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Evolution of carbonic anhydrase in C_4 plants

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During the evolution of C_4 photosynthesis, the intracellular location with most carbonic anhydrase (CA) activity has changed. In *Flaveria*, the loss of the sequence encoding a chloroplast transit peptide from an ancestral C_3 CA ortholog confined the C_4 isoform to the mesophyll cell cytosol. Recent studies indicate that sequence elements and histone modifications controlling the expression of C_4-associated CAs were likely present in the C_3 ancestral chromatin, enabling the evolution of the C_4 pathway. Almost complete abolishment of maize CA activity yields no obvious phenotype at ambient CO_2 levels. This contrasts with results for *Flaveria* CA mutants, and has opened discussion on the role of CA in the C_4 carbon concentrating mechanism.

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Introduction

Multiple genes encoding distinct carbonic anhydrase (CA; EC 4.2.1.1) isoforms are found in all higher plants so far examined [1–3]. These proteins are divided into three diverse families, α, β, γ, with members of all the families shown to play roles in CO_2 uptake, fixation or recycling, or there is evidence implicating them in these functions [1–4]. The β-CAs are the most abundant CAs in higher plants, with cytosolic, membrane-associated, and organelle-specific isoforms identified. The evolution of β-CAs involved in the C_4 photosynthetic pathway will be the focus of this review.

In the leaves of C_4 plants, the highest β-CA activity is found in the cytosol of mesophyll cells [5,6]. Like all other known CAs, these C_4-associated isoforms catalyze the reversible conversion of carbon dioxide and bicarbonate ($CO_2 + H_2O \Leftrightarrow HCO_3^- + H^+$). In the C_4 mesophyll, the enzyme converts atmospheric CO_2 to bicarbonate, which is then used to carboxylate phospho*enol*pyruvate (PEP) by the primary carboxylase of C_4 plants, PEP carboxylase (PEPC). This reaction initiates the C_4 acid transfer cycle that is integral to the carbon concentrating mechanism (CCM) of C_4 plants, and leads to CO_2 concentrations surrounding ribulose-1,5-bisphospahte carboxylase/oxygenase (Rubisco) in neighboring bundle-sheath cells (BSC) that are at least 10-times that of the surrounding atmosphere [7].

C_3 plants do contain orthologs of the gene encoding the cytosolic C_4 CA isoform; however, in the leaves of C_3 plants, most β-CA activity localizes to the chloroplast stroma of the mesophyll cells [8,9]. This indicates that unlike what is seen for other enzymes in C_4 photosynthesis, the intracellular location with the highest CA activity changed during the evolution of the C_4 pathway from the ancestral C_3 biochemistry.

Interestingly, a significant role for β-CAs in C_3 photosynthesis remains unsettled. It has been suggested that in C_3 plants, a stromal CA would facilitate CO_2 diffusion across the chloroplast envelope and ensure adequate supply of CO_2 to Rubisco [10]. However, mature tobacco [11–13] and *Arabidopsis thaliana* [14] plants with reduced levels of the major stromal β-CA, generated through antisense or knockout technologies, showed no obvious phenotype, or changes in photosynthetic characteristics [11–14]. Instead, C_3 stromal β-CAs have been shown to be involved in pathogen resistance [15–17], seedling survival [14], and lipid biosynthesis [18]. In *A. thaliana*, stromal (*At*βCA1) and membrane-associated (*At*βCA4) β-CA isoforms function in stomatal development through a CO_2-controlled signaling pathway [19,20•]. Overexpression of the mitochondrial β-CA (*AtβCA6*; [21]) in *Arabidopsis* resulted in an increase in plant biomass, but the transformants demonstrated no significant change in photosynthetic rates compared to wild type plants [22•]. The *AtβCA6* overexpression lines, however, did show a decrease in respiration rates. These results support the idea of a basal CCM in C_3 plants whereby CO_2 released from respiration (or photorespiration) is converted to bicarbonate by a mitochondrial β-CA, and then transported to the chloroplast for re-fixation [4].

In addition to the cytosolic β-CA that catalyzes the first step in the C_4 pathway, C_4 plants contain other cytosolic and organellar CA isoforms [3,23]. Although little direct work has been done on the functions of these non-C_4-associated forms of β-CA, it is likely that they carry out at least some of the ancestral C_3 roles described above.

This review will summarize our current knowledge of the molecular changes that occurred during the evolution of C_4 β-CAs from their C_3 ancestors. Recent work on the regulation of CA expression in C_4 plants, and the significance of the enzyme for the C_4 CCM will also be considered.

Molecular evolution of C_4 β-carbonic anhydrases

To date, insights into the evolution of a β-CA involved in C_4 photosynthesis, and dissection of the alterations that occurred at the molecular level to give rise to this cytosolic isoform have been obtained from only a single C_4 lineage. This work was done using the genus *Flaveria*, which contains congeners representing the evolutionary continuum from C_3 to C_4, including proto-Kranz, C_3–C_4 intermediates, and C_4-like species [24,25,26•].

In the C_4 species *Flaveria bidentis* and C_3 congener *Flaveria pringlei*, cDNAs encoding three distinct β-CAs (CA1, CA2, CA3) have been isolated from leaf tissue and characterized [23,27]. Transcript analyses showed CA3 mRNA was at least 50-times more abundant than CA1 or CA2 transcripts in mature leaves of *F. bidentis*, while transcripts encoding CA1 were the most abundant in the leaves of the C_3 *F. pringlei*. Localization experiments using isolated pea chloroplasts and radiolabelled CA precursor proteins showed that CA1 from both *F. bidentis* and *F. pringlei* were imported into chloroplasts [23,27], whereas the CA2 isoforms from both species were not [23,27], and consequently appear to be cytosolic CAs in both species. The localization results for the CA3 isoforms were enlightening with respect to the evolution of the C_4 form of CA3: *F. pringlei* CA3 was imported into isolated pea chloroplasts, whereas *F. bidentis* CA3 was not [23,27]. Comparison of the predicted amino acid sequences of the two CA3 isoforms showed that *F. bidentis* CA3 lacks the first 71 residues relative to the CA3 of *F. pringlei*; however, the predicted polypeptides show 95% amino acid identity over the region they do share [27]. A high proportion of Ser and Thr residues and a low number of charged amino acids are predicted in the *F. pringlei* CA3 N-terminus — properties consistent with the region encoding a chloroplast transit peptide. These results as well as those of the localization experiments were supported by *in silico* protein localization analyses [3]. Taken together, these results indicate that the highly abundant CA3 transcripts in *F. bidentis* code for the cytosolic CA that catalyzes the first committed step of C_4 photosynthesis, and that during evolution of the C_4 pathway, the ancestral C_3 CA3 gene lost the sequence encoding the chloroplast transit peptide, essentially trapping the protein in the cytosol of C_4 mesophyll cells [27].

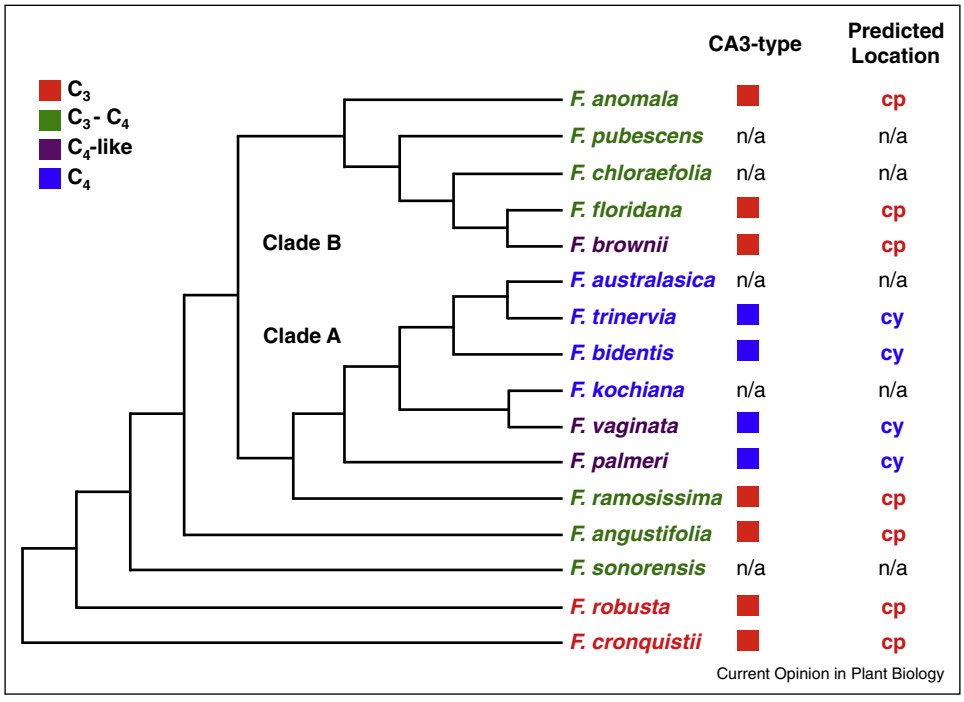
In subsequent work, the predicted amino acid sequences of the cDNAs encoding CA3 from two other C_3 *Flaveria* species, *F. cronquistii* [3] and *F. robusta* (Figure 1), were found to be homologous with that of *F. pringlei*, suggesting a chloroplast location for these proteins. The putative amino acid sequence of CA3 in the C_4 *Flaveria trinervia* contains an N-terminus homologous to that of *F. bidentis* CA3, with no evidence of a chloroplast transit peptide, and consequently a cytosolic location is predicted (Figure 1). The predicted N-terminal sequences of the CA3 isoforms from all *Flaveria* C_3–C_4 intermediate species sequenced to date appear to encode chloroplast transit peptides, and therefore are likely to be chloroplast isoforms ([3]; Figure 1). In the C_4-like species, *Flaveria palmeri* (Figure 1) and *Flaveria vaginata* [3], the putative CA3 polypeptide sequences have N-termini that are homologous to those of C_4 *Flaveria* congeners, and are expected to be cytosolic proteins. In contrast, the CA3 cDNA from *Flaveria brownii*, also considered to be a C_4-like species, encodes a C_3-type N-terminus, suggesting that *F. brownii* may represent an earlier step in the *Flaveria* C_3 to C_4 evolutionary continuum [3]. These results are consistent with the more C_4-like leaf anatomy [28] and gas exchange properties [29] demonstrated by *F. vaginata* than *F. brownii*.

Other groups containing closely related species demonstrating different photosynthetic biochemistries do exist [25]. However somewhat surprisingly, comparative characterization of the β-CA orthologs, the mRNAs and isoforms they encode has not been done. Consequently, there is no information as to whether the mechanism for the evolution of the C_4-associated CA in *Flaveria* is common to other lineages, or if alternative processes were used. Currently no information exists regarding the mechanism of C_4 CA evolution in monocots, which to some extent is due to the lack of lineages containing congeners using different photosynthetic pathways. In this regard, the Australian grass tribe Neurachninae, which contains C_3, C_4 and C_3–C_4 species, holds great promise [30].

Insights into the evolution of C_4 β-CA gene expression

A comparison of the leaf transcriptomes of the Cleomaceae C_4 species *Gynandropsis gynandra* and the closely related C_3 *Tarenaya hassleriana* found transcripts encoding the homolog of the *Arabidopsis* membrane-associated β-CA (AtβCA4; [21]) exhibited an increase in abundance of the same level as transcripts of genes encoding C_4 pathway proteins [31]. Analysis of the *G. gynandra* 5′-untranslated and 3′-untranslated regions (UTRs) using β-glucuronidase (GUS) fusion constructs showed elements in these regions contained information for the mesophyll-specific accumulation of GUS in *G. gynandra* [32]. Information in either UTR was sufficient for this activity. Interestingly, *cis*-elements in the homologous 5′-UTR and 3′-UTR of *AtβCA4* were also able to independently direct the accumulation of GUS in the mesophyll of *G. gynandra*. These results suggest that, for at least some lineages, and some genes encoding C_4-associated

Figure 1



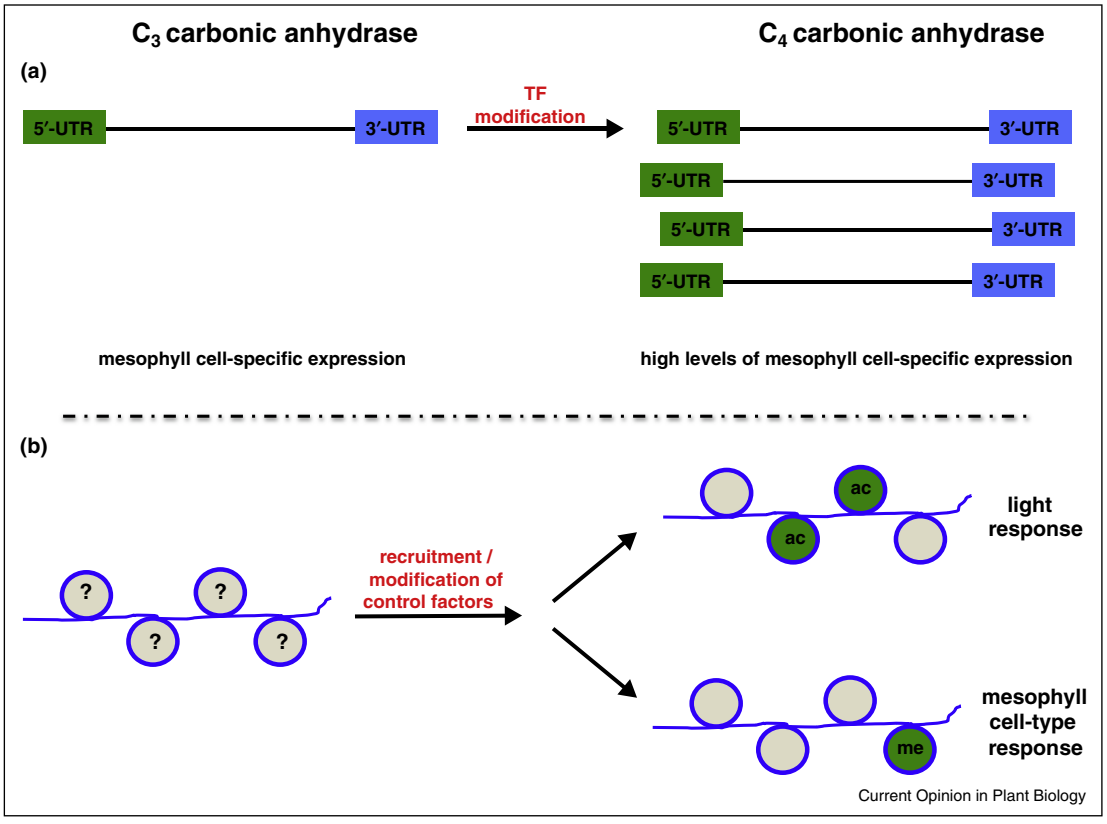
The types of CA3 and their predicted intracellular locations mapped to the *Flaveria* phylogeny. Based on the results of CA3 targeting experiments, the enzymes from all C_4 species are predicted to localize to the mesophyll cytosol (cy) [23], whereas CA3 isoforms from all C_3 species are mesophyll chloroplast (cp) proteins [27]. Sequence analyses indicate all *Flaveria* C_3–C_4 intermediate species contain a C_3-type CA3 and are predicted to have a chloroplast location. For the three C_4-like *Flaveria* species examined, the CA3 homolog from *F. brownii* is also a C_3-type CA, with most likely a chloroplast location; however, *F. vaginata* and *F. palmeri* have C_4-type CA3s that are predicted to be cytosolic proteins. *Flaveria* phylogeny modified from Lyu *et al*. [26•].

CAs, the information for cell-specific expression is present in the orthologous genes of close C_3 relatives (Figure 2), and this may have expedited the evolution of the C_4 syndrome [32].

A recent study looked at the levels of histone H3 with K9ac and K4me3 modifications, and their positioning relative to the transcription initiation site (TIS) of genes encoding several C_4-associated proteins, including *CA1* (GRMZM2G121878), in maize leaves [33••]. These modified histones, along with H4K5ac, had been shown previously to be associated with the activation of the gene encoding the maize C_4 PEPC [34–36]. Like the C_4 PEPC gene, *CA1* showed enrichment of H3K9ac and H3K4me3 and comparable positioning of the nucleosomes containing them. In addition, *CA1* demonstrated H3K9ac and H4K5ac enhancement both at and upstream of the TIS in response to light, and the distribution patterns were similar to those of five other genes encoding C_4-associated proteins. In chromatin from mesophyll cells, enrichment of trimethylated H3K4 relative to the dimethylated protein was found at sites downstream of the *CA1* gene TIS, which correlated with the findings for genes encoding other C_4-associated proteins showing mesophyll-specific expression. These are intriguing results regarding the evolution of maize genes coding for C_4-associated enzymes, including *CA1*, as they suggest that the chromatin containing these genes share a common histone code (Figure 2), and therefore some common mechanism controlling gene expression with respect to environmental signals and cell specificity [33••].

Although Heimann *et al*. [33••] did not examine the *CA1* orthologs of sorghum or *Setaria*, the finding that the orthologs encoding C_4 PEPC and NADP-malic enzyme (NADP-ME) in the distinct maize/sorghum and *Setaria* lineages have similar histone modification patterns led to the suggestion that the modifications were present in ancestral C_3 grasses and were co-opted during the evolution of the C_4 pathway [33••]. This idea of predisposition of ancestral C_3 gene regulatory components for adoption into an evolving C_4 pathway is consistent with the results of the Cleomaceae CA study described above [31], as well as the findings of other work focused on the control of genes encoding C_4-associated pyruvate, orthophosphate dikinase, NAD-ME, and glycine decarboxylase [32,37,38•]. Changes in *trans*-acting or other regulatory factors would enable the cell compartmentation and levels of expression seen in present day C_4 plants (Figure 2).

Figure 2



Control elements and histone modifications in C_3 CA genes were adopted for C_4 CA gene expression. **(a)** Sequences in the 5′-untranslated and 3′-untranslated regions (UTRs) of CA genes control mesophyll cell expression in C_3 and C_4 plants [32]. The modification of *trans*-acting factors (TF) is thought to have enabled expression in C_4 species, for example, high levels of expression. **(b)** Common histone modifications are found in several maize genes coding for C_4-associated proteins, including CA, as a result of exposure to light and cell type expression [33••]. Similar modifications were found in different C_4 lineages, suggesting that the marks were present in the ancestral C_3 genes. However, the histones of the C_3 orthologs have not been examined, which is indicated in the figure by the questions marks. Additional or modified control factors were likely recruited to yield the expression pattern and levels seen for present day C_4 CA genes.

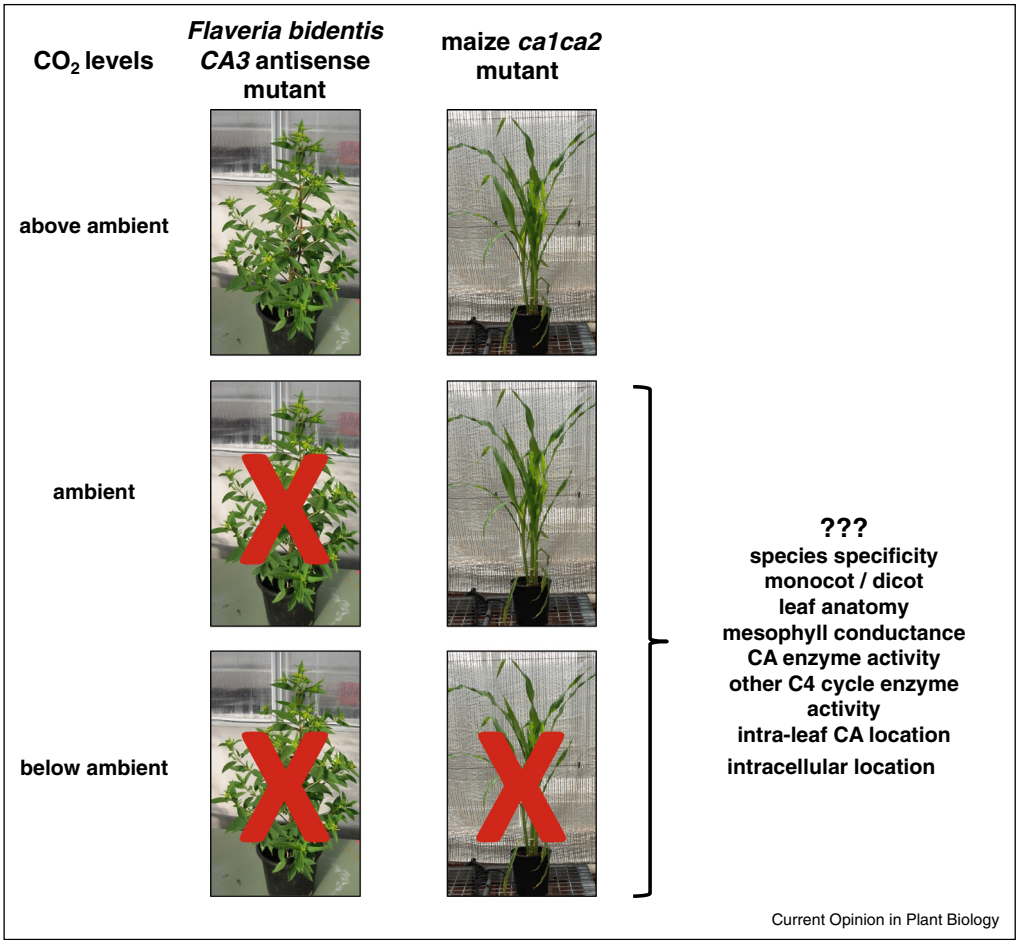
Significance of β-CA in the C_4 carbon concentrating mechanism

To determine the importance of CA in C_4 photosynthesis, *F. bidentis* plants were transformed with a CA3 antisense construct [39]. Transformants with 20% of wild type *F. bidentis* CA activity had decreased rates of steady-state CO_2 assimilation at ambient levels of CO_2, and those with 10% or less wild type CA activity required high CO_2 for growth (Figure 3). These results indicated that while CA activity is not limiting in wild type *F. bidentis*, it is a definite requirement for the C_4 pathway to function as a CCM in this dicotyledonous species.

Recently maize lines carrying single and double mutations in two highly expressed genes encoding distinct *β*-CAs, *CA1* (GRMZM2G121878) and *CA2* (GRMZM2G348512), were generated through insertional mutagenesis [40••]. Homozygous *ca1* mutants showed about 10% of wild type maize CA activity, while the *ca1ca2* double mutant contained just 3% of wild type activity. These mutant lines demonstrated no change in CO_2 assimilation rates at ambient (or higher) CO_2 levels; however, at low intercellular CO_2 concentrations, assimilation rates of both mutants were decreased relative to those of wild type plants (Figure 3). A corresponding decrease in dry mass was also detected in the single and double mutants compared to wild type maize plants when grown under sub-ambient CO_2 conditions [40••].

Clearly, the results of the maize work contrast with those of the *F. bidentis* study. While greatly reduced levels of CA activity led to severe impairment of photosynthesis and growth in *F. bidentis* at ambient CO_2 levels [39], it was only at sub-ambient CO_2 concentrations that CA activity appeared to be required for the maize C_4 pathway to operate as a CCM [40••]. All C_4 plants are predicted to have evolved within the last 30 million years, under comparable low atmospheric CO_2 conditions [41]. In this low-CO_2 world, a CA, working in tandem with PEPC in the mesophyll cytosol, would have been advantageous to

Figure 3



The importance of CA in the C_4 carbon concentrating mechanism differs between *Flaveria bidentis* and maize. *F. bidentis* plants containing reduced amounts of the cytosolic C_4-associated CA show impairment of photosynthesis and growth at ambient levels of CO_2, indicating CA activity is required for the proper functioning of the C_4 pathway. In contrast, maize knockout mutants demonstrate reduced photosynthetic rates and growth only when CO_2 concentrations levels are below ambient levels. The mechanism underlying this difference is not clear, indicated by the question marks in the figure. Some avenues of investigation that might resolve the discrepancy are listed. Note: The images are representative of the species, not the mutants or the phenotypes resulting from the different CO_2 levels.

maintain efficient and high rates of photosynthesis. However, the actual molecular mechanisms and influence of local environmental factors underlying the evolution of the C_4 CA isoforms in maize and *Flaveria* are likely to have differed. Future work should consider whether the difference in CA contribution to the C_4 CCM seen between the species is species specific; or a difference between C_4 monocots and dicots; or is related to inherent CA activity, which is quite variable among C_4 monocots and dicots ([42] and references therein), other C_4 cycle enzyme activities, leaf structure, specific CA location, mesophyll conductance; or a combination of one or more of these factors (Figure 3).

Conclusions

The intracellular location of the majority of CA activity has changed during the evolution of C_4 plants from their C_3 ancestors. This has facilitated the provision of bicarbonate for the primary carboxylase of C_4 plants in the mesophyll cell cytosol, and the evolution of the C_4 CCM. Changes in coding and non-coding regions of CA genes responsible for converting a C_3 CA into a C_4 enzyme are only now being identified, as are differences in contributions of CA to the CCMs of diverse C_4 lineages. Work thus far allows the evolution of the CA isoform important in the C_4 pathway to be mapped on the recently described five-stage model of C_4 evolution [25]. Orthologs encoding this CA can be identified in ancestral C_3 and proto-Kranz species and code for proteins with chloroplast transit peptides (stages a and b [25]). C_3–C_4 intermediate species that carry out C_2 photosynthesis, with its photorespiratory pump (stage c [25]), also express a chloroplast-located CA homolog. Stages d and e [25] of the model are characterized by complete C_4 acid transfer cycle activity, limitation

of Rubisco activity to the BSC, and overall optimization of the C_4 pathway. Advanced C_4-like and full C_4 species represent these stages, and contain orthologs encoding C_4-associated CAs that do not have chloroplast transit peptides and functions in the mesophyll cytosol, providing bicarbonate to PEPC. Multiple lineages with closely related species that use different photosynthetic biochemistries representing the continuum from C_3 to C_4 offer excellent opportunities to further pinpoint mechanisms that account for the evolution of cytosolic C_4 CAs and their involvement in the C_4 CCM, as well as distinguish elements that control expression of these enzymes at the transcriptional and post transcriptional levels. These studies will build on the knowledge of processes already recognized in *Flaveria*, Cleomaceae, and maize, and will determine whether common mechanisms governed the evolution of C_4 CAs. This will inform us of the extent of parallelism and convergence in C_4 pathway evolution, and contribute to efforts directed at identifying the essential components with which to augment C_3 plants for sustainable crop and biofuel production.

Acknowledgements

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References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Moroney JV, Bartlett SG, Samuelsson G: **Carbonic anhydrase in plants and algae**. *Plant Cell Environ* 2001, **24**:141-153.
2. Tiwari A, Kumar P, Singh S, Ansari SA: **Carbonic anhydrase in relation to higher plants**. *Photosynthetica* 2005, **43**:1-11.
3. Ludwig M: **Carbonic anhydrase and the molecular evolution of C_4 photosynthesis**. *Plant Cell Environ* 2012, **35**:22-37.
4. Zabaleta E, Martin MV, Braun H-P: **A basal carbon concentrating mechanism in plants?** *Plant Sci* 2012, **187**:97-104.
5. Ku MSB, Edwards GE: **Photosynthesis in mesophyll protoplasts and bundle sheath cells of various types of C_4 plants. V. Enzymes of respiratory metabolism and energy utilizing enzymes of photosynthetic pathways**. *Z Pflanzenphysiol* 1975, **77**:16-32.
6. Burnell JN, Hatch MD: **Low bundle sheath carbonic anhydrase is apparently essential for effective C_4 pathway operation**. *Plant Physiol* 1998, **86**:1252-1256.
7. Jenkins CLD, Furbank RT, Hatch MD: **Mechanism of C_4 photosynthesis. A model describing the inorganic carbon pool in bundle sheath cells**. *Plant Physiol* 1989, **91**:1372-1381.
8. Everson RG, Slack CR: **Distribution of carbonic anhydrase in relation to the C_4 pathway of photosynthesis**. *Phytochemistry* 1968, **7**:581-584.
9. Poincelot RP: **Intracellular distribution of carbonic anhydrase in spinach leaves**. *Biochim Biophys Acta* 1972, **258**:637-642.
10. Jacobson BS, Fong F, Heath RL: **Carbonic anhydrase of spinach. Studies on its location, inhibition, and physiological function**. *Plant Physiol* 1975, **55**:468-474.
11. Majeau N, Arnoldo M, Coleman JR: **Modification of carbonic anhydrase activity by antisense and over-expression constructs in transgenic tobacco**. *Plant Mol Biol* 1994, **25**:377-385.
12. Price GD, von Caemmerer S, Evans JR, Yu J-W, Lloyd J, Oja V, Kell P, Harrison K, Gallagher A, Badger MR: **Specific reduction of chloroplast carbonic anhydrase activity by antisense RNA in transgenic tobacco plants has a minor effect on photosynthetic CO_2 assimilation**. *Planta* 1994, **193**:331-340.
13. Williams TG, Flanagan LB, Coleman JR: **Photosynthetic gas exchange and discrimination against $^{13}CO_2$ and $C^{18}O^{16}O$ in tobacco plants modified by an antisense construct to have low chloroplastic carbonic anhydrase**. *Plant Physiol* 1996, **112**:319-326.
14. Ferreira FJ, Guo C, Coleman JR: **Reduction of plastid-localized carbonic anhydrase activity results in reduced Arabidopsis seedling survivorship**. *Plant Physiol* 2008, **147**:585-594.
15. Slaymaker DH, Navarre DA, Clark D, del Pozo O, Martin GB, Klessig DF: **The tobacco salicylic acid-binding protein 3 (SABP3) is the chloroplast carbonic anhydrase, which exhibits antioxidant activity and plays a role in the hypersensitive defense response**. *Proc Natl Acad Sci U S A* 2002, **99**:11640-11645.
16. Restrepo S, Myers KL, del Pozo O, Martin GB, Hart AL, Buell CR, Fry WE, Smart CD: **Gene profiling of a compatible interaction between *Phytophthora infestans* and *Solanum tuberosum* suggests a role for carbonic anhydrase**. *Mol Plant Microbe Interact* 2005, **18**:913-922.
17. Wang Y-Q, Feechan A, Yun B-W, Shafiei R, Hofmann A, Taylor P, Xue P, Yang F-Q, Xie Z-S, Pallas JA, Chu C-C, Loake GJ: **S-nitrosylation of AtSABP3 antagonizes the expression of plant immunity**. *J Biol Chem* 2009, **284**:2131-2137.
18. Hoang CV, Chapman KD: **Biochemical and molecular inhibition of plastidial carbonic anhydrase reduces the incorporation of acetate into lipids in cotton embryos and tobacco cell suspensions and leaves**. *Plant Physiol* 2002, **128**:1417-1427.
19. Hu H, Boisson-Dernier A, Israelsson-Nordström M, Böhmer M, Xue S, Ries A, Godoski J, Kuhn JM, Schroeder JI: **Carbonic anhydrases are upstream regulators of CO_2-controlled stomatal movements in guard cells**. *Nat Cell Biol* 2010, **12**:87-93.
20. • Engineer CB, Ghassemian M, Anderson JC, Peck SC, Hu H, Schroeder JI: **Carbonic anhydrases, EPF2 and a novel protease mediate CO_2 control of stomatal development**. *Nature* 2014, **513**:246-250.

This paper reports a regulatory role for chloroplast and membrane-associated carbonic anhydrases in the CO_2-mediated signaling pathway controlling stomatal development. It contributes to our understanding of the role of chloroplast carbonic anhydrase in C_3 plants, which has been controversial with respect to an involvement in photosynthesis. The work was done in *Arabidopsis*, but it is likely CA homologs in C_4 plants carry out the same functions.

21. Fabre N, Reiter IM, Becuwe-Linka N, Genty B, Rumeau D: **Characterization and expression analysis of genes encoding α and β carbonic anhydrases in *Arabidopsis***. *Plant Cell Environ* 2007, **30**:617-629.
22. • Jiang C, Tholen D, Xu JM, Xin C, Zhang H, Zhu X, Zhao Y: **Increased expression of mitochondria-localized carbonic anhydrase activity resulted in an increased biomass accumulation in *Arabidopsis thaliana***. *J Plant Biol* 2014, **57**:366-374.

Arabidopsis plants overexpressing a mitochondrial carbonic anhydrase showed decreased respiration rates and increased biomass. This work supports the idea that all plants have a basal carbon concentrating mechanism through which CO_2 that is released from respiration in mitochondria is re-fixed in the chloroplasts.

23. Tetu SG, Tanz SK, Vella N, Burnell JB, Ludwig M: **The *Flaveria bidentis* β-carbonic anhydrase gene family encodes cytosolic and chloroplastic isoforms demonstrating distinct organ-specific expression patterns**. *Plant Physiol* 2007, **144**:1316-1327.
24. McKown AD, Moncalvo J-M, Dengler NG: **Phylogeny of *Flaveria* (Asteraceae) and inference of C_4 photosynthesis evolution**. *Am J Bot* 2005, **92**:1911-1928.

25. Sage RF, Sage TL, Kocacinar F: **Photorespiration and the evolution of C_4 photosynthesis**. *Annu Rev Plant Biol* 2012, **63**: 19-47.

26. • Lyu M-JA, Gowik U, Kelly S, Covshoff S, Mallmann J, Westhoff P, Hibberd JM, Stata M, Sage RF, Lu H, Wei X, Wong GK-S, Zhu X-G: **RNA-Seq based phylogeny recapitulates previous phylogeny of the genus *Flaveria* (Asteraceae) with some modifications**. *BMC Evol Biol* 2015, **15**:116.

This paper describes a proof of concept that robust phylogenetic relationships can be reconstructed using RNA-Seq data. The resulting relationships among 16 species of *Flaveria* correlated with previous analyses based on morphological and DNA markers; however, higher support was obtained for a number of branches in this study. It was also shown that the *F. pringlei* lines used in a number of laboratories is a hybrid between pure *F. pringlei* and *F. angustifolia*.

27. Tanz SK, Tetu SG, Vella NGF, Ludwig M: **Loss of the transit peptide and an increase in gene expression of an ancestral chloroplastic carbonic anhydrase were instrumental in the evolution of the cytosolic C_4 carbonic anhydrase**. *Plant Physiol* 2009, **150**:1515-1529.

28. McKown AD, Dengler NG: **Key innovations in the evolution of Kranz anatomy and C_4 vein pattern in *Flaveria* (Asteracese)**. *Am J Bot* 2007, **94**:382-399.

29. Ku MSB, Wu J, Dai Z, Scott RA, Chu C, Edwards GE: **Photosynthetic and photorespiratory characteristics of *Flaveria* species**. *Plant Physiol* 1991, **96**:518-528.

30. Christin PA, Wallace MJ, Clayton H, Edwards EJ, Furbank RT, Hattersley PW, Sage RF, Macfarlane TD, Ludwig M: **Multiple photosynthetic transitions, polyploidy, and lateral gene transfer in the grass subtribe Neurachninae**. *J Exp Bot* 2012, **63**:6297-6308.

31. Bräutigam A, Kajala K, Wullenweber J, Sommer M, Gagneul D, Weber KL, Carr KM, Gowik U, Maß J, Lercher MJ, Westhoff P, Hibberd JM, Weber APM: **An mRNA blueprint for C_4 photosynthesis derived from comparative transcriptomics of closely related C_3 and C_4 species**. *Plant Physiol* 2011, **155**: 142-156.

32. Kajala K, Brown NJ, Williams BP, Borrill P, Taylor LE, Hibberd JM: **Multiple Arabidopsis genes primed for recruitment into C_4 photosynthesis**. *Plant J* 2012, **69**:47-56.

33. •• Heimann L, Horst I, Perduns R, Dreesen B, Offermann S, Peterhansel C: **A common histone modification code on C_4 genes in maize and its conservation in sorghum and *Setaria italica***. *Plant Physiol* 2013, **162**:456-469.

The gene encoding the maize C_4-associated carbonic anhydrase shows similar histone modifications and positioning relative to the transcriptional start site as the genes encoding other C_4-associated proteins. These histone marks were shown to be associated with light induction and mesophyll cell-specific expression. Because the similarities were seen in genes coding for C_4 proteins from two distinct lineages, it was suggested that the marks were present in the C_3 ancestors, and adopted for C_4 evolution.

34. Offermann S, Danker T, Dreymüller D, Kalamajka R, Töpsch S, Weyand K, Peterhänsel C: **Illumination is necessary and sufficient to induce histone acetylation independent of transcriptional activity at the C_4-specific phosphoenolpyruvate carboxylase promoter in maize**. *Plant Physiol* 2006, **141**:1078-1088.

35. Offermann S, Dreesen B, Horst I, Danker T, Jaskiewicz M, Peterhansel C: **Developmental and environmental signals induce distinct histone acetylation profiles on distal and proximal promoter elements of the C4-Pepc gene in maize**. *Genetics* 2008, **179**:1891-1901.

36. Danker T, Dreesen B, Offermann S, Horst I, Peterhänsel C: **Developmental information but not promoter activity controls the methylation state of histone H3 lysine 4 on two photosynthetic genes in maize**. *Plant J* 2008, **53**:465-474.

37. Brown NJ, Newell CA, Stanley S, Chen JE, Perrin AJ, Kajala K, Hibberd JM: **Independent and parallel recruitment of preexisinting mechanisms underlying C_4 photosynthesis**. *Science* 2011, **331**:1436-1439.

38. • Schulze S, Mallmann J, Burscheidt J, Koczor M, Streubel M, Bauwe H, Gowik U, Westhoff P: **Evolution of C_4 photosynthesis in the genus *Flaveria*: establishment of a photorespiratory CO_2 pump**. *Plant Cell* 2013, **25**:2522-2535.

Two genes encoding glycine decarboxylase subunit P are expressed in leaves of C_3 *Flaveria* species with one containing promoter elements that control bundle-sheath cell-specific expression (*GLDPA*), while the other is ubiquitously expressed (*GLDPB*). C_3–C_4 *Flaveria* species show a significant decrease in *GLDPB* transcripts and *GLDPA* is the only expressed *GLDP* gene in the bundle-sheath cells of C_4 *Flaveria* species — *GLDPB* gene is a pseudogene. These results indicate the evolution of C_2 photosynthesis, and consequently the C_4 pathway in *Flaveria* proceeded in a stepwise manner.

39. von Caemmerer S, Quinn V, Hancock NC, Price GD, Furbank RT, Ludwig M: **Carbonic anhydrase and C_4 photosynthesis: a transgenic analysis**. *Plant Cell Environ* 2004, **27**:697-703.

40. •• Studer AJ, Gandin A, Kolbe AR, Wang L, Cousins AB, Brutnell TP: **A limited role for carbonic anhydrase in C_4 photosynthesis is revealed by a *ca1ca2* double mutant in maize**. *Plant Physiol* 2014, **165**:608-617.

Maize plants containing only 3% of wild type carbonic anhydrase activity showed no impairment of photosynthesis or growth at ambient CO_2 levels; however, decreased photosynthetic rates and growth effects were exhibited when plants were grown under low CO_2 conditions. These results contrast with those found for CA antisense mutants of the C_4 plant *Flaveria bidentis*.

41. Christin PA, Osborne CP, Sage RF, Arakaki M, Edwards EJ: **C_4 eudicots are not younger than C_4 monocots**. *J Exp Bot* 2011, **62**:3171-3181.

42. Cousins AB, Badger MR, von Caemmerer S: **C_4 photosynthetic isotope exchange in NAD-ME- and NADP-ME-type grasses**. *J Exp Bot* 2008, **59**:1695-1703.