What Does It Take to Be C_4? Lessons from the Evolution of C_4 Photosynthesis

Gerald E. Edwards, Robert T. Furbank, Marshall D. Hatch, and C. Barry Osmond*

School of Biological Sciences, Washington State University, Pullman, Washington 99164–4236 (G.E.E.); Division of Plant Industry, Commonwealth Scientific and Industrial Research Organization, Box 1600, Canberra, Australian Capital Territory 2601, Australia (R.T.F., M.D.H.); and Photobioenergetics Group, Research School of Biological Sciences, Australian National University, Box 3252, Weston Creek, Australian Capital Territory 2611, Australia (C.B.O.)

Twenty-five years ago research had already established a firm biochemical and physiological understanding of the CO_2-concentrating mechanism that creates a high CO_2 environment (1,000–3,000 μbar) in bundle-sheath cells in leaves of C_4 plants and accounts for most of their distinctive photosynthetic properties (5). It was then clear that the minimum requirements for this CO_2 concentrating mechanism included: (a) cell-specific amplification of enzymes of C_4 photosynthesis (i.e. phospho*enol*pyruvate carboxylase [PEPC] in mesophyll, and C_4 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 CO_2 diffusion between the site of CO_2 fixation by PEPCase in mesophyll cells and sites of CO_2 release and refixation by Rubisco in bundle-sheath cells.

These requirements have been met in a great variety of ways during the evolution of C_4 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 C_4.

INSIGHTS IN C_4 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 C_4 pathways (Kellogg in 12), all of which share PEPCase as the primary carboxylase, but which engage diverse decarboxylases to regenerate CO_2 for Rubisco in various structural arrangements of mesophyll and bundle-sheath cells. Leaf and cotyledon anatomies and organelle arrangements are especially diverse in C_4 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 $\delta^{13}C$ value of -13.1% (more typical of C_4 plants) and differentiated chloroplasts at the poles of radially arranged single large cells. We know little of the efficiency of the CO_2-concentrating mechanism in diverse natural variants of C_4 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 C_4 plant biology from the molecule to the biosphere (12). We will highlight them here by citations from then and now.

EVOLUTION OF DIVERSE C_4 PHOTOSYNTHETIC PATHWAYS REFLECTS EVOLUTIONARY OUTCOMES IN THE FACE OF ONE DOMINANT SELECTIVE PRESSURE, THE DECLINING CO_2, AND HIGH O_2 CONCENTRATIONS IN THE ATMOSPHERE THROUGHOUT THE TERTIARY

It is believed that the C_4 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 $\delta^{13}C$ value of -13.7% (Cerling in 12). Much $\delta^{13}C$ evidence from many indirect sources (soil carbonates deposited about grass roots, tooth enamel of herbivores, etc.) dates the explosion of C_4 plant biomass at some six to eight million years ago when atmospheric CO_2 concentrations fell to about 200 μbar in air with 20

* Corresponding author; e-mail osmond@rsbs.anu.edu.au; fax 61–2–6287–4241.

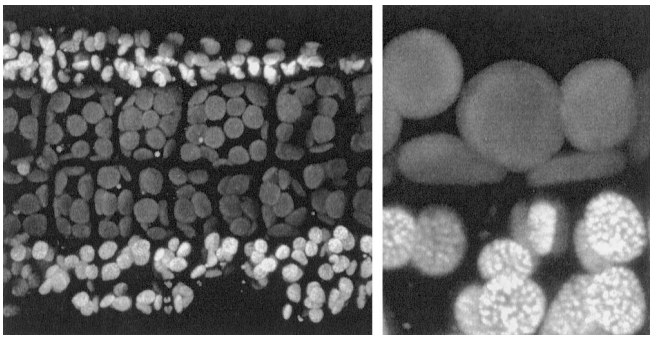

Figure 1. Confocal microscope image of chlorophyll autofluorescence from mesophyll and bundle-sheath cells in *Sorghum bicolor*. Mesophyll cell chloroplasts (outer rows, left) that have a stroma devoid of Rubisco and thylakoids with high activity of both photosystems show strong fluorescence from photosystem 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). Noncyclic electron transport in mesophyll chloroplasts sustains PEP synthesis, the substrate for initial CO_2 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 CO_2 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 C_4 photosynthesis.

mbar O_2. 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 C_3 photosynthesis beyond the cost of the CO_2 concentrating mechanisms that evolved in C_4 photosynthesis. Thus C_4 plants gained a competitive edge during the low CO_2 atmospheres and warmer periods of the Palaeozoic (Sage in 12). The subsequent evolutionary success of C_4 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 CO_2-concentrating mechanism. The productivity of C_4 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 C_3-C_4 intermediates has been proposed (11), the significance of these plants remains controversial. All extant C_4 plants use a 4C acid-decarboxylase-based CO_2-concentrating mechanism in bundle-sheath cells, but the partial C_4 cycle in some C_3-C_4 intermediates does not seem to contribute to a CO_2-concentrating mechanism (Monson in 12). Other C_3-C_4 intermediates show higher Gly decarboxylase in bundle-sheath mitochondria and lower CO_2 compensation points (11), but it seems unlikely that relocation of the photorespiratory CO_2 evolving apparatus into bundle-sheath cells could be a prelude to development of a CO_2-concentrating mechanism to inhibit photorespiration. Some ask if *Moricandia* is a failed experiment (Kellogg in 12), and others suggest reversions from C_4 to the C_3 pathway in *Salsola* (10).

DIFFERENTIATION OF COOPERATIVE PHOTOSYNTHETIC PROCESSES IN ADJACENT CELLS OF C_4 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 C_4 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 C_4 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 posttranscriptional regulations, and the function of novel transcription factors." One plausible overview (Monson in 12) suggests that C_4 biochemical profiles may arise from genes for anaplerotic or housekeeping functions in C_3 metabolism through up- and down-regulation of activities by cis-acting promoters. Specific catalytic functions in C_4 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 C_4 PEPCase in *Flaveria*. Much less is known of the evolution of distinctive decarboxylation systems in bundle-sheath cells of different C_4 plants or of the lower specificity factor of C_4 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-carboykinase 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 CO_2 concentrating mechanism.

Accepting that "C_4 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 interphotosystem 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 C_3 to C_4 photosynthesis in culms of *Eleocharis* in which C_4 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 C_3 metabolism in the cotyledons of C_4 Chenopodiaceae (19) suggests that genotypic variation and environmental-selective pressures have explored most of the conceivable options in C_4 metabolism.

In the meantime, notions that crop yields can be improved through greater photosynthetic capacity and that C_4 metabolism alone may boost yield of C_3 crops continue to stimulate creative research. Such projects are exposing the consequences of introducing C_4 photosynthetic traits into C_3 plants, but evidence of functional C_4 metabolism has yet to be published. Achievement of high levels of expression of C_4 enzymes in *Oryza* (8) suggests that trans-acting factors present in rice recognize C_4 genomic clones, and that mechanisms for up-regulation of "housekeeping genes" such as *Ppc* and *Pdk* still exist in C_3 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 C_4 plants. Anti-sense experiments with C_4 *Flaveria* show that in spite of the CO_2 concentrating mechanism, Rubisco remains the major determinant of carbon flux at high light and moderate temperature in C_4 plants (18), with PEPCase and pyruvate-orthophosphate dikinase showing lower control coefficients. The complex regulatory cascades of many C_4 enzymes may be exercised more commonly as light-dark switches than as flux control systems during photosynthetic CO_2 fixation. We still lack understanding of what it takes to be C_4 in anything but the most general terms, and building functional C_4 traits into C_3 plants remains an immense challenge, especially in terms of the structural components.

RECREATION OF CRETACEOUS CO_2 CONCENTRATIONS IN BUNDLE-SHEATH CELLS THROUGH DIVERSE C_4 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 C_4 plants during the last full Glacial, 20,000 to 30,000 years ago (Cerling in 12). Another contraction of C_4 plants may begin in the lifetime of our grandchildren—perhaps in the time it may take to transfer C_4 traits effectively into C_3 crops and to see them accepted by consumers. It is obvious that the low atmospheric CO_2 concentration that was the major selective pressure favoring C_4 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 CO_2 in the course of a few hundred years. Doubling of atmospheric CO_2 concentration, confidently expected to occur in the second one-half of the 21st century, may itself mitigate the Rubisco penalty in many C_3 plants in many habitats (except perhaps where accompanied by higher temperatures and drought), with little impact on assimilation or growth of C_4 plants (7). This global experiment will certainly test our assumptions as to what it means to be C_4, and what value C_4 *Oryza* then? Quo vadis, C_4?

ACKNOWLEDGMENTS

The authors thank Hans Heldt and Peter Westhoff for advice during the preparation of the manuscript.

LITERATURE CITED

1. **Bläsing OE, Westhoff P, Svensson P** (2000) J Biol Chem
2. **Chitty JA, Furbank RT, Marshall JS, Chen Z, Taylor WC** (1994) Plant J **6:** 949–956
3. **Flügge U-I** (1999) Annu Rev Plant Physiol Plant Mol Biol **50:** 27–45
4. **Freitag H, Stichler W** (2000) Plant Biol **2:** 154–160

5. **Hatch MD, Osmond CB** (1976) *In* U Heber and CR Stocking eds. Encyclopedia of Plant Physiology (New Series), Vol 3. Springer Verlag, Heidelberg, pp. 144–184
6. **Hattersley PW, Watson L** (1976) Austral J Bot **24:** 297–308
7. **Henderson S, Hattersley PW, von Caemmerer S, Osmond CB** (1994) *In* E-D Schulze, MM Caldwell eds, Ecophysiology of Photosynthesis. Ecological Studies, Vol 100. Springer Verlag Heidelberg. pp 530–549
8. **Ku MSB, Agarie S, Nomura M, Hirose S, Toki S, Miyao-Tokutomi M, Matsuoka M** (1999) Nature Biotech **17:** 76–80
9. **Pfundel EE, Neuborn B** (1999) Plant Cell Environ **22:** 1569–1577
10. **P'yankov VI, Voznesenskaya EV, Konratschuk AV, Black CC** (1999) Amer J Bot **84:** 597–606
11. **Rawsthorne S, Bauwe H** (1998) *In* AS Ragavendra ed. Photosynthesis: A Comprehensive Treatise. The University Press, Cambridge, pp 150–172
12. **Sage RF, Monson RK eds** (1999) C_4 Plant Biology. Academic Press, San Diego
13. **Sheen J** (1999) Annu Rev Plant Physiol Plant Mol Biol **50:** 187–217
14. **Takeuchi Y, Akagi H, Kamasawa N, Osumi M, Honda H** (2000) Planta **111:** 265–274
15. **Ueno O** (1998) Plant Cell **10:** 517–538
16. **von Caemmerer S** (2000) Biochemical Models of Leaf Photosynthesis. CSIRO Publishing, Melbourne
17. **von Caemmerer S, Furbank RT. eds** (1997) C_4 Photosynthesis 30 (or 40) Years On. Aust J Plant Physiol **24:** (Special Issue) 409–554
18. **von Caemmerer S, Millgate A, Farquhar GD, Furbank RT** (1997) Plant Physiol **113:** 469–477
19. **Voznesenskaya EV, Franceschi VR, P'yankov VI, Edwards GE** (1999) J Exp Bot **50:** 1779–1795
20. **Walbot V** (1977) Cell **11:** 729–737