

Minireview

NADP-malic enzyme from plants: a ubiquitous enzyme involved in different metabolic pathways

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Abstract NADP-malic enzyme (NADP-ME) is a widely distributed enzyme that catalyzes the oxidative decarboxylation of L-malate. Photosynthetic NADP-MEs are found in C₄ bundle sheath chloroplasts and in the cytosol of CAM plants, while non-photosynthetic NADP-MEs are either plastidic or cytosolic in various plants. We propose a classification of plant NADP-MEs based on their physiological function and localization and we describe recent advances in the characterization of each isoform. Based on the alignment of amino acid sequences of plant NADP-MEs, we identify putative binding sites for the substrates and analyze the phylogenetic origin of each isoform, revealing several features of the molecular evolution of this ubiquitous enzyme. © 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: NADP-malic enzyme; C₄ plant; CAM plant; C₃ plant; Molecular evolution

1. Introduction

NADP-malic enzyme (NADP-ME; L-malate:NADP oxidoreductase [oxaloacetate decarboxylating], EC 1.1.1.40) is a widely distributed enzyme involved in different metabolic pathways in prokaryotic and eukaryotic microorganisms. It catalyzes the oxidative decarboxylation of L-malate to yield pyruvate, CO₂ and NADPH in the presence of a bivalent cation. Since the last (and only) review of this enzyme in plants [1], which mostly focused on the catalytic function of the enzyme, a remarkable advance has been made in the characterization of NADP-ME isoforms from a wide variety of sources, and a considerable number of nucleotide sequences of cDNAs or genomic structures are now available. Thus, the purpose of the present review is to classify NADP-ME isoforms from different plant sources and to analyze information on the evolution of this enzyme in plants. The comparison of C₄, C₃ and CAM NADP-MEs made in the present review helps to determine the mechanisms that might have been involved in the evolution of C₄ and CAM photosynthesis.

The best studied isoform of NADP-ME is the one involved in C₄ photosynthesis. In these plants, the enzyme plays a specialized role in bundle sheath chloroplasts, where it pro-

vides CO₂ for fixation by RuBisCO. Another photosynthetic isoform of NADP-ME is found in certain CAM plants, where it performs an analogous role. Nevertheless, both isoforms differ in the subcellular localization, as the C₄-type enzyme is chloroplastic and the CAM-type enzyme is cytosolic. Apart from this specialized role, the enzyme has been found in varied tissues of C₃ plants, where it plays non-photosynthetic roles, and has also different subcellular localizations depending on the species. Non-photosynthetic isoforms of NADP-ME have also been found in tissues of C₄ and CAM plants. In order to classify the NADP-MEs that have been characterized to date, we refer to the isoforms of NADP-ME as follows: C₄₍₁₎-NADP-ME: photosynthetic isoform found in bundle sheath chloroplasts of some C₄ plants; C₄₍₂₎-NADP-ME: non-photosynthetic isoform of NADP-ME found in plastids of C₄ plants; C₄₍₃₎-NADP-ME: cytosolic NADP-ME found in C₄ plants; CAM₍₁₎-NADP-ME: photosynthetic isoform of NADP-ME found in the cytosol of some CAM plants; CAM₍₂₎-NADP-ME: non-photosynthetic isoform of NADP-ME found in the cytosol of CAM plants; C₃₍₁₎-NADP-ME: non-photosynthetic isoform of NADP-ME found in the cytosol of some C₃ plants; and C₃₍₂₎-NADP-ME: non-photosynthetic isoform of NADP-ME present in plastids of some C₃ plants.

2. C₄₍₁₎-NADP-ME

This isoform is found in some C₄ plants such as maize, sugar cane, sorghum and C₄ *Flaveria* species, where it is used for the decarboxylation of malate in bundle sheath chloroplasts; the CO₂ produced is then fixed by RuBisCO.

The best studied C₄₍₁₎-NADP-ME is the enzyme from maize leaves. This enzyme has been purified and its kinetic parameters (Table 1), molecular properties and amino acid residues essential for catalysis have been determined. The corresponding cDNA of this enzyme was the first to be determined among plant NADP-ME [2], and the N-terminal sequence of the mature protein was also determined [3]. Immunolocalization studies have shown that this protein is specifically located in bundle sheath chloroplasts [4] and its expression is regulated by light [3]. As is the case for other bundle sheath-specific proteins, compartmentalization of this enzyme in this type of cells seems to be regulated at both the transcriptional and posttranscriptional levels in maize [5]. Recent studies have also shown a novel physiological function of this enzyme in repairing UV-induced damage [6,7].

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Transgenic rice expressing high levels of maize NADP-ME [8] under the control of the rice light-harvesting chlorophyll *a/l* binding protein promoter was recently obtained. Interestingly, the chloroplasts in the transgenic plants were agranal without stacking of thylakoids. As bundle sheath chloroplasts of NADP-ME type C₄ plants are unique in having reduced grana formation, the authors postulated that high levels of chloroplastic NADP-ME activity in transgenic C₃ plants could strongly affect the development of this organelle by generating excessive reducing power [8].

C₄₍₁₎-NADP-ME has also been studied from the dicot species *Flaveria*, specifically *Flaveria trinervia* and *Flaveria bidentis*. The primary structure of the mature NADP-ME from *F. trinervia* [9] shows an extensive sequence similarity with the maize enzyme. However, this is not the case for the putative transit peptides of the two enzymes, suggesting that this part of the genes arose independently during evolution of monocot and dicot C₄ plants. A partial genomic clone for the C₄₍₁₎-NADP-ME was identified from *F. bidentis* (*Me1*) [10], whose expression parallels the degree of C₄ photosynthesis in *Flaveria* species. Transgenic *F. bidentis* plants obtained with a set of constructs using 5' and 3' regions of the *Me1* gene revealed that the 5' region determines bundle sheath specificity and the 3' region contains enhancer-like elements that confer high expression level in leaves [11]. Further studies localized the quantitative regulator sequences in the 3' untranslated sequences and the N-terminal coding region of the gene, and demonstrated that the *Me1* 3' region has an enhancing function in other species (W.C. Taylor, personal communication). On the other hand, Western screening of 13 *Flaveria* species, including C₃, C₃–C₄ and C₄ species, as well as in situ immunolocalization studies, indicated that the degree of C₄ photosynthesis corresponded with the occurrence of the C₄₍₁₎-NADP-ME isoform and its localization to bundle sheath chloroplasts [12].

Recently, other forms of C₄₍₁₎-NADP-ME were characterized from *Haloxylon persicum*, an unusual C₄ NADP-ME type tree species [13], and from *Flaveria floridana*, an intermediate C₃–C₄ species [14]. These enzymes, although localized in bundle sheath chloroplasts [14,15], do not share all the kinetic and structural features of the maize C₄₍₁₎-NADP-ME (Table 1).

3. C₄₍₂₎-NADP-ME

A non-photosynthetic isoform of NADP-ME in plastids of

C₄ plants was identified in the monocot maize [3,16] and in the dicot *F. bidentis* [10] by different approaches.

In maize, the C₄₍₂₎-NADP-ME was purified from both etiolated leaves [3] and roots [16], and the plastidic localization of the protein was determined [4]. The isolation of the root cDNA NADP-ME by reverse transcription-polymerase chain reaction using mRNA from green leaves as template indicated that the C₄₍₂₎-NADP-ME represents a protein with a constitutive pattern of expression in plastids of maize [16]. This enzyme is under transcriptional or post-transcriptional regulation by effectors related to plant defense responses, as is the case for other NADP-ME from C₃ plants (see below) [16].

In *F. bidentis*, a second NADP-ME genomic clone (*Me2*) was characterized [10]. The two exons present in this clone inferred a similar protein to that encoded by *Me1*, including an apparent chloroplast transit peptide. *Me2* was expressed in leaves and roots of *F. bidentis* and it probably represents a housekeeping enzyme required for some non-photosynthetic process. Apparently, this gene is induced at a specific stage in chloroplast biogenesis (T. Nelson, personal communication). In accordance with these results, Western blotting in different C₄ *Flaveria* species also indicated the existence of a second immunoreactive band apart from the photosynthetic NADP-ME (the C₄₍₁₎-NADP-ME), which may be the product of expression of this gene [12].

4. C₄₍₃₎-NADP-ME

From both subcellular fractionation and in situ immunolocalization studies in different tissues of maize, the occurrence of a cytosolic isoform of this enzyme could not be ruled out [4]. To date, it has not been possible to isolate or characterize a cytosolic isoform in maize. However, recently, a gene coding for a cytosolic NADP-ME in maize was cloned (GenBank AJ224847). This gene is apparently expressed in the embryo root epidermis from *Zea mays*, although the particular function of this enzyme has not yet been studied (P. Puigdomenech, personal communication).

Genomic Southern blotting analysis of NADP-ME in the C₄ plants *F. bidentis* and *F. trinervia*, showed that both plants contain three, or possibly four, NADP-ME genes [10]. Thus, apart from the characterized *Me1* and *Me2* genes, the occurrence of a cytosolic isoform of NADP-ME is highly probably in *Flaveria* C₄ plants. This was recently confirmed in *F. trinervia*, where the presence of a single cytosolic NADP-ME, which apparently produces multiple transcripts in leaves that

Table 1
Some kinetic and molecular properties of purified NADP-ME isoforms

Type	C ₄₍₁₎ -NADP-ME			C ₄₍₂₎ -NADP-ME	CAM ₍₁₎ -NADP-ME	C ₃₍₂₎ -NADP-ME	
Source	maize [42]	<i>H. persicum</i> [13]	<i>F. floridana</i> [14]	etiolated leaves and roots from maize [3,16]	ice plant [18]	wheat [35]	<i>Egeria densa</i> [34]
Mass (SDS-PAGE)	62	67	62	72	64	72	72
pH optimum	8.0	8.2	7.5	7.5	7.2	7.2	7.3
Specific activity (U/mg)	30.9	59.1	15	1.4	68.8	0.98	1.16
K _m malate (mM) ^a	0.19	0.08	0.46	0.2	0.35	0.96	4.5 ^b
Malate-inhibition at pH 7.0	yes	yes	no	no	no	no	no
K _m NADP (μM) ^a	8.6	3.15	12	6.5	9.9 ^b	37	47.2
K _m Mg ²⁺ (mM) ^a	0.23–0.05	3.5–0.011	0.16–0.005	0.22–0.099	N.D.	0.20–0.006	1.46

N.D.: Not determined.

^aMeasured at pH optimum.

^bS_{0.5}.

Table 2

Conserved regions among the 18 deduced amino acid sequences of NADP-ME

	CONSENSUS MOTIF	Positions in maize C ₄₍₁₎ -NADP-ME and differences with the consensus
SITE I	VYTPTVGEACQKYG 1 2	<ul style="list-style-type: none"> ▪ Positions 183-196 ▪ 1 is I in C₃₍₂₎ from <i>A. thaliana</i> ▪ 2 is G in CAM₍₁₎ from ice plant
SITE II	<u>IQVIVVTDGERILGLGLGCGMGIPVGKL</u> 3 4 5 6	<ul style="list-style-type: none"> ▪ Positions 227-256 ▪ 3 is C in C₄₍₂₎ and C₄₍₁₎ from maize ▪ 4 is A and 5 is S in C₄₍₂₎ from maize ▪ 6 is I in C₃₍₁₎ from poplar ▪ The underlined amino acids are not found in C₃₍₂₎ from <i>A. thaliana</i>
SITE III	QFEDFANHNAF 7	<ul style="list-style-type: none"> ▪ Positions 325-335 ▪ 7 is Y in CAM₍₁₎ from <i>A. arborescens</i>
SITE IV	FNDDIQGTASVVL 8	<ul style="list-style-type: none"> ▪ Positions 348-360 ▪ 8 is A in C₃₍₁₎ from poplar
SITE V	LFLGAGEAGTGIAEL 9	<ul style="list-style-type: none"> ▪ Positions 380-394 ▪ 9 is F in C₄₍₃₎ from maize

The indicated positions correspond to the C₄₍₁₎-NADP-ME from maize, which was the first NADP-ME sequenced in plants [2]. Differences within the consensus sequence are indicated in each case. The following sequences were used to perform the alignment by Clustal W (1.81): C₄₍₁₎-NADP-ME from maize [2] and *F. trinervia* [9]; C₄₍₂₎-NADP-ME from maize ([16] and GenBank U39958); C₄₍₃₎-NADP-ME from maize (GenBank AJ224847); CAM₍₁₎-NADP-ME from the ice plant [17] and *A. arborescens* [19]; CAM₍₂₎-NADP-ME from *A. arborescens* [21]; C₃₍₁₎-NADP-ME from bean [22], poplar [23], grape berries [24], tomato (GenBank AF001270) and *A. graveolens* (GenBank AJ132257); C₃₍₂₎-NADP-ME from rice [30], *F. pringlei* [31], tomato (GenBank AF001269), grape berries (GenBank U67426), *A. thaliana* (GenBank AC010793) and *R. communis* (GenBank AF262997).

are differentially expressed in response to leaf development, injury and illumination, was determined (T. Nelson, personal communication).

5. CAM₍₁₎-NADP-ME

Some CAM plants use cytosolic NADP-ME, along with mitochondrial NAD-ME, for the decarboxylation of malate that is stored during the nocturnal fixation in the vacuoles. The released CO₂ enters the reductive-pentose phosphate pathway during the daytime. Although this enzyme has an analog function to the C₄₍₁₎-NADP-ME, both enzymes differ in subcellular localization.

Besides some kinetic studies with the NADP-ME from some CAM plants (reviewed in [1]), not much work on this enzyme has been done. A cDNA for a cytosolic NADP-ME was cloned from the common ice plant (*Mesembryanthemum crystallinum*) [17], a plant that shifts from C₃ photosynthesis to CAM under high salinity and drought. The enzyme was purified from CAM-induced leaves and its kinetic and structural properties studied (Table 1) [18].

More recently, a cDNA clone encoding a leaf-abundant NADP-ME (AME1) was isolated from *Aloe arborescens*, which is an obligate CAM plant [19]. Although this species is a PEP carboxykinase-type CAM plant and mainly uses this enzyme for C₄ acid decarboxylation, it is proposed that MEs

(NADP-ME and NAD-ME) may be responsible for a smaller portion of malate decarboxylation in this species. Although no diurnal rhythm was found on the transcript level, the occurrence of this enzyme in leaves is indicative for a photosynthetic role [19].

6. CAM₍₂₎-NADP-ME

Evidence for the presence of non-photosynthetic isoforms of NADP-ME in CAM plants are found in the facultative CAM plant *M. crystallinum* [20] and in the obligate CAM plant *A. arborescens* [19,21].

Genomic Southern blot analysis of *M. crystallinum* showed the presence of more than one NADP-ME genes [17]. The existence of two isoforms of NADP-ME, which differ in their expression during induction of CAM, was later found in this plant [20]. Both enzymes were distinguished by their electrophoretic mobility and antigenic differences [20]. Although the enzyme from C₃-mode leaves was not characterized and its subcellular localization has not yet been determined, this enzyme is possibly involved in non-photosynthetic functions. It remains to be determined whether this enzyme is also expressed in leaves of the CAM-mode.

On the other hand, a multigene family of NADP-ME was found in *A. arborescens* by Southern blot analysis [21]. A second full length cDNA (AME2), coding also for a cytosolic

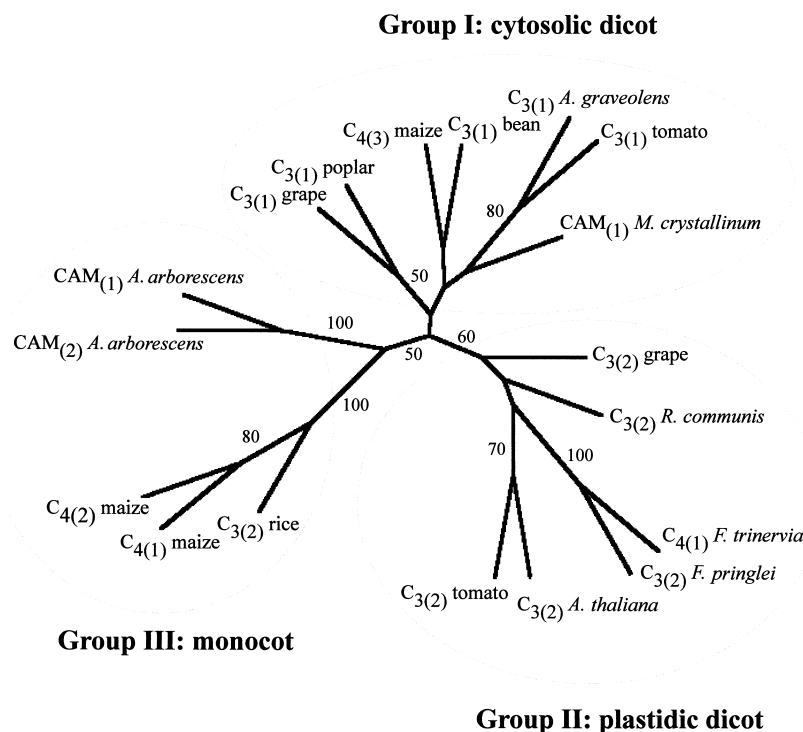


Fig. 1. Phylogenetic tree of plant NADP-ME. The existence of transit peptides in each NADP-ME sequence (listed in Table 2) was predicted by ChloroP1.1 software. Mature proteins were aligned using ClustalW (1.81) and the alignment obtained was modified by visual inspection to exclude the sites containing gaps. The phylogenetic tree was constructed by the neighbor-joining (NJ) method. Statistical significance of each branch of the tree was evaluated by bootstrap analysis by 100 iterations of bootstrap samplings and reconstruction of trees by the NJ method. The topology obtained by this method is shown, along with statistical significance higher than 50%.

NADP-ME, which is constitutively expressed in all organs at low and nearly equal level, was isolated [21]. Although the physiological function of this isoform has not yet been determined, it is probably involved in non-photosynthetic functions.

7. $C_{3(1)}$ -NADP-ME

Different cytosolic NADP-MEs have been characterized in C_3 plants by the isolation of their respective cDNA clones lacking transit peptides. These include the NADP-ME from bean [22], poplar [23], grape berries [24], tomato (GenBank AF001270) and *Apium graveolens* (GenBank AJ132257).

The expression of bean NADP-ME gene has been extensively studied. The promoter of this gene presented *cis*-regulatory elements possibly involved in the activation of the gene by fungal elicitors and UV [25]. Later, fusions of this promoter to the β -glucuronidase reporter gene were analyzed in transgenic tobacco plants, indicating that the promoter was activated by different effectors related to plant defense responses and agents that produce redox perturbations [26]. Direct evidence of the induction of bean NADP-ME by UV-B radiation was also obtained [27]. From these studies, it was concluded that this cytosolic enzyme is involved in plant defense responses, possibly by providing NADPH for the biosynthesis of lignin and flavonoids.

NADP-ME in fruit tissues of tomato and grape berries was implicated in respiration during ripening, providing pyruvate and/or NADPH as a substrate for respiration [1,28]. In earlier work, the enzyme from tomato fruit was found in the non-

mitochondrial fraction and suggested to be cytosolic, although the plastidic localization was not ruled out [29]. Later, both cytosolic and plastidic NADP-ME cDNA clones were identified in both tomato (GenBank AF001270 and AF001269) and grape berries ([24] and GenBank U67426), indicating that more than one NADP-ME is expressed in these species although the specific role of each isoform is not known.

8. $C_{3(2)}$ -NADP-ME

A plastidic isoform of NADP-ME has been identified in C_3 plants by either cloning of the corresponding cDNAs having putative plastidic transit peptides (rice [30], *Flaveria pringlei* [31], tomato (GenBank AF001269), grape berries (GenBank U67426), *Arabidopsis thaliana* (GenBank AC010793) and *Ricinus communis* (GenBank AF262997)), detecting the enzyme in isolated chloroplasts (*Cucurbita pepo* and *Glycine max* [32], *Hydrilla verticillata* [33] and *Egeria densa* [34]) or by in situ immunolocalization studies (wheat [4] and C_3 *Flaveria* species [12]).

The plastidic NADP-ME cDNA clone from the C_3 *F. pringlei* was expressed with similar levels in leaves, stems and roots [31]. Analogous results in C_3 *Flaveria* species were obtained when the same immunoreactive band of NADP-ME was found in stems, roots and leaves [12]. In this way, this isoform seems to be rather constitutive, at least in *Flaveria* C_3 plants.

NADP-ME from wheat stems was purified and its kinetic parameters determined (Table 1) [35]. The enzyme was induced by different effectors which cause lignification of

wounded leaves, suggesting that this enzyme could provide NADPH to support lignin biosynthesis in defense mechanisms, as is the case for the $C_{3(1)}$ -NADP-ME in bean [26].

NADP-ME activity was detected in chloroplasts isolated from cotyledons of *C. pepo* and from suspensions cultures of *G. max* [32]. In this work, the NADP-ME activity in different C_3 plants was higher in tissues where biosynthesis was most marked, with a close correlation to the synthesis of amino acids, although participation in fatty acid biosynthesis was not ruled out.

The enzyme was also studied from the submersed aquatic macrophytes *H. verticillata* and *E. densa* [33,34]. When these plants are grown under CO_2 limiting conditions, a C_4 -like cycle is induced, with an increase in C_4 enzymes, including NADP-ME, which is located in the chloroplasts of the photosynthetic cells in both species. NADP-ME from *E. densa* was further characterized and the kinetic and molecular properties were similar to the C_3 isoform (Table 1). Thus, the increase in NADP-ME by low CO_2 availability in these species may facilitate maintenance of high rates of decarboxylation of malate and delivery of CO_2 to RuBisCO in the chloroplasts.

NADP-ME activity is also associated with the plastids from developing embryos of oilseed rape [36] and castor bean endosperm [37]. In these systems, the enzyme was postulated to be involved in lipid biosynthesis by providing pyruvate and NADPH. This physiological role was also postulated for the enzyme from *R. communis* cotyledons, as the enzyme from this tissue responds to metabolites of the fatty acid synthesis pathway [38].

9. Comparison of the different plant NADP-ME isoforms

In the present review, we identify seven groups of NADP-ME in plant systems based on their different physiological roles and localization. Although the role for the photosynthetic NADP-MEs ($C_{4(1)}$ -NADP-ME and $CAM_{(1)}$ -NADP-ME) has been known for a long time, recent studies have dealt with the particular physiological role of non-photosynthetic isoforms. Based on the existence of several metabolic pathways in non-photosynthetic plastids that consume reductive power, NADP-ME in these organelles ($C_{4(2)}$ -NADP-ME and $C_{3(2)}$ -NADP-ME) may have an important and universal role in the provision of reducing power. On the other hand, cytosolic NADP-ME from C_3 plants ($C_{3(1)}$ -NADP-ME) has undoubtedly been linked to plant defense responses, although this function has also been described for plastidic NADP-MEs ($C_{4(2)}$ -NADP-ME and $C_{3(2)}$ -NADP-ME) and may be a universal feature for non-photosynthetic NADP-MEs [39].

A comparison of the kinetic parameters between representative isoforms of NADP-ME that have been purified (Table 1) indicates that the isoform involved in photosynthetic CO_2 assimilation ($C_{4(1)}$ -NADP-ME and $CAM_{(1)}$ -NADP-ME) is the form that possesses the higher specific activity, although the optimum pH for activity depends on the photosynthetic pathway in which it participates (C_4 or CAM) and, thus, its subcellular localization. The K_m values for the substrates NADP and L-malate (Table 1) do not change substantially between each enzyme indicating that their binding sites have been conserved among NADP-MEs; however, the values are slightly higher for the C_3 enzyme. Inhibition by the substrate malate at pH 7.0 is a particular feature of the enzyme in C_4

metabolism, and it is possibly a way of regulation of this cycle.

The accumulation of sequence information (18 complete nucleotide cDNA sequences, see Table 2) has enabled us to investigate the divergence of plant NADP-MEs during the course of molecular evolution. A multiple alignment of the deduced amino acid sequences (not shown) allowed us to identify several conservative sites (Table 2). The first conservative site (site I, Table 2) was previously identified [2] as a NADP-binding site by comparing it to other NADP-binding enzymes. Nevertheless, this site does not contain the characteristic amino acid sequence motif typically associated with this kind of site [40,41]. The second site (site II, Table 2), displays homology to a functional $\beta\alpha\beta$ -fold domain responsible for ADP-binding including recurrent Gly residues which is indicative for a typical NADP-binding site [40,41]. Nevertheless, this site is in part absent in the $C_{3(2)}$ -NADP-ME sequence from *A. thaliana* (Table 2). If this sequence is correct, the *Arabidopsis* enzyme may display unique characteristics in the binding of NADP. The functional significance of the third and fourth conservative sites (sites III and IV, Table 2) has not been determined yet, although they display a high level of amino acid conservation. The last site (site V, Table 2) also shows the typical pattern of amino acids of NADP-binding sites [40,41] and it is well conserved among the 18 NADP-MEs sequences.

The phylogenetic tree constructed with the performed alignment (Fig. 1) clearly shows three groups. Group I includes cytosolic dicot NADP-MEs; group II, the plastidic dicot NADP-MEs and group III, the monocot NADP-MEs. The existence of two separate groups within the dicot NADP-MEs suggests that they may have originated from two different ancestral genes. It is possible that one of the genes may become silent during the evolution of some species or that all the dicot plants have both the cytosolic ($C_{3(1)}$) and plastidic ($C_{3(2)}$) enzyme, such as tomato and grape. It is also clear that both cytosolic and plastidic ancestral genes have the potential to originate photosynthetic NADP-MEs independently, as CAM and C_4 NADP-ME are found in different groups. On the other hand, both plastidic and cytosolic monocot NADP-MEs are found within group III, except for the $C_{4(3)}$ NADP-ME of maize which is found in group I. The presence of this enzyme within group I is also supported by the fact that it contains some common amino acid residues typical for the dicot NADP-MEs (Asp in position 100 instead of Glu which is found in all monocot NADP-MEs and Gln in position 512 and Asp in position 574 instead of His and Glu, respectively, which are both typical for the plastidic monocot branch). In this way, this enzyme may represent an ancestral gene that has been maintained in the monocot maize during evolution. More sequence data of cytosolic monocot NADP-MEs are necessary to clarify this point. With regard to the C_4 and CAM photosynthetic NADP-ME isoforms, the tree shows that these enzymes appear several times in different branches of the tree, supporting the polyphyletic origin of the C_4 and CAM photosynthesis. The tree obtained accords also with the statement that the divergence between dicots and monocots occurred earlier than the appearance of both C_4 and CAM photosynthesis, as the specific forms of NADP-ME associated with those pathways appear in well different branches of the tree.

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