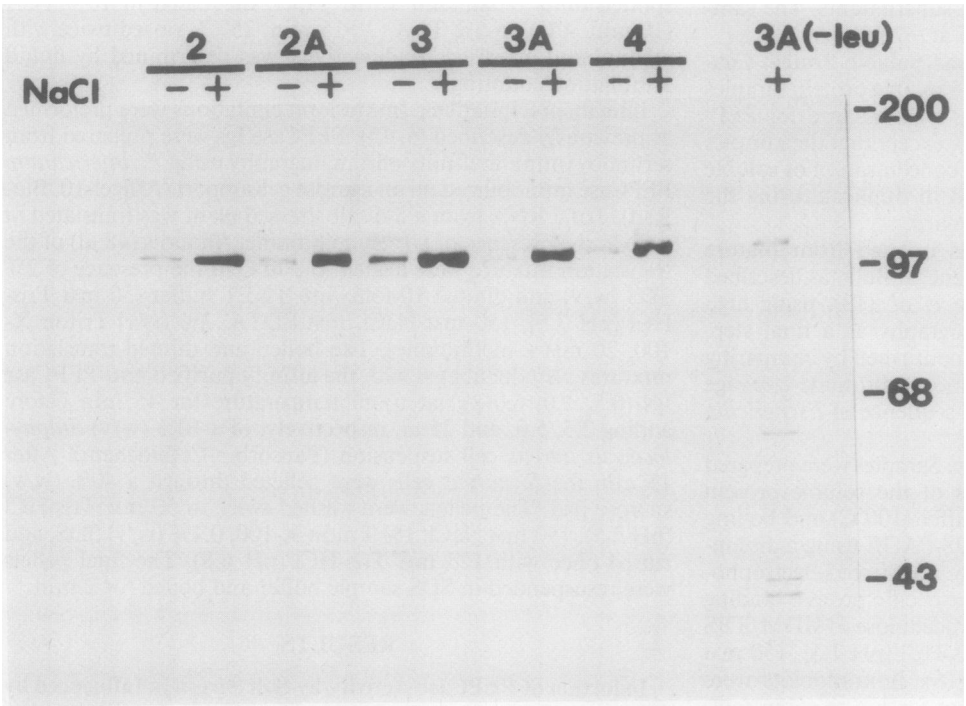

FIG. 2. Immunoblot identification of PEPCase in samples from leaf and axillary shoot positions before (−), and after (+), 5 d of salt stress. Frozen tissue from leaf positions 2, 3, 4, and axillary shoot positions 2A and 3A was extracted in TND buffer containing 2 mM leupeptin. An additional sample of frozen tissue from axillary shoot 3A (after 5 d salt stress) was extracted in TND buffer without leupeptin (3A (−leu)). The extracts were clarified by centrifugation and aliquots of each supernatant were combined with an equal volume of preheated (100°C) SDS sample buffer. Soluble proteins (5 μg/lane) were resolved by SDS-PAGE in a 10% polyacrylamide gel, and transferred to nitrocellulose. PEPCase polypeptides were identified using anti-PEPCase antibodies as described under Materials and Methods. The position of molecular mass standards (in kD) are shown at the right margin.

determine if young plants with low endogenous levels of PEPCase activity could be used to characterize the process of CAM induction during salt stress. Soluble protein samples were prepared from unstressed plants and plants watered with 0.5 M NaCl for 5 d during the fourth, fifth, and sixth week after germination and assayed for extractable PEPCase activity. Although PEPCase activity was present at a level of 56 $\mu mol \cdot pg^{-1}$ protein $\cdot min^{-1}$ in extracts from the youngest unstressed plants (22 d old, approximately 250 mg fresh weight/plant), there was no significant increase in PEPCase activity in either salt-stressed or unstressed plants at the end of the first 5-d induction period (not shown). Plants that were watered with 0.5 M NaCl during the fifth week after germination also did not have higher levels of PEPCase activity compared to unstressed controls (Fig. 1A). However, PEPCase activity increased fourfold in plants which were salt stressed during the sixth week after germination (Fig. 1B). Immunoblot analysis of soluble protein samples from these plants revealed that salt stress induced the accumulation of a 107 kD polypeptide that was recognized by anti-PEPCase antibodies (not shown). This confirmed previous reports that the level of PEPCase protein increased in parallel with PEPCase enzymatic activity (6, 13).

NaCl Stress Induces the Synthesis of PEPCase Polypeptides at All Leaf and Axillary Shoot Positions. *M. crystallinum* produced five pairs of leaves during the first 6 weeks after germination when grown under our conditions. The leaf pairs initiated sequentially from the stem apex, with the result that leaves varied in age from the youngest expanding leaf pair at the stem apex to the older more fully expanded leaves near the base of the plant. Axillary shoots typically began to develop during the sixth week after germination and contributed from 10 to 15% of the total fresh weight by the end of the sixth week. We tested the possibility that the induction of PEPCase synthesis during salt stress was influenced either by the age of the leaf tissue or by tissue source

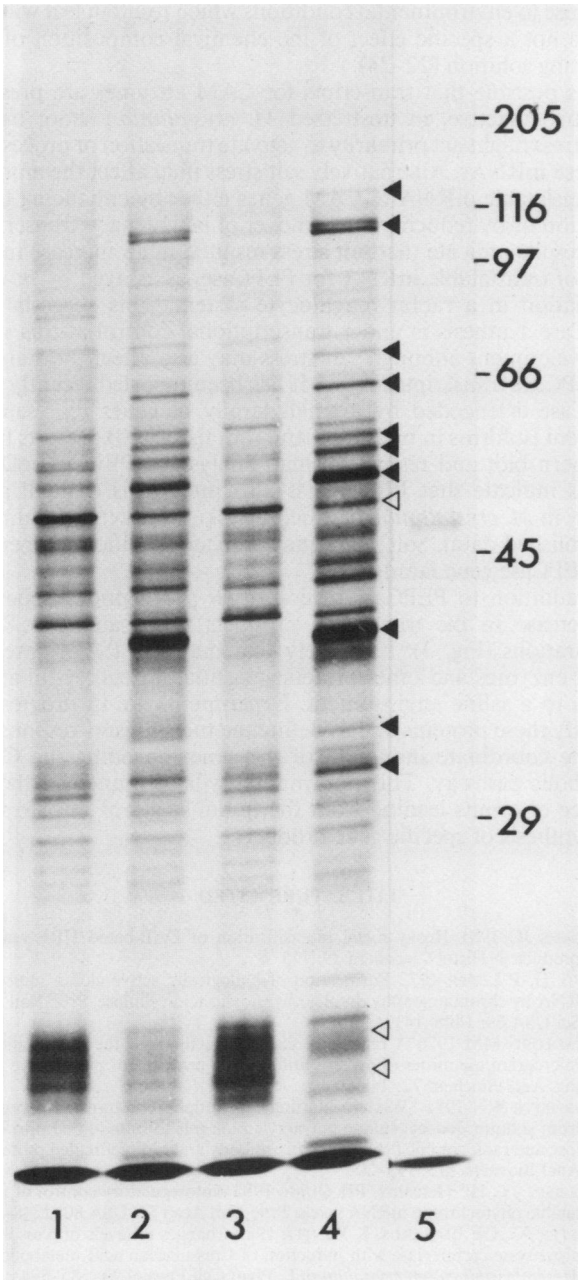

FIG. 3. *In vitro* translation of mRNA isolated from axillary shoot and leaf tissue of *M. crystallinum* before and after 5 d of salt stress. A+ mRNA was isolated as described under "Materials and Methods" and translated in rabbit reticulocyte lysates in the presence of L-[^{35}S]methionine. A fluorograph is shown of the labeled proteins resolved by SDS-PAGE in a 10% polyacrylamide gel. Lanes 1 and 2, axillary shoot 3A before and after 5 d of salt stress; lanes 3 and 4, leaf position 4 before and after 5 d of salt stress; lane 5 (−) mRNA control, an equivalent volume of sterile water was added to the translation mixture. Solid arrowheads and open arrowheads indicate, respectively, polypeptides which either increase or decrease in translation products from salt-stressed plants. Lanes were loaded with equal amounts of TCA precipitable radioactivity based on duplicate 1 ul aliquots of the translation reaction mixtures. Molecular mass standards are indicated in kD.

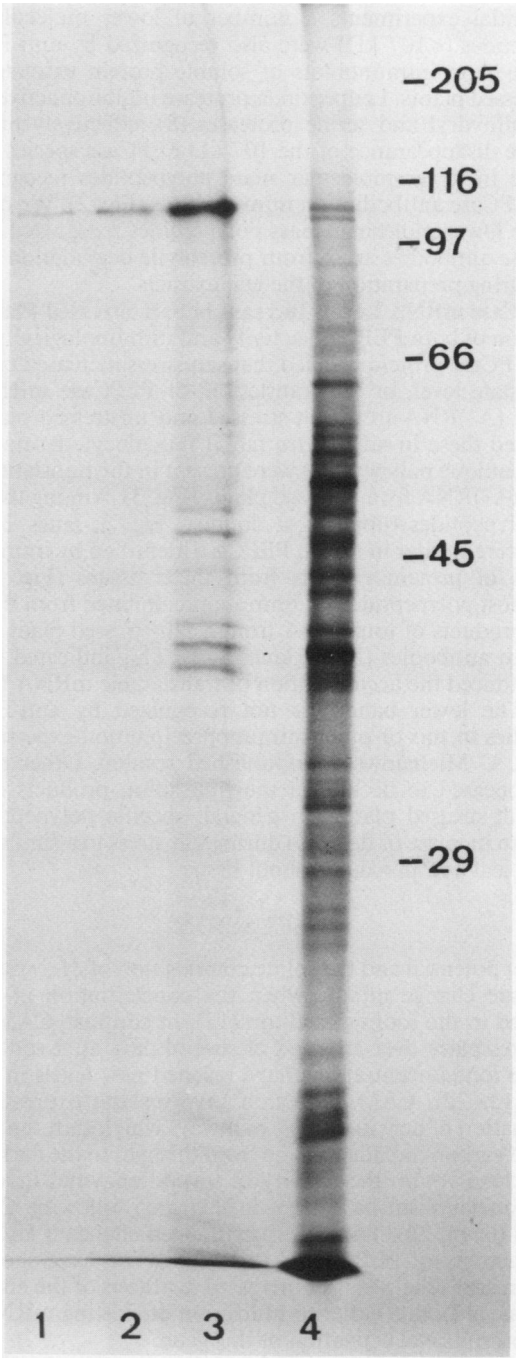

FIG. 4. Immunoprecipitation of PEPCase from *in vitro* translation products of RNA from salt stressed tissue. Total RNA was isolated from leaf 4 after 5 d of salt stress and translated in a rabbit reticulocyte lysate preparation. The translation mixture was divided into 48 μl aliquots. Lane 1, 0.5 μg; lane 2, 1.0 μg; lane 3, 5 μg of affinity-purified anti-PEPCase antibodies and *S. aureus* cells were used to immunoprecipitate L-[^{35}S]methionine labeled PEPCase as described under "Materials and Methods." Lane 4 contains 1.5 μl of the total translation products from the reticulocyte lysate. A fluorograph is shown of the labeled proteins resolved by SDS-PAGE in a 10% polyacrylamide gel. The position of molecular mass standards (in kD) are indicated at the right margin.

within the plant by extracting soluble protein from individual leaf and axillary shoot positions of unstressed and salt-stressed plants. Immunoblot analysis of these extracts with anti-PEPCase antibodies demonstrated that PEPCase synthesis was induced in all plant tissues regardless of age or position within the plant (Fig. 2). Similar amounts of PEPCase were induced in both expanded leaf tissue and young rapidly growing axillary shoot tissue (Fig. 2, lanes 2 *versus* 2A, 3 *versus* 3A).

In initial experiments a number of lower molecular mass polypeptides (<107 kD) were also recognized by anti-PEPCase antibodies on immunoblots of soluble protein extracted from salt-stressed plants. Leupeptin, a protease inhibitor active against both sulfhydryl and serine proteases (8), effectively prevented both the disappearance of the 107 kD PEPCase species and the increase in lower molecular mass polypeptides recognized by anti-PEPCase antibodies on immunoblots (Fig. 2). We conclude that the lower molecular mass polypeptides recognized by anti-PEPCase antibodies arose from proteolytic degradation of PEPCase during preparation of the leaf extracts.

PEPCase mRNA Levels Increase in Salt-Stressed Plants. The induction of both PEPCase activity and immunologically detectable PEPCase protein implied that salt stress increased either the steady-state level, or the translation, of PEPCase mRNA. We isolated (A^+)RNA from salt-stressed and unstressed plants and translated these in an *in vitro* rabbit reticulocyte lysate system. Several unique polypeptides were present in the translation products of (A^+)RNA from stressed plants (Fig. 3). Among these were two polypeptides (uppermost doublet, Fig. 3, lanes 2 and 4) which were similar in size to PEPCase identified by immunoblot analysis of protein extracts from these tissues (Fig. 2). The uppermost polypeptide was immunoprecipitated from the translation products of total RNA from a salt-stressed plant by anti-PEPCase antibodies (Fig 4, lanes 1–3). This indicated that salt stress induced the accumulation of translatable mRNA for PEPCase. The lower band was not recognized by anti-PEPCase antibodies in this or other immunoprecipitation experiments (J Ostrem, C Michalowski, unpublished results). Other polypeptides appeared to decline in the translation products of RNA from salt-stressed plants. In general, specific polypeptides appeared to increase or decrease during salt stress to a similar extent both in leaf and in axillary shoot tissue.

DISCUSSION

Water potential and the solute composition of *M. crystallinum* leaf tissue change rapidly when the concentration of NaCl is increased in the rooting medium (12). In contrast, CAM induction takes place over a period of several days after sodium and chloride ion concentrations have reached new levels in the leaf tissue (12, 22). CAM induction involves the expression and coordination of new metabolic pathways which shift the primary phase of carbon dioxide fixation from the light to the dark period. In *M. crystallinum* the activity of several enzymes involved in carbon metabolism have been investigated following CAM induction (6, 14, 26). The most pronounced change is an increase in the activity of PEPCase (14). Recently we have shown that this increase is the result of increased synthesis of the enzyme in leaf tissue (13), due either to utilization of existing mRNA or to the transcriptional activation of this gene.

Here we show that the age of the plant, but not the age of the tissue source within the plant, influenced the induction of PEPCase in response to salt stress. We were unable to detect any increase in PEPCase activity or PEPCase polypeptide levels after plants were irrigated with 0.5 M NaCl for 5 d during the fourth or fifth week after germination. However, PEPCase activity increased within 2 to 3 d when plants were irrigated with 0.5 M NaCl during the sixth week after germination. Furthermore, PEPCase polypeptide and mRNA levels increased both in older leaf tissue and in young rapidly growing axillary shoot tissue. Possible explanations for the apparent link between development and CAM induction in response to salt stress are: (a) the elongation of axillary shoots signals a change in the levels of plant growth regulators (19) which play a role in the control of CAM induction or, (b) structural or physiological characteristics of the young root and leaf tissue resist the dehydrating effect of the salt solution. It is clear from previous work that CAM is induced in response to environmental conditions which result in leaf wilting, and is not a specific effect of the chemical composition of the irrigating solution (22–24).

It is possible that transcripts for CAM enzymes are present, although inactive, in unstressed *M. crystallinum* shoot tissue. Salt stress might act primarily to activate translation or processing of these mRNAs. Alternatively salt stress may affect the amount of translatable mRNA of CAM genes either by enhancing transcription or by reducing the turnover of newly made transcripts. Our results indicate that salt stress resulted in an increase in the level of translatable mRNA for PEPCase, as assayed by *in vitro* translation in a rabbit reticulocyte system. This suggests that PEPCase synthesis is under transcriptional control at this stage of development although salt stress may also affect the stability of PEPCase transcripts *in vivo*. It has been reported recently that PEPCase is encoded by a small family of genes representing different isoforms in maize leaf and root tissue (10). Results from Southern blot and restriction map analysis of PEPCase cDNA clones indicate that PEPCase is also present as a small gene family in *M. crystallinum* (C Michalowski, J Rickers, H Bahnert, unpublished data). Salt stress may activate specific members of the PEPCase gene family.

In addition to PEPCase several other polypeptides appeared to increase in the translation products from salt-stress RNA preparations (Fig. 3). It is likely that many of these represent CAM enzymes and other proteins essential for successful adaptation to a saline environment. Experiments are in progress to identify these proteins and to define the mechanisms responsible for the coordinate induction of the genes encoding the CAM metabolic pathway. This information will help uncover the sequence of events leading from the initial stages of salt stress to the synthesis of specific gene products.

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