Annals of Botany **93**: 13–23, 2004
doi:10.1093/aob/mch003, available online at www.aob.oupjournals.org

INVITED REVIEW

Evolution of C4 Phosphoenolpyruvate Carboxylase. Genes and Proteins: a Case Study with the Genus *Flaveria*

PETER WESTHOFF* and UDO GOWIK

Institut für Entwicklungs- und Molekularbiologie der Pflanzen, Heinrich-Heine-Universität, Universitätsstraße 1, D-40225 Düsseldorf, Germany

Received: 6 August 2003 Returned for revision: 8 September 2003 Accepted: 16 September 2003 Published electronically: 26 November 2003

C4 photosynthesis is characterized by a division of labour between two different photosynthetic cell types, mesophyll and bundle-sheath cells. Relying on phosphoenolpyruvate carboxylase (PEPC) as the primary carboxylase in the mesophyll cells a CO_2 pump is established in C4 plants that concentrates CO_2 at the site of ribulose 1,5-bisphosphate carboxylase/oxygenase in the bundle-sheath cells. The C4 photosynthetic pathway evolved polyphyletically implying that the genes encoding the C4 PEPC originated from non-photosynthetic PEPC progenitor genes that were already present in the C3 ancestral species. The dicot genus *Flaveria* (Asteraceae) is a unique system in which to investigate the molcular changes that had to occur in order to adapt a C3 ancestral PEPC gene to the special conditions of C4 photosynthesis. *Flaveria* contains not only C3 and C4 species but also a large number of C3–C4 intermediates which vary to the degree in which C4 photosynthetic traits are expressed. The C4 PEPC gene of *Flaveria trinervia*, which is encoded by the *ppcA* gene class, is highly expressed but only in mesophyll cells. The encoded PEPC protein possesses the typical kinetic and regulatory features of a C4-type PEPC. The orthologous *ppcA* gene of the C3 species *Flaveria pringlei* encodes a typical non-photosynthetic, C3-type PEPC and is weakly expressed with no apparent cell or organ specificity. PEPCs of the *ppcA* type have been detected also in C3–C4 intermediate *Flaveria* species. These orthologous PEPCs have been used to determine the molecular basis for C4 enzyme characteristics and to understand their evolution. Comparative and functional analyses of the *ppcA* promoters from *F. trinervia* and *F. pringlei* make it possible to identity the *cis*-regulatory sequences for mesophyll-specific gene expression and to search for the corresponding *trans*-regulatory factors.

Key words: Phosphoenolpyruvate carboxylase, C4 photosynthesis, evolution, *Flaveria*.

INTRODUCTION

The C4 photosynthetic carbon cycle is a sophisticated addition to the C3 photosynthetic pathway and enables C4 plants to cope well with high light intensities, high temperatures and dryness. For this reason, C4 plants dominate grassland floras and biomass production in the warmer climates of the tropical and subtropical regions. It is no surprise that C4 plants, such as sugar cane, maize and sorghum are among the most productive crops in agriculture (Brown, 1999; Sage, 1999).

The high photosynthetic capacity of C4 plants is due to their unique mode of carbon assimilation which involves two different photosynthetic cell types, mesophyll and bundle-sheath cells. CO_2 is initially fixed by phosphoenolpyruvate carboxylase (PEPC) in the mesophyll cells into the C4 acids malate and/or aspartate, which are then transported to the bundle-sheath. In the bundle-sheath cells the C4 acids are decarboxylated, and the CO_2 is refixed by ribulose 1,5-bisphosphate carboxylase/oxygenase (Hatch, 1987).

As a consequence of this CO_2 concentration at the site of ribulose 1,5-bisphosphate carboxylase/oxygenase, the competitive inhibition of this enzyme by oxygen, which becomes prominent at higher temperatures, is largely excluded and C4 plants show drastically reduced rates of photorespiration. In C3 plants this process may be responsible for a reduction in photosynthesis of up to 40 % (Ehleringer and Monson, 1993). The CO_2 pump ensures high rates of photosynthesis even when CO_2 concentrations are low in the intercellular air spaces of the leaf. With this, C4 plants are able to limit the opening of their stomata and thereby minimize water loss due to transpiration. As the CO_2 pump delivers saturating concentrations of CO_2 to the site of ribulose 1,5-bisphosphate carboxylase/oxygenase high photosynthetic rates are maintained with less enzyme than is required in C3 species. This is reflected in a higher nitrogen use efficiency (Long, 1999).

The functioning of C4 photosynthesis depends upon strict compartmentation of the CO_2 assimilatory enzymes into the photosynthetic cell types, mesophyll and bundle sheath cells. The primary carboxylating enzyme, phosphoenolpyruvate carboxylase, occurs exclusively in the mesophyll cells while the secondary carboxylase, ribulose 1,5-bisphosphate carboxylase/oxygenase, and the decarboxylating enzymes NADP-dependent malic enzyme, NAD-dependent malic enzyme or phosphoenolpyruvate carboxykinase are restricted to the bundle-sheath cells (Hatch, 1987; Kanai and Edwards, 1999). Enzymes of the photorespiratory C2 carbon cycle (Baldy and Cavalié, 1984), the nitrogen (Rathnam and Edwards, 1976) and sulphur (Schmutz and Brunold, 1984) assimilation pathways and of carbohydrate

* For correspondence. E-mail west@uni-duesseldorf.de

metabolism (Lunn and Hatch, 1995) also accumulate differentially in mesophyll and bundle-sheath cells. Even the photosynthetic electron transport chains of mesophyll and bundle sheath chloroplasts of the NADP-malic enzyme type of C4 plants differ. While thylakoid membranes of mesophyll chloroplasts possess a fully developed linear electron transport chain, those of the bundle-sheath chloroplasts are devoid of grana and severely depleted in photosystem II (Woo *et al*., 1970; Meierhoff and Westhoff, 1993). These facts demonstrate that the C4 mode of photosynthesis involves a restructuring of the entire metabolic pathway of the mesophyll and bundle-sheath cells and leads to a single, highly integrative metabolic system.

The vast majority of C4 plants uses this two-cell mode, i.e. Kranz anatomy, to concentrate CO_2 at the site of ribulose 1,5-bisphosphate carboxylase/oxygenase. However, there are plants where inducible or permanent CO_2 pumps have been established within a single cell (Holaday and Bowes, 1980; Freitag and Stichler, 2000, 2002). These one-cell types of C4 photosynthesis are rare exceptions which apparently evolved only under very special environmental conditions.

The division of labour between mesophyll and bundle-sheath cells is governed by differential gene expression. It is known that this differential expression of the genes encoding the C4 cycle enzyme is largely due to transcriptional control (Dengler and Nelson, 1999; Sheen, 1999). Differential expression has also been reported for nuclear as well as plastid-encoded genes for photosystem II proteins (Sheen *et al*., 1987; Kubicki *et al*., 1994). Other genes are expressed differentially in C4 photosynthesis too (Wyrich *et al*., 1998; Furumoto *et al*., 2000; Renné *et al*., 2003) but the extent has still to be elucidated.

All gene expression data indicate that the two cell types follow distinct but complementary differentiation pathways. To date it is not known how this interdependence of mesophyll and bundle sheath cells is achieved during leaf development. Cell-lineage analyses indicate that the two cells do not necessarily depend on a single clonal relationship. The available data suggest that positional information and light play important roles in determining the differentiation of mesophyll and bundle sheath cells (Dengler and Nelson, 1999).

C4 plants occur in at least 18 families of mono- and dicotyledonous plants (Sage *et al*., 1999). These families are phylogenetically quite separate from each other. This indicates that C4 plants must have evolved several times independently from C3 ancestors during the evolution of angiosperms. There is strong evidence that even within a single taxon, for instance the Gramineae, this transition from C3 to C4 may have occurred more than once (Kellogg, 1999; Monson, 1999). The multiple independent origin of C4 photosynthesis suggests that the evolution of a C3 into a C4 species must have been relatively easy in genetic terms and that just a few master genes, if any, would have been involved. The available molecular data on the C4 cycle enzymes support this point of view. None of the C4 enzymes are unique to C4 plants. Non-photosynthetic isoforms of these enzymes are also present in C3 species and in the non-photosynthetic tissues of C4 species. The ubiquity of these non-photosynthetic isoforms of the C4 cycle enzymes in C3 plants strongly indicates that these ‘C3 isoforms’ served as the starting point for the evolution of the C4 genes (Monson, 1999).

At least three major changes must have occurred during the evolution to adapt the C4 progenitor gene for its function in C4 photosynthesis. First, C4 isoform genes are highly expressed (Harpster and Taylor, 1986; Hermans and Westhoff, 1990; Crétin *et al*., 1991) but C3 isoform genes are only moderately transcribed (Crétin *et al*., 1991; Kawamura *et al*., 1992; Ernst and Westhoff, 1996). The effectiveness of gene expression had therefore to be increased. Secondly, the C4 isoform genes had to evolve an organ- and cell-specific expression pattern, because strict compartmentation of the C4 enzymes is imperative for proper functioning of the C4 cycle (Hatch, 1987). Thirdly, it is known, at least for PEPC, that the C4 cycle enzymes differ from their C3 counterparts in kinetic and regulatory characteristics (Ting and Osmond, 1973*a*; Svensson *et al*., 2003). Therefore, the coding regions had to be changed to achieve the required adaptations of the enzymatic properties.

To gain insight into the evolution of C4 genes the entry enzyme of the C4 cycle, PEPC is being used as our model C4 enzyme/gene and the genus *Flaveria* (Asteraceae; Powell, 1978) as our experimental system. *Flaveria* contains C3 and C4 species and, in addition, a large number of C3–C4 photosynthetic intermediates (Edwards and Ku, 1987). These intermediates differ quantitatively in the expression of C4 photosynthetic traits, and there is convincing evidence that at least some of these are true evolutionary intermediates (Monson and Moore, 1989). Their presence suggests that evolution towards C4 photosynthesis is still continuing in this genus.

Phosphoenolpyruvate carboxylase was selected for this evolutionary analysis because the biochemistry and molecular biology of this enzyme have been studied intensively both in C4 and C3 plants (Rajagopalan *et al*., 1994; Chollet *et al*., 1996). PEPC catalyses the irreversible carboxylation of phosphoenolpyruvate (PEP) to form oxaloacetate. The enzyme needs Mg^{2+} as an essential cofactor and requires that the inorganic carbon be supplied as bicarbonate. Consequently, mesophyll cells of C4 plants contain high amounts of carbonic anhydrase to fulfil the demands of PEPC when the carbon flux through the C4 cycle is high (Badger and Price, 1994). Native PEPC is a tetramer comprising four identical subunits each with a molecular mass of about 100 000 Da (Kai *et al*., 2003). PEPC activity is regulated by metabolites (Kai *et al*., 2003), but also post-translationally by phosphorylation (Nimmo, 2003).

This brief review is summary of our work on the evolution of the enzymatic characteristics of C4 PEPC and of the transcriptional regulation of its gene in *Flaveria*.

THE GENUS *FLAVERIA* AND C4 PHOTOSYNTHESIS

The PEPC gene family

The photosynthetic and non-photosynthetic PEPCs of the C4 plant *F. trinervia* are encoded by a small gene family which consists of three distinct classes, named *ppcA*, *ppcB*

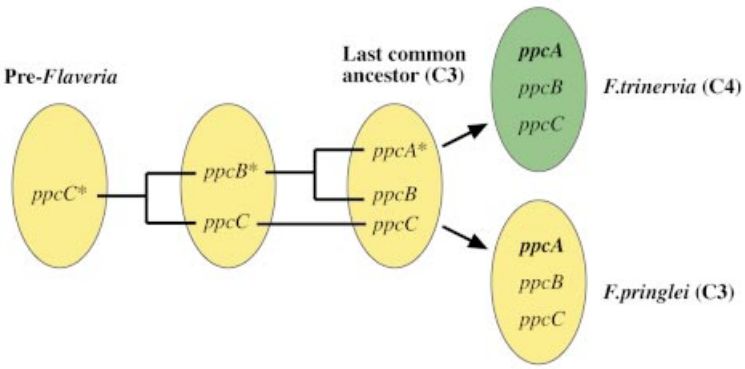

FIG. 1. Evolution of PEPC genes (*ppc*) in the genus *Flaveria*. Sequence analyses suggest that the *ppcA* gene class was formed by gene duplication of a *ppcB*-type PEPC gene (*ppcB**) already present in the last common ancestral C3 plant that gave rise to *F. pringlei* (C3) and *F. trinervia* (C4) (Bläsing *et al.*, 2002). The *ppcA* genes of *F. trinervia* (C4) encode the C4 PEPC of this species. The orthologous *ppcA* gene class of the closely related C3 species *F. pringlei* is used as a reference C3 gene for studying the evolution of C4 PEPC.

and *ppcC* (Fig. 1). The *ppcA* gene class contains two members and encodes the C4 isoform of PEPC (Poetsch *et al*., 1991; Hermans and Westhoff, 1992). The *ppcB* and *ppcC* gene classes probably consist of only one gene each and code for non-photosynthetic PEPC isoforms. The exact physiological roles of the *ppcB* and *ppcC* PEPCs have not been determined (Ernst and Westhoff, 1996). Phylogenetic analysis of cDNA sequences revealed that *ppcA* and *ppcB* genes are sister gene classes. This indicates that they were derived from an ancestral *ppcB*-like gene by gene duplication (Fig. 1; Bläsing *et al*., 2002). There may be a fourth gene class, named *ppcD*. However, the existence of this remains doubtful since neither genomic nor cDNA sequences have been analysed and no expression has been detected (Hermans and Westhoff, 1990).

The same three classes of *ppc* genes are also present in the C3 plant *F. pringlei* (Hermans and Westhoff, 1990, 1992). This indicates that the last common ancestor of *F. pringlei* and *F. trinervia* contained the same set of genes as those in present C3 and C4 flaverias (Fig. 1). The nearest neighbour to the *ppcA* genes of *F. trinervia* is the *ppcA* gene class of *F. pringlei*, which appears to be represented by a single copy gene (Hermans and Westhoff, 1990; Bläsing *et al*., 2002). The *ppcA* gene classes of *F. trinervia* and *F. pringlei* therefore represent evolutionary orthologues. If it is assumed that the *ppcA* gene of *F. pringlei* (C3) was similar, both in function and expression behaviour, to the *ppcA* gene of the last common C3 ancestor of present C3 and C4 *Flaveria* species, the *ppcA* gene class of *F. pringlei* provides the reference C3 gene that defines the starting point of C4 evolution.

The systematics of the genus Flaveria *and the evolution of C4 photosynthesis*

Based on the number of phyllaries, Powell (1978) divided the genus *Flaveria* into two major branches (Fig. 2A). The section with species that possess three to four phyllaries contains C3, C3–C4 and C4 species. Within this group are *F. trinervia* and *F. pringlei*, which serve as model C4 and C3 species of this genus, respectively. The three to four phyllaries branch also contains *F. bidentis* which is the only C4 *flaveria* that is amenable to genetic engineering by tissue-culture-based *Agrobacterium tumefaciens*-mediated transformation techniques (Chitty *et al*., 1994). The group with five to six phyllaries is composed of only C3–C4 intermediate species. Within this group is *F. brownii*, a C4-like species but with the expression of C4 photosynthesis dependent on environmental conditions, i.e. light intensity and growth temperature (Cheng *et al*., 1989). The group with five to six phyllaries also contains the C3–C4 intermediate species *F. pubescens* which is the only transformable C3–C4 intermediate *flaveria* (Chu *et al*., 1997).

Kopriva *et al*. (1996) proposed a molecular phylogeny of the genus *Flaveria* by using the H-protein of the glycine cleavage system as a gene marker. Their findings indicate that the group with five to six phyllaries forms a separate clade, thus confirming Powell's classification (Powell, 1978). Two to three thousand base pairs of *ppcA1* promoter sequences were isolated from the C4 plants *F. trinervia* and *F. bidentis*, the C4-like species *F. palmeri*, *F. vaginata* and *F. brownii*, the C4–C3-like species *F. pubescens*, *F. floridana, F. anomala* and *F. chloraefolia*, and the C3 plants *F. pringlei* and *F. cronquistii*. The overall structure of the promoters clearly separates the species with five to six phyllaries, i.e. *F. brownii*, *F. pubescens*, *F. floridana* and *F. chloraefolia* from the other *Flaveria* species analysed (U. Gowik and P. Westhoff, unpublished data). The 500 base pairs of proximal promoter sequences which can be aligned to each other without major gaps were used to construct a phylogenetic tree. The phylogram obtained supports the above classification and confirms that the species with five to six phyllaries form a distinct group (Fig. 2B). Based on these *ppcA* PEPC data one may propose that the evolution from C3 to C4 photosynthesis was initiated at least twice in this genus. However, to confirm this conclusion additional C4-related genes should be investigated at the phylogenetic level.

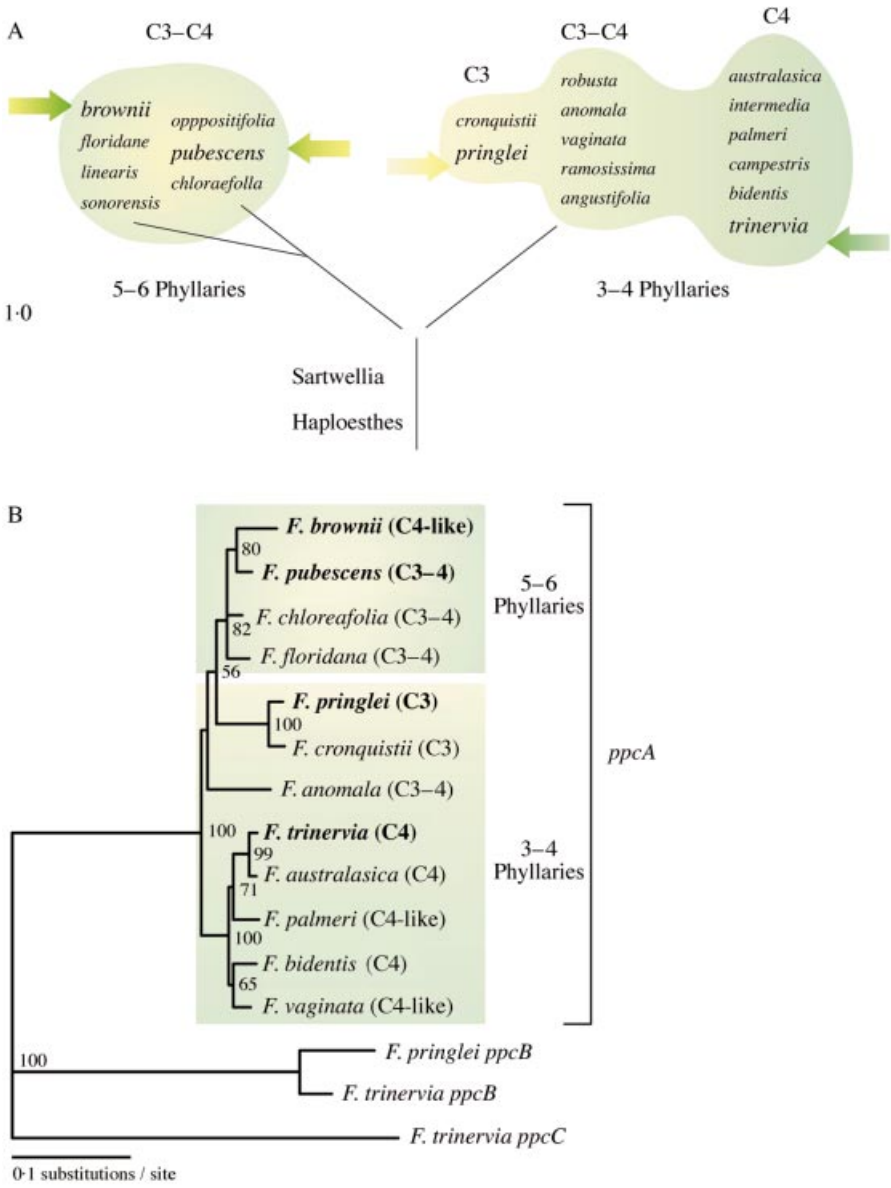

FIG. 2. Systematics and evolution of the genus *Flaveria*. A, Occurrence of C3, C3–C4 and C4 species. The figure is based on the systematics of the genus *Flaveria* as presented by Powell (1978). B, Phylogenetic tree of *Flaveria* species based on *ppcA1* promoter sequences. The 500 base pairs of proximal *ppcA1* promoter sequences were aligned using Clustal W (Thompson *et al.*, 1994). The phylogenetic tree was generated by the neighbour-joining method as implemented in PAUP 4·0 (Swofford, 2002).

EVOLUTION OF THE PEPC PROTEIN

Differences between C3 and C4 PEPCs

PEPC is positively and negatively controlled by its metabolic context. The enzyme is activated by hexose- and triose-phosphates and feedback inhibited by malate and aspartate (Fig. 3; Kai *et al.*, 2003). In addition, PEPC is regulated by phosphorylation at a specific serine residue near the amino terminus thereby increasing its general activity and decreasing its inhibition by malate (Nimmo, 2003).

The PEPCs involved in C4 photosynthesis differ from their non-photosynthetic isoforms in C3 plants (collectively named C3 PEPCs) in kinetic and regulatory properties. C4 PEPCs exhibit substrate saturation constants (K_m) for PEP that are usually about ten times larger than those of their C3 counterparts (Ting and Osmond, 1973*b*). On the other hand, the saturation constants for bicarbonate, the second substrate, are lower in C4 than C3 PEPCs (Bauwe, 1986). Finally, C4 PEPCs are more tolerant to malate when compared with the C3 PEPC isozymes (Dong *et al.*, 1998; Bläsing *et al.*, 2002).

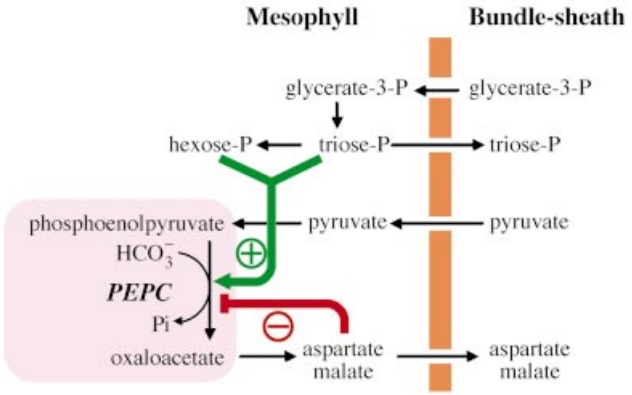

FIG. 3. The physiological context of C4 PEPC and its metabolic regulation. Metabolite products of photosynthesis, i.e. sugar phosphates, stimulate the enzyme and decrease K_m for the substrate PEP. Malate and other four-carbon organic acids (oxaloacetate, aspartate) are feedback inhibitors.

Species	*F. pringlei*	*F. pubescens*	*F. brownii*	*F. trinervia*
Photosynthesis	C3	C3–4	C4-like	C4
$K_{0.5}$(PEP) [μM]	41	60	128	393
K_i (malate) [μM]	200	600	1500	2500

C4 Photosynthesis

FIG. 4. Evolutionary changes of the affinity of *ppcA* PEPCs for the substrate PEP and in the inhibition by malate. Data from Engelmann *et al.* (2003).

Molecular determinants of C4 properties

The differences in substrate affinity and inhibition by malate suggest that C4 PEPCs harbour specific C4 determinants that were acquired during the evolution of C4 photosynthesis. The use of the genus *Flaveria* as a model system permitted the first analyses of the molecular nature of these determinants. Recombinant C4, C3–C4 and C3 *ppcA* PEPCs and their chimeras were produced by functional expression of the respective cDNAs in a PEPC-negative *E. coli* strain, and the enzymatic properties of the purified proteins were investigated (Svensson *et al.*, 2003).

The *ppcA* PEPC of *F. trinervia* (C4) revealed a K_m(PEP) which is about nine-fold higher than the K_m(PEP) of the *ppcA* PEPC of *F. pringlei* (C3). The C4 enzyme is also ten times more tolerant to malate than its C3 counterpart (Fig. 4). The two orthologous PEPC enzymes, therefore, show the expected differences in kinetic and regulatory properties which are typical for C4 and C3 PEPC isoforms (Svensson *et al.*, 1997).

To unravel how PEPC enzyme characteristics changed during evolution towards C4 photosynthesis, *ppcA* PEPCs from the C3–C4 intermediate plant *F. pubescens* and the C4-like C3–C4 intermediate *F. brownii* were investigated. Both the K_m(PEP) values and the malate inhibition constants, *Ki*, are intermediate between the C3 and C4 *ppcA* PEPCs (Fig. 4). This indicates that the C3 PEPC evolved step by step into a C4 enzyme (Engelmann *et al.*, 2003).

Since the C3 and C4 *ppcA* isoforms share 96 % identical amino acid positions it should be feasible to pinpoint changes in the amino acid sequence responsible for the C4 characteristics (Svensson *et al.*, 1997). Therefore, reciprocal domain swapping experiments were conducted with the two *ppcA* PEPCs to locate regions in the enzyme that influence K_m(PEP) (Bläsing *et al.*, 2000). With this approach two regions, from amino acids 296 to 437 (region 2) and from amino acids 645 to 966 (region 5), were identified that contain the major C4 determinants for the saturation kinetics of the substrate PEP (Fig. 5). This was confirmed by inserting region 2 of the C4 enzyme and the C4 determinant of region 5 (see below) into an otherwise C3 background. The resulting chimeric enzyme possessed about two thirds of C4 PEPC characteristics with respect to K_m(PEP) (Engelmann *et al.*, 2002).

In region 5, the C4-specific properties were confined to a single amino acid, serine 774 (Fig. 5). All C4 enzymes studied to date contain a serine at this position while in all non-photosynthetic and CAM PEPCs this site is occupied by an alanine (Svensson *et al.*, 2003). It has to be concluded that serine 774 is of central importance for the evolution of C4 characteristics, at least with regard to the K_m(PEP). All investigated C3–C4 intermediate PEPCs, even from the C4-like species *F. brownii*, still show an alanine at this position (Engelmann *et al.*, 2003). This suggests that the change from alanine to serine occurred only recently during evolution from C3 to C4 photosynthesis.

In region 2, 16 differences were detected between the C3 and C4 *ppcA* PEPCs (Fig. 5). There is only one amino acid residue, a lysine at position 347, which both *F. trinervia* and *F. brownii* have in common and which differs from the arginine in this position in *F. pubescens* and *F. pringlei* (Fig. 5; Engelmann *et al.*, 2003). This lysine is also conserved in the C4 PEPC of maize where it is located between helices 12 and 13 (Matsumura *et al.*, 2002). This suggests that a lysine at this position is essential for C4 enzyme properties and hence this C4-associated lysine residue is a prime candidate for a C4 determinant of region 2.

Determinants for malate sensitivity and the affinity constant of bicarbonate have not been identified. Since the chimeric C3–C4 enzyme strategy in combination with phylogenetic comparisons of C3–C4 intermediate PEPCs, was very successful in identifying molecular determinants for K_m(PEP) at the amino acid level, this approach should also give new insights into the evolution of C4 PEPC characteristics for malate sensitivity and K_m(bicarbonate).

EVOLUTION OF C4 PEPC GENE REGULATION

Expression patterns of ppcA *genes*

The *ppcA* genes of *F. trinervia* are highly expressed but only in leaves (Ernst and Westhoff, 1996), where expression is

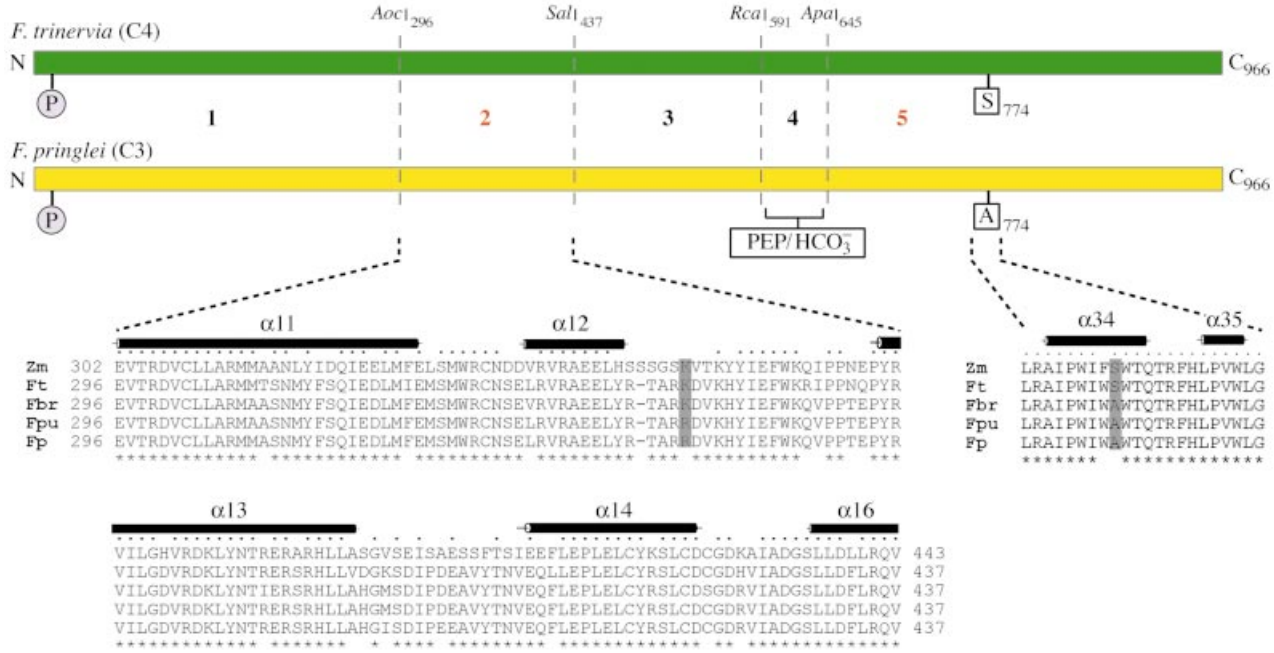

FIG. 5. An evolutionary model of C4 PEPC in *Flaveria*. From five investigated enzyme domains, region 2 (positions 296–437) and region 5 (amino acids 645–966) contain the major C4 determinants for the saturation kinetics of PEP. P indicates the target phosphorylation site at position 11. The secondary structures indicated on top of the sequence alignments (black bars) were obtained from the recently published 3D structure of the C4 PEPC of *Zea mays* (Matsumura *et al.*, 2002). The dots above the maize sequence show those amino acid residues that are conserved in all displayed sequences. Sequence positions which are identical in all four *Flaveria ppcA* PEPCs are marked by stars below the strings of sequences. Note that position 347 (grey column) of the PEPC isoenzymes harbours a lysine in the C4 species *F. trinervia* and *Z. mays*, and in the C4-like plant *F. brownii*, while the C3 species *F. pringlei* and the C3–C4 intermediate plant *F. pubescens* both instead hold an arginine at this site. At position 774 (grey column) serine occurs only in C4 PEPCs, while PEPCs from C3 and C3–C4 intermediate plants contain an alanine at this position. The amino acid numbering follows that of the *F. trinervia* protein.

restricted to the mesophyll cells (Höfer *et al.*, 1992; Koprivova *et al.*, 2001). This contrasts with the *ppcA* genes of *F. pringlei* which are weakly expressed similarly in leaves, stems and roots (Ernst and Westhoff, 1996). The increased gene expression in only the leaves, and the restriction of expression to the mesophyll cells were therefore two major steps taken during the evolution of the C3 ancestral *ppcA* gene into the present C4 *ppcA* genes. Elevated amounts of *ppcA* RNAs can already be detected in less advanced C3–C4 intermediates, i.e. *F. chloraefolia* (Engelmann *et al.*, 2003), while the corresponding *ppcA* PEPC is still almost C3-like (Bauwe and Chollet, 1986). This suggests that an increase in *ppcA* gene expression occurred first during C4 evolution and preceded the conversion of the C3 PEPC into the C4 enzyme. As far as the specificity of *ppcA* gene expression is concerned, no detailed *in situ* studies have been conducted on proteins and RNAs and it remains to be shown when the specific elements controlling mesophyll expression evolved.

Cis-*regulatory elements for mesophyll specific* ppcA *gene expression*

The mesophyll-specific expression of the *ppcA1* gene of *F. trinervia* is controlled at the transcriptional level. About 2200 base pairs of 5′ flanking sequences (with reference to the AUG translational start codon) are sufficient to cause high β-glucuronidase (GUS) expression exclusively in the mesophyll cells of the closely related C4 plant *F. bidentis*. In contrast, the 2538 base pairs (with reference to the AUG start codon) of the 5′ flanking sequences of the *ppcA1* gene of *F. pringlei* were found to be a weak promoter and did not direct any organ- or cell-specific expression (Stockhaus *et al.*, 1997; Fig. 6). Both promoters thus exhibited all the attributes expected from the accumulation patterns of their corresponding RNAs and proteins. The increase in gene expression, but exclusively in the leaves, and the confinement of expression to the mesophyll cells were, therefore, the two prominent steps taken to convert the C3 ancestral *ppcA* gene into the present C4 *ppcA* genes.

Promoter deletion and recombination studies (U. Gowik, J. Burscheidt, M. Akyildiz and P. Westhoff, unpublished data) revealed that the distal sequences between base pairs –1565 to –2188 (DR segment) in combination with the proximal 570 base pairs (PR segment) of 5′ flanking sequences of the *ppcA1* gene (Fig. 7A) are sufficient to achieve elevated mesophyll expression of the GUS reporter gene, i.e. the nucleotide sequences between –570 and –1566 are essentially dispensable for *ppcA1* promoter activity. The C4-DR functions both in the correct and the inverse orientation; this *cis*-regulatory region, therefore, shows the typical features of a transcriptional enhancer

(Blackwood and Kadonaga, 1998). When the C4-DR was inserted into the *ppcA1* promoter of *F. pringlei* a mesophyll expression component was added to that promoter, but its expression strength was only slightly increased (U. Gowik, J. Burscheidt, M. Akyildiz and P. Westhoff, unpublished data). Thus the C4-DR contains mesophyll expression determinants which are lacking in the C3 *ppcA1* promoter. However, a high expression in the mesophyll cells is only observed, when the C4-DR is combined with its corresponding PR segment. It also follows that the PR segments of the two promoters differ in mesophyll expression determinants.

The following scenario for the evolution of the C3 into the C4 *ppcA1* promoter may be derived from these findings. The evolutionary changes occurred both in the DR and PR segments of the promoter (Fig. 7A), and resulted in a high and mesophyll-specific expression of the C4 *ppcA* gene (Fig. 7A). First, *cis*-regulatory elements had to evolve that led to mesophyll specificity. Since the C3 *ppcA1* promoter is active in all leaf cells (Stockhaus *et al*., 1997; Fig. 6) mesophyll specificity required that *cis*-regulatory elements for expression in non-mesophyll cells became inactivated. In addition, novel *cis*-regulatory elements for mesophyll expression may have been created. Secondly and concomitantly, the quantity of *ppcA* gene expression had to be increased. This could have been achieved by adding novel mesophyll specificity elements to the promoter whose interaction with the corresponding *trans*-regulatory factors was optimized step by step when progressing from C3 to C4 photosynthesis. Alternatively, and/or in addition, novel quantitative elements were created that interacted specifically with the mesophyll quality elements.

Detailed analyses allowed more precise definition of determinants in the C4-DR responsible for mesophyll expression. A 41-base-pair segment, named MEM1 (mesophyll expression module 1), was identified in the DR segment of the *F. trinervia* promoter that, in combination with the PR segment of that promoter, was sufficient to confer specificity for expression of the GUS reporter gene in the mesophyll (U. Gowik, J. Burscheidt, M. Akyildiz and P. Westhoff, unpublished data).

MEM1 homologous sequences were also detected in the *ppcA1* promoter of *F. pringlei* (Fig. 7B) and in other C3, C4 and C4-like *Flaveria* species (Gowik *et al*., 2003). Their comparison revealed that MEM1 sequences consist of two parts, A and B, which are contiguous in *F. trinervia*, but are separated by 97–108 base pairs in the C3 species *F. pringlei* and *F. cronquistii*, the C4 plant *F. bidentis* (C4) and the C4-like species *F. palmeri* and *F. vaginata* (U. Gowik, J. Burscheidt, M. Akyildiz and P. Westhoff, unpublished data). The A parts of all C4 and C4-like species show a guanine at their first nucleotide position, while an adenine is present in the A-homologues of the two C3 species. A similar C4-to-C3 associated difference is also found for the tetra nucleotide CACT. This assemblage is present in the B parts of all C4 and C4-like species but absent in both C3 promoters. These C4-to-C3 correlated differences in MEM1 composition are likely candidates for *cis*-regulatory elements governing mesophyll specific gene expression.

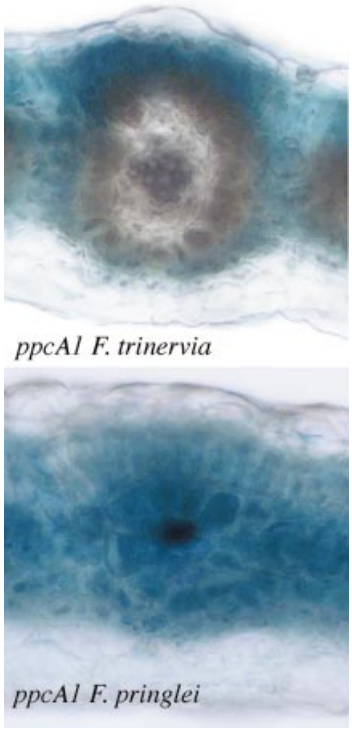

FIG. 6. Histochemical analysis of the activities of the *ppcA1* promoters of *F. trinervia* (C4) and *F. pringlei* (C3) in transgenic *F. bidentis* (C4) (cf. Stockhaus *et al.*, 1997). The β-glucuronidase gene (*GUS*) was used as a reporter gene (Jefferson *et al.*, 1987).

Trans-*regulatory factors for mesophyll specific* ppcA *gene expression*

Analysis of *trans*-regulatory factors that are necessary for mesophyll-specific expression of the *ppcA* gene is in its infancy. By using the yeast one-hybrid system (Vidal and Legrain, 1999), homeodomain proteins were identified that belong to a hitherto uncharacterized class containing a zinc finger domain. Such proteins specifically interact with the PR segment of the *ppcA1* promoter of *F. trinervia* but not with the corresponding region of the *ppcA1* promoter of *F. pringlei* (Windhövel *et al*., 2001; Fig. 8). They are, therefore, prime candidates for transcription factors that are required for establishing the C4 specific expression pattern of the C4 *ppcA* genes. However, *in planta* experiments to probe their participation in C4 *ppcA1* gene expression are still lacking.

Similarly, one-hybrid approaches have been performed with MEM1 of *F. trinervia* as the bait (M. Akyildiz and P. Westhoff, unpublished data). The isolated MEM1-binding proteins still need to be completely characterized and their specific binding properties verified (Fig. 8). Taken together, the data suggest that the one-hybrid screening assay is a good heuristic tool to use in the search for transcription factor candidates. Reverse genetic approaches based on RNAi-triggered gene inactivation (Waterhouse and Helliwell, 2003) or 'gain-of-function' experiments which ectopically express the putative transcription factor (Schwechheimer *et al*., 1998) are required to verify the

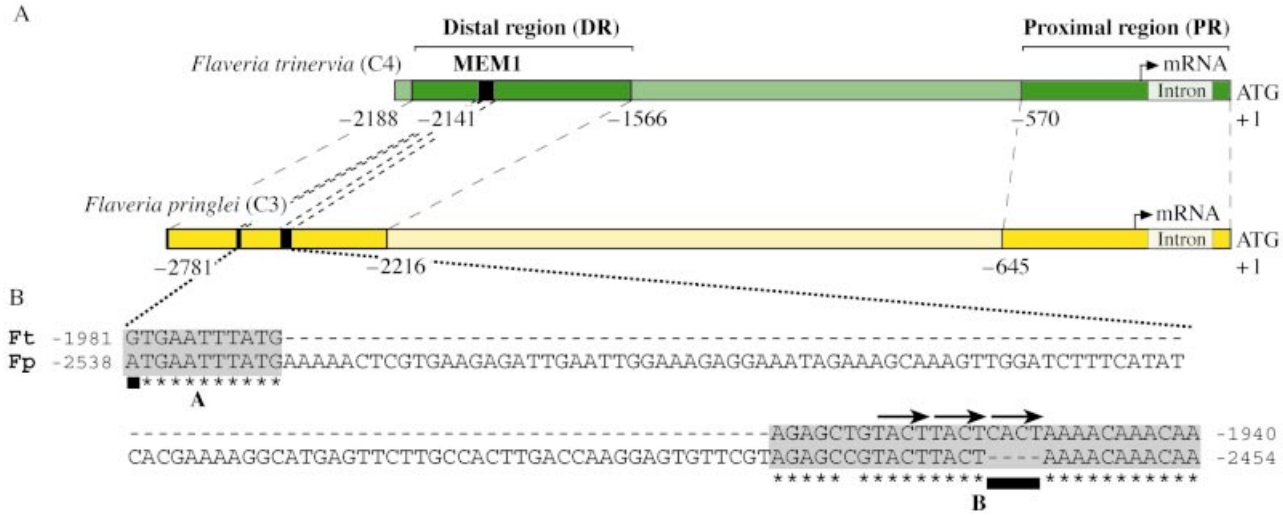

FIG. 7. The structures of the *ppcA1* promoters from *F. trinervia* (C4) and *F. pringlei* (C3) and the nucleotide composition of the *mesophyll expression module* MEM1. A, Schematic comparisons of the 5′ flanking sequences of the *ppcA1* genes of *F. trinervia* and *F. pringlei*. The numbers of nucleotides refer to the translation initiation codon. The proximal (PR) and distal regions (DR) of the two promoters are strongly coloured. The positions of MEM1 and its homologue in *F. pringlei* are marked by black boxes. B, Sequence comparison of MEM1 of *F. trinervia* (Ft) and its homologue in *F. pringlei* (Fp). Asterisks label identical nucleotides in the A or B segments of MEM1. Black bars indicate the single nucleotide difference in A and the CACT tetra nucleotide in B. The two tandem TACT repeats and the third imperfect CACT repeat are marked by arrows. The C/T difference in the B segment is not correlated with C3/C4 photosynthesis, because all C4 flaverias except *F. trinervia* contain a C at that position. Data from U. Gowik, J. Burscheidt, M. Akyildiz and P. Westhoff, unpublished data

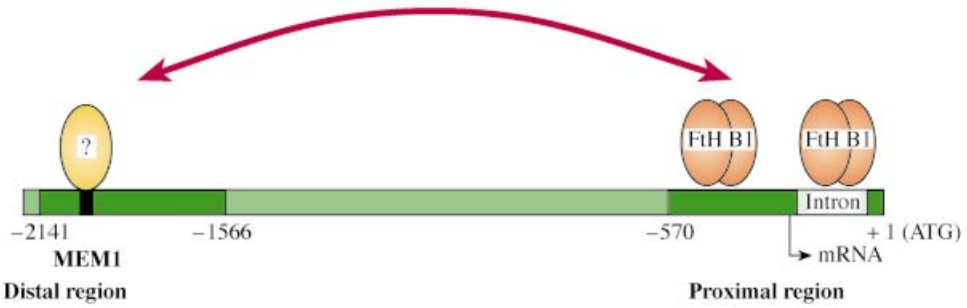

FIG. 8. A working model of the C4 *ppcA1* promoter of *F. trinervia*. FtHB1 denotes the zinc finger homeodomain transcription factors that have been shown to bind to the proximal region of the C4 *ppcA1* promoter (Windhövel *et al.*, 2001). The *trans*-regulatory factors which bind to the enhancer-like distal region are unknown.

in planta relevance of these possible *trans*-regulatory factors.

CONCLUSIONS AND OUTLOOK

C4 photosynthesis is a fascinating system for studying the evolution of a new metabolic capability. The C4 photosynthetic pathway evolved polyphyletically and provides insight into the flexibility of the genetic system of angiosperms. This system independently created C4 photosynthesis several times, the main feature—indeed the central pillar—of which is the efficient CO_2 pump, based on PEPC, that concentrates CO_2 where ribulose 1,5-bisphosphate carboxylase/oxygenase is located. The CO_2 pump normally requires two different cell-types, mesophyll and bundle sheath cells, which unite to a metabolic system that is characterized by a division of labour.

The differentiation of these two photosynthetic cell-types required an alteration of the developmental programmes. This makes C4 photosynthesis an attractive system which reveals how regulatory circuits of gene expression are modified to give new, specific expression patterns. Also, evolution of the C4 assimilatory pathway requires adaptation of enzymes to a new metabolic role which involves changes in enzyme kinetics and their regulation. The case study with C4 PEPC, which is reviewed here, demonstrates that the pursuit of this evolution-oriented approach leads to new insights into structure–function relationships of this enzyme (Svensson *et al.*, 2003) and the molecular nature of *cis*- and *trans*-regulatory factors that are required for the mesophyll specific expression of the corresponding gene.

The polyphyletic origin of C4 photosynthesis implies that, in genetic terms, it has been comparatively simple to evolve C4 plants from C3 ancestors. The data obtained with *Flaveria* support this point of view. Small alterations sufficed to convert a C3 into a C4 PEPC enzyme and change a C3 into a C4 PEPC promoter. Comparison of the enzymatic properties of C3, C3–C4 and C4 PEPC enzymes shows that the changes proceed gradually. To date comparable data on the changes within the gene regulation system are not available. Investigation of *ppcA* RNA amounts in C3, C3–C4, C4-like and C4 *Flaveria* species suggests that at least the changes in the promoter strength also happened step by step (Engelmann *et al*., 2003).

This gradual transition from C3 to C4 photosynthesis, which occurred in *Flaveria*, can easily be explained if the C4 syndrome is viewed as a quantitative trait. Quantitative traits (Mauricio, 2001) are of a polygenic nature and their gene components are called quantitative trait loci (QTL). In contrast to Mendelian traits, which are discrete, quantitative traits are affected by multiple genes and by environmental factors. Individual QTL may have only small effects on the expression of the trait, i.e. are minor genes. However, recent studies with crop and model plants show that individual loci may behave as major genes and determine a large part of the trait variation. These studies also reveal that regulatory loci play a prominent role within the genetic architecture of quantitative traits (Remington and Purugganan, 2003).

Unfortunately, the quantitative trait concept of C4 photosynthesis cannot be easily tested experimentally, because there is no genetic system consisting of easily crossable C3 and C4 species. A segregating population between *Atriplex hastata* (C3) and *A. rosea* (C4) was constructed and individual C4 traits segregated in that population (Björkman *et al*., 1971). However, the F_2 plants were aneuploid and a genetic mapping of the C4 QTL failed for this reason (Nobs, 1976). Within the genus *Flaveria* crosses between C3 and C3–C4 species have been achieved, but crosses between C3 and C4 *Flaveria* species have not succeeded (Brown and Bouton, 1993). Clearly, a genetic system which allows the construction of segregating populations from C3 and C4 species is highly desirable. The number of genes which make up the genetic architecture of C4 photosynthesis, and their molecular nature, could be identified with this strategy. Whether mutational studies, for instance with maize, will be a good substitute for the QTL approach remains to be seen.

In his influential review on biochemical evolution, A. C. Wilson pointed out that 'quantitative mutations affecting enzyme levels may have had a major role in the adaptative metabolic evolution of multicellular organisms', and that 'these quantitative effects can result from point mutations in control genes' (Wilson *et al*., 1977). He believed that these regulatory mutations happened before mutations in protein coding regions. The studies with the molecular evolution of C4 PEPC in *Flaveria* are in line with this concept. These investigations have shown that a C4-type pattern of gene expression evolved before the C4 enzyme characteristics became established. This is best illustrated by the C4-like species *F. brownii*. The *ppcA* PEPC of this species is rather C3-like, most notably its amino acid position 774 is still occupied by an alanine and not by the C4-typical serine. On the other hand, the expression levels of the *ppcA* mRNA (Engelmann *et al*., 2003) and the distribution of the PEPC protein (Cheng *et al*., 1988) are essentially of C4 type.

Evolutionary biologists have collected convincing evidence which support the view that changes in the spatio-temporal expression patterns of genes are the principal mechanisms for novel morphological and biochemical traits (Doebley and Lukens, 1998; Carroll, 2000). *Flaveria* is a unique system which allows detailed tests of this concept by using C4 photosynthesis as the model trait.

ACKNOWLEDGEMENTS

Work summarized here was supported by the Deutsche Forschungsgenmeinschaft (DFG) within the Sonderforschungsbereich SFB590 and the Graduiertenkolleg Molekulare Physiologie both at the Heinrich-Heine-Universität of Düsseldorf. Additional support from the Fonds der Chemischen Industrie is greatfully acknowledged.

LITERATURE CITED

Badger MR, Price GD. 1994. The role of carbonic anhydrase in photosynthesis. *Annual Review of Plant Physiology and Plant Molecular Biology* **45**: 369–392.

Baldy P, Cavalié G. 1984. Compartmentation of photorespiratory enzymes in a C_4 photosynthesis plant, *Zea mays*. *Zeitschrift für Pflanzenphysiologie* **114**: 255–259.

Bauwe H. 1986. An efficient method for the determination of Km values for HCO_3^- of phosphoenolpyruvate carboxylase. *Planta* **169**: 356–360.

Bauwe H, Chollet R. 1986. Kinetic properties of phosphoenolpyruvate carboxylase from C_3, C_4, and C3–C4 intermediate species of *Flaveria* (Asteraceae). *Plant Physiology* **82**: 695–699.

Björkman O, Nobs MA, Berry JA. 1971. Further studies on hybrids between C3 and C4 species of *Atriplex*. *Carnegie Institution of Washington, Year Book* 70: Carnegie Institution, 507–511.

Blackwood EM, Kadonaga JT. 1998. Going the distance: a current view of enhancer action. *Science* **281**: 60–63.

Bläsing OE, Ernst K, Streubel M, Westhoff P, Svensson P. 2002. The non-photosynthetic phosphoenolpyruvate carboxylases of the C4 dicot *Flaveria trinervia* – implications for the evolution of C4 photosynthesis. *Planta* **215**: 448–456.

Bläsing OE, Westhoff P, Svensson P. 2000. Evolution of C4 phosphoenolpyruvate carboxylase in *Flaveria*, a conserved serine residue in the carboxyl-terminal part of the enzyme is a major determinant for C4-specific characteristics. *Journal of Biological Chemistry* **275**: 27917–27923.

Brown RH. 1999. Agronomic implications of C4 photosynthesis. In: Sage RF, Monson RK, eds. *C4 Plant Biology*. San Diego: Academic Press, 473–507.

Brown RH, Bouton JH. 1993. Physiology and genetics of interspecific hybrids between photosynthetic types. *Annual Review of Plant Physiology and Plant Molecular Biology* **44**: 435–456.

Carroll SB. 2000. Endless forms: the evolution of gene regulation and morphological diversity. *Cell* **101**: 577–580.

Cheng SH, Moore Bd, Edwards GE, Ku MSB. 1988. Photosynthesis in *Flaveria brownii*, a C_4-like species. Leaf anatomy, characteristics of CO_2 exchange, compartmentation of photosynthetic enzymes, and metabolism of $^{14}CO_2$. *Plant Physiology* **87**: 867–873.

Cheng SH, Moore Bd, Wu J, Edwards GE, Ku MSB. 1989. Photosynthetic plasticity in *Flaveria brownii*. Growth irradiance and the expression of C_4 photosynthesis. *Plant Physiology* **89**: 1129–1135.

Chitty JA, Furbank RT, Marshall JS, Chen Z, Taylor WC. 1994.

Genetic transformation of the C_4 plant, *Flaveria bidentis*. *Plant Journal* **6**: 949–956.

Chollet R, Vidall J, O'Leary MH. 1996. Phosphoenol pyruvate carboxylase: a ubiquitous, highly regulated enzyme in plants. *Annual Review of Plant Physiology and Plant Molecular Biology* **47**: 273–298.

Chu CC, Qu N, Bassüner B, Bauwe H. 1997. Genetic transformation of the C3–C4 intermediate plant, *Flaveria pubescens* (Asteraceae). *Plant Cell Reports* **16**: 715–718.

Crétin C, Santi S, Keryer E, Lepiniec L, Tagu D, Vidal J, Gadal P. 1991. The phosphoenolpyruvate carboxylase gene family of *Sorghum*: promoter structures, amino acid sequences and expression of genes. *Gene* **99**: 87–94.

Dengler NG, Nelson T. 1999. Leaf structure and development in C4 plants. In: Sage RF, Monson RK, eds. *C4 Plant Biology*. San Diego: Academic Press, 133–172.

Doebley J, Lukens L. 1998. Transcriptional regulators and the evolution of plant form. *Plant Cell* **10**: 1075–1082.

Dong LY, Masuda T, Kawamura T, Hata S, Izui K. 1998. Cloning, expression, and characterization of a root-form phosphoenolpyruvate carboxylase from *Zea mays*: comparison with the C4-form enzyme. *Plant and Cell Physiology* **39**: 865–873.

Edwards GE, Ku MSB. 1987. Biochemistry of C3–C4 intermediates. In: Hatch MD, Boardman NK, eds. *The Biochemistry of Plants, Vol. 10*. New York: Academic Press, 275–325.

Ehleringer JR, Monson RK. 1993. Evolutionary and ecological aspects of photosynthetic pathway variation. *Annual Review of Ecology and Systematics* **24**: 411–439.

Engelmann S, Bläsing OE, Gowik U, Svensson P, Westhoff P. 2003. Molecular evolution of C4 phosphoenolpyruvate carboxylase in the genus *Flaveria* – a gradual increase from C3 to C4 characteristics. *Planta* **217**: 717–725.

Engelmann S, Bläsing OE, Westhoff P, Svensson P. 2002. Serine 774 and amino acids 296 to 437 comprise the major C4 determinants of the C4 phosphoenolpyruvate carboxylase of Flaveria trinervia. *FEBS Letters* **524**: 11–14.

Ernst K, Westhoff P. 1996. The phosphoenolpyruvate carboxylase (*ppc*) gene family of *Flaveria trinervia* (C_4) and *F. pringlei* (C_3): molecular characterization and expression analysis of the *ppcB* and *ppcC* genes. *Plant Molecular Biology* **34**: 427–443.

Freitag H, Stichler W. 2000. A remarkable new leaf type with unusual photosynthetic tissue in a Central Asiatic genus of Chenopodiaceae. *Plant Biology* **2**: 154–160.

Freitag H, Stichler W. 2002. *Bienertia cycloptera* Bunge ex Boiss**.** Chenopodiaceae, another C4 plant without Kranz tissues. *Plant Biology* **4**: 121–134.

Furumoto T, Hata S, Izui K. 2000. Isolation and characterization of cDNAs for differentially accumulated transcripts between mesophyll cells and bundle sheath strands of maize leaves. *Plant and Cell Physiology* **41**: 1200–1209.

Harpster MH, Taylor WC. 1986. Maize phosphoenolpyruvate carboxylase. Cloning and characterization of mRNAs encoding isozymic forms. *Journal of Biological Chemistry* **261**: 6132–6136.

Hatch MD. 1987. C_4 photosynthesis: a unique blend of modified biochemistry, anatomy and ultrastructure. *Biochimica et Biophysica Acta* **895**: 81–106.

Hermans J, Westhoff P. 1990. Analysis of expression and evolutionary relationships of phosphoenolpyruvate carboxylase genes in *Flaveria trinervia* (C_4) and *F. pringlei* (C_3). *Molecular & General Genetics* **224**: 459–468.

Hermans J, Westhoff P. 1992. Homologous genes for the C_4 isoform of phosphoenolpyruvate carboxylase in a C_3- and a C_4-*Flaveria* species. *Molecular & General Genetics* **234**: 275–284.

Höfer MU, Santore UJ, Westhoff P. 1992. Differential accumulation of the 10-, 16- and 23-kDa peripheral components of the water-splitting complex of photosystem II in mesophyll and bundle-sheath chloroplasts of the dicotyledonous C_4 plant *Flaveria trinervia* (Spreng.) C. Mohr. *Planta* **186**: 304–312.

Holaday AS, Bowes G. 1980. C4 acid metabolism and dark CO_2 fixation in a submersed aquatic macrophyte (*Hydrilla verticillata*). *Plant Physiology* **65**: 331–335.

Jefferson RA, Kavanagh TA, Bevan MW. 1987. GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO Journal* **6**: 3901–3907.

Kai Y, Matsumura H, Izui K. 2003. Phosphoenolpyruvate carboxylase: three-dimensional structure and molecular mechanisms. *Archives of Biochemistry and Biophysics* **414**: 170–179.

Kanai R, Edwards GE. 1999. The biochemistry of C4 photosynthesis. In: Sage RF, Monson RK, eds. *C4 Plant Biology*. San Diego: Academic Press, 49–87.

Kawamura T, Shigesada K, Toh H, Okumura S, Yanagisawa S, Izui K. 1992. Molecular evolution of phosphoenolpyruvate carboxylase for C4 photosynthesis in maize: comparison of its cDNA sequence with a newly isolated cDNA encoding an isozyme involved in anaplerotic function. *Journal of Biochemistry* **112**: 147–154.

Kellogg EA. 1999. Phylogenetic aspects of the evolution of C4 photosynthesis. In: Sage RF, Monson RK, eds. *C4 Plant Biology*. San Diego: Academic Press, 411–444.

Kopriva S, Chu CC, Bauwe H. 1996. Molecular phylogeny of *Flaveria* as deduced from the analysis of nucleotide sequences encoding the H-protein of the glycine cleavage system. *Plant Cell and Environment* **19**: 1028–1036.

Koprivova A, Melzer M, vonBallmoos P, Mandel T, Brunold C, Kopriva S. 2001. Assimilatory sulfate reduction in C-3, C-3-C-4, and C-4 species of Flaveria. *Plant Physiology* **127**: 543–550.

Kubicki A, Steinmüller K, Westhoff P. 1994. Differential transcription of plastome-encoded genes in the mesophyll and bundle-sheath chloroplasts of the monocotyledonous NADP-malic enzyme type C4 plants maize and Sorghum. *Plant Molecular Biology* **25**: 669–679.

Long SP. 1999. Environmental responses. In: Sage RF, Monson RK, eds. *C4 Plant Biology*. San Diego: Academic Press, 215–249.

Lunn JE, Hatch MD. 1995. Primary partitioning and storage of photosynthate in sucrose and starch in leaves of C_4 plants. *Planta* **197**: 385–391.

Matsumura H, Xie Y, Shirakata S, Inoue T, Yoshinaga T, Ueno Y, Izui K, Kai Y. 2002. Crystal structures of C4 form maize and quaternary complex of *E. coli* phosphoenolpyruvate carboxylases. *Structure* **10**: 1721–1730.

Mauricio R. 2001. Mapping quantitative trait loci in plants: uses and caveats for evolutionary biology. *Nature Reviews Genetics* **2**: 370–381.

Meierhoff K, Westhoff P. 1993. Differential biogenesis of photosystem II in mesophyll and bundle-sheath cells of monocotyledonous NADP-malic enzyme-type C_4 plants: the non-stoichiometric abundance of the subunits of photosystem II in the bundle-sheath chloroplasts and the translational activity of the plastome-encoded genes. *Planta* **191**: 23–33.

Monson RK. 1999. The origins of C4 genes and evolutionary pattern in the C4 metabolic phenotype. In: Sage RF, Monson RK, eds. *C4 Plant Biology*. San Diego: Academic Press, 377–410.

Monson RK, Moore Bd. 1989. On the significance of C3–C4 intermediate photosynthesis to the evolution of C_4 photosynthesis. *Plant Cell and Environment* **12**: 689–699.

Nimmo HG. 2003. Control of the phosphorylation of phosphoenolpyruvate carboxylase in higher plants. *Archives of Biochemistry and Biophysics* **414**: 189–196.

Nobs MA. 1976. Hybridizations in *Atriplex*. *Carnegie Institution of Washington, Year Book* 75: Carnegie Institution, 421–423.

Poetsch W, Hermans J, Westhoff P. 1991. Multiple cDNAs of phosphoenolpyruvate carboxylase in the C_4 dicot *Flaveria trinervia*. *FEBS Letters* **292**: 133–136.

Powell AM. 1978. Systematics of *Flaveria* (Flaveriinae-Asteraceae). *Annals of the Missouri Botanical Garden* **65**: 590–636.

Rajagopalan AV, Devi MT, Raghavendra AS. 1994. Molecular biology of C4 phosphoenolpyruvate carboxylase: structure, regulation and genetic engineering. *Photosynthesis Research* **39**: 115–135.

Rathnam CKM, Edwards GE. 1976. Distribution of nitrate-assimilating enzymes between mesophyll protoplasts and bundle sheath cells in leaves of three groups of C4 plants. *Plant Physiology* **57**: 881–885.

Remington DL, Purugganan MD. 2003. Candidate genes, quantitative trait loci, and functional trait evolution in plants. *International Journal of Plant Sciences* **164**: Suppl. S7–S20.

Renné P, Dreßen U, Hebbeker U, Hille D, Flügge U-I, Westhoff P, Weber A. 2003. The Arabidopsis mutant *dct* is deficient in the

plastidic glutamate/malate translocator DiT2. *Plant Journal* **35**: 316–341.

Sage RF. 1999. Why C4 photosynthesis? In: Sage RF, Monson RK, eds. *C4 Plant Biology*. San Diego: Academic Press, 3–16.

Sage RF, Li M, Monson RK. 1999. The taxonomic distribution of C4 photosynthesis. In: Sage RF, Monson RK, eds. *C4 Plant Biology*. San Diego: Academic Press, 551–584.

Schmutz D, Brunold C. 1984. Intercellular localization of assimilatory sulfate reduction in leaves of *Zea mays* and *Triticum aestivum*. *Plant Physiology* **74**: 866–870.

Schwechheimer C, Zourelidou M, Bevan MW. 1998. Plant transcription factor studies. *Annual Review of Plant Physiology and Plant Molecular Biology* **49**: 127–150.

Sheen J. 1999. C4 gene expression. *Annual Review of Plant Physiology and Plant Molecular Biology* **50**: 187–217.

Sheen JY, Sayre RT, Bogorad L. 1987. Differential expression of oxygen-evolving polypeptide genes in maize leaf cell types. *Plant Molecular Biology* **9**: 217–226.

Stockhaus J, Schlue U, Koczor M, Chitty JA, Taylor WC, Westhoff P. 1997. The promoter of the gene encoding the C4 form of phosphoenolpyruvate carboxylase directs mesophyll specific expression in transgenic C4 *Flaveria* spp. *Plant Cell* **9**: 479–489.

Svensson P, Bläsing O, Westhoff P. 1997. Evolution of the enzymatic characteristics of C4 phosphoenolpyruvate carboxylase: a comparison of the orthologous *ppcA* phosphoenolpyruvate carboxylases of *Flaveria trinervia* (C4) and *F. pringlei* (C3). *European Journal of Biochemistry* **246**: 452–460.

Svensson P, Bläsing OE, Westhoff P. 2003. Evolution of C4 phosphoenolpyruvate carboxylase. *Archives of Biochemistry and Biophysics* **414**: 180–188.

Swofford DL. 2002. *PAUP*: Phylogenetic Analysis Using Parsimony (and other methods) 4.0*. Sinauer Associates, Sunderland, MA, USA.

Thompson JD, Higgins DG, Gibson TJ. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research* **22**: 4673–4680.

Ting IP, Osmond CB. 1973*a*. Multiple forms of plant phosphoenolpyruvate carboxylase associated with different metabolic pathways. *Plant Physiology* **51**: 448–453.

Ting IP, Osmond CB. 1973*b*. Photosynthetic phosphoenolpyruvate carboxylase. Characteristics of allozymes from leaves of C3 and C4 plants. *Plant Physiology* **51**: 439–447.

Vidal M, Legrain P. 1999. Yeast forward and reverse 'n'-hybrid systems. *Nucleic Acids Research* **27**: 919–929.

Waterhouse PM, Helliwell CA. 2003. Exploring plant genomes by RNA-induced gene silencing. *Nature Reviews Genetics* **4**: 29–38.

Wilson AC, Carlson SS, White TJ. 1977. Biochemical evolution. *Annual Review of Biochemistry* **46**: 573–639.

Windhövel A, Hein I, Dabrowa R, Stockhaus J. 2001. Characterization of a novel class of plant homeodomain proteins that bind to the C4 phosphoenolpyruvate carboxylase gene of *Flaveria trinervia*. *Plant Molecular Biology* **45**: 201–214.

Woo KC, Anderson JM, Boardman NK, Downton WJS, Osmond CB, Thorne SW. 1970. Deficient photosystem II in agranal bundle sheath chloroplasts of C4 plants. *Proceedings of the National Academy of Sciences of the USA* **67**: 18–25.

Wyrich R, Dreßen U, Brockmann S, Streubel M, Chang C, Qiang D, Paterson AH, Westhoff P. 1998. The molecular basis of C4 photosynthesis in sorghum: isolation, characterization and RFLP mapping of mesophyll- and bundle-sheath-specific cDNAs obtained by differential screening. *Plant Molecular Biology* **37**: 319–335.