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Photosynthesis in C₃–C₄ intermediate Moricandia species

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Abstract

Evolution of C₄ photosynthesis is not distributed evenly in the plant kingdom. Particularly interesting is the situation in the Brassicaceae, because the family contains no C₄ species, but several C₃–C₄ intermediates, mainly in the genus Moricandia. Investigation of leaf anatomy, gas exchange parameters, the metabolome, and the transcriptome of two C₃–C₄ intermediate Moricandia species, M. arvensis and M. suffruticosa, and their close C₃ relative M. moricandioides enabled us to unravel the specific C₃–C₄ characteristics in these Moricandia lines. Reduced CO₂ compensation points in these lines were accompanied by anatomical adjustments, such as centripetal concentration of organelles in the bundle sheath, and metabolic adjustments, such as the balancing of C and N metabolism between mesophyll and bundle sheath cells by multiple pathways. Evolution from C₃ to C₃–C₄ intermediacy was probably facilitated first by loss of one copy of the glycine decarboxylase P-protein, followed by dominant activity of a bundle sheath-specific element in its promoter. In contrast to recent models, installation of the C₃–C₄ pathway was not accompanied by enhanced activity of the C₄ cycle. Our results indicate that metabolic limitations connected to N metabolism or anatomical limitations connected to vein density could have constrained evolution of C₄ in Moricandia.

Key words: Bundle sheath, C₃–C₄ intermediacy, C₄ photosynthesis, evolution, glycine decarboxylase, Moricandia.

Introduction

C₄ plants evolved in warm, open, and often arid regions, where the C₄ concentrating mechanism leads to enhanced photosynthetic carbon fixation efficiency (Sage, 2004). In most C₄ species, this is achieved by upstream CO₂ fixation through phosphoenolpyruvate carboxylase (PEPC) in the mesophyll (MS) and transport of the synthesised C₄ metabolites to the bundle sheath (BS) cells (Hatch, 1987). Decarboxylation of C₄ metabolites in the BS cells increases CO₂ concentration around Ribulose-1,5 bisphosphate carboxylase/oxygenase (Rubisco), thus promoting the carboxylase reaction while reducing photorespiration (Hatch and Slack, 1970; Bowes et al., 1971; Hatch, 1987). Division of photosynthetic
biochemistry between two different cell types requires anatomical adaptation including high vein density, reduction of MS tissue, and enlarged, chloroplast-rich BS cells (Dengler et al., 1994; McKown and Dengler, 2007; Christin et al., 2013). Despite its complexity, the C₄ trait evolved independently more than 60 times in flowering plants (Sage et al., 2012; Sage, 2016). Soon after the discovery of C₄ photosynthesis, it became apparent that transition from C₃ to C₄ photosynthesis could not have been realised in one giant step, but more likely evolved via a series of transitory states (Kennedy and Laetsch, 1974). Potential C₃–C₄ intermediates were identified by their CO₂ compensation point, which lay between the values of C₃ and C₄ species, as well as some C₄-like anatomical features in the BS cells (Kennedy and Laetsch, 1974; Krenzer et al., 1975). The Brassicaceae species *Moricandia arvensis* was among the first species classified as a potential C₃–C₄ intermediate (Krenzer et al., 1975).

The genus *Moricandia* consists of eight accepted species (www.theplantlist.org), all originating from Mediterranean or Saharo-Sindian areas (Tahir and Watts, 2010). Based on CO₂ compensation points, *Moricandia* includes C₃ species (*M. moricandioides*, *M. foetida*, and *M. foleyi*) as well as C₃–C₄ intermediates (*M. arvensis*, *M. suffruticosa*, *M. nitens*, *M. spinosa*, and *M. sicaica*; Brown and Hattersley, 1989; Apel et al., 1997). Besides a low CO₂ compensation point, the C₃–C₄ candidates exhibit lower sensitivity to O₂ (Holday et al., 1982) and high incorporation of ¹⁴C into glycine and serine (Holaday and Chollet, 1983; Hunt et al., 1987). The BS area per cell profile is increased as well as the number of chloroplasts, mitochondria, and peroxisomes in the BS (Holaday and Chollet, 1983; Hunt et al., 1987). In contrast to C₄ species, these potential intermediates possess a C₄-like δ¹⁴C signature, C₄-like Rubisco kinetics (Bauwe and Apel, 1979; Holbrook et al., 1985), and low activities of typical C₄ enzymes such as PEPC, pyruvate phosphate dikinase (PDPK), NADP malic enzyme (NADP-ME), NAD-ME, and phosphoenopyruvate carboxykinase (PEPCK) (Holaday et al., 1981; Holaday and Chollet, 1983).

Rawsthorne and colleagues showed that the P-subunit of the glycine decarboxylase multi-enzyme system (GLDP) is exclusively localised to the BS of the leaf of *M. arvensis* (Rawsthorne et al., 1988a, b; Rylott et al., 1998), while all other enzymes of the photorespiratory or photosynthetic pathways, such as the L, H, and T subunits of GLD, serine hydroxymethyltransferase (SHMT), glycolate oxidase (GOX), and Rubisco, are found in MS as well as BS tissues (Rawsthorne et al., 1988b, Morgan et al., 1993). These findings led to the first experimentally verified model of photosynthesis in C₃–C₄ intermediates (Rawsthorne, 1992). The interruption of the photorespiratory cycle in the MS caused by the absence of the functioning GLD/SHMT complex leads to an accumulation of glycine and its diffusion to the BS cells. There, its decarboxylation creates a local CO₂ enrichment, thus increasing the carboxylation activity of Rubisco in the BS and therefore reducing the CO₂ compensation point of the leaf (Rawsthorne, 1992). This process is also named the glycine shuttle, photorespiratory CO₂ pump, or C₄ photosynthesis (Sage et al., 2014).

Species with C₃–C₄ intermediate characteristics have been identified in diverse groups of plants (Krenzer et al., 1975; Rajendrudu et al., 1986; Moore et al., 1987; Hylton et al., 1988; Apel et al., 1997; Muhaidat et al., 2011; Sage et al., 2011b; Wen and Zhang, 2015; Khosh ravesh et al., 2016). Phylogenetic studies have shown that many of these C₃–C₄ plants are closely related to C₃ siblings, and it is therefore likely that intermediates served as transitory states on the evolutionary path from C₃ to C₄ (McKown et al., 2005; Christin et al., 2011a; Fisher et al., 2015; Lyu et al., 2015). The possibility that C₃–C₄ intermediates bridge the evolutionary gap between C₃ and C₄ states has also recently been supported by different computational modelling approaches (Heckmann et al., 2013; Williams et al., 2013; Mallmann et al., 2014; Brautigam and Gowik, 2016). Experimental as well as computational models predict that under favourable genetic and anatomic pre-conditions, the shift of GLD to the BS is a decisive step for installation of the glycine shuttle and the transition from C₃ to C₃–C₄ photosynthesis. Because the GLD/SHMT reaction in the BS releases not only CO₂ but also NH₃, rebalancing of the N metabolism between the two cell types is required (Mallmann et al., 2014). For re-balancing of N metabolism, the current model suggests additional metabolite shuttles, e.g. glutamate–2-oxoglutarate, alanine–pyruvate, and aspartate–malate. Parts of these shuttles and the associated biochemical enzymes also play an important role in C₄ photosynthesis. Existing C₄ enzymes and transporters can create a primordial C₄ cycle in the C₃–C₄ background on which selection can act as long as selective pressure for efficient C₃ assimilation persists (Aubry et al., 2011; Mallmann et al., 2014; Brautigam and Gowik, 2016). In essence, altered GLD localisation is predicted to initiate a smooth path to C₄ (Heckmann et al., 2013; Brautigam and Gowik, 2016; Schluter and Weber, 2016). In support of this model, over 90% of plant lineages with C₃–C₄ intermediates also contain C₄ species (Mallmann et al., 2014).

The model, however, fails to explain the presence of evolutionary stable C₃–C₄ intermediates. The situation in the Brassicaceae is therefore particularly interesting; to date no *sensu stricto* C₄ species have been identified in this family, but it contains multiple lines of C₃–C₄ intermediates, including members of the genus *Moricandia* (Hylton et al., 1988), *Diplotaxis tenuifolia* (Apel et al., 1997; Ueno et al., 2006), and *Brassica graminia* (Ueno, 2011). In this current study, the C₃–C₄ metabolism in the genus *Moricandia* was investigated in more detail by simultaneous analyses of phylogeny, leaf anatomy, gas exchange, and metabolite and transcript patterns in closely related C₃ (*M. moricandioides*) and C₃–C₄ species (*M. arvensis* line MOR1 and *M. suffruticosa*). The data were used for testing hypotheses related to C₄ evolution or lack thereof: (i) phylogenetic patterns of GLDP explain the evolution of intermediacy in just one specific branch of the Brassicaceae; (ii) metabolic differences between C₃ and intermediate species relate to the N-shuttle; and (iii) differences with the genus *Flaveria*, which evolved full C₄, indicate where *Moricandia* species might be restricted in evolution towards C₄.
Material and methods

Plant cultivation

Seeds for different *Moricandia* lines were obtained from botanic gardens and seed collections (*Moricandia moricandioides*, 04-0393-10-00 from Osnabrück Botanic Gardens; *M. arvensis*, line 12-0020-10-00 from Osnabrück Botanic Gardens, lines 0119708, 0000321, 0084187 from the Royal Botanic Gardens in Kew, lines MOR1 and MOR3 from IPK Gatersleben; *M. suffruticosa*, line 0105433 from the Royal Botanic Gardens in Kew; *M. nitens*, 0209858 from the Royal Botanic Gardens in Kew). Seeds were vapour-sterilised by incubation in a desiccator together with a freshly prepared mix of 100 ml 13% Na-hypochloride with 3 ml of 37% HCl for 2 h. The sterilised seeds were then germinated on plates containing 0.22% (w/v) Murashige Skoog medium, 50 mM MES pH 5.7 and 0.8% (w/v) Agar. After about a week, seedlings were transferred to pots containing a mixture of sand and soil (BP substrate, Klasmann & Deilmann GmbH, Germany) at a ratio of 1:2. In the first experiment, testing all the *Moricandia* lines, plants were grown in a greenhouse (Heinrich Heine University, Düsseldorf) in September 2013, where they received natural light ranging from 300 and 600 µmol s⁻¹ m⁻². For our more detailed studies of C₃–C₄ intermediate metabolism in *Moricandia*, we chose one species from each *Moricandia* C₃–C₄ subgroup presented in Fig. 1A (*M. suffruticosa* from group I and *M. arvensis* line MOR1 from group II) and compared them to their C₃ relative *M. moricandioides*. For all following experiments, plants were cultivated in a climate chamber (CLF Mobilux Growbanks, Wertingen, Germany) under 12-h day conditions with 23/20 °C day/night temperatures. The plants were exposed to ambient CO₂ concentrations and irradiance at plant level was ~200 µmol s⁻¹ m⁻². All experiments were conducted before the transition to the reproductive state. Mature leaf material was harvested from *M. moricandioides*, *M. arvensis* (line MOR1), and *M. suffruticosa* ~2 h after the start of the light period by flash-freezing in liquid nitrogen. The material was homogenised into a fine powder by grinding in liquid nitrogen. The material was stored at ~80 °C and used for analysis of elements, metabolites, transcripts, and proteins.

Phylogeny

Relationships between *Moricandia* species and their closest relatives were determined using sequences from the ITS nuclear region. DNA was extracted from all the *Moricandia* lines available in our study, *Diplotaxis tenuifolia* (line ‘Wilde Rauke’, origin: N.L. Chrestensen Erfurter Samen-und Pflanzenzucht GmbH), and *D. viminea* (line GB.31066, origin: Rijk Zwaan Distribution B.V., Netherlands). The ITS sequences were amplified and sequenced using the primers ITS1 (*O’Kane* et al., 1996) and ITS4 (*White* et al., 1990). Additional ITS sequences were retrieved from the NCBI database (see Supplementary Table S1 at *JXB* online). The sequences were aligned with Muscle (*Edgar*, 2004), and the alignment was used to infer a time-calibrated phylogeny following a relaxed molecular clock.
approach, as implemented in Beast (Drummond and Rambaut, 2007). The analysis was run for 10 000 000 generations, sampling a tree every 1000 generations under a GTR+G+I substitution model, a log-normal relaxed clock, and a Yule speciation prior. The tree was rooted by forcing the monophyly of both the outgroup (the two ‘Cleome’ species) and the ingroup (Brassicaceae). The tree was calibrated by setting the age of the crown of Brassicaceae with a normal distribution with a mean of 29.3 Ma and a standard deviation of 3.0, based on estimates from markers across nuclear genomes (Christin et al., 2014). The convergence of the analysis and adequacy of the burn-in period were verified using Trace (Drummond and Rambaut, 2007). Medians of node ages for tree samples after a burn-in period of 1 000 000 generations were mapped on the maximum-credibility tree using Treeannotator (Drummond and Rambaut, 2007).

GLDP-specific mRNA sequences were retrieved from online databases (see Supplementary Table S2). GLDP-specific coding sequences for Moricandia and Diplotaxis lines were obtained from the assembly of next-generation sequencing reads produced in our own lab (see below). The phylogenetic tree was constructed with the help of the Phylogeny.fr webtool (http://phylogeny.lirmm.fr) in the default mode consisting of alignment by Muscle, G-block building, maximum-likelihood tree generation by PhyML, and visualisation by TreeDyn.

Plant anatomy

Vein density measurements were done on the top third of fully grown rosette leaves. The leaf material was cleared in an acetic acid:ethanol mix (1:3) overnight followed by staining of cell walls in 5% safranin O in ethanol, and de-staining in 70% ethanol. Pictures were taken using a Nikon Eclipse Ti-U microscope equipped with a ProgRes MF camera (Jenoptik, Jena, Germany), at 4× magnification. At least six leaves were analysed for vein density per line, always with five pictures measured and averaged per leaf using ImageJ open software (https://imagej.nih.gov/ij/).

For histological and ultrastructural analysis 2-mm² sections from mature rosette leaves were used for combined conventional and microwave-proceeded fixation, dehydration, and resin embedding in a PELCO BioWave®34700-230 (Ted Pella, Inc., Redding CA, USA) as described in Supplementary Table S3. Semi-thin sections with a thickness of approximately 2.5 μm were mounted on slides and stained for 2 min with 1% methylene blue / 1% Azur II in 1% aqueous borax at 60 °C prior to light microscopical examination in a Zeiss Axio Imager M2 microscope (Carl Zeiss Microscopy GmbH, Göttingen, Germany). Ultra-thin sections with a thickness of approximately 70 nm were cut with a diamond knife, transferred onto TEM-grids and contrasted in a LEICA EM STAIN (Leica Microsystems, Vienna, Austria) with uranyl acetate and Reynolds’ lead citrate prior to analysis using a Tecnai Sphera G2 transmission electron microscope (FEI, Eindhoven, Netherlands) at 120 kV.

Photosynthetic gas exchange

Mature rosette leaves were used for gas exchange measurements with a LICOR 6400XT (LI-COR Biosciences, Lincoln, USA). The conditions were a flow of 300 μmol s⁻¹, light of 1500 μmol m⁻² s⁻¹, leaf temperature of 25 °C, and the vapour pressure deficit was kept below 1.5 kPa. Initial analysis of the Moricandia lines and comparison with the related C₃ plants Brassica oleracea and Tarenaya haastleri, the C₃-C₄ intermediate Diplotaxis tenuifolia, and the C₄ plant Gynandropsis gynandra were done with A–C₃ curves, with measurements at 400, 50, 100, 200, 400, 800, 1200, and 1800 ppm CO₂. Significance of the differences in the CO₂ compensation points between lines was tested using a one-way ANOVA followed by a post-hoc Tukey’s multiple comparison test.

More detailed A–C₄ curves were measured on the selected species M. moricandoides, M. arvensis MOR1, and M. suffruticosa. After acclimation, an A–C₄ curve was determined starting at a CO₂ concentration (C₄) of 400 ppm, then going down to 20 ppm in nine steps, then going back to 400 ppm and raising the CO₂ concentration stepwise up to 1800 ppm. Measurements at the lowest six CO₂ concentrations were used to extract the CO₂ compensation point and the initial slope of the graph corresponding to the carboxylation efficiency. Maximal assimilation was determined at CO₂ concentrations of 1600 to 1800 ppm. At least six plants were measured per line, and statistical significance between values for the different species was evaluated using Student’s t-test.

Metabolite and element analysis

The homogenized leaf material was extracted for metabolite analysis by gas chromatography-mass spectrometry (GC-MS) according to Fiehn et al. (2000) using a 7200 GC-QTOF (Agilent, Santa Clara, USA). Data analysis was conducted with the Mass Hunter Software (Agilent, Santa Clara, USA). For relative quantification, all metabolite peak areas were normalized to the peak area of an internal standard of ribitol added prior to extraction. The same homogenised leaf material was used for determination of δ¹³C and CN ratios. After lyophilisation, the material was analysed using an Isoprime 100 isotope ratio mass spectrometer coupled to an ISOTOPE cube elemental analyzer (both from Elementar, Hanau, Germany) according to Gowik et al. (2011). Measurements were always done on ten biological replicates. Statistical significance was analysed using Student’s t-test.

Transcript analysis

Total RNA was extracted from the homogenized leaf material using the GeneMatrix Universal RNA purification kit (Roboklon GmbH, Berlin, Germany). The RNA was treated with DNase for a few seconds only and quality controlled on a Bioanalyzer 2100 (Agilent, Santa Clara, USA). Subsequently, mRNA purification and adapter ligation was performed with the TruSeq RNA Sample Preparation Kit (Illumina, San Diego, USA) using 1 μg of total RNA. After a second quality control on the Bioanalyzer, samples were sent to Beckmann Coulters Genomics (Danvers, MA, USA) and sequenced on a HiSeq2500 sequencer (Illumina, San Diego, CA) as single-end 100-bp reads. Three to four biological replicates were used per Moricandia species with between 13 and 17 million reads per sample. The reads were aligned to a minimal set of coding sequences of the TAIR 10 release of the Arabidopsis genome (http://www.arabidopsis.org/) using BLAT (Kent, 2002) in protein space. The best BLAT hit for each read was determined by (1) the lowest e-value and (2) the highest bit score (Brautigam et al., 2011). Raw read counts were transformed to reads per million (rpm) to normalize for the number of reads available at each sampling stage. Cross-species mapping takes advantage of the completeness and annotation of the Arabidopsis genome. In all samples, 80 to 86% of reads mapped to the reference genome. Comparison of the transcript pattern between species was performed with the edgeR tool (Robinson et al., 2010) in R (www.R-project.org) using the Benjamini–Hochberg false discovery test with a cut-off at false discovery rate (FDR) ≤0.01 for significant differences. The agriGO webtool was employed for gene ontology (GO) term analysis (Du et al., 2010). The transcriptome data was deposited at the GEO repository (www.ncbi.nlm.nih.gov/geo/) under the accession number GSE87343.
For construction of the specific *Moricandia* transcript assemblies, one sample per line was sequenced as pair-end 150-bp reads on an Illumina HiSeq2000 platform at the BMFZ (Biologisch-Medizinisches Forschungszentrum) of the Heinrich Heine University (Düsseldorf, Germany). The resulting reads were trimmed using the trimmomatic tool (Bolger et al., 2014) followed by assembly using Trinity (Haas et al., 2013), which yielded between 68,000 and 91,000 contigs. Sequences for transcripts from *D. tenuifolia*, *D. viminea*, and *D. muralis* were obtained following the same protocol.

**PEPC activity**

Total soluble proteins were extracted from the homogenised leaf material in 50 mM Hepes-KOH pH 7.5, 5 mM MgCl₂, 2 mM DTT, 1 mM EDTA, 0.5% Triton-X-100, 0.1% B-mercaptoethanol. For the PEPC assay, 20 μl of the extract were mixed with assay buffer consisting of 100 mM Tricine-KOH pH 8.0, 5 mM MgCl₂, 2 mM DTT, 1 mM KHC₄O₃, 0.2 mM NADH, 5 mM glucose-6-phosphate, 2 U ml⁻¹ malate dehydrogenase in a microtitre plate (Ashton et al., 1990). The reaction was started after addition of phosphoenolpyruvate to a final concentration of 5 mM in the assay. Absorbance at 340 nm was measured with a Synergy HT microplate reader (BioTek Instruments, USA). Protein content of the solutions was determined with the Bradford assay (Quick Start Bradford Protein Assay kit, BioRad). The ratio between fresh and dry weight of the leaf material was determined by weighing a second mature leaf from the same plants before and after drying it at 70 °C overnight.

**Physiological features allow differentiation of C₃ and C₄–C₃ Moricandia lines**

Many details of the C₃–C₄ intermediate photosynthesis character were investigated in the 1980s and 1990s at the University of Nebraska (see Holaday et al., 1981, 1982; Holaday and Chollet, 1983), in Gatersleben in Germany (see Bawe and Apel, 1979; Apel et al., 1997), and at the John Innes Centre in Norwich, UK (see Rawsthorne et al., 1988a, b; Rylott et al., 1998). Since the stock seed material from these initial analyses could no longer be obtained, CO₂ compensation points in genotyped greenhouse-grown lines were characterized (Fig. 1B) and compared with data from the C₃ plant *T. hastleriana* and the C₄ plant *G. gynandra* from the neighbouring Cleomaceae family. The measurements of the CO₂ compensation points clearly allowed classification of the tested lines as a C₃, C₃–C₄, or C₄ species (Fig. 1B). All C₃–C₄ intermediate lines had CO₂ compensation points that were significantly lower than in the C₃ species, but also significantly higher than in the C₄. On the other hand, no significant differences could be observed among the C₃–C₄ intermediate lines.

The selected accessions *M. moricandioides*, *M. arvensis* MOR1, and *M. suffruticosa* were then grown under controlled environmental conditions in a climate chamber. Under these conditions, the differences in the CO₂ compensation point of the C₃ and C₃–C₄ *Moricandia* species were even more pronounced (Fig. 2A, B). A closer inspection of the shape of A–C₄ curves showed that, despite the low CO₂ compensation point, the curve of the intermediates looked much more similar to the C₃ curve than the one of the C₄ species *G. gynandra*, which had a very steep initial ΔA/ΔC₄ slope of 0.557 (Fig. 2A). The initial slope of the A–C₄ curve is connected to the carboxylation efficiency in the photosynthetic system, the PEPC efficiency in C₄, and the Rubisco carboxylation efficiency in C₃ (von Caemmerer, 2000). A comparison of the A–C₄ curves in the C₃ and C₃–C₄ *Moricandia* showed that the initial slope was actually steeper in the C₃ species than in the C₃–C₄ intermediate lines (Fig. 2A, C).

The assimilation at ambient CO₂ was lower in the C₃–C₄ intermediate species than in the C₃ species (Fig. 2D). Differences in conductance were not responsible for this variation, and the maximal CO₂ assimilation at high CO₂ reached similar levels in all species tested (see Supplementary Table S4). In addition, measurements of PEPC activity in extracts

**Results**

**Occurrence of C₃–C₄ intermediates in the Brassicaceae**

The phylogenetic relationships between all the *Moricandia* accessions available in our study were investigated by sequencing their ITS region and in comparison with data available in the NCBI database (Fig. 1A). We aimed at testing whether the C₃–C₄ character evolved independently or in one single event in different *Moricandia* lines. With the exception of *M. foetida*, all *Moricandia* species formed a monophyletic group in the phylogenetic tree (Fig. 1A). Within this clade, a C₃ group (*M. moricandioides*, *M. foetida*) was sister to the C₃–C₄ intermediate species (*M. arvensis*, *M. suffruticosa*, *M. niten*, *M. sinaica*, *M. spinosa*; Fig. 1A), indicating that the evolution of the C₃–C₄ intermediate character is most parsimoniously explained by a single event in the *Moricandia* genus. Intermediates with smaller, more ellipse-shaped leaves (group I, Fig. 1A; Supplementary Fig. S1) formed a monophyletic group, while the intermediates with mainly longer petioles formed a paraphyletic clade (group II). Lines taxonomically assigned to *M. arvensis* could be found within both groups.

Besides the *Moricandia* C₃–C₄ species, very similar features, such as significantly reduced CO₂ compensation points, occur in *Diplotaxis tenuifolia* and *Brassica graveina* (Apel et al., 1997; Ueno, 2003, 2011). The development of C₃–C₄ intermediacy in these species was clearly independent from the events in *Moricandia* (Fig. 1A). Phylogenetic trees of the Brassicaceae with higher species density (Warwick and Sauder, 2005; Arias et al., 2014) suggest that these species belong to different branches of the tree and that C₃–C₄ intermediacy also evolved independently in *D. tenuifolia* and *B. graveina*. In the list of Apel et al. (1997), single measurements of CO₂ compensation points in *D. muralis* and *D. erucoides* also indicated low CO₂ compensation points. *Diplotaxis muralis* is a hybrid between *D. viminea* (C₃) and *D. tenuifolia* (C₃–C₄) and usually closer to C₃ in its gas exchange characteristics (Ueno et al., 2006), but detailed studies are lacking for *D. erucoides*. It is also remarkable that C₃–C₄ intermediates were only found in the Oleracea group of the Brassicaceae tribe, in the lineage II Brassicaceae (Apel et al., 1997; Warwick and Sauder, 2005; Arias et al., 2014), but no C₃–C₄ intermediates have so far been identified in any other subgroup of this large family.
from mature leaves indicated that PEPC did not play a major role in the primary fixation of CO₂ in C₃-C₄ intermediate *Moricandia* species (Fig. 2G). No significant differences could be observed in the carbon isotope ratio of the C₃ and C₃-C₄ intermediate species (Fig. 2E). The protein content per total dry weight tended to be lower in the intermediates as compared to the related C₃ species *M. moricandioides*; however, these results were only significant for one C₃-C₄ line, *M. arvensis* (Fig. 2F).

**Differences in CO₂ compensation points are connected to anatomical changes**

The development of C₃-C₄ intermediate physiology relies on functional specification of metabolism in the MS and BS cells and an increase in the metabolite exchange between the two cell types. Differences among photosynthetic types were therefore expected to be closely connected to changes in the anatomy, which were evaluated on a histological and ultrastructural level.

Measurements of vein length per area revealed that the C₃-C₄ *Moricandia* species had lower or equal vein density when compared to the C₃ relative (Fig. 2H). In the top view of cleared leaves, veins of C₃-C₄ intermediate species appeared thicker than in the C₃ species (Fig. 3A–C), probably connected to the large number of chloroplasts, which were centripetally arranged around the veins in the leaf cross-sections (Fig. 3D–I). In-depth ultrastructural analysis showed a high number of mitochondria located predominantly in the cytoplasm between the chloroplasts and the cell wall of adjacent cells of the vascular tissue of C₃-C₄ plants (Fig. 3J–L). The C₃ species *M. moricandioides* had eight cell layers, while

![Fig. 2. Physiological features in the C₃ species *M. moricandioides*, and the C₃-C₄ intermediate species *M. suffruticosa* and *M. arvensis* line MOR1.](image-url)
M. suffruticosa and M. arvensis both had one MS cell layer fewer (Supplementary Table S4, Fig. 3D–F). Such a reduction in the MS tissue alone could be responsible for a shift in the leaf cell profile towards a higher proportion of BS cells (McKown and Dengler, 2007).

**Specific metabolite pattern in C$_3$–C$_4$ intermediates**

The metabolite pattern of the leaves is expected to be influenced by species-specific differences as well as by the photosynthesis type. The overall metabolite patterns in *M. moricandioides*, *M. suffruticosa*, and *M. arvensis* were first assessed by principal component analysis (PCA) (see Supplementary Fig. S2A). In the first principal component (PC1), samples from the C$_3$ species *M. moricandioides* localised predominantly to the right, next to samples of C$_3$–C$_4$ intermediate species. PC2 mainly separated the two C$_3$–C$_4$ intermediate species. The samples from both C$_3$–C$_4$ intermediates were also characterised by high variation. Three metabolites showed significantly ($P<0.01$) different concentrations in all tested comparisons (*M. arvensis* vs *M. moricandioides*, *M. suffruticosa* vs *M. moricandioides*, and *M. suffruticosa* vs *M. arvensis*): maleic acid, serine, and threonine (Supplementary Fig. S2B). To distinguish between C$_3$ and C$_3$–C$_4$ intermediate species, we screened...
for metabolites that significantly differed between the C$_3$ and the two C$_3$–C$_4$ species, but not between the two intermediates. Among the nine metabolites in this category were alanine, glycine, GABA, gluconic acid, leucine, malate, malonic acid, and valine (Supplementary Fig. S2B, Supplementary Table S4).

The predicted N shuttle metabolites (Mallmann et al., 2014), glutamate, alanine, and malate, had higher concentrations in both intermediate Moricandia lines. Aspartate was only increased in M. suffruticosa (Fig. 4). Glycolate and glycerate are part of the photorespiratory pathway, but no significant differences in the concentration of these two metabolites were detected in leaves of the C$_3$ and C$_3$–C$_4$ Moricandia species (Fig. 4), confirming that these metabolites do not play a major role in coordination of metabolism between MS and BS cells.

Transcript patterns do not show a strong specific C$_3$–C$_4$ signature

Next-generation sequencing allows an analysis and comparison of the transcriptomes of species for which no reference genome is available by mapping the reads against the minimal transcriptome of Arabidopsis thaliana (Brautigam et al., 2011). In all Moricandia samples, between 79% and 86% reads were mapped, which is higher than in previous work with Asterales species (Gowik et al., 2011). PCA showed that the transcript pattern was most prominently influenced by the species (see Supplementary Fig. S3A). PC1 clearly separated samples from M. moricandioides, M. arvensis, and M. suffruticosa, while PC2 only separated M. moricandioides and M. arvensis from M. suffruticosa (Supplementary Fig. S3A). Subsequent PCs were already influenced by replicate-specific differences. An influence of the photosynthesis type was not detected in the first three PCs (Supplementary Fig. S3B).

The abundance of 1671 transcripts was significantly different in the C$_3$ and the C$_3$–C$_4$ intermediate leaves, but not between the two intermediates. All of these had changed in both C$_3$–C$_4$ intermediate species to the same direction: 797 were commonly enhanced in C$_3$–C$_4$ while 874 were commonly reduced in C$_3$–C$_4$ (Supplementary Fig. S3C, Supplementary Table S5). GO term analysis of both groups of transcripts revealed quite diverse categories. Only two GO terms were enriched among transcripts enhanced in C$_3$–C$_4$, while 21 process GO-terms were enriched in transcripts reduced in C$_3$–C$_4$. The latter included high-level terms such as cellular compound, nitrogen metabolism, and carbohydrate metabolism. The cellular compartment mainly affected by transcript reduction in C$_3$–C$_4$ intermediates appeared to be the chloroplast (see Supplementary Table S6).

The metabolism in C$_3$–C$_4$ intermediate leaves is predicted to differ from C$_3$ leaves mainly with respect to cellular distribution of photorepiratory processes and subsequent re-adjustment of C and N balance by metabolite shuttle mechanisms (Mallmann et al., 2014). Transcripts predicted to be involved in all these processes were therefore studied in more detail. No changes were observed for transcripts encoding components of the photorespiratory pathway, in particular the GLDP protein, which is only present in the BS cell of C$_3$–C$_4$ intermediates (Fig. 5). Not all transcripts of the Calvin–Benson–Bassham (CBB) cycle did show significant differences between C$_3$–C$_4$, and the C$_3$ species as determined by a t-test (P≤0.01). MMori, M. moricandioides; Marv, M. arvensis line MOR1; Msuff, M. suffruticosa. (This figure is available in colour at JXB online.)

Figure 4. Selected metabolites in the C$_3$ species M. moricandioides, and the C$_3$–C$_4$ intermediate species M. suffruticosa and M. arvensis line MOR1. The box-whisker plots represent summaries of 10–12 biological replicates. The asterisks indicate significant differences between the C$_3$–C$_4$ and the C$_3$ species as determined by a t-test (P≤0.01). MMori, M. moricandioides; Marv, M. arvensis line MOR1; Msuff, M. suffruticosa. (This figure is available in colour at JXB online.)
(Brautigam et al., 2011). The evolutionary trajectory of pathway expression from C₃ via C₃-₄ to C₄ metabolism can be followed in *Flaveria* species and compared to the results from *Moricandia*. No large changes in the average transcript abundance of the photorespiratory pathway were detected in the intermediate *Flaveria* species. The abundance of photorespiratory transcripts decreased only in the C₄-like *F. brownii* and then even more in the C₄ species (Fig. 6). The same pattern was also observed for the CBB cycle. A decrease of CBB cycle transcripts in intermediates without increased C₄ cycle activity, as we found in the two investigated *Moricandia* species, was not observed in *Flaveria* (Fig. 6).

Transcripts encoding enzymes with potential functions in the metabolite shuttles between MS and BS cells were inspected in detail. Only the aspartate aminotransferase (*AspAT*) encoding genes were enhanced in both intermediates compared to the C₃ species (Fig. 5). Furthermore, transcripts that could potentially be recruited into a C₄ cycle were tested for their pattern (Fig. 5). In *Flaveria*, basic C₃–C₄ intermediates were characterised by increases in alanine aminotransferase (*AlaAT*) and NADP-malic enzyme (*NADP-ME*) transcripts, in the evolutionary series this was followed by increases in *PEPC* transcripts, which were enhanced in all but one of the basic *Flaveria* intermediates (*F. chloraeofolia*; Fig. 6). In C₃–C₄ intermediate *Moricandia* species, *AlaAT* transcripts were not enhanced, and only slight increases in *NADP-ME* transcripts were observed (Fig. 5). Another potential C₄ decarboxylating enzyme, *PEPCK*, showed enhanced transcript abundance in the C₃–C₄ *Moricandia* intermediates (Fig. 5; Supplementary Table S7). The same was true for the *PPDK* transcripts. Two *PEPC* transcripts with C₃-like characteristics (Paulus et al., 2013) were found in noticeable amounts in *Moricandia* leaves. The higher-abundant form was actually reduced compared to the C₃ transcriptome, and only the lower-abundant form was enhanced in the intermediates (Fig. 5; Supplementary Table S7).

The GLD/SHMT reaction in the BS also produces NADH and this may require adjustments of the redox balance in the cells. Strong increases were observed for transcripts encoding alternative oxidases (*AOX*) in the C₃–C₄ intermediates. The same tendency was found for the uncoupling protein (*UCP*) and the dicarboxylate transporter *DIC2* (see Supplementary Table S7). A NADH dehydrogenase transcript on the other hand was reduced in the C₃–C₄ leaves (Fig. 5). Increases in *AOX* and *UCP* transcripts were not unique to *Moricandia*, but were also observed in the C₃–C₄ *Flaveria* intermediates. In the C₃-like and C₄ *Flaveria* species, the *AOX* and *UCP* transcripts decreased again compared to the C₃ plant. Hence the increase in *AOX* transcript abundance was a common feature in C₃–C₄ intermediate *Moricandia* and *Flaveria* species (Figs 5 and 6).

**Moricandia** species possess only a single copy of the GLDP gene

The number of GLDP copies and their phylogenetic relationship was investigated in the Brassicaceae. Only species where full genome information was available were considered for the comparison. *Arabidopsis thaliana* has two copies of the gene, *AtGLDP1* and *AtGLDP2* (Engel et al., 2007). Comparison with other Brassicaceae revealed that other species from the Brassicaceae lineage I (*Arabidopsis lyrata, Camelina sativa, Capsella rubella*) as well as species from the extended lineage II (*Eutrema salsugineum, Thellungiella halophila*) also possess transcripts with high similarity to both *Arabidopsis* genes (Fig. 7). From *Arabis alpina* only the *AtGLDP2*-like gene was identified. In the *Brassica* species *B. rapa, B. oleracea*, and *B. napus* on the
other hand, only AtGLDP1-like copies were found (Fig. 7). Transcriptomes from mature leaves of *M. moricandioides*, *M. arvensis*, *M. suffruticosa*, *Dipoltaxis tenuifolia*, *D. viminea*, and *D. muralis* also assembled only copies with high similarity to the AtGLDP1 gene. In all these species, GLDP was represented by one unique transcript. Two transcripts were assembled only in *D. muralis*, one with a high similarity to the sequence of *D. tenuifolia* and the other one with high similarity to the *D. viminea* sequence. Since *D. muralis* is a hybrid between these two species, this was expected and underlines the successful assembly of the GLDP gene sequences in our study. An assembly of gene sequences from a leaf transcriptome could still miss copies that are simply not expressed at all in the leaves examined. The complete absence of AtGLDP2-like sequences also in the genome of the sequenced *Brassica* species, however, indicated that the AtGLDP2 copy was absent in the whole Brassicaceae subgroup containing *Brassica*, *Moricandia*, and *Dipoltaxis* species, most likely by loss at the base of Brassicaceae subgroup.

**Discussion**

Evolution from C₃ to C₃–C₄ was promoted by lack of a second GLDP copy in the Brassicaceae

Evolution of C₄ photosynthesis is not equally distributed across the plant kingdom, being frequent in some groups but completely absent from many others with similar growth forms (Sage *et al.*, 2011a; Sage, 2016). In the Brassicaceae we find no true C₄, but possibly three independent C₃–C₄ lines (*Moricandia*, *Dipoltaxis tenuifolia*, *Brassica graminifolia*), and all these lines belong to the Oleracea group of the Brassicaceae (Warwick and Sauder, 2005; Arias *et al.*, 2014).

The decisive step from C₃ to C₃–C₄ intermediacy is associated with a shift of the activity of photorespiratory GLD from ubiquitous expression to almost exclusive expression in the BS (Heckmann *et al.*, 2013; Khoshravesh *et al.*, 2016). Detailed studies of the promoter sequences of the GLDP in *Flaveria* have shown that the regulatory elements mediating BS-specific expression were already present in the C₃ ancestors (Schulze *et al.*, 2013). *Flaveria* species possess two
copies of the GLDP gene: one is ubiquitously expressed in the leaf tissue (GLDPB), while transcripts of the other one were found exclusively in the BS (GLDPA). The transition from C\(_3\) to C\(_3\)–C\(_4\) photosynthesis in \textit{Flaveria} was then realised via a gradual decrease and finally pseudogenization of the ubiquitously expressed copy and exclusive expression of the BS-specific gene (Schulze \textit{et al.}, 2013).

\textit{Arabidopsis thaliana} belongs to the lineage I of the Brassicaceae and also possesses two copies of the GLDP gene, \textit{AtGLDP1} and \textit{AtGLDP2}, and both are abundantly expressed in leaf tissue (Engel \textit{et al.}, 2007). Orthologs of the genes were also detected in species from the same lineage and also in the extended lineage II of Brassicaceae. Only in the Brassicaceae subgroup, which includes all known C\(_3\)–C\(_4\) Brassicaceae species, was the \textit{AtGLDP2}-like copy missing (Fig. 7). The step from \textit{C\(_3\)} to \textit{C\(_3\)}–\textit{C\(_4\)} photosynthesis in the Brassicaceae was apparently facilitated by loss of the \textit{GLDP2} copy. Analysis of the promoter region from the \textit{AtGLDP1} gene revealed the presence of an MS (M-box) as well as a BS/vein (V-box) -specific element and these were highly conserved throughout the Brassicaceae family (Adwy \textit{et al.}, 2015). Changes in the M-box of the promoter could therefore easily lead to loss of gene function in the mesophyll, and without a second copy of the gene, BS-specific \textit{GLDP} expression typical for the \textit{C\(_3\)}–\textit{C\(_4\)} species could be realised, driven by the V-box. This scenario is supported by the absence of the M-box but the conserved presence of the V-box in the \textit{GLDP} promoter of the \textit{C\(_3\)}–\textit{C\(_4\)} species \textit{Moricandia nitens} (Zhang \textit{et al.}, 2004; Adwy \textit{et al.}, 2015). In \textit{Flaveria}, it has been hypothesised that a gradual decrease of MS \textit{GLDP} expression might have been crucial for the adjustment of intercellular metabolism (Schulze \textit{et al.}, 2013). It will be interesting to investigate whether single nucleotide changes are sufficient to completely disrupt the function of the Brassicaceae M-box.

\textit{C\(_3\)}–\textit{C\(_4\)} characteristics are stable in different \textit{Moricandia} species

\textit{Moricandia} species had been characterised for their specific physiological, anatomical, and biochemical properties (Bauwe and Apel, 1979; Rawsthorne \textit{et al.}, 1988a, b; Brown and Hattersley, 1989), but direct comparisons of the results from the different laboratories could be problematic because the features investigated might vary among different accessions of the same species (Sayre and Kennedy, 1977). We therefore tested CO\(_2\) compensation points and phylogenetic relationships between
one C₃ *M. moricandioides* line and eight different C₃–C₄ intermediate lines. In the phylogenetic tree, all C₃-C₄ intermediates of *Moricandia* formed a monophyletic clade, indicating that the evolution of C₃–C₄ photosynthesis probably occurred once, with subsequent speciation (Fig. 1A). The CO₂ compensation points of all the C₃-C₄ lines tested were significantly lower than in C₃ relatives and higher than in the C₄ species *G. gynandra*, but the lines did not differ amongst each other (Fig. 1B).

In the C₃-C₄ accessions *M. arvensis* line MOR1 and *M. sufruticosa*, the reduction in CO₂ compensation point was closely associated with an increase in organelle number and their centripetal arrangement in the BS cells of the mature leaf (Fig. 3). A very similar picture has been described for the C₃-C₄ species *Moricandia spinosa* (Brown and Hattersley, 1989) and *M. arvensis* (Holaday et al., 1981), as well as C₃-C₄ species from other plant families (McKown and Dengler, 2007; Muhaidat et al., 2011; Khoshravesh et al., 2016). BS cells in the C₃-C₄ intermediates are still in direct contact with the intercellular space and CO₂ can diffuse in and out of the cell. Therefore, the efficiency of the glycine shuttle and re-fixation of the released CO₂ depends on the close arrangement of the mitochondria, where CO₂ is released, and the chloroplasts, where the carboxylation reaction of Rubisco can profit from the proximate increase in CO₂ concentration.

After establishment of the photosynthetic carbon shuttle, further fitness gains are predicted by support of the glycine shuttle by C₄ acids, which serve as carbon backbones for re-assimilation of photosynthetic ammonia (Heckmann et al., 2013; Mallmann et al., 2014). The majority of *Flaveria* C₃-C₄ intermediates are characterised by enhanced PEPC activity and a limited C₄ cycle (Vogan and Sage, 2011; Mallmann et al., 2014). In C₃-C₄ intermediate *Moricandia* species, the PEPC activity was not changed compared to the C₃ species (Fig. 2G). Earlier measurements of PEPC activity in *M. arvensis* showed a slight two-fold increase in comparison to the C₃ species *M. foetida* (Holaday et al., 1981). However, ¹⁴C labelling experiments gave no further evidence for a significant contribution of C₄ acids to the photosynthetic carbon assimilation (Holaday and Chollet, 1983; Hunt et al., 1987). Values for δ¹³C, which would indicate a substantial contribution of PEPC to primary CO₂ fixation, were also indistinguishable between C₃ and C₃-C₄ *Moricandia* species. In *Flaveria*, the installation of the glycine shuttle led to implementation of different degrees of the C₄ cycle, including true C₄ photosynthesis, but in the *Moricandia* species analysed similar developments were not accompanied by a substantial C₄ pathway contribution.

**C₃–C₄ specific metabolism influences metabolite but not transcript patterns in Moricandia**

The absence of GLD is thought to induce enhanced glycine concentration in the MS, followed by diffusion of the metabolite to the BS (Rawsthorne and Hylton, 1991). As expected, an increase in the glycine concentration was detectable in total leaf extracts from *M. arvensis* and *M. sufruticosa* (Fig. 4). Serine, as the direct product of the GLD/SHMT reaction, is most likely one of the metabolites transported back to the MS cell. Just like glycine, its steady-state pool is typically increased in C₃-C₄ intermediate leaf extracts and it is characterised by a high turnover rate in the illuminated leaf (Fig. 4; Rawsthorne and Hylton, 1991). The pools of metabolites suggested to maintain N-balance (e.g. glutamate, alanine, malate) were also increased in the C₃-C₄ *Moricandia* lines when compared to the C₃ relative species; only aspartate was enhanced in just one intermediate line. A strong preference for one of the suggested shuttle mechanisms could, however, be detected. The results tend to suggest that many shuttles contribute to the metabolic balancing between the cells (Fig. 8), and it is very well possible that further metabolites are involved.

In contrast to the metabolite patterns, a C₃-C₄-related transcript pattern could not be detected in the PCA of *Moricandia* transcriptomes. The partly random enrichment of GO-terms in the commonly up- and down-regulated transcripts also suggested that species-specific differences had a major influence on the transcript pattern. The results differed considerably from the picture obtained for comparisons of transcriptomes from C₃ and C₄ species (Brautigam et al., 2011, 2014; Gowik et al., 2011), which were always characterised by a very strong C₄ signature with very high abundance of all transcripts encoding the C₄ photosynthesis proteins, including PEPC, PPDK, ME, NADP-MDH, AspAT, and adenosine monophosphate kinase (Brautigam et al., 2014). Changes in the *Moricandia* lines were on a much smaller scale, but some transcripts encoding enzymes associated with C₄ metabolism, such as PPDK, PEPC, a plastidic NADP-MDH, a cytosolic NAD-MDH, and three copies of AspAT, were also enhanced in both C₃-C₄ intermediate species. The same was true for one PEPC copy (Supplementary Table S5).

In *Flaveria*, only *F. chorifoliae* displayed a similar level of C₃–C₄ metabolism as the *Moricandia* species tested, with no significant contribution of PEPC and the C₄ cycle. However, even this basic C₃–C₄ intermediate species showed increases in transcript abundance of AlaAT and NADP-ME, and these changes were associated with the operation of the N-balancing shuttle mechanisms. The fact that the transcript changes in the C₃–C₄ intermediate *Moricandias* were usually moderate or small compared with true C₄ species supported the hypothesis that there was not one main shuttle operating. Not all steps predicted in the model shown in Fig. 8 were accompanied by increases in transcript levels. This might not be necessary because the required enzymes are not only present in a C₃ background (Aubry et al., 2011), but also of high enough abundance. It is furthermore possible that transcripts did not change in their general abundance between C₃ and C₃–C₄ species, but that they were affected in their post-transcriptional regulation or cellular distribution instead. Overall, metabolite as well as transcript patterns indicated that the N metabolism between MS and BS was adjusted by multiple pathways.

**Redox balance requires transcriptional changes**

When the GLD reaction is shifted to the BS, it increases not only the release of CO₂ and NH₃ in the BS mitochondria,
but also produces high amounts of NADH (Fig. 8). In *Moricandia*, three AOX and one UCP transcripts were more abundant in the leaves of C₃–C₄ intermediate species than in the C₃ relative, suggesting that re-balancing the redox metabolism of the mitochondria is supported by alternative electron transport. Increases in AOX are usually found under stress, but AOX expression is also affected in photorespiratory mutants (Voss et al., 2013). The association of enhanced alternative electron transport in C₃–C₄ photosynthesis could be verified by comparisons with the transcript patterns in different *Flaveria* species. Increases in AOX transcripts were also present in the intermediate species but they returned to C₃ levels in the C₄ species (Fig. 6), indicating that redox balance is harmonised again after full implementation of the C₄ cycle. This model predicts the irretrievable loss of the mitochondrial NADH transported by the photorespiratory pump.

Fig. 8. Model of metabolite shuttle network active between the mesophyll cells (left) and bundle sheath cells (right) of C₃–C₄ intermediate *Moricandia* species. The inactivity of the photorespiratory glycine decarboxylating complex in the mesophyll cells leads to glycine accumulation and transport to the bundle sheath. In the mitochondria of bundle sheath cells two molecules of glycine are converted by the GLD/SHMT complex to serine, CO₂, NH₃, and NADH. In the adjacent chloroplasts the bundle sheath Rubisco is exposed to enhanced CO₂ conditions. The imbalance created by parallel release of NH₃ requires further adjustment of C and N metabolism that is probably realised by a whole network of reactions, including additional shuffling of amino acids from the bundle sheath to the mesophyll (dark blue arrows), and re-shuttling of organic acids (light blue arrows). Enzymes highlighted in bold could be associated with an increased abundance of at least one transcript copy.

Anatomical and environmental constrains could be responsible for impeded evolution towards C₄ in *Moricandia*

In the model presented by Mallmann et al. (2014), the initial shift of the GLD in the C₃–C₄ intermediates promotes a smooth transition to C₄ by gradual enhancement of the C₄ cycle, but it does not provide a straightforward explanation why some species remain stuck in intermediacy. In *Moricandia*, the analysis of potential C₃ cycle genes indicated that they are expressed, albeit at low level, in the intermediates and are theoretically capable of forming a C₄ cycle. So possible reasons for abidence of *Moricandia* in the C₃–C₄ state could be lack of time or the absence of some genetic, anatomic, or environmental drivers for the transition to C₄ to take place (Mallmann et al., 2014; Heckmann, 2016).

Estimates of the time of split between C₃ and C₃–C₄ *Moricandia* are between 11 Ma (Fig. 1A) and 2 Ma (Arias et al., 2014). The same period was predicted to have passed since the separation of C₃ and C₃–C₄ intermediate *Diplotaxis* species (Fig. 1A). In *Flaveria*, one of the youngest lines evolving C₄ photosynthesis, C₃–C₄ metabolism is thought to have evolved about 3 Ma ago and, in at least one line, evolution to full C₄ photosynthesis was completed about 1–0.5 Ma ago (Christin et al., 2011a). Although accurate timing of these evolutionary events is difficult, the results indicate that evolution from C₃–C₄ to C₄ might have been generally possible in the 2–11 Ma that elapsed since the origin of C₃–C₄ in *Moricandia*, but it would probably depend on several beneficial pre-conditions. Stability of C₃–C₄ metabolism for several Ma has also been described for a second lineage in *Flaveria* (*F. sonorensis*; Christin et al., 2011a) and *Mollugo* (Christin et al., 2011b).

Environmental conditions promoting C₄ evolution can generally be associated with conditions of high photorespiration, such as high temperatures and C limitation of photosynthesis, which can be found in hot, open environments with water limitation and high salinity (Osborne and Sack, 2012; Brautigam and Gowik, 2016). In many habitats, nutrients other than carbon, for example bio-available nitrogen or phosphorus, restrict plant growth (Korner, 2015). The low carboxylation efficiency of *Moricandia* intermediates, as indicated by low initial slopes in the A–C₄ curves (Fig. 2C), point to low N-content in leaves (Sage et al., 1987), probably due to reduced levels of Rubisco and CBB cycle enzymes (Fig. 5). Lower leaf N-content was supported by higher C/N ratios and lower protein content per leaf dry-weight (Fig. 2). The advantages of intermediate *Moricandia* were thus probably limited to very low CO₂ partial pressures, as occur when stomata are closed due to water limitations, while C₃ *Moricandia* reached higher assimilation rates under ambient CO₂, as encountered
when stomata are open. The low leaf protein content pointed to an evolutionary history of adaptation to N-limited environments. A comparison with $A-C_i$ curves from other $C_3-C_4$ intermediate species showed that the phenotype is specific for Moricandia. In intermediate species of Heliotropium, the carboxylation efficiency of $C_3-C_4$ intermediates was slightly higher than in related $C_3$ species (Vogan and Sage, 2011), and the carboxylation efficiency presented for $C_3-C_4$ intermediate Flaveria species are also similar to the $C_3$ relatives (Dai et al., 1996). Expression of CBB cycle genes was only reduced in Flaveria species with at least $C_4$-like metabolism, while transcription remained comparable to $C_3$ species in all $C_3-C_4$ intermediates (Fig. 6; Mallmann et al., 2014). In both the $C_3-C_4$ intermediate Moricandia lines that were tested, on the other hand, the CBB cycle genes were already reduced at the basic intermediate state (Figs 5 and 6). Transcripts belonging to nitrogen as well as carbohydrate metabolism are enriched in the group of genes commonly reduced in the intermediate species. Thus, possibly both C and N limitation promoted the evolution of $C_3-C_4$ intermediacy in these species. Finally, Moricandia provided new insights into the importance of anatomic enablers not only for the transition from $C_3$ to $C_3-C_4$ but also for further evolution towards $C_4$. Activation of BS cells and high vein density are essential pre-conditions for establishment of an efficient $C_4$ cycle (Christin et al., 2013; Khoshravesh et al., 2016). The efficiency of the glycine shuttle and connected C- and N-balancing mechanisms depend on enhanced metabolite exchange between the MS and BS cells and are therefore also dependent on a limited distance between the two cell types. The importance of the narrow vein spacing increases with the increasing contribution of a $C_4$ cycle in advanced $C_3-C_4$ intermediates and finally through to full $C_4$ (McKown and Dengler, 2007). Plant families in which $C_4$ photosynthesis evolved such as Flaveria and Heliotropium are generally characterised by vein densities considerably higher than in Moricandia (McKown et al., 2005; Muhaidat et al., 2011). It is therefore possible that limited anatomical pre-conditions hampered evolution to $C_4$ in the Brassicaceae.

Conclusions

Current models suggest that after implementation of the photorespiratory CO$_2$ pump, re-balancing of N and C metabolism promotes further shuttle mechanisms involving $C_4$ metabolites between the MS and BS cells, and finally installation of highly efficient $C_4$ photosynthesis (Mallmann et al., 2014; Brautigam and Gowik, 2016). In Moricandia, the installation of a glycine shuttle was definitely successful, and they possessed BS-specific GLDP expression, low CO$_2$ compensation points, and BS cells with a high number of centripetally arranged organelles. The metabolite pattern also suggests the activity of additional metabolite shuttles in the intermediates leaves. Establishment of the $C_4$ cycle was apparently not hampered as the $C_3$ cycle genes were present and expressed. Thus far, the situation in Moricandia does not look very different from Flaveria, but while some Flaveria lines progressed to $C_4$, all Moricandia lines remained at the basic intermediate state. Lack of progression to $C_4$ in the Brassicaceae could still be connected to chance, but our Moricandia data now provide evidence for possible constrains on the path to $C_4$, namely anatomical limitation of efficient metabolite exchange or insufficient evolutionary pressure due to limitation in nutrients other than carbon, i.e. nitrogen. In contrast to $C_3-C_4$ lines with $C_4$ relatives, intermediate Brassicaceae grow in colder climates (MR Lundgren and PA Christin, unpublished data), so pressure to reduce photorespiration might also be limited. In the end, limited environmental pressure and anatomical constrains might have led to metabolic balancing by multiple pathways rather that continued promotion of the $C_4$ cycle in Moricandia. The analyses of additional intermediates with no closely related $C_4$ species, especially with respect to their leaf architecture and N metabolism, will hopefully provide further glimpses into the evolution of intermediacy and of $C_4$.

Supplementary data

Supplementary data are available at JXB online.

Figure S1. Phenotypes of the tested Moricandia lines.

Figure S2. Statistical summary of Moricandia metabolite patterns.

Figure S3. Statistical summary of Moricandia transcript patterns.

Table S1. ITS sequences extracted from the NCBI database.

Table S2. Sequences for the glycine decarboxylase P-protein.

Table S3.: Protocol for combined conventional and micro-wave-proceeded fixation, dehyration, and resin embedding of Moricandia leaf sections for histological and ultrastructural analysis.

Table S4. Summary of metabolite, element, gas exchange, anatomy, and protein measurements.

Table S5. Transcripts significantly different in abundance between the three Moricandia species (M. moricandioides, M. arvensis line MOR1 and M. suffructicosus).

Table S6. GO-terms enriched in commonly regulated transcripts in the comparison between $C_3-C_4$ and $C_3$ Moricandia species, but not different between the two $C_3-C_4$ Moricandia species.

Table S7. Changes in abundance of transcripts belonging to selected pathways.

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