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# Molecular cloning and expression of chloroplast NADP-malate dehydrogenase during Crassulacean acid metabolism induction by salt stress*

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## Abstract

A full-length cDNA clone for NADP$^+$-dependent malate dehydrogenase (NADP-MDH; EC 1.1.1.82) from the facultative CAM plant, *Mesembryanthemum crystallinum* has been isolated and characterized. NADP-MDH is responsible for the reduction of oxaloacetate to malate in the chloroplasts of higher plants. The cDNA clone is 1747 bp in size and contains a single open reading frame encoding a 441 amino acid long precursor polypeptide with a predicted molecular weight of 47 949. The predicted, mature NADP-MDH polypeptide sequence from *M. crystallinum* shares 82.7% to 84% amino acid identity with other known higher plant sequences. Genomic Southern blot analysis of *M. crystallinum* DNA indicates that MDH is encoded by a small gene family. Steady-state transcript levels for chloroplast NADP-MDH decrease transiently in the leaves after salt stress and then increase to levels greater than two-fold higher than in unstressed plants. Transcript levels in roots are extremely low and are unaffected by salt-stress treatment. In vitro transcription run-on experiments using isolated nuclei from leaf tissue confirm that the accumulation of NADP-MDH transcripts is, at least in part, the result of increased transcription of this gene during salt stress. The salt-stress-induced expression pattern of this enzyme suggests that it may participate in the $CO_2$ fixation pathway during CAM.

*Abbreviations:* CAM – Crassulacean acid metabolism; DHAP – dihydroxyacetonephosphate; NADP-MDH – NADP-malate dehydrogenase; NAD-MDH – NAD-malate dehydrogenase; OAA – oxaloacetate; PEPC – phospho*enol*pyruvate carboxylase

## Introduction

Higher plants contain various NAD- and NADP-dependent isoforms of malate dehydrogenase that catalyze the reduction of oxaloacetate (OAA) to malate in different subcellular locations in the context of several different metabolic pathways. Chloroplasts contain a unique, nuclear encoded, NADP-specific malate dehydrogenase (malate oxidoreductase, EC 1.1.1.82; NADP-MDH) that exhibits light-dependent activation via the thioredoxin/ferredoxin system (Ferte 1986, Scheibe 1987). In $C_3$ plants, NADP-MDH is essential for balancing reducing equivalents between the chloroplast and the cytoplasm via the malate/oxaloacetate shuttle (Heber 1974). In $C_4$ plants, an abundant NADP-MDH activity is located exclusively in the mesophyll chloroplasts

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* The nucleotide sequence data reported in this paper will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number X63727.

where it functions to reduce oxaloacetate (OAA), the product of primary $CO_2$ fixation by phosphoenolpyruvate carboxylase (PEPC), to malate which is then transported into adjacent bundle sheath cells. The $CO_2$ released during the decarboxylation of malate is then refixed by RUBISCO to enter the Calvin–Benson cycle (Hatch and Slack 1969, Ray and Black 1979). The partial amino acid sequence of the $C_3$ form of NADP-MDH has been determined in pea (Fickenscher et al. 1987, Scheibe et al. 1991). In $C_4$ plants, NADP-MDH has been extensively studied (Kagawa and Bruno 1988) and complete amino acid sequences have been deduced from cDNA or genomic clones from maize (Metzler et al. 1989) and *Sorghum* (Cretin et al. 1990, Luchetta et al. 1990, Luchetta et al. 1991).

In Crassulacean acid metabolism (CAM) plants, the exact role of NADP-dependent malate dehydrogenases in photosynthetic $CO_2$ fixation has not been clearly established. CAM plants separate carboxylation reactions temporally, rather than spatially, as in $C_4$ plants. The majority of $CO_2$ uptake and fixation into $C_4$ acids occurs at night. During the day, plants close their stomata which results in reduced evaporative water loss and draw upon the stored malate as a carbon source for refixation via the Calvin–Benson ($C_3$) photosynthesis cycle.

In the facultative CAM plant, *Mesembryanthemum crystallinum*, the ability to perform CAM can be brought about by water stress in the form of high salinity or drought (Winter 1985). During the transition from $C_3$ to CAM, the activities of both NADP- and NAD-dependent malate dehydrogenase increase (Holtum and Winter 1982, Winter et al. 1982). The chloroplast localized NADP-MDH activity (OAA → MAL) shows a pH optimum of 8.0 and is dependent upon preincubation with dithiothreitol. The extrachloroplastic (mitochondrial and cytosolic) NAD-MDH activity (OAA → MAL) has a pH optimum of 6.5 and does not require dithiothreitol pretreatment. The reduction of OAA to malate during dark $CO_2$ fixation is thought to be catalyzed primarily by the NAD-dependent form of MDH. Thus, the increased NADP-MDH activity that occurs during CAM induction is difficult to explain because a specific role for this enzyme in CAM has not been described. To better understand the role this important enzyme plays in CAM, a full-length cDNA clone encoding a chloroplast localized form of $NADP^+$-dependent malate dehydrogenase (NADP-MDH) from *M. crystallinum* was isolated and characterized. The expression of this gene increases as a result of transcriptional induction during the transition from $C_3$ photosynthesis to CAM brought about by salt stress. The elevated expression levels of NADP-dependent MDH in CAM suggest that this enzyme may participate in $CO_2$ fixation reactions both in the dark and the light.

## Materials and methods

### Plant material

Common ice plants (*Mesembryanthemum crystallinum*) were grown from seed in vermiculite irrigated with half-strength Hoagland's solution in a growth chamber on a 12-h light (23 °C)/12-h dark (18 °C) cycle. Fluorescent lighting provided a photon flux density of 300–350 $\mu E\ m^{-2}\ s^{-1}$. Three-week-old seedlings were transferred to 25 liter black tubs containing Hoagland's solution and grown hydroponically with constant aeration. Six-week-old plants were stressed by the addition of NaCl to a final concentration of 400 mM. Plant material was harvested at various times after the stress treatment (as indicated in figure legends), frozen in liquid nitrogen and stored at −70 °C until use.

### Construction and screening of cDNA libraries

cDNA libraries were constructed from $polyA^+$ RNA isolated from leaf tissue from unstressed or plants in λ-Uni-ZAP XR (Stratagene, Inc., La Jolla, CA) according to manufacturer's instructions. Libraries were prepared from leaf tissue from plants that had been stressed for 30 h and from root tissue from plants that had been stressed for 6 or 30 h. Libraries were screened using a partial cDNA clone for NADP-MDH kindly provided by Dr T. Nelson (Yale) by three rounds of plaque hybridization (Benton and Davis 1977). Positive λ Uni-ZAP-XR clones were excised and propagated as plasmids according to manufacturer's instructions.

### *DNA sequencing and sequence data analysis*

Single-stranded Bluescript SK-plasmid DNA was made according to Vieira and Messing (1987). Deletion subclones for sequence analysis were generated using the technique of Dale et al. (1985). A 'bandaid' oligomer having the sequence: 5′-GAACAAAAGCTGGAGCTCCC-CCC-3′ was used to linearize the single-stranded DNA with SacI prior to digestion with the 3′–5′ exonuclease activity of T4 polymerase. To sequence the opposite strand, the full-length insert was excised using KpnI/SpeI and recloned into these same sites in Bluescript KS-. Sequencing reactions were then conducted using single-stranded DNA and two synthetic primers whose complementary sequences are shown (italicized) in Fig. 2. All sequencing was performed using the dideoxy chain termination method with a modified form of T7 DNA polymerase (Sequenase 2.0) (Tabor and Richardson 1987). Oligonucleotides were synthesized by the University of Arizona Macromolecular Facility, Department of Biotechnology.

DNA sequence data was compiled using the DNA Inspector IIe sequence analysis program (Textco, Inc., W. Lebanon, NH). Database searches were conducted using the FASTA search algorithm described by Pearson and Lipman (1988) as part of the University of Wisconsin Genetics Computer Group program package (UWGCG) (Devereux et al. 1985). Pairwise sequence alignments were conducted using the GAP program of the UWGCG package (Devereux et al. 1985). Multiple sequence alignments were conducted using the CLUSTAL program (Higgins and Sharp 1989).

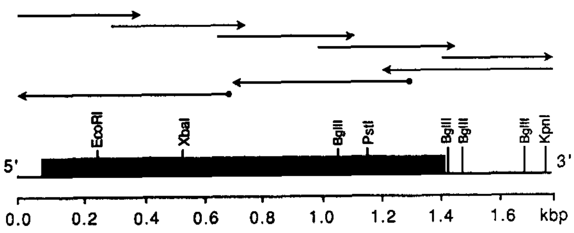

*Fig. 1.* Physical map and sequencing strategy of the full-length NADP-malate dehydrogenase cDNA clone from *Mesembryanthemum crystallinum*. The predicted protein coding region is indicated by a solid box and the putative transit sequence is designated by a shaded box. Selected restriction endonuclease sites are shown as indicated. Arrows (→) indicate the direction and extent of sequencing of deletion clones or of synthetic oligonucleotide primed sequencing (←●).

### *RNA isolation and Northern and Slot-blot analysis*

Total RNA was isolated as described (Ostrem et al. 1987). For Northern blot analysis, 10 $\mu$g total RNA per lane was denatured and resolved on 1.2% agarose gels containing 2.2 M formaldhyde prior to capillary transfer to nitrocellulose (Maniatis et al. 1982). Total RNA was slot-blotted onto nitrocellulose using a Minifold II apparatus according to the protocol supplied by the manufacturer (Schleicher and Schuell, Keene, NH). The baked (2 h at 80 °C) nitrocellulose filters were then prehybridized for 1 h at 42 °C in 6X SSC (SSC = 0.15 M NaCl, 0.01 M $Na_3$ Citrate) containing 50% (v/v) formamide and 0.25% (w/v) non-fat dry milk and then hybridized overnight with a 1.8 kbp KpnI/SpeI insert of MDH-4R that was radiolabeled using the random-priming method (Feinberg and Vogelstein 1983). Filters were washed twice for 15 min in 2X SSC/0.1% SDS and then twice for 15 min in 0.1X SSC/0.1% SDS at 60 °C . Filters were then exposed to X-ray film (X-Omat AR-5, Kodak) at −70 °C with intensifying screens.

### *Genomic Southern blot analysis*

Total genomic DNA was isolated according to the method of Steinmüller and Apel (1986). After restriction digestion and separation on 1% agarose gels, DNA was blotted onto 0.45 $\mu$m nylon membranes (Magna®, MSI) followed by 3 min cross-linking with UV (254 nm) light (Stratalinker, Stratagene, La Jolla, CA). Filters were then prehybridized in 5X SSC, 1% blocking reagent (Boehringer Mannheim), 0.1% N-lauroylsarcosine, 0.02% SDS for 1 h at 65 °C. Prehybridization solution was discarded and replaced with hybridization solution containing random-primed probe and hybridized overnight. Filters were washed for 15 min in 2X SSC/0.1% SDS at RT (low stringency) and then twice for 15 min in 0.1X SSC/0.1% SDS at 65 °C (high stringency).

### *In vitro transcription assays*

Nuclei isolation and in vitro transcription assays were conducted as previously described (Cushman et al. 1989). Blots were quantitated directly using a Betascope 603 Blot Analyzer (Betagen, Inc., Waltham, MA).

## Results

### *Isolation and nucleotide sequence determination of ice plant NADP-MDH*

cDNA libraries were screened using a heterologous cDNA probe from *Zea mays* (Metzler et

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         .        20         .        40         .        60         .        80         .       100
CTCACTCAGTCCCCTGTTTTCTCCTTCTGAACCAGAAAATTCATTTCTCTCTCCTGTTTAGTATTCTCTGCTGCCATGGCAGTGGCAGAGCTTTCTCCTT
                                                                            M  A  V  A  E  L  S  P
         .       120         .       140         .       160         .       180         .       200
CATATAAAACCCAACTTAAAACTTGCCAGCAGCTCTCATCATCACTATCGACCCGCCTTTCCGATCACCGAAAATTCTCTCTTCGCCTCCTTCCTCGTCC
S  Y  K  T  Q  L  K  T  C  Q  Q  L  S  S  S  L  S  T  R  L  S  D  H  R  K  F  S  L  R  L  L  P  R  P
         .       220         .       240         .       260         .       280         .       300
TGTAAGTGTAAGGGGTGGAATTCGTTGCTCGGTTGCACCAAATCAAGTTCAAGCTCCGGTCGCTGTTCCCGCTGAAGGGCAGACTGGCAAGCCGGAGTGC
  V  S  V  R  G  G  I  R  C  S  V  A  P  N  Q  V  Q  A  P  V  A  V  P  A  E  G  Q  T  G  K  P  E  C
         .       320         .       340         .       360         .       380         .       400
TATGGAATCTTTTGCTTGACGTATGATTTGAAAGCCGAAGAGGAGACTAAAACATGGAAAAAAATGATCACGATTGCAGTTTCAGGTGCTGCTGGGATGA
 Y  G  I  F  C  L  T  Y  D  L  K  A  E  E  E  T  K  T  W  K  K  M  I  T  I  A  V  S  G  A  A  G  M
         .       420         .       440         .       460         .       480         .       500
TATCAAACCATCTGTTATTCAAACTTGCTTCTGGTGAGGTATTTGGACCAGATCAACCAATTGCTTTGAAGTTACTGGGCTCCGAGCGGTCATTCAATGC
I  S  N  H  L  L  F  K  L  A  S  G  E  V  F  G  P  D  Q  P  I  A  L  K  L  L  G  S  E  R  S  F  N  A
         .       520         .       540         .       560         .       580         .       600
TCTAGAAGGAGTAGCTATGGAACTTGAGGACTCGTTATATCCTTTGTTGAGGGCAGTCAGCATTGGTATTGACCCTTATGACATATTCCAAGATGCGGAA
  L  E  G  V  A  M  E  L  E  D  S  L  Y  P  L  L  R  A  V  S  I  G  I  D  P  Y  D  I  F  Q  D  A  E
         .       620         .       640         .       660         .       680         .       700
TGGGCCCTTCTAATTGGAGCAAAACCTCGTGGGCCTGGCATGGAACGAGCTGACTTATTAGATATAAATGGGCAGATTTTTGCAGAGCAGGGGAAAGCTC
 W  A  L  L  I  G  A  K  P  R  G  P  G  M  E  R  A  D  L  L  D  I  N  G  Q  I  F  A  E  Q  G  K  A
         .       720         .       740         .       760         .       780         .       800
TTAATGCTGTAGCATCTCGTAATGTGAAAGTCATAGTGGTGGGAAATCCCTGCAACACCAATGCATTGATCTGCTTGAAAAATGCTCCAAATATACCTGC
L  N  A  V  A  S  R  N  V  K  V  I  V  V  G  N  P  C  N  T  N  A  L  I  C  L  K  N  A  P  N  I  P  A
         .       820         .       840         .       860         .       880         .       900
AAAGAATTTTCATGGTCTCACTAGATTAGATGAAAACCGAGCAAAGTGCCAGCTGGCTCTCAAAGCTGGTGTCTTTTACGATAAGGTATCAAATATGACC
  K  N  F  H  G  L  T  R  L  D  E  N  R  A  K  C  Q  L  A  L  K  A  G  V  F  Y  D  K  V  S  N  M  T
         .       920         .       940         .       960         .       980         .      1000
ATTTGGGGGAACCACTCGACCACTCAGGTCCCAGATTTTTTAAATGCCAAAATTGATGGATTACCTGTCAAAACTGTCATCAAGGACCACAAGTGGTTGG
 I  W  G  N  H  S  T  T  Q  V  P  D  F  L  N  A  K  I  D  G  L  P  V  K  T  V  I  K  D  H  K  W  L
         .      1020         .      1040         .      1060         .      1080         .      1100
AAGAGGAGTTCACAGTGATGATTCAAAAAAGAGGTGGGGCGCTTATTCAGAAATGGGGAAGATCTTCTGCTGCTTCAACTGCTGTGTCAATTGCTGATGC
E  E  E  F  T  V  M  I  Q  K  R  G  G  A  L  I  Q  K  W  G  R  S  S  A  A  S  T  A  V  S  I  A  D  A
         .      1120         .      1140         .      1160         .      1180         .      1200
CATTAAATCTCTGGTCACCCCTACTCCAGAGGGTGACTGGTTTTCTTCTGCAGTATATACGAATGGGAATCCTTACGGTATAGCTGAGGATCTTGTTTTC
  I  K  S  L  V  T  P  T  P  E  G  D  W  F  S  S  A  V  Y  T  N  G  N  P  Y  G  I  A  E  D  L  V  F
         .      1220         .      1240         .      1260         .      1280         .      1300
AGTATGCCATGCAGATCAAAGGGAGATGGTGATTATGAGCTTGTCAAGGATGTGGTGTTTGATGACTACCTTCGTCAGCGAATAAAGAAGAGTGAGGAGG
 S  M  P  C  R  S  K  G  D  G  D  Y  E  L  V  K  D  V  V  F  D  D  Y  L  R  Q  R  I  K  K  S  E  E
         .      1320         .      1340         .      1360         .      1380         .      1400
AACTGTTGGCTGAGAAGCGATGCACGGCACACCTCACAGGAGAGGGTGTGGCTGTTTGTGATCTTCCTGCTGGAGACACAATGCTTCCAGGAGAAATGTA
E  L  L  A  E  K  R  C  T  A  H  L  T  G  E  G  V  A  V  C  D  L  P  A  G  D  T  M  L  P  G  E  M  *
         .      1420         .      1440         .      1460         .      1480         .      1500
GATCTTATATGTCATACCCTTCCAACTCAACTAGAAATCGGCATTGATATTATTGACCTACATAGAAGATCTCTACTGTGTGATTTATTCAGCACAAATA
         .      1520         .      1540         .      1560         .      1580         .      1600
AATGTACCTTTTTTGCAATAAATTGAGAGAGAGATGGGGACTGAGATGCTCATTGAGCCCTTGGCCTGTACAATACCCAGCAAACTGGAGGAAGCGGCGG
         .      1620         .      1640         .      1660         .      1680         .      1700
GTAGGACTGTGGGGGTTGCAGCAACCTGCGTTTACAATTCATTCTTTTTCGGGATCTCGATTTGAAAACAAGATCTTGGTTGGTTAGGATTGTCACCTTC
         .      1720         .      1740
CCTTTTCAATGGTACCCTTGTGTTCTTACAAAAAAAAAAAAAAAAAA
```

*Fig. 2.* Nucleotide sequence and deduced amino acid residues of a full-length chloroplastic *M. crystallinum* NADP-MDH. The deduced single letter amino acid sequence is given below the center nucleotide of each codon triplet. An asterisk (*) designates the TAG termination codon. Two putative polyadenylation signals are underlined along with a conserved YGTGTTYY motif thought to be involved in efficient transcript processing. *Italicized* sequences designate complementary oligonucleotides used for sequencing the opposite strand.

al. 1989). DNA was isolated from positively hybridizing plaques from root (6 h stress) and leaf libraries (30 h stress) and screened by restriction analysis for insert lengths equivalent to the expected size of a full-length transcript of approximately 1.8 kbp. One clone designated MDH-4R was sequenced according to the strategy outlined in Fig. 1 and was found to be full-length. This cDNA is 1747 nucleotides long including a poly-A tail of 18 adenine residues. A single open reading frame of 1323 bp begins with an AUG initiation codon at position 76 and ends with a TAG termination codon at position 1399 as shown in Fig. 2. The nucleotide sequence that surrounds the initiation codon at position 76 is identical to the consensus sequence for plant translational start sites (AACAAUGGC) at six out of nine places (Lütcke et al. 1987). Two polyadenylation signals that exactly match the consensus polyadenylation signal AATAAA described for higher plants (Joshi et al. 1987) are located 95 and 116 bp downstream of the TAG termination codon. A sequence located 196 bp downstream of the 3′ polyadenylation signal matches perfectly the YGTGTTYY motif regarded as being important for efficient transcript processing (Ingelbrecht et al. 1989).

### *Genomic Southern blot analysis*

To assess the complexity of the NADP-MDH gene family in *M. crystallinum*, genomic Southern blots of DNA digested with four different restriction endonucleases were probed with the full-length cDNA clone. Duplicate blots were washed at low (room temperature) or high (65 °C) stringency as shown in Fig. 3. At low stringency, multiple fragments are detected, whereas at high stringency many of these bands are absent. At high stringency, one major band remains along with one or two weaker bands. These results suggest that there is probably a low copy number of NADP-MDH genes encoding the chloroplast form described here and encoding the mitochondrial and cytosolic isoforms present on the common ice plant genome.

### *Amino acid sequence alignments with higher plant NADP-MDH*

The open reading frame shown in Fig. 2 encodes a 441 amino acid long polypeptide with a predicted molecular weight of 47 949. This predicted protein sequence was compared to other known higher plant NADP$^+$-malate dehydrogenase sequences as shown in Fig. 4. The ice plant NADP-MDH preprotein is slightly larger than the *Sorghum* (429 or 432 AA) or maize (432 AA) forms. This difference is accounted for almost entirely by the predicted NH$_2$-terminal transit peptide in the ice plant. The amino acid sequence surrounding the experimentally determined cleavage site for *Sorghum* (RC^SV, positions 51–54 in Fig. 4)(Cretin et al. 1990) is completely conserved in all five amino acid se-

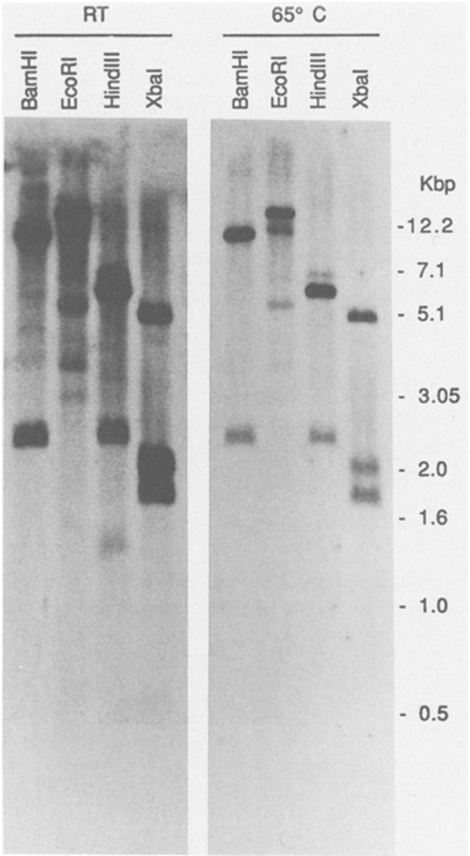

*Fig. 3.* Genomic Southern blot analysis of the NADP-MDH gene family in the common ice plant. Total genomic DNA isolated from ice plant leaf tissue was digested with BamHI, EcoRI, HindIII and XbaI. The DNA samples (5 μg/lane) were then separated on a 1% agarose gel, blotted onto nylon membranes and hybridized as described in Methods and materials. Duplicate blots were then washed at low (room temperature, RT) or high (65 °C) stringency conditions.

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        ..................20..................40..................60
MC      MAVAEL-SPSYKTQLKTCQQLSSSLSTRLSDHRKFSLRLLPRPVSVRGGIRCSV-APN-Q
PS      xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx
SVI1     GLSTAY  VGSH- ---------APAP GHR SAQ HR-  RALL-ATV    D AK-
SVI2     GLSTAY  AGSG- ---------VPAP ARAARRRSVQ-V RPRL-ATV    VDAAK
SVT      GLSTAY  VGSH- ---------APAP GHR SAQ HR-  RALL-ATV    D AK-
ZM       GLSTVY  AGPR- ---------VPAP GRC SAQP R-  RAPL-ATV    D TK-

        ..................80.................100.................120
MC      VQAPVA--VPAEGQTGKPECYGIFCLTYDLKAEEETKTWKKMITIAVSGAAGMISNHLLF
PS      xxxxxxxAQTQDPK   D   V          TxxxxxxxL N
SVI1      DG  TA--- APATRKD F V  T       DK  S   LVN
SVI2      DG  TA-AGG AASGN  F V  NI      DK  S   LV
SVT       DG  TA--- APATRKD F V  T       DK  S   LVN
ZM      A DG  TA AT APASRK  F V  T       DK  S R LVNV

        .................140.................160.................180
MC      KLASGEVFGPDQPIALKLLGSERSFNALEGVAMELEDSLYPLLRAVSIGIDPYDIFQDAE
PS                       xxxxxx IQ             F    E V S    EV
SVI1             Q               Q                  E        EV E VD
SVI2             Q               Q     R            E     G  EV   VD
SVT              Q               Q                  E        EV E VD
ZM               Q               Q                  E        VV   VD

        .................200.................220.................240
MC      WALLIGAKPRGPGMERADLLDINGQIFAEQGKALNAVASRNVKVIVVGNPCNTNALICLK
PS                xxxxxx A              xxxxxxxxxxx
SVI1                     A          D          K    L
SVI2                     A          D               L
SVT                      A          D          K    L
ZM                       A          D            DE L

        .................260.................280.................300
MC      NAPNIPAKNFHGLTRLDENRAKCQLALKAGVFYDKVSNMTIWGNHSTTQVPDFLNAKIDG
PS      xxxxxxxx   A   xxxxxxx                                  RL
SVI1       D       A                          V
SVI2     T         A                          V
SVT      P H       A                          V
ZM                 A                          V

        .................320.................340.................360
MC      LPVKTVIKDHKWLEEEFTVMIQKRGGALIQKWGRSSAASTAVSIADAIKSLVTPTPEGDW
PS      I   xxxxxxx       EKxxxxxxxxxxxxxx      S   V   R  I
SVI1    R   E    T        ITV
SVI2    R   EI Q T        MTV     L                 L             E
SVT     R   E    T        ITV
ZM      R   E    T        LTV     V                 V   R

        .................380.................400.................420
MC      FSSAVYTNGNPYGIAEDLVFSMPCRSKGDGDYELVKDVVFDDYLRQRIKKSEEELLAEKR
PS         G     V       I  G              N  I      xxxxxxxxxxxxxxx
SVI1      TG   T         I                AT  SM  F WE      A      K
SVI2      TG   T         I                AT  SM  F WE      A      K
SVT       TG   T         I                AT  SM  F WE      A      K
ZM        TG   T         I                AS  LM  F WE      A      K

        .................440......
MC      CTAHLTGEGVAVCDLPAGDTMLPGEM
PS       V       I      -
SVI1     V       N Y  V E-       V
SVI2     V       D F    E-       N
SVT      V       N Y  V E-       V
ZM       V       N F    E-       V
```

*Fig. 4.* Comparison of the predicted amino acid sequences of higher plant chloroplast NADP-MDH. The predicted amino acid sequences of higher plant chloroplast NADP-MDH are aligned to the *Mesembryanthemum crystallinum* (MC) sequence. Amino acid sequence from *Pisum sativum* (PS) was derived from peptide sequencing (Fickenscher et al. 1987, Scheibe et al. 1991) with x's denoting unsequenced portions of the protein. Amino acid sequences from *Sorghum vulgare* cv. INRA 450 (SVI1) (Luchetta et al. 1990, 1991), *Sorghum vulgare* cv. INRA 450 (SVI2) (Luchetta et al. 1991), *Sorghum vulgare* cv. Tamaran (SVT) (Cretin et al. 1990) and *Zea mays* (ZM) (Metzler et al. 1989), were deduced from nucleotide sequences. Sequences were aligned using the CLUSTAL multialignment program (Higgins and Sharp 1989). Gaps to optimize alignments are designated by (-). Residues identical to the MC sequence are not shown for simplicity. Conserved cysteine residues at positions 80 and 85 that participate in disulfide bridge formation are underlined.

quences determined to date. Furthermore, the region proximal to the processing site in *M. crystallinum* has the sequence (SVRGG-IRC^), which resembles the conserved region just before the small subunit of RUBISCO cleavage site (SN-GGRVRC^) (Keegstra et al. 1989). If this processing site is used, the predicted ice plant transit peptide would be 51 amino acids long. In maize, however, the N-terminal amino acid sequence of the mature NADP-MDH was determined to occur at position 70 in Fig. 4 that predicts a 57 amino acid transit peptide. The predicted, mature ice plant NADP-MDH polypeptide shares between 82.7% to 84% amino acid identity (96.9% to 97.9% similarity) with the mature $C_4$ enzymes from maize (Metzler et al. 1989) and *Sorghum* (Cretin et al. 1990, Luchetta et al. 1990, 1991). Comparable levels of amino acid identity and similarity to the Pea sequence are expected if a complete amino acid sequence were available (Fickenscher et al. 1987, Schiebe et al. 1991).

### *Salt-induced expression of NADP-MDH*

Plants were grown hydroponically until six weeks old and then stressed with 400 mM NaCl to characterize the expression pattern of NADP-MDH in the common ice plant during the initial stages of the transition from $C_3$ photosynthesis to CAM. Northern blot analysis shows a single transcript of about 1.8 kb in size that decreases after 6 h of salt stress. After a transient decrease at 6 h of stress, transcript levels begin to increase again by 12 h of stress and continue to accumulate to a maximum level after 78 h of stress (Fig. 5A). Unstressed control plants harvested over the same time course did not display any changes in transcript levels (data not shown). To quantitate the increases in steady-state mRNA levels in leaf and root tissues, serial dilutions of total RNA isolated during the same time-course of salt stress were slot-blotted and hybridized with a NADP-MDH radiolabeled cDNA probe and quantitated by laser densitometry (Fig. 5B). Steady-state mRNA levels in leaves decline transiently after 6–12 h of salt stress and then increase to reach maximal levels by 78 h. In contrast, NADP-MDH transcripts were present at very low levels in root tissue harvested from the same plants and did not change during the salt stress treatment (Fig. 5B).

### *Transcriptional activation of NADP-MDH by salt stress*

To verify that the observed increases in NADP-MDH transcript accumulation were due to transcriptional activation of this gene, changes in transcription rates during stress were examined. Amounts of NADP-MDH transcripts synthesized by nuclei isolated from leaves of unstressed or 78-h salt-stressed plants were quantitated by RNA-DNA slot-blot hybridization. Filters were washed at high stringency (0.1X SSC/0.1% SDS at 60 °C) to distinguish only those transcripts specific for the NADP-dependent MDH gene in ice plant. As shown in Fig. 6, salt stress caused over a 100% increase in transcription rates for NADP-MDH in comparison to rates in unstressed leaves. This transcriptional activation is like that previously observed for a CAM specific isogene (*Ppc*1) of phosphoenolpyruvate carboxylase, which is also transcriptionally induced by salt stress (Cushman et al. 1989). In contrast, the transcription rates of constitutively expressed genes such as the $C_3$ form of PEPC (*Ppc*2) and the chloroplast localized phosphoribulokinase (PRK) remains relatively unchanged by salt stress (Cushman et al. 1989, Michalowski et al. 1992). However, the transcription rate of a second chloroplast localized enzyme, ferredoxin-$NADP^+$ reductase (FNR), decreased almost two-fold as the result of the salt stress treatment in agreement with previous findings (Michalowski et al. 1992).

## Discussion

In response to salinity or drought stress, the facultative, halophytic CAM plant *M. crystallinum* will shift from a $C_3$ to a CAM mode of photosynthesis. This transition provides a unique opportunity to study the molecular basis of CAM photosynthetic carbon fixation. A stress-induced cDNA clone for the NADP-dependent form of malate dehydrogenase from *M. crystallinum* was isolated and characterized. Steady-state transcript levels of NADP-MDH in the common ice

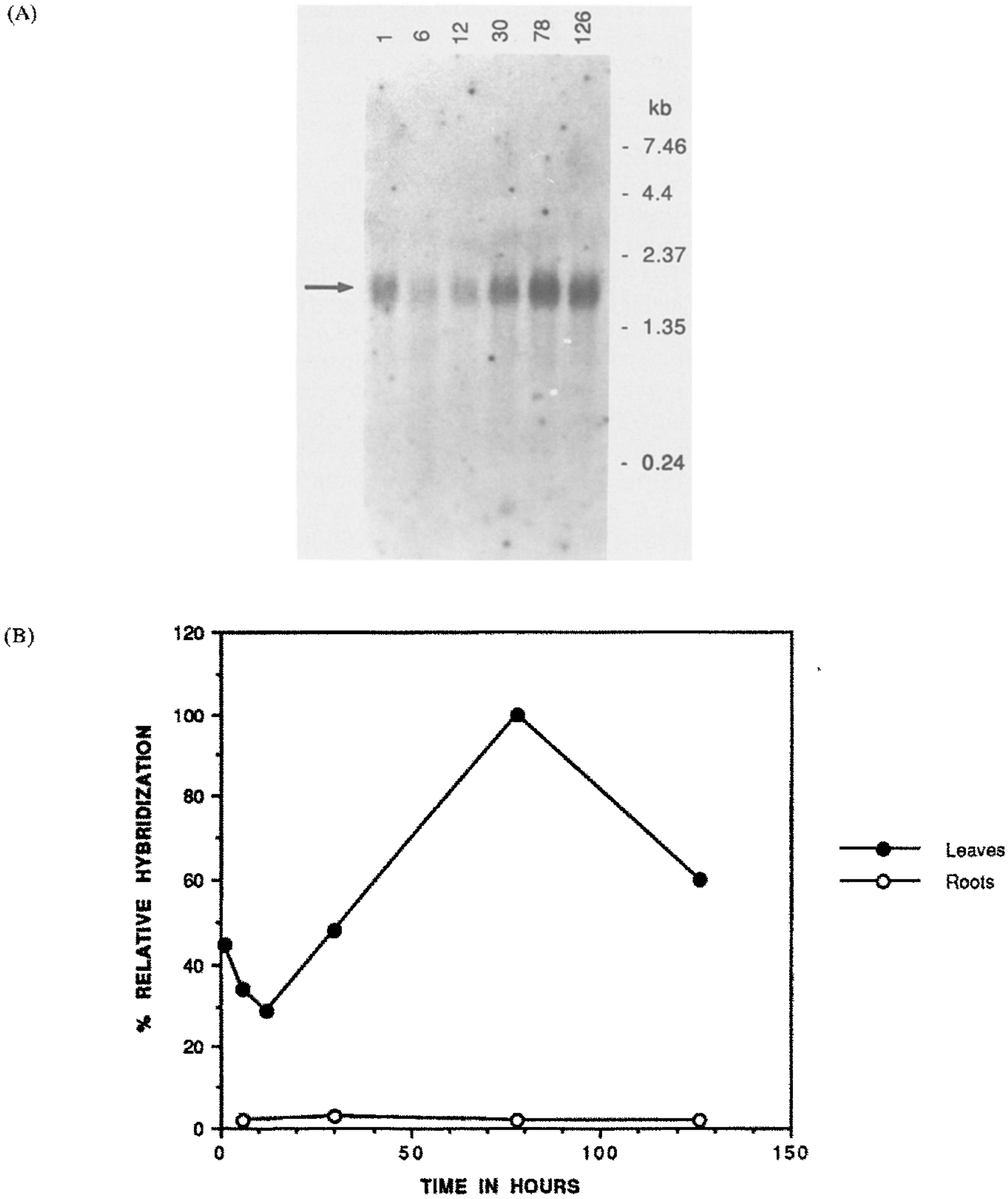

*Fig. 5.* Northern and slot blot analysis of NADP-MDH steady-state transcript levels during salt stress. (A) Total RNA was isolated from leaves of six-week-old plants that had been irrigated for 1, 6, 12, 30, 72 and 126 h with 0.4 M NaCl. Equal amounts (10 μg) of total RNA were loaded in each lane and resolved by electrophoresis through a 1.2% Agarose gel containing formaldyhyde. RNA was then transferred to nitrocellulose and probed with the full-length 4R cDNA clone of NADP-MDH. Arrow indicates the single major transcript of about 1.8 kb. (B) Quantitation of the accumulation of steady-state transcript levels in ice plant leaves and roots was carried out by slot-blot hybridization. Equal amounts of total RNA isolated from leaf and root tissue isolated from ice plants stressed with 0.4 M NaCl for 1, 6, 12, 30, 72 and 126 h were slot-blotted onto nitrocellulose filters as two-fold serial dilutions (5.0 μg to 0.15 μg), hybridized and washed as described in methods and materials. Filters were then exposed to X-ray film and quantitated by laser desitometry. The relative percent hybridization signal was then plotted over time of salt stress.

plant increase in leaf tissue during the transition from $C_3$ to CAM initiated by high salinity. Prior to this increase, transcript levels declined transiently in a manner similar to that observed for transcripts of other chloroplast localized proteins such as SSU of Rubisco, chlorophyll *a*/*b* binding protein (Michalowski et al. 1989a) and PRK (Michalowski et al. 1992). This transient decline may be the result of changes in transcript stability or a decline in transcription rates immediately after the initial stress treatment. Fluctuations in steady-state transcript levels, however, are not due to diurnal expression patterns of NADP-MDH (DeRocher EJ, Michalowski CB and Cushman JC, unpublished). Roots contained low, but detectable levels of NADP-MDH message that did not increase as a result of salt stress. This result is not unexpected considering that the chloroplast localized gene product of NADP-MDH may function primarily in a photosynthetic capacity.

To confirm that increases in NADP-MDH transcript accumulation in leaves were due to transcriptional activation, in vitro transcription run-on experiments using nuclei isolated from leaves of unstressed or 78-h stressed plants were conducted. Salt stress leads to an increase in transcription rates for NADP-MDH (Fig. 6). The induction for NADP-malate dehydrogenase transcription is less pronounced than *Ppc*1 transcription (Cushman et al. 1989) and correspondingly leads to lower levels of transcript accumulation (2–3-fold), when compared to the very large accumulation of *Ppc*1 transcripts (over 30-fold) induced by salt stress (Cushman et al. 1989). The transcriptionally induced expression of NADP-MDH resembles that of other CAM genes up-regulated by salt stress such as *Ppc*1 (Cushman et al. 1989) and NADP-malic enzyme (Cushman 1992). This observation suggests that a common mechanism is involved in the induction of certain CAM pathway enzymes by salt stress in the common ice plant. In comparison, the expression patterns for the constitutively expressed *Ppc*2 gene (Cushman et al. 1989) and the chloroplast localized phosphoribulokinase (PRK), another

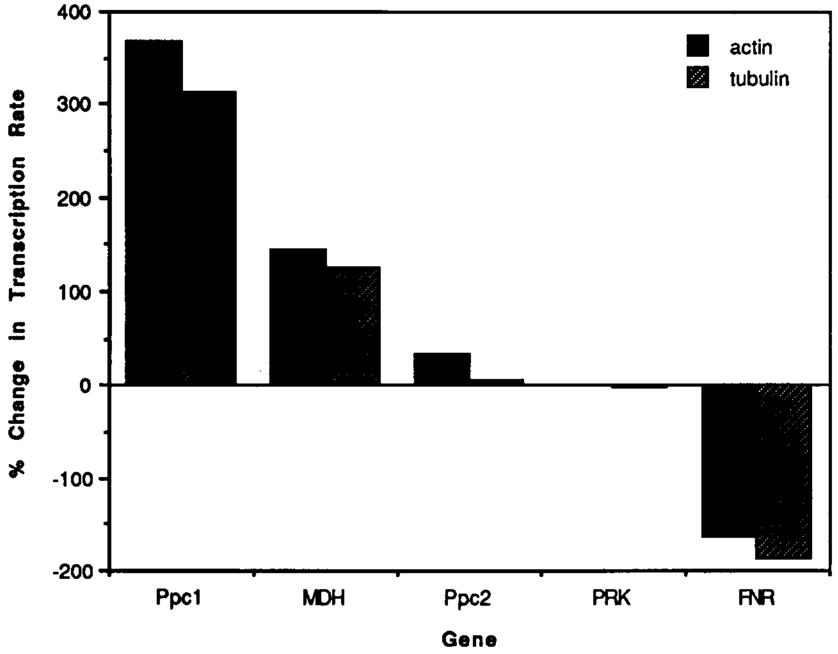

*Fig. 6.* Salt-stress induction of NADP-MDH transcription. Radiolabeled in vitro transcripts synthesized by nuclei isolated from unstressed or 78-h salt-stressed plants were hybridized to nitrocellulose filters containing 5 μg cloned DNA sequences from *Ppc*1, NADP-MDH (MDH), *Ppc*2, phophoribulokinase (PRK), and ferredoxin-NADP + reductase (FNR). The fold increases in transcription rates were normalized to both actin or tubulin (from *M. crystallinum*, unpublished), the transcription rates of which do not change during salt stress. The data represent the average of two independent experiments.

thioredoxin/ferredoxin regulated enzyme, remain relatively unaffected by the salt stress treatment (Michalowski et al. 1992). In contrast, ferredoxin-NADP$^+$ reductase (FNR) transcription rates decline almost two-fold during salt stress (Michalowski et al. 1992) despite constant levels of steady-state messenger RNA (Michalowski et al. 1989b).

Two malate dehydrogenase activities, one NAD-dependent and the other NADP-dependent, increase during CAM induction, suggesting that both enzymes participate in CAM (Holtum and Winter 1982, Winter et al. 1982). The NAD-dependent form is thought to carry out the majority of malate synthesis during the period of nocturnal $CO_2$ fixation by PEPC. In contrast, the NADP-dependent form of the enzyme may participate in $CO_2$ fixation reactions under both dark and light conditions. NADP-MDH activity requires that disulfide bridges on the protein be reduced by thioredoxin, which receives electrons from ferredoxin and the photosynthetic electron transport system (Buchanan 1980). This covalent modification system for regulating the enzyme operates in CAM plants via the ferredoxin/thioredoxin system (Hutcheson and Buchanan 1983a,b). Two highly conserved cysteine residues located at positions 80 and 85 (see Fig. 4) participate in the formation of covalent disulfide bridges that are responsible for the activation of the enzyme under reducing conditions in the light (Dicottignies et al. 1988, Scheibe et al. 1991). The light activation of NADP-MDH by reduction of disulfide linkages suggests that the highest activities of the enzyme occur primarily during the daytime.

NADP-MDH involvement in photosynthetic $CO_2$ fixation in the light is further supported by the observation that CAM plants typically display a large burst of $CO_2$ uptake just after illumination under laboratory (Winter and Tenhunen 1982, Kluge et al. 1982) and under natural conditions (Lange et al. 1975, Osmond et al. 1979, Schuber and Kluge 1981). During the early part of this burst, the majority of $CO_2$ fixation results in malate formation via the combined actions of PEPC and NAD(P)-MDH. This 'morning burst' of $CO_2$ fixation may contribute substantially to total net carbon gain (Winter 1980). The light-induced activation of the NADP-dependent form of MDH in CAM plants (Gupta and Anderson 1978, Osmond and Holtum 1981, Hutcheson and Buchanan 1983a) suggests that it may be responsible for this burst of $CO_2$ fixation. Direct evidence for the apparent light activation of NADP-MDH has been documented in *Opuntia erinacea* where the enzyme showed increased activity during the morning burst (Littlejohn and Ku 1984). The light-inducible nature of NADP-MDH and the salt-stress-inducible expression pattern documented in this work both support the suggestion that this enzyme may contribute to $CO_2$ fixation by catalyzing malate formation during certain times of the daily diurnal CAM cycle.

The suggestion that NADP-MDH is also functional in CAM plants in the dark is supported by the observation that chloroplasts can fix $CO_2$ in the dark via the DHAP shuttle if they are supplied with DHAP and OAA (Werdan et al. 1975). This shuttle yields ATP production and is dependent upon NADP-MDH activity for the reoxidation of NADPH in the stroma. This process would require 3-phosphoglycerate, a product of glycolysis, which is a process normally operative during nocturnal $CO_2$ fixation in *M. crystallinum*. The operation of the DHAP shuttle has recently been proposed to contribute to the generation of intraplastidic ATP for fatty acid biosynthesis in root plastids (Kleppinger-Sparace et al. 1992). Furthermore, inhibition of NADP-MDH in both $C_3$ and $C_4$ plants can be overcome in the dark by anaerobic conditions (Nakamoto and Edwards 1983, Vivkanandan and Edwards 1987). The source of reducing power for activating NADP-MDH under dark conditions comes from glycolysis presumably via the DHAP shuttle. Despite the light-activated regulation of NADP-MDH, there is apparently enough activity in various CAM species to sustain sufficient levels of carbon flow in the dark (Gupta and Anderson 1978, Hutcheson and Buchanan 1983a). These observations suggest that NADP-MDH is functional in the dark in CAM plants and can potentially participate in dark $CO_2$ fixation reactions. The detection of NADP-MDH specific transcripts in roots and the isolation of cDNA clones for NADP-MDH from root libraries of *M. crystallinum* serves to reinforce the possible participation of this enzyme in dark $CO_2$

fixation reactions. More detailed analysis of the activity of NADP-MDH in CAM plants grown under dark conditions is needed.

A third possible role for NADP-MDH, which is similar to the role of the enzyme in $C_3$ plants, may be to balance reducing equivalents between the chloroplast and the cytosol via the malate/OAA shuttle (Heber 1974). In this capacity, NADP-MDH is not directly involved in photosynthetic carbon metabolism, but instead serves as a 'malate valve' that readily transports reducing equivalents in the form of malate via the dicarboxylate translocator located on the inner chloroplast envelope membrane, which is normally impermeable to pyridine nucleotides (Scheibe 1987). During CAM, starch breakdown via glycolysis occurs in the dark to supply PEP as a substrate for $CO_2$ fixation into OAA by PEPC. If PEP supply during glycolysis involves the export of 3-phosphoglycerate rather than glyceraldehyde 3-phosphate from the chloroplasts, then NADP-MDH may serve to regenerate chloroplastic NADP (Osmond and Holtum 1981). In $C_3$ plants, the activity of NADP-MDH is correlated with electron flux through Photosystems I and II (Harbinson et al. 1990). In *Mesembryanthemum*, there is no evidence of any pronounced changes in the photosynthetic apparatus or electron transport activities during CAM induction brought about by salt stress (Köster and Anderson 1988) that might alter redox status within the plastid stroma. Thus, the increased activity of this enzyme during salt stress is likely to be the direct result of increased amounts of this enzyme caused by the transcriptional induction of the NADP-MDH gene described here and not the result of changes in redox state.

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## References

Buchanan BB (1980) Role of light in the regulation of chloroplast enzymes. Ann Rev Plant Physiol 31: 341–374

Benton WD and Davis RW (1977) Screening lambda *gt* recombinant clones by hybridization to single plaques *in situ*. Science 196: 180–182

Cretin C, Luchetta P, Joly C, Decottignies P, Lepiniec L, Gadal P, Sallantin M, Huet J-C and Pernollet J-C (1990) Primary structure of sorghum malate dehydrogenase (NADP) deduced from cDNA sequence. Eur J Biochem 192: 299–303

Cushman JC (1992) Characterization and expression of a NADP-Malic Enzyme cDNA induced by salt stress from the facultative CAM plant, *Mesembryanthemun crystallinum*. Eur J Biochem (in press)

Cushman JC, Meyer G, Michalowski CB, Schmitt JM and Bohnert HJ (1989) Salt stress leads to the differential expression of two isogenes of phospho*enol*pyruvate carboxylase during Crassulacean acid metabolism induction in the common ice plant. Plant Cell 1: 715–725

Dale RMK, McClure BA and Houchins JP (1985) A rapid single-stranded cloning strategy for producing a sequencial series of overlapping clones for use in DNA sequencing: Application to sequencing the corn mitochondrial 18S rDNA. Plasmid 13: 31–40

Decottignies P, Schmitter J-M, Miginiac-Maslow M, Le Marechal P, Jacquot J-P and Gadal P (1988) Primary structure of the light-dependent regulatory site of corn NADP-malate dehydrogenase. J Biol Chem 263: 11780–11785

Devereux J, Haeberli P and Smithies O (1985) A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res 12: 387–393

Feinberg AP and Vogelstein B (1983) A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal Biochem 132: 6–13

Ferte N, Jacquot J-P and Meunier J-C (1986) Structural, immunological and kinetic comparisons of NADP dependent malate dehydrogenases from spinach (C3) and corn (C4) chloroplasts. J Biochem 154: 587–595

Fickenscher K, Scheibe R and Marcus F (1987) Amino acid sequence similarity between malate dehydrogenases (NAD) and pea chloroplast malate dehydrogenase (NADP). Eur J Biochem 168: 653–658

Gupta VK and Anderson LE (1978) Light modulation of the activity of carbon metabolism enzymes in the Crassulacean acid metabolism plant *Kalanchoë*. Plant Physiol 61: 469–471

Harbinson J, Genty B and Foyer CH (1990) Relationship between photosynthetic electron transport and stromal enzyme activity in pea leaves. Plant Physiol 94: 545–553

Hatch MD and Slack CR (1969) NADP-specific malate dehydrogenase and glycerate kinase in leaves and evidence for their location in chloroplasts. Biochem Biophys Res Comm 34: 589-593

Heber U (1974) Metabolite exchange between chloroplasts and cytoplasm. Ann Rev Plant Physiol 24: 393–421

Higgins DG and Sharp PM (1989) Fast and sensitive multiple sequence alignments on a microcomputer. CABIOS Communications 5: 151–153

Holtum JAM and Winter K (1982) Activity of enzymes of carbon metabolism during the induction of Crassulacean acid metabolism in *Mesembryanthemum crystallinum* L. Planta 155: 8–16

Hutcheson SW and Buchanan BB (1983a) Enzyme regulation in Crassulacean acid metabolism photosynthesis: Studies of the ferredoxin/thioredoxin system of *Kalanchoë daigremontiana.* Plant Physiol 72: 870–876

Hutcheson SW and Buchanan BB (1983b) Enzyme regulation in Crassulacean acid metabolism photosynthesis. Studies on thioredoxin-linked enzymes of *Kalanchoë daigremontiana.* Plant Physiol 72: 877–885

Ingelbrecht ILW, Herman LMF, Dekeyser RA, Van Montagu MC and Depicker AG (1989) Different 3′ end regions strongly influence the level of gene expression in plant cells. Plant Cell 1: 671–680

Joshi CP (1987) Putative polyadenylation signals in nuclear genes of higher plants: A compilation and analysis. Nucleic Acids Res 15: 9627–9640

Kagawa T and Bruno PL (1988) NADP-malate dehydrogenase from leaves of *Zea mays*: Purification and physical, chemical, and kinetic properties. Arch Biochem Biophys 260: 674–695

Keegstra K, Olson LJ and Theg SM (1989) Chloroplastic precursors and their transport across the envelope membranes. Annu Rev Plant Physiol 40: 471–501

Kleppinger-Sparace KF, Stahl RJ and Sparace SA (1992) Energy requirements for fatty acid and glycerolipid biosynthesis from acetate by isolated pea root plastids. Plant Physiol 98: 723–727

Kluge M, Fischer A and Buchanan-Bollig IC (1982) Metabolic control of CAM. In: Ting IP and Gibbs M (eds) Crassulacean Acid Metabolism, pp 31–50. Amer Soc Plant Physiol. Rockville, MD

Köster S and Anderson JM (1988) The photosynthetic apparatus of C3 and CAM-induced *Mesembryanthemum crystallinum* L. Photosynth Res 19: 251–264

Lange OL, Schulze ED, Kappen L, Evenari M and Buschbom U (1975) $CO_2$ exchange pattern under natural conditions of *Caralluma negevensis*, a CAM plant of the Negev desert. Photosynthetica 9: 318–326

Littlejohn RO and Ku MSB (1984) Characterization of early morning Crassulacean acid metabolism in *Opuntia erinacea* var. Columbiana (Griffiths) L. Benson. Plant Physiol 74: 1050–1054

Luchetta P, Cretin C and Gadal P (1990) Structure and characterization of the *Sorghum vulgare* gene encoding NADP-malate dehydrognase. Gene 89: 171–177

Luchetta P, Cretin C and Gadal P (1991) Organization and expression of the two homologous genes encoding the NADP-malate dehydrognase in *Sorghum vulgare* leaves. Mol Gen Genet 228: 473–481

Lütcke HA, Chow KC, Mickel FS, Moss KA, Kern HF and Scheele GA (1987) Selection of AUG initiation codons differs in plants and animals. EMBO J 6: 43–48

Maniatis T, Fritsch EF and Sambrook J (1982) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

Metzler MC, Rothermel BA and Nelson T (1989) Maize NADP-malate deydrogenase: cDNA cloning, sequence, and mRNA characterization. Plant Mol Biol 12: 713–722

Michalowski CB, Olson SW, Piepenbrock M, Schmitt JM and Bohnert HJ (1989a) Time course of mRNA induction elicited by salt stress in the common ice plant (*Mesembryanthemum crystallinum*). Plant Physiol 89: 811–816

Michalowski CB, Schmitt JM and Bohnert HJ (1989b) Expression during salt stress and nucleotide sequence of cDNA for ferredoxin-NADP$^+$ reductase from *Mesembryanthemum crystallinum.* Plant Physiol 89: 817–822

Michalowski CB, DeRocher EJ, Bohnert HJ and Salvucci ME (1992) Phosphoribuloskinase from ice plant: Transcription, transcripts and proteins expression during environmental stress. Photosynth Res 31: 127–138

Nakamoto H and Edwards GE (1983) Dark activation of maize leaf NADP-malate dehydrogenase and pyruvate, orthophosphate dikinase in vivo under anaerobic conditions. Plant Sci Lett 32: 139–146

Osmond CB (1984) CAM: Regulated photosynthetic metabolism for all seasons. In: Sybesma C (ed) Advances in Photosynthesis Research, Vol III, pp 557–564. Martinus Nijhoff/Dr W. Junk publishers, The Hague

Osmond CB and Holtum JAM (1981) Crassulacean acid metabolism. In: Hatch MD and Boardman NK (eds) The Biochemistry of Plants: A Comprehensive Treatise, Vol 8, pp 283–328. Academic Press, London/New York

Osmond CB, Nott DL and Firth PM (1979) Carbon assimilation patterns and growth of the introduced CAM plant *Opuntia inermis* in Eastern Australia. Oecologia 40: 331–350

Ostrem JA, Olson SW, Schmitt JM and Bohnert HJ (1987) Salt stress increases the level of translatable mRNA for Phospho*enol*pyruvate carboxylase in *Mesembryanthemum crystallinum.* Plant Physiol 84: 1270–1275

Pearson WR and Lipman DJ (1988) Improved tools for biological sequence comparison. Proc Natl Acad Sci USA 85: 2444–2448

Ray TB and Black CC (1979) The C4 pathway and its regulation. In: Gibbs M and Latzko E (eds) Encyclopedia of Plant Physiology, New Series Vol 6, pp 77–112. Springer-Verlag, New York

Scheibe R (1987) NADP$^+$-malate dehydrogenase in C3-plants: Regulation and role of a light-activated enzyme. Physiol Plant 71: 393–400

Scheibe R, Kampfenkel K, Wessels R and Tripier D (1991) Primary structure and analysis of the location of the regulatory disulfide bond of pea chloroplast NADP-malate dehydrogenase. Biochem Biophys Acta 1076: 1–8

Schuber M, Kluge M (1981) In situ studies on Crassulacean acid metabolism in *Sedum acre* L. and *Sedum mite* Gil Oecologia 50: 82–87

Steinmüller K and Apel K (1986) A simple and efficient procedure for isolating chromatin which is suitable for studies of DNase I-sensitive and hypersensitive sites. Plant Molec Biol 7: 87–94

Tabor S and Richardson CC (1987) DNA sequence analysis with a modified bacteriophage T7 DNA polymerase Proc Natl Acad Sci USA 84: 4767–4771

Vieira J and Messing J (1987) Production of single-stranded plasmid DNA. Meth Enzymol 153: 3–12

Vivkanandan M and Edwards GE (1987) Light and dark anaerobic activation of NADP-malate dehydrogenase in pea leaves and chloroplasts. In: Biggins J (ed) Progress in Photosynthesis Research, Vol III, pp 257–260. Martinus Nijhoff Publishers, Dordrecht

Werdan K, Heldt HW and Milovancev M (1975) The role of pH in the regulation of carbon fixation in the chloroplast stroma. Studies on $CO_2$ fixation in the light and dark. Biochim Biophys Acta 396: 276–292.

Winter K (1980) Carbon dioxide and water vapor exchange in the Crassulacean acid metabolism plant *Kalanchoë pinnata* during a prolonged light period. Plant Physiol 66: 917–921

Winter K (1985) Crassulacean acid metabolism. In: Barber J and Baker NR (eds) Photosynthatic Mechanisms and the Environment, pp 329–387. Elsevier, Amsterdam

Winter K and Tenhunen JD (1982) Light-stimulated burst of carbon dioxide uptake following nocturnal acidification in the Crassulacean acid metabolism plant *Kalanchoë daigremontiana*. Plant Physiol 70: 1718–1722

Winter K, Foster JG, Edwards GE and Holtum JAM (1982) Intracellular localization of enzymes of carbon metabolism in *Mesembryanthemum crystallinum* exhibiting C3 photosynthetic characteristics or performing Crassulacean acid metabolism. Plant Physiol 69: 300–307