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Salt Stress Leads to Differential Expression of Two Isogenes of Phosphoenolpyruvate Carboxylase during Crassulacean Acid Metabolism Induction in the Common Ice Plant

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The common ice plant is a facultative halophyte in which Crassulacean acid metabolism, a metabolic adaptation to arid environments, can be induced by irrigating plants with high levels of NaCl or by drought. This stress-induced metabolic transition is accompanied by up to a 50-fold increase in the activity of phosphoenolpyruvate carboxylase (PEPCase). To analyze the molecular basis of this plant response to water stress, we have isolated and characterized two members of the PEPCase gene family from the common ice plant. The PEPCase isogenes, designated *Ppc1* and *Ppc2*, have conserved intron-exon organizations, are 76.4% identical at the nucleotide sequence level within exons, and encode predicted polypeptides with 83% amino acid identity. Steady-state levels of mRNAs from the two genes differ dramatically when plants are salt-stressed. Transcripts of *Ppc1* increase about 30-fold in leaves within 5 days of salt stress. In contrast, steady-state levels of *Ppc2* transcripts decrease slightly in leaf tissue over the same stress period. Steady-state levels of transcripts of both genes decrease in roots over 5 days of salt stress. We have used in vitro transcription assays with nuclei isolated from leaves to demonstrate that the increased expression of *Ppc1* caused by water stress occurs in part at the transcriptional level.

INTRODUCTION

Crassulacean acid metabolism (CAM) is a well-characterized physiological adaptation to water stress found in plants of tropical origin that typically grow in warm, dry climates (Kluge and Ting, 1978). CAM is defined by diurnal fluctuation of organic acids (mainly malate) and reciprocal diurnal fluctuation of storage carbohydrates. CAM plants open their stomata primarily at night, when the majority of CO_2 uptake occurs, and close them during the day to avoid excess evaporative water loss (Osmond and Holtum, 1981; Ting, 1985). CAM is also characterized by high activities of phosphoenolpyruvate carboxylase (PEPCase, EC 4.1.1.31), the enzyme responsible for the primary fixation of CO_2 into oxaloacetate, which is subsequently converted to malate (O'Leary, 1982). During the day, accumulated C_4 acids (mainly malate) are decarboxylated to provide CO_2 for later fixation by ribulose-bisphosphate carboxylase (Ting, 1985).

PEPCase is present in bacteria, algae, and higher plants. In addition to its role in photosynthesis, several other diverse anapleurotic functions have been postulated for the enzyme, such as replenishment of tricarboxylic acid cycle intermediates, NADPH regeneration, and recapture of respired CO_2 (Andreo, Gonzalez, and Iglesias, 1987). Numerous plant species switch from a C3 mode of photosynthetic carbon metabolism to CAM in response to various environmental stimuli (Ting and Rayder, 1982). In some plants, this switch can be induced by salt or water stress (Winter and von Willert, 1972; von Willert et al., 1976a, 1976b; Ting and Hanscom, 1977), by changes in photoperiod (Brulfert et al., 1982), or as a part of their normal developmental program (Jones, 1975; Brulfert, Guerrier, and Queiroz, 1982; Sipes and Ting, 1985). In the common ice plant, this metabolic transition is accompanied by substantial increases in the activity of a set of carbon metabolism enzymes of the glycolytic pathway (Holtum and Winter, 1982). Among those enzymes whose activities are known to increase, PEPCase has been most intensively studied (Foster, Edwards, and Winter, 1982). The increase of PEPCase enzyme activity in the common ice plant is the result of de novo protein synthesis (Höfner et al., 1987). The increase in PEPCase protein is paralleled

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by an increase in PEPCase mRNA as assayed by immunoprecipitation of PEPCase from the products of in vitro translation of RNA isolated from stressed plants (Ostrem et al., 1987; Michalowski et al., 1989). PEPCase mRNA levels drop rapidly upon removal of salt (Vernon et al., 1988).

Multiple forms of PEPCase are known to exist in higher plants (Ting and Osmond, 1973). Different classes of the enzyme have apparently evolved to perform the same enzymatic step associated with different metabolic pathways. At least four different molecular species of the enzyme have been identified on the basis of kinetic and chromatographic properties in different species, including a C3 photosynthetic form, a nonautotrophic or "root" form, a C4 photosynthetic form, and a CAM photosynthetic form (Ting and Osmond, 1973). Recently, there has been progress in the analysis of the genetic basis for different forms of PEPCase in the C4 plant maize. PEPCase cDNA clones have been used to identify different PEPCase mRNAs that encode isozymic forms present in roots and leaves (Harpster and Taylor, 1986). cDNA clones, including a full-length cDNA clone for the C4 PEPCase (Yanagisawa et al., 1988) and two different genomic PEPCase clones, have been isolated from maize (Hudspeth et al., 1986). The maize PEPCase gene family consists of five distinct genes (Grula and Hudspeth, 1987) with only the C4 photosynthetic version of the genomic PEPCase clone having been characterized thus far (Hudspeth and Grula, 1989). PEPCase is expressed in a developmental, light- and tissue-specific manner in maize leaves (Nelson and Langdale, 1989, and references therein).

In contrast, very little is known at the molecular level about different forms of PEPCase present in CAM plants and the metabolic roles that each of these forms plays. While there have been many studies that have revealed the existence of different PEPCase isoforms (von Willert et al., 1976b; Brulfert and Queiroz, 1982; Brulfert, Guerrier, and Queiroz, 1982; Brulfert et al., 1982; Müller and Kluge, 1983; Höfner et al., 1989), little is known about the genes encoding these different forms. We have recently characterized a full-length PEPCase cDNA clone (Rickers et al., 1989) isolated from a cDNA library made from mRNA isolated from salt-stressed common ice plants (Schmitt, Michalowski, and Bohnert, 1988). We have now characterized two distinct genomic PEPCase genes that differ markedly in their expression during CAM induction. One of the genes, *Ppc1*, encodes a form of the enzyme whose expression is induced by salt stress. This gene corresponds to the CAM-specific cDNA described by Rickers et al. (1989). The second gene encodes an alternate form of the enzyme whose expression is not enhanced by stress. We show by in vitro transcription run-on experiments using isolated nuclei from the ice plant that the differences in accumulation of steady-state transcripts are controlled in part at the level of transcription.

RESULTS

Isolation and Characterization of Genomic Clones

A portion (90,000 plaques) of a primary genomic library constructed in λ FIX (Stratagene, Inc.) was screened using a CAM-specific PEPCase cDNA isolated from a cDNA library made from poly(A+) RNA isolated from salt-stressed plants (Rickers et al., 1989). During purification of plaques by hybridization, two different classes of plaques became evident. One class hybridized very strongly with the CAM-specific PEPCase cDNA probe even after high-stringency (60°C) washes of filters. Eight independent genomic clones for this class of gene were isolated. The second class of plaques hybridized only weakly with the cDNA probe and not at all under conditions of high-stringency washing. Six independent clones of this type were isolated.

To further characterize and distinguish these two classes of genomic PEPCase clones, restriction maps of the two groups of clones were constructed. All of the clones from each class were found to possess identical restriction maps. DNA blots were used to determine the position of restriction fragments with respect to the coding regions of the genes, and similar differences in hybridization characteristics between the types of clones were observed as described above (data not shown).

Characterization of PEPCase Isogenes

Restriction fragments containing regions that hybridized to the cDNA probe were subcloned into pTZ18u or 19u, and deletion subclones for sequence analysis were generated (Dale, McClure, and Houchins, 1985). The restriction maps and structure of the two different PEPCase isogenes are illustrated in Figure 1. The *Ppc1* gene spans a region of 6920 bp and is interrupted by nine introns. The *Ppc2* gene is also interrupted by nine introns and extends for 7416 bp. Since both genes have coding regions of similar size (966 codons for *Ppc1* versus 960 codons for *Ppc2*), most of the difference in gene size is accounted for by differences in intron size. The placement of introns within the coding regions of the two genes is completely conserved (Figure 2). All of the introns exhibit the conserved dinucleotides GT and AG at their 5′ ends and 3′ ends, respectively, common to eukaryotic intron-exon splice junctions (Mount, 1982). Introns in *Ppc1* range in size from 86 to 1638 bp and from 103 to 1463 bp for *Ppc2*. The complete nucleotide sequences for *Ppc1* and *Ppc2* will appear in the EMBL/GenBank/DDBJ nucleotide sequence databases under accession numbers X14587 and X14588, respectively.

The predicted amino acid sequences of the *Ppc1* and

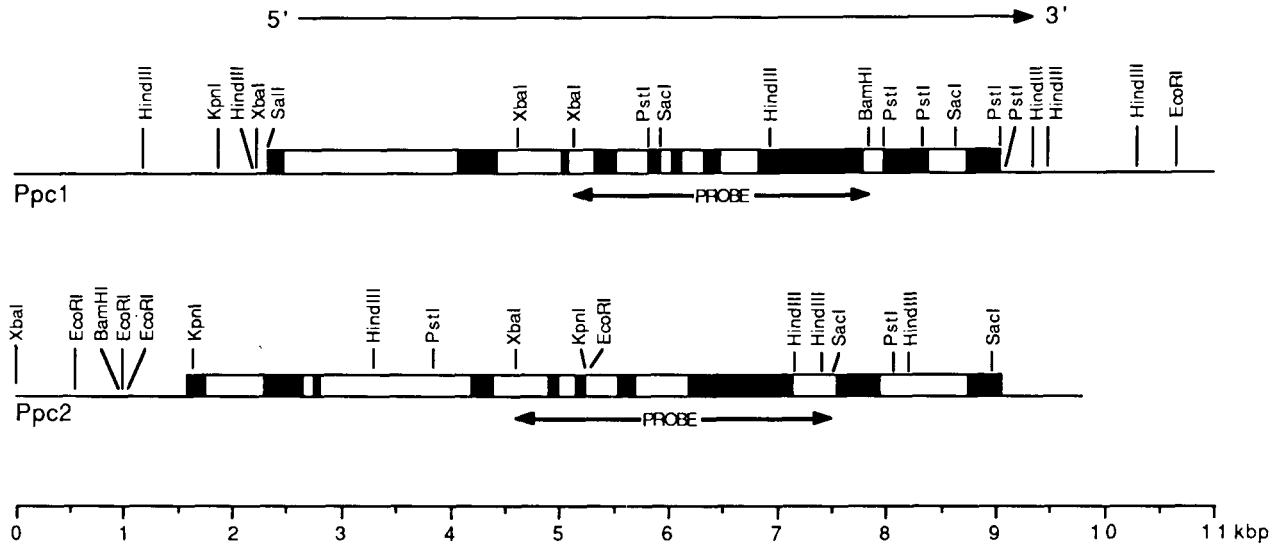

Figure 1. Structure of PEPCase Isogenes from the Common Ice Plant.

Restriction maps and gene organization of two distinct members of the PEPCase (*Ppc1* and *Ppc2*) gene family are shown. Exons are designated as black boxes and introns are designated as open boxes. The direction of transcription for both genes is designated by an arrow above the gene maps. Regions designated as "probe" were used as hybridization probes in Figures 4, 5, and 6.

Ppc2 genes are depicted in Figure 3. These amino acid sequences are 83% identical to one another. If conservative amino acid exchanges are included, they are 97% related. The predicted *Ppc1* amino acid sequence is 6 amino acids longer than that of *Ppc2*. The sequences are essentially colinear, except for an 8-amino acid deletion close to the amino terminus in the *Ppc2* gene product corresponding to positions 2 to 10 in the *Ppc1* gene product, and a 2-amino acid deletion in the *Ppc1* gene product corresponding to positions 919 and 920 in the *Ppc2* gene product (Figure 3). The predicted molecular weight of the polypeptide encoded by the 966 codons of *Ppc1* is 110,533. This predicted weight is slightly larger than the size of the 100- to 105-kD PEPCase subunit that is observed to accumulate preferentially during CAM induction (Höfner et al., 1989; Michalowski et al., 1989). The difference between the predicted and apparent molecular weights might be due to inaccurate size determination by SDS-PAGE or aberrant electrophoretic mobility. Alternatively, differences between predicted and apparent molecular weights could be the result of NH_2- or COOH-terminal processing or covalent modifications. NH_2-terminal sequencing data from the different forms are needed before these differences can be understood fully. The predicted molecular weight of the 960-amino acid polypeptide encoded by *Ppc2* is 109,041. This predicted protein size corresponds to a 110-kD protein found in unstressed leaves of plants in the C3 mode of photosynthesis and in roots (Höfner et al., 1989). The predicted pI values for the *Ppc1* and *Ppc2* gene products (7.76 and 7.68, respectively) differ by 0.08 units. This predicted difference in pI agrees with the difference (0.09 units) between experimentally determined pI values for two different forms of PEPCase from the common ice plant (L. Vazquez-Moreno, unpublished data).

PEPCase Is Encoded by a Small Gene Family in the Common Ice Plant

Blots of total genomic DNA isolated from leaf tissue were probed with radiolabeled gene-specific DNA fragments isolated from either *Ppc1* (XbaI-BamHI fragment) or *Ppc2* (XbaI-SacI fragment) as indicated in Figure 1. The *Ppc1*- and *Ppc2*-specific probes yielded distinct hybridization patterns on genomic DNA gel blots, as would be expected from restriction mapping data (Figure 4). Under both low-stringency (room temperature) and high-stringency (60°C) wash conditions, *Ppc1* gave strong hybridization to single restriction fragments and fainter hybridizations to one or two other fragments (Figure 4A). This supports earlier observations using both coding region and 3′-specific probes (Rickers et al., 1989) that indicate that *Ppc1* is present as a single copy on the ice plant genome. The *Ppc2*-specific probe, however, hybridized to a multitude of bands at conditions of low stringency and to several spe-

	EXON	INTRON	EXON
Ppc1-1	AAAGAAACG	GTATA -1628bp- ATCAG	GTTCAAGAA
	K E T		V Q E
Ppc2-1	CGTGAAACA	GTATG - 559bp- TGTAG	GTCCAAGAG
	R E T		V Q E
Ppc1-2	ACATGGAAG	GTTTG - 603bp- CCCAG	GATAAGGGA
	H G R		I R D
Ppc2-2	GCATGGCAG	GTATG - 93bp- ATCAG	GATAAGGAA
	H G R		I R N
Ppc1-3	CAAAGAGAG	GTATG - 240bp- TGTAG	ATACAAGCT
	Q R E		I Q A
Ppc2-3	CAGAGAGAG	GTAAA -1453bp- TGCAG	ATCCAAGCG
	Q R E		I Q A
Ppc1-4	ATCGAGATG	GTAAG - 292bp- TGCAG	GAAATCCGA
	D R D		G N P
Ppc2-4	ATCGTGATG	GTATG - 520bp- TACAG	GTAATCCGA
	D R D		G N P
Ppc1-5	ATGTTTGAG	GTATA - 76bp- AACAG	CTCTCAATG
	M F E		L S M
Ppc2-5	ATGTTTGAG	GTACA - 97bp- GGCAG	TTATCTATG
	M F E		L S M
Ppc1-6	ACTACATTG	GTATG - 192bp- TGAAG	AGTTTTGGA
	H Y I		E F W
Ppc2-6	ATTACATAG	GTACC - 314bp- TGCAG	AGTTCTGGA
	H Y I		E F W
Ppc1-7	ATTGATCAG	GTTTG - 311bp- AGTAG	TTTCTGGAG
	I D Q		F L E
Ppc2-7	TTGGAGCAG	GTATA - 250bp- TTCAG	TTCCTGGAA
	L E Q		F L E
Ppc1-8	TTCCGTCTC	GTAAG - 206bp- TGCAG	GCAACGCCA
	F R L		A T P
Ppc2-8	TTCCGCCTG	GTAAG - 368bp- CACAG	GCCACACCA
	F R L		A T P
Ppc1-9	CTCCTCGAG	GTAAG - 345bp- TACAG	GTCGCTGGG
	L L E		V A G
Ppc2-9	ATCCTCAAG	GTAAA - 795bp- GCCAG	ATTGCTGGA
	I L K		I A G

Figure 2. Summary of Intron-Exon Splice Junctions of *Ppc1* and *Ppc2*.

The single-letter amino acid code is shown below the middle nucleotide of each codon of the mRNA-like strand of the nucleotide sequence. Consensus sequences of intron splice boundaries are underlined.

cific fragments at high stringency (Figure 4B). At low stringency, there is limited cross-hybridization to the fragments that are highlighted with the *Ppc1*-specific gene probes. In addition, we observed hybridization signals that cannot be accounted for by either the *Ppc2*- or the *Ppc1*-specific genes at high stringency, suggesting that there might exist yet another distinct member of the PEPCase gene family. We have been able to isolate and identify only genomic clones belonging to the two classes of genes described here.

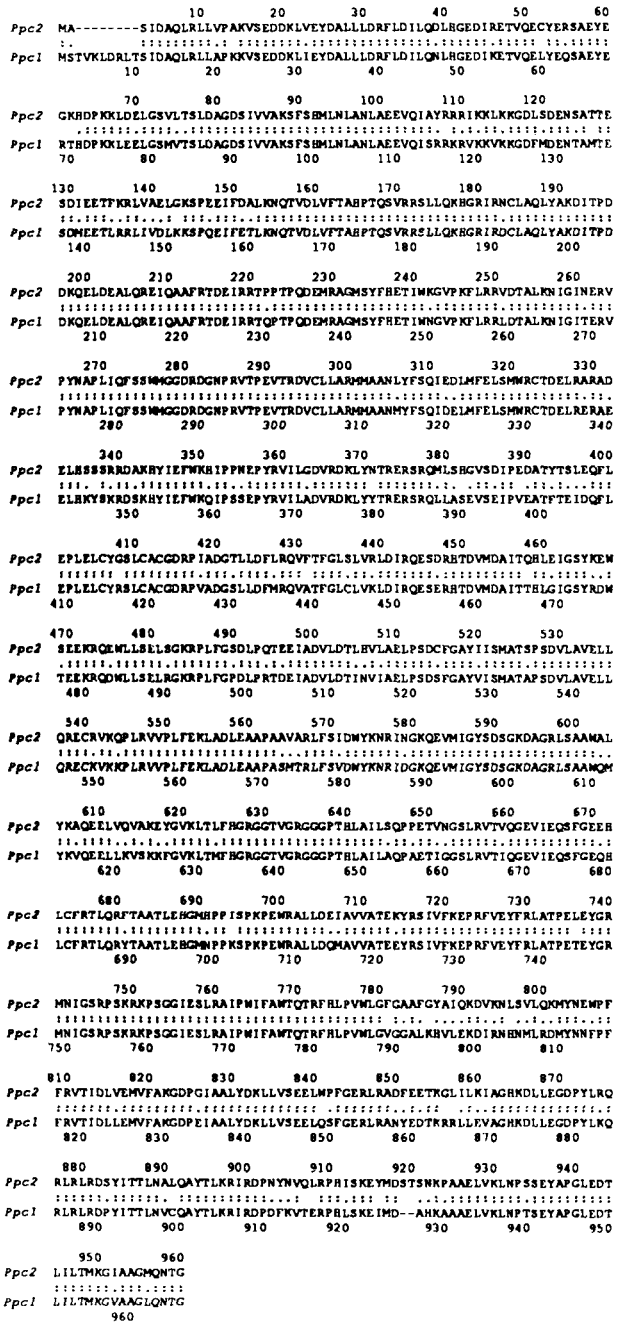

Figure 3. Comparison of the Predicted Amino Acid Sequences from *Ppc1* and *Ppc2*.

The predicted amino acid sequences for the two PEPCase isozymes were aligned using the FASTP program of Lipman and Pearson (1985). Amino acid identity is designated by a colon (:) and conservative replacement is designated by a period (.). Deletions in the amino acid sequences are denoted by dashes (–).

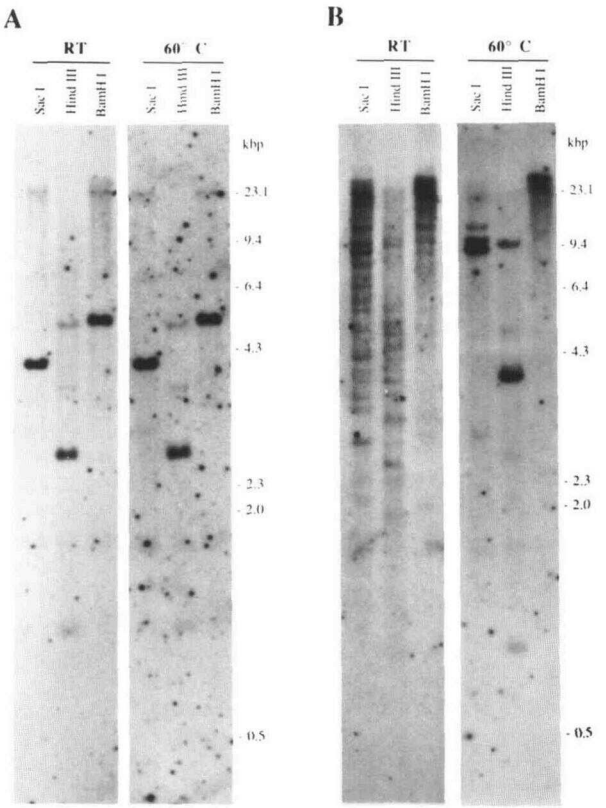

Figure 4. Identification of Different Members of the PEPCase Gene Family in the Common Ice Plant.

(A) Genomic DNA gel blot hybridized with a *Ppc1*-specific probe. Total genomic DNA isolated from ice plant leaf tissue was digested with HindIII, SacI, and BamHI. The DNA samples (4 μg) were separated on a 0.8% agarose gel, blotted onto nitrocellulose, hybridized as described in Methods, and washed at room temperature (RT) in 0.1% SDS, 2 × SSC or 60°C in 0.1% SDS, 0.1 × SSC.

(B) Genomic DNA gel blot hybridized with a *Ppc2*-specific probe. The blots were processed as in **(A)**.

Differential Expression of *Ppc1* and *Ppc2*

To characterize the expression patterns of each of the PEPCase isogenes isolated from ice plant, plants were grown until they were 6 weeks old and then irrigated with 0.5 M NaCl in nutrient solution. Unstressed control plants were irrigated with nutrient solution alone. Leaves were harvested daily for 5 days, and total RNA was isolated as described in Methods. To quantitate the increases in steady-state mRNA levels in leaf tissue, serial dilutions of total RNA, isolated during the time course of salt stress, were applied to nitrocellulose filters using a slot-blot apparatus (Schleicher & Schuell, Inc.) and hybridized with ^{32}P-labeled DNA probes from *Ppc1* or *Ppc2* (see Figure 1). *Ppc1*-specific PEPCase transcripts begin to increase after 29 hr of exposure to high salinity and continue to increase over the 5-day stress period (Figure 5A). By comparison, PPC2 transcripts show a slight transient increase after 1 day of stress, followed by a decline during subsequent days of stress (Figure 5A). Levels of PEPCase transcripts were quantified by densitometric scanning of autoradiograms and plotting the relative levels of hybridization intensity (Figure 5B). The steady-state level of PPC1 transcripts increased over 30-fold, whereas the level of PPC2 transcripts decreased by about twofold after 5 days of stress. Under conditions of high stringency, no cross-hybridization between PPC1 and PPC2 transcripts was detected.

Differential Expression of *Ppc1* and *Ppc2* in Roots and Leaves

To determine whether differential gene expression of the PEPCase isogenes occurs in different organs during stress, slot-blot analysis was conducted on total RNA isolated from root and leaf tissue collected from hydroponically grown unstressed plants and from plants that had been stressed for 1, 3, or 5 days with 0.5 M NaCl. As expected, we observed an accumulation of PPC1 transcripts in leaves of salt-stressed plants (Figure 6). In root tissue, however, the amount of PPC1 transcript declines with increasing duration of stress. Levels of PPC2 transcripts decrease in leaves during stress as shown above. Like PPC1 transcripts, PPC2 transcripts are clearly present in roots, but their levels decrease during stress.

Nuclear Run-On Transcription by Isolated Nuclei

The observed differences in transcript accumulation between the two PEPCase genes could be due to enhanced rates of transcription or to changes in mRNA stability. We examined changes in transcription rates of *Ppc1* and *Ppc2* during stress by nuclear run-on experiments with isolated nuclei (Hagen and Guilfoyle, 1985). The incorporation of α-^{32}P-UTP into RNA by equal amounts of isolated ice plant nuclei is essentially linear for at least 15 min, except for nuclei isolated from plants stressed for 1 day (Figure 7). Nuclei isolated from plants stressed for more than 1 day consistently had lower rates of incorporation (Figure 7) as did nuclei isolated from drought stressed plants (data not shown).

The amounts of PEPCase transcripts synthesized by isolated nuclei during salt stress were quantitated by RNA-DNA slot-blot hybridization. The results of hybridization between DNA from PPC1 and PPC2 PEPCase clones and ^{32}P-RNA synthesized in vitro by isolated nuclei from unstressed plants at 4 weeks of age and 6 weeks of age and

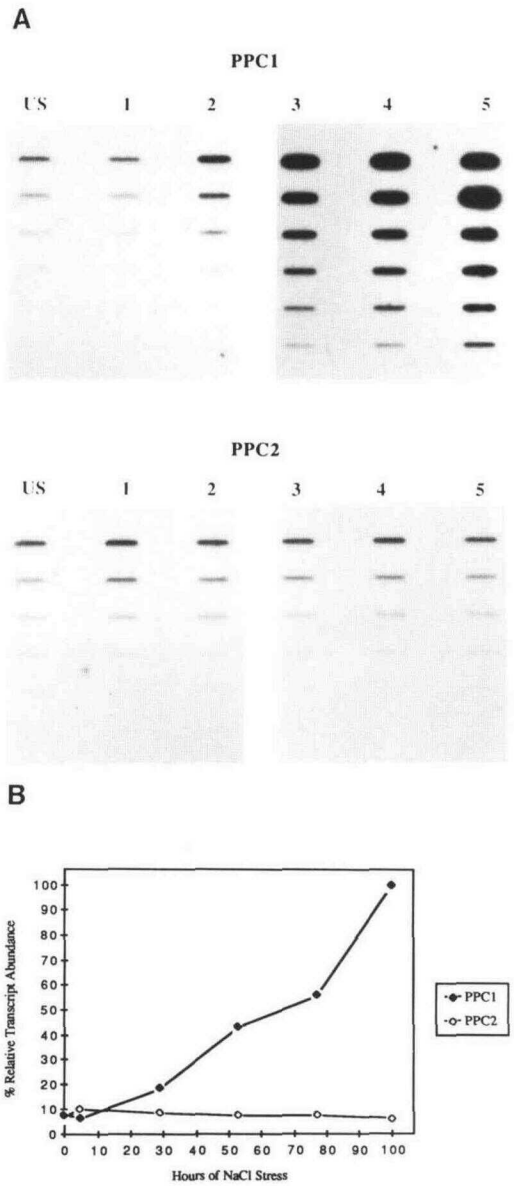

Figure 5. Differential PPC Transcript Accumulation during Salt Stress.

(A) Slot-blot hybridization of differential PPC1 and PPC2 transcript accumulation during salt stress. Equal amounts of total RNA from ice plants stressed with 0.5 M NaCl for various lengths of time (US, unstressed; 1, 5 hr; 2, 29 hr; 3, 53 hr; 4, 77 hr; 5, 100 hr) were slot-blotted onto nitrocellulose filters as twofold serial dilutions (5.0 μg to 0.18 μg). Duplicate filters were hybridized with equal counts of radiolabeled *Ppc1*- or *Ppc2*-specific probes (Figure 1), washed under high-stringency (60°C) conditions, and exposed to x-ray film.

(B) Quantitation of the differential accumulation of steady-state levels of PEPCase transcripts during salt stress. Autoradiograms were scanned with a laser densitometer and the values from three dilutions in the linear range were averaged. The relative percent hybridization signal was plotted.

6-week-old plants salt-stressed for 1, 3, or 5 days are shown in Figure 8A. The amounts of hybridizing mRNAs were compared with control DNAs, including ferredoxin NADP-reductase (FNR) from the common ice plant, whose steady-state levels of transcripts are known to remain constant during the stress procedure (Michalowski, Schmitt, and Bohnert, 1989); small subunit of ribulose bisphosphate carboxylase (SSU) from the common ice plant, whose steady- state mRNA levels decrease slightly during salt stress (J. DeRocher, unpublished data); and 18S rDNA from soybean (Eckenrode, Arnold, and Meagher, 1985). The hybridizing bands were cut out and quantitated by liquid scintillation counting and plotted as a relative percent of rRNA hybridization (Figure 8B). λ DNA used as a negative control for nonspecific hybridization gave no detectable hybridization signal. The rates of transcription of the SSU and FNR controls showed a small increase during stress. *Ppc2* transcription remained relatively unchanged and accounted for less than 7% of detectable transcripts. In contrast, transcription rates for *Ppc1* increased greater than sixfold during salt stress. Nuclei isolated from plants that had been drought-stressed (not watered) for 9 days also exhibited an induction in

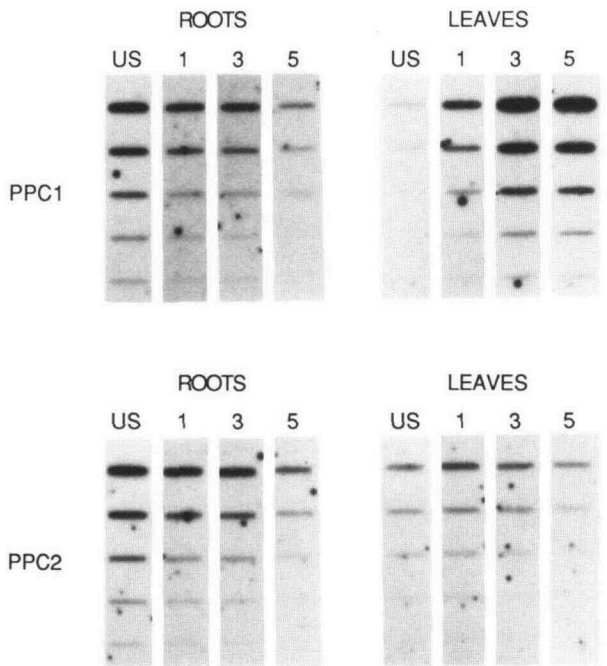

Figure 6. Differential Expression of *Ppc* Genes in Roots and Leaves.

Serial dilutions (5.0 μg to 0.31 μg) of total RNA were analyzed by slot-blot hybridization as described in Figure 5. RNA was isolated from hydroponically grown plants that were unstressed (US) or stressed for 1, 3, or 5 days with 0.5 M NaCl.

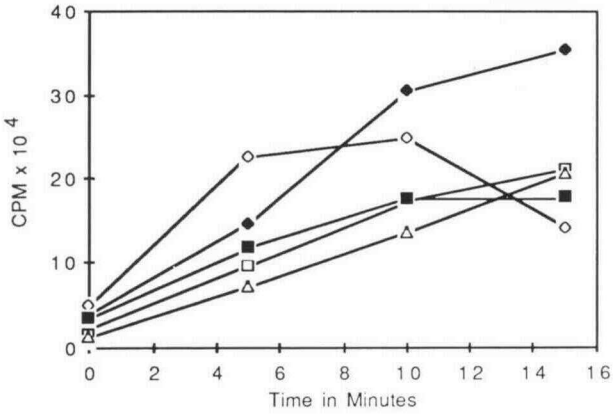

Figure 7. In Vitro Transcription Rates of Isolated Ice Plant Nuclei during Salt Stress.

In vitro transcription rates of nuclei isolated from either unstressed plants of 4 weeks (■) or 6 weeks (♦) of age or plants that had been stressed for 1 (◇), 3 (□), or 5 (△) days with 0.5 M NaCl were plotted over a 15-min time period. Incorporation kinetics were assayed as described in Methods. Values shown are the average of two independent experiments.

Ppc1 transcription rates at a level half that of plants salt-stressed for 5 days (data not shown).

DISCUSSION

The salt stress- or drought-inducible transition from a C3 mode of photosynthetic carbon metabolism to CAM in the facultative halophyte *Mesembryanthemem crystallinum* provides a model system for studying adaptation to environmental stress in higher plants (Michalowski et al., 1989). We are interested in understanding the underlying molecular mechanisms responsible for this metabolic transition and the factors responsible for conferring the ability of this plant to adapt to conditions of water stress. CAM, a relatively widespread phenomenon occurring in many plant families, is clearly one of the ways in which these plants have adapted to arid environments. Despite an impressive amount of physiological and biochemical knowledge about CAM, relatively little is known about the genetic basis of this complex metabolic process. As a first step toward understanding CAM induction, we have isolated two different genes for PEPCase, the enzyme responsible for the primary fixation of CO_2 in C4 and CAM, and characterized their differential expression.

Two different members of the PEPCase family in ice plant were characterized by sequence analysis. Both genes share identical intron placement within their coding regions (Figures 1 and 2) and are 76.4% identical at the

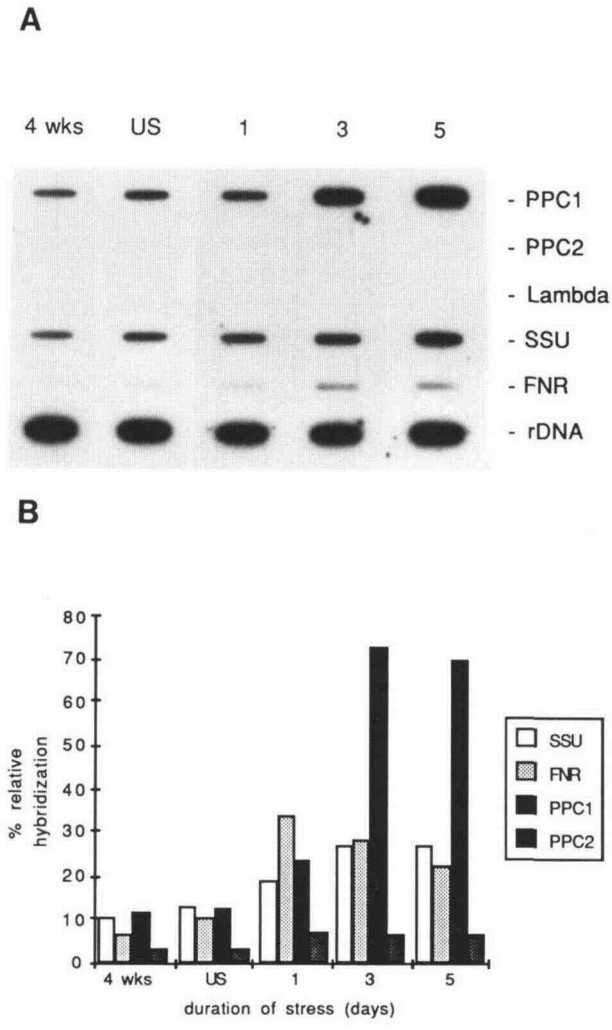

Figure 8. Characterization of in Vitro Synthesized Transcripts by Isolated Ice Plant Nuclei.

(A) Autoradiogram of DNA-RNA slot-blot hybridizations. Radiolabeled in vitro transcripts synthesized by nuclei isolated from unstressed plants 4 weeks old (4 wks) or 6 weeks old (US) or plants stressed for 1, 3, or 5 days with 0.5 M NaCl were hybridized to 5 μg of DNA from *Ppc1* (λMc-2C), *Ppc2* (λMc-4C) genomic clones, λ, SSU (pC3-3) from ice plant, FNR (pFNR-1) from ice plant, and 18S rDNA (pSRI.2B3) from soybean (Eckenrode, Arnold, and Meagher, 1985) and immobilized onto nitrocellulose. Replica filters were washed at high stringency and exposed to x-ray film.

(B) Quantitation of transcripts synthesized in vitro by isolated nuclei. Slots from nitrocellulose filters were cut out and quantitated by liquid scintillation counting. Radioactive counts were plotted as a percent of relative hybridization to rDNA. The average values of two independent experiments are shown.

nucleotide sequence level within exons. The predicted polypeptides of the PEPCase isozymes are 83% identical at the amino acid level (Figure 3). Biochemical analysis of PEPCase proteins isolated from ice plant confirms that two closely related isoforms of the enzyme are present in this facultative CAM plant. These two isozymes were judged to be very similar to one another on the basis of digestion by V8 protease and acidic hydrolysis analysis (Höfner et al., 1989).

The ice plant PEPCase gene family consists of two or perhaps three members. Each of the PEPCase genes described here is present as a single copy per genome equivalent. Hybridization by the *Ppc2*-specific probe at low stringency to other restriction fragments not highlighted by *Ppc1* and *Ppc2* at high stringency suggests the possibility that additional PEPCase genes similar to *Ppc2* may exist in ice plant (Figure 4). Similarly, the PEPCase gene family in the C4 plant maize consists of five members (Grula and Hudspeth, 1987). From these two examples, it would seem likely that the different forms of PEPCase that have been identified in various plant species (Ting and Osmond, 1973) are encoded by different members of a PEPCase gene family. Furthermore, specific members of the gene family are likely to be preferentially expressed to account for the major isozyme species associated with C4 and CAM photosynthesis.

Upon salt stress, steady-state transcript levels of one member of the PEPCase gene family (*Ppc1*) increase by about 30-fold, paralleling enzyme and activity increases during CAM induction. In contrast, a second member of the PEPCase gene family (*Ppc2*) exhibits a slight decrease in its mRNA levels after 5 days of salt stress (Figure 5). To determine whether the PEPCase isogenes are expressed differentially in specific organs, steady-state transcript levels were examined in roots and leaves during stress. When total RNA isolated from leaves and roots was hybridized to a *Ppc1*-specific probe, steady-state transcript levels increased in leaves during stress as expected. This observation is consistent with the biochemical analysis that an isozyme of PEPCase having an apparent molecular weight of 100 accumulates in response to salt stress during CAM induction (Höfner et al., 1989). In roots, transcripts for both genes are clearly present in unstressed plants, but then decrease as the duration of the stress period increases. The initial presence of transcripts for both PEPCase isogenes in roots is consistent with the finding of two isozymes in root extracts (Höfner et al., 1989). We propose that *Ppc1* encodes the photosynthetic CAM form of PEPCase, which is responsible for primary CO_2 fixation under salt stress. The expression of *Ppc2* decreases slightly during salt stress. This pattern of expression is consistent with the observation of a second isoform of the enzyme with an apparent molecular weight of 110 found in both roots and leaves of C3 plants that does not increase appreciably in leaves during CAM induction (Höfner et al., 1989). This second PEPCase gene may encode an enzyme that carries out nonphotosynthetic anapleurotic "housekeeping" functions (Andreo, Gonzalez, and Iglesias, 1987) or may represent another form of the enzyme conducting CAM-related CO_2 fixation in roots.

CAM may not be solely a response to water stress, but a more general metabolic adaptation to environments that have low concentrations of CO_2 (Ting, 1985). Certain aquatic species that experience CO_2 depletion during the day take up CO_2 at night and store it as malate. CO_2 in the form of bicarbonate is then assimilated the following day in a photosynthetic CAM manner (Keeley and Morton, 1982; Keeley, 1983). CAM has also been shown to be operative in orchid roots that lack stomata (Goh, Arditti, and Avadani, 1983). The presence of transcripts and protein arising from *Ppc1* in root tissues of ice plant suggests that CAM processes may be operative there. A more detailed analysis of the molecular species of PEPCase functioning in roots (during stress) is required.

Previous experiments using in vitro translation of isolated mRNA from unstressed and stressed leaves suggested that the induction of PEPCase during salt stress was due to increased levels of PEPCase mRNA (Ostrem et al., 1987). To confirm directly that these increases in PEPCase transcript levels, specifically transcripts from *Ppc1*, are the result of transcriptional activation, we conducted nuclear run-on transcription experiments using isolated nuclei of ice plant. With increasing duration of stress, the overall rates of incorporation of radiolabeled UTP declined (Figure 7). The transcription rates for *Ppc1* were much greater than those of *Ppc2* (Figure 8B). Furthermore, the pattern of increased transcription rates for *Ppc1* mirrors the pattern of overall *Ppc1* transcript accumulation (Figures 5B and 8B). The transcriptional induction of *Ppc1* can also be induced simply by withholding water from the plants. Thus, increased rates of transcription from *Ppc1* appear not to be a specific result of NaCl stress, but rather a general response to water stress. The activation of *Ppc1* is slow in comparison with many other plant responses brought about by environmental stimuli. This delayed response suggests the possibility that secondary messengers might be involved in the signal transduction pathway linking water stress to CAM induction.

While the expression of *Ppc1* is clearly under transcriptional control, one cannot rule out the possibility that additional levels of gene regulation such as changes in mRNA turnover rates or alternate processing of large pre-mRNA molecules are involved in regulating the expression of *Ppc1*. In fact, we observed that very young plants (4 weeks old) can accumulate *Ppc1*-specific transcripts when salt-stressed (J.M. Schmitt, unpublished data), but fail to accumulate the CAM form of the PEPCase enzyme (Ostrem et al., 1987). This response indicates that post-transcriptional regulatory mechanisms may be important for the regulation of CAM induction.

We expect that other genes encoding enzymes, particularly those of the glycolytic pathway whose activity is

induced during CAM induction (Holtum and Winter, 1982), will exhibit similar kinetic patterns of transcriptional activation. We have isolated via differential screening of mRNA from unstressed and stressed plants a number of cDNA clones that exhibit patterns of transcript accumulation during salt stress that are similar to that of *Ppc1*. One of these clones encodes a cytosolic form of glyceraldehyde-3-phosphate dehydrogenase, an enzyme that plays a major role in the glycolytic pathway (J.A. Ostrem, D.M. Vernon, and H.J. Bohnert, submitted). These genes may share common 5′ sequence elements that are involved in their coordinate regulation. Characterization of the upstream sequences of genes whose expression is induced by environmental stresses will allow the dissection of the mechanisms that regulate gene expression of this important plant metabolic pathway. In addition to the specific activation of *Ppc1* by water stress demonstrated here, the promoter for this gene is likely to be very complex, as PEPCase expression is also modulated by a diurnal rhythmicity (Vernon et al., 1988) that is one of the central characteristics of CAM (Ting, 1985). We are currently studying this diurnal regulation at the mRNA and transcriptional level. Further study of *cis*-acting regulatory elements and putative transcription factors will enable us eventually to understand the signal transduction pathway involved in the perception of water stress and the metabolic adjustments implemented by the ice plant to cope with such stress.

METHODS

Growth and Harvesting of Plant Material

Common ice plants (*Mesembryanthemum crystallinum*) were grown from seedlings as previously described (Ostrem et al., 1987). Plants were stressed when 6 weeks to 7 weeks old by irrigation each day with a nutrient solution containing 0.5 M NaCl. Plant material was collected at various times after stress (as indicated in figure legends), frozen in liquid nitrogen, and stored at −70°C. Hydroponically grown plants for organ-specific analyses were grown in 0.5 X Hoagland solution with aeration and stressed by adding NaCl solution to a final concentration of 0.5 M to the nutrient solution.

Construction and Screening of Genomic Library

Common ice plants were grown in the greenhouse for 6 weeks. Eighty grams of plant material (leaves and stems) were harvested, and the DNA was isolated according to the method of Bedbrook (1981). When analyzed by electophoresis in a 0.5% agarose gel, the isolated DNA migrated slightly slower than intact bacteriophage T4 DNA (~165 kb).

Aliquots of 12 μg of total genomic DNA were partially digested for various times with 6 units of MboI and separated on a 0.5% agarose gel. The fragments between 23 kb and 9 kb (λ HindIII markers) were electroeluted from gels using a Biotrap apparatus (Schleicher & Schuell, Inc.). The eluted DNA was subjected to a partial fill-in reaction and ligated into the XhoI site of λ FIX (Stratagene, Inc.) according to manufacturer's instructions. 0.1 μg to 0.3 μg of ligated DNA was packaged in vitro using packaging extracts prepared according to method B (Maniatis, Fritsch, and Sambrook, 1982). 0.5 to 0.8 × 10^5 recombinant phage were obtained per packaging reaction. The primary library was screened for PEPCase clones using a nick-translated (Maniatis, Fritsch, and Sambrook, 1982) 2.5-kb XhoI fragment of a PEPCase cDNA clone designated p1211, which contains most of the coding region of the gene from the common ice plant (Rickers et al., 1989). Individual hybridizing plaques were purified by three rounds of plaque hybridization (Benton and Davis, 1977).

Isolation and Characterization of λ DNA from Clones

Large-scale isolation of λ DNA was conducted according to the method of Carlock (1987) except that the CsCl gradient purification step was omitted. Restriction analysis of isolated DNA was conducted according to manufacturer's recommendations.

DNA Sequencing and Sequence Data Analysis

DNA fragments from λ clones that hybridized to p1211 and thus were likely to contain PEPCase coding regions were subcloned into plasmid vectors pTZ18u or pTZ19u (Mead and Kemper, 1986). Single-stranded plasmid DNA was made according to Vieira and Messing (1987). Deletion subclones for sequence analysis were generated using the technique of Dale, McClure, and Hutchins (1985) and sequenced using the dideoxy chain termination method with a modified form of T7 DNA polymerase (Sequenase) (Tabor and Richardson, 1987).

DNA sequence data were compiled using the DNA Inspector IIe sequence analysis program (Textco, Inc., Keene, NH) and analyzed using the programs of the University of Wisconsin Genetics Computer Group (Devereux, Haeberli, and Smithies, 1985) on a Digital Equipment Corporation microVAX2. Amino acid sequence alignments were conducted using the FASTP search algorithm described by Lipman and Pearson (1985).

Genomic DNA Blots

Total genomic DNA was isolated according to the method of Steinmüller and Apel (1986). DNA fragments were transferred from 0.8% agarose gels onto nitrocellulose and hybridized to nick-translated probes as described below.

RNA Blots and Slot-Blots

Total RNA was isolated and slot-blotted onto nitrocellulose as previously described (Michalowski et al., 1989). Nitrocellulose filters were prehybridized briefly and then hybridized at 42°C in 6 × SSC (SSC = 0.15 M NaCl, 0.01 M Na_3 citrate) containing 50% (v/v) formamide and 0.25% (w/v) nonfat dry milk (Johnson et al., 1984) with the appropriate radioactive probe labeled by nick translation (Maniatis, Fritsch, and Sambrook, 1982). Filters were washed in 2 × SSC/0.1% SDS at room temperature or at 60°C

in 0.1 × SSC/0.1% SDS. The filters were exposed to x-ray film (X-omat AR-5, Kodak) at −70°C with intensifying screens. Slot-blots were quantitated with a GS 300 scanning densitometer (Hoefer Scientific Instruments) coupled to a Shimadzu Chromatapac CR-1 integrator.

In Vitro Transcription Assays

Nuclei were isolated at the beginning of the dark period (6 PM) according to the method of Hagen and Guilfoyle (1985) with some modifications. Briefly, after ether treatment, 20 g of fresh leaf tissue was ground with mortar and pestle in 40 mL of nucleus isolation buffer. The homogenate was filtered through two layers of Miracloth and a 90-μm nylon filter. Triton X-100 was added dropwise with stirring to the filtrate to a final concentration of 0.25%. The filtrate was then applied directly to Percoll gradients. The average yield of nuclei was about 1.8×10^5/g fresh weight of tissue. In vitro transcription assays containing 5×10^5 nuclei were conducted as described (Hagen and Guilfoyle, 1985). Incorporation of ^{32}P-UTP (>3000 Ci/mM) was assayed by spotting duplicate 2-μL samples removed during various periods of incubation at 30°C onto Whatman DE81 filters. Filters were washed five times in 5% $NaHPO_4$, two times in dionized H_2O, and two times in 95% ethanol, dried, and counted in 5-mL of nonaqueous scintillation mixture.

DNA Slot-Blot Hybridization

Plasmid or λ DNA (5 μg) was denatured by heating for 1 hr at 65°C in 0.3 M NaOH, neutralized with 0.5 M NH_4OAc (pH 4.7), and diluted with 5 volumes of 10 × SSC (pH 7.0). DNA was then applied to nitrocellulose using a slot-blot device, and membranes were baked for 2 hr at 80°C. Filters were then hybridized to ^{32}P-RNA synthesized by isolated nuclei (1×10^7 cpm/2 mL) as described for RNA slot-blot analysis.

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