Plant Cell Physiol. 35(8): 1165–1171 (1994)

Isoforms of NADP-Malic Enzyme from *Mesembryanthemum crystallinum* L. That are Involved in C_3 Photosynthesis and Crassulacean Acid Metabolism

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Exposure of the facultative halophyte *Mesembryanthemum crystallinum* L. to salt stress induces a shift from C_3 photosynthesis to Crassulacean acid metabolism (CAM). During induction of CAM, the activity of NADP-malic enzyme (EC 1.1.1.40) increased as much as 12-fold in leaves, while the enzymatic activity in roots fell to half of the original level. These changes in the activity of the enzyme corresponded to changes in levels of the enzyme protein. NADP-malic enzymes extracted from leaves in the C_3 and CAM modes could be distinguished by differences in electrophoretic mobility during electrophoresis on a non-denaturing polyacrylamide gel. NADP-malic enzyme extracted from roots in the C_3 mode and in the CAM mode migrated as fast as the enzyme extracted from leaves in the CAM mode on the same gel. Although the pattern of peptide fragments from NADP-malic enzyme from CAM-mode leaves was similar to that from C_3-mode leaves, as indicated by peptide mapping, both immunoprecipitation and an enzyme-linked immunosorbent assay revealed some antigenic differences between the enzymes extracted from leaves in the C_3 and the CAM modes. These results suggest the existence of at least two isoforms of NADP-malic enzyme that differ in their levels of expression during induction of CAM.

Key words: Crassulacean acid metabolism (inducible) — Isozymes — *Mesembryanthemum crystallinum* L. — NADP-malic enzyme (EC 1.1.1.40).

NADP-malic enzyme (L-malate: NADP oxidoreductase/decarboxylase; EC 1.1.1.40) is widespread in nature and catalyzes the reversible oxidative decarboxylation of L-malate, in the presence of a divalent cation, to produce CO_2, pyruvate and NADPH. In plants, two forms of this enzyme are known to occur and both have important metabolic roles. The cytosolic form is thought to participate in the regulation of intracellular pH (Pupillo and Bussi 1979, Davies and Patil 1974) or in the provision of reducing power that can be used in processes that require NADPH (Edwards and Huber 1981). The chloroplast stromal form is found specifically in the bundle sheath chloroplasts of NADP-malic enzyme-type C_4 plants, such as maize. The enzyme plays a key role in photosynthesis by providing CO_2 for fixation in the Calvin cycle in bundle sheath cells (Edwards and Andreo 1992, Edwards and Huber 1981).

In the leaves of certain CAM plants that are designated malic enzyme-type species, both NADP-malic enzyme and NAD-malic enzyme (EC 1.1.1.38) are believed to decarboxylate L-malate during deacidification in the light (Winter 1985, Osmond and Holtum 1981). In the CAM-inducible plant *Mesembryanthemum crystallinum* L. (Winter and von Willert 1972), the significance of NADP- and NAD-malic enzymes in the decarboxylation of L-malate is indicated by the considerable increases in extractable activities observed during the induction of CAM (Holtum and Winter 1982, Winter et al. 1982). However, there are few reports about purified NADP-malic enzymes from CAM plants and it has not been clearly established whether any special type of NADP-malic enzyme is associated with CAM.

We recently purified NADP-malic enzyme from leaves of *M. crystallinum* in the CAM mode (Saitou et al. 1992, 1993). The analysis of subunits by SDS-PAGE and the

Abbreviations: BSA, bovine serum albumin; CAM, Crassulacean acid metabolism; ELISA, enzyme-linked immunosorbent assay; PAGE, polyacrylamide gel electrophoresis; PEPCase, phosphoenolpyruvate carboxylase; PVP, polyvinylpolypyrrolidone; TTBS, Tris-buffered saline containing 0.05% (v/v) Tween 20.

determination of native molecular mass indicated that the enzyme was a hexamer of identical subunits. The purified enzyme exhibited no cooperativity for binding of L-malate and positive cooperativity for binding of NADP. The enzyme purified from leaves of *M. crystallinum* in the CAM mode had different structural and enzymatic properties from those reported for the enzymes from C_3 and C_4 plants. The present report describes an immunochemical comparison of NADP-malic enzymes isolated from *M. crystallinum* in the C_3 mode and in the CAM mode.

Materials and Methods

Plant materials—Plants were grown from seeds of *Mesembryanthemum crystallinum* L. as described previously (Saitou et al. 1992). Six-week-old plants that had been maintained under hydroponic conditions were used for induction of CAM. CAM was induced by including 400 mM NaCl in the growth medium for two weeks.

Preparation of antibodies—NADP-malic enzyme was purified from leaves of *M. crystallinum* in the CAM mode as described previously (Saitou et al. 1993). Antiserum was raised in the standard manner by inoculating a New Zealand white rabbit with a homogeneous preparation of the enzyme.

Preparation of crude extract—All procedures were performed at 0 to 4°C. The second and third (fully expanded) leaf pairs or the roots were ground with a chilled mortar and pestle in 1.5 volumes of ice-cold medium that contained 100 mM Tris-HCl (pH 8.0 for leaves, pH 7.4 for roots), 10 mM $MgCl_2$, 1 mM EDTA, 20% glycerol, 10 mM 2-mercaptoethanol and 2% (w/v) insoluble PVP. The homogenate was filtered through four layers of gauze. The crude extract was centrifuged at $10,000 \times g$ for 10 min, and the supernatant was used directly for assays of enzymatic activity and PAGE. The protein in the supernatant was precipitated with $(NH_4)_2SO_4$ at 50% saturation and pelleted by centrifugation at $23,000 \times g$ for 10 min. The resulting supernatant was further adjusted to 70% saturation with solid $(NH_4)_2SO_4$ and centrifuged as before. The pellet was resuspended in buffer A which contained 50 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 0.1 mM EDTA, 10% (w/v) glycerol and 10 mM 2-mercaptoethanol, and the suspension was centrifuged at $23,000 \times g$ for 10 min. The supernatant fraction was passed through an NAP column (Pharmacia LKB, Uppsala, Sweden), which had previously been equilibrated with buffer A. The eluate was used for PAGE, immunoprecipitation and ELISA.

Assay of NADP-malic enzyme and units of enzymatic activity—The enzyme was assayed by following the malate-dependent reduction of NADP at 340 nm (Saitou et al. 1992). The reaction mixture (1.0 ml) contained 50 mM Tris-TES (pH 7.5), 5 mM $MgCl_2$ and 0.5 mM NADP. The reaction was initiated by the addition of L-malate to a final concentration of 5 mM and carried out at 30°C. One unit of enzyme was defined as the amount of enzyme that catalyzed the reduction of 1 μmol of NADP per min under these conditions.

Electrophoresis—Native PAGE and SDS-PAGE were carried out by the methods of Laemmli (1970). The activity of NADP-malic enzyme was located on non-denaturing gels by incubating the gels in a solution that contained 50 mM HEPES-KOH (pH 7.5), 4 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM NADP, 5 mM L-malate, 0.01% (w/v) phenazinemethosulfate and 0.2% (w/v) nitroblue tetrazolium. NADP-malic enzyme protein was detected by Western blot analysis (Saitou et al. 1991). After blocking with TTBS that contained 5% (w/v) non-fat dry milk, blots were incubated for 1 h in TTBS with antiserum raised against NADP-malic enzyme that had been purified from *M. crystallinum* in the CAM mode. After washing, the filter was incubated for 1 h in TTBS with alkaline phosphatase-conjugated antibodies raised in goat against rabbit IgG. After further washing of the filter, protein bands were visualized by incubation in the color-development solutions specific for alkaline phosphatase.

Peptide mapping—Proteins were separated by native PAGE and the activity of NADP-malic enzyme was located as described above. The bands of NADP-malic enzyme were cut out of the gel. The proteins in the gel slices were digested by incubation with 100 $\mu g\ ml^{-1}$ *Staphylococcus aureus* V_8 protease in 0.125 M Tris-HCl buffer (pH 6.8) that contained 0.1% SDS at 37°C for 30 min. The samples were then heated at 100°C for 3 min. The resulting peptides were resolved as described by Cleveland et al. (1977) and were detected by silver staining (Oakley et al. 1980) or by protein blotting as described above.

Immunoprecipitation of NADP-malic enzyme—NADP-malic enzyme was immunoprecipitated by incubation with the antiserum raised against NADP-malic enzyme from *M. crystallinum* in the CAM mode (Matsuoka and Hata 1987). The preparation of enzyme was incubated for 15 min at room temperature with 15 mM HEPES-KOH (pH 7.2) that contained antiserum (4 μl), 12.5% glycerol, 3.75 mM $MgCl_2$ and 15.5 mM 2-mercaptoethanol in a total volume of 224 μl. The incubation was continued for another 15 min after the further addition of 120 μl of a suspension of Protein A-Sepharose CL-4B (Pharmacia LKB, Uppsala, Sweden) in 10 mM HEPES-KOH (pH 7.2). After centrifugation at $10,000 \times g$ for 15 min, 150 μl of the supernatant were subjected to the assay for NADP-malic enzyme.

ELISA—ELISA was performed by the method described by Kawarabata and Hayasaka (1987). Duplicate wells of microtitration plates (Flow Lab., McLean, VA) were used. The antiserum was used at a dilution of 1 : 10,000. Alkaline phosphatase-conjugated goat antibodies against rabbit IgG (TAGO, Burlingame, CA) were used at

a dilution of 1 : 2,000 and the volume of the assay mixture was 100 μl.

Quantitation of protein—Protein concentrations were determined spectrophotometrically at 595 nm by the method of Bradford (1976) with Coomassie brilliant blue G-250 (Bio-Rad Co., Richmond, CA). A standard curve was established using BSA.

Results

Changes in the activity of NADP-malic enzyme upon exposure to salt stress—After the increase in the concentration of NaCl in the nutrient solution, the activity of NADP-malic enzyme in extracts from leaves continued to increase until the eighth day and remained constant thereafter (Fig. 1). As a consequence, the activity of NADP-malic enzyme on the twelfth day was twelve-fold higher than the original level. By contrast, the activity of NADP-malic enzyme in extracts from roots decreased gradually to half of the original level on the sixth day and remained constant thereafter. The activities of NADP-malic enzyme in extracts from leaves and roots of unstressed plants on the twelfth day were almost identical to those before the increase in the concentration of NaCl in the nutrient solution.

Immunoblot analysis of NADP-malic enzyme—Extracts of soluble proteins from leaves and roots harvested during salt stress were subjected to SDS-PAGE and analyzed by immunoblotting with the antiserum raised against NADP-malic enzyme from leaves of *M. crystallinum* in the CAM mode (Fig. 2). Each lane shown in Figures 2A and 2B was loaded with an equal amount of soluble protein. All the lanes loaded with extracts from leaves and roots yielded one major band of an immunoreactive protein of approximately 64 kDa. In extracts from leaves, the intensity of the band of protein that reacted with the antibody increased after the increase in the concentration of NaCl in the nutrient solution (Fig. 2A). In extracts from roots, the intensity of the band decreased (Fig. 2B). These changes paralleled the changes in the activity of NADP-malic enzyme during salt stress.

Western blots of proteins after native PAGE revealed one major band for extracts from leaves in both the C_3 and the CAM modes (Fig. 3B). NADP-malic enzyme extracted

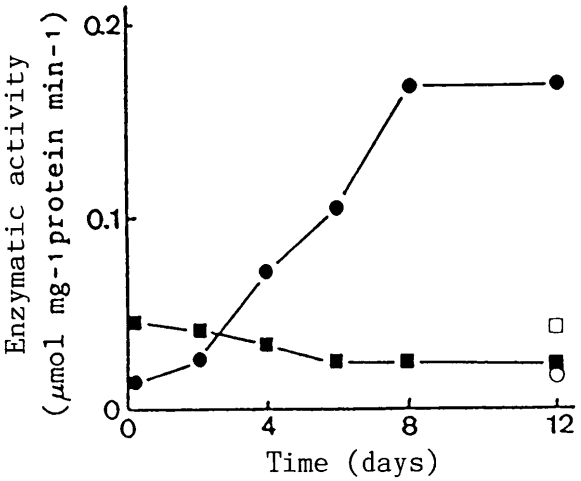

Fig. 1 Changes in the activity of NADP-malic enzyme in leaves (●) and roots (■) of *M. crystallinum* during salt stress. ○, The activity of NADP-malic enzyme in leaves of unstressed plants on the twelfth day; □, that in roots of unstressed plants on the twelfth day.

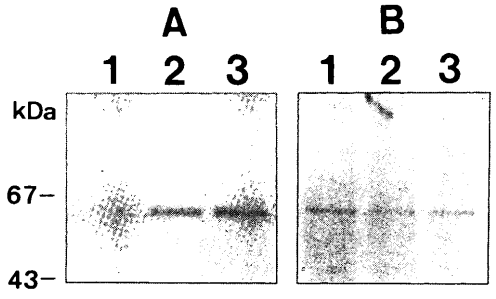

Fig. 2 Changes in levels of NADP-malic enzyme protein in leaves (A) and roots (B) of *M. crystallinum* during salt stress, as visualized by immunoblot analysis. An amount of protein in each fraction corresponding to 0.6 mg of total protein was loaded onto the gel for SDS-PAGE. Lane 1, non-stressed plants; lane 2, plants stressed for 4 days; lane 3, plants stressed for 8 days.

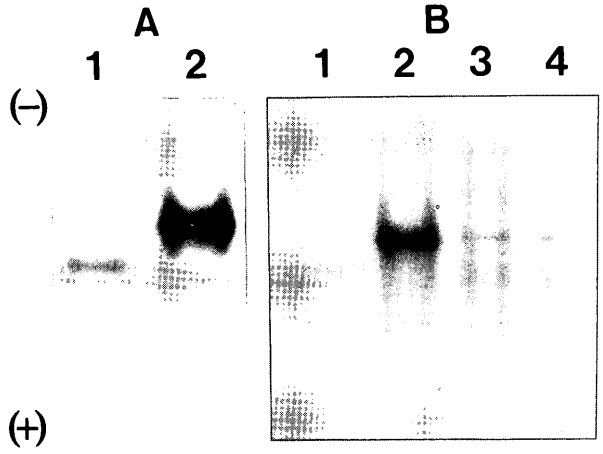

Fig. 3 Detection of NADP-malic enzyme activity and protein on a zymogram (A) and by immunoblot analysis (B). A total of 2.1 mg of total protein was used for each lane in both the zymogram and the immunoblot analysis. Bands of NADP-malic enzyme protein were visualized as described in the text. Lane 1, extract from C_3-mode leaves; lane 2, extract from CAM-mode leaves; lane 3, extract from C_3-mode roots; lane 4, extract from CAM-mode roots.

from C_3-mode leaves migrated more rapidly than that extracted from CAM-mode leaves. This profile corresponded to the zymogram (Fig. 3A). However, we could not ignore the possibility that the difference in electrophoretic mobility of the enzymes was due to a proteolytic artifact during extraction. In order to examine this possibility, the enzymes were extracted from each source with an extraction buffer that contained inhibitors of proteases (1 mM phenylmethylsulfonyl fluoride, 0.1 mg ml^{-1} soybean trypsin inhibitor and 2 mM leupeptin). When the extracts were subjected to native PAGE and analyzed by Western blotting, exactly the same results as those in Figure 3 were obtained (data not shown). The enzyme extracted from leaves of eight-week-old plants without prior exposure to NaCl stress migrated at the same rate as that from leaves of six-week-old plants during PAGE on a non-denaturing gel (data not shown).

In roots of *M. crystallinum* in both the C_3 and the CAM modes, immunoblot analysis after native PAGE revealed one band of enzyme with the same mobility as that of the enzyme extracted from CAM-mode leaves (Fig. 3B). No difference in electrophoretic mobility on a native gel was observed between the enzymes extracted from roots of six-week-old plants and of eight-week-old plants without exposure to NaCl stress (data not shown).

Peptide mapping—Peptide mapping was carried out to compare the primary structures of the NADP-malic enzyme proteins in C_3- and CAM-mode leaves (Fig. 4A). The two NADP-malic enzyme proteins yielded similar peptide maps and, therefore, they were deduced to be homologous in terms of amino acid sequence. To examine in further detail the similarity between these proteins, we examined the reactivities of the various peptide fragments with the polyclonal antibodies raised against NADP-malic enzyme from leaves of *M. crystallinum* in the CAM mode (Fig. 4B). Almost all the peptide fragments of the two NADP-malic enzyme proteins from C_3- and CAM-mode leaves were recognized by the antibodies. The patterns of immunoreactive fragments were similar. However, the peptide fragment denoted by arrow 1 in Figure 4B was detectable among the fragments of the enzyme extracted from CAM-mode leaves, but it was not detectable among those of the enzyme extracted from C_3-mode leaves. The peptide fragment denoted by arrow 2 was detectable among the fragments of the enzyme extracted from C_3-mode leaves, but it was not detectable among those of the enzyme extracted from CAM-mode leaves. These results indicate that the NADP-malic enzyme protein in CAM-mode leaves is similar to that in C_3-mode leaves in terms of primary structure but has some limited regions that are immunologically distinct.

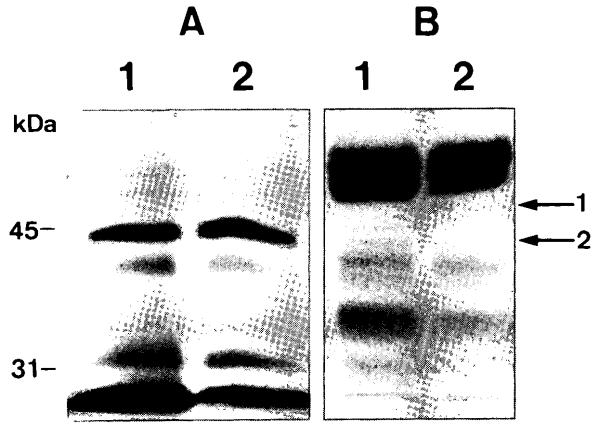

Fig. 4 Peptide mapping of NADP-malic enzyme from C_3- and CAM-mode leaves. The bands of NADP-malic enzyme were cut out from non-denaturing gels. Proteins were partially digested with V_8 protease and then fractionated by SDS-PAGE. A, The bands were detected by silver staining. B, The bands were detected by immunoblotting. Arrow 1 indicates a peptide fragment that is detectable in lane 2 but not in lane 1. Arrow 2 indicates a peptide fragment that is detectable in lane 1 but not in lane 2. Lane 1, NADP-malic enzyme from C_3-mode leaves; lane 2, the enzyme from CAM-mode leaves.

Immunological characterization—The specificity of the antiserum and the relative antigenicity of NADP-malic enzyme from C_3- and CAM-mode leaves were determined by immunoprecipitation and ELISA. The antiserum effectively precipitated the activity of NADP-malic enzyme in extracts from both C_3- and CAM-mode leaves (Fig. 5). The amounts of antiserum required for 50% precipitation were about 10 and 17 μl of antiserum per unit of enzyme from C_3- and CAM-mode leaves, respectively. This result reveals some antigenic differences between the enzymes extracted from the C_3- and CAM-mode leaves.

The ELISA was also used to evaluate the immunological difference between the NADP-malic enzymes extracted from C_3- and CAM-mode leaves (Fig. 6). The slopes repre-

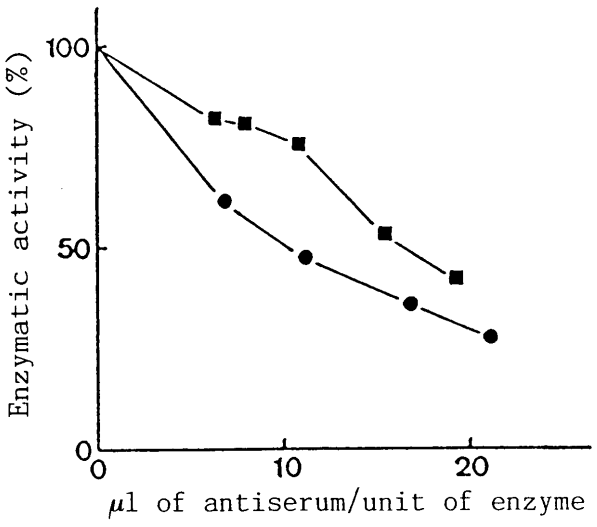

Fig. 5 Immunoprecipitation curves for NADP-malic enzyme from C_3-mode (■) and CAM-mode (●) leaves.

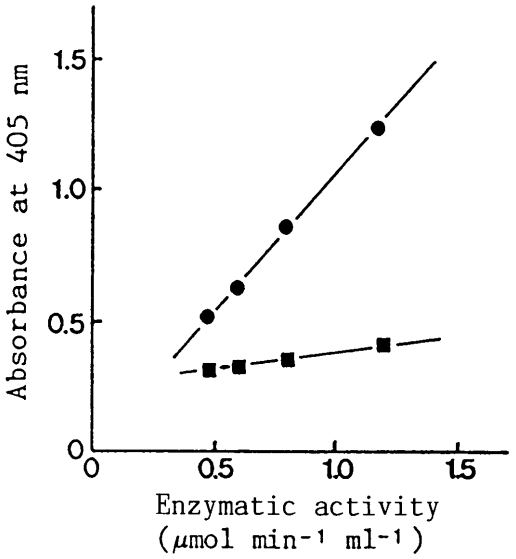

Fig. 6 ELISA of proteins extracted from C_3-mode (■) and CAM-mode (●) leaves.

senting the avidity of the enzyme for the antibodies were very different for the two forms of the enzyme. The ELISA assay confirmed the data from immunoprecipitation experiments.

Discussion

Plant NADP-malic enzymes have been categorized as being of the "C_3 and CAM type" and the "C_4 type" on the basis of their respective affinities for L-malate and their pH optima (Nishikido and Wada 1974). In immunotitration tests, NADP-malic enzymes from maize and sugar cane (both C_4 monocots) showed complete identify of epitopes, while the enzymes from other groups of photosynthetic plants (C_3 and CAM) showed a lower degree of cross-reactivity (Fathi and Schnarrenberger 1990). In the present study, we compared the molecular properties of NADP-malic enzymes extracted from *M. crystallinum* in the C_3 and CAM modes by immunochemical procedures and peptide mapping.

A difference in electrophoretic mobility on a non-denaturing polyacrylamide gel was observed between the NADP-malic enzymes extracted from leaves in the C_3 and CAM modes (Fig. 3). The difference was not likely to have been caused by an artifact because the possibility that the difference in electrophoretic mobility of the enzymes might have been due to a proteolytic artifact was eliminated by experiments with protease inhibitors. Furthermore, the pattern of peptide fragments for the enzyme from CAM-mode leaves was not identical to that from C_3-mode leaves when peptide mapping was combined with immunological detection (Fig. 4B). Finally, studies by both immunoprecipitation and ELISA revealed some antigenic differences between the enzymes extracted from C_3- and CAM-mode leaves (Figs. 5, 6). These results suggest that two isoforms of NADP-malic enzyme, which differ in their expression during induction of CAM, exist in *M. crystallinum*. Recently, Cushman (1992) reported that there are at least two members of the family of genes for NADP-malic enzyme and that the rate of transcription of one gene is greatly enhanced during induction of CAM. It would seem likely that the different forms of NADP-malic enzyme are encoded by different members of this gene family.

It has been demonstrated that two isoforms of PEPCase, which play a role in the initial carbon-fixation reaction in CAM plants, are present in *M. crystallinum* and an isozyme of PEPCase with an apparent molecular mass of 100 kDa accumulates in response to salt stress during induction of CAM (Höfner et al. 1989). The results for the two isoforms of NADP-malic enzyme are rather similar to those for the two isoforms of PEPCase. However, the C_3 form of NADP-malic enzyme was not detectable in CAM-mode leaves (Fig. 4), while the level of the C_3 form of PEPCase increased slightly in leaves during induction of CAM (Höfner et al. 1989). The results show that regulation of the expression of C_3-specific NADP-malic enzyme is different from that of the expression of C_3-specific PEPCase during induction of CAM.

While the NADP-malic enzyme extracted from C_3-mode leaves migrated more rapidly than that extracted from CAM-mode leaves on a non-denaturing gel (Fig. 3), we did not find any appreciable difference in the molecular mass of the subunits of the enzymes from C_3- and CAM-mode leaves (Fig. 2). Thorniley and Dalziel (1988) reported that the enzyme in maize leaves could be isolated in different forms, depending on the buffer used, and that it dissociated from a tetramer to dimers in the absence of 1,4-dithiothreitol. The state of oligomerization of the enzyme from CAM-mode leaves (hexameric) was different from that reported for the enzyme from C_3 or C_4 plants and did not change as a function of either the buffer used or the pH (Saitou et al. 1992). The possibility remains that the state of oligomerization of the enzyme from C_3-mode leaves of *M. crystallinum* is different from that of the enzyme from CAM-mode leaves.

The activity of NADP-malic enzyme in roots fell to half of the original level after the increase in the concentration of NaCl in the nutrient solution. The fall in the activity of the enzyme was accompanied by a fall in levels of the enzyme protein. Recently, levels of transcripts of the gene(s) for NADP-malic enzyme in roots were shown to be unaffected by salt stress (Cushman 1992). The level of stress-induced proteins is determined not only by rates of transcription, but also by post-transcriptional mechanisms such as selective translation or stabilization of transcripts. There is indirect evidence that post-transcriptional events

are involved in the regulation during development of expression of *Ppc1*, which encodes the CAM form of PEPCase (Cushman et al. 1990). Determination of whether similar mechanisms are involved in controlling the expression of NADP-malic enzyme will require an assessment of the stability of transcripts in vivo during induction of CAM.

NADP-malic enzyme extracted from roots of *M. crystallinum* in both the C_3 and the CAM modes migrated as rapidly as that extracted from leaves in the CAM mode on denaturing and non-denaturing gels. Cushman et al. (1989) reported that transcripts and proteins encoded by both C_3- and CAM-specific genes (*Ppc2* and *Ppc1*) were present in root tissues of *M. crystallinum*. A more detailed analysis of the molecular species of NADP-malic enzyme in roots is now required.

The authors thank Dr. T. Kawarabata of Kyushu University for technical advice on the ELISA.

References

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Anal. Biochem.* 72: 248–254.

Cleveland, D.W., Fisher, S.G., Kirschner, M.W. and Laemmli, U.K. (1977) Peptide mapping by limited proteolysis in sodium dodecyl sulfate and analysis by gel electrophoresis. *J. Biol. Chem.* 252: 1102–1106.

Cushman, J.C. (1992) Characterization and expression of a NADP-malic enzyme cDNA induced by salt stress from the facultative crassulacean acid metabolism plant, *Mesembryanthemum crystallinum*. *Eur.J. Biochem.* 208: 259–266.

Cushman, J.C., Meyer, G., Michalowski, C.B., Schmitt, J.M. and Bohnert, H.J. (1989) Salt stress leads to differential expression of two isogenes of phosphoenolpyruvate carboxylase during Crassulacean acid metabolism induction in the common ice plant. *The Plant Cell* 1: 715–725.

Cushman, J.C., Michalowski, C.B. and Bohnert, H.J. (1990) Developmental control of Crassulacean acid metabolism inducibility by salt stress in the common ice plant. *Plant Physiol.* 94: 1137–1142.

Davies, D.D. and Patil, K.D. (1974) Regulation of 'malic' enzyme of *Solanum tuberosum* by metabolites. *Biochem. J.* 137: 45–53.

Edwards, G.E. and Andreo, C.S. (1992) NADP-malic enzyme from plants. *Phytochemistry* 31: 1845–1857.

Edwards, G.E. and Huber, S.C. (1981) The C_4 pathway. *In* The Biochemistry of Plants. A Comprehensive Treatise. Edited by Hatch, M.D. and Boardman, N.K. Vol. 8. pp. 237–281. Academic Press, New York.

Fathi, M. and Schnarrenberger, C. (1990) Purification by immunoadsorption and immunochemical properties of NADP-dependent malic enzymes from leaves of C_3, C_4, and Crassulacean acid metabolism plants. *Plant Physiol.* 92: 710–717.

Höfner, R., Vazquez-Moreno, L., Abou-Mandour, A.A., Bohnert, H.J. and Schmitt, J.M. (1989) Two isoforms of phosphoenolpyruvate carboxylase in the facultative CAM plant *Mesembryanthemum crystallinum*. *Plant Physiol. Biochem.* 27: 803–810.

Holtum, J.A.M. 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.

Kawarabata, T. and Hayasaka, S. (1987) An enzyme-linked immunosorbent assay to detect alkali-soluble spore surface antigens of strains of *Nosema bombycis* (Microspora: Nosematidae). *J. Invertebr. Pathol.* 50: 118–123.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680–685.

Matsuoka, M. and Hata, S. (1987) Comparative studies of phosphoenolpyruvate carboxylase from C_3 and C_4 plants. *Plant Physiol.* 85: 947–951.

Nishikido, T. and Wada, T. (1974) Comparative studies of NADP-malic enzyme from C_4- and C_3-plants. *Biochem. Biophys. Res. Commun.* 61: 243–249.

Oakley, B.R., Kirsch, D.R. and Morris, N.R. (1980) A simplified ultrasensitive silver stain for detecting proteins in polyacrylamide gels. *Anal. Biochem.* 105: 361–363.

Osmond, C.B. and Holtum, J.A.M. (1981) Crassulacean acid metabolism. *In* The Biochemistry of Plants. A Comprehensive Treatise. Edited by Hatch, M.D. and Boardman, N.K. Vol. 8. pp. 283–327. Academic Press, New York.

Pupillo, P. and Bossi, P. (1979) Two forms of NADP-dependent malic enzyme in expanding maize leaves. *Planta* 144: 283–289.

Saitou, K., Agata, W., Asakura, M. and Kubota, F. (1992) Structural and kinetic properties of NADP-malic enzyme from the inducible Crassulacean acid metabolism plant *Mesembryanthemum crystallinum* L. *Plant Cell Physiol.* 33: 595–600.

Saitou, K., Agata, W., Asakura, M. and Kubota, F. (1993) Purification of NADP-malic enzyme with the high yield from leaves of *Mesembryanthemum crystallinum* exhibiting Crassulacean acid metabolism. *Jpn. J. Crop Sci.* 62: 134–136.

Saitou, K., Nakamura, Y., Kawamitsu, Y., Matsuoka, M., Samejima, M. and Agata, W. (1991) Changes in activities and levels of pyruvate, orthophosphate dikinase with induction of Crassulacean acid metabolism in *Mesembryanthemum crystallinum* L. *Jpn. J. Crop Sci.* 60: 146–152.

Thorniley, M.S. and Dalziel, K. (1988) NADP-linked malic enzyme. Purification from maize leaves, M_r and subunit composition. *Biochem. J.* 254: 229–233.

Winter, K. (1985) Crassulacean acid metabolism. *In* Photosynthetic Mechanisms and the Environment. Edited by Barber, J. and Baker, N.R. pp. 329–387. Elsevier, Amsterdam.

Winter, K., Foster, J.G., Edwards, G.E. and Holtum, J.A.M. (1982) Intracellular localization of enzymes of carbon metabolism in *Mesembryanthemum crystallinum* exhibiting C_3 photosynthetic characteristics or performing Crassulacean acid metab-

olism. *Plant Physiol.* 69: 300–307.
Winter, K. and von Willert, D.J. (1972) NaCl-induzierter Crassulaceen-Säurestoffwechsel bei *Mesembryanthemum crystallinum*. *Z. Pflanzenphysiol.* 67: 166–170.

(Received April 21, 1994; Accepted September 5, 1994)