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REVIEW PAPER

The molecular evolution of β-carbonic anhydrase in *Flaveria*

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Abstract

Limited information exists regarding molecular events that occurred during the evolution of C_4 plants from their C_3 ancestors. The enzyme β-carbonic anhydrase (CA; EC 4.2.1.1), which catalyses the reversible hydration of CO_2, is present in multiple forms in C_3 and C_4 plants, and has given insights into the molecular evolution of the C_4 pathway in the genus *Flaveria*. cDNAs encoding three distinct isoforms of β-CA, CA1–CA3, have been isolated and examined from *Flaveria* C_3 and C_4 congeners. Sequence data, expression analyses of CA orthologues, and chloroplast import assays with radiolabelled CA precursor proteins from the C_3 species *F. pringlei* Gandoger and the C_4 species *F. bidentis* (L.) Kuntze have shown that both contain chloroplastic and cytosolic forms of the enzyme, and the potential roles of these isoforms are discussed. The data also identified CA3 as the cytosolic isoform important in C_4 photosynthesis and indicate that the C_4 *CA3* gene evolved as a result of gene duplication and neofunctionalization, which involved mutations in coding and non-coding regions of the ancestral C_3 *CA3* gene. Comparisons of the deduced CA3 amino acid sequences from *Flaveria* C_3, C_4, and photosynthetic intermediate species showed that all the C_3–C_4 intermediates investigated and *F. brownii*, a C_4-like species, have a C_3-type CA3, while *F. vaginata*, another C_4-like species, contains a C_4-type CA3. These observations correlate with the photosynthetic physiologies of the intermediates, suggesting that the molecular evolution of C_4 photosynthesis in *Flaveria* may have resulted from a temporally dependent, stepwise modification of protein-encoding genes and their regulatory elements.

Key words: C_4 photosynthesis, carbonic anhydrase, *Flaveria*, molecular evolution, photosynthetic intermediate.

Introduction

Plants using the C_4 photosynthetic pathway have evolved from C_3 ancestors in multiple angiosperm lineages, with falling CO_2 concentrations in the atmosphere during the Oligocene likely to be the primary driver for the evolution of the syndrome (Sage, 2004; see other contributions to this volume). Further expansion of the C_4 condition during the Miocene and Pleistocene occurred due to changes in other environmental factors that promote photorespiration in C_3 plants such as heat, aridity, and soil salinity (Sage, 2004; Osborne and Beerling, 2006; Tipple and Pagani, 2007; Christin *et al.*, 2008; Vicentini *et al.*, 2008; Osborne and Freckleton, 2009; Edwards and Smith, 2010; Edwards *et al.*, 2010). These atmospheric and climatic pressures led to the evolution of a combination of anatomical and biochemical traits that enable C_4 plants to concentrate CO_2 around ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), almost eliminating photorespiration. As a consequence, C_4 plants outcompete C_3 species in hot, high light, dry, and saline environments—environments that are expanding in some regions of the world due to global climate change.

While the remarkable similarities in the structural and biochemical characteristics of C_4 plants of different ancestry have been recognized for years, relatively little is known about the molecular mechanisms underlying the evolution of the C_4 pathway. Determining how the genes controlling the advantageous survival traits of C_4 species evolved—that is identification of gene duplication events and modifications leading to neofunctionalization—and how the enzymatic steps came together to give a functional C_4 plant will expand our understanding of what molecular mechanisms were used and required in the evolution of C_4 pathways,

and this will contribute to targeted strategies for transferring these traits to C_3 crop plants.

Flaveria: a model genus to study the molecular evolution of C_4 photosynthesis

Flaveria is one of several plant genera that contain individual species that perform C_3 photosynthesis, others that use the C_4 pathway, and still others that carry out C_3–C_4 or C_4-like intermediate types of photosynthesis (Powell, 1978). *Flaveria* photosynthetic intermediates demonstrate differing leaf anatomies, biochemistries, and photosynthetic physiologies that appear to form a continuum from the C_3 ancestral condition to the more highly derived C_4 state (Powell, 1978; Edwards and Ku, 1987; McKown *et al.*, 2005; McKown and Dengler, 2007). Phylogenetic information indicates that C_4 photosynthesis may have evolved at least three times in the genus (McKown *et al.*, 2005). This diversity of photosynthesis amongst a group of closely related plants greatly assists molecular evolutionary studies; for example, orthologous and paralogous genes are typically easily recognized, which facilitates the identification of differences (mutations) in coding and/or regulatory regions. Not surprisingly, a number of research groups have taken advantage of this collection of plants to study the molecular evolution of C_4 photosynthetic enzymes (Hermans and Westhoff, 1990; Rajeevan *et al.*, 1991; Lipka *et al.*, 1994; Rosche *et al.*, 1994; Ludwig and Burnell, 1995; McGonigle and Nelson, 1995; Marshall *et al.*, 1996; Drincovich *et al.*, 1998; Lai *et al.*, 2002). The work from these groups has shown that the genes encoding enzyme isoforms important in *Flaveria* C_4 photosynthesis evolved from the duplication of an ancestral C_3 gene and/or the acquisition of regulatory sequences that led to increased levels of cell-dependent expression (Marshall *et al.*, 1997; Rosche *et al.*, 1998; Ali and Taylor, 2001; Lai *et al.*, 2002; Gowik *et al.*, 2004; Engelmann *et al.*, 2008).

Carbonic anhydrases (CAs) in C_3 and C_4 plants

CAs (EC 4.2.1.1) of land plants are zinc metalloenzymes that catalyse the reversible hydration of CO_2. Three classes of CA, α, β, and γ, have been identified in land plants (Hewett-Emmett and Tashian, 1996; Moroney *et al.*, 2001; Ivanov *et al.*, 2007) and, while no conservation of amino acid sequence or higher orders of structure is seen between enzymes from the different classes, all catalyse the interconversion of CO_2 and bicarbonate.

Currently little information exists regarding γ- and α-CA isoforms in flowering plants. Higher plant γ-CA isoforms are part of the mitochondrial complex I (Parisi *et al.*, 2004), and involvement in complex I assembly has been shown for one *Arabidopsis* γ-CA isoform using knockout mutants (Perales *et al.*, 2005). No CA activity has been detected biochemically for these proteins, although they have been shown to bind inorganic carbon (Martin *et al.*, 2009). Eight *α-CA* genes have been identified in *Arabidopsis*; however, Fabre *et al.* (2007) detected transcripts from only *AtαCA1*, *AtαCA2*, and *AtαCA3*, and suggested that the transcripts from the other genes may be unstable, or their expression may be low, induced under particular conditions, or in specific cell types. *AtαCA1* mRNA was found in all aboveground organs tested (Fabre *et al.*, 2007) and this CA is transported to the chloroplast through the secretory pathway (Villarejo *et al.*, 2005). *AtαCA2* transcripts, found in stems and roots, and *AtαCA3* mRNA, detected in flowers and siliques, increased in abundance when plants were grown in a low CO_2 environment (Fabre *et al.*, 2007). Although roles for γ- and α-CA isoforms in photosynthesis cannot be ruled out, currently there is no clear support for these proteins being involved in either the C_3 or the C_4 pathway.

Multiple forms of β-CAs have been found in all investigated angiosperms (Burnell, 2000; Coleman, 2000; Moroney *et al.*, 2001; Ivanov *et al.*, 2007). In C_3 plants, most CA activity localizes to the chloroplast stroma of the mesophyll cells (Poincelot, 1972; Jacobson *et al.*, 1975; Tsuzuki *et al.*, 1985) where it has been proposed to facilitate the diffusion of CO_2 across the chloroplast envelope or catalyse the rapid dehydration of bicarbonate to CO_2, ensuring adequate concentrations of CO_2 for Rubisco and carbohydrate production (Reed and Graham, 1981; Cowan, 1986; Price *et al.*, 1994). However, this role is somewhat controversial as no significant impairment in CO_2 assimilation was detected in mature leaves of β-CA antisense tobacco plants containing <1% of wild-type CA levels (Majeau *et al.*, 1994; Price *et al.*, 1994; Williams *et al.*, 1996) or *Arabidopsis* chloroplastic β-CA gene knockout lines (Ferreira *et al.*, 2008). Recently, C_3 plastidial β-CAs have been shown to play a role in stomatal closure (Hu *et al.*, 2010), and to have non-photosynthetic functions, including lipid synthesis (Hoang and Chapman, 2002) and disease resistance (Slaymaker *et al.*, 2002; Restrepo *et al.*, 2005; Wang *et al.*, 2009).

Whereas solid evidence for chloroplastic β-CA playing a role in C_3 photosynthesis remains elusive, the function of β-CA in the C_4 photosynthetic pathway is unequivocal. The greatest CA activity in C_4 plants also localizes to leaf mesophyll cells; however, the enzyme is active in the cytosol of these cells (Gutierrez *et al.*, 1974; Ku and Edwards, 1975; Burnell and Hatch, 1988). In the C_4 mesophyll, CA catalyses the first reaction in the C_4 photosynthetic pathway (Hatch and Burnell, 1990)—the hydration of atmospheric CO_2—producing bicarbonate for phospho*enol*pyruvate carboxylase (PEPC), the primary carboxylase of C_4 plants.

Chloroplastic forms of β-CA are also found in the leaves of C_4 plants (Tetu *et al.*, 2007), and C_3 plants contain cytosolic forms of the enzyme (Kachru and Anderson, 1974; Reed, 1979; Fett and Coleman, 1994; Rumeau *et al.*, 1996; Fabre *et al.*, 2007; Tanz *et al.*, 2009). Chloroplastic β-CA isoforms in C_4 plants are likely to carry out at least some of the same functions as those described above for C_3 plant plastidial

β-CAs, although ensuring adequate concentrations of CO_2 are available for Rubisco and carbohydrate production is not likely to be a primary role in C_4 species due to their biochemical CO_2-concentrating mechanism (CCM). Non-photosynthetic, cytosolic forms of β-CA are thought to provide non-photosynthetic PEPC isoforms with bicarbonate for anaplerotic roles such as replenishment of tricarboxylic acid intermediates, amino acid synthesis, and maintenance of cellular pH (Fett and Coleman, 1994; Raven and Newman, 1994).

β-CA isoforms in *Flaveria*

Most of what is currently known about the molecular evolution of plant β-CAs has come from work using *F. pringlei* Gandoger, a C_3 species (Tanz *et al.*, 2009), and the C_4 species *F. bidentis* (L.) Kuntze (Tetu *et al.*, 2007). cDNAs encoding three distinct β-CA isoforms have been isolated from leaf tissues of these species, and the orthologous genes encoding CA1, CA2, and CA3 have been identified through sequence analyses. Greater than 90% amino acid sequence identity is seen between the isoforms encoded by orthologues (Ludwig and Burnell, 1995; Tetu *et al.*, 2007; Tanz *et al.*, 2009), and amino acid residues involved in substrate binding and catalysis in pea (Provart *et al.*, 1993; Kimber and Pai, 2000) and spinach (Bracey *et al.*, 1994) β-CAs are highly conserved in all *Flaveria* CA sequences published to date (Cavallaro *et al.*, 1994; Ludwig and Burnell, 1995; Tetu *et al.*, 2007; Tanz *et al.*, 2009).

The location of CA in the leaves of *F. pringlei* and *F. bidentis* was resolved using immunocytochemistry with an anti-*F. bidentis* CA3 antiserum. Labelling was detected in the choroplasts and in the cytosol of leaf mesophyll cells from both *F. pringlei* (Tanz *et al.*, 2009) and *F. bidentis* (Tetu *et al.*, 2007). Chloroplast import assays, using isolated pea chloroplasts and radiolabelled CA precursor proteins, were used to determine which *F. pringlei* and *F. bidentis* β-CAs were chloroplastic enzymes. The results of these assays demonstrated that for *F. pringlei*, two CA isoforms, CA1 and CA3, were imported into isolated pea chloroplasts with a concomitant decrease in the molecular masses of the CA1 and CA3 precursor molecules, indicating processing to the mature forms of the enzymes through removal of chloroplast transit peptides during import. In contrast, *F. pringlei* CA2 was not imported into isolated pea chloroplasts and is presumably a cytosolic form of the enzyme in this C_3 species (Tanz *et al.*, 2009). When *F. bidentis* CA precursor proteins were used in the assays, only CA1 was imported into the pea chloroplasts, indicating that this C_4 species contains two cytosolic forms of CA, CA2 and CA3 (Tetu *et al.*, 2007). All these localization results are supported by protein targeting prediction programs, which predict that the N-terminal ends of *F. pringlei* CA1 and CA3 and *F. bidentis* CA1 encode chloroplast transit peptides, but the N-termini of *F. pringlei* CA2 and *F. bidentis* CA2 and CA3 encode no organelle targeting information (Tetu *et al.*, 2007; Tanz *et al.*, 2009).

The *CA1–CA3* genes from *F. pringlei* and *F. bidentis* show differential expression in leaves, roots, and flowers. In *F. pringlei* leaves, quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) assays showed that *CA1* and *CA3* gene transcripts are of equal abundance, and at least 10 times more abundant than *CA2* mRNA (Fig. 1; Tanz *et al.*, 2009). In the leaves of the C_4 *F. bidentis*, *CA3* mRNA levels are at least 50 times greater than those of *CA1* and *CA2* transcripts (Fig. 1; Tetu *et al.*, 2007). On a leaf total RNA basis, *F. bidentis CA3* gene transcript levels are about a magnitude greater than those of the *F. pringlei CA3* gene (Fig. 1). In roots and flowers of *F. pringlei*, *CA2* mRNAs are the most abundant CA gene transcript, with *CA3* transcripts below the level of detection in these non-photosynthetic organs, and *CA1* mRNA just detectable in flowers but not in *F. pringlei* roots (Table 1; Tanz *et al.*, 2009). *Flaveria bidentis CA2* transcript levels are also higher than those of *CA1* and *CA3* mRNAs in roots; however, in *F. bidentis* flowers, *CA2* and *CA3* transcript levels are nearly equal, most probably reflecting the presence of photosynthetic tissues surrounding the still maturing florets in the composite flower of this species (Table 1; Tetu *et al.*, 2007).

Potential roles of CA1 and CA2 in *Flaveria* C_3 and C_4 species

Table 1 summarizes the CA isoform localization and gene expression patterns described above, which indicate that CA1 is a chloroplastic form of the enzyme that is expressed in the leaves of both C_3 and C_4 *Flaveria* species. In the mesophyll cells of *F. pringlei* and in the bundle-sheath cells

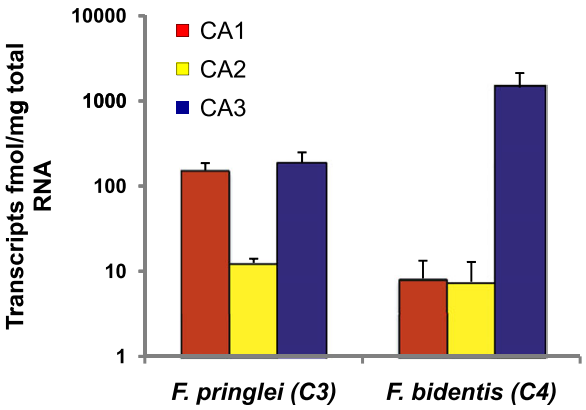

Fig. 1. *CA1*, *CA2*, and *CA3* gene transcript abundance in leaves of *Flaveria pringlei* and *Flaveria bidentis*. Transcript levels were determined using quantitative reverse transcription PCR assays and *F. pringlei* or *F. bidentis CA1–CA3* gene-specific primers, and are based on the total amount of RNA used in the assays. Error bars represent the SE of at least three independent reactions for *F. pringlei* and the SD of two independent replicates for *F. bidentis*. Data were originally reported in Tetu *et al.* (2007) and Tanz *et al.* (2009). www.plantphysiol.org, Copyright American Society of Plant Biologists.

of *F. bidentis*, CA1 isoforms are likely to be responsible for one or more of the functions described above for plastidial β-CAs (Fig. 2). As these results were collected from *in vitro* import assays and whole leaf mRNA, the possibility that CA1 may also carry out the non-photosynthetic roles of plastidial β-CAs in the chloroplasts of *F. bidentis* mesophyll cells, and/or regulate stomatal movement (Hu *et al.*, 2010) in *F. pringlei* and *F. bidentis* guard cells cannot be ruled out.

Flaveria bidentis and *F. pringlei CA2* gene transcripts, which encode cytosolic forms of β-CA, are of relatively low abundance in both photosynthetic and non-green tissues, suggesting that this CA isoform may be responsible for supplying bicarbonate to housekeeping forms of PEPC in both photosynthetic subtypes (Fig. 2). Interestingly, when the deduced amino acid sequences of the *F. pringlei* and *F. bidentis* CA2 isoforms are compared with those of the six *Arabidopsis* β-CAs (Fabre *et al.*, 2007), the highest identity is seen with *At*βCA4 (Tanz *et al.*, 2009), which localizes to *Arabidopsis* (Fabre *et al.*, 2007) and tobacco (Hu *et al.*, 2010) cell plasma membranes, and along with *At*βCA1 has recently been shown to be involved in stomatal movement (Hu *et al.*, 2010). A number of associations between cytosolic β-CAs and plant plasma membranes have been documented (Utsunomiya and Muto, 1993; Santoni *et al.*, 1998; Kawamura and Uemura, 2003; Alexandersson *et al.*, 2004; Mongrand *et al.*, 2004; Benschop *et al.*, 2007; Mitra *et al.*, 2007; Tang *et al.*, 2008), and in one of these (Mongrand *et al.*, 2004), the β-CA was also found closely associated with a plasma membrane-located aquaporin shown to have CO_2 transport activity (Uehlein *et al.*, 2003). The current localization and expression data for CA2 in C_3 and C_4 *Flaveria* congeners certainly leave open the possibility that this cytosolic CA isoform may play a role in cellular CO_2 conductance and/or diffusion. The possibility that CA2

Table 1. *Carbonic anhydrase isoform location and gene expression patterns in* Flaveria pringlei *and* Flaveria bidentis

	CA isoform location[a]		Relative CA transcript abundance[b]		
	Chloroplast	Cytosol	Leaves	Roots	Flowers
F. pringlei (C_3)	CA1; CA3	CA2	CA1=CA3>>CA2	CA2	CA2>>CA1
F. bidentis (C_4)	CA1	CA2; CA3	CA3>>>CA1=CA2	CA2>CA3>CA1	CA2=CA3>CA1

[a] Localization results are based on *in vitro* chloroplast import assays (Tetu *et al.*, 2007; Tanz *et al.*, 2009).
[b] Relative transcript abundance data are from qRT-PCR assays (Tetu *et al.*, 2007; Tanz *et al.*, 2009).

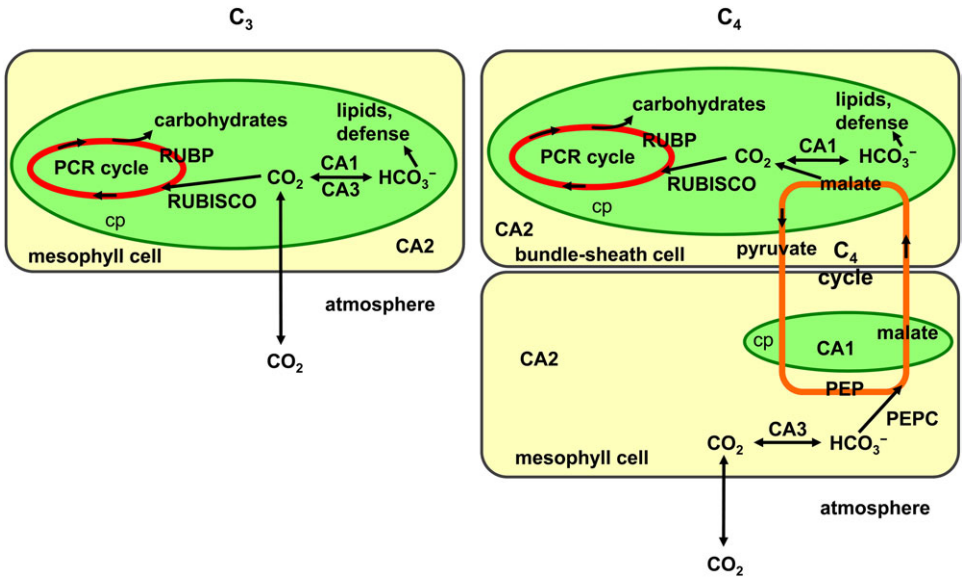

Fig. 2. Schematic diagram of the C_3 and C_4 photosynthetic pathways and the locations of carbonic anhydrase (CA) isoforms. In C_3 plants most CA activity localizes to the chloroplasts (cp) of the mesophyll cells where it plays a role in lipid biosynthesis, defence, and potentially in ensuring adequate concentrations of CO_2 are available for carbohydrate production via ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) and the other enzymes of the photosynthetic carbon reduction (PCR) cycle. In C_3 *Flaveria* species, CA1 and CA3 are chloroplastic enzymes, while CA2 is cytosolic. In the leaves of C_4 plants, most CA activity is found in the cytosol of the mesophyll cells where it catalyses the conversion of atmospheric CO_2 to bicarbonate, which is then used to carboxylate phospho*enol*pyruvate (PEP) by PEP carboxylase (PEPC), producing a four-carbon compound, such as malate, that then diffuses into the bundle-sheath cell where it is decarboxylated. In C_4 *Flaveria* species, decarboxylation occurs in the bundle-sheath chloroplasts through NADP-malic enzyme activity. The released CO_2 is fixed by Rubisco and the pyruvate is used to regenerate PEP. In C_4 *Flaveria* species, CA1 localizes to the chloroplast while CA2 and CA3 are cytosolic, with the latter catalysing the first step in the C_4 pathway. RUBP, ribulose-1,5-bisphosphate.

may contribute bicarbonate to the *F. bidentis* C_4 pathway cannot be dismissed; however, the low steady-state transcript levels of this CA isoform compared with those of CA3 in *F. bidentis* leaves argue against a major role for CA2 in the pathway.

As indicated above, it is likely that in C_4 *Flaveria* species, the ancestral C_3 roles of the CA1 and CA2 isoforms have been maintained; however, the functions of these isoforms in *Flaveria* species, as well as those of their homologues in other C_3 and C_4 plants, still await clarification. Resolution of the cell type-specific expression patterns of these isoforms and identification of the proteins interacting with each in C_3 and C_4 species will fill some of these gaps, and will result in a more complete picture of the physiological processes involving β-CAs in C_3 and C_4 plants, and the molecular evolution of the C_4 pathway in *Flaveria*.

Flaveria CA3: how a chloroplastic C_3 CA became the cytosolic isoform necessary for the C_4 biochemical CCM

Unlike CA1 and CA2 from the *Flaveria* congeners, the CA3 isoforms from *F. pringlei* and *F. bidentis* show striking differences with respect to their cellular locations and the expression patterns of their cognate genes (Table 1). The high levels of *CA3* transcripts in *F. bidentis* leaves relative to other *F. bidentis* organs and relative to the levels of *F. pringlei CA3* transcripts (Fig 1; Table 1), and the demonstration that this isoform is not imported into isolated pea chloroplasts (Tetu *et al.*, 2007) but *F. pringlei* CA3 is (Tanz *et al.*, 2009) strongly argue that this is the enzyme catalysing the formation of bicarbonate for PEPC in the *F. bidentis* mesophyll cytosol. This premise is further supported by results of experiments in which an antisense construct targeted against *F. bidentis CA3* was used to transform wild-type *F. bidentis* plants. It was shown that although CA activity is not limiting for CO_2 assimilation in wild-type plants, high activity is required in the C_4 mesophyll cytosol for the C_4 pathway to function as a biochemical CCM (von Caemmerer *et al.*, 2004).

When the deduced amino acid sequences of the *F. pringlei* and *F. bidentis* CA3 isoforms are compared, 93% identity is seen over the shared regions when the initiating methionine of *F. bidentis* CA3 is aligned with the second methionine of the *F. pringlei* isoform (Met72; Fig. 3). The N-terminal 71 amino acids of *F. pringlei* CA3 encode a chloroplast transit sequence with the characteristically high proportion of serine and threonine residues and a low number of charged amino acids (von Heijne *et al.*, 1989).

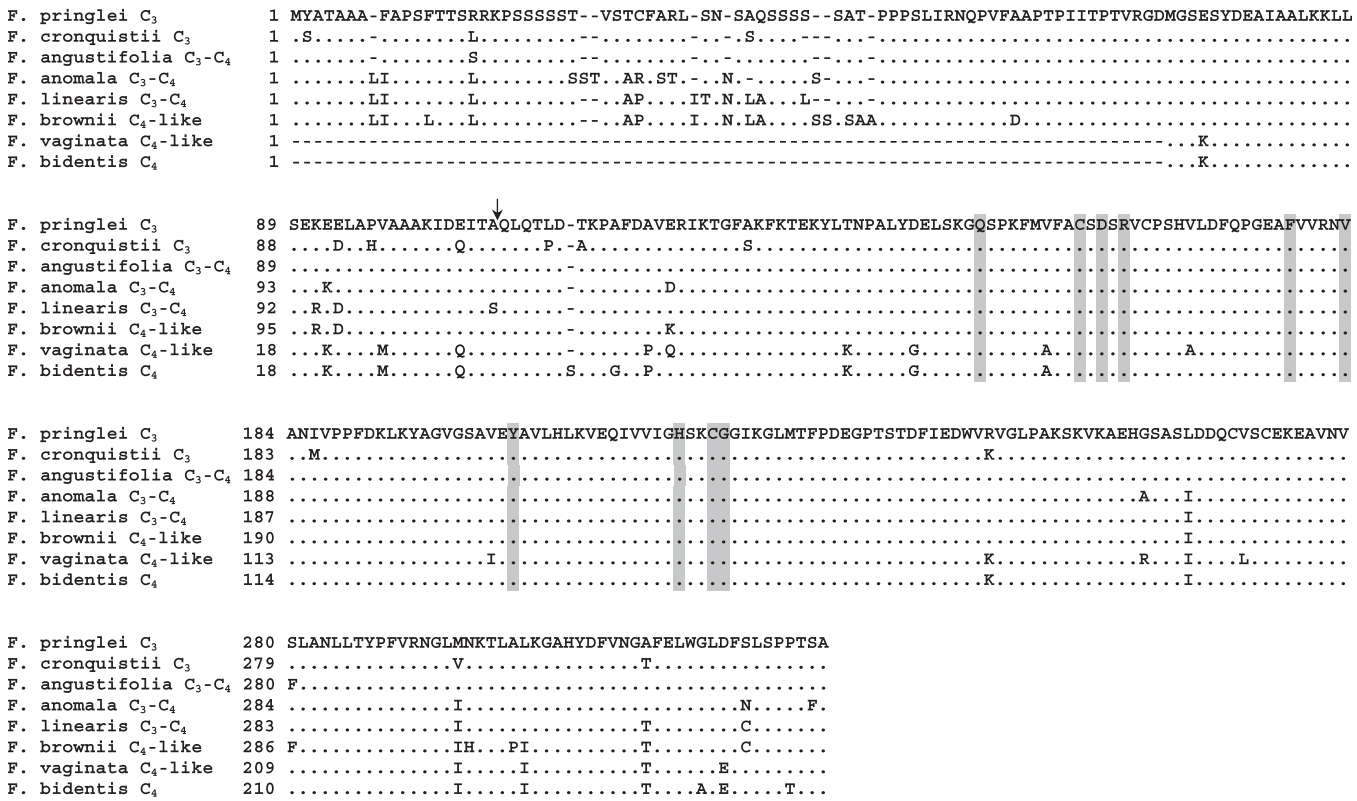

Fig. 3. Alignment of the deduced amino acid sequences of CA3 isoforms from selected *Flaveria* species. Identical resides are depicted as dots, while gaps inserted to maximize the alignment are represented by dashes. The predicted cleavage site of plastidial C_3 dicot β-CAs is indicated by the arrow (Burnell *et al.*, 1990; Roeske and Ogren, 1990). Residues involved in Zn^{2+} binding and the active site of pea CA are shaded grey (Provart *et al.*, 1993; 1994; Kimber and Pai, 2000). GenBank accession numbers: *F. pringlei* CA3, DQ273587; *F. cronquistii* CA3, JF313387; *F. angustifolia* CA3, JF313384; *F. anomala* CA3, JF313385; *F. linearis* CA3, DQ273588; *F. brownii* CA3, JF313386; *F. vaginata* CA3, JF313388; *F. bidentis* CA3, AY16711.

Altogether, the results of the localization, expression, transgenic, and sequence analyses indicate that the C_3 *CA3* gene was the evolutionary template for the cytosolic C_4 form of the enzyme. During the evolution of the C_4 pathway in *Flaveria*, mutation(s) occurred in the sequence encoding the chloroplast transit peptide of the ancestral C_3 *CA3* gene, which prevented the import of the isoform into the chloroplasts of C_4 species, and instead allowed the hydration of atmospheric CO_2 to bicarbonate in the mesophyll cytosol (Fig. 2).

Changes in the control regions of the ancestral C_3 *CA3* gene must also have occurred to account for the mesophyll-dependent, high level of CA3 expression in present-day C_4 *Flaveria* species. Sequence analysis of the region 2 kb upstream of the *F. bidentis* CA3 translation initiation site identified a sequence similar to that of the *Mesophyll expression module 1* (*Mem1*; SK Tanz and M Ludwig, unpublished results), which directs mesophyll-specific expression of *ppcA*, the C_4 PEPC gene in *Flaveria* (Gowik *et al.*, 2004), and, in combination with a proximal promoter region, regulates high levels of PEPC expression in the C_4 mesophyll (Engelmann *et al.*, 2008). No *Mem1*-like sequence was found in the 4 kb region upstream of the *F. pringlei* CA3 translation initiation site (SK Tanz and M Ludwig, unpublished results). Preliminary work has indicated that the 2 kb upstream region of the *F. bidentis CA3* gene directs mesophyll-specific expression of a β-glucuronidase (GUS)–reporter construct (U Gowik, P Westhoff, and M Ludwig, unpublished results). The possibility that the expression of the *Flaveria ppcA* and *CA3* genes, which encode the enzymes that catalyse the first two steps in the C_4 pathway, resulted from the co-option of a similar regulatory strategy and control elements during evolution is intriguing, and identification of specific regulatory elements within the 2 kb *F. bidentis CA3* upstream region is underway.

Although the role of CA3 in C_4 *Flaveria* species is unambiguous, this is not the case for the CA3 isoform in *F. pringlei*. As described above, diverse roles have been proposed and shown for plastidial β-CAs. As *F. pringlei* CA3 transcripts were detected in only the photosynthetic tissues examined by Tanz *et al.* (2009), this isoform may function in CO_2 diffusion to Rubisco in C_3 *Flaveria* chloroplasts (Reed and Graham, 1981; Cowan, 1986; Price *et al.*, 1994). This role would have been expendable in an evolving C_4 system because of the developing biochemical CCM, presenting the opportunity for neofunctionalization of the *CA3* gene in C_4 lineages. Non-photosynthetic roles described above for C_3 plastidial β-CAs, such as lipid synthesis and defence, which are also necessary in C_4 species, would be catalysed by the chloroplastic CA1 isoform.

Chloroplastic and cytosolic CA isoforms in *Flaveria* photosynthetic intermediates

Sequence information and protein targeting prediction data accumulated thus far for CA1 and CA2 isoforms from the C_3–C_4 intermediates *F. angustifolia* (Cav.) Persoon, *F. anomala* B.L. Robinson, and *F. linearis* Lagasca, the C_4-like species *F. brownii* A.M. Powell and *F. vaginata* B.L. Robinson & Greenman, and another C_3 species, *F. cronquistii* A.M. Powell, indicate that these proteins are chloroplastic and cytosolic isoforms, respectively, in all these species (ML, unpublished results).

Complete open reading frames encoding CA3 isoforms have been isolated from rapid amplification of cDNA ends (RACE) libraries made from leaf mRNA of the above intermediates and *F. cronquistii*, using primers that bind to highly conserved regions of *F. pringlei* and *F. bidentis* CA3 cDNAs. Like the CA3 isoforms from *F. pringlei* and *F. bidentis*, the predicted protein sequences of these recently acquired CA3 cDNAs show high identity (at least 88%) and contain all the amino acid residues required for zinc binding and β-CA active site formation (Fig. 3; Provart *et al.*, 1993; Bracey *et al.*, 1994; Kimber and Pai, 2000).

The N-termini of the CA3 isoforms from the C_3 *F. cronquistii* and all the photosynthetic intermediates except *F. vaginata* contain a high number of hydroxylated amino acids and few charged residues, and all are predicted by the protein targeting prediction program Predotar (Small *et al.*, 2004) to encode chloroplast transit peptides (data not shown). In contrast, the N-terminus of the deduced *F. vaginata* CA3 amino acid sequence aligns with the initiating methionine of the *F. bidentis* CA3 isoform, the CA supplying PEPC with bicarbonate in the cytosol of C_4 mesophyll cells.

Mapping the type of CA3 found in the C_3, C_3–C_4, C_4-like, and C_4 *Flaveria* species so far examined onto the *Flaveria* phylogeny re-constructed by McKown *et al.* (2005) shows that the cytosolic (C_4) and chloroplastic (C_3) CA3 isoforms cluster in different clades (Fig 4). The C_3 isoforms are found in the basal C_3 species and in species clustering in clade B, which, with the exception of *F. brownii*, contains only C_3–C_4 intermediate species. In contrast, the C_4 isoforms are found in species in clade A, which contains C_3–C_4, C_4-like, and true C_4 *Flaveria* species (Fig. 4). Of particular interest is the finding that *F. brownii* and *F. vaginata*, which are both recognized as C_4-like intermediates (Table 2; Smith and Powell, 1984; Monson *et al.*, 1986; Moore *et al.*, 1987, 1989; Cheng *et al.*, 1988; Chastain and Chollet 1989; Ku *et al.*, 1991; McKown and Dengler 2007), contain forms of CA3 that are structurally characteristic of C_3 and C_4 *Flaveria* species, respectively, and consequently, localize to different intracellular compartments and perform different biochemical roles.

Flaveria brownii and *F. vaginata*: C_4-like intermediates at different steps on the pyramid of C_4 photosynthesis

Both *F. brownii* and *F. vaginata* demonstrate Kranz leaf anatomy, and few quantitative differences are detected between their leaves and those of true C_4 *Flaveria* species, although the numbers of mesophyll chloroplasts and cell

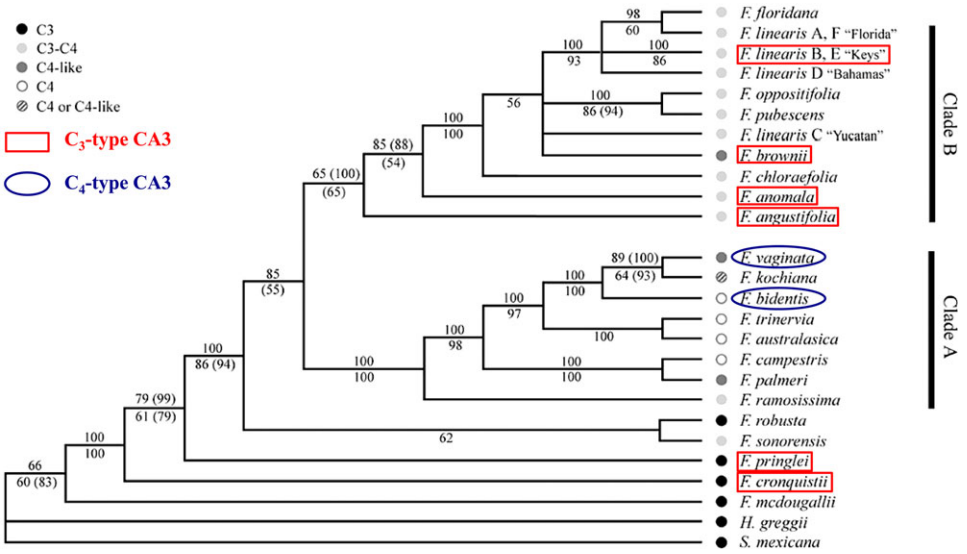

Fig. 4. The distribution of CA3 isoform types in *Flaveria*. C_3-type CA3 isoforms (red boxes) are found in the ancestral C_3 *Flaveria* species, the C_3–C_4 intermediates in Clade B examined so far, and *F. brownii*, a C_4-like *Flaveria* species also in Clade B. C_4-type CA3 isoforms (blue circles) are found in the C_4 species *F. bidentis* and *F. vaginata*, a C_4-like species, both in Clade A. Diagram modified from McKown *et al.* (2005) used by permission of the *American Journal of Botany*.

Table 2. *Phylogenetic clustering, type of CA3 isoform, and gas exchange properties of selected C_3, C_4, and photosynthetic intermediate species of* Flaveria

Species	Clade[a]	CA3 type	CO_2 assimilation rate[b] (μmol m^{-2} s^{-1})	CO_2 compensation point[b] (μbar)	O_2 inhibition of CO_2 assimilation[b] (%)	$\delta^{13}C$[c] (‰)
F. cronquistii (C_3)	Basal	C_3	16.8±0.9	60.4±1.7	30.2±0.7	–23.0
F. pringlei (C_3)	Basal	C_3	20.9±0.8	62.0±0.3	32.2±1.0	–26.5
F. angustifolia (C_3–C_4)	B	C_3	24.4±0.3	24.1±0.4	26.8±0.2	–26.8
F. anomala (C_3–C_4)	B	C_3	13.9±0.3	15.5±0.7	22.4±1.7	–28.5
F. linearis (C_3–C_4)	B	C_3	17.0±1.7	27.0±1.7	25.9±0.9	–27.4
F. brownii (C_4-like)	B	C_3	25.2±0.6	6.0±1.3	11.4±0.5	–17.3
F. vaginata (C_4-like)	A	C_4	27.2±2.1	3.0±1.2	7.1±0.3	–15.3
F. bidentis (C_4)	A	C_4	32.4±0.5	3.2±0.3	–1.2±0.4	–16.5

[a] Data from McKown *et al.* (2005).
[b] Data from Ku *et al.* (1991).
[c] Data from Smith and Powell (1984).

layers are greater in *F. brownii* (McKown and Dengler, 2007). As well as having a C_4-type CA3, *F. vaginata* has a characteristic C_4 *Mem1* regulatory element upstream of the *ppcA* gene, with a guanine in the first position of the A submodule and a CACT tetranucleotide in the B submodule (Gowik *et al.*, 2004; Akyildiz *et al.*, 2007). In contrast, *F. brownii* has the C_4 *ppcA* B submodule CACT sequence, but a C_3-specific adenine in the first position of the A submodule, and consequently has an intermediate *Mem1* sequence like that of the C_3–C_4 intermediate *F. pubescens* Rydberg (Akyildiz *et al.*, 2007). Interestingly, however, neither species shows complete compartmentation of PEPC and Rubisco in the mesophyll and bundle-sheath, respectively (Cheng *et al.*, 1988; Moore *et al.*, 1989).

From the above characteristics, a more C_4-like photosynthetic physiology is predicted for *F. vaginata* than *F. brownii*, and the gas exchange data shown in Table 2 support this idea; the CO_2 compensation point of *F. vaginata* is the same as that of the C_4 *F. bidentis*, and about half that of *F. brownii*. *Flaveria vaginata* CO_2 assimilation rates, carbon isotope ratios ($\delta^{13}C$), and oxygen inhibition of CO_2 assimilation values are also more C_4 like than those of *F. brownii*.

A seven-phase model describing the evolution of C_4 photosynthesis proposes that the earliest phase involved gene duplication events and other general modifications to a C_3 ancestor that predisposed it to subsequent steps toward C_4-ness (Sage, 2004). Phases two and three involved changes to leaf anatomy, including reducing the distance between veins and development of a C_4-like bundle sheath, that then allowed biochemical changes to occur in phases four and five. In the last two phases, the derived biochemistry was integrated and coordinated with the ancestral pathway, and then ultimately a full C_4 pathway was optimized (Sage, 2004). In light of this scheme, it appears that *F. vaginata* has ascended more steps of the C_4 pyramid than *F. brownii*, having acquired the C_4 regulatory components for

mesophyll-specific expression of PEPC and a C_4-type CA3. Several other C_3–C_4 and C_4-like intermediates cluster in clade A of the *Flaveria* phylogeny with *F. vaginata* and the true C_4 *Flaveria* species (McKnown *et al.*, 2005), and their inclusion in future comparative studies at the molecular level may give further insights into the pre-conditions that were necessary and the steps that were taken during the evolution of the C_4 photosynthetic pathway in *Flaveria*.

Conclusions

The identification of sequences encoding β-CA orthologues in C_3 and C_4 *Flaveria* species, resolution of their expression patterns, and intracellular localization of the isoforms they encode have provided evidence for gene duplication and neofunctionalization events during the evolution of the C_4 pathway in the genus. From these results, the current working model for the evolution of β-CA in C_4 *Flaveria* species envisages a duplication of the gene encoding a chloroplastic CA in the ancestral C_3 *Flaveria* species, with one of the duplicates subsequently accumulating mutations in coding and non-coding regions. These modifications led to the loss of the sequence encoding the chloroplast transit peptide and gain of regulatory regions that direct high levels of mesophyll cell-specific expression, and resulted in the β-CA isoform that synthesizes bicarbonate in the first step in CO_2 assimilation in C_4 *Flaveria* species.

During the evolution of the C_4 syndrome in *Flaveria*, changes to C_3 leaf anatomy, which resulted in characteristic Kranz anatomy, were acquired stepwise, with particular traits preceding others, and these structural modifications evolved prior to C_4 biochemistry (McKown and Dengler, 2007). The studies described above suggest that the evolutionary route to C_4 biochemistry in *Flaveria* may have also depended on a specific stepwise acquisition of modifications. Further work on C_4 enzymes as well as metabolite transporters in *Flaveria* congeners using recent advances in cell type separation methods, cell and protein labelling techniques, and '-omics' technologies is needed to elucidate fully the underlying molecular biology and biochemistry responsible for the photosynthetic physiologies of the *Flaveria* intermediates, and to resolve the molecular evolutionary path to full C_4 photosynthetic biochemistry in this genus. This information will generate a blueprint for what it took to evolve, and what it will probably take to construct, a C_4 plant from a C_3 ancestor.

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