Malate decarboxylases: evolution and roles of NAD(P)-ME isoforms in species performing C₄ and C₃ photosynthesis

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Abstract

In the C₄ pathway of photosynthesis two types of malate decarboxylases release CO₂ in bundle sheath cells, NADP- and NAD-dependent malic enzyme (NADP-ME and NAD-ME), located in the chloroplasts and the mitochondria of these cells, respectively. The C₄ decarboxylases involved in C₄ photosynthesis did not evolve de novo; they were recruited from existing housekeeping isoforms. NADP-ME housekeeping isoforms would function in the control of malate levels during hypoxia, pathogen defence responses, and mesosporereparation, while NAD-ME participates in the respiration of malate in the tricarboxylic acid cycle. Recently, the existence of three enzymatic NAD-ME entities in Arabidopsis, occurring by alternative association of two subunits, was described as a novel mechanism to regulate NAD-ME activity under changing metabolic environments. The C₄ NADP-ME is thought to have evolved from a C₃ chloroplastic ancestor, which in turn would have evolved from an ancient cytosolic enzyme. In this way, the C₄ NADP-ME would have emerged through gene duplication, acquisition of a new promoter, and neo-functionalization. In contrast, there would exist a unique NAD-ME in C₄ plants, which would have been adapted to perform a dual function through changes in the kinetic and regulatory properties of the C₃ ancestors. In addition to this, for the evolution of C₄ NAD-ME, insertion of promoters or enhancers into the single-copy genes of the C₃ ancestors would have changed the expression without gene duplication.

Key words: C₄ photosynthesis, C₄ subtype, chloroplast, CO₂ pump, evolution, isoforms, malate decarboxylases, NAD-ME, NADP-ME, TCA cycle.

Introduction: three major biochemical subtypes for C₄ carbon fixation

C₄ photosynthesis has evolved to increase the photosynthetic efficiency in conditions where photorespiration would be enhanced (e.g. salinity, drought, elevated temperatures, and low CO₂ levels). This was achieved through the introduction of a series of anatomical and biochemical features that allow the concentration of CO₂ around ribulose 1,5-bisphosphate oxygenase-carboxylase (Rubisco). Except for single-cell C₄ photosynthesis in Chenopodiaceae (Edwards et al., 2004) and the facultative C₄ plant Hydrida verticillata (Estavillo et al., 2007), this photosynthetic pathway results from the metabolic interaction of two distinct cell types, mesophyll cells and bundle sheath cells (BSCs). In the first step of the CO₂ pump operating in C₄ plants, CO₂ is fixed in the form of HCO₃⁻ by phosphoenolpyruvate carboxylase in mesophyll cells producing oxaloacetate (Fig. 1). This C₄ acid is converted to malate and/or aspartate, which function as transient stores of fixed CO₂. In the C₄ lineages that transport primarily malate to the BSCs, the release of CO₂ is mediated by chloroplastic NADP-dependent malic enzyme (NADP-ME, EC1.1.1.40) (Fig. 1A). Other C₄ plants transport mainly aspartate to BSCs, which is further deaminated and reduced to malate (Fig. 1B). This C₄ acid is decarboxylated in mitochondria by NAD-dependent malic enzyme (NAD-ME; EC1.1.1.39). In the third C₄ lineage, aspartate and malate are transported to the BSCs (Fig. 1C). Aspartate is decarboxylated by the consecutive action of cytosolic aspartate aminotransferase and phosphoenolpyruvate carboxykinase (PEP-CK), while malate is decarboxylated via mitochondrial
insights into their roles in C₄ and C₃ plants. In light of recent knowledge, differences in the mechanisms that may have driven the evolution of the C₄ function of these decarboxylases, apart from their selective and high level of expression in BSC chloroplasts, are discussed. Data on PEP-CK function and evolution are summarized in the accompanying review by Aubry and co-workers.

**NADP-ME isoforms in species performing C₄ photosynthesis**

In species performing C₄ photosynthesis three isoforms of NADP-ME have been identified and characterized to date: (i) the photosynthetic chloroplastic NADP-ME; (ii) a non-photosynthetic plastidic NADP-ME; and (iii) a cytosolic NADP-ME (Table 1).

The photosynthetic NADP-ME from different C₄ species such as the monocots maize (Zea mays) and sugar cane (Saccharum sp.) (Mauroino et al., 1996, 1997; Saigo et al., 2004; Detarsio et al., 2003), the dicot Flaveria (Casati et al., 1999), and the tree species Haloxylon persicum (Casati et al., 1999) has a very high catalytic efficiency (Table 1). This isoform has an optimal activity at a pH of 8.0 where it forms a homotramer. At pH 7.0 it forms a less active dimer and is inhibited by malate (Iglesias and Andreo, 1990; Mauroino et al., 1996; Ashton, 1997). These structural and kinetic features were postulated to be a possible way of regulating the activity in vivo, as NADP-ME would be more active when light is driving photosynthesis (Iglesias and Andreo, 1990). However, this claim was challenged by recent work showing that a cytosolic non-photosynthetic isoform also presents this feature (Gerrard-Wheeler et al., 2008).

The non-photosynthetic plastidic NADP-ME is expressed constitutively at low levels in maize and Flaveria (Mauroino et al., 1997, 2001; Lai et al., 2002a). The housekeeping role of this isoform, although still not conclusively proved, might be the provision of substrates for plastidic-localized lipid and protein biosynthesis (Lai et al., 2002a) and is likely to accompany defence responses (Mauroino et al., 2001). Moreover, it assembles as dimer and is not inhibited by malate at pH 7.0 (Mauroino et al., 2001; Lai et al., 2002a; Saigo et al., 2004). In recent work using chimeras of both plastidic proteins it was shown that the regions flanked by amino acid residues 102 and 247 and from residue 248 to the C-terminal end of the C₄ isoform are involved in the tetrameric oligomerization and the inhibition by high malate concentrations at pH 7.0, respectively (Detarsio et al., 2007). The fact that both plastidic NADP-ME isoforms have a similar high catalytic efficiency (Table 1; Saigo et al., 2004) and share a high degree of sequence homology (Mauroino et al., 1997) allows it to be postulated that the non-photosynthetic isoform represents the most recent and direct ancestor of the photosynthetic isoform (Tausta et al., 2002). With regards to this, both proteins cluster in the monocots group in a phylogenetic tree constructed with known NADP-ME sequences (Fig. 2). In this group, plastidic and cytosolic NADP-MEs group

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**Fig. 1.** The three major biochemical subtypes for C₄ carbon fixation. 1, carbonic anhydrase; 2, phosphoenolpyruvate carboxylase; 3, NADP-malate dehydrogenase; 4, NADP-malic enzyme; 5, pyruvate orthophosphate dikinase; 6, aspartate aminotransferase; 7, NAD-malic enzyme; 8, alanine aminotransferase; 9, phosphoenolpyruvate carboxykinase. RPCC, reductive photosynthetic carbon cycle. Chloroplasts and mitochondria are represented in green and red, respectively.

NAD-ME. Although the predominance of one of these decarboxylases defines the C₄ photosynthetic subtypes (NADP-ME, NAD-ME, or PEP-CK subtype; Fig. 1), current knowledge indicates that they may operate simultaneously in many species (Furumoto et al., 1999; Wingler et al., 1999; Calsa and Filgueira, 2007; Muhaitat et al., 2007).

The C₄ decarboxylases involved in C₄ photosynthesis did not evolve de novo to assist this pathway; instead they were recruited and evolved from existing housekeeping isoforms. The preferred model to explain this evolutionary achievement would comprise gene duplication followed by modification within promoter regions to achieve high levels of expression in BSCs, neo-functionalization of the coding region, and further selection for traits such as carbon conservation and enhanced water and nitrogen-use efficiency under adverse conditions (Monson, 2003).

This review summarizes recent research on the biochemical, structural, and functional characterization of NADP- and NAD-dependent malate decarboxylases and provides...
together and sequences from CAM and facultative C4 plants are also included (Fig. 2).

Recently, the first cytosolic NADP-ME in a plant species performing C4 photosynthesis was identified and characterized at the molecular level. In the phylogenetic tree this isoform belongs to a group composed of cytosolic monocot and dicot sequences (Fig. 2). This isoform has low catalytic efficiency (Table 1) and is specifically expressed in embryo and emerging roots where it may be involved in the control of malate levels during hypoxia (Detarsio et al., 2008). The existence of additional cytosolic isoforms in C4 plants was also reported (Lai et al., 2002b) and recently confirmed through next-generation sequencing of the transcriptome of leaves of *Flaveria bidentis* that showed the presence of transcripts coding for several isoforms (U Gowik and P Westhoff, personal communication). Moreover, at least two more isoforms were detected in maize, one of which is constitutively expressed (E Detarsio, MF Drincovich, and VG Maurino, unpublished results). Thus, it remains a future challenge to clone and characterize these isoforms at the biochemical and functional level to clarify evolutionary events further.

### NADP-ME isoforms in species performing C3 photosynthesis

C3 plants such as *Arabidopsis thaliana* and *Oryza sativa* (rice) possess four NADP-ME isoforms, three located to the cytosol (NADP-ME1 to NADP-ME3) and one to plastids (NADP-ME4) (Table 1; Chi et al., 2004; Gerrard Wheeler et al., 2005). Although these proteins share a high degree of identity, the *Arabidopsis* isoforms show different biochemical properties and patterns of expression, suggesting that each isoform should possess a specific biological function (Gerrard Wheeler et al., 2005, 2008; Maurino et al., 2009).

While the expression of *Arabidopsis* NADP-ME2 and NADP-ME4 is rather ubiquitous, NADP-ME1 is highly expressed in the last stages of embryogenesis, in emerging roots, and in some secondary roots of adult plants, and NADP-ME3 is exclusively found in developing lateral roots and pollen (Gerrard Wheeler et al., 2005).

The kinetic characterization of all *Arabidopsis* isoforms revealed that NADP-ME1 resembles the recently characterized maize cytosolic NADP-ME (Table 1), which also has a similar expression pattern (Detarsio et al., 2008) and clusters in the same group in a phylogenetic tree (Fig. 2). *Arabidopsis* NADP-ME2 is responsible for the major part of the total NADP-ME activity in mature tissues and is highly regulated by metabolic effectors (Gerrard Wheeler et al., 2005, 2008). At the protein level it shares 90% identity with NADP-ME3, and both group with cytosolic dicot sequences in a phylogenetic tree (Fig. 2). The characterization of chimeras and several mutated and truncated versions of both isoforms allowed the identification of amino acid residues and segments of the primary structure involved in their differential kinetics and metabolic regulation (Gerrard Wheeler et al., 2009). In this way, although the active sites of NADP-ME2 and NADP-ME3 are highly conserved,

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### Table 1. Properties of NADP-ME isoform from the C3 plant *A. thaliana* and the C4 plant *Z. mays*

<table>
<thead>
<tr>
<th>Isoform</th>
<th>Localization</th>
<th>Kinetic constants $K_{\text{cat}}$ (s$^{-1}$)</th>
<th>$K_m$ NADP (μM)</th>
<th>$K_m$ malate (mM)</th>
<th>$K_{\text{cat}}/K_m$ malate</th>
<th>Optimum pH</th>
<th>Oligomeric state</th>
<th>Expression pattern/biological role</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. thaliana</em> (C3)</td>
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<td></td>
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<tr>
<td>NADP-ME1</td>
<td>Cytosol</td>
<td>38.7 ± 3.5$^a$</td>
<td>205.1 ± 22.6$^a$</td>
<td>2.96 ± 0.33$^a$</td>
<td>13.0</td>
<td>6.8$^b$</td>
<td>Tetramer$^b$</td>
<td>Emerging roots$^b$/unknown</td>
</tr>
<tr>
<td>NADP-ME2</td>
<td>Cytosol</td>
<td>324.1 ± 29.2$^a$</td>
<td>72.1 ± 7.2$^a$</td>
<td>3.33 ± 0.37$^a$</td>
<td>97.3</td>
<td>6.8$^b$</td>
<td>Tetramer$^b$</td>
<td>Constitutive$^b$/defence responses$^b$</td>
</tr>
<tr>
<td>NADP-ME3</td>
<td>Cytosol</td>
<td>268.1 ± 24.1$^a$</td>
<td>6.5 ± 0.62$^a$</td>
<td>0.83 ± 0.09$^a$</td>
<td>323.0</td>
<td>7.7$^b$</td>
<td>Tetramer$^b$</td>
<td>Secondary roots, pollen$^b$/microspore separation$^b$</td>
</tr>
<tr>
<td>NADP-ME4</td>
<td>Plastids$^a$</td>
<td>151.3 ± 12.1$^a$</td>
<td>10.2 ± 1.2$^a$</td>
<td>0.23 ± 0.025$^a$</td>
<td>657.8</td>
<td>7.7$^b$</td>
<td>Tetramer/ dimer$^b$</td>
<td>Constitutive$^b$/unknown</td>
</tr>
<tr>
<td><em>Z. mays</em> (C4)</td>
<td></td>
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<tr>
<td>CyNADP-ME</td>
<td>Cytosol</td>
<td>53.1 ± 2.8$^a$</td>
<td>19.1 ± 0.5$^a$</td>
<td>1.4 ± 0.60$^a$</td>
<td>37.9</td>
<td>7.0$^a$</td>
<td>Hexamer–octamer$^a$</td>
<td>Embryo and emerging roots$^a$/control of malate levels during hypoxia$^a$</td>
</tr>
<tr>
<td>Non-C4$^{-}$NADP-ME</td>
<td>Plastids$^f$</td>
<td>105.6 ± 8.9$^b$</td>
<td>70.2 ± 0.3$^b$</td>
<td>0.42 ± 0.039$^b$</td>
<td>251.4$^b$</td>
<td>7.8$^b$</td>
<td>Dimer$^f$</td>
<td>Constitutive/unknown</td>
</tr>
<tr>
<td>C4$^{-}$NADP-ME</td>
<td>Plastids$^f$</td>
<td>201 ± 9.8$^b$</td>
<td>8.0 ± 0.3$^b$</td>
<td>0.22 ± 0.02$^b$</td>
<td>913.6$^b$</td>
<td>8.0$^f$</td>
<td>Tetramer$^f$</td>
<td>Green tissues/C4 photosynthesis</td>
</tr>
</tbody>
</table>

Data are taken from

- $^a$ Gerrard Wheeler et al. (2005)
- $^b$ Gerrard Wheeler et al. (2008)
- $^c$ LM Voll, MB Zell, and VG Maurino, unpublished results
- $^d$ VG Maurino, unpublished results
- $^e$ Detarsio et al. (2008)
- $^f$ Maurino et al. (1997)
- $^g$ Saigo et al. (2004)
- $^h$ Detarsio et al. (2003)
- $^i$ Not conclusively proved.

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minimal structural changes yielded different allosteric sites, leading to the creation of proteins with unique regulatory mechanisms. Interestingly, the kinetic properties of Arabidopsis NADP-ME4 resemble those of both maize plastidic isoforms (Table 1). Moreover, Arabidopsis NADP-ME4 groups with all plastidic dicot sequences, while the plastidic isoform of rice clusters with the monocot sequences in a phylogenetic tree (Fig. 2). These data together suggest that a C3 plastidic isoform could have been an ancestor of the photosynthetic and non-photosynthetic plastidic isoforms found in C4 plants. Moreover, while the Arabidopsis cytosolic isoforms are active as tetramers, NADP-ME4 assembles in an equilibrium of dimers and tetramers, a capacity that was also described for the maize plastidic isoforms (Table 1) (Gerrard-Wheeler et al., 2005, 2008; Detarsio et al., 2008).

Arabidopsis single, double, and triple loss-of-function mutants of NADP-ME develop normally under standard growth conditions (Gerrard Wheeler et al., 2005), indicating that these isoforms may play specific roles at particular developmental stages and/or under specific physiological situations (Maurino et al., 2009). Although the roles of NADP-ME isoforms are still not defined, it was speculated that NADP-ME4 could be associated with the provision of pyruvate and NADPH for plastidic lipid or protein biosynthesis, NADP-ME1 could be involved in the control of malate levels during hypoxia (as its counterpart in maize), NADP-ME2 would generate reducing power in the cytosol for anabolic processes, possibly assisting the oxidative pentose phosphate pathway, and NADP-ME3 could be involved in providing NADPH during detoxification of cytotoxins and xenobiotics via glutathione conjugation in...
trichomes (Gerrard-Wheeler et al., 2005). Recently, it was shown experimentally that the expression of rice NADP-ME2 was induced by several environmental stresses and its overexpression conferred high salt and osmotic stress tolerance (Liu et al., 2007). Interestingly, the orthologous enzyme in Arabidopsis did not show these characteristics but was instead found to be associated with pathogen defence responses as its expression and activity increased after infection with the hemibiotrophic ascomycete fungus Colletotrichum higginsianum and diverse elicitors (LM Voll, MB Zell, and VG Maurino, unpublished results). A role for NADP-ME3 in microspore separation is also emerging as loss-of-function mutants in this enzyme showed changes in pollen morphology (VG Maurino, unpublished results). The biological function of NADP-ME1 and NADP-ME4 in C3 plants still remains to be elucidated. The similarity in biochemical and expression properties of these two isoforms with those of isoforms found in C4 plants together with the use of loss-of-function mutants and complementary approaches such as metabolic profiling and flux analysis under changing conditions will enable further exploration of the still unknown biological roles of these isoforms in C3 plants.

**NAD-ME in C3 species: a housekeeping enzyme with alternative subunit associations as regulatory mechanism**

Plant NAD-ME is found in mitochondria of all cells, where it is involved in the respiration of malate, a function shared with malate dehydrogenase (MDH). In addition to this universal role, some C4 species use NAD-ME to release CO2 in the BSCs during photosynthesis.

In C3 plants, NAD-ME is a multimer composed of two subunits, \( \alpha \) and \( \beta \), which are 65% identical and immunologically different. In a phylogenetic tree constructed with known sequences, this protein groups separately, \( \alpha \) subunits form group 1 and \( \beta \) subunits form group 2 (Fig. 3). In Solanum tuberosum, the formation of the heterodimer is required for the enzymatic activity (Grover and Wedding, 1982; Willeford and Wedding, 1987). Arabidopsis also contains two genes encoding proteins belonging to the \( \alpha \) and \( \beta \) group (Tronconi et al., 2008). By alternative association of these proteins three different enzymatic entities with similar basic kinetic properties but differential kinetic mechanisms and regulation by metabolic effectors are generated: the heterodimer (NAD-MEH) and the homodimers (NAD-ME1 and NAD-ME2) (Tronconi et al., 2010a, b). The existence of these three enzymatic entities in vivo was confirmed through the use of single and double loss-of-function mutants (Tronconi et al., 2008). Recently, the characterization of chimeric enzymes between NAD-ME1 and NAD-ME2 allowed the identification of specific regions in the primary structure that are involved in the differential allosteric regulation (Tronconi et al., 2010a). Moreover, NAD-MEH, NAD-ME1, and NAD-ME2 present a differing pattern of accumulation in separated components of flowers (Tronconi et al., 2010b). These different properties of NAD-MEH, NAD-ME1, and NAD-ME2 suggest that NAD-ME activity may be regulated by variations in the native association in vivo, rendering enzymatic entities with distinct allosteric regulation that could be best suited to specific physiological conditions. In this regard, plants completely lacking NAD-ME activity showed modifications of metabolite levels specifically during the dark period (Tronconi et al., 2008) that were different from those found in plants lacking NAD-ME1 or NAD-ME2 (A Maier and VG Maurino, unpublished results), indicating different contributions of each NAD-ME to plant metabolism.

**Did a specific C4 NAD-ME evolve by duplication of an original metabolic enzyme or has one enzyme got a dual function?**

NAD-ME from the C4 dicotyledoneous Amaranthus hypochondriacum is composed of \( \alpha \) and \( \beta \) subunits (Long et al., ...
1994), while those of *Amaranthus tricolor* and the grasses *Eleusine coracana* and *Panicum dichotomiflorum* were identified by electrophoresis as octamers of only one type of subunit (Oshugi and Murata, 1980; Murata et al., 1989). In these plants, the photosynthetic NAD-ME may represent a unique and specialized isofrom. In this regard, it is intriguing that non-photosynthetic isoforms of NAD-ME in C₄ plants have not been characterized at all and it is still not clear if the isofrom detected in non-photosynthetic tissues of C₄ plants corresponds to the C₄ NAD-ME isofrom or to a C₃-like isofrom.

The question of how NAD-ME evolved and is regulated in C₄ plants is currently being approached using *Cleome*, a genus closely related to *Arabidopsis*, which contains species spanning a developmental progression from C₃ to C₄ plants and uses NAD-ME in the C₄ photosynthetic pathway (Brown et al., 2005; Marshall et al., 2007). Through next-generation sequencing the transcriptomes of leaves of the closely related C₃ (*Cleome spinosa*) and C₄ (*Cleome gyroandra*) species were recently analysed (Brätügum et al., 2011). In both species only two distinct transcripts, derived from each orthologous gene in *Arabidopsis*, were found. In C₄ *Cleome* the transcript abundances of NAD-ME1 and NAD-ME2 are 20- and 27-fold higher than in C₃ *Cleome* during the light period. The full-length cDNAs corresponding to NAD-ME1 and NAD-ME2 are often present in all mature plant organs of C₃ and C₄ *Cleome* (A Maier and VG Maurino, unpublished results). These two full-length transcripts were present in all mature plant organs by assembling the expressed partial sequences into contigs and further rapid amplification of cDNA ends (RACE). The putative full-length cDNA sequences were then acquired by reverse transcription-PCR (RT-PCR) with specific primer pairs and were sequenced again for confirmation (A Maier, JM Hibberd, and VG Maurino, unpublished results). These two full-length transcripts are present in all mature plant organs of C₃ and C₄ *Cleome* (A Maier and VG Maurino, unpublished results) and the deduced protein sequences group with the corresponding orthologous sequences in a phylogenetic tree (Fig. 2). Thus, it could be possible that in C₄ plants there may exist only one major NAD-ME isofrom present as a heterodimer, which may carry out both the basic metabolic role in all cell types and the photosynthetic role exclusively in green tissues, where it is most abundantly expressed. This dual function of the enzyme may be achieved through strict regulatory properties that may differ from those isoforms exclusively found in C₃ plants. On the other hand, this would indicate that for the evolution of C₄ NAD-ME insertion of promoters or enhancers into the single-copy genes of the C₃ ancestors would have changed the expression without gene duplication.

However, as mentioned before, the existence of three enzymatic NAD-ME entities in *Arabidopsis* occurring by alternative association of two subunits would be a means to regulate the enzymatic activity under changing metabolic environments. In this way, it is also conceivable that in C₄ plants the function of NAD-ME as a basic metabolic enzyme and as a specific photosynthetic decarboxylase could be regulated by structural changes (induced by changes in pH, light, levels of certain metabolites, or a combination of them), which in turn would produce isoforms with different kinetics and regulatory properties. This idea is also backed up by the fact that the presence of a higher proportion of a less aggregated form of NAD-ME in a mutant of the C₄ plant *Amaranthus edulis* containing only 5% of the wild-type NAD-ME activity is unable to carry out photosynthetic CO₂ assimilation in normal air (Dever et al., 1998). Interestingly, no changes in the content of α and β NAD-ME were detected in these plants, indicating that the inability to form the octameric more active form of the enzyme, found in the wild type, is responsible for the reduced rate of CO₂ assimilation. Further studies are needed to determine whether changes in the aggregation state of NAD-ME are a common regulatory property of this enzyme and if a C₄ isofrom of NAD-ME is expressed in all tissues in NAD-ME C₄ plants or whether other isoforms exist.

**Is there a requirement for malate decarboxylases for photosynthesis in mid-veins of *A. thaliana***?

Cells associated with veins of petioles of C₃ plants such as tobacco, celery, and *Arabidopsis* possess relatively high activities of NAD-ME and NADP-ME (Hibberd and Quick, 2002; Brown et al., 2010). In mid-veins of *Arabidopsis*, transcripts for NADP-ME2, NADP-ME4, NAD-ME1, and NAD-ME2 were detected, and analysis of insertion mutants in these genes revealed that cytosolic NADP-ME2 is responsible for 80% of the activity in mid-veins and that both NAD-ME1 and NAD-ME2 are required for high NAD-ME activity (Brown et al., 2010).

It was suggested that these decarboxylases may release CO₂ from malate for photosynthesis in this type of cells as radionlabelled bicarbonate and malate fed to the xylem stream of C₃ plants were incorporated into insoluble material (e.g. starch and cell wall material and amino acids) in the mid-veins (Hibberd and Quick, 2002; Brown et al., 2010). However, when this experiment was performed with loss-of-function mutants of NAD- and NADP-ME, no differences in the incorporation patterns were observed, indicating that the individual isoforms of these decarboxylases have relatively little impact on the fixation of carbon from the xylem stream (Brown et al., 2010). In addition, removing the activity of the individual decarboxylases affected the abundance of sugars, amino acids, and glucoamine in mid-veins (Brown et al., 2010), suggesting that NAD-ME1, NAD-ME2, NADP-ME2, and NADP-ME4 may not predominantly function in supplying CO₂ to photosynthesis as the photosynthetic counterparts do in C₄ plants but they may primarily regulate the use of carbon for specific metabolic pathways. On the other hand, it cannot be ruled out that the combined action of these decarboxylases would be needed to support CO₂ fixation in the mid-veins.
Different evolutionary scenarios of NADP- and NAD-ME

$C_4$ photosynthesis originated at least 45 times independently in 19 families (Sage, 2004). Interestingly, in eudicots, a similar number of genera belong to the NADP- and NAD-ME subtypes, but in monocots 11 genera belong to the NADP-ME subtype and only four genera belong to the NAD-ME subtype, all this meaning that more $C_4$ species belong to the NADP-ME subtype. The NADP-ME decarboxylating pathway would confer some advantages over the NAD-ME pathway as it was shown that NADP-ME grasses have superior nitrogen-use efficiency relative to NAD-ME grasses. NADP-ME grasses achieved this higher nitrogen-use efficiency with less leaf nitrogen content and with Rubisco having a higher catalytic efficiency (Ghannoum et al., 2005).

As in some $C_4$ plants, more than one decarboxylase functions at the same time in the mid-veins of $C_3$ plants (Hibbert and Quick, 2004; Brown et al., 2010). This distribution could have been the starting point of the polyphyletic evolutionary origin of $C_4$ photosynthesis. Current knowledge indicates that the evolutionary scenarios suggested for both types of decarboxylases starkly differ. The $C_4$ NADP-ME is thought to have evolved from a $C_3$ chloroplastic ancestor, which in turn would have evolved from an ancient cytosolic enzyme. In this way, the $C_4$ chloroplastic NADP-ME would have emerged through gene duplication, followed by acquisition of a new promoter to direct its specific localization and neo-functionalization. In contrast, there would exist a unique NAD-ME in $C_4$ plant mitochondria, which would have been adapted through evolution to perform a dual function: the respira-
tion of malate in the tricarboxylic acid (TCA) cycle in all plant cells and malate decarboxylation to release CO$_2$ at the site of Rubisco in photosynthetic BSCs. This mechanism would imply changes in the kinetics and regulation of the enzyme, which would have occurred through successive mutations in a trial-and-error mechanism.

A crucial pre-condition for the initiation of $C_4$ bio-
chemistry evolution in certain taxa would have been the capacity to create and maintain a large number of duplicated genes (Monson, 2003; Sage, 2004). In the case of NADP-ME, the housekeeping functions of the enzyme and thus normal metabolism would not have been affected and thus evolution of this $C_4$ subtype could have been favoured.

Conclusions

Malate decarboxylases involved in $C_4$ photosynthesis were recruited from existing housekeeping isoforms. The previously suggested roles of these non-photosynthetic isoforms are being broadened through the use of many alternative approaches. These functions include the control of malate levels during hypoxia, the participation in pathogen defence responses and microspore separation, and the combined action of the decarboxylases to regulate the use of carbon for specific metabolic pathways in the mid-veins.

$C_4$ NADP-ME would have originated by gene duplication, a selective and high level of expression, and specialization of function through the gaining of unique kinetic and structural properties. In contrast, insertion of functional promoters or enhancers into single-copy genes of NAD-ME may have changed its expression without gene duplication, and the specialization of the $C_4$ function may have arisen through specific changes in regulatory and structural properties. This is supported by the existence of three enzymatic NAD-ME entities in Arabidopsis occurring by alternative association of two subunits as a means to regulate the enzymatic activity. Alternatively, the regulation of NAD-ME activity in $C_4$ tissues may take place at other points to match the amount of malate produced during a diurnal cycle. In this way, it is possible that in $C_4$ green tissues both NAD-ME1 and NAD-ME2 would be differentially regulated during the day and night, either at the kinetic or at the expression levels, or at both. Further studies, using the recently sequenced NAD-ME1 and NAD-ME2 from Cleome $C_1$ and $C_4$ species should determine whether changes in the aggregation state and levels of NAD-ME are involved in the regulation of the $C_4$ activity or if these mechanisms are a common regulatory property of this enzyme.

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