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RESEARCH PAPER

The dicotyledonous NAD malic enzyme C_4 plant Cleome gynandra displays age-dependent plasticity of C_4 decarboxylation biochemistry

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ABSTRACT

The C_4 photosynthetic pathway enriches carbon dioxide in the vicinity of Rubisco, thereby enabling plants to assimilate carbon more efficiently. Three canonical subtypes of C_4 exist, named after their main decarboxylating enzymes: NAD-dependent malic enzyme type, NADP-dependent malic enzyme type and phospho*enol*pyruvate carboxykinase type. *Cleome gynandra* is known to perform NAD-ME type C_4 photosynthesis. To further assess the mode of C_4 in *C. gynandra* and its manifestation in leaves of different age, total enzyme activities of eight C_4-related enzymes and the relative abundance of 31 metabolites were measured. *C. spinosa* was used as a C_3 control. *C. gynandra* was confirmed as an NAD-ME type C_4 plant in mid-aged leaves, whereas a mixed NAD-ME and PEPCK type was observed in older leaves. Young leaves showed a C_3-C_4 intermediate state with respect to enzyme activities and metabolite abundances. Comparative transcriptome analysis of mid-aged leaves of *C. gynandra* and *C. spinosa* showed that the transcript of only one aspartate aminotransferase (AspAT) isoform is highly abundant in *C. gynandra*. However, the canonical model of the NAD-ME pathway requires two AspATs, a mitochondrial and a cytosolic isoform. Surprisingly, our results indicate the existence of only one highly abundant AspAT isoform. Using GFP-fusion, this isozyme was localised exclusively to mitochondria. We propose a revised model of NAD-ME type C_4 photosynthesis in *C. gynandra*, in which both AspAT catalysed reactions take place in mitochondria and PEPCK catalyses an alternative decarboxylating pathway.

INTRODUCTION

Green plants produce organic matter from gaseous carbon dioxide (CO_2) using photosynthesis. While the core reactions of the Calvin-Benson cycle are conserved, different carbon enrichment mechanisms have evolved to improve the efficiency of carbon fixation. Although many seed plants assimilate CO_2 using only the Calvin-Benson cycle (*i.e.*, the C_3 pathway), employing ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) as the sole CO_2 assimilating enzyme, some plants use C_4 photosynthesis (Hatch & Slack 1966). C_4 photosynthesis has evolved independently multiple times from C_3 photosynthesis and leads to accumulation of CO_2 in the bundle sheath layer, where regular Rubisco fixation takes place. This makes C_4 plants more efficient at carbon assimilation, leading to higher water use efficiency (Black 1973), higher nitrogen use efficiency and/or faster accumulation of biomass. C_4 photosynthesis is a remarkable example of convergent evolution. The pathway has evolved independently over 60 times and independent origins exist both in eudicots and monocots (Sage 2004; Sage *et al.* 2011).

In principle, C_4 photosynthesis needs a primary fixation enzyme, which is specific for carbon assimilation and does not react with O_2, as is the case for Rubisco. In all known C_4 species, phospho*enol*pyruvate (PEP) carboxylase (PEPC) is the primary CO_2-fixing enzyme. The CO_2 is transported to the site of the Calvin cycle in the form of a C_4 acid and released from the C_4 acid by a decarboxylation enzyme, either PEP carboxykinase (PEPCK), NAD-dependent malic enzyme (NAD-ME) or NADP-dependent malic enzyme (NADP-ME) (Hatch 1987). Traditionally, C_4 plants are classified according to which is the major decarboxylation enzyme. Recently, however, it was hypothesised that plasticity exists in the decarboxylation biochemistry in response to environmental cues (Furbank 2011). The dicot *Cleome gynandra* is classified as a NAD-ME plant (Marshall *et al.* 2007). The NAD-ME pathway was primarily elaborated using the monocot model *Panicum miliaceum* and it was assumed to also operate in dicotyledonous NAD-ME C_4 plants, such as *C. gynandra* (Bräutigam *et al.* 2011a). Based on the *P. miliaceum* model, CO_2 is fixed into oxaloacetate (OAA), which is subsequently converted to Asp by cytosolic aspartate aminotransferase (AspAT) in mesophyll cells. Asp is then transferred to the bundle sheath, where it is transaminated to OAA, reduced to malate, and finally decarboxylated, releasing the CO_2 and pyruvate. Pyruvate is transaminated to alanine, thus serving as the terminal amino group acceptor for the AspAT reaction. The C_3 amino acid returns to the mesophyll, where it is converted over several steps into PEP, thus providing the precursor for a new round of carboxylation and decarboxylation. The transfer of a C_4 amino acid to the bundle sheath and the return of a C_3 amino acid to the mesophyll cells

balances the amino group transfer. The canonical model of NAD-ME type C_4 photosynthesis assumes that two isoforms of AspAT exist: one AspAT in the mesophyll cell cytosol and one AspAT in the bundle sheath mitochondria (as summarised in Hatch 1987). In the NAD-ME plant *P. miliaceum*, total AspAT activity is higher than in C_3 plants and distributed equally between mesophyll and bundle sheath (Hatch & Mau 1973). The AspAT activity in the bundle sheath is localised to mitochondria, while the localisation of mesophyll AspAT is not organelle associated (Hatch & Mau 1973). In a recent comparative transcriptome analysis of *C. spinosa* and *C. gynandra* using mRNA-Seq, only one out of five AspAT isozymes, a mitochondrial AspAT, was found to be upregulated in C_4 plants (Bräutigam *et al.* 2011a).

Compared to C_4 monocot model species, relatively little is known about dicotyledonous C_4 models, especially the new model *C. gynandra* (Brown *et al.* 2005). For example, in *Z. mays* (maize) it has been shown that C_4 photosynthesis develops with leaf age and with light exposure (Bassi & Passera 1982). Cross-sections of leaves show that Kranz anatomy develops during maturation, and it was concluded that C_4 metabolism develops during maturation of leaves and continuously takes over primary carbon fixation (Miranda *et al.* 1981). In the dicot *Flaveria trinervia*, young leaves fix a higher proportion of $^{14}CO_2$ directly through Rubisco, as compared to older leaves (Moore *et al.* 1986). Hence, establishment of Kranz anatomy limits young leaves of both monocots and dicots in conducting C_4 photosynthesis (Nelson & Dengler 1992). Whether such changes in C_4 enzyme activity occur during maturation of the dicot *C. gynandra* has not yet been tested.

In this work, two species of the genus *Cleome* were used, *Cleome gynandra* as a C_4 plant and *Cleome spinosa* as a C_3 plant (Marshall *et al.* 2007) to investigate the biochemistry of dicotyledonous NAD-ME type C_4 plants. The genus *Cleome* belongs to the Cleomaceae, which is phylogenetically close to the Brassicaceae model plant *Arabidopsis thaliana* (Hall *et al.* 2002; Brown *et al.* 2005; Inda *et al.* 2008). Our experiments addressed two major questions: is the C_4 pathway of the dicot *C. gynandra* modulated with age, and which enzymes play a role in NAD-ME type C_4 photosynthesis performed in the dicot *C. gynandra*?

MATERIAL AND METHODS

Plant growth

Cleome gynandra, *C. spinosa* and *Nicotiana benthamiana* plants were grown in soil in a glasshouse. Natural sunlight was supplemented by artificial 400 W spotlights mounted ~1.5 m above the ground level from 05:30 to 09:30 h to extend the daily light period to 16 h. Natural illumination was limited to 60,000 lux using shading. The temperature was kept constant at 24 °C through artificial ventilation. The humidity was measured between 75% and 90%.

Harvesting leaf material

Leaf material was harvested from mature green leaves, when not used for leaf age gradient experiments. For all experiments, leaves were snap-frozen in liquid nitrogen immediately after harvesting. For age gradient experiments, plants were grown for 4 weeks, at which point eight to ten true leaves were developed. Material from ten plants was pooled. The second youngest leaf was taken as the young leaf sample, the fifth youngest leaf was taken as the mid-aged leaf sample and the second oldest leaf was taken as the old leaf sample. Leaves were harvested after 6 h of illumination (at 11:30 h).

Enzyme assays

Leaves were cut at the base and immediately frozen in liquid nitrogen. Samples were ground to a fine powder under constant supply of liquid nitrogen, using a mortar and pestle. Leaves were harvested according to the described routine for the age gradient. Aliquots of 10 mg of leaf powder for each developmental stage were transferred into pre-chilled reaction tubes, and 1 ml of extraction buffer containing 25 mM Tris HCl (pH 7.5), 1 mM magnesium sulphate, 1 mM ethylenediaminetetraacetic acid, 5 mM DTT, 0.2 mM phenylmethylsulphonyl fluoride and 10% (v/v) glycerol were added to each sample and immediately vortexed to mix the ingredients before the extraction buffer froze. Tubes were thawed at room temperature for 10 min. After inverting the tubes four to six times, samples were centrifuged for 5 min at 4 °C and 16,000 *g*, and 500 µl of the supernatant were transferred into a fresh tube.

Enzyme activity measurements were conducted using coupled assays. PEPC activity was determined as described in Jiao & Chollet (1988). NAD-ME and NADP-ME were assayed as described in Hatch & Mau (1977). The protocol of Walker *et al.* (1995) was used to determine PEPCK activity in the reverse direction. The method of Hatch & Mau (1973) was performed to determine AspAT and AlaAT activity. MDH assays were performed according to Johnson & Hatch (1970). Leaf extracts of each developmental stage were desalted using size exclusion chromatography on NAP5 columns [Sephadex (GE Healthcare, Barrington, IL, USA) G-25 DNA Grade] prior to all enzyme assays.

Determination of chlorophyll and protein

Total chlorophyll was measured according to Porra (2002). Total protein determination was carried out using the BCA™ Protein Assay Kit (Thermo Scientific, Rockford, IL, USA). Protein concentration was determined in leaf extracts of each development stage according to manufacturer's instructions. Leaf extract was generated as described above.

Metabolite profiling

The extraction of leaves was performed according to Fiehn (2007) and GC/MS was performed according to Lee & Fiehn (2008). Analysis of metabolites was done *via* GC/MS-TOF [Autosampler: Gerstel, (Mülheim an der Ruhr, Germany), MPS2 XL; GC: Agilent Technologies (Santa Clara, CA, USA) 7890A; MS-TOF: Waters (Milford, MA, USA) GCT premier] and analysed using the MassLynx software package supplied with the instrument (Waters). Ribitol was added as an internal standard and the ratio of the area of each metabolite and the corresponding ribitol area was referred to as the ribitol response factor (RRF).

Native polyacrylamide gel electrophoresis

Aliquots of 50 mg of leaf powder were transferred into pre-chilled 1.5-ml reaction tubes, with 200 µl of extraction buffer (50 mM Tris HCl pH 6.8, 10% (v/v) glycerol, 5% 2-mercaptoethanol, 0.001% bromophenol blue) added to each sample. Samples were vortexed immediately to avoid freezing of the extraction buffer. Tubes were thawed for 10 min at room temperature and centrifuged at 16,000 *g* for 5 min at 4 °C. Supernatants were transferred into fresh tubes. Gels consisted of a stacking gel, which was ~1-cm wide, followed by a running gel, which was ~6-cm wide (Ferl *et al.* 1979). AspAT gels were stained with L-cysteine sulphinic acid as described in Stejskal (1994).

Transient expression of proteins in *N. benthamiana*

Cleome gynandra AspAT was cloned into pMDC83 *via* pDONR207. The fragment was amplified using primer A (GGGGACAAGTTTGTACAAAAAAGCAGGCTGAGAG ATGG CTATGGCTATGG) and primer B (GGGGACCACTTTGTAC AAGAAAGCTGG GTCAGACTTAGAGACCTCATGGATCGC). To detect the cellular localisation of proteins, vectors containing expression cassettes were transformed into *N. benthamiana* leaves *via* agrobacterial infection (Bendahmane *et al.* 1999). The co-infiltration with a mitochondrial reporter construct (ScCOX4: mCherry; Nelson *et al.* 2007) and the AspAT-containing construct was achieved by mixing liquid *Agrobacterium* cultures prior to infiltration (Walter *et al.* 2004). Localisation was observed in epidermal peels using epifluorescence microscopy with appropriate filters for mCherry and for GFP.

RESULTS

Activity of C_4 enzymes

To assess possible age-related differences in the mode of C_4, the activity of enzymes involved in C_4 photosynthetic pathways was determined in extracts of *Cleome* leaves of different age. The three decarboxylating enzymes NAD-ME, NADP-ME and PEPCK were tested, as well as PEPC, AspAT, alanine aminotransferase (AlaAT) and both NAD-dependent and NADP-dependent malate dehydrogenase (NAD-MDH and NADP-MDH).

In mid-aged mature leaves, the activity of PEPC was 1.8 U g^{-1} FW in the C_4 species and 0.02 U g^{-1} FW in the C_3 species. NAD-ME showed the highest activity among the decarboxylating enzymes (0.59 U g^{-1} FW) and exceeded the activity in *C. spinosa* (0.04 U g^{-1} FW) by an order of magnitude (Fig. 1A). Also, AspAT (1.51 U g^{-1} FW) and AlaAT (2.89 U g^{-1} FW) in mid-aged *C. gynandra* leaves were significantly ($P < 0.05$) higher than the corresponding activities in *C. spinosa* leaves (0.53 and 0.86 U g^{-1} FW, respectively). There was no significant difference between *C. gynandra* and *C. spinosa* in the activity of NAD-MDH, which is also part of the C_4 shuttling pathway in NAD-ME-dependent C_4 plants. Mid-aged leaves of *C. gynandra* showed substantial activity of PEPCK, reaching two-thirds of the NAD-ME activity in the same sample (Fig. 1). NADP-ME showed low activity in mid-aged leaves of *C. gynandra* (0.03 U g^{-1} FW) when compared to the other decarboxylating enzymes. In mid-aged leaves of *C. spinosa*, NADP-ME activity was significantly higher (0.26 U g^{-1} FW).

It was then tested whether differences in enzyme activity exist in different aged leaves. In old leaves of *C. gynandra*, the NAD-ME activity (0.28 U g^{-1} FW) decreased significantly ($P < 0.05$) by 50% when compared to the mid-aged leaf sample (Fig. 1; Table 1). Also the AlaAT activity in old *C. gynandra* leaves decreased significantly (Table 1). In contrast, the PEPCK activity increased by 30% in old *C. gynandra* leaf samples and the AspAT activity increased significantly ($P < 0.05$) by 60% (Table 1). PEPC and both NAD- and NADP-dependent MDHs showed only minor changes in activity in old as compared to mid-aged leaf samples. NADP-ME activity increased by 40% (Table 1), but NADP-ME was still the least active decarboxylating enzyme in *C. gynandra* (Fig. 1). Old leaves of *C. spinosa* showed a general age-related reduction in enzyme activities (Fig. 1). NADP-ME showed lower activity compared to mid-aged leaves. Also, both NAD- and NADP-dependent MDH showed significantly decreased activity (Fig. 1).

In young *C. gynandra* leaves, PEPC showed 50% less activity when compared to mid-aged leaf samples. PEPC activity

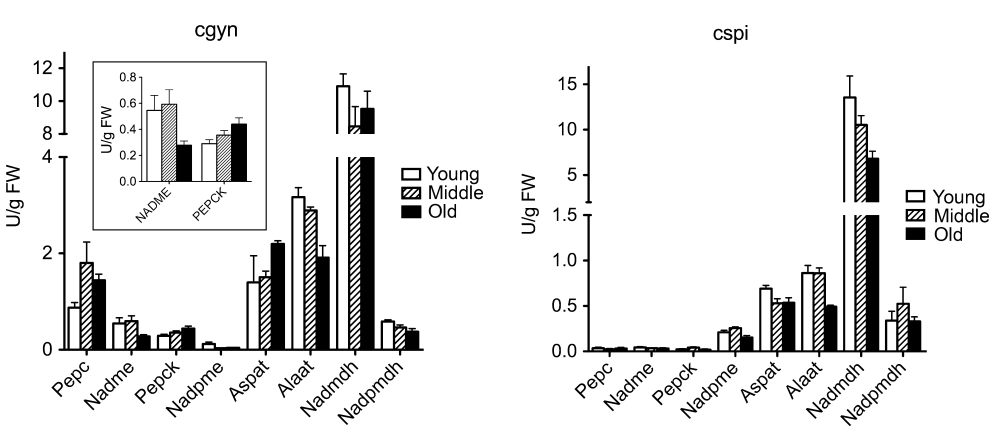

Fig. 1. Enzyme activity in young, mid-aged and old leaves. Means and standard errors of n = 5 are plotted. cgyn *C. gynandra*; cspi *C. spinosa*; pepc phosphoenolpyruvate carboxylase; nadme, NAD-dependent malic enzyme; pepck PEP carboxykinase; nadpme, NADP-dependent malic enzyme; aspat, aspartate aminotransferase; alaat, alanine aminotransferase; nadmdh, NAD-dependent malate dehydrogenase; nadpmdh, NADP-dependent malate dehydrogenase.

Table 1. Age-related fold-change of enzyme activity in *C. gynandra* leaves.

fold change	young/middle	middle/old	young/old
PEP carboxylase	2.0	0.9	1.8*
NAD malic enzyme	1.3	0.5*	0.6
PEP carboxykinase	1.3	1.3	1.7*
NADP malic enzyme	0.4	1.4	0.7
aspartate aminotransferase	1.6	1.6**	2.2
alanine aminotransferase	0.9	0.7**	0.6**
NAD malate dehydrogenase	0.8	1.1	0.9
NADP malate dehydrogenase	0.8	0.9	0.6*

Asterisks mark significant fold changes. *P < 0.05; **P < 0.01; ***P < 0.001.

in young leaves was significantly lower than in old leaves. Also, the AspAT activity in young *C. gynandra* leaves was 40% lower as compared to mid-aged leaves, albeit this difference was not statistically significant. The activities of other enzymes showed only minor changes. In young *C. spinosa* leaves, the only significantly different activity compared to mid-aged leaves was observed for AspAT, which showed increased activity in young leaves.

Although NAD-ME activity was reduced significantly from mid-aged to old leaves, the sum of activities of all decarboxylating enzymes (NAD-ME, NADP-ME and PEPCK) did not change significantly between leaves of different ages.

Total chlorophyll and protein content

Ratios of total protein per total chlorophyll and total protein per fresh weight in *C. gynandra* and *C. spinosa* were compared. *C. spinosa* contained significantly more protein than *C. gynandra*, both on the basis of chlorophyll and fresh weight in mid-aged leaves (Fig. 2). No significant difference was detected between the C_3 and the C_4 plant in young and old leaves.

Metabolite analysis

Metabolites were analysed to obtain a more detailed view of the differences between C_3 and C_4 metabolism. In total, 49 distinct metabolites were analysed, of which 31 could be detected and quantified reliably.

In young leaf samples, nine of 31 metabolites were more abundant in *C. gynandra*; this number increased to 24 in old leaves. The main metabolites of the C_4 shuttling pathway, *e.g.*, alanine, aspartate and malate, were significantly more abundant in mid-aged and old leaves of *C. gynandra*, as compared to *C. spinosa* (Fig. 3B,C). However, no significant difference was detected for aspartate and malate in young leaves (Fig. 3A). Also, asparagine, which can be synthesised from aspartate by aspartate-ammonia ligase, fumarate, which can be synthesised from malate by fumarate hydratase, and glutamate, which is a central metabolite of nitrogen metabolism, are significantly more abundant in mid-aged and old *C. gynandra* leaves compared to levels in *C. spinosa*. However, there was no significant difference in these metabolites in young leaves (Fig. 3). The contents of the sugars glucose, fructose, mannose, xylose and sucrose did not differ; only maltose showed a significantly higher abundance in *C. spinosa* in all leaf ages (Fig. 3). Xylose abundance appeared to be greater in young *C. spinosa* samples. Sucrose abundance was almost identical when comparing *C. gynandra* and *C. spinosa* leaves of the same age (Fig. 3). Among the 11 amino acids that could be reliably detected and quantified, aspartate, alanine and asparagine were more abundant in C_4 samples, whilst others were detected in higher concentrations in the C_3 plant, *e.g.*, methionine and leucine.

Aspartate aminotransferase in *C. gynandra*

After analysing age-related differences in enzyme activity and steady state metabolite pools, AspAT function was analysed in detail in mature mid-aged leaves. Transcriptome analysis detected five AspAT isoforms in leaf transcriptomes of both *C. gynandra* and *C. spinosa* (Bräutigam & Weber 2011a,b). Native protein gels followed by activity staining were performed to determine the numbers of isozymes and their individual activities in these two species. For both species, five distinct bands stained for AspAT activity (Fig. 4). In *C. gynandra*, the five bands were spread wider as compared to in *C. spinosa*. The *C. gynandra* band migrating furthest into the gel showed the highest activity of all bands, as evidenced by the intensity of staining. The four other bands of *C. gynandra* and the five bands of *C. spinosa* showed comparable activity.

Subcellular localisation of the highly abundant AspAT isoform of *C. gynandra*

Combining quantitative (Bräutigam *et al.* 2011a) and qualitative (Bräutigam *et al.* 2011b) next generation sequencing information, as proposed in Bräutigam & Gowik (2010), identified the sequence of the upregulated CgAspAT. A CgAspAT-GFP fusion construct based on the full-length contig of

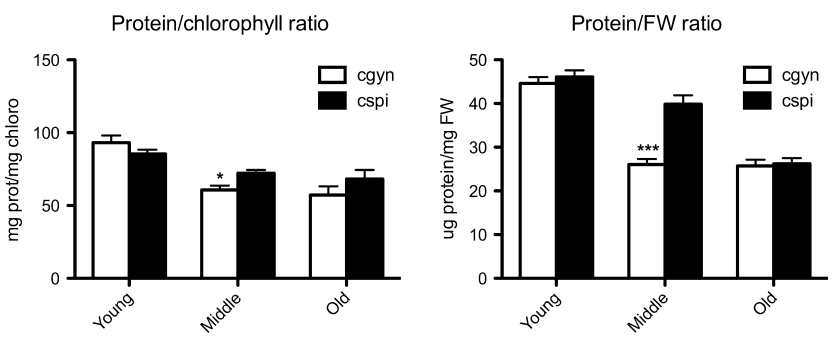

Fig. 2. Total chlorophyll and protein in a C_3 and a C_4 plant. Significant changes are marked with asterisks, *P < 0.05; ***P < 0.001.

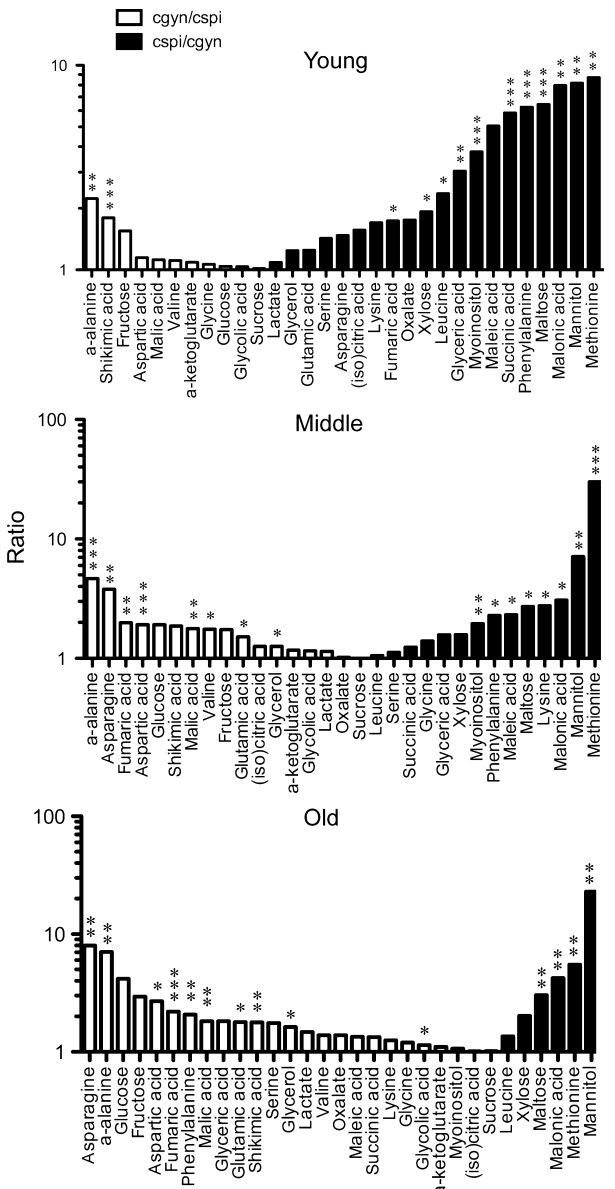

Fig. 3. Metabolite analysis in three leaf age stages. The ratios between C_3 and C_4 leaves are plotted. Significant changes were calculated with Student's *t*-tests (n = 5) and are marked with asterisks, *P < 0.05; **P < 0.01; ***P < 0.001.

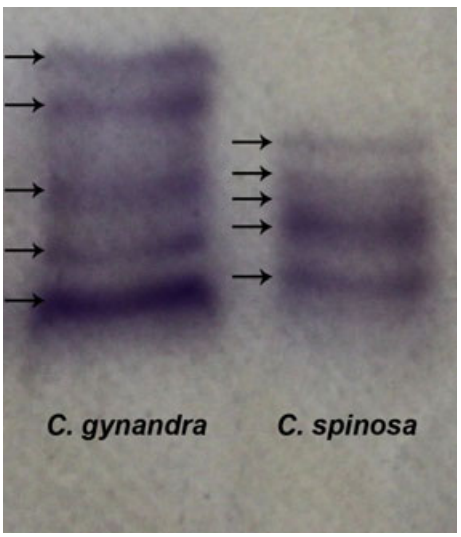

Fig. 4. Isozyme analysis of AspATs in *C. gynandra* and *C. spinosa*. Arrowheads point to isozymes.

this upregulated AspAT was created to investigate the subcellular localisation of the transcriptionally abundant isozyme of AspAT (Bräutigam *et al.* 2011a) in *N. benthamiana*, which maps to the mitochondrial AspAT1 of *A. thaliana*. Mitochondria were tagged with a fluorescent marker as a positive control (Nelson *et al.* 2007). The CgAspAT::GFP fusion protein was detected as small dots, both in photosynthetic cells as well as epidermal cells of *N. benthamiana* leaves. Co-localisation was determined in epidermal cells to minimise interference from chlorophyll fluorescence. The organelles labelled with GFP were detected on the periphery of the cell (Fig. 5B). Structures in the same position were labelled with the mitochondrial marker (Fig. 5A) and the overlay revealed perfect congruence of the fluorescence signals (Fig. 5D). Chloroplasts in the sample (Fig. 5C) caused minor signals in the mCherry channel for the mitochondrial marker (Fig. 5A, C).

DISCUSSION

Leaf age-related changes in C_4 photosynthesis in *C. gynandra*

Photosynthetic C_3 and C_4 plant tissues have distinct biochemical properties that can be assessed using enzyme activity assays. In Cleomaceae, *C. gynandra* performs C_4 photosynthesis of the NAD-ME type, whereas *C. spinosa* performs C_3 photosynthesis (Marshall *et al.* 2007). Enzymatic tests were performed to qualify and quantify the activity of C_4 marker enzymes, including PEPC and the main decarboxylating enzyme NAD-ME, and to provide information about establishment of the C_4 syndrome during leaf development. In addition, metabolite analysis was performed *via* GC/MS-TOF to assess the steady state metabolite pools associated with the photosynthetic subtypes in *C. gynandra* and *C. spinosa*.

The soluble sugar contents in the C_4 and the C_3 species were comparable. Sucrose was found at nearly the same concentration in both species (*C. gynandra/C. spinosa* ratio, young: 1.0, mid: 1.0, old: 1.0). Only maltose, which is exported from the chloroplast following metabolic conversion of transitory starch at night (Weise *et al.* 2004), showed consistently higher abundance in *C. spinosa*. Both C_4-related metabolite pools as well as C_4-related enzyme activities, however, clearly differed between the two species. NAD-ME had highest activity among the decarboxylating enzymes in mid-aged leaves of *C. gynandra*, which confirms *C. gynandra* as an NAD-ME type plant (Marshall *et al.* 2007). The ratios between NAD-ME and NADP-ME (18:1) and between NAD-ME and PEPCK (2:1) were also comparable to earlier results (Marshall *et al.* 2007; Bräutigam *et al.* 2011a). Minor activities of secondary decarboxylating enzymes in C_4 plants or general decarboxylating enzymes in C_3 plants are probably related to their function in housekeeping processes, such as amino acid biosyntheses (Brown *et al.* 2010), or in other

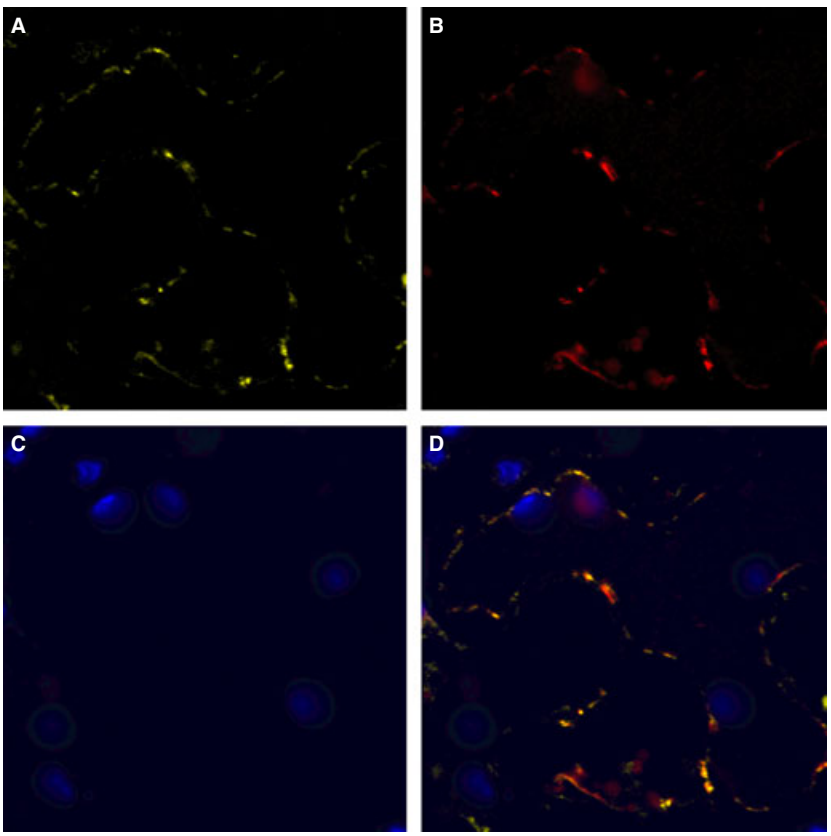

Fig. 5. AspAT::GFP co-localised with the mitochondrial marker. A mCherry fluorescence of the mitochondrial reporter construct; B GFP fluorescence of the AspAT::GFP fusion construct; C chloroplast autofluorescence; D merged image of A–C.

processes, such as defence (Maurino *et al.* 2001). Intermediates of the NAD-ME type C_4 pathway, namely aspartate and malate, showed higher ribitol response factors (RRFs; see Material and Methods) in mid-aged *C. gynandra* leaves (aspartate: 1.9-fold, malate: 1.8-fold, alanine: 4.7-fold). The high abundances of intermediates of NAD-ME type C_4 photosynthesis is in agreement with predominant activity of enzymes of the NAD-ME cycle. Both MDH activities did not significantly increase in *C. gynandra* (Fig. 1; Table 1) when comparing mid-aged leaf samples, which is surprising, given that the expected increase in MDH activity is necessary for proper C_4 carbon shuttling in *C. gynandra*. However, the activity of NAD-MDH was already very high in the C_3 species (Fig. 1). This may indicate that the MDH activity is high enough to support C_4 photosynthesis in addition to its housekeeping function. Alternatively, since total activity was measured, mitochondrial activity may have been higher, which may have been offset by lower peroxisomal, cytosolic and/or plastidic activities. Transcriptome analysis of *C. gynandra* and *C. spinosa* did not reveal an increase in mitochondrial NAD-MDH (Bräutigam *et al.* 2011a), which favours the first hypothesis. Although the activity of NAD-MDHs was steady, the pool size of malate was larger in the C_4 species.

The pattern of C_4-related enzyme activities changes with leaf age. Analysis of the decarboxylating enzymes revealed that *C. gynandra* NAD-ME activity decreased in old leaves by 50%, while PEPCK activity was increased significantly compared to that of young leaves and exceeded the NAD-ME activity by 50%. These results are unexpected for an NAD-ME type plant, since previous data indicated only minor PEPCK activity in NAD-ME plants (Hatch 1987). Significant PEPCK activity in leaves is in contrast to a recent report, which includes immunostaining of *C. gynandra* crude leaf extract (Voznesenskaya *et al.* 2007); no binding of PEPCK-specific antibody to the extract was detected in this experiment. In this previous study, the developmental stage of harvested leaves was referred to as mature. Possibly, the leaf age was younger compared to what we call mid-age. Only PEPCK type C_4 plants are known to show major secondary decarboxylating enzyme activity for NAD-ME (Hatch *et al.* 1988). The observed gradient in decarboxylating enzyme activity can be explained by the hypothesis that *C. gynandra* gradually shifts its C_4 pathway during leaf development from NAD-ME type to a mixed NAD-ME and PEPCK type. If this hypothesis holds, the activity of other enzymes involved in the C_4 shuttling pathway ought to change as well, namely less activity of NAD-ME type specific enzymes and constant activity of enzymes that are required for both types of C_4 photosynthesis. Indeed, AlaAT, which is required for NAD-ME type C_4 photosynthesis but has no use in the PEPCK pathway, showed significantly lower activity in old *C. gynandra* leaves when compared to mid-aged leaves. The shift from NAD-ME type to PEPCK type may decrease the demand for this enzyme. At the same time, PEPC, which is required for all types of C_4 photosynthesis, did not show decreased activity when comparing old and mid-aged leaves of *C. gynandra*. However, the pool size of intermediates in the NAD-ME pathway further increases in old C_4 leaves compared to those in the C_3 plant. The C_4/C_3 ratio of alanine increased from 4.7-fold in mid-aged leaves to 7.0 in old leaves. This indicates that although the decarboxylating activity is at least in part taken over by PEP-CK, the C_3 amino acid alanine remains

important. If aspartate continues to be shuttled for a PEP-CK type of cycle and PEP is regenerated, the amino group ratio between mesophyll and bundle sheath becomes unbalanced. Possibly, glutamate (increased significantly in the C_4 species in mid-aged and old leaves) and 2-oxoglutarate (not significantly increased) play a role in the C_4 cycle (Fig. 3). A shift from NADP-ME type to a mixed NADP-ME and PEPCK type has been observed in old leaves for the monocot plant *Zea mays* (Wingler *et al.* 1999). Our analysis of protein to chlorophyll and protein to fresh weight ratios supports the notion that major changes occur from young to mid-aged old leaves, which altered the protein/chlorophyll and protein/fresh weight ratios observed in mature mid-aged leaves (Fig. 2). The significantly lower protein content in mid-aged C_4 leaves is most likely due to the efficiency of the C_4 pathway. It has been shown that C_4 plants contain less Rubisco protein compared to C_3 plants (Ku *et al.* 1979). Transcriptome analysis of *Cleome* species also revealed that functional clusters of proteins, which are directly or indirectly involved in photosynthesis, are differentially expressed in C_4 photosynthesis (Bräutigam *et al.* 2011a). That is, transcripts encoding Rubisco subunits and enzymes of photorespiration are expressed at lower levels in the C_4 plant. In addition, it was found that genes encoding components of cytosolic and plastidial ribosomes were also expressed at lower levels, which likely also contributes to a lower protein content in these leaves. Further, it was found in a recent study using C_3, C_3-C_4 intermediate and C_4 species of *Flaveria* that the C_4 species always displayed a higher carbon-to-nitrogen ratio than the C_3 species, which is indicative of a lower protein content in the C_4 species (Gowik *et al.* 2011). Thus, since changes in total leaf protein concentration are apparently a consequence of functional adaptations in C_4 photosynthesis rather than natural variation, total protein content was not used in this study to normalise enzyme activity data.

The PEPC activity in young leaves was low compared to that in other leaf ages (Fig. 1). This indicates that C_4 carbon fixation is not yet fully expressed at this developmental stage. Also, the C_4/C_3 ratio of metabolites involved in the NAD-ME pathway, *i.e.*, aspartate (young: 1.1-fold, mid: 1.9-fold), malate (young: 1.1-fold, mid: 1.8-fold) and alanine (young: 2.2-fold, mid: 4.7-fold), is lower in young compared to mid-aged leaves. This finding is consistent with the hypothesis that C_4 photosynthesis is not fully developed in young *C. gynandra* leaves compared to mid-aged and old leaves. Instead, young leaves may use C_3 type photosynthesis or a mix of C_4 and C_3 photosynthesis. It is also possible that young leaves perform less total photosynthesis. Based on the protein ratios, major changes occur when leaves mature and fully implement C_4 photosynthesis. However, the current results do not allow the rejection of either hypothesis.

AspAT isozymes

The aspartate aminotransferases (AspATs) were investigated in detail in mid-aged *Cleome* leaves. They convert OAA to aspartate and *vice versa*. The canonical model for NAD-ME type C_4 plants requires two isoforms of this enzyme, a cytosolic isoform in mesophyll cells and a mitochondrial isoform in bundle sheath cells (Taniguchi & Sugiyama 1990; Taniguchi *et al.* 1995). This model was based on activity gels of *P. miliaceum* (Hatch & Mau 1973). mRNA-Seq data (Bräutigam *et al.* 2011a), however, indicate that in *C. gynandra* only one isoform is upregulated at the transcriptional level, which contradicts the canonical model.

In both *C. gynandra* and *C. spinosa*, a total of five AspAT isozymes were detected in native enzyme activity gels (Fig. 4), which is in accordance with the number of different cDNAs detected at the transcriptome level (Bräutigam *et al.* 2011a). This number is also identical to the number of AspAT isozymes encoded by the genome of *A. thaliana* (Schultz & Coruzzi 1995; Wilkie *et al.* 1995). Only one of these five isozymes showed very high activity in *C. gynandra* compared to any of the other isozymes in both species (Fig. 4). This result is in agreement with findings of mRNA-Seq, where only a single upregulated AspAT transcript was detected (Bräutigam *et al.* 2011a). In models of NAD-ME type C_4 photosynthesis, two reactions catalysed by AspAT take place, one of them in the cytosol and one in mitochondria. Usually proteins are targeted to only one location in the cell; however, some proteins are present in multiple locations due to dual targeting (Small *et al.* 1998). Subcellular localisation studies of CgAspAT1 were performed to assess the localisation of the highly abundant AspAT isoform in *C. gynandra*. The overlay of the signals of GFP-tagged CgAspAT with a mitochondrial-localised control fused to the mCherry protein confirmed co-localisation of both gene products. Hence, the highly abundant AspAT isozyme is targeted to mitochondria. Multiple targeting was excluded since no GFP signal was detected in other compartments of the cell or in the cytosol. In *P. miliaceum* NAD-ME type C_4 photosynthesis is concomitant with increased activity of two different AspAT isoforms in bundle sheath and mesophyll cells (Hatch & Mau 1973). Since mesophyll and bundle sheath cells cannot be easily separated in dicots, in contrast to monocots, only whole leaf extracts were examined (Fig. 4). However, in *P. miliaceum*, even in whole leaf extracts the change in activity of two isoforms, not just one, was apparent on activity gels of whole leaf extracts. It is therefore concluded that CgAspAT1 performs both AspAT-catalysed reactions of the C_4 pathway in mitochondria, which indicates that the NAD-ME C_4 pathway in *C. gynandra* differs from that of *P. miliaceum*.

The mitochondrial localisation of AspAT requires additional transport processes across the mitochondrial membrane. The substrates of the AspAT reaction, namely OAA and glutamate, have to be imported, whereas the products, aspartate and 2-oxoglutarate, have to be exported. Despite detailed knowledge on soluble proteins in C_4 photosynthesis, the adaption of membrane proteins remains largely unknown (Bräutigam *et al.* 2008; Weber & von Caemmerer 2010; Bräutigam & Weber 2011a; b; Furumoto *et al.* 2011). Since it is known that proteins involved in C_4 photosynthesis are modified in localisation and abundance rather than newly invented (Sage 2004), it is assumed that transporters that already exist in C_3 plants are re-dedicated to C_4-related transport processes in *C. gynandra* (Furumoto *et al.* 2011). Two transporters were characterised biochemically in mitochondria of *Vigna sinensis* and *Pisum sativum*; one catalyses the counter-exchange of 2-oxoglutarate and OAA (Desantis *et al.* 1976) and the other catalyses the counter-exchange of aspartate and glutamate (Vivekananda & Oliver 1989). Taken together, both carriers are able to shuttle all required sub-

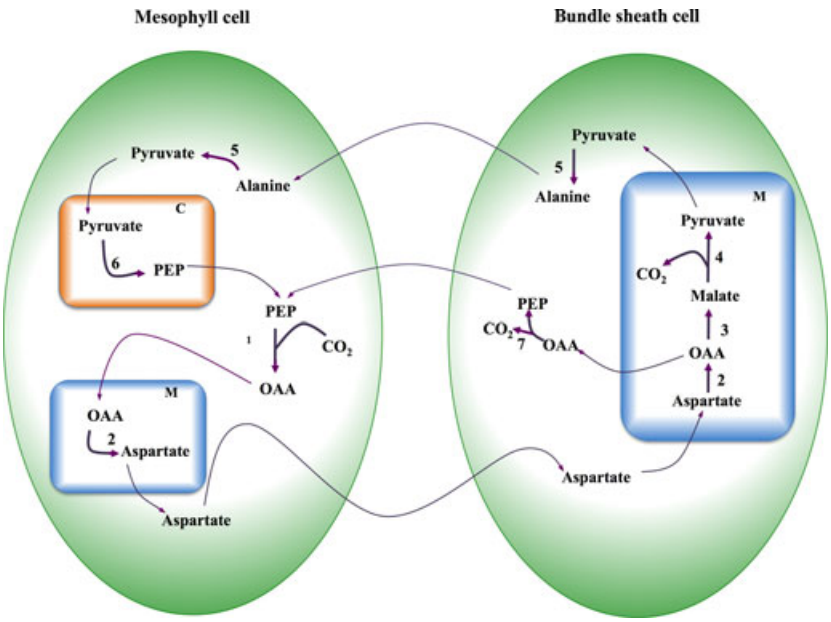

Fig. 6. New model of the NAD-ME pathway. Thick arrows indicate reaction, thin arrows indicate transport process. M=mitochondrium. C=chloroplast. 1 = PEPC, 2 = AspAT, 3 = MDH, 4 = NAD-ME, 5 = AlaAT, 6 = PPDK, 7 = PEPCK.

strates and products for AspAT-catalysed transamination, and this balances the amino groups between the cytosol and the mitochondria. It is currently not known whether and how the transport capacities of mitochondria are adjusted in NAD-ME plants. In evolutionary terms, establishing the *P. miliaceum* model requires the upregulation of two different genes and the restriction of one gene to the mesophyll and of a second gene to bundle sheath cells. Establishing the *C. gynandra* model requires the general increase in expression of one gene and the up-regulation of two transport proteins, if transport capacity is indeed limiting. mRNA-Seq data showed increased expression levels of only three mitochondrial transport proteins in *C. gynandra*, namely the dicarboxylate carriers DIC1 and DIC2, and a phosphate transporter (Bräutigam *et al.* 2011a). Neither of the dicarboxylate carriers shows transport activity for aspartate or any other amino acid in the C_3 plant or the close relative *A. thaliana* (Palmieri *et al.* 2008). The phosphate transporter is a *bona fide* phosphate transporter in grasses (Takabatake *et al.* 1999). It remains unclear whether one of the DIC transporters can transport aspartate in C_4 plants. In summary, it is proposed that *C. gynandra* performs a modified C_4 cycle in which not only the AspAT-catalysed reaction occurs in bundle sheath cells, but also in the mitochondria of mesophyll cells. Required transporters were not found in this work, but the required transport processes can be performed by equivalents of at least partially described transporters from C_3 plant mitochondria. The evolutionary changes required are not exceptionally larger than those required for the *P. miliaceum* model.

A model of NAD-ME in the dicot *C. gynandra*

This work shows that *C. gynandra* does not perform NAD-ME type C_4 photosynthesis exclusively, but also uses PEPCK as a decarboxylation enzyme. PEPCK use is more pronounced in older leaves, while very young leaves show only limited PEPC activity. An increase in PEPC in mid-aged leaves suggests that C_4 photosynthesis may be used predominantly in more mature leaves, while young leaves use either C_3, a mix of C_3 and C_4 photosynthesis or perform less total photosynthesis. Based on the results of age-dependent analyses, it is proposed that *C. gynandra* passes through key changes in the carbon assimilation mechanism during leaf development, namely showing a shift from C_3 photosynthesis or a mix of C_3 and C_4 photosynthesis in young leaves to exclusively, or almost exclusively, C_4 photosynthesis in old leaves, and at the same time, a shift from NAD-ME C_4 type in young leaves to a combination of NAD-ME and PEPCK C_4 type in old leaves. The decarboxylation enzymes are indeed flexible, although plasticity based on developmental changes rather than environmental changes as proposed in (Furbank 2011) was demonstrated.

In addition, the presence of only a single up-regulated isoform of AspAT, which is localised to mitochondria, leads to a new model for C_4 photosynthesis in mesophyll cells. Instead of a cytosolic conversion of OAA to aspartate, the reaction takes place in mitochondria. Consequently, a new model for NAD-ME type C_4 photosynthesis is proposed for *C. gynandra* (Fig. 6).

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