

What Does It Take to Be C₄? Lessons from the Evolution of C₄ Photosynthesis

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Twenty-five years ago research had already established a firm biochemical and physiological understanding of the CO₂-concentrating mechanism that creates a high CO₂ environment (1,000–3,000 μbar) in bundle-sheath cells in leaves of C₄ plants and accounts for most of their distinctive photosynthetic properties (5). It was then clear that the minimum requirements for this CO₂ concentrating mechanism included: (a) cell-specific amplification of enzymes of C₄ photosynthesis (i.e. phosphoenolpyruvate carboxylase [PEPC] in mesophyll, and C₄ acid decarboxylases and Rubisco in bundle-sheath cells), with complementary adjustments of photosystem and electron transport activities; (b) novel cell-specific organelle metabolite translocators; (c) symplastic connections of the spatially separated sources and sinks of 4C-dicarboxylic acid transport metabolites; and (d) barriers to CO₂ diffusion between the site of CO₂ fixation by PEPCase in mesophyll cells and sites of CO₂ release and refixation by Rubisco in bundle-sheath cells.

These requirements have been met in a great variety of ways during the evolution of C₄ plants, through diverse cooperative pathways of carbon metabolism and integrated photoreactions in adjacent, differentiated photosynthetic cells. Perhaps the most simple, highly evolved system is that in *Sorghum* (detailed in the legend of Fig. 1), but it is in the diversity of other systems that we can expect to discover clues as to what it takes to be C₄.

INSIGHTS IN C₄ PHOTOSYNTHESIS HAVE TRADITIONALLY ARISEN FROM CLOSE WORKING RELATIONSHIPS BETWEEN TAXONOMISTS, ANATOMISTS, ECOPHYSIOLOGISTS, BIOCHEMISTS, AND MOLECULAR BIOLOGISTS

Phylogenetic analysis confirms the multiple origins of the diverse C₄ pathways (Kellogg in 12), all of which share PEPCase as the primary carboxylase, but

which engage diverse decarboxylases to regenerate CO₂ for Rubisco in various structural arrangements of mesophyll and bundle-sheath cells. Leaf and cotyledon anatomies and organelle arrangements are especially diverse in C₄ members of the Chenopodiaceae, revealed recently following better access to the organisms and research expertise from Central Asia (19). The paradigm of spatial separation of PEPCase and Rubisco in different cells has been challenged by recent findings concerning *Borszczowia* (4), which has a δ¹³C value of –13.1% (more typical of C₄ plants) and differentiated chloroplasts at the poles of radially arranged single large cells. We know little of the efficiency of the CO₂-concentrating mechanism in diverse natural variants of C₄ photosynthesis, but rely instead on the interpretation of stable isotope data and the use of models to detect leakiness (16).

Although some wild plants such as *Flaveria* and *Eleocharis* have been amenable to molecular genetic analysis (2), most progress has been made with maize and *Amaranthus* sp. These advances can be followed in a collection of research reports (17), in specialist reviews (3, 14), and in a book that comprehensively integrates C₄ plant biology from the molecule to the biosphere (12). We will highlight them here by citations from then and now.

EVOLUTION OF DIVERSE C₄ PHOTOSYNTHETIC PATHWAYS REFLECTS EVOLUTIONARY OUTCOMES IN THE FACE OF ONE DOMINANT SELECTIVE PRESSURE, THE DECLINING CO₂, AND HIGH O₂ CONCENTRATIONS IN THE ATMOSPHERE THROUGHOUT THE TERTIARY

It is believed that the C₄ pathway has probably existed at low abundance for much of the past 12 to 13 million years, since the time of the fossil grass *Tomlinsonia*, which has Kranz anatomy and a δ¹³C value of –13.7% (Cerling in 12). Much δ¹³C evidence from many indirect sources (soil carbonates deposited about grass roots, tooth enamel of herbivores, etc.) dates the explosion of C₄ plant biomass at some six to eight million years ago when atmospheric CO₂ concentrations fell to about 200 μbar in air with 20

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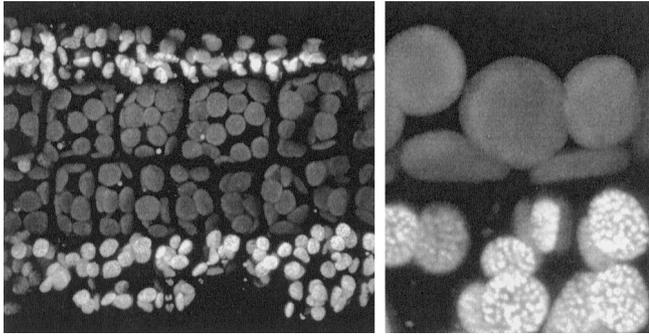


Figure 1. Confocal microscope image of chlorophyll autofluorescence from mesophyll and bundle-sheath cells in *Sorghum bicolor*. Mesophyll cell chloroplasts (outer rows, left) that have a stroma devoid of Rubisco and thylakoids with high activity of both photosystems show strong fluorescence from photosystem II in grana (bottom chloroplasts, right). Adjacent bundle-sheath cells that contain larger chloroplasts (inner rows, left) with Rubisco replete stroma, but photosystem II-deficient thylakoids, lack grana and show diffuse fluorescence from photosystem I alone (top chloroplasts, right). Non-cyclic electron transport in mesophyll chloroplasts sustains PEP synthesis, the substrate for initial CO₂ fixation by PEPCase in the mesophyll cell cytosol, and the reduction of its product to malic acid (5). Symplastic metabolite exchange between the two cell layers delivers malic acid for decarboxylation by NADP-ME, generating high CO₂ concentrations that minimize the oxygenase activity of Rubisco. This decarboxylase also generates one-half the reductant needed by 3-PGA, compensating for the photosystem I deficiency in bundle-sheath chloroplasts (the remainder of the 3-PGA is returned for reduction in mesophyll chloroplasts). Distinctive mesophyll chloroplast translocators for pyruvate, PEP, and 3-PGA (3) are critical components of cooperative C₄ photosynthesis.

mbar O₂. Under these conditions the catalytic shortcomings of Rubisco favor the oxygenation of RuBP and energetically wasteful photorespiratory carbon recycling in the photorespiratory carbon oxidation (PCO) and photosynthetic carbon reduction (PCR) cycles. This so-called Rubisco penalty increases the energy cost of C₃ photosynthesis beyond the cost of the CO₂ concentrating mechanisms that evolved in C₄ photosynthesis. Thus C₄ plants gained a competitive edge during the low CO₂ atmospheres and warmer periods of the Palaeozoic (Sage in 12). The subsequent evolutionary success of C₄ photosynthesis was due to their improved water use efficiency and nutrient use efficiency, as well as their high photosynthetic capacity at higher temperature, all of which follow from Rubisco function in bundle-sheath cells served by a CO₂-concentrating mechanism. The productivity of C₄ crops today also stems from their longer growth cycles in the tropics, and their success as weeds owes much to their aggressive reproductive strategies.

Although a plausible series of evolutionary steps through different C₃-C₄ intermediates has been proposed (11), the significance of these plants remains controversial. All extant C₄ plants use a 4C acid-decarboxylase-based CO₂-concentrating mechanism in bundle-sheath cells, but the partial C₄ cycle in

some C₃-C₄ intermediates does not seem to contribute to a CO₂-concentrating mechanism (Monson in 12). Other C₃-C₄ intermediates show higher Gly decarboxylase in bundle-sheath mitochondria and lower CO₂ compensation points (11), but it seems unlikely that relocation of the photorespiratory CO₂ evolving apparatus into bundle-sheath cells could be a prelude to development of a CO₂-concentrating mechanism to inhibit photorespiration. Some ask if *Moricandia* is a failed experiment (Kellogg in 12), and others suggest reversions from C₄ to the C₃ pathway in *Salsola* (10).

DIFFERENTIATION OF COOPERATIVE PHOTOSYNTHETIC PROCESSES IN ADJACENT CELLS OF C₄ PLANTS DEPENDS ON DIVERSE TRANSCRIPTIONAL, POSTTRANSCRIPTIONAL, AND TRANSLATIONAL PROCESSES, AND SOMEHOW ON POSITION

Relatively small changes in gene regulation may be required for the assembly of the catalytic systems of C₄ photosynthesis. Advances in our understanding of the regulated expression of identical genomes (20) in nuclei and chloroplasts of adjacent cells have been summarized by Sheen (13): "... pre-existing genes were recruited for the C₄ pathway after acquiring potent and surprisingly diverse regulatory elements... consisting of synergistic and combinatorial enhancers and silencers, the use of 5' and 3' untranslated regions for transcriptional and post-transcriptional regulations, and the function of novel transcription factors." One plausible overview (Monson in 12) suggests that C₄ biochemical profiles may arise from genes for anaplerotic or housekeeping functions in C₃ metabolism through up- and down-regulation of activities by cis-acting promoters. Specific catalytic functions in C₄ photosynthesis may emerge through gene duplication, and differential expression in adjacent cells may be dominated by 3'-promoter sequences and posttranscriptional events.

The molecular evolution of PEPCase and the control of its expression is reasonably well understood (Westhoff et al. in 17). Bläsing et al. (1) recently used site-directed mutagenesis to confirm the identity of two interacting regions that confer the distinctive kinetic properties of C₄ PEPCase in *Flaveria*. Much less is known of the evolution of distinctive decarboxylation systems in bundle-sheath cells of different C₄ plants or of the lower specificity factor of C₄ Rubisco. Single decarboxylase systems such as NADP-ME in *Sorghum* (Fig. 1) may be less common than multiple pathways involving NAD-malic enzyme (ME)/PEP-carboxykinase type, as well as NAD-ME/NADP-ME and NADP-ME/PEP-carboxykinase type (e.g. Walker et al. in 17). Diversity in decarboxylation types is matched by diversity of photosystem II/photosystem I ratios in mesophyll and bundle-

sheath cells (9) that accommodates the varied energy demands of the CO₂ concentrating mechanism.

Accepting that “C₄ genes are independently regulated by multiple control mechanisms in response to developmental, environmental and metabolic signals” (Berry et al. in 17), two large questions remain far from resolution. First, the paramount importance of positional information in relation to vascular development is clear (6), but the positional signals that guide differentiation of complementary cell types remain elusive (Dengler and Nelson in 12). Second, the importance of environmental signals in cell-specific expression of key genes has been recognized, but the effects of light, for example, in different species are as different as day and night (required in *Zea*, but not in *Amaranthus*; 13). Regulatory signals such as inter-photosystem redox status clearly produce differential responses in different gene expression systems in different species. It may be sometime before gene regulation can be reduced to suites of “. . . unique or universal mechanisms underlying cell-type specificity, coordinate nuclear-chloroplast actions, hormonal, metabolic, stress and light responses” (13).

Environmental responsiveness is most obvious in the submersed-to-emergent transition from C₃ to C₄ photosynthesis in culms of *Eleocharis* in which C₄ metabolism can be induced by abscisic acid while submerged (15), illustrating the importance of simultaneous evaluation of genotypic and environmental diversity. The organ-specific control of photosynthetic pathways such as C₃ metabolism in the cotyledons of C₄ Chenopodiaceae (19) suggests that genotypic variation and environmental-selective pressures have explored most of the conceivable options in C₄ metabolism.

In the meantime, notions that crop yields can be improved through greater photosynthetic capacity and that C₄ metabolism alone may boost yield of C₃ crops continue to stimulate creative research. Such projects are exposing the consequences of introducing C₄ photosynthetic traits into C₃ plants, but evidence of functional C₄ metabolism has yet to be published. Achievement of high levels of expression of C₄ enzymes in *Oryza* (8) suggests that trans-acting factors present in rice recognize C₄ genomic clones, and that mechanisms for up-regulation of “house-keeping genes” such as *Ppc* and *Pdk* still exist in C₃ plants. The discovery that the over expression of *Zea* NADP-ME in rice chloroplasts is accompanied by reduction in photosystem II activity and reduced granal stacking (14) opens astonishing possibilities for research into coregulation of unrelated genes.

As emphasized in the beginning, getting the enzymes in the right place is a first step, but we know next to nothing about regulatory interactions that determine assimilatory flux in C₄ plants. Anti-sense experiments with C₄ *Flaveria* show that in spite of the CO₂ concentrating mechanism, Rubisco remains the major determinant of carbon flux at high light and

moderate temperature in C₄ plants (18), with PEP-Case and pyruvate-orthophosphate dikinase showing lower control coefficients. The complex regulatory cascades of many C₄ enzymes may be exercised more commonly as light-dark switches than as flux control systems during photosynthetic CO₂ fixation. We still lack understanding of what it takes to be C₄ in anything but the most general terms, and building functional C₄ traits into C₃ plants remains an immense challenge, especially in terms of the structural components.

RECREATION OF CRETACEOUS CO₂ CONCENTRATIONS IN BUNDLE-SHEATH CELLS THROUGH DIVERSE C₄ PATHWAYS IN 8,000 TO 10,000 SPECIES IN 31 ANGIOSPERM FAMILIES HAS BEEN A SIGNAL, BUT PERHAPS TRANSIENT, EVENT IN PHOTOSYNTHETIC EVOLUTION

There have been well-defined advances and contractions in the distribution of C₄ plants during the last full Glacial, 20,000 to 30,000 years ago (Cerling in 12). Another contraction of C₄ plants may begin in the lifetime of our grandchildren—perhaps in the time it may take to transfer C₄ traits effectively into C₃ crops and to see them accepted by consumers. It is obvious that the low atmospheric CO₂ concentration that was the major selective pressure favoring C₄ photosynthesis is vanishing, in an instant as it were, on geological time scales. The industrial revolution is returning several billion years of fossil photosynthesis to the atmosphere as CO₂ in the course of a few hundred years. Doubling of atmospheric CO₂ concentration, confidently expected to occur in the second one-half of the 21st century, may itself mitigate the Rubisco penalty in many C₃ plants in many habitats (except perhaps where accompanied by higher temperatures and drought), with little impact on assimilation or growth of C₄ plants (7). This global experiment will certainly test our assumptions as to what it means to be C₄, and what value C₄ *Oryza* then? Quo vadis, C₄?

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