NADP-malic enzyme: immunolocalization in different tissues of the C₄ plant maize and the C₃ plant wheat

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Abstract

In situ immunolocalization and Western blot analysis of separated cellular and subcellular fractions, were used to determine the localization of different isoforms of NADP-malic enzyme in both wheat (C₃) and maize (C₄) plants. In both techniques, an affinity purified anti-(maize 62 kDa NADP-ME) IgG from the maize green leaf isoform also reacted with a 72 kDa protein in tissues of C₄ plants as well as C₃ plants. The light-inducible 62 kDa isoform is located in bundle sheath chloroplasts of maize leaves. In etiolated leaves and in roots of maize there is evidence for the occurrence of a 72 kDa isoform which co-migrates on 2-D (SDS and isoelectric focusing) PAGE. The 72 kDa isoform is also present in low levels in green leaves. This form may occur in multiple intracellular compartments; but in situ immunolocalization experiments and Western blot and activity assays on fractionated protoplasts indicate that a significant amount of this isoform occurs in plastids. With regards to C₃ plants such as wheat, a 72 kDa isoform in leaves is largely confined to the chloroplasts based on in situ immunolocalization and Western blots and enzyme activity assays with fractionated protoplasts. In maize, it appears that the constitutive expression pattern of a possible C₃ ancestral gene for NADP-malic enzyme has been maintained, and a high level expression of a light-inducible isoform located in bundle sheath chloroplasts (62 kDa) has been acquired during its evolution.

Key words: NADP-malic enzyme, Triticum aestivum, Zea mays.

Introduction

Higher plants can be divided into groups, e.g. C₃ and C₄ based on differences in the mechanism utilized for photosynthetic carbon assimilation. Photosynthesis by C₃ plants involves only one photosynthetic cell type, and in these plants atmospheric CO₂ is fixed directly by the primary carbon fixation enzyme ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco). In contrast, C₄ plants, such as the monocot maize, possess a Kranz-type anatomy consisting of two cell types, mesophyll and bundle sheath, that differ in their photosynthetic activities (Edwards and Huber, 1981; Hatch, 1992). The genes encoding the C₄ pathway enzymes, including pyruvate, orthophosphate dikinase (PPDK), phosphoenolpyruvate carboxylase (PEPC), NADP-malate dehydrogenase, and NADP-malic enzyme (NADP-ME), are highly expressed in C₄ plants and, in addition, they are differentially expressed in the two photosynthetic cell types (Sheen and Bogorad, 1987a; Langdale et al., 1988; Nelson and Langdale, 1989). PPDK, PEPC and NADP-malate dehydrogenase are utilized in mesophyll cells for efficient fixation of CO₂ into malate via the carboxylation phase of the C₄ pathway. The CO₂ trapped by fixation into malate is transferred to bundle sheath cells and released by NADP-ME. The Calvin cycle, which is active only in bundle sheath cells, then refixes the released CO₂ through Rubisco in a relatively high CO₂ environment that greatly reduces photorespiration (Edwards and Walker, 1983; Hatch, 1987).

At present, information on the molecular mechanisms for the evolution and regulation of C₄ photosynthetic...
genes is limited. It has been suggested that either new C₄ isogenes were created and/or the expression of existing genes was modified in the development of the C₄ cycle (Aogayi and Bassham, 1986; Rothermel and Nelson, 1989). A number of studies have been made which are relevant to the evolution of genes of the C₄ pathway in maize. With respect to PEPC, different isoforms exist in maize: the C₄-specific, C₅ or etiolated, and the root forms (see Rajagopalan et al., 1994). It was suggested that the acquisition of a 'new' promoter by a pre-existing PEPC gene was a key factor in the evolution of C₄ plants (Schaffner and Sheen, 1992). With regards to PPDK, the gene for the C₄ chloroplastic form of the enzyme in maize may be derived from a gene encoding a cytosolic PPDK (Glackin and Grula, 1990; Sheen, 1991). Three endogenous PPDK genes, one encoding the C₄ chloroplast enzyme and two encoding cytosolic PPDK forms are present, and the C₄ PPDK gene was apparently created by genomic rearrangement (Sheen, 1991). With respect to NADP-ME, which is the topic of the current study, two predominant isoforms were reported in maize based on differences in isoelectric points of the protein, one in etiolated tissue and the other in green leaves (Pupillo and Bassi, 1979; Scaglia-rini et al., 1988). The enzyme exists as a tetramer in higher plants with a Mₑ of c. 220-280 kDa (Edwards and Andreo, 1992). Purified NADP-ME from etiolated leaves of maize has a higher Mₑ than the purified enzyme from green leaves on native PAGE as observed from silver stains, activity stains, and Western blots using antibody against the green leaf NADP-ME (Maurino et al., 1996). This is consistent with the monomer of the purified etiolated form on SDS-PAGE having a higher Mₑ (72 kDa) than the major green leaf form (62 kDa), the latter being involved in C₄ photosynthesis. However, the inter- and intracellular localization of these isoforms is not clear. In the present work, the localization of both isoforms in different tissues of maize was studied by immunogold labelling and by analyses of different cellular and subcellular fractions during greening of etiolated leaves and in normal green leaves. Moreover, the presence and localization of a 72 kDa isoform of the enzyme in the C₃ plant wheat is reported.

Materials and methods

Plant material

Growth of plant material for Western blots and enzyme assays with cellular preparations: Etiolated maize seedlings (Zea mays) were obtained by germination and growth in vermiculite under complete darkness for 10 d at 28°C. For greening experiments, the etiolated seedlings were illuminated continuously under fluorescent light (c. 50 μmol quanta m⁻²s⁻¹) for the time specified. For normal green maize, following germination the plants were illuminated continuously under fluorescent lamps (75 μmol quanta m⁻²s⁻¹). Wheat plants were grown on a mixture of soil and vermiculite for 10 d at 20°C under natural daylight.

Growth of plant material for immunolocalization and two-dimensional electrophoresis: For growth of etiolated maize seedlings, a sheet of aluminium foil was overlaid with a paper towel, seeds placed across the middle, and overlaid with another towel. After wetting with water, the material was rolled up and placed upright in plastic pans containing c. 3 cm of water. The seedlings were grown in complete darkness in a growth room at 28°C. Etiolated seedlings were illuminated continuously under fluorescent light (200 μmol quanta m⁻²s⁻¹) for greening experiments. Normal green plants of maize and wheat were grown in a mixture of peat moss:vermiculite:sand in a 2:1:1 ratio (watered twice a day and supplemented every 2 or 3 d with a nutrient solution: 1 g l⁻¹; Peter's fertilizer, Grace-Sierra Hort. Products Co., Milpitas, CA). Plants were cultivated in a growth room, under a 14 h light (25°C, 50-60% relative humidity)/8 h dark (18°C, 70-80% relative humidity) cycle. The irradiance provided by a combination of mercury vapour and high-pressure sodium lamps was 500 μmol quanta m⁻²s⁻¹ at the plant canopy. The fully expanded third or fourth leaves of light-grown plants were used for the experiments.

Enzyme activity and protein measurement

NADP-ME activity was determined spectrophotometrically at 30°C by following NADPH production at 340 nm in a Hitachi 150-20 spectrophotometer. The standard assay medium contained 50 mM TRIS-HCl, pH 8, 10 mM MgCl₂, 0.5 mM NADP, and 4 mM L-malate in a final volume of 1 ml.

PEP carboxylase activity was measured spectrophotometrically at 30°C monitoring NADH oxidation at 340 nm by coupling the carboxylase reaction with malate dehydrogenase. The standard assay medium contained 50 mM TRIS-HCl, pH 7.3, 5 mM MgCl₂, 0.15 mM NADH, 10 mM NaHCO₃, 2 IU malate dehydrogenase, and 4 mM PEP in a final volume of 1 ml.

NADP-malate dehydrogenase was assayed spectrophotometrically in a reaction medium containing 100 mM TRIS, pH 8.0, and 1 mM EDTA. NADPH was added, the base line was checked, and the reaction was initiated by addition of 1 mM oxaloacetate.

Protein concentration was determined by the method of Sedmak and Grossberg (1977), using BSA as standard.

In situ immunolocalization of NADP-malic enzyme

TEM: TEM samples were fixed for 12-24 h at 4°C in 2% (v/v) paraformaldehyde and 1.25% (v/v) glutaraldehyde in 50 mM PIPES buffer, pH 7.2. The sections were dehydrated with an ethanol series and embedded in L.R. White acrylic resin. Thin sections on uncoated nickel grids were incubated for 1 h in TBST (10 mM TRIS-HCl, 150 mM NaCl, 0.1% Tween 20 (v/v), pH 7.2)/BSA (1% w/v) to block non-specific protein binding sites on the sections. They were then incubated for 16 h with either pre-immune serum (without dilution) or affinity purified anti-(maize leaf 62 kDa NADP-ME) IgG (using 10-fold concentrated purified antibody) using the purification method of Plaxton (1989). After extensive washing with TBST/BSA, the sections were incubated for 1 h with Protein A-gold (15 nm) diluted 1:100 with TBST/BSA. The sections were washed with TBST/BSA, TBST, and distilled water prior to post-staining with a 1:4 mix of 1% (w/v) potassium permanganate and 2% (w/v) aqueous uranyl acetate.

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Light microscopy. Sections, 1 μm thick, from the same samples prepared for TEM were dried on to gelatin-coated slides and blocked for 1 h with TBST/BSA. They were then incubated for 16 h with the purified antibodies or pre-immune serum with TBST/BSA. The slides were then treated 1 h with protein A-gold (Amersham). The sections were subsequently exposed to a silver enhancement reagent according to the manufacturer’s directions (Amersham), stained with 1% (w/v) Safranin O and photographed using a Leitz Aristoplan microscope.

Density of gold labelling among samples prepared for immunolocalization was determined using NIH Image 1.56 software. Images from light micrographs and TEM were digitized, and point counting combined with automated planimetry was used to determine the intracellular distribution of gold particles. The pre-immune controls were largely free of gold labelling, which makes corrections for non-specific binding difficult on an entire section. Thus the results reported on compartmentation are semi-quantitative. The distribution of particles between and within cells is given in the figure legends, which give an unambiguous, although not statistical read out.

PAGE and immunoblotting

Protein samples, prepared from various tissues or cellular fractions, were analysed by SDS-PAGE (Laemmli, 1972) with a 5% (w/v) acrylamide stacking gel and 10% (w/v) acrylamide separating gel; prestained MW markers were used for molecular mass determinations. The MW of the polypeptides were estimated from a plot of log MW of marker standards versus migration distance (a linear relationship). The markers and the samples were run on the same gel.

For two-dimensional PAGE, total protein from the different tissues was extracted according to van Etten et al. (1979). The pH gradient used for isoelectrofocusing was from 4.2 to 7.5 and a gradient polyacrylamide gel (7.5 to 15% (w/v)) containing SDS was used for separating proteins by size in the second dimension. Prestained MW markers were used and the pH's of the reacting bands were calculated from a pH calibration curve. The markers and the samples were run on the same gel.

For isolation and purification of bundle sheath (BS) strands and mesophyll protoplasts (MP)

BS strands and MP were isolated using a modification of the procedures described by Kanai and Edwards (1973) and Ngerprapsiritsiri et al. (1989). Fully expanded leaves were cut perpendicular to the midrib in sections of 1 mm and suspended in enzyme digestion medium containing 0.5% (w/v) Sumizyme (gift from Dr R Kanai), 0.15% (w/v) Macerozyme R-10 (Yakult Biochemical Co., Nishinomiya, Japan), 0.5 M mannitol, 1 mM CaCl₂, 1 mM MgCl₂, 0.1% (w/v) BSA, and 10 mM MES, pH 5.3. After 2 h of incubation at 25°C under illumination by an incandescent lamp (75 μmol quanta m⁻² s⁻¹ for green tissue) or in the dark (etiolated tissue), the digestion medium was removed by filtration through a tea strainer. The leaf sections were washed several times with a sucrose medium consisting of 0.7 M sucrose in Buffer A (1 mM CaCl₂, 1 mM MgCl₂, 0.1% (w/v) BSA, 5 mM HEPES, pH 7.0), to release the BS strands and MP. The washes were pooled and filtered successively through a tea strainer and an 80 μm mesh net to collect the BS strands. The filtrate was poured into centrifuge tubes, overlaid with mannitol medium consisting of 0.5 M mannitol in Buffer A, and centrifuged at 250 g for 10 min. Intact MP which floated to the interface were collected, diluted with mannitol medium, pelleted by centrifugation at 100 g for 5 min, and washed twice with 0.5 M mannitol in Buffer A. The material collected on the 80 μm net was resuspended in mannitol medium, vortexed, and the purified BS strands were collected on the 80 μm net and washed several times. The MP and BS strand preparations were examined under a light microscope for purity. The procedure for isolation of MP from wheat was similar to that of maize.

Fractionation of mesophyll protoplasts and bundle sheath strands

BS strands of maize were ground at 4°C in a mortar with 10 mM TRIS-HCl, pH 7.5 and 10 mM DTT. The homogenate was filtered through a 200 μm net. To prepare the total MP extract of maize, an aliquot of MP was centrifuged (100 g), the protoplasts were ruptured by osmotic shock by resuspending protoplasts in 10 mM TRIS-HCl, pH 8.0 and 10 mM mercaptoethanol. Another aliquot of MP was centrifuged, resuspended in 50 mM TRIS-HCl, pH 8.0, and 300 mM sucrose; the protoplasts were broken by passage (4 times) through a 20 μm net and centrifuged at 2500 g for 3 min. A small aliquot of the preparation was examined under a microscope for completeness of rupture of protoplasts. The supernatant was taken as the extract for isolation of MP from wheat was similar to that of maize.

For analysis of chloroplast preparations on SDS gels, chloroplasts were resuspended in 10 mM TRIS-HCl, pH 8.0, 0.1% (v/v) Triton X-100 and incubated at 0°C for 30 min. To remove Triton X-100 prior to electrophoresis, the Triton X-100 treated chloroplasts were brought up to 30% saturation with solid ammonium sulphate and then centrifuged at 15 800 g for
30 min. This resulted in an aqueous phase which contained the chloroplast proteins which was collected and desalted against 50 mM TRIS, pH 7.5 and 10 mM DTT (or 10 mM mercaptoethanol); a very small upper phase containing Triton X-100 and membranes was discarded. PMSF (1 mM) was added to preparations to protect against proteases.

**Extraction and purification of maize root plastids**

Plastids were prepared by a modification of a method previously described (Bowsher et al., 1989). Maize roots were cut into small pieces and homogenized in a mortar with Buffer B (50 mM TRIS–HCl, pH 7.9, 400 mM sucrose, 1 mM EDTA). The homogenate was filtered through four layers of muslin and centrifuged at 200 g for 5 min. The supernatant was centrifuged at 1500 g for 20 min in order to obtain a crude plastid pellet. The resulting supernatant was taken as the 'non-plastid' fraction. The pellet containing plastids was resuspended with Buffer B, layered on to a solution containing 0.58 M sucrose in Buffer B and centrifuged at 4000 g for 6 min. The pellet containing plastids was resuspended with Buffer B, resuspended in 10 mM TRIS–HCl, pH 7.5, 0.1% Triton X-100, brought to 30% saturation with solid ammonium sulphate and centrifuged at 15800 g for 30 min. The sample was then processed for analysis on SDS gels as described above for the isolated mesophyll chloroplasts.

**Results**

**Western blots with anti-(maize leaf 62 kDa NADP-ME) IgG and activity stains using native gels with protein extracts from green and etiolated maize leaves, maize roots and wheat leaves**

Western blots using anti-(maize 62 kDa NADP-ME) and activity stains for NADP-ME on native gels show extracts from green maize leaves have a smaller isoform of the enzyme than extracts from etiolated maize leaves or maize roots (Fig. 1). This is consistent with results which show the enzyme purified from green leaves have a smaller isoform than that purified from etiolated leaves (Maurino et al., 1996) or maize roots (Maurino and Andreo, unpublished results) on both native and SDS–PAGE. Extracts from the C₃ plant, wheat, show reactivity on native gels which indicates that the protein is similar in size to that from etiolated maize leaves and roots (Fig. 1); which is consistent with Western blots of SDS–PAGE which show a 72 kDa isoform for these sources of the enzyme (see results below) compared to the predominant 62 kDa form in green maize leaves.

**Localization of NADP-malic enzyme in maize tissues**

The affinity purified anti-(maize 62 kDa NADP-ME) IgG was used for in situ immunolocalization of NADP-ME and Western blot studies using SDS–PAGE on fractions from purified bundle sheath and mesophyll cells of etiolated, greening and green maize leaves. Similar studies were also performed in maize roots. Cross-contamination in preparations of mesophyll protoplasts and bundle sheath strands was checked by light microscopy. The degree of cross-contamination from green leaves was also checked by assay of PEP carboxylase as a marker of mesophyll cells and Western blot analysis with Rubisco as a marker of bundle sheath. Only preparations having negligible cross-contamination were used. PEPC activity was also measured as a marker for the cytosolic fraction of mesophyll cells and ADP-glucose pyrophosphorylase and Rubisco were assayed, by Western blot, to evaluate the degree of purity and intactness of mesophyll chloroplasts.

Using the antibodies against the 62 kDa NADP-ME from green maize leaves, Western blot analysis of protein extracts from separated mesophyll protoplasts and bundle sheath cells of etiolated leaves were made to determine the location of the 72 kDa protein. The results revealed that in etiolated leaves most of the 72 kDa protein is present in the mesophyll cells; a faint reactive band at 72 kDa was also present in bundle sheath cells (only visible in the original gel) along with a light band of the 62 kDa isoform (Fig. 2). With greening of the etiolated leaves for 24 h the 62 kDa isoform increased in content specifically in BS strands, and this was the major isoform in BS strands isolated from normal green leaves (Fig. 2); although there is detectable 72 kDa isoform in normal green leaves.

Fractionation studies with maize mesophyll protoplasts isolated from green leaves show NADP-ME activity is associated with the chloroplast fraction similar to that of the chloroplast marker NADP-malate dehydrogenase, while PEPC a cytosolic marker occurs mainly in the supernatant (Table 1). Although not a quantitative analysis, Western blots of fractionated mesophyll protoplasts isolated from etiolated and green leaves also showed that the 72 kDa protein was predominantly associated with
the plastid fraction as was the plastid marker ADPG pyrophosphorylase (results not shown).

Immunolocalization with antibodies to the 62 kDa isofrom of NADP-ME was studied by light and electron microscopy to analyse distribution of NADP-ME among cell types in mature leaves and in time-dependent greening of leaves. In etiolated maize, leaf label was about equally distributed among bundle sheath and mesophyll cells (Fig. 3A). On average about half of the label appeared in the plastids of etiolated and 6 h greening leaves. High resolution immunolocalization with electron microscopy shows that labelling occurred in both the plastids and cytosol in bundle sheath and mesophyll cells of etiolated leaves (Fig. 4A, B). Since the background labelling with pre-immune serum is low (see Materials and methods) the results suggest the 72 kDa isoform occurs in both the plastid and cytosol. However, infrequent patches of background occur including label in vacuoles when using pre-immune serum (not shown) which can result in an underestimation of the percentage localization of the protein in plastids. Some background was apparent in the etiolated leaf light micrograph (Fig. 3A), where gold particles appear in vacuoles of a few mesophyll cells. After 26 h of greening (Fig. 3E, F) and in normal green leaves (Fig. 3G, H) the amount of labelling increased and the majority of the label in both mesophyll and bundle sheath cells was associated with plastids, although some label could be found in the cytosol of mesophyll cells (Fig. 4C). A significant amount of the label in bundle sheath chloroplasts appeared to be associated with the thylakoids (about 40% based on gold particle distribution, Fig. 4D).

While labelling in plastids in maize was apparent in both light and electron micrographs (Figs. 3, 4), gold particles were generally not found in other organelles (mitochondria, vacuoles, nuclei or microbodies, Fig. 4 and results not shown) in etiolated tissue or at any stage of greening. These results provide evidence for localization of both the 62 kDa and 72 kDa isoforms of NADP-ME in plastids of maize; and also suggest the 72 kDa isoform occurs in the cytosol, particularly in etiolated tissue. In etiolated leaves, the cytosolic labelling may represent a native active form of NADP-ME or inactive monomers which are targeted to the plastids.

Evidence was also obtained for the occurrence of NADP-ME in plastids of cortical cells of maize roots in

### Table 1. Distribution of NADP-malic enzyme, phosphoenolpyruvate carboxylase and NADP-malate dehydrogenase in fractionated mesophyll protoplasts of wheat and maize

<table>
<thead>
<tr>
<th>Species</th>
<th>Enzyme</th>
<th>Protoplast extracts (IU mg⁻¹ protein)</th>
<th>Percentage distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Chloroplasts</td>
</tr>
<tr>
<td>Wheat</td>
<td>NADP-ME</td>
<td>0.065</td>
<td>70.5</td>
</tr>
<tr>
<td></td>
<td>NADP-MDH</td>
<td>0.73</td>
<td>67.9</td>
</tr>
<tr>
<td></td>
<td>PEPC</td>
<td>0.064</td>
<td>11.0</td>
</tr>
<tr>
<td>Maize</td>
<td>NADP-ME</td>
<td>0.040</td>
<td>70.7</td>
</tr>
<tr>
<td></td>
<td>NADP-MDH</td>
<td>3.5</td>
<td>68.3</td>
</tr>
<tr>
<td></td>
<td>PEPC</td>
<td>7.6</td>
<td>12.5</td>
</tr>
</tbody>
</table>

Part of the protoplasts were used to determine total activity; the remainder was used for preparation of a chloroplast and supernatant fraction (see Materials and methods). Protoplasts were fractionated in TRIS-HCl pH 8.0 with 10 mM mercaptoethanol, 330 mM sucrose and chloroplasts were resuspended in the same medium. In the case of NADP-MDH, an aliquot of the enzyme was pre-incubated for 10 min at room temperature prior to assay. The total units of activity in each fraction was determined and the percentage distribution between the chloroplast pellet and supernatant calculated by taking the sum of the two as 100% (the sum of the activities in the supernatant and chloroplast fractions were 80–90% of that of the total protoplast extract). Results are average of two experiments. The deviations from the means was usually no greater than 2%.
immunolocalization studies (Fig. 4E). Western blots of SDS gels of the plastid fraction isolated from maize roots also show the presence of a 72 kDa isoform of NADP-ME (equivalent density of bands in plastid versus supernatant fraction), although a quantitative intercellular distribution was not possible due to substantial plastid breakage by mechanical isolation (as shown by Western blots using the putative plastid marker ADPG pyrophosphorylase, results not shown).

In order to compare the isoelectric point versus molecular mass of the different isoforms of maize NADP-ME, two-dimensional PAGE coupled with Western immunodetection was performed with the total protein extracted from maize roots, etiolated and green leaves (Fig. 5). The total protein extracted from green leaves exhibited two immunoreactive proteins: a 72 kDa polypeptide with an apparent \( pI \) of 5.4 and a 62 kDa polypeptide with an apparent \( pI \) of 4.6 (Fig. 5A). On 2-D gels the 62 kDa isoform consistently appeared as a smaller band compared to the 72 kDa band (Fig. 5A) while Western blots of proteins extracted from green leaves of maize and separated in one dimension on SDS gels show a strong reactivity with the 62 kDa and a lower reactivity with the 72 kDa isoform (see Fig. 2C and Maurino et al., 1996). This suggests that the green leaf specific tetramer form is susceptible to some loss during extraction and processing on 2-D electrophoresis. Either the 62 kDa isoform may have a poorer efficiency of partitioning into the phenol phase than the 72 kDa isoform during extraction, or the phenol may cause adverse effects on the 62 kDa isoform resulting in a partial loss. In the 2-D Western blots only the 72 kDa polypeptide \( (pI \ 5.4) \) was observed in the protein samples from etiolated leaves and roots (Fig. 5B, C, respectively); when these two samples were mixed, only one protein band was identified in the 2-D Western blot (Fig. 5D). The results show that the NADP-ME in maize roots and etiolated leaves have the same mobility as they come to the same position on the 2-D gel with respect to isoelectric point and molecular mass.

**Localization of NADP-malic enzyme in wheat**

Western blots of protein extracts from normal green wheat leaves revealed a 72 kDa isoform (not shown); localization studies were performed using isolated MP. The occurrence of the 72 kDa isoform primarily in the chloroplast fraction (Fig. 6A) and the activity of the native enzyme predominantly in this fraction (Fig. 6D; Table 1), corresponds to the localization of chloroplast marker proteins primarily in the chloroplast fraction (Fig. 6B, C; Table 1). NADP-ME immunocytochemistry in wheat leaves was performed in conjunction with the western blotting studies. Light microscopy immunolocalization demonstrated abundant label in mesophyll cells (Fig. 7A), and this label is predominantly associated with the chloroplasts (Fig. 7A, B). This was further demonstrated by TEM level immunolocalization (Fig. 8A, B). As in maize, a significant amount of the label in wheat chloroplasts is associated with the thylakoid membranes of chloroplasts as can be seen in low magnification views by electron microscopy (Fig. 8B).

Interestingly, light microscopy images of immunolabelled stomatal complexes of wheat indicated that in this cell type NADP-ME is predominantly in the cytosol (Fig. 7D). This was seen for both guard cells and their subsidiary cells (Fig. 7D), while almost no labelling could be seen in the adjacent epidermal cells (Fig. 7C).

**Discussion**

**Presence of 62 and 72 kDa isoforms of NADP-ME in maize**

Scagliarini et al. (1988) identified an isoform of NADP-ME in etiolated leaves of maize with a \( pI \) of 5.4 and a green leaf form with a \( pI \) of 4.6 based on column isoelectric focusing and activity assays. Using affinity purified antibodies to the 62 kDa isoform from green maize leaves, two isoforms of NADP-ME were recently demonstrated in maize leaves which have very different molecular masses (a 72 kDa form found in etiolated leaves and a 62 kDa form found in green leaves) (Maurino et al., 1996). In the present study, the predominant green leaf 62 kDa isoform has an apparent isoelectric point of 4.6 while a constitutive 72 kDa isoform has an apparent isoelectric point of 5.4. Although Scagliarini et al. (1988) have evidence for a very minor isoform with a \( pI \) of 6.5 which appeared in the latter stages of greening (based on activity assays); no evidence for this form was observed in the present study from 2-D Western blots. If this protein is present, either its abundance or cross-reactivity is too low to react with the antibodies from the 62 kDa isoform.

From our previous work, antibodies prepared against the 62 kDa isoform show only 30% cross-reactivity with the 72 kDa isoform from maize as measured by densitometric analysis using the same amount of purified protein (Maurino et al., 1996). However, in an earlier study by Langdale et al. (1987) with maize, only one isoform was apparent in Western blots using anti-(maize green leaf

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Fig. 3. Light microscopy of in situ immunolocalization of NADP-ME in maize leaves. Etiolated leaves (A, B); leaves greening for 6 h (C, D), leaves greening for 26 h (E, F), and mature leaves developed under normal light (G, H). M = mesophyll cell, BS = bundle sheath cell, E = epidermal cell. The bars equal 10 μm. The percentage of the gold particles appearing in the plastid fraction was determined in plates A, C, E, and G with the following results: etiolated (A): M (35%), BS (58%); 6 h greening (C): M (66%), BS (41%); 26 h greening (E): M (67%), BS (77%); normal green leaves (G): M (62%), BS (85%).
Fig. 5. Two-dimensional Western blots of total protein extracts (300 ng) from green (A) and etiolated (B) maize leaves, maize roots (C), and a mixture of etiolated maize leaves and maize roots (D). The membranes were treated with purified anti-(maize 62 kDa NADP-ME) IgG. The estimated isoelectric points for the 72 kDa and 62 kDa proteins are 5.4 and 4.6, respectively.

62 kDa NADP-ME) IgG. This may be due to differences in the epitopes which the antibodies recognize, i.e. the antibodies used by Langdale et al. (1987) may have a very weak reactivity toward the 72 kDa form. It is unlikely that the identification of a 72 kDa NADP-ME isoform is the result of an artefact of reactivity with related enzymes since: (a) the antibodies used were immuno-purified against the 62 kDa protein from green maize leaves (via the method of Plaxton, 1989), and (b) the 72 kDa protein, which reacts with the antibodies, has been purified to homogeneity from etiolated leaves of maize and showed NADP-ME activity (Maurino et al., 1996).

**Intercellular and intracellular localization of two isoforms of NADP-ME in maize leaves**

The main purpose of this study was to determine the localization of two isoforms of NADP-ME in leaves and roots of maize and to compare this with the form of the enzyme found in the C3 plant wheat. In etiolated leaves of maize the 72 kDa isoform is the only form detectable in mesophyll cells. A combination of techniques (activity assays and Western blots on fractionated protoplast extracts and in situ immunolocalization) indicated the 72 kDa occurs in plastids. Immunolocalization results also suggest that part of the 72 kDa isoform is located in the cytosol in etiolated tissue. Low amounts of both the 62 and 72 kDa...
forms are found in bundle sheath cells of etiolated leaves. With greening there is a major increase in the 62 kDa isoform in the bundle sheath cells. This form is not observed in mesophyll protoplasts of green leaves or in maize roots. The 62 kDa form is located in bundle sheath chloroplasts based on in situ immunolocalization. The localization of NADP-ME in the bundle sheath chloroplasts of green leaves of maize is consistent with the
results of previous studies. High activities of NADP-ME were observed in isolated bundle sheath chloroplasts of maize (Jenkins and Boag, 1985). An in situ immunolocalization study by Langdale et al. (1987) also indicates that NADP-ME localization in the bundle sheath chloroplasts in maize as does a recent study on two C_4 grasses (Madhavan et al., 1996).

Possible role of the 72 kDa isoform in maize

Based on the occurrence of the 72 kDa protein in maize roots, and etiolated and green leaves it seems that the expression of this protein is constitutive in maize and may be involved in a non-autotrophic function. Moreover, the in situ immunolocalization data, as well as localization by fractionation of mesophyll protoplasts, clearly shows that an isoform of 72 kDa occurs in plastids. Possible functions of the enzyme in plastids is provision of NADPH and pyruvate for the biosynthesis of lipids, as recently suggested for plastids from developing embryos of oilseed rape (Kang and Rawsthorne, 1994), or isoprenoids. The specific activity and kinetic constants of the purified 72 kDa isoform of NADP-ME from maize exhibit characteristics that resemble those in the NADP-ME found in C_3 plants in both photosynthetic and nonphotosynthetic tissues (Maurino et al., 1996; Maurino and Andreo, unpublished results). This isoform exhibits higher K_m values for the substrates, a much lower specific activity, and a lower pH optimum than the 62 kDa isoform in C_4 plants (Nishikido and Wada, 1974; Das et al., 1986; Dhillon et al., 1985, Edwards and Andreo, 1992).

Occurrence of the 72 kDa isoform of NADP-ME in the C_3 species wheat

It is clear from the present study that in the C_3 plant wheat a 72 kDa isoform of NADP-ME occurs mainly in chloroplasts of mesophyll cells. NADP-ME has also been found in chloroplasts isolated from cotyledons of *Cucurbita pepo* and in chloroplasts isolated from suspension cultures of *Glycine max*, both C_3 species (El-Shora and ap Rees, 1991). The physiological role of this isoform in plastids of C_3 and C_4 plants may be the same.

There is also some evidence for a cytosolic isoform of NADP-ME in a few studies on C_3 and CAM plants. This comes in part from sequence information on NADP-ME cDNA clones of poplar and bean (C_3) which indicates they lack a chloroplast targeting transit peptide sequence (van Doorsselaere et al., 1991; Walter et al., 1994). In the present study, results from in situ immunolocalization indicate the occurrence of a cytosolic form in the guard cell complex of wheat (the size of this cytosolic isoform remains to be determined). Malate is one of the metabolites for generating the required turgor pressure in guard cells for stomatal opening during the day; NADP-ME may play a key role in controlling the diurnal fluctuations of malate in the guard cells. In the CAM plant *Mesembryanthemum crystallinum*, fractionation of mesophyll protoplasts (Winter et al., 1982) and sequencing information on cDNA clone of NADP-ME showing lack of a transit peptide (Cushman, 1992) indicate a cytosolic isoform functions in CAM. The predicted size of the mature protein, based on the deduced amino acids from cDNA is 64.3 kDa.

The 62 kDa isoform of NADP-ME in maize in relation to evolution of C_4 photosynthesis

Although all C_4 enzymes appear to be developmentally regulated, the expression of each in maize is greatly enhanced by light (Nelson et al., 1984; Sheen and Bogorad, 1987b; Nelson and Langdale, 1992). In this study with NADP-ME in maize, levels of the 62 kDa protein in bundle sheath cells of etiolated leaves was very low. Whether a small amount of the 62 kDa protein appears in etiolated leaves, or whether there was sufficient exposure of etiolated tissue to light to induce a small amount of this isoform is uncertain. Care was taken to grow the tissue in complete darkness, to prepare it for enzymatic digestion under minimum light, and to digest it in darkness. Light induces a specific increase of the 62 kDa protein in bundle sheath cells indicative of its role in C_4 photosynthesis. Furthermore, this isoform is presumed to be associated with the evolution of C_4 photosynthesis in NADP-ME type species because it is not found in C_3 plants (Fig. 6 for wheat and other C_3 species examined but not shown, including Flaveria pringlei, tobacco, and tomato leaves and fruit). It appears that the constitutive expression pattern of a possible C_3 ancestral gene for NADP-ME has been maintained in C_4 plants and that a high level expression of a light-inducible isoform of NADP-ME located in bundle sheath chloroplasts has been acquired. Modification of a pre-existing gene encoding for a plastid isoform of NADP-ME could account for evolution of the C_4 specific isoform. Such evolution would require modification of the promoter element to allow light dependence and specificity in expression in bundle sheath cells.

Evidence exists for at least two genes in maize that encode for NADP-ME (Rothermel and Nelson, 1989, Maurino et al., 1996). The 62 kDa isoform is encoded from a gene expressed only in green leaves while the 72 kDa isoform is apparently a product of at least one other gene. The presence of more than one gene for NADP-ME in maize (Maurino et al., 1996) is consistent with the occurrence of different isoforms of this enzyme; one gene may be expressed constitutively and the other only expressed in bundle sheath cells of green maize leaves in a light-dependent fashion. Further studies are needed in maize on the large isoform
(72 kDa) found in plastids in etiolated leaves and root tissues to evaluate the transit peptide sequence for import, and to characterize the promoter element and the catalytic site relative to that of the C₄ isomeric. Such studies will help to shed further light on the molecular mechanism of evolution of C₄ photosynthesis.

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References


differential regulation of phosphoenolpyruvate carboxylase genes. The Plant Journal 2, 221–32.


