Increased Expression of a Gene Coding for NAD:Glyceraldehyde-3-phosphate Dehydrogenase during the Transition from C₃ Photosynthesis to Crassulacean Acid Metabolism in Mesembryanthemum crystallinum*

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We utilized differential plaque hybridization to identify three cDNA clones for transcripts which increase in abundance during the salinity-induced transition from C₃ photosynthesis to crassulacean acid metabolism (CAM) in Mesembryanthemum crystallinum. Although there are differences in the abundance of these transcripts in unstressed tissue, steady-state levels of all three increased within 30 h following irrigation with 0.5 M NaCl. One cDNA encodes the cytosolic form of glyceraldehyde-3-phosphate dehydrogenase (D-glyceraldehyde-3-phosphate:NAD⁺ oxidoreductase (phosphorylating)) (NAD-GAPDH), an enzyme involved in the production of phosphoenolpyruvate for CO₂ fixation at night and the conversion of pyruvate to storage carbohydrate during the day. Coding region and 3'-noncoding sequence probes were used to examine the expression of NAD-GAPDH transcripts in leaf and root tissue. We show that the gene encoding the NAD-GAPDH cDNA is expressed in both leaf and root tissue during C₃ photosynthesis and CAM. NAD-GAPDH transcript levels increase rapidly in leaf (but not in root) tissue during the transition to CAM. Our data indicate that the predominant NAD-GAPDH transcript expressed during C₃ photosynthesis and CAM is encoded by a single gene in M. crystallinum. These results imply that the transition to CAM in some cases involves an upward readjustment in the level of a gene product expressed during C₃ photosynthesis, rather than the expression of a CAM-specific isoform with unique regulatory or kinetic properties.

A small number of plant species switch from C₃ photosynthesis to crassulacean acid metabolism (CAM) as an adaptive response to water stress. A number of changes accompany this transition. During CAM, the normal diurnal cycle of stomatal opening is reversed. Stomata open at night, and the initial carboxylation step utilizes phosphoenolpyruvate carboxylase (EC 4.1.1.31). The product, oxaloacetate, is reduced to malate and stored temporarily in the cell vacuole. Malate is released into the cytoplasm during the following light period and decarboxylated, and the CO₂ is fixed by ribulose-1,5-bisphosphate carboxylase (EC 4.1.1.30) and the Calvin cycle (Osmond and Holtum, 1981).

The transition from C₃ photosynthesis to CAM has been characterized most thoroughly in the halophyte Mesembryanthemum crystallinum (Bohnert et al., 1988). Water stress brought about by increased salinity in the rooting medium ("salt stress") results in a 10–20 fold increase in the activity of P-enolpyruvate carboxylase and a 3–4 fold increase in the activity of several other enzymes involved in carbohydrate metabolism (Holtum and Winter, 1982; Winter et al., 1982). P-enolpyruvate carboxylase is synthesized de novo in response to salt stress (Foster et al., 1982; Hoefner et al., 1987) due at least in part to an increase in P-enolpyruvate carboxylase transcript levels in both fully expanded and rapidly growing leaf tissue (Ostrem et al., 1987). More recent results show that the increased expression of P-enolpyruvate carboxylase is reversible (Vernon et al., 1988) and that stress increases the expression of one member of a multigene P-enolpyruvate carboxylase family (Cushman et al., 1989).

We are characterizing cDNA clones for transcripts which increase in abundance during CAM induction as a first step toward understanding the mechanisms underlying the shift from C₃ photosynthesis to CAM (Schmitt et al., 1988; Rickers et al., 1989). We have now identified clones for several stress-regulated transcripts by differential screening of a cDNA library. One encodes the cytosolic form of glyceraldehyde-3-phosphate dehydrogenase (D-glyceraldehyde-3-phosphate:NAD⁺ oxidoreductase (phosphorylating), EC 1.2.1.12) (NAD-GAPDH), an enzyme that catalyzes an essential step in the production of substrate for nighttime CO₂ fixation in CAM. We report here the sequence of a full-length cDNA and the results from an analysis of the regulation of this gene in M. crystallinum. We show that transcript levels for NAD-GAPDH, P-enolpyruvate carboxylase, and two cDNAs coding for unidentified proteins increase in leaf tissue at similar but not identical rates following irrigation with 0.5 M NaCl. NAD-GAPDH transcript levels increase more rapidly in leaf (but not in root) tissue during the transition to CAM. These results support previous work (Ostrem et al., 1987; Schmitt et al., 1988; Michalowski et al., 1989) showing that CAM induction involves a coordinate increase in steady-state transcript levels for a select subset of genes in M. crystallinum.

EXPERIMENTAL PROCEDURES

Plant Material—Seeds of M. crystallinum were germinated and grown for 6 weeks in potting soil as described (Ostrem et al., 1987). Two h after the start of the light period, plants were watered with...
150 ml of either nutrient solution (control) or nutrient solution containing 0.5 M NaCl (stressed). For construction of the cDNA library, plants were harvested at 8 h (2 h before the first light period) and 32 h (2 h before the end of the light period) after irrigation with 0.5 M NaCl.

**Construction and Screening of cDNA Library—**RNA was extracted, and poly(A)^+ RNA (poly(A)^+) was prepared as described previously (Ottrem et al., 1987). Poly(A)^+ RNAs (2.5 µg) from the 8- and 32-h time points were combined. First- and second-strand cDNA synthesis, ligation with λgt10 arms, and in vitro packaging were carried out according to the manufacturer’s directions (Amer sham Corp.). The filters were screened by differential plaque hybridization (Maniatis et al., 1982) using single-stranded cDNA probes generated by in vitro transcription of plasmid cDNA containing the coding region (H/S) and 3'-noncoding region (A/X) probes to then washed at high stringency and exposed to film as described.

**Electrophoresis, the RNA was electroblotted onto Nytran (Schleicher & Schuell) in 25 mM sodium phosphate buffer, pH 7.0, 0.25% nonfat dry milk, 50% formamide at 42 °C (Johnson et al., 1984) in the presence of 1−3× 10^6 cpm of labeled probe. Filters were washed twice for 15 min at room temperature in 2× SSC, 0.1% (w/v) SDS (low stringency) and then twice for 15 min at 60 °C in 0.1× SSC, 0.1% (w/v) SDS (high stringency) and exposed to film for 24−48 h.

**Construction of Subclones and Nucleotide Sequencing—**Phage DNA was digested with EcoRI and separated in 1.2% (w/v) agarose using 10 mM methyl mercury as the denaturant in the gel and sample buffers. Following digestion with RNase-free DNase (CooperBiomedical, Inc.), unincorporated nucleotides were removed by ethanol precipitation, followed by spin column chromatography (Maniatis et al., 1982). The sizes of the transcription products were checked by gel electrophoresis. The concentration of the transcripts used for the serial dilutions was determined by spectrophotometric measurement.

**NAD-GAPDH Expression during CAM Induction**

**Results**

We identified several cDNA clones for transcripts which either increase or decrease in abundance during CAM induction. One clone (McUA1) contained an open reading frame coding for the carboxyl terminus of NAD-GAPDH. The identity of the coding region of the two other cDNAs coding for transcripts which increased during CAM induction (McUB4 and McUB5) is unknown. A cDNA clone for a transcript which decreases during CAM induction (McDB6) hybridized with a probe for a chlorophyll a/b-binding protein gene isolated from *M. crystallinum* (Michalowski et al., 1989a) and was not characterized further.

**Steady-state Transcript Levels Increase with Similar but Not Identical Kinetics during CAM Induction—**Total RNA was blotted onto nitrocellulose and hybridized with cDNA inserts prepared from McUA1, McUB4, McUB5, and the coding region of a P-enolpyruvate carboxylase cDNA (clone 12,12, Rickers et al., 1989) to compare the kinetics of the induction of NAD-GAPDH and the genes encoded by the two unidentified cDNAs with that of P-enolpyruvate carboxylase.

**Analysis of NAD-GAPDH Gene Structure in Genomic DNA** was carried out by Southern hybridization. Aliquots (8 µg) of *M. crystallinum* DNA were prepared as described by Steinhull and Apel (1986) and digested overnight in 100 µl of KB buffer (McClelland et al., 1988) with EcoRI, HindIII, or SalI. The digests were split into two 50-µl aliquots and electrophoresed overnight at 25 V in a 0.5% (w/v) agarose gel. Air-dried gels were hybridized overnight onto 0.45-µm nitrocellulose by capillary blotting (Southern, 1975). Filters were baked and hybridized at 42 °C as described above with either a coding region (H/S) or a 3'-noncoding region (A/X) probe. Hybridization and washing steps were conducted as described in the legend of Fig. 4.

**Materials and Methods**

**NAD-GAPDH transcript levels were quantitated by hybridization the coding region (H/S) and 3'-noncoding region (A/X) probes to dilutions of total RNA alongside dilutions of full-length and coding region transcripts prepared as described previously (Ottrem et al., 1987). Poly(A)^+ RNAs (2.5 µg) from the 8- and 32-h time points were combined: First- and second-strand cDNA synthesis, ligation with λgt10 arms, and in vitro packaging were carried out according to the manufacturer’s directions (Amer sham Corp.). The filters were screened by differential plaque hybridization (Maniatis et al., 1982) using single-stranded cDNA probes generated by in vitro transcription of plasmid cDNA containing the 8- and 32-h time points were combined: First- and second-strand cDNA synthesis, ligation with λgt10 arms, and in vitro packaging were carried out according to the manufacturer’s directions (Amer sham Corp.). The filters were screened by differential plaque hybridization (Maniatis et al., 1982) using single-stranded cDNA probes generated by in vitro transcription of plasmid cDNA containing the coding region (H/S) and 3'-noncoding region (A/X) probes to then washed at high stringency and exposed to film as described.

**Electrophoresis, the RNA was electroblotted onto Nytran (Schleicher & Schuell) in 25 mM sodium phosphate buffer, pH 7.0, 0.25% nonfat dry milk, 50% formamide at 42 °C (Johnson et al., 1984) in the presence of 1−3× 10^6 cpm of labeled probe. Filters were washed twice for 15 min at room temperature in 2× SSC, 0.1% (w/v) SDS (low stringency) and then twice for 15 min at 60 °C in 0.1× SSC, 0.1% (w/v) SDS (high stringency) and exposed to film for 24−48 h.

**Construction of Subclones and Nucleotide Sequencing—**Phage DNA was digested with EcoRI and separated in 1.2% (w/v) agarose using 10 mM methyl mercury as the denaturant in the gel and sample buffers. Following digestion with RNase-free DNase (CooperBiomedical, Inc.), unincorporated nucleotides were removed by ethanol precipitation, followed by spin column chromatography (Maniatis et al., 1982). The sizes of the transcription products were checked by gel electrophoresis. The concentration of the transcripts used for the serial dilutions was determined by spectrophotometric measurement.

**Serial dilutions for slot blot analysis began with 100 ng of either the full-length or the coding region transcript.

**Analysis of NAD-GAPDH gene structure in genomic DNA was carried out by Southern hybridization. Aliquots (8 µg) of *M. crystallinum* DNA were prepared as described by Steinhull and Apel (1986) and digested overnight in 100 µl of KB buffer (McClelland et al., 1988) with EcoRI, HindIII, or SalI. The digests were split into two 50-µl aliquots and electrophoresed overnight at 25 V in a 0.5% (w/v) agarose gel. Air-dried gels were hybridized overnight onto 0.45-µm nitrocellulose by capillary blotting (Southern, 1975). Filters were baked and hybridized at 42 °C as described above with either a coding region (H/S) or a 3'-noncoding region (A/X) probe. Hybridization and washing steps were conducted as described in the legend of Fig. 4.
The results from Northern analysis of total RNA probed with the 3'-noncoding region probe (A/X) are shown in Fig. 3. The probe hybridized with a 1.4-kb transcript present in total RNA isolated from control plants (Fig. 3, 0 h). Steady-state levels of the 1.4-kb transcript increased ~10-fold in abundance following irrigation with 0.5 M NaCl (Fig. 3, upper). The relative abundance of this transcript in control leaf tissue and the kinetics of its increase following irrigation with 0.5 M NaCl were virtually identical to that observed previously using a probe consisting of both coding and 2'-noncoding sequences (Fig. 3, lower). These results suggested that GAPal encodes the predominant NAD-GAPDH transcript present in M. crystallinum leaf tissue during both C₃ photosynthesis and CAM. To make a more quantitative estimate of NAD-GAPDH transcript levels during C₃ photosynthesis and CAM, we compared hybridization signals obtained from total RNA with those obtained from known quantities of RNA prepared by transcription of plasmid templates containing either the full-length cDNA (GAPal) or the majority of the coding region for NAD-GAPDH (GAPCOD). We estimated the total amount of NAD-GAPDH transcript present in leaf and root tissue RNA by hybridization of the coding region probe (H/S) to total RNA. The 3'-noncoding region probe (A/X) was used to determine the proportion of the total NAD-GAPDH hybridization signal that could be attributed to the stress-induced transcript encoded by GAPal.

Both probes give equivalent hybridization signals in serial dilutions of total RNA from three separate experiments. NAD-GAPDH transcripts were present at ~100 ng/µg of total RNA in root tissue from unstressed plants and at 40 ng/µg of total RNA in unstressed leaf tissue. Transcript levels in root tissue decreased within 6 h following irrigation with 0.5 M NaCl, but increased to levels equal to or slightly above those present in unstressed root tissue at 30- and 54-h time points. In contrast, NAD-GAPDH transcript levels in leaf tissue increased to ~125 ng/µg of total RNA at 30 h and 160 ng/µg of total RNA at 54 h following irrigation with 0.5 M NaCl. After 12 days of irrigation with 0.5 M NaCl, NAD-GAPDH transcript levels (as determined by hybridization with both coding region and 3'-specific probes) were ~10-fold greater in stressed than in unstressed leaf tissue. Hybridization to ribosomal RNA dilutions included on each blot, and to coding region transcripts probed with A/X, was insignificant (data not shown).

The results from Southern analysis of genomic DNA using the H/S and A/X probes are presented in Fig. 4. The blot probed with H/S was washed at low stringency to identify, if possible, all NAD-GAPDH gene family members. This blot was compared with the hybridization pattern from an identical blot probed with A/X and washed at high stringency to identify restriction fragments hybridizing with the 3'-portion of the stress-regulated NAD-GAPDH transcript. The hybridization patterns of these two blots are remarkably similar (Fig. 4). The gene encoding NAD-GAPDH (including the 3'-untranslated region of GAPal) is located on a 15-kb EcoRI fragment, whereas an adjacent 15-kb HindIII fragment contains the 3'-portion of GAPal, but none of the coding region. A 15-kb genomic clone derived from a partial MboI digest of M. crystallinum DNA contains the entire NAD-GAPDH cDNA described here.2 Preliminary analysis of this clone shows that there is an EcoRI site located ~700 bp upstream of the first exon-containing coding sequence, a 2.0-kb SacI fragment containing the majority of the coding region (Fig. 4, SacI digest probed with H/S), and an adjacent 1.5-kb SacI fragment containing the 3'-noncoding region (faintly visible in Fig. 4, SacI digest probed with A/X). We believe that the 3.5-kb fragment hybridizing with both A/X and H/S probes in the SacI digest is due to incomplete digestion of the SacI site linking these adjacent SacI fragments. These data provide evidence that NAD-GAPDH transcripts expressed in leaf and root tissue during C₃ photosynthesis and CAM are encoded by a single gene in M. crystallinum.

**DISCUSSION**

CAM conserves plant water resources in arid environments by allowing CO₂ fixation to occur primarily at night when water loss due to transpiration is minimal. Although some species exhibit characteristics of CAM (e.g. nighttime sto-

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1. J. A. Ostrem, H. B. Bohnert, J. C. Cushman, and G. Meyer, unpublished data.
FIG. 2. Nucleotide sequence and predicted amino acid sequence of stress-induced NAD-GAPDH cDNA from *M. crystallinum*. The amino acid sequence, in one-letter code, is aligned with NAD-GAPDH from *Z. mays* (Brinkmann et al., 1987), *N. tabacum* (Shih et al., 1986), and *S. alba* (Martin and Cerff, 1986). The numbering system used is that of Harris and Waters (1976). Differences in the peptide sequences of other plant NAD-GAPDH enzymes are shown directly above the amino acid residue in the crystalhum polypeptide; identities are indicated (.). Restriction endonuclease sites are shown below the nucleotide sequence.

Despite opening and diurnal fluctuation in malate levels throughout their lifespan, other species adapt to seasonal changes in water availability by switching between C3 photosynthesis and CAM (Ting and Hanscom, 1977; Winter et al., 1978; Guralnick and Ting, 1986). The salinity-induced transition to CAM in *M. crystallinum* provides a simple and reproducible system for investigating the mechanisms underlying this response.

CAM induction entails a complex metabolic readjustment in carbon flow within the plant cell. During CAM, glycolysis plays a major role in providing substrate for P-enolpyruvate carboxylase activity at night; during the day, gluconeogenesis regenerates storage carbohydrates which are utilized during the subsequent period of nighttime CO2 fixation (Holtum and Osmond, 1981). Our finding that stress increases the steady-state level of NAD-GAPDH transcripts is not surprising in light of the increased importance of glycolysis/gluconeogenesis during CAM. Previous work has shown that the activity of several enzymes involved in carbon metabolism increases in CAM leaf tissue from *M. crystallinum* (Holtum and Winter, 1982; Winter et al., 1982; Fahrendorf et al., 1987). Although it is tempting to speculate that CAM induction is regulated primarily via transcriptional control of the genes encoding these enzymes, post-transcriptional controls may also help regulate the balance between C3 photosynthesis and CAM. We have shown recently that P-enolpyruvate carboxylase transcript levels decline rapidly (t1/2 = 2.5 h) in salt-stressed leaf tissue when the rooting medium is flushed with distilled H2O (Vernon et al., 1988). This indicates that control of transcript stability may play a major role in maintaining CAM.

Does stress alter the level of existing enzymes or induce the expression of CAM-specific isotypes? The P-enolpyruvate carboxylase gene family in *M. crystallinum* is made up of at least two members, one of which responds to salt stress and another which is not regulated in response to stress (Cushman et al., 1989). Our data show that stress acts primarily by altering the level of expression of a NAD-GAPDH gene expressed during both C3 photosynthesis and CAM, rather than by inducing the expression of a new CAM-specific isotype. In support of this interpretation, we show that a probe (A/X) unique to a 148-bp sequence in the 3'-noncoding region of GAPDH hybridized with transcripts which were present in unstressed tissue (Fig. 3, upper). These transcripts increased in abundance with kinetics identical to those obtained with a probe consisting of coding region and 3'-noncoding sequences.
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**FIG. 3.** Northern blot analysis of total RNA hybridized with probe for 3'-noncoding region of NAD-GAPDH cDNA. Upper, RNA (10 µg/lane) isolated from leaf tissue at the times indicated (hours) was hybridized with the 3'-specific probe (A/X) and washed at high stringency as described under “Experimental Procedures.” A photograph of the autoradiogram is shown. Lower, the film images were quantitated by densitometry as described in the legend of Fig. 1. The increase in the relative abundance of the transcript hybridizing with the 3'-noncoding probe (●) in this Northern blot is compared with the increase in transcripts which hybridized with a probe consisting of both coding and 3'-noncoding regions (○) in Fig. 1.

(Fig. 3, lower). Furthermore, equivalent quantities of transcripts were detected using either a coding region probe (H/S) that should hybridize with all NAD-GAPDH-related mRNA or a 3'-specific probe that should hybridize only with the stress-regulated NAD-GAPDH transcript described here. The nucleotide sequences of two P-enolpyruvate carboxylase genes from *M. crystallinum* show very little similarity outside the coding region (Cushman and Bohnert, 1989a, 1989b). These results suggest that a single gene encodes the primary NAD-GAPDH transcript present in leaf and root tissue during C₃ photosynthesis as well as during the stress-induced shift to CAM. Increased expression of this gene occurs primarily in leaf tissue, where glycolysis and gluconeogenesis are essential for nighttime CO₂ fixation.

The cDNA for NAD-GAPDH described here has two interesting features. First, the 55-bp untranslated leader sequence is CT-rich (44% C, 43% T, 11% A, 2% G) compared with the 3'-noncoding region (15% C, 38% T, 24% A, 23% G) and is characterized by CT and CTT repeats throughout its length. CT-rich leader sequences are present in other cDNAs from *M. crystallinum* (Michalowski et al., 1989b). The possible functions of these leader sequences in regulating mRNA translation or stability are unknown at present. Second, there is a striking difference in cysteine content among the plant NAD-GAPDH enzymes. The cDNA from *M. crystallinum* encoded 7 cysteine residues. In the other two full-length sequences (*Zea mays* and *Sinapis alba*), cysteine residues are present only in a highly conserved region which contains the catalytically active Cys¹⁴⁰. Four of the additional cysteines in *M. crystallinum* replace valine (Cys²⁵ and Cys²⁸⁵), tryptophan (Cys²⁵⁵), or isoleucine (Cys²⁴⁰) residues which are conserved in *Z. mays*, *S. alba*, and *Nicotiana tabacum* NAD-GAPDH sequences. However, these and other conspicuous changes (Ser²⁴⁰, Thr²⁴⁵, Gln²⁶⁰, Ser²⁷⁰, Iso²⁴⁰) occur in variable regions in the polypeptide (Fabry and Hensel, 1988). The additional cysteines in the *M. crystallinum* polypeptide are located at the surface of the subunit in the crystal structure of the enzyme (Moras et al., 1975).

Future work should provide a more detailed description of the mechanisms underlying the rapid and coordinate increase in transcript levels during CAM induction. We are in the process of characterizing genomic clones for NAD-GAPDH and P-enolpyruvate carboxylase to determine whether conserved genetic elements are present in the promoter region of these genes. The availability of cloned genes for proteins induced in response to stress will facilitate a more systematic investigation of CAM as an adaptive response to water stress and contribute to our understanding of factors which differentiate C₃ photosynthesis and CAM in higher plants.

**Acknowledgments**—We thank Linda Thompkins for help with subcloning and sequencing and Drs. John Cushman and Cathy Wasmann for advice and critical reading of the manuscript.

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J. Biol. Chem. 1990, 265:3497-3502.