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REVIEW

C_4 Cycles: Past, Present, and Future Research on C_4 Photosynthesis

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In the late 1960s, a vibrant new research field was ignited by the discovery that instead of fixing CO_2 into a C_3 compound, some plants initially fix CO_2 into a four-carbon (C_4) compound. The term C_4 photosynthesis was born. In the 20 years that followed, physiologists, biochemists, and molecular and developmental biologists grappled to understand how the C_4 photosynthetic pathway was partitioned between two morphologically distinct cell types in the leaf. By the early 1990s, much was known about C_4 biochemistry, the types of leaf anatomy that facilitated the pathway, and the patterns of gene expression that underpinned the biochemistry. However, virtually nothing was known about how the pathway was regulated. It should have been an exciting time, but many of the original researchers were approaching retirement, C_4 plants were proving recalcitrant to genetic manipulation, and whole-genome sequences were not even a dream. In combination, these factors led to reduced funding and the failure to attract young people into the field; the endgame seemed to be underway. But over the last 5 years, there has been a resurgence of interest and funding, not least because of ambitious multinational projects that aim to increase crop yields by introducing C_4 traits into C_3 plants. Combined with new technologies, this renewed interest has resulted in the development of more sophisticated approaches toward understanding how the C_4 pathway evolved, how it is regulated, and how it might be manipulated. The extent of this resurgence is manifest by the publication in 2011 of more than 650 pages of reviews on different aspects of C_4. Here, I provide an overview of our current understanding, the questions that are being addressed, and the issues that lie ahead.

INTRODUCTION

The Discovery

In 1956, the pathway through which plants fix CO_2 into organic acids was elucidated (Bassham et al., 1956). The subsequently named Calvin-Benson or C_3 cycle uses the enzyme ribulose-1,5-bis-phosphate carboxylase/oxygenase (Rubisco) to fix CO_2 into the three-carbon compound 3-phosphoglycerate (Figure 1A). At the time, it was generally assumed that the Calvin-Benson cycle accounted for CO_2 assimilation in all plants. However, further $^{14}CO_2$ labeling experiments revealed that in maize (*Zea mays*) and sugarcane (*Saccharum officinarum*), the four-carbon compounds malate and Asp were among the earliest labeled products (Karpilov, 1960; Kortschak et al., 1965). The significance of these findings was not fully understood until M.D. Hatch and C.R. Slack proposed a model for the C_4 dicarboxylic acid pathway, wherein CO_2 is initially fixed into a four-carbon compound, subsequently decarboxylated, and then refixed into a three-carbon compound (Hatch and Slack, 1966; Hatch, 2002). These three steps define the canonical C_4 photosynthetic pathway.

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Variations on a Theme

Variants of C_4 biochemistry have been found in a marine macroalga (*Udotea flabellum*) (Reiskind and Bowes, 1991), a diatom (*Thalassiosira weissflogii*) (Roberts et al., 2007), and in both aquatic (reviewed in Bowes, 2011) and terrestrial angiosperms. Some of these variants operate in the context of a single cell, but in most cases, the C_4 pathway is partitioned between two morphologically distinct cell types known as bundle sheath (BS) and mesophyll (M) cells (reviewed in Edwards et al., 2004). In C_4 plants, these BS and M cells surround the leaf veins in concentric circles, leading to a wreath-like appearance. This specialized arrangement was named Kranz anatomy (from the German word for wreath) many years before its association with the C_4 pathway was elucidated (Haberlandt, 1896), but the link is now very well established, and as with the biochemistry, many variations on the Kranz theme exist (Brown, 1975; reviewed in Edwards and Voznesenskaya, 2011).

In the context of the two-cell C_4 pathway, three biochemical subtypes have been defined that differ in the subcellular localization and type of C_4 acid decarboxylase used in the BS cells (reviewed in Drincovich et al., 2011). The first to be discovered was the NADP-malic enzyme (ME) type, in which the decarboxylation step is performed in BS chloroplasts by NADP-dependent ME (Figure 1B). In this pathway, CO_2 enters the M cell cytoplasm where it is first converted to bicarbonate ions by carbonic anhydrase (CA) and is then fixed by phospho*eno*lpyruvate

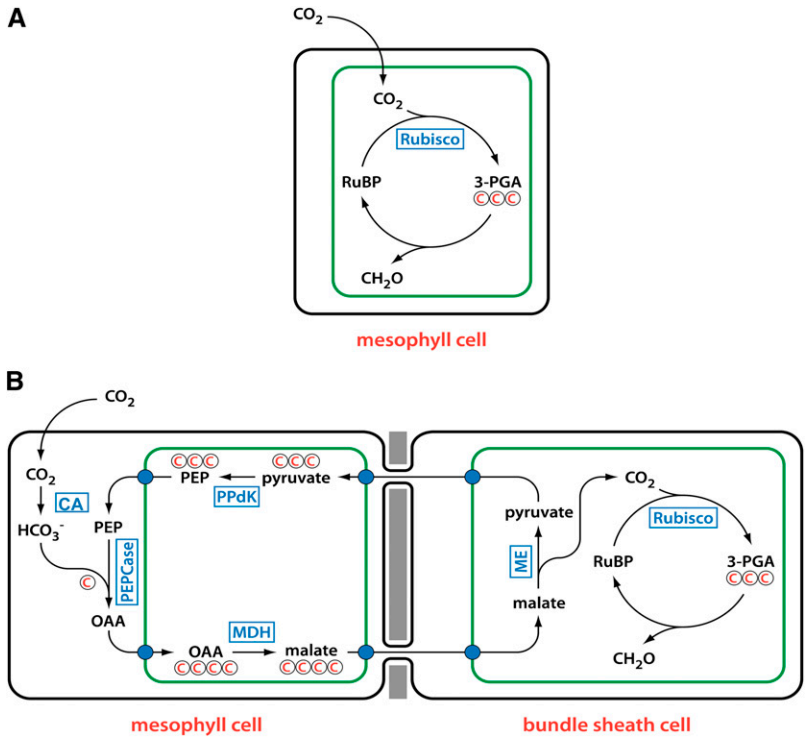

Figure 1. Schematic of C_3 Calvin-Benson and NADP-ME C_4 Cycles.

Calvin-Benson **(A)** and NADP-ME C_4 **(B)** cycles. The green box represents the chloroplast. Blue dots represent active transport steps.

carboxylase (PEPCase) to form oxaloacetate (OAA). OAA is subsequently transported from the M cytoplasm to the M chloroplast where it is converted to malate by NADP-malate dehydrogenase. Malate is then transported out of the M chloroplast and into the BS chloroplast, a process that requires transport across the chloroplast and plasma membranes of both cell types. In the BS cell chloroplast, malate is decarboxylated by NADP-ME, and the released CO_2 is refixed by Rubisco in the Calvin-Benson (C_3) cycle. The pyruvate generated by the decarboxylation reaction is transported back from the BS chloroplast to the M chloroplast where it acts as a substrate for pyruvate orthophosphate dikinase (PPdK) to regenerate phosphoenolpyruvate (PEP). The cycle is restarted when PEP is transported from the M chloroplast to the M cell cytoplasm to combine once again with CO_2.

The key features of the NADP-ME subtype are movement of malate and pyruvate between M and BS cells and decarboxylation of malate in the BS chloroplasts. By contrast, the NAD-ME and phosphoenolpyruvate carboxykinase (PEP-CK) subtypes both move Asp and Ala between M and BS cells. Asp is converted to either malate or OAA, and then malate is decarboxylated by NAD-ME in the BS cell mitochondria or OAA is decarboxylated by PEP-CK in the BS cell cytoplasm. Notably, the NAD-ME and PEP-CK pathways have higher energy requirements than the NADP-ME pathway, and both have more intracellular transport steps. In the PEP-CK subtype, PEP-CK and NAD-ME decarboxylases can operate in parallel, placing an even greater energetic load on the process (Burnell and Hatch, 1988). PEP-CK activity has also been detected in maize, which is classically considered as an NADP-ME subtype, raising the question of whether the subtype classification is actually robust (Furbank, 2011).

The energetic cost of the C_4 pathway is offset by the fact that all forms of the pathway act to concentrate CO_2 at the site of Rubisco. This carbon-concentrating mechanism prevents oxygen from competing for the active site of Rubisco and thus reduces the energetically wasteful process of photorespiration, which in C_3 plants can reduce photosynthetic output by up to 40% (Ehleringer et al., 1991). However, these recognized gains demand the development of specialized leaf anatomy and the compartmentalization of biochemical reactions. This in turn requires sophisticated regulatory processes to operate at all levels of gene expression and protein function.

EVOLUTION

Phylogenetic Diversity

In land plants, the C_4 pathway is found only in angiosperms. In this group, there are 62 C_4 taxa that comprise 36 eudicots, 6 sedges, 18 grasses, and 2 aquatic lineages in the genera *Hydrilla* and *Egeria* (Sage et al., 2011). While the evolutionary independence of all of these lineages is not clear, it is indisputable that the C_4 pathway arose multiple independent times from the ancestral C_3 pathway (Christin et al., 2010). In most cases (58 lineages), the pathway evolved in association with Kranz

anatomy, but in the aquatic lineages and in two Chenopod lineages (*Binertia* and *Suaeda*), the pathway operates in a single cell. In the aquatic species, CO_2 is concentrated from the cytoplasm to the chloroplast (Bowes, 2011), whereas in the Chenopods, CO_2 is concentrated from an outer to an inner region of the cell (Edwards and Voznesenskaya, 2011). In total, there are ~7500 C_4 species, most of which use the NADP-ME pathway and most of which (~4600 species) are grasses (Sage et al., 2011).

The phylogenetic distribution of C_4 grasses is notable in that they all occur in the so-called PACMAD clade (Christin et al., 2009a). This group comprises the six subfamilies Panicoideae, Arundinoideae, Chloridoideae, Micrairoideae, Aristidoideae, and Danthonioideae and thus includes the agronomically important crop plants maize, sorghum (*Sorghum bicolor*), and sugarcane. With at least 17 independent origins of C_4 proposed in this clade (Christin et al., 2007, 2008a), and none in the other seven grass families, it is tempting to speculate that a preconditioning event occurred in the last common PACMAD ancestor. In this regard, it may not be a coincidence that low levels of CA, the first enzyme of the C_4 shuttle, are a characteristic of the entire clade (Edwards et al., 2007).

Ecological Drivers

Given the multiple independent origins of C_4, it is not easy to identify the evolutionary drivers. However, because the C_4 pathway concentrates CO_2 at the site of Rubisco and because it is only energetically favorable in warm arid climates, three paleoclimatic drivers have been proposed: declines in CO_2, increases in temperature, and periods of drought. Notably, C_3 photosynthesis evolved in a CO_2-rich atmosphere of well over 1000 ppm, but atmospheric CO_2 levels dropped around 32 to 25 million years ago in the Oligocene, to ~500 ppm (Pagani et al., 2005). Molecular dating of the C_4 grass lineages suggests that the first transition from C_3 to C_4 occurred around 30 million years ago, coincident with this reduction in atmospheric CO_2 levels (Christin et al., 2008a; Vicentini et al., 2008). However, C_4 lineages continued to appear over the subsequent 20 million years (Christin et al., 2008a; Vicentini et al., 2008) and the ecological dominance of C_4 grasslands did not occur until 8 to 6 million years ago (Cerling et al., 1997). Thus, while declining CO_2 levels may have facilitated C_4 evolution, other factors influenced its expansion.

Biogeographical and phylogenetic studies have attempted to characterize the emergence and ultimate dominance of C_4 plants (particularly grasses) in certain environmental niches. Crucially, the level of atmospheric CO_2 at which C_4 outcompetes C_3 is dependent on temperature. C_4 is favored at 550 ppm CO_2 at 35°C, 450 ppm at 30°C, and 350 ppm at 25°C (Ehleringer et al., 1997). Given this interdependence, it might be predicted that C_4 plants evolved first in the tropics and only moved north as atmospheric CO_2 levels dropped to levels of ~250 ppm in the Miocene. However, although most C_4 species are found growing in high-temperature climates, the analysis of a 1200-taxon grass phylogeny alongside climate data for each of the species failed to correlate C_4 with any of a number of temperature parameters (Edwards and Smith, 2010) (Figure 2). Instead, there was compelling evidence to suggest that 18 of the 20 C_4 origins examined were correlated with marked reductions in annual rainfall.

Despite the inference that the evolution of C_4 was influenced by reduced water availability (Edwards and Smith, 2010), the issue remains far from resolved. Other reports suggest that although extant C_4 species are preferentially localized in arid environments, drought was not a driver for C_4 evolution. Instead, it is suggested that once the pathway had evolved, C_4 as opposed to C_3 grasses were more likely to make the transition into arid habitats (Osborne and Freckleton, 2009). The Miocene-Pliocene expansion of C_4 grasslands, to the point where 3% of vascular plant species account for 25% of terrestrial photosynthesis, is further proposed to have resulted from combinations of coevolution with grazing mammals (Bouchenak-Khelladi et al., 2009), increased temperature, increased summer rainfall, and more frequent occurrence of fire (discussed in Osborne, 2011).

The difficulty of trying to understand the complex interplay between paleoclimatic factors that favored C_4 versus C_3 physiology is illustrated by the results of a long-term elevated CO_2 experiment. Although elevated CO_2 is predicted to favor productivity in C_3 plants, when combined with an increase in temperature, the opposing effects of CO_2 and temperature on soil water content led to enhanced productivity in C_4 rather than C_3 prairie grasses (Morgan et al., 2011). Enhanced photosynthetic activity was also seen in C_4 maize plants when exposed to elevated CO_2 levels in the field (Leakey et al., 2004). In a similar paradox, despite the fact that C_4 species generally occupy drier habitats than C_3 species, a comparison of physiological properties in a range of grass species demonstrated that the performance advantages of C_4 photosynthesis are actually reduced by drought (Taylor et al., 2011). In light of such apparent contradictions, it seems that we may have overestimated our ability to identify the ecological drivers for C_4 evolution and to predict how the pathway will respond to future climate change.

DEVELOPMENT

Developmental Innovations

The evolution of C_4 photosynthesis required the modification of leaf development programs. In single-cell C_4 systems, intracellular partitioning mechanisms evolved, while in two-cell systems, specialized Kranz leaf anatomy developed. Insight into how these developmental pathways may have evolved has been obtained from comparisons between development in extant C_3 and C_4 species and by the examination of species that develop traits intermediate between C_3 and C_4. Such intermediates have been identified in a number of genera, most of which are eudicots (reviewed in Sage et al., 2011). In families such as *Flaveria*, C_3, C_3-C_4 intermediate, C_4-like, and C_4 species have all been identified (Ku et al., 1983). Intermediate *Flaveria* species may thus represent a transitional phase of C_4 evolution. However, other intermediates, such as *Moricandia arvensis* (Holaday et al., 1981), occur in families with no known C_4 species. Although it is possible that C_4 species have yet to evolve in these families, it is perhaps more likely that such intermediates define a distinct developmental state.

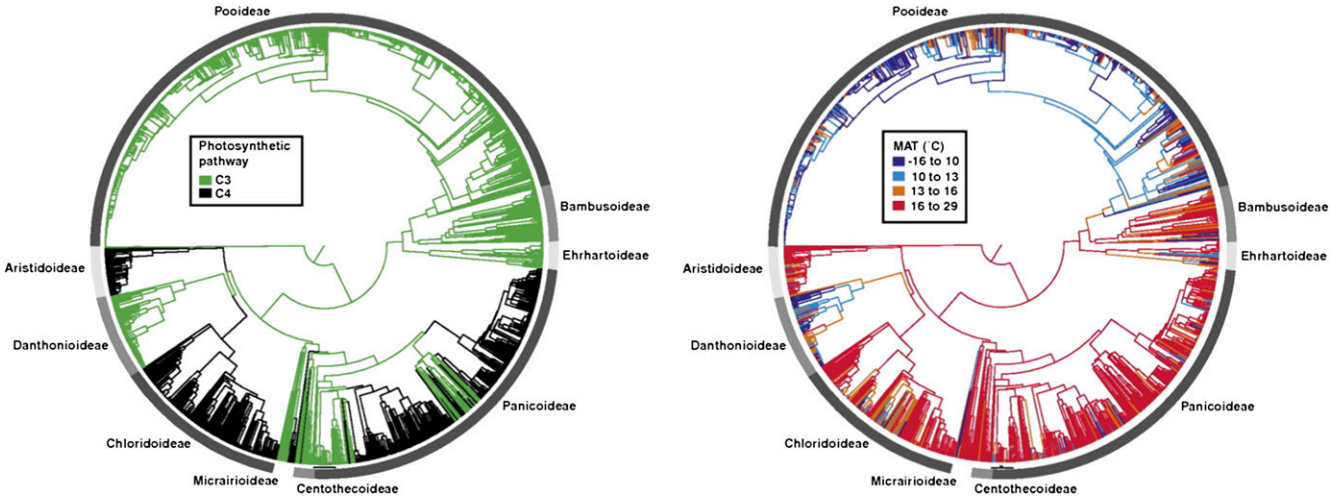

Figure 2. Comparison between Photosynthetic Pathway and Mean Annual Temperature for 1200 Grass Species Representing 20 Origins of C_4.

(Reprinted with permission from *Edwards and Smith [2010]*, Figure 2.)

In addition to obligate C_3-C_4 intermediates, there are a number of examples where C_4 development is induced by environmental cues. These facultative C_4 systems provide an opportunity to examine the developmental transition from C_3 to C_4 in the context of individual plants. Examples of this type include *Eloecharis vivipara*, an aquatic species that develops C_3 anatomy in submerged leaves and C_4 anatomy in aerial leaves (Ueno et al., 1988). Interestingly, in this system, the transition from C_3 to C_4 can also be induced by abscisic acid (Ueno, 1998). Another well-studied example is *Flaveria brownii*, in which the extent of C_4 induction is correlated with light intensity; plants grown in higher light intensities are more C_4-like than those grown at lower intensities (Monson et al., 1987; Cheng et al., 1989).

C_3 Development Is Default

The single-cell C_4 pathway operates in aquatic C_4 species and in the terrestrial chenopods *Binertia* and *Suaeda*. Leaf development in these species is quite remarkable in that chlorenchyma cells are organized into two distinct cytoplasmic compartments that are maintained by an organized network of actin filaments and microtubules (Chuong et al., 2006). In *Binertia*, there is a centrally located compartment surrounded by a more peripheral compartment (Voznesenskaya et al., 2002; Offermann et al., 2011), whereas in *Suaeda*, the two compartments are distal (toward the outside of the leaf) and proximal in the cell (Voznesenskaya et al., 2001). In each of the two compartments, chloroplasts accumulate a distinct complement of photosynthetic enzymes with the peripheral/distal chloroplasts analogous to M cell chloroplasts of the Kranz system and the central/proximal chloroplasts analogous to the BS cell chloroplasts. Crucially, this dimorphism is not apparent early in development in that a monomorphic C_3 chloroplast state develops by default and the C_4 pattern is induced by later developmental cues (Voznesenskaya et al., 2005; Lara et al., 2008).

The development of a default C_3 state in C_4 plants is not confined to species with single-cell systems. A similar situation occurs in both the monocot maize and the eudicot amaranth, where Rubisco accumulates in both BS and M cell chloroplasts unless light and/or developmental cues restrict accumulation to BS cells (Langdale et al., 1988; Wang et al., 1993). In maize, it has been concluded that the C_4-inducing signals are only perceived in cells that are within a two-cell radius of a vein (Langdale and Nelson, 1991). This deduction is based on the observation that in leaf-like organs, such as the husk leaf sheath, where up to 20 cells separate vein pairs, dimorphic chloroplast development is only observed in cells immediately surrounding the vasculature (Langdale et al., 1988; Pengelly et al., 2011).

Veins Act as Organizing Centers

It is perhaps not surprising that veins play a key role in the differentiation of C_4 leaf anatomy since one of the most obvious differences between leaf morphology in C_3 and C_4 plants is leaf venation pattern. Measurements of vein density in a range of C_3 and C_4 species demonstrated that veins are consistently more closely spaced in C_4 species (Crookston and Moss, 1974). Furthermore, quantitative measurements of BS-to-M cell ratios in C_3 and C_4 leaves showed that in C_4 plants the ratio approaches 1:1 (Hattersley and Watson, 1975; Dengler et al., 1994; Muhaidat et al., 2007). This ratio equates to veins (V) being separated by only four photosynthetic cells in C_4 leaves as opposed to up to 20 cells in C_3 leaves (Figure 3). As such, the repeating V-BS-M-M-BS-V unit of Kranz anatomy is generated. One notable exception to this repeating pattern is found in *Arundinella hirta*, a C_4 grass that exhibits an atypical anatomy where wreaths of so-called distinctive (D) cells are found between V-BS-M-M-BS-V units (Crookston and Moss, 1973; Dengler and Dengler, 1990). The D cells carry out the same function as BS cells but are not themselves associated with veins (Reger and Yates, 1979; Dengler et al., 1996; Wakayama et al., 2006). Notably, if the number of BS and D cells is combined, the 1:1 ratio is also observed in *A. hirta*.

A comparison of vascular development in C_3 and C_4 *Flaveria* species showed that both the major and minor veins were

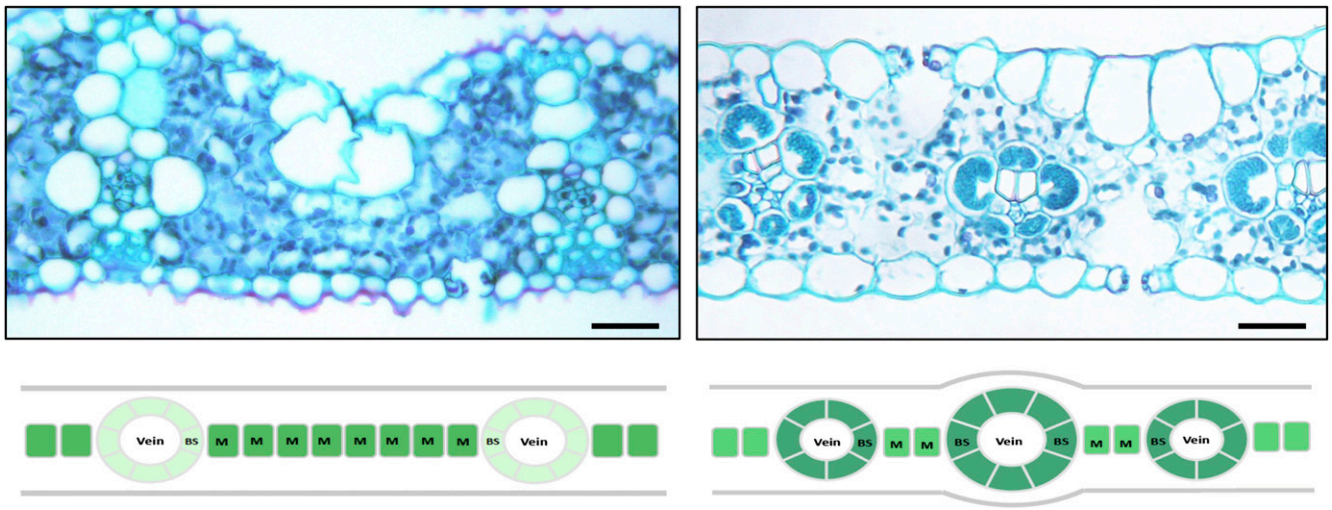

Figure 3. Transverse Leaf Sections and Corresponding Schematics of C_3 Rice and C_4 Maize.

Rice (left) and maize (right). Bars = 30 μm.

initiated at comparable stages in development but that a greater number of minor veins were initiated in the C_4 species (McKown and Dengler, 2009). A first step in the evolution of Kranz anatomy may thus have been the acquisition of a mechanism to induce procambium at more regular intervals across the leaf. Given the established role of auxin in vascular development, it is likely that such a mechanism was adapted from existing auxin pathways. A study that compared anatomical and biochemical differences between 16 *Flaveria* species that encompassed C_3, C_3-C_4 intermediate, C_4-like, and C_4 types further supported the suggestion that altered vein patterning was an early modification in the evolution of C_4. Based on the phylogeny of *Flaveria*, it was concluded that C_4 vein pattern traits were acquired prior to either intermediate or C_4-like biochemistry (McKown and Dengler, 2007). Because the presence of extra veins leads to an effective increase in BS cell area and a decrease in M cell area, it is likely that these traits also preceded biochemical changes.

BIOCHEMISTRY

Metabolic Modifications

Most of the enzymes involved in the C_4 pathway play housekeeping roles in C_3 plants (reviewed in Aubry et al., 2011). For example, chloroplast-localized CA ensures a supply of CO_2 into the Calvin-Benson cycle (Price et al., 1994), and PEPCase generates malate as a photosynthetic product (Ting and Osmond, 1973). PEPCase-generated malate is used to provide carbon skeletons to the TCA cycle (Miyao and Fukayama, 2003) and for ammonium assimilation (Masumoto et al., 2010). In addition, PEPCase activity contributes to the extension of fibers in cotton (*Gossypium hirsutum*; Li et al., 2010b) and to salt and drought responses in wheat (*Triticum aestivum*; González et al., 2003) and *Arabidopsis thaliana* (Sánchez et al., 2006). The decarboxylase PEP-CK has similarly diverse roles in C_3 plants. These roles include mobilization of sugars from lipids in seeds during germination (Leegood and ap Rees, 1978), provision of PEP to the shikimate pathway, and metabolism of nitrogenous compounds (Walker et al., 1999). PPdK-generated PEP has also been shown to contribute to seed metabolism (Kang et al., 2005), the shikimate pathway (Hibberd and Quick, 2002), and nitrogen remobilization (Lin and Wu, 2004). C_4 biochemistry thus evolved through modification of existing functions rather than de novo. This conclusion is supported by the fact that C_3 remains the default developmental state in C_4 plants (discussed above) and that biochemical characteristics of C_4 photosynthesis are found around the vascular bundles of C_3 plant stems (Hibberd and Quick, 2002; Brown et al., 2010).

One of the main advantages of the C_4 pathway is a reduction in photorespiration because O_2 cannot effectively compete for the active site of Rubisco in the CO_2-enriched environment of the BS cells. However, it is a misconception that C_4 plants eliminate the photorespiratory pathway entirely. Maize mutants that are deficient in glycolate oxidase, a key enzyme in the pathway, are seedling lethal at ambient CO_2 (Zelitch et al., 2009). When grown at higher CO_2 levels that inhibit photorespiration, however, the seedlings survive. This suggests that the early stages of the pathway are functional in the mutant and that a buildup of glycolate is toxic for the plant. Most of the photorespiratory pathway is localized to the BS cells of C_4 plants (Majeran et al., 2005), and as a consequence, the released CO_2 further enriches the environment for Rubisco. The use of the photorespiratory pathway as a shuttle to enrich CO_2 in the BS cells is also found in C_3-C_4 intermediates, where the final step of the pathway is restricted to BS cells (reviewed in Bauwe, 2011). This step is catalyzed by Gly decarboxylase and as such it has been proposed that one of the first steps in the evolution of C_4 metabolism was the localization of Gly decarboxylase to the BS (Sage, 2004; Gowik and Westhoff, 2011). This would have enriched the BS

environment with CO_2 and may have acted as a driver to induce the Calvin-Benson cycle in this cell type.

Metabolite Transport

Increased photosynthetic efficiency in C_4 plants results from the CO_2-enriched BS cell environment in which Rubisco operates. This environment can only be maintained if the CO_2 that is generated by the BS-localized decarboxylation reaction cannot diffuse back out of the cell. It is generally assumed that the suberized BS cell wall prevents CO_2 leakage. However, the situation cannot be that simple, not least because NAD-ME C_4 species do not have suberized BS cell walls and the different C_4 subtypes carry out the decarboxylation reaction in different sub-cellular compartments. It is thus likely that the diffusion kinetics are also affected by chloroplast and mitochondrial position in the cell and by the distance of the decarboxylation site from the BS-M cell interface (von Caemmerer and Furbank, 2003). A role for porins in CO_2 movement across intracellular membranes has been discussed, but their importance in C_4 plants is far from clear (Weber and von Caemmerer, 2010). Regardless of the exact mechanism, mathematical modeling has shown that the efficiency of the C_4 pathway can only be maintained (through development or in different environmental conditions) if BS cell resistance to leakage increases as the amount of C_4 acid that is decarboxylated decreases (and vice versa) (von Caemmerer and Furbank, 2003). The mechanisms that regulate this dynamic process are far from clear.

Although CO_2 must be prevented from moving between BS and M cells, many metabolic intermediates of the pathway must diffuse between the two cell types and must be actively transported between compartments in individual cells. In C_3 plants, one transport process has to occur across the chloroplast envelope for every three CO_2 molecules assimilated into triose phosphate (TP). By contrast, 30 transport steps are required per TP generated in NADP-ME C_4 plants (reviewed in Weber and von Caemmerer, 2010). This difference has implications in terms of the energetic cost of photosynthesis, the establishment of plasmodesmatal connections between the two cell types, and the proteins that had to be modified during C_4 evolution. Until recently, the identities of the transporter proteins were not known, and even now there remain big gaps in our knowledge (reviewed in Majeran and van Wijk, 2009). The only two transporter proteins that have been unambiguously identified are the TP transporter, which moves TP from the M chloroplast to M cytoplasm and from the BS cytoplasm to the BS chloroplast, and the PEP/phosphate translocator, which moves PEP from the M chloroplast into the M cytoplasm (Bräutigam et al., 2008). Candidates for the M cell malate/OAA antiporter (dicarboxylate transporter) (Taniguchi et al., 2004; Majeran et al., 2008) and for a sodium-dependent pyruvate transporter (bile acid:sodium symporter family protein 2) have also been identified (Furumoto et al., 2011). Other transporters (including all of the BS cell–specific transporters) remain to be identified. Given the quantitative and cell-specific proteomic data available, however, it is presumably only a matter of time before functional assays (Nozawa et al., 2007) of potential candidates (Bräutigam et al., 2008; Majeran et al., 2008) provide insight.

Physiological Efficiencies

Although the transition from C_3 to C_4 can be considered at the level of individual genes and proteins (see below), C_4 is in effect a complex trait. In addition to the modified photosynthetic pathway, aspects of nitrogen and sulfur metabolic pathways are also altered or localized in specific cell types (Friso et al., 2010; Bräutigam et al., 2011). Key physiological enhancements include greater radiation, nitrogen, and water use efficiencies (RUE, NUE, and WUE) than C_3 plants. For example, measured at 30°C and 380 ppm CO_2, estimates for the maximum conversion efficiency of solar energy to biomass is 4.6% for C_3 plants and 6% for C_4 plants (Zhu et al., 2008). The relatively higher CO_2 assimilation rates in C_4 plants result from increased efficiency of Rubisco, and this in turn means that only 8% of leaf N needs to be allocated to the enzyme. This contrasts with a >20% allocation to Rubisco in some C_3 plants, leading to a much higher proportion of N required per CO_2 fixed (reviewed in Ghannoum et al., 2011). Increased WUE has also been proposed to result from increased CO_2 assimilation rates (Wong et al., 1985), although decreased stomatal conductance has also been implicated (Taylor et al., 2010).

REGULATION OF GENE FUNCTION

Gene Families

A comparison of photosynthetic gene expression patterns in independently evolved C_4 grass lineages demonstrated that the only patterns common to all origins were an upregulation of PEPCase and a downregulation of Rubisco in M cells (Sinha and Kellogg, 1996). All other gene expression patterns varied between different lineages and different C_4 subtypes. The recruitment of PEPCase into an M cell–specific photosynthetic role was thus a key step in the evolution of the C_4 pathway. PEPCase genes are members of a multigene family that encodes multiple isoforms of the enzyme, only one of which is involved in the C_4 pathway (Lepiniec et al., 1994). Phylogenetic analyses of these gene families in the grasses have shown that the C_4 gene evolved eight independent times from the same non-C_4 gene (Christin et al., 2007). During this transition, 21 amino acids evolved under positive selection and converged to similar or identical amino acids. In some amino acid positions, identical changes have also been recorded in non-grass C_4 species (Bläsing et al., 2000; Gowik et al., 2006; Christin et al., 2007, 2011). At some sites, such convergence appears to reflect the need for a specific amino acid for C_4 function, whereas at other sites, there appears to be a requirement for loss of the C_3-associated amino acid.

In addition to PEPCase, examples of positive selection and gene convergence during the evolution of C_4 have also been reported for genes encoding Rubisco and PEP-CK (Christin et al., 2008b, 2009b). In the case of PEP-CK, there is evidence for initial acquisition of the C_4 gene followed by recurrent losses and at least three independent reacquisitions. All of these examples point to gene duplication in C_3 ancestors being a prerequisite for C_4 evolution. Neofunctionalization then presumably occurred either in the context of the C_3 ancestor or, at least in the case of PEP-CK, within the C_4 lineages (for a discussion, see Monson,

2003). Support for this evolutionary trajectory has been provided by a comparative analysis of C_3 (rice [*Oryza sativa*]) and C_4 (maize and sorghum) genomes (Wang et al., 2009).

While in some cases, the recruitment to C_4 involved changes in protein function, in other cases, protein targeting mechanisms were altered. For example, there are three genes encoding chloroplast-localized CA in the C_3 species *Flaveria pringlei*. In the C_4 species *Flaveria bidentis*, two genes also encode chloroplast-localized proteins, as in *F. pringlei*, whereas the third has lost the chloroplast-targeting signal, facilitating CA function in the M cell cytoplasm (Tanz et al., 2009).

Cis- and *Trans*-Regulators of Transcription

Over the last 25 years, considerable effort has been invested into understanding how the cell-specific and light-induced regulation of C_4 enzymes is achieved. These studies have examined the activity of *cis*- and *trans*-regulatory factors through the use of biochemical assays, transient expression assays in protoplasts, transgenic manipulation of gene expression in both C_3 plants and C_4 plants, and mutant analysis. Two substantial reviews, written 10 years apart, cover the detailed information for each gene and by comparison illustrate how the field has advanced in recent years (Sheen, 1999; Hibberd and Covshoff, 2010). A few key points emerge from a synthesis of the data, and they can be grouped according to level of gene regulation (Wang et al., 2011).

At the epigenetic level, both nucleotide and histone methylation have been associated with the M cell–specific regulation of genes encoding PEPCase (Ngernprasirtsiri et al., 1989; Langdale et al., 1991; Offermann et al., 2006; Danker et al., 2008) and histone methylation with BS cell–specific regulation of NADP-ME (Danker et al., 2008). However, such examples are limited both with respect to the generality across C_4 species and in terms of how epigenetic mechanisms interact with other levels of gene regulation. Information about the epigenetic regulation of other C_4 genes is similarly lacking.

In terms of transcriptional control, *cis*-regulatory elements that direct M or BS cell–specific expression have been identified for a number of genes (reviewed in Hibberd and Covshoff, 2010). In the case of any individual C_4 gene, however, the identified elements differ between C_4 species in terms of both sequence composition and position within the gene (particularly between monocots and eudicots). With few exceptions, these *cis*-regulators of transcription have yet to be proven sufficient for cell-specific expression. One exception is the 41-bp mesophyll expression module 1 (MEM1) element from the C_4 species *Flaveria trinervia ppcA* gene promoter. MEM1 is both necessary and sufficient to drive M cell–specific accumulation of *ppcA* gene transcripts in both C_4 and C_3 *Flaveria* species (Gowik et al., 2004; Akyildiz et al., 2007). Two other exceptions have been reported for genes of the NAD-ME C_4 species *Cleome gynandra*. The 5′ and 3′ untranslated region sequences from the *C. gynandra* genes encoding PPdK and CA have been shown to be sufficient for M cell–specific expression in transient assays (Kajala et al., 2011). Interestingly, these sequences are conserved in the orthologous genes of the C_3 species *Arabidopsis*. This observation suggests that cell specificity in the C_4 species evolved through changes in *trans*-regulatory mechanisms.

A similar scenario of altered *trans*-regulators in C_4 species relates to the gene encoding NAD-ME. In this case, a novel mechanism of gene regulation has been revealed. Specifically, a 240-bp sequence of the coding region of the gene encoding NAD-ME, which must be transcribed to be functional, is necessary and sufficient to direct BS cell–specific expression (Brown et al., 2011). As with the PPdK and CA examples discussed above, this sequence is also present in the C_3 orthologs of *Arabidopsis*, where expression is not cell specific.

Putative *trans*-regulators of cell-specific gene expression in maize have been identified by gel retardation assays with 5′ promoter sequences of genes encoding PEPCase (Taniguchi et al., 2000), Rubisco small subunit (Xu et al., 2001), and PPdK (Matsuoka and Numazawa, 1991). However, the context in which these proteins act is not understood, and the properties of the proteins are not known. The only known transcription factors that have been proposed to play a role in C_4 regulation are members of the DNA binding with one finger (DoF) and *Golden2*-like (*GLK*) gene families. DoF1 is a zinc finger DNA binding protein that was shown to bind to the promoter of the maize *PEPC* gene and was proposed to play a role in regulating cell-specific gene expression (Yanagisawa and Sheen, 1998). While this may be the case, subsequent analyses showed that DoF proteins also perform a more general role in the transcriptional activation of non-photosynthesis-related genes in maize (Yanagisawa, 2000). *Golden2* (*G2*) is a GARP transcription factor that was initially identified by mutant analysis in maize, where loss of function led to impaired BS cell development (Hall et al., 1998a; Rossini et al., 2001). The first mutant that was isolated exhibited rudimentary chloroplast development and reduced accumulation of transcripts for C_4 enzymes in the BS cells, leading to the suggestion that G2 was a global regulator of C_4 development in BS cells (Langdale and Kidner, 1994). However, subsequent analysis of an allelic series of *g2* mutations determined that the effects on C_4 gene expression were a secondary consequence of perturbed plastid development and thus showed that G2 is not a direct regulator of genes encoding C_4 enzymes (Cribb et al., 2001).

Although *G2* is not a direct regulator of C_4 gene expression, it nevertheless functions specifically in maize BS cells, whereas its paralog Zm-*Glk1* functions specifically in M cells. By contrast, *GLK* gene pairs in C_3 plants act redundantly in a single photosynthetic cell type (Rossini et al., 2001; Fitter et al., 2002; Yasumura et al., 2005). It is now known that in the C_3 plant *Arabidopsis*, GLK proteins act cell autonomously to directly regulate the expression of a suite of genes encoding chlorophyll biosynthesis enzymes, light harvesting, and electron transport components (Waters et al., 2008, 2009). As such, they are proposed to synchronize photosynthetic gene expression in response to environmental and developmental cues. This suggestion is supported by the number of pathways in which GLK proteins have been shown to play a role (Savitch et al., 2007; Gutiérrez et al., 2008; Yu et al., 2011). Importantly, overexpression of *GLK1* in the C_3 plant rice leads to the light-induced development of chloroplasts in most cell types (Nakamura et al., 2009). However, a similar response is not seen in *Arabidopsis* (Waters et al., 2008). As such, GLK proteins are sufficient to induce the proplastid-to-chloroplast transition, but only in certain developmental contexts. Given the cell-autonomous action of

GLK proteins and cell-specific accumulation of *G2* and *Glk1* transcripts in maize (Rossini et al., 2001) and sorghum (unpublished transcriptome data; U. Gowik and P. Westhoff, personal communication), it remains possible that the compartmentalization of GLK function played a critical role in the evolutionary transition to C_4 photosynthetic development.

Posttranscriptional Regulation

Mechanisms that posttranscriptionally regulate gene expression can be divided into those that regulate transcript turnover, translation, or posttranslational activation. Genes encoding the large (*rbcL*) and small (*RbcS*) subunits of Rubisco are regulated at all of these levels. This observation is perhaps not surprising given that Rubisco function in C_4 plants requires the integration of nuclear and chloroplast gene expression programs in addition to BS cell–specific regulation of subunit assembly. The DNAJ-like chaperone BUNDLE SHEATH DEFECTIVE2 (BSD2) has been shown to bind polysome-associated *rbcL* RNA and is thought to mediate Rubisco assembly and stability in maize (Brutnell et al., 1999). Loss of BSD2 function leads to absence of Rubisco protein and to ectopic accumulation of *rbcL* transcripts in M cells (Roth et al., 1996). Although cell-specific posttranscriptional mRNA turnover has been implicated for both *rbcL* and *RbcS* genes, it is not understood how the failure to assemble Rubisco in BS cells of the *bsd2* mutant leads to a failure to repress *rbcL* transcript accumulation in the M cells. Similarly, there is nothing known about the mechanism of mRNA turnover that operates during normal development. In this regard, it is somewhat surprising that there have been no reports of C_4 gene regulation by noncoding RNAs, given that such RNAs are regulatory components of so many developmental processes (Vaucheret, 2006).

Because research into C_4 photosynthesis was founded in biochemistry, it has been known for many years that posttranslational mechanisms play a key role in the regulation of at least two enzymes of the pathway. PEPCase is posttranslationally and diurnally regulated by the enzyme PEPCase kinase (PEPCk) (not to be confused with PEP-CK, which is the decarboxylase PEP carboxykinase) (Nimmo et al., 1987; Saze et al., 2001). The relatively rapid activation and inactivation that is demanded for the diurnal activity of PEPCase is facilitated by the rapid turnover and degradation of PEPCk by the ubiquitin-proteosome pathway (Agetsuma et al., 2005). PPdK is also reversibly light activated by a protein kinase, but in this case, rapid deactivation is facilitated by the same protein. PPdK regulatory protein is a bifunctional Ser/Thr kinase phosphatase that catalyzes both the ADP-dependent inactivation and Pi-dependent activation of PPdK (Burnell and Hatch, 1985; Burnell and Chastain, 2006).

GENETICS

Although maize has been a model genetic organism for almost a century, genetic approaches to understand C_4 have yielded limited information. Screens for maize mutants with perturbed vein spacing patterns were unsuccessful (J. Langdale, unpublished data), while those for disrupted BS or M cell development led to the identification of only a handful of examples (Langdale and Kidner, 1994; Roth et al., 1996; Hall et al., 1998b; Covshoff et al., 2008). Of those that were characterized in depth, *bsd2* and *high chlorophyll fluorescence136* were shown to be perturbed in the assembly and/or stabilization of BS (Rubisco) and M (photosystem II) cell–specific proteins, respectively (Brutnell et al., 1999; Covshoff et al., 2008), whereas the *bsd1* mutant phenotype resulted from loss of G2 transcription factor activity (Hall et al., 1998a) (see above). In *Panicum maximum*, a potential vein spacing mutant was identified in an ethyl methanesulfonate–mutagenized population, but the pleiotropic nature of the phenotype led to lethality and the line was lost (Fladung, 1994).

Other approaches to identify genetic regulators of C_4 include the generation of hybrids between C_3 and C_3-C_4 *Flaveria* species and the characterization of oat (*Avena sativa*) lines with single maize chromosomes added. While the *Flaveria* experiments provided some insight into whether aspects of C_4 were dominant or recessive in F1 hybrids, the sterility of the hybrids precluded quantitative trait loci analysis for C_4 traits (Brown et al., 1986, 1993; Cameron and Bassett, 1988; Holaday et al., 1988; Cameron et al., 1989). Similarly, the oat-maize addition lines provided insight into certain aspects of C_4 regulation but failed to reveal global regulators of the pathway. In particular, oat-maize addition lines that contained maize chromosomes 6 and 9 were shown to accumulate maize PEPCase and PPdK (Kowles et al., 2008). Notably, both enzymes were active, suggesting that oat PEPCk and PPdK regulatory protein can phosphorylate the maize proteins. However, even in lines with both chromosomes present, photosynthesis was more C_3-like than C_4.

The introduction of *Setaria viridis* as a new model organism for studying the C_4 pathway in monocots provides hope that future genetic analyses will be informative because the plant is relatively small and the generation time is short (Brutnell et al., 2010). This will allow mutant screens to be performed on a much larger scale than has been possible in maize and other C_4 large plants. That said, the dearth of insight thus far provided by genetic approaches may simply be a reflection of the quantitative nature of C_4 traits, and it will be some time before molecular and genetic tools are sufficiently advanced to make substantive progress in *S. viridis*.

C_4 SYSTEMS

Over the last few years, a number of approaches have been taken to assess C_4 at a systems level. These include proteome comparisons between isolated BS and M cell chloroplasts (Majeran et al., 2005; Friso et al., 2010), microarray analysis of BS and M cell transcriptomes (Sawers et al., 2007), transcriptome profiling of mature sugarcane leaves (Calsa and Figueira, 2007), transcriptome and proteome profiling in a single-cell C_4 species (Park et al., 2010) and across a developmental gradient in the maize leaf (Li et al., 2010a; Majeran et al., 2010), comparative transcriptomics between closely related C_4 and C_3 species (Bräutigam et al., 2011; Gowik et al., 2011), and genome-scale models of flux distribution between BS and M cells (Dal'Molin et al., 2010). All of these studies have generated a substantial amount of data, and more is on the way.

For now, we can say that 64% of maize genes are differentially expressed along the developing leaf gradient and that 21% (i.e.,

3441 genes) are differentially expressed between BS and M cells (Li et al., 2010a). Included in the 21% are members of 180 transcription factor families. Proteomes of a similar developmental gradient elucidate key metabolic and structural transitions along five phases of leaf development (phase 1 being the youngest basal leaf section and phase 5 being the oldest tip section) (Majeran et al., 2010). Three key features emerge from this analysis. First, BS cells (with associated vascular strands) can be isolated from whole-leaf tissue at all phases along the gradient. Second, distinct BS and M cell plastids are observed at phase 2. Third, distinct proteome specialization only becomes apparent in the regions of the leaf that are autotrophic (i.e., phases 3 to 5). In combination, these observations demonstrate that BS and M cell identity is determined early in development and that photosynthetic/metabolic distinctions are mapped onto this anatomical template much later in development.

Transcriptome comparisons between closely related species are harder to analyze for C_4-specific signatures because of background species differences. However, as more pairwise comparisons are added to the data set, the signal-to-noise ratio will increase. Thus far, a comparison between fully expanded *Cleome spinosa* (C_3) and *C. gynandra* (C_4) leaves has shown that 603 transcripts (2.8% of those identified) are more abundant in C_4 leaves (Bräutigam et al., 2011). These include genes encoding transport proteins, putative plasmodesmata-related proteins, cell wall–modifying enzymes, and 17 transcription factors. At a pathway level, the C_4 species had lower levels of transcripts associated with one-carbon metabolism, the shikimate pathway, amino acid metabolism, the Calvin-Benson cycle, photorespiration, and protein synthesis (both cytoplasmic and plastidic). By contrast, starch metabolism, cofactor synthesis, and nitrogen metabolism–associated transcripts were elevated in the C_4 species. Similar observations were made when comparing five *Flaveria* species with C_3, intermediate, or NADP-ME C_4 photosynthesis (Gowik et al., 2011). In this study, the authors placed an upper limit of 3582 expression changes required for the transition to C_4. Of course the key will be to determine which of those changes are necessary and sufficient for the transition.

FUTURE PERSPECTIVES

The renewed interest in C_4 biology results from increased global awareness of the difficulty we face in trying to provide food and fuel for a growing population. One way to increase yields while simultaneously improving WUE and NUE could be to introduce C_4 traits into C_3 crops. This idea was first proposed in the late 1990s when transgenic experiments to understand C_4 gene function were initiated (reviewed in Matsuoka et al., 2001) and Japan Tobacco was granted a U.S. patent on the generation of PEP-CK type C_4 cycle in rice (Arai et al., 2003).

One of the most promising reports at the time showed that introducing the intact maize *Ppc* gene into rice led to high levels of transgene expression, PEPCase enzyme activity two- to threefold of that found in maize, and reduced O_2 inhibition of photosynthesis (Ku et al., 1999). Introduction of the maize *PPdk* gene also produced increases in enzyme activity (as much as 40-fold in some lines) and that activity was light/dark regulated as normal (Fukayama et al., 2001). Similarly, introduction of the sorghum NADP-ME gene led to elevated transcript and protein levels and a 1.7-fold increase in enzyme activity (Chi et al., 2004). However, neither the PPdK nor NADP-ME transgenics showed changes in carbon assimilation, and in the case of the PEPCase transgenics, subsequent reports went on to show that the reduced O_2 inhibition was due to reduced rates of photosynthesis. This reduction was in part because of Pi limitation (Agarie et al., 2002) but also because the enzyme was phosphorylated in the dark (in the same way as the endogenous rice enzyme) instead of in the light (like the maize enzyme) (Fukayama et al., 2003). These findings highlighted the complexity of trying to alter the activity of just one enzyme, and when subsets of the different transgenes were combined, the picture became even more complicated (Taniguchi et al., 2008). In no case was a CO_2 concentrating mechanism generated, and in the case of PEPCase and NADP-ME, overexpression led to stunted growth that was only slightly mitigated by overexpression of NADP-malate dehydrogenase.

So why would the more recently formed C_4 rice consortium (http://irri.org/c4rice) and its funders, The Bill and Melinda Gates Foundation, once again consider introducing C_4 traits into rice? The rationale is straightforward: C_4 plants have higher RUE than C_3 plants, and yield increases in C_3 cereal crops are becoming limited by RUE (Hibberd et al., 2008). In addition, technology has improved significantly over the last few years. Phenotypes can now be assessed at the whole-plant level (Furbank et al., 2009), gene interactions can be diagnosed at a systems level (Zhu et al., 2010; Wang et al., 2011), and mutated genes associated with specific phenotypes can be identified through whole-genome sequencing. Even with these advances, however, the project remains a grand challenge.

Another driver of the current C_4 research agenda is the global focus on biofuels. Two of the current major biofuel crops, sugarcane and maize, are both C_4 species. Whereas the future of sugarcane as a fuel crop is almost certain, the use of maize can only be defended in a future where lignocellulosic fermentation means that grain is not used to produce ethanol. However, another C_4 species may hold the key to biofuel demands, at least in the US. The perennial grass *Miscanthus* × *giganteus* is capable of producing higher biomass than maize, primarily because it can photosynthesize efficiently for a longer period during the growing season. This increased efficiency is achieved in two ways. First, *Miscanthus* can photosynthesize at cooler temperatures than maize as a consequence of cold-tolerant PPdK activity (Wang et al., 2008). Second, its perennial habit means that it is able to capture more light early in the season because at that time the canopy is bigger than that of annual crops, such as maize (Dohleman and Long, 2009). Current estimates suggest that 9.7 million hectares of *Miscanthus* would provide enough biomass to meet the annual U.S. energy mandate (Somerville et al., 2010). Given that long-term field trials have shown that *Miscanthus* yields highly even on poor soils and that 14 million hectares of land dropped out of agricultural use in the US between 1997 and 2007 (http://www.ers.usda.gov/statefacts/us.htm), this C_4 perennial could resolve the food versus fuel dilemma in the US for the foreseeable future.

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