The molecular evolution of β-carbonic anhydrase in Flaveria

Martha Ludwig*

School of Biomedical, Biomolecular and Chemical Sciences, University of Western Australia, 35 Stirling Highway, Crawley, WA 6009, Australia

* To whom correspondence should be addressed. E-mail: martha.ludwig@uwa.edu.au

Received 17 November 2010; Accepted 21 February 2011

Abstract

Limited information exists regarding molecular events that occurred during the evolution of C4 plants from their C3 ancestors. The enzyme β-carbonic anhydrase (CA; EC 4.2.1.1), which catalyses the reversible hydration of CO2, is present in multiple forms in C3 and C4 plants, and has given insights into the molecular evolution of the C4 pathway in the genus Flaveria. cDNAs encoding three distinct isoforms of β-CA, CA1–CA3, have been isolated and examined from Flaveria C3 and C4 congensers. Sequence data, expression analyses of CA orthologues, and chloroplast import assays with radiolabelled CA precursor proteins from the C3 species F. pringlei Gandoger and the C4 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 C4 photosynthesis and indicate that the C4 CA3 gene evolved as a result of gene duplication and neofunctionalization, which involved mutations in coding and non-coding regions of the ancestral C3 CA3 gene. Comparisons of the deduced CA3 amino acid sequences from Flaveria C3, C4, and photosynthetic intermediate species showed that all the C3–C4 intermediates investigated and F. brownii, a C4-like species, have a C3-type CA3, while F. vaginata, another C4-like species, contains a C4-type CA3. These observations correlate with the photosynthetic physiologies of the intermediates, suggesting that the molecular evolution of C4 photosynthesis in Flaveria may have resulted from a temporally dependent, stepwise modification of protein-encoding genes and their regulatory elements.

Key words: C4 photosynthesis, carbonic anhydrase, Flaveria, molecular evolution, photosynthetic intermediate.

Introduction

Plants using the C4 photosynthetic pathway have evolved from C3 ancestors in multiple angiosperm lineages, with falling CO2 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 C4 condition during the Miocene and Pleistocene occurred due to changes in other environmental factors that promote photorespiration in C3 plants such as heat, aridity, and soil salinity (Sage, 2004; Osborne and Beering, 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 C4 plants to concentrate CO2 around ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), almost eliminating photorespiration. As a consequence, C4 plants outcompete C3 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 C4 plants of different ancestry have been recognized for years, relatively little is known about the molecular mechanisms underlying the evolution of the C4 pathway. Determining how the genes controlling the advantageous survival traits of C4 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 C4 plant will expand our understanding of what molecular mechanisms were used and required in the evolution of C4 pathways.
and this will contribute to targeted strategies for transferring these traits to C₃ crop plants.

Flaveria: a model genus to study the molecular evolution of C₄ photosynthesis

Flaveria is one of several plant genera that contain individual species that perform C₃ photosynthesis, others that use the C₄ pathway, and still others that carry out C₃–C₄ or C₄-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 C₃ ancestral condition to the more highly derived C₄ state (Powell, 1978; Edwards and Ku, 1987; McKown et al., 2005; McKown and Dengler, 2007). Phylogenetic information indicates that C₄ 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 C₄ 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; Drincovic et al., 1998; Lai et al., 2002). The work from these groups has shown that the genes encoding enzyme isoforms important in Flaveria C₄ photosynthesis evolved from the duplication of an ancestral C₃ 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 C₃ and C₄ plants

CAs (EC 4.2.1.1) of land plants are zinc metalloenzymes that catalyse the reversible hydration of CO₂. 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 CO₂ 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 AtxCA1, AtxCA2, and AtxCA3, 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. AtxCA1 mRNA was found in all above-ground organs tested (Fabre et al., 2007) and this CA is transported to the chloroplast through the secretory pathway (Villarejo et al., 2005). AtxCA2 transcripts, found in stems and roots, and AtxCA3 mRNA, detected in flowers and siliques, increased in abundance when plants were grown in a low CO₂ 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 C₃ or the C₄ 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 C₃ 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 CO₂ across the chloroplast envelope or catalyse the rapid dehydration of bicarbonate to CO₂, ensuring adequate concentrations of CO₂ 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 CO₂ 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, C₃ 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 C₃ photosynthesis remains elusive, the function of β-CA in the C₄ photosynthetic pathway is unequivocal. The greatest CA activity in C₄ 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 C₄ mesophyll, CA catalyses the first reaction in the C₄ photosynthetic pathway (Hatch and Burnell, 1990)—the hydration of atmospheric CO₂—producing bicarbonate for phosphoenolpyruvate carboxylase (PEPC), the primary carboxylase of C₄ plants.

Chloroplastic forms of β-CA are also found in the leaves of C₄ plants (Tetu et al., 2007), and C₃ 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 C₄ plants are likely to carry out at least some of the same functions as those described above for C₃ plant plastidial
β-CAs, although ensuring adequate concentrations of CO₂ are available for Rubisco and carbohydrate production is not likely to be a primary role in C₄ species due to their biochemical CO₂-concentrating mechanism (CCM). Non-photosynthetic, cytosolic forms of β-CA are thought to provide non-photosynthetic PEPC isoforms with bicarbonate for anaerobic 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 C₃ species (Tanz et al., 2009), and the C₄ 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 identified through sequence analyses. Greater than 90% gous genes encoding CA1, CA2, and CA3 have been bcdNA sequences published to date (Cavallaro et al., 1994; 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 antisera. Labelling was detected in the chloroplasts 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 C₃ 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 C₄ 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 C₄ 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 C₃ and C₄ 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 C₃ and C₄ 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.](http://jxb.oxfordjournals.org/)...
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 *AtβCA4* (Tanz *et al.*, 2009), which localizes to *Arabidopsis* (Fabre *et al.*, 2007) and tobacco (Hu *et al.*, 2010) cell plasma membranes, and along with *AtβCA1* 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 CO2 transport activity (Uehlein *et al.*, 2003). The current localization and expression data for CA2 in *C3* and *C4 Flaveria* congeners certainly leave open the possibility that this cytosolic CA isoform may play a role in cellular CO2 conductance and/or diffusion. The possibility that CA2

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

<table>
<thead>
<tr>
<th>CA isoform location</th>
<th>Relative CA transcript abundance</th>
</tr>
</thead>
<tbody>
<tr>
<td>F. pringlei (C3)</td>
<td>Leaves: ∆∆CA3&gt;CA2 &gt; CA1, Roots: CA2, Flowers: CA2&gt;CA1</td>
</tr>
<tr>
<td>F. bidentis (C4)</td>
<td>Leaves: ∆∆CA1&gt;CA2&gt;CA3, Roots: CA2&gt;CA3&gt;CA1, Flowers: CA2=CA3&gt;CA1</td>
</tr>
</tbody>
</table>

*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 C3 and C4 photosynthetic pathways and the locations of carbonic anhydrase (CA) isoforms. In C3 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 CO2 are available for carbohydrate production via rubulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) and the other enzymes of the photosynthetic carbon reduction (PCR) cycle. In C3 *Flaveria* species, CA1 and CA3 are chloroplastic enzymes, while CA2 is cytosolic. In the leaves of C4 plants, most CA activity is found in the cytosol of the mesophyll cells where it catalyses the conversion of atmospheric CO2 to bicarbonate, which is then used to carboxylate phosphoenolpyruvate (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 C4 *Flaveria* species, decarboxylation occurs in the bundle-sheath chloroplasts through NADP-malic enzyme activity. The released CO2 is fixed by Rubisco and the pyruvate is used to regenerate PEP. In C4 *Flaveria* species, CA1 localizes to the chloroplast while CA2 and CA3 are cytosolic, with the latter catalysing the first step in the C4 pathway. RUBP, ribulose-1,5-bisphosphate.*

![Diagram](http://jxb.oxfordjournals.org/figs/2013/08/suppl_3/3074_fig2.png)
may contribute bicarbonate to the *F. bidentis* C₄ 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 C₄ *Flaveria* species, the ancestral C₃ 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 C₃ and C₄ plants, still await clarification. Resolution of the cell type-specific expression patterns of these isoforms and identification of the proteins interacting with each in C₃ and C₄ species will fill some of these gaps, and will result in a more complete picture of the physiological processes involving β-CAs in C₃ and C₄ plants, and the molecular evolution of the C₄ pathway in *Flaveria*.

**Flaveria CA3: how a chloroplastic C₃ CA became the cytosolic isoform necessary for the C₄ 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 CO₂ assimilation in wild-type plants, high activity is required in the C₄ mesophyll cytosol for the C₄ 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 residues are depicted as dots, while gaps inserted to maximize the alignment are represented by dashes. The predicted cleavage site of plastidial C₃ dicot β-CAs is indicated by the arrow (Burnell et al., 1990; Roeske and Ogren, 1990). Residues involved in Zn²⁺ 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.](http://jxb.oxfordjournals.org/)

Downloaded from http://jxb.oxfordjournals.org/ at QP site access on April 22, 2013
Altogether, the results of the localization, expression, transgenic, and sequence analyses indicate that the C₃ CA3 gene was the evolutionary template for the cytosolic C₄ form of the enzyme. During the evolution of the C₄ pathway in *Flaveria*, mutation(s) occurred in the sequence encoding the chloroplast transit peptide of the ancestral C₃ CA3 gene, which prevented the import of the isoform into the chloroplasts of C₄ species, and instead allowed the hydration of atmospheric CO₂ to bicarbonate in the mesophyll cytosol (Fig. 2).

Changes in the control regions of the ancestral C₃ CA3 gene must also have occurred to account for the mesophyll-dependent, high level of CA3 expression in present-day C₄ *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 C₄ PEPC gene in *Flaveria* (Gowik et al., 2004), and, in combination with a proximal promoter region, regulates high levels of PEPC expression in the C₄ 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 C₄ 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 C₄ *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 CO₂ diffusion to Rubisco in C₃ *Flaveria* chloroplasts (Reed and Graham, 1981; Cowan, 1986; Price et al., 1994). This role would have been expendable in an evolving C₄ system because of the developing biochemical CCM, presenting the opportunity for neofunctionalization of the CA3 gene in C₄ lineages. Non-photosynthetic roles described above for C₃ plastidial β-CAs, such as lipid synthesis and defence, which are also necessary in C₄ 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 C₃–C₄ intermediates *F. angustifolia* (Cav.) Persoon, *F. anomala* B.L. Robinson, and *F. linearis* Lagasca, the C₄-like species *F. brownii* A.M. Powell and *F. vaginata* B.L. Robinson & Greenman, and another C₃ 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 C₃ *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 C₄ mesophyll cells.

Mapping the type of CA3 found in the C₃, C₃–C₄, C₄-like, and C₄ *Flaveria* species so far examined onto the *Flaveria* phylogeny re-constructed by McKown et al. (2005) shows that the cytosolic (C₄) and chloroplastic (C₃) CA3 isoforms cluster in different clades (Fig. 4). The C₃ isoforms are found in the basal C₃ species and in species clustering in clade B, which, with the exception of *F. brownii*, contains only C₃–C₄ intermediate species. In contrast, the C₄ isoforms are found in species in clade A, which contains C₃–C₄, C₄-like, and true C₄ *Flaveria* species (Fig. 4). Of particular interest is the finding that *F. brownii* and *F. vaginata*, which are both recognized as C₄-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 C₃ and C₄ *Flaveria* species, respectively, and consequently, localize to different intracellular compartments and perform different biochemical roles.

**Flaveria brownii** and *F. vaginata*: C₄-like intermediates at different steps on the pyramid of C₄ photosynthesis

Both *F. brownii* and *F. vaginata* demonstrate Kranz leaf anatomy, and few quantitative differences are detected between their leaves and those of true C₄ *Flaveria* species, although the numbers of mesophyll chloroplasts and cell...
layers are greater in *F. brownii* (McKown and Dengler, 2007). As well as having a C₄-type CA3, *F. vaginata* has a characteristic C₄ 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 C₄ *ppcA* B submodule CACT sequence, but a C₃-specific adenine in the first position of the A submodule, and consequently has an intermediate Mem1 sequence like that of the C₃–C₄ 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 C₄-like photosynthetic physiology is predicted for *F. vaginata* than *F. brownii*, and the gas exchange data shown in Table 2 support this idea; the CO₂ compensation point of *F. vaginata* is the same as that of the C₄ *F. bidentis*, and about half that of *F. brownii*. *Flaveria vaginata* CO₂ assimilation rates, carbon isotope ratios (δ¹³C), and oxygen inhibition of CO₂ assimilation values are also more C₄ like than those of *F. brownii*.

A seven-phase model describing the evolution of C₄ photosynthesis proposes that the earliest phase involved gene duplication events and other general modifications to a C₃ ancestor that predisposed it to subsequent steps toward C₄-ness (Sage, 2004). Phases two and three involved changes to leaf anatomy, including reducing the distance between veins and development of a C₄-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 C₄ pathway was optimized (Sage, 2004). In light of this scheme, it appears that *F. vaginata* has ascended more steps of the C₄ pyramid than *F. brownii*, having acquired the C₄ regulatory components for

---

**Table 2.** Phylogenetic clustering, type of CA3 isoform, and gas exchange properties of selected C₃, C₄, and photosynthetic intermediate species of *Flaveria*

<table>
<thead>
<tr>
<th>Species</th>
<th>Cladeieber</th>
<th>CA3 type</th>
<th>CO₂ assimilation rateb (µmol m⁻² s⁻¹)</th>
<th>CO₂ compensation pointb (µbar)</th>
<th>O₂ inhibition of CO₂ assimilationb (%)</th>
<th>δ¹³Cc (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>F. cronquistii</em> (C₃)</td>
<td>Basal</td>
<td>C₃</td>
<td>16.8±0.9</td>
<td>60.4±1.7</td>
<td>30.2±0.7</td>
<td>-23.0</td>
</tr>
<tr>
<td><em>F. pringlei</em> (C₃)</td>
<td>Basal</td>
<td>C₃</td>
<td>20.9±0.8</td>
<td>62.0±0.3</td>
<td>32.2±1.0</td>
<td>-26.5</td>
</tr>
<tr>
<td><em>F. angustifolia</em> (C₃–C₄)</td>
<td>B</td>
<td>C₃</td>
<td>24.4±0.3</td>
<td>24.1±0.4</td>
<td>26.8±0.2</td>
<td>-26.8</td>
</tr>
<tr>
<td><em>F. anomala</em> (C₃–C₄)</td>
<td>B</td>
<td>C₃</td>
<td>13.9±0.3</td>
<td>15.5±0.7</td>
<td>22.4±1.7</td>
<td>-28.5</td>
</tr>
<tr>
<td><em>F. linearis</em> (C₃–C₄)</td>
<td>B</td>
<td>C₃</td>
<td>17.0±1.7</td>
<td>27.0±1.7</td>
<td>25.9±0.9</td>
<td>-27.4</td>
</tr>
<tr>
<td><em>F. brownii</em> (C₃–like)</td>
<td>B</td>
<td>C₃</td>
<td>25.2±0.6</td>
<td>6.0±1.3</td>
<td>11.4±0.5</td>
<td>-17.3</td>
</tr>
<tr>
<td><em>F. vaginata</em> (C₃–like)</td>
<td>A</td>
<td>C₄</td>
<td>27.2±2.1</td>
<td>3.0±1.2</td>
<td>7.1±0.3</td>
<td>-15.3</td>
</tr>
<tr>
<td><em>F. bidentis</em> (C₄)</td>
<td>A</td>
<td>C₄</td>
<td>32.4±0.5</td>
<td>3.2±0.3</td>
<td>-1.2±0.4</td>
<td>-16.5</td>
</tr>
</tbody>
</table>

* Data from McKown et al. (2005).
* Data from Ku et al. (1991).
* Data from Smith and Powell (1984).
mesophyll-specific expression of PEPC and a C₄-type CA3. Several other C₃-C₄ and C₄-like intermediates cluster in clade A of the Flaveria phylogeny with F. vaginata and the true C₄ Flaveria species (McKown 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 C₄ photosynthetic pathway in Flaveria.

Conclusions

The identification of sequences encoding β-CA orthologues in C₃ and C₄ 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 C₄ pathway in the genus. From these results, the current working model for the evolution of β-CA in C₄ Flaveria species envisages a duplication of the gene encoding a chloroplastic CA in the ancestral C₃ 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 CO₂ assimilation in C₄ Flaveria species.

During the evolution of the C₄ syndrome in Flaveria, changes to C₃ leaf anatomy, which resulted in characteristic Kranz anatomy, were acquired stepwise, with particular traits preceding others, and these structural modifications evolved prior to C₄ biochemistry (McKown and Dengler, 2007). The studies described above suggest that the evolutionary route to C₄ biochemistry in Flaveria may have also depended on a specific stepwise acquisition of modifications. Further work on C₄ 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 pathway to full C₄ 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 C₄ plant from a C₃ ancestor.

Acknowledgements

I thank Peter Westhoff for stimulating discussions and hosting me during my study leave.

References


Kimber MS, Pai EF. 2000. The active site architecture of Pisum sativum β-carbonic anhydrase is a mirror image of that of α-carbonic anhydrases. EMBO Journal 19, 1407–1418.


Tanz SK, Tetu SG, Vella NGF, Ludwig M. 2009. Loss of the transit peptide and an increase in gene expression of an ancestral chloroplastic carbonic anhydrase were instrumental in the evolution of


