

COMMENTARY

Exploiting the engine of C₄ photosynthesis

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Abstract

Ever since the discovery of C₄ photosynthesis in the mid-1960s, plant biologists have envisaged the introduction of the C₄ photosynthetic pathway into C₃ crops such as rice and soybeans. Recent advances in genomics capabilities, and new evolutionary and developmental studies indicate that C₄ engineering will be feasible in the next few decades. Furthermore, better understanding of the function of C₄ photosynthesis provides new ways to improve existing C₄ crops and bioenergy species, for example by creating varieties with ultra-high water and nitrogen use efficiencies. In the case of C₄ engineering, the main enzymes of the C₄ metabolic cycle have already been engineered into various C₃ plants. In contrast, knowledge of the genes controlling Kranz anatomy lags far behind. Combining traditional genetics, high-throughput sequencing technologies, systems biology, bioinformatics, and the use of the new C₄ model species *Setaria viridis*, the discovery of the key genes controlling the expression of C₄ photosynthesis can be dramatically accelerated. Sustained investment in the research areas directly related to C₄ engineering has the potential for substantial return in the decades to come, primarily by increasing crop production at a time when global food supplies are predicted to fall below world demand.

Key words: Crop improvement, C₄ engineering, C₄ evolution, photosynthesis.

Introduction

C₄ photosynthesis is a relatively recent evolutionary phenomenon, arising many times over the past 30 million years with major consequences for the modern biosphere. Although C₄ species are relatively few compared with the much more numerous C₃ plants (~7500 C₄ species to nearly 250 000 C₃ species), they account for approximately a quarter of the primary productivity on the planet, and dominate the grassland and savannah biomes of warm-temperate to tropical latitudes (Sage *et al.*, 1999; Still *et al.*, 2003; Edwards *et al.*, 2010). The rise to dominance of C₄ grasses and sedges probably drove the expansion of the grassland biome in warm climates, and contributed to the evolutionary origin of the great grazing fauna within these grasslands (Cerling, 1999; Bobe and Behrensmeier, 2004; Edwards *et al.*, 2010; Osborne, 2011). Even the origin of our genus, *Homo*, may be related to the success of C₄ plants, because the expansion of savannah and grassland in eastern Africa that has been linked to humanity's origin followed the ascent of C₄ grasses (Sage, 1999; van der Merwe and Tschauner, 1999; Bobe and Behrensmeier, 2004; deMenocal, 2011).

While knowledge of C₄ photosynthesis is important for understanding the origin and function of the modern biosphere, the imperative to meet humanity's growing food, fuel, and fibre needs has promoted a resurgence in photosynthesis research in general, and C₄-related research in particular. On a global level, humanity faces a looming food crisis brought on by an increasing population and a rapid rise in living standards in the developing world (Mitchell and Sheehy, 2006; Hibberd *et al.*, 2008; Dawe *et al.*, 2010; Zhu *et al.*, 2010). In addition, demands for bioenergy are rising, potentially diverting significant land and raw material away from food production and natural habitat (FAO, 2008; Field *et al.*, 2008; Tilman *et al.*, 2009; Dauber *et al.*, 2010). With many agricultural regions now approaching their yield ceiling (the peak productive potential), existing options for enhancing yield will be limited, unless novel ways are found to boost productivity (Mitchell and Sheehy, 2006; Zhu *et al.*, 2010). One of the most promising ways to increase the yield ceiling and hence global productivity is to better exploit the superior engine of C₄ photosynthesis, both by using it on a greater scale and by improving the ability of C₄ plants to resist environmental stress (Sheehy *et al.*, 2000; 2007b; Hibberd *et al.*, 2008; Byrt *et al.*, 2011). In recognition of this possibility, the *Journal of Experimental Botany* has devoted this special issue to the theme 'Exploiting the engine of C₄ photosynthesis' as a way of highlighting some of the recent developments and future promise in the area of C₄ plant biology. In particular, this

special issue presents numerous treatments of past and present efforts to engineer the C₄ photosynthetic pathway into C₃ crops (Aubry *et al.*, 2011; Furbank, 2011; Kajala *et al.*, 2011; Miyao *et al.*, 2011; Nelson, 2011; Peterhansel, 2011). C₄ engineering represents one of the most ambitious undertakings in the life sciences, and, if successful, would significantly enhance agricultural production and provide humanity with new capacity to address the competing challenges of increasing food and bioenergy production simultaneously, while maintaining natural landscapes. In this commentary on the special issue, we provide background context and a perspective on humanity's efforts to better exploit the productivity advantage of the C₄ photosynthetic pathway.

The engine of C₄ photosynthesis

C₄ photosynthesis is the most efficient form of photosynthesis on the terrestrial Earth, due to its ability to concentrate CO₂ around ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) and thus suppress ribulose 1,5-bisphosphate (RuBP) oxygenation and photorespiration. The ability to concentrate CO₂ is a result of the C₄ metabolic cycle and the separation of the C₃ and C₄ functions into separate compartments, as outlined by Kajala *et al.* (2011) and Aubry *et al.* (2011) in this issue. With the cost of one to two ATPs per turn of the C₄ cycle, the C₄ engine increases bundle sheath (BS) CO₂ levels sufficiently to suppress photorespiration by $\geq 80\%$, depending upon the temperature (Kanai and Edwards, 1999; Sage *et al.*, 2011b). In addition, elevating the BS CO₂ allows Rubisco to operate close to its CO₂ saturation point, increasing its *in vivo* catalytic activity 2- to 5-fold in warmer climates (Seemann *et al.*, 1984; Sage, 2002; Ghannoum *et al.*, 2011). Because Rubisco in C₄ plants functions in a higher CO₂ setting than in C₃ plants, C₄ plants are able to have elevated photosynthetic capacities at warmer temperatures compared with ecologically similar C₃ plants, and realize instantaneous water use efficiency (WUE) and nitrogen use efficiency (NUE) that are typically 1.3–4 times greater than in C₃ plants (Long, 1999; Sage and Pearcy, 2000; Kocacinar *et al.*, 2008; Ghannoum *et al.*, 2011).

While the direct effects of CO₂ concentration on photosynthesis, WUE, and NUE are well described for C₄ plants, there are a number of secondary effects of the C₄ pathway that further contribute to growth enhancement and ecological performance in warm environments. Because C₄ plants can operate Rubisco more efficiently, they require 50–80% less Rubisco for a given photosynthetic rate (Sage and Pearcy, 1987b, 2000; Sage *et al.*, 1987; Ghannoum *et al.*, 2011). This enables them to allocate more of their N reserves to the most critical environmental challenge. For example, in agricultural weeds where competition for light can be severe, C₄ species can maintain a higher leaf area production rate at lower leaf N levels than C₃ weeds (Sage and Pearcy, 1987a). Elevated WUE delays drought

and can allow for longer growing seasons because soil water is less rapidly depleted (Markelz *et al.*, 2011). High WUE may also allow C₄ plants to exhibit more flexible allocation patterns, for example allocating proportionally more biomass to shoots in moist environments, or to roots in dry environments (Long, 1999; Taylor *et al.*, 2010). C₄ plants have a lower leaf-specific conductivity (hydraulic conductivity per unit leaf area) than similar C₃ species, possibly as a result of their superior WUE (Kocacinar *et al.*, 2008). In dry environments, C₄ plants exploit this difference by having safer xylem that is less prone to cavitation, while in moist environments they produce more leaf area per unit xylem, potentially allowing for faster canopy growth (Kocacinar and Sage, 2003, 2004).

Secondary benefits of C₄ photosynthesis also extend to the business and environmental side of crop production. Enhanced WUE and NUE have the potential to reduce agronomic costs and environmental impacts by lowering the diversion of water from cities and natural ecosystems, and decreasing the use of fertilizers per unit of yield (Barker *et al.*, 2010; Gregory *et al.*, 2010). Fertilizer use is one of the leading causes of biodiversity loss and water pollution around the planet, and is a major source of nitrous oxide, one of the leading anthropogenic greenhouse gases (Vitousek, 1997). Dead zones along coastlines near crop production regions are a direct result of fertilizer runoff (Robertson and Vitousek, 2009). In addition, improving the ability to feed humanity reduces pressure to convert rainforest and other natural habitat to agriculture. Increasing the efficiency of photosynthesis by better exploiting the C₄ pathway may thus be one of the best ways to increase profitability while reducing the detrimental impacts of agriculture on the global environment.

When crops are provided with adequate nutrients and water, the conversion of radiant energy into biomass is the most limiting environmental factor for crop growth (Zhu *et al.*, 2010). Absorption of sunlight is directly correlated with biomass yield in most crops, with the slope of the relationship reflecting the efficiency of radiation (=light) use (Fig. 1A). Radiation use efficiency (RUE) is dependent on two variables (Zhu *et al.*, 2010). The first is maximum quantum yield, which is greater in C₄ plants above 25 °C due to the high cost of photorespiration in C₃ species (Ehleringer and Pearcy, 1983). Secondly, as light levels increase towards saturation, quantum yields decline, due to the inability of carbon metabolism to utilize fully the light energy absorbed by the leaf (Zhu *et al.*, 2010). In warm climates with adequate water and fertilizer, C₄ plants have less of a decline in the instantaneous quantum yield at higher light levels, in part because Rubisco capacity does not become limited by CO₂ supply to the degree that occurs in C₃ plants (Sage, 2002). As a result, in equivalent environments above daytime temperatures of 25–30 °C, C₄ species can exhibit RUEs that are 50% greater than those of C₃ species (Fig. 1A; Long, 1999).

The combined effects of higher photosynthetic capacity and greater WUE, NUE, and RUE explain the differences in the yield ceiling observed between C₃ and C₄ crops. This

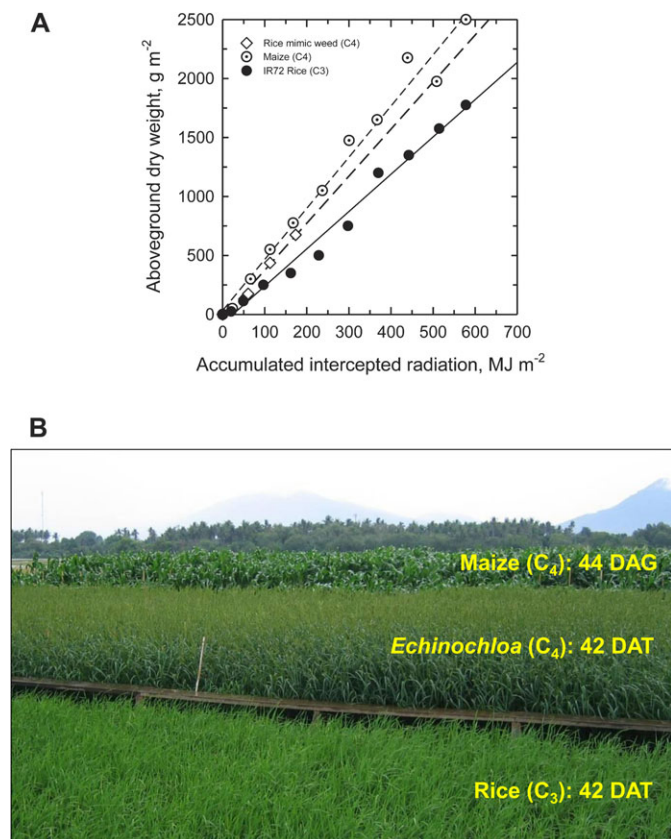


Fig. 1. (A) The relationship between above-ground dry matter and accumulated intercepted radiation for rice (variety IR72, filled circles, solid line), maize (open circles with centre dot and short dashed line), and *Echinochloa glabrescens*, a weed of rice paddies (often termed rice mimic; open diamonds, long-dashed line). The slopes of the relationships represent the radiation use efficiency for each crop. Redrawn from Sheehy *et al.* (2007a) by permission of John Sheehy and the International Rice Research Institute. (B) A photograph of the field trial shown in A. The photograph was taken when the rice and *Echinochloa* stands were at 42 d after transplanting of seedlings (42 DAT) and the maize stand was at 44 d after germination (44 DAG). Photo courtesy of John Sheehy of IRRI.

is well illustrated by a comparison of peak biomass yields measured for a range of bioenergy grasses (Byrt *et al.*, 2011), crops (Monteith, 1978; Loomis, 1983; Sheehy *et al.*, 2007a), tropical grasslands (Long, 1999), and forage species (Snaydon, 1991). These studies consistently show that C₄ species are the peak performers in warm conditions when comparisons are made between plants of similar growth form and ecological type, and results are standardized for accumulated light interception or growing season length. Below 30° latitude, Snaydon's (1991) review of annual biomass production lists only one C₃ species producing >44 t ha⁻¹ year⁻¹. In contrast, Snaydon (1991) lists 12 C₄ species with annual biomass yields >50 t ha⁻¹; seven of these C₄ species produced >60 t ha⁻¹ of biomass. Only C₄ species have been recorded as producing >80 t ha⁻¹, and the record yield for plant production per year is near 100 t

ha⁻¹, by tropical C₄ grasses such as *Echinochloa polystachya* and *Pennisetum purpureum* (Napier grass) (Snaydon, 1991; Long, 1999; Byrt *et al.*, 2010). Not all yield comparisons show C₄ species outperforming C₃ species, which has engendered some controversy regarding the superior productivity of the C₄ pathway (Monteith, 1978; Snaydon, 1991). C₄ species are less productive in cooler climates, and many C₄ plants are not inherently high producers (Eagles and Wilson, 1982; Loomis, 1983). For example, Bryt *et al.* (2011) lists three C₄ species grown in summer that produced >40 g plant⁻¹ and three that produced <5 g plant⁻¹; in all of these species, winter yields were an order of magnitude less than summer yields. These comparisons highlight the importance of evaluating species of equivalent growth form under identical conditions, and interpreting the results in the context of the prevailing environmental conditions (Percy and Ehleringer, 1984).

To evaluate C₃ versus C₄ production under identical field conditions in a warm tropical climate, Sheehy *et al.* (2007a) grew maize, a high yielding rice variety (IR72), and the major rice weed *Echinochloa glabrescens* side by side on well fertilized soils at the International Rice Research Institute (IRRI) in the Philippines during the dry season of 2006 (Fig. 1B). *Echinochloa glabrescens* was selected due to its identical growth form to rice, which is reflected in its common name, rice mimic. Maize and *E. glabrescens* exhibited RUEs that were 4.4 g and 4.0 g dry matter per MJ of radiation, compared with 2.9 g MJ⁻¹ for rice (Fig. 1). Final dry mass for maize was 28.8 t ha⁻¹, while that of rice harvested on the same day was 17.9 t ha⁻¹. The rice mimic matured weeks earlier and was not included in the final harvest. Significantly, maize and *E. glabrescens* closed the leaf canopy 1–2 weeks earlier than rice, demonstrating an ability of young C₄ plants to build up a leaf canopy sooner in warm conditions than C₃ plants of similar growth form. As a consequence of these differences, Sheehy *et al.* (2007a) concluded that in tropical to subtropical environments, C₄ rice could outproduce C₃ rice by 50%, or, viewed differently, C₄ rice at 60 d after planting could equal the yield of C₃ rice at 100 d after planting.

Improving the C₄ engine

To enhance the yield potential of global agriculture dramatically, the simple solution would be to adopt C₄ cropping systems as far and wide as climate conditions allow. This, however, is not practical because there are relatively few C₄ crop species that humanity can exploit. Of the leading 12 crops in the world, only two—maize and sugar cane—are C₄ plants (Table 1). Of ~150 crops listed by the United Nations Food and Agricultural Organization in their 2008 productivity tables, only five listings represent C₄ crops (maize, sugar cane, sorghum, fonio, and the group of C₄ species termed millets; Table 2). The major grains rice, wheat, barley, oats, and rye are C₃ species, as are all legumes, root crops, fibre crops, and fruit crops (Tables 1, 2). Notably, many of the major crops of warm regions are C₃,

Table 1. The photosynthetic pathway of the 12 most valuable crops in the world

Crop rank is based on 2008 production values in US dollars (given in parentheses), according to the United Nations Food and Agriculture Organization (FAOstat 2011, <http://faostat.fao.org>).

C ₃ crop	Global rank (US\$billion)	C ₄ crops	Global rank (US\$billion)
Rice, paddy	1 (136)	Maize	4 (40)
Wheat	2 (83)	Sugar cane	6 (35)
Soybeans	3 (47)		
Potatoes	5 (38)		
Cotton, lint	7 (34)		
Tomatoes	8 (32)		
Grapes	9 (31)		
Apples	10 (20)		
Peanuts	11 (18)		
Cassava	12 (16)		

Table 2. The photosynthetic pathway of leading crops in the world exclusive of the 12 most valuable crops shown in Table 1

C₃ crops are listed alphabetically (FAOstat 2011, <http://faostat.fao.org>).
C₄ crops are ranked by area under cultivation (after Brown, 1999).

C ₃ crops	C ₄ crops
Asparagus	Sorghum (<i>Sorghum bicolor</i>)
Banana and plantain (musas)	Pearl millet (<i>Pennisetum glaucum</i>)
Barley	Foxtail millet (<i>Setaria italica</i>)
Beans (all types)	Proso millet (<i>Panicum milliaceum</i>)
Berries (all types)	Finger millet (<i>Eragrostis tef</i>)
Cabbage	Tef (<i>Eragrostis tef</i>)
Carrots and turnips	Fonio (<i>Digitaria exilis</i>)
Cauliflower and broccoli	Amaranth (<i>Amaranthus spp.</i>)
Chilies and peppers	
Citrus (all types)	
Coconut	
Coffee	
Flax	
Hemp	
Hops	
Jute	
Lettuce	
Lentils	
Mango and guava	
Melons (all types)	
Oats	
Olives	
Onions	
Papaya	
Peas (all types)	
Plantains	
Pumpkins, squashes, and gourds	
Rape seed	
Safflower	
Spinach	
Sugar beet	
Sunflower	
Sweet potato	
Taro	
Yams	

with rice, soybeans, cotton, peanuts, and cassava standing out as the leading C₃ crops of low latitude (Table 1). All species used in agroforestry are also C₃. The major

exception to this pattern is forage and energy crops (Brown, 1999). In warm climates, forage grasses are largely C₄, and the leading bioenergy grass crops now produced—maize and sugar cane—are C₄, as are the majority of the second-generation bioenergy crops in development (e.g. *Miscanthus* and switchgrass) (Table 1; Brown, 1999; Jones, 2011). Thus, to be able to switch food production in warm climates from C₃ to C₄ systems, the options are to encourage wider consumption of existing C₄ crops, create new C₄ food crops, or to engineer the C₄ pathway into existing C₃ crops. Despite the technological challenge, the engineering option appears to be the most feasible, given longstanding cultural preferences for most C₃ foods over C₄ equivalents. One major impediment to changing food sources is that traditional foods such as rice often have a sacred status that partially defines the culture (van der Merwe and Tschauner, 1999; Plant Cultures, 2011). Perhaps of greater significance in modern times is that C₄ grains other than maize are widely viewed as less appealing from a culinary point of view.

The engineering of C₄ photosynthesis into C₃ crops has long been a goal of plant biologists. Within 3 years of the discovery of the C₄ pathway in 1966, efforts had begun to cross C₃ and C₄ species in the hope of identifying important genes that control the expression of C₄ photosynthesis (Björkman *et al.*, 1969; Brown and Bouton, 1993). With the advent of molecular biology and efficient transformation technologies, it became feasible to insert C₄ genes into C₃ plants, and in the 1990s concerted efforts were underway to create a single-celled C₄ rice plant (Matsouka *et al.*, 2001; Burnell, 2011; Miyao *et al.*, 2011). This work demonstrated that a C₄ cycle could be inserted into rice mesophyll cells; but, without the rest of the C₄ engine, such as the cellular compartmentalization and the transport systems, the single-celled efforts have not dramatically enhanced crop photosynthesis (Matsouka *et al.*, 2001; Miyao *et al.*, 2011). Miyao *et al.* (2011) in this issue provide a perspective of the efforts to engineer a single-celled C₄ rice plant, and they identify five specific lessons that the new generation of C₄ engineers should study carefully. In the last 10 years, John Sheehy at IRRI drew attention to the looming rice shortages in Asia, and predicted that converting rice into a C₄ plant could produce sufficient yield increases to meet the expected needs of Asia by 2050 (Sheehy *et al.*, 2000, 2007b; Mitchell and Sheehy, 2006). Under his leadership, a C₄ engineering consortium made up of the world's leading C₄ plant biologists was formed in 2006 to assess the feasibility of introducing the C₄ pathway into rice. With an initial grant of US\$11 million dollars from the Bill and Melinda Gates foundation, the C₄ engineering consortium in 2009 initiated a 3 year programme to begin anew the engineering of the C₄ pathway into rice (<http://C4rice.irri.org>).

The focus of the first section of this special issue is this effort to engineer the C₄ pathway into rice, with five reviews from some of the principle labs engaged in C₄ engineering (Aubry *et al.*, 2011; Kajala *et al.*, 2011; Li and Brutnell, 2011; Miyao *et al.*, 2011; Peterhansel, 2011). These papers are followed by reviews of the function, regulation, and

molecular biology of some of the key enzymes in the C₄ metabolic cycle (Chastain *et al.*, 2011; Furbank, 2011; Ludwig *et al.*, 2001; Maier *et al.*, 2011). As previously shown for phosphoenolpyruvate carboxylase (PEPC; Gowik and Westhoff, 2011), understanding the molecular biology of major C₄ enzymes will be important for successfully introducing functional proteins into C₃ plants. It is not sufficient simply to introduce the structural gene into a C₃ species, but it is also necessary to transform in appropriate targeting sequences and regulatory factors so that the foreign enzymes can effectively function in the proper compartment in the leaf (Miyao *et al.*, 2011). The treatments of C₄ enzyme function in this issue focus on carbonic anhydrase (CA; Ludwig, 2011), the decarboxylating enzymes (Maier *et al.*, 2011), and pyruvate, orthophosphate dikinase (PPDK; Chastain *et al.*, 2011), all of which are critical to the success of C₄ engineering but have not received as much attention as PEPC. CA, in particular, is not commonly thought of as being a major C₄ enzyme, yet the enhancement of the mesophyll-specific form, and loss of BS forms, are essential for the function of the C₄ pathway (Ludwig, 2011). The mesophyll form of CA provides a steady supply of bicarbonate for PEPC, while the absence of BS CA prevents the formation of bicarbonate that can quickly leak out through the plasmodesmata (Burnell and Hatch, 1988; Hatch and Burnell, 1990). The example of CA highlights the special challenge inherent in all engineering projects—one has to consider the whole system if the basic concept is to work (see also Miyao *et al.*, 2011 in this issue). In light of this, systems-level modelling will be key to C₄ engineering (Zhu *et al.*, 2010). Already, systems modelling has identified biochemical and anatomical controls over carbon gain and mesophyll cell function in C₃ plants (Zhu *et al.*, 2007; Tholen and Zhu, 2011). In C₄ plants, systems modelling should also be able to identify critical points of metabolic control that could be used to guide C₄ engineers. This work will be able to exploit metabolic flux studies using *Flaveria* transgenics and *Amaranthus* mutants that have already identified control coefficients for major enzymes of the C₄ pathway (Dever *et al.*, 1997; Furbank *et al.*, 1997; Kubien *et al.*, 2003).

One of the advantages the present generation of C₄ engineers have over their predecessors is high-throughput sequencing technology (Bräutigam and Gowik, 2010). C₄ researchers have recently exploited the high-throughput technologies to compare the transcriptomes of closely related C₃ and C₄ species of *Cleome* (Bräutigam *et al.*, 2011a). As outlined by Bräutigam *et al.* (2011b) in this issue, comparisons of related C₃ and C₄ species have great promise for identifying unknown genes that control the many aspects of C₄ function. With the advent of the next-generation sequencing technologies, whole-genome comparisons will become financially feasible, potentially allowing for a rapid increase in the rate of gene discovery (Bräutigam and Gowik, 2010; Li and Brutnell, 2011). Given the possibility that many distinct controlling elements of C₄ photosynthesis will need to be identified, the high-throughput capability of whole-genome sequencing will be

an invaluable tool for all aspects of C₄ bioengineering in the foreseeable future.

Evolutionary perspectives

The complexity of C₄ photosynthesis relative to the C₃ pathway leads many to assume that the engineering of C₄ photosynthesis is well beyond current capabilities. However, the high number of distinct C₄ origins in nature is evidence that converting a C₃ genotype into a C₄ genotype could be relatively straightforward and thus feasible (Mitchell and Sheehy, 2006; Hibberd *et al.*, 2008; Kajaja *et al.*, 2011). As Sage *et al.* (2011a) describe in this issue, there are at least 62 independent origins of the C₄ pathway in the angiosperms. Eighteen of these origins are in the grasses, six in the sedges, and 38 in the eudicots. This number of independent C₄ lineages provides many opportunities for comparative genomics of closely related C₃ and C₄ species, and, increasingly, species that express intermediate traits between fully formed C₃ and C₄ species (Bräutigam *et al.*, 2011a, b; Gowik and Westhoff, 2011). Sage *et al.* (2011c) report two newly identified C₃–C₄ intermediate species in the genus *Euphorbia*; both are phylogenetically close to the C₃ sister species as well as a large clade of C₄ *Euphorbia*. These additional C₃–C₄ species add to the collection of C₃–C₄ intermediates that can be studied to better understand how nature was able to evolve C₄ photosynthesis with such frequency, as well as providing C₄ engineers with additional species to genetically dissect in their hunt for the critical genes related to C₄ traits.

Molecular phylogenetics has been of great value to the study of C₄ evolution. It has delineated many of the independent lineages of C₄ plants (Kellogg, 1999; Christin *et al.*, 2011b), and recently has been used to estimate the timing of the various C₄ origins. In two seminal contributions, Christin *et al.* (2008) and Vicentini *et al.* (2008) estimated that the earliest C₄ grass lineages date to the late-Oligocene period, ~30 million years ago. These origins corresponded to a period when atmospheric CO₂ levels dropped to the low levels of recent geological time, supporting the hypothesis that low atmospheric CO₂ facilitated the origin of the C₄ pathway (Ehleringer *et al.*, 1991). In this issue, Christin *et al.* (2011a) follow up the grass studies with an evaluation of the timing of C₄ origins in the eudicots. As with the grasses, the origin of C₄ photosynthesis in the eudicot lineages is estimated to have occurred in the past 30 million years. Notably, Christin *et al.* (2008, 2011a) identify the youngest C₄ genera (*Flaveria* and *Neurachne*). This is useful for gene discovery efforts because the most recently diverged groups should have the least amount of genetic differentiation. As a result, C₄-specific genes should be easier to identify.

Physiological perspectives

While bioengineering is often focused on molecular issues, the complexity of C₄ engineering is such that an integrative, multidisciplinary approach is needed. Such an approach has

been useful in understanding the evolutionary origin of the decarboxylating enzymes, which were co-opted from ancestral C_3 forms late in the evolution of the C_4 pathway (Sage, 2004; Aubry *et al.*, 2011). Three distinct C_4 decarboxylating enzymes are known, and these form the basis for subdividing the C_4 pathway into one of three subtypes: the NADP-malic enzyme subtype, the NAD-malic enzyme subtype, and the PEP carboxykinase subtype (Kanai and Edwards, 1999; Maier *et al.*, 2011). Due to this nomenclature, there has been a tendency to presume the three types are distinct; however, and as pointed out by Furbank (2011) in this issue, there is considerable overlap, particularly between the NADP-malic enzyme type and the PEP carboxykinase type. The adaptive significance of using multiple decarboxylating enzymes is unclear at this time. Sharing the decarboxylation function between two or three enzymes could be just a chance event that evolution has yet to optimize; however, as Furbank (2011) points out, there could be significant complementarity between these enzymes that increases the efficiency of C_4 photosynthesis.

Whole plant approaches continue to provide insights into the sequence of changes during the evolution of C_4 photosynthesis. Pinto *et al.* (2011) in this issue demonstrate that C_3 - C_4 intermediate species lack WUE and NUE enhancements relative to their C_3 congeners, indicating that major improvements in resource use efficiency come late in the evolutionary transition to C_4 photosynthesis. Organelle distribution is important in C_4 species, particularly in the BS, and thus may have to be modified from the ancestral C_3 condition in any engineering effort. However, it is currently not understood how organelle location is controlled in C_4 species. Maai *et al.* (2011) demonstrate that chloroplast position is variable in the mesophyll of C_4 grasses, but tends to be fixed in the BS. Light quality, light quantity, and abscisic acid (ABA) are key factors influencing the position of chloroplasts in the mesophyll (Maai *et al.*, 2011). Another area of uncertainty is developmental patterning of leaves. Apart from maize and *Flaveria*, there are relatively few developmental studies that document the development of Kranz anatomy (McKown and Dengler, 2009; Nelson, 2011). Developmental issues are paramount to the C_4 engineering effort, as highlighted in this issue by Nelson (2011). In particular, genetic control over vein patterning, BS properties, and mesophyll cell number must be understood if Kranz anatomy is to be engineered into C_3 species. How best to identify these critical developmental controls is still unresolved. Kajala *et al.* (2011) describe how the C_4 Rice Project at IRRI is using mutagenesis of rice and sorghum to identify developmental controls. The objective of this approach is to identify gain-of-function mutants in rice that express incipient forms of Kranz anatomy, and loss-of-function mutants in sorghum lacking Kranz expression. Alternatively, the natural diversity of C_4 species provides many opportunities for discovering Kranz anatomy genes, since there are many variations of Kranz anatomy in the 62 known C_4 lineages. For example, in *Sueada*, a genus with four independent C_4 lineages, there are four anatomical versions, including the two known

single-celled C_4 variations (Edwards and Vosnesenskaya, 2011). Koteyeva *et al.* (2011) in this issue describe how different patterns of Kranz anatomy arise during leaf maturation in the two Kranz-type *Sueada* lineages. By comparing anatomical developments in these two relatively closely related lines, it may be possible to identify key points in the developmental sequence that could be later linked to altered patterns of gene expression using transcriptomics or whole genome comparisons.

While C_4 engineering is a high-profile programme with great potential payoff, there is much to be gained from improving the performance of existing C_4 crops, which account for ~30% of the grains produced by humanity (Brown, 1999). A need to better understand and improve C_4 photosynthesis is critical for the nascent biofuels industry. Although fossil fuel costs are rising, they are still cheaper than biofuels, and will probably remain competitive for the next few decades, at least. To compete with fossil fuels, dedicated bioenergy crops will need to be as efficient as possible, and grown in areas where costs are low and land is inexpensive. This indicates that biofuel production will be centred on marginal land, using low fertilizer and pesticide inputs (Field *et al.*, 2008). Exploiting the C_4 engine to maximize yields and resource use efficiencies in marginal environments could thus be critical for the success of the biofuel industry. Lopez *et al.* (2011) in this issue discuss a number of the options for improving drought tolerance in maize; some strategies involve improving photosynthesis, some do not. Markelz *et al.* (2011) show that limiting nitrogen aggravates drought injury in maize while elevated CO_2 reduces drought impacts on C_4 photosynthesis. Rising CO_2 will still benefit C_4 plants, despite their reduced photosynthetic response to CO_2 enrichment relative to C_3 plants (Markelz *et al.*, 2011). In particular, optimizing C_4 species for high CO_2 environments could produce varieties with very high WUE and NUE. Because C_4 species are often grown in hot, water-limited environments, impacts of future CO_2 levels could be greater than commonly predicted from studies under optimal conditions (Sage and Kubien, 2003; Lopez *et al.*, 2011; Markelz *et al.*, 2011).

Besides drought and nutrient deficiency, C_4 performance could also be improved by enhancing photosynthesis in low light, and improving allocation of carbohydrate to the economic product. While C_4 plants are commonly described as requiring high light, many routinely experience shade (Sage and Pearcy, 2000). For example, all of the productive C_4 crop and forage grasses shade their lower leaves following canopy closure. To maintain efficient function, leaves from the interior of the canopy will require enzyme investment strategies different from those for leaves at the top of the canopy. This will necessitate engineering strategies that consider the overall canopy, not simply the photosynthetic efficiency at the top of the canopy. Improving performance of shaded, interior leaves is thus another way to increase light use and carbon gain, but to do so requires understanding of the mechanisms governing C_4 light use efficiency in the shade. In C_4 plants, leakage of CO_2 out of the BS increases in shaded leaves, reducing

photosynthetic efficiency at the lower parts of the canopy (Evans *et al.*, 2007). Ubierna *et al.* (2011) in this issue evaluate increases in leakiness at low light using theoretical modelling and online measurements of carbon isotope discrimination, which potentially estimates leakiness. With respect to carbohydrate allocation, Weise *et al.* (2011) compare transitory starch metabolism in plants of different photosynthetic pathways. C₄ plants have a high potential for starch production, which is valuable in a biofuel context because starch can be easily degraded into fermentable sugars; however, mature C₄ leaves cease to store transitory starch, reducing the value of this material for silage and bioenergy production. Boosting starch reserves in mature leaves and other vegetative tissues could be one way to increase the bioethanol yield in plants such as sugar cane, because more carbon can be stored in starch than in sucrose, which is the main sugar used for ethanol production in sugar cane. Weise *et al.* (2011) provide a good example of how thinking beyond current systems could lead to novel strategies that reduce food versus fuel trade-offs in agricultural production.

The need for model C₄ systems

Modern biology is heavily dependent on model organisms that enable forward and reverse genetics to identify the genes controlling physiological function, developmental patterning, and environmental responses. In plant biology, *Arabidopsis thaliana* has been the primary model organism since the mid-1980s. The success of the *Arabidopsis* model is due to its being tractable from both a genetic and a logistics standpoint. Ideally, a model organism has a small genome, is easily transformable, can be readily crossed, and can be cycled through a generation within a month or two (Westhoff and Gowik, 2010; Li and Brutnell, 2011). Model plants should be small, allowing for large numbers of plants in limited growth space, have high seed production, and be able to be grown at modest light levels indoors. If a plant does not meet these requirements, then research costs and time requirements rapidly escalate. *Arabidopsis* meets all of these requirements; however, it is a C₃ eudicot, and as a result has limited utility for studying questions associated with C₄ photosynthesis. Until recently, no C₄ plant has gained traction as a genetic model for C₄ photosynthesis, a constraint that contributed to a decline in C₄-related research over the past quarter century. C₃–C₄ intermediates and C₄ species in the genus *Flaveria* have supported extensive C₄ research since the early 1980s (Brown *et al.*, 2005); however, its genomic resources are limited, and the plants are large at maturity. Consequently, *Flaveria* has been more valuable as an evolutionary and biochemical model, rather than as a genetic model. *Cleome gynandra* has recently been proposed as a tractable C₄ model, since it is closely related to *Arabidopsis* and its well-studied genome could thus be exploited (Brown *et al.*, 2005; Marshall *et al.*, 2007; Bräutigam *et al.*, 2011a, b). The usefulness of *C. gynandra* as a genetic model is also limited because it is not small like *Arabidopsis* and can be difficult to grow in low light. Also, since they are eudicots, *Flaveria* and *Cleome*

species have limited utility for grass systems such as rice, maize, and wheat. With the rapid, next-generation sequencing technologies now becoming available, maize and sorghum could potentially serve as C₄ models; however, these large grasses require substantial growth space, elevated light levels, and months to set and mature seed (Brutnell *et al.*, 2010). Thus, large-scale genetic screens are impractical for the most part with the major C₄ crops.

Li and Brutnell (2011) in this issue, and Brutnell *et al.* (2010), propose *Setaria viridis* as an ideal model to serve as the genetic workhorse in support of C₄ research. *Setaria* has a small genome (510 Mb), is short statured, easy to grow in growth cabinets, and produces large numbers of seeds in 6 weeks (Li and Brutnell, 2010). *Setaria viridis* has also been sequenced, and now has a working transformation system (Brutnell *et al.*, 2010). *Setaria* is the only genetic model proposed for the PACMAD clade of grasses, which includes all C₄ grasses and many important crops species, including the leading C₄ bioenergy crops sugar cane, maize, switchgrass, big bluestem, and *Miscanthus*. In addition, *Setaria* is widely distributed, with many genotypes adapted to cold and drought stress, indicating it will be a valuable resource for understanding the molecular biology of abiotic stress in C₄ plants (Li and Brutnell, 2011). The advent of *Setaria* as a model species should promote a resurgence of C₄ research, particularly by the many labs now focusing on the improvement of C₄ bioenergy crops.

How to build a C₄ plant: a module-based approach

In the first special issue of the *Journal of Experimental Botany* devoted to CO₂-concentrating mechanisms (April 2002 issue), Leegood (2002) identified seven functional requirements for efficient C₄ photosynthesis (Table 3). These requirements are met by a series of modifications to leaf structure and numerous enzyme systems present in C₃ species. Here, the approach of Leegood (2002) is built upon by proposing a module-based, hierarchical framework that can guide C₄ engineering and the dissection of C₄ evolution. The hierarchy is comprised of four organizational levels (Fig. 2). The first hierarchical level is the goal (in an engineering context) or end result (in an evolutionary context). The second level describes the traits being altered, and the third the physiological mechanisms by which the traits are altered. The fourth level presents the underlying

Table 3. Seven requirements for a CO₂-concentrating system in C₄ plants (Leegood, 2002)

1. A CO₂ capture system centred around PEP carboxylase
2. Coupling of photosynthetic energy to the C₄ metabolic pump
3. An intermediate metabolite pool to hold and transport captured CO₂
4. A mechanism to release CO₂ from the intermediate metabolite pool
5. A compartment in which to concentrate the released CO₂ around Rubisco
6. A means to reduce leakage of CO₂ from the site of CO₂ elevation
7. Modification of Rubisco to operate in a high CO₂ environment

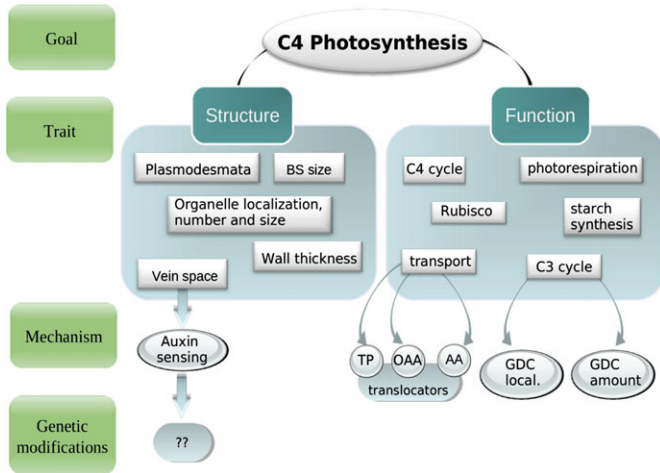


Fig. 2. A hierarchical framework for converting a C_3 plant to a C_4 plant, via either bioengineering or natural evolution. The top level identifies the engineering goal (or in an evolutionary context, the end result). The second level identifies some of the key traits that will need to be modified to introduce a C_4 system into a C_3 plant. Each of the listed traits can be further represented as a module which can be expanded to show the mechanisms underlying each trait. For example, the transport function module can be expanded to describe changes in triose phosphate (TP), oxaloacetate (OAA), or amino acid (AA) transporters. The fourth level will describe the genetic changes underpinning the mechanistic changes, as they become known.

changes to the genome that explain the mechanism. For organizational clarity, the traits were subdivided into two categories, termed structure and function. In a simple sense, the structural changes reflect the alterations to the photosynthetic factory building that enable the specialization of labour behind C_4 photosynthesis, while the function category lists the changes to the machines within the factory. Each trait within the structure and function categories is represented as a module that can be expanded first to highlight the mechanistic changes that explain the trait, and then further expanded to delineate the associated genetic changes, if known (Fig. 3). If the mechanism and genetic changes are unknown, they can be identified as such to emphasize the need to target these areas for future research. For example, changes in C_4 vein pattern are thought to involve changes in auxin signalling (Nelson, 2011). It is not known how such changes are controlled at the genetic level, so the genetic alterations underpinning auxin signalling are highlighted with question marks. For many structural traits, such as wall thickness, BS size, and plasmodesmatal frequency, the underlying mechanisms controlling these traits are unknown, and thus must also be highlighted with a question mark.

On the function side, more is known about the mechanisms and, in numerous cases, the genetic control over these mechanisms. The best information is present in the C_4 cycle module, where research on the C_4 enzyme systems has provided extensive understanding of the mechanism and, in a few cases, the genetic control. Some of these changes are highlighted in articles in this issue (Aubry *et al.*, 2011; Chastain *et al.*, 2011; Kajala *et al.*, 2011; Ludwig, 2011;

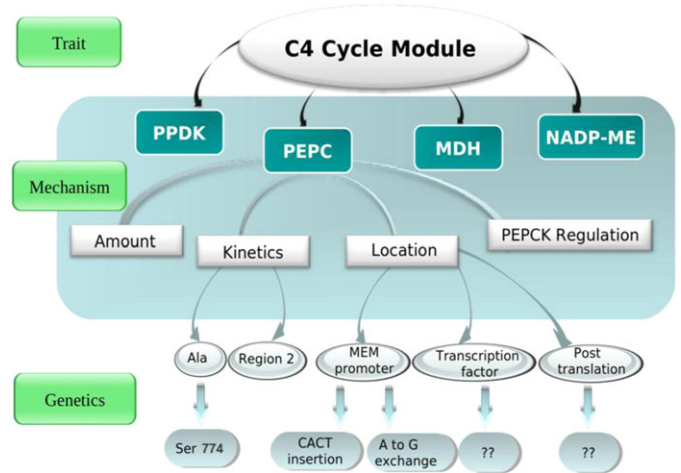


Fig. 3. An expanded description of the C_4 cycle module shown in Fig. 2. Mechanisms shown can be expanded into submodules to identify changes to key enzyme systems in the C_4 metabolic cycle, for example pyruvate, orthophosphate dikinase (PPDK), PEP carboxylase (PEPC), malate dehydrogenase (MDH), and the NADP-malic enzyme (NADP-ME), as would occur in an NADP-ME species such as maize. In this example, the PEPC submodule is expanded to show some of the genetic level changes that alter its kinetics and location of expression, as described by Peterhansel (2011) in this issue and Gowik and Westhoff (2011).

Maier *et al.*, 2011; Peterhansel, 2011) and elsewhere (Hibberd and Covshoff, 2010; Gowik and Westhoff, 2011; Kaprolov *et al.*, 2011). The best understood system is the evolution of the C_4 PEPC, where evolutionary changes altered the location and amount of PEPC accumulation, its kinetics, and its regulatory properties (Gowik and Westhoff, 2011). As indicated in Fig. 3, these changes were brought about by changes to the amino acid sequences, for example by the substitution of a serine for an alanine near position 774 in the PEPC protein, and alterations to the promoter region, notably the formation of a mesophyll expression module (MEM) in the promoter of *ppcA* that confers mesophyll-specific gene expression in the C_4 leaf. Although not shown in Fig. 3, similar layers of understanding are being developed for the other major C_4 cycle enzymes (see, for example the articles in this issue by Ludwig for CA, Maier *et al.* for the decarboxylating enzymes, and Chastain for PPDK). When a detailed engineering schematic is developed, it would be possible to quickly identify areas of progress versus knowledge gaps that require investigation. It may also be possible to identify knowledge gaps that can be ignored or circumvented should transcription factors and other regulatory elements be shown to control the expression of multiple genes (Westhoff and Gowik, 2010). Once the modules are understood, the framework could guide the assembly of a functional C_4 pathway in a C_3 crop.

Conclusion: the future of C_4 research

The coming decade holds much promise for C_4 photosynthetic research. The combination of increasing needs, new

model species, high-throughput technologies, and systems biology provides confidence that financial resources will be available and exciting results forthcoming. The financial cost of a major biotechnology project such as C₄ engineering is not clear, although it is not thought to be large in comparison with many big science programmes which often cost billions of dollars. At a workshop on C₄ engineering in July 2009, nearly 50 C₄ researchers were asked to estimate the cost and time required to introduce C₄ photosynthesis into a C₃ plant. Their estimates ranged from a total of US\$50 million to US\$10 billion over about 20 years—most estimates were in the US\$100 million to US\$1 billion range. The return from such an investment would easily be in the tens of billions of dollars per year. In the case of rice, for example, a 50% enhancement in current global yield could produce ~US\$68 billion per year in 2008 dollars from increased crop value (US\$136 billion×0.5, Table 1), and an estimated US\$7 billion per year in reduced input costs associated with improved WUE and NUE (JE Sheehy, unpublished data). Put another way, the ~US\$75 billion enhancement per year in the value of the global rice crop would equate to US\$ 1–1.5 trillion over 15–20 years, the time frame most frequently estimated to accomplish C₄ engineering. In addition to this financial gain would be the humanitarian benefit of meeting the food needs expected in Asia by the year 2050 (Mitchell and Sheehy, 2006; Hibberd *et al.*, 2008). Once C₄ is engineered into the first C₃ crops, the knowledge gained would facilitate introducing the C₄ pathway into other C₃ crops. C₄ soybean, peanuts, and other legumes, for example, could join the C₄ engine with nitrogen fixation. If the yields of the warm climate crops (rice, soybeans, cotton, tomatoes, peanuts, and cassava) from the top 12 list in Table 1 are increased 50% by the introduction of the C₄ pathway, the added value of these crops in 2008 dollars would approximate US\$141 billion per year, and probably >US\$150 billion per year when reduced costs from higher WUE and NUE are factored in. In summary, C₄ engineering appears to represent a good bet for the research dollar, and it will be an important challenge for the C₄ research community to convince others of this promise, through organizing efforts, public outreach, and, importantly, demonstrating feasibility through their science. This special issue on the engine of C₄ photosynthesis is one important step towards this larger goal.

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References

- Aubry S, Brown NJ, Hibberd JM.** 2011. The role of proteins in C₃ plants prior to their recruitment into the C₄ pathway. *Journal of Experimental Botany* **62**, 3049–3059.
- Barker R, Meinzen-Dick R, Shah T, Tuong TP, Levine G.** 2010. Managing irrigation in an environment of water scarcity. In: Pandey S, Byerlee D, Dawe D, Dobermann A, Mohanty S, Rozelle S, Hardy B, eds. *Rice in the global economy. Strategic research and policy issues for food security*. Los Banos, Philippines: International Rice Research Institute, 265–296.
- Björkman O, Gauhl E, Nobs MA.** 1969. Comparative studies of *Atriplex* species with and without B-carboxylation photosynthesis and their first-generation hybrid. *Carnegie Institute of Washington Yearbook* **68**, 620–633.
- Bobe R, Behrensmeier AK.** 2004. The expansion of grassland ecosystems in Africa in relation to mammalian evolution and the origin of the genus *Homo*. *Palaeogeography, Palaeoclimatology, Palaeoecology* **207**, 399–420.
- Bräutigam A, Gowik U.** 2010. What can next generation sequencing do for you? Next generation sequencing as a valuable tool in plant research. *Plant Biology* **12**, 831–841.
- Bräutigam A, Kajala K, Wullenweber J, et al.** 2011a. An mRNA blueprint for C₄ photosynthesis derived from comparative transcriptomics of closely related C₃ and C₄ species. *Plant Physiology* **155**, 142–156.
- Bräutigam A, Mullick T, Schliesky S, Weber APM.** 2011b. Critical assessment of assembly strategies for non-model species mRNA-Seq data and application of next-generation sequencing to the comparison of C₃ and C₄ species. *Journal of Experimental Botany* **62**, 3093–3102.
- Brown NJ, Parsley K, Hibberd JM.** 2005. The future of C₄ research—maize, *Flaveria* or *Cleome*? *Trends in Plant Science* **10**, 215–221.
- Brown RH.** 1999. Agronomic implications of C₄ photosynthesis. In: Sage RF, Monson RK, eds. *C₄ plant biology*. San Diego, CA: Academic Press, 473–507.
- Brown RH, Bouton J.** 1993. Physiology and genetics of interspecific hybrids between photosynthetic types. *Annual Review of Plant Physiology and Plant Molecular Biology* **44**, 435–456.
- Brutnell TP, Wang L, Swartwood K, Goldschmidt A, Jackson D, Zhu XG, Kellogg E, Van Eck J.** 2010. *Setaria viridis*: a model for C₄ photosynthesis. *The Plant Cell* **22**, 2537–2544.
- Burnell JN.** 2011. Hurdles to engineering greater photosynthetic rates in crop plants: C₄ rice. In: Raghavendra AS, Sage RF, eds. *C₄ photosynthesis and related CO₂ concentrating mechanisms*. Dordrecht, The Netherlands: Springer, 361–378.
- Burnell JN, Hatch MD.** 1988. Low bundle sheath carbonic anhydrase is apparently essential for effective C₄ pathway operation. *Plant Physiology* **86**, 1252–1256.
- Byrt CS, Grof CPL, Furbank RT.** 2011. C₄ plants as biofuel feedstocks: optimising biomass production and feedstock quality from a lignocellulosic perspective. *Journal of Integrative Plant Biology* **53**, 120–135.
- Cerling TE.** 1999. Paleorecords of C₄ plants and ecosystems. In: Sage RF, Monson RK, eds. *C₄ plant biology*. San Diego, CA: Academic Press, 445–469.

- Chastain CJ, Failing CJ, Manandhar L, Zimmerman M, Lakner M, Nguyen TTHT.** 2011. Functional evolution of C₄ pyruvate, orthophosphate dikinase. *Journal of Experimental Botany* **62**, 3083–3091.
- Christin PA, Besnard G, Samaritani E, Duvall MR, Hodkinson TR, Savolainen V, Salamin N.** 2008. Oligocene CO₂ decline promoted C₄ photosynthesis in grasses. *Current Biology* **18**, 37–43.
- Christin PA, Osborne CP, Sage RF, Arakaki M, Edwards EJ.** 2011a. C₄ eudicots are not younger than C₄ monocots. *Journal of Experimental Botany* **62**, 3171–3181.
- Christin PA, Sage TL, Edwards EJ, Ogburn RM, Khoshravesh R, Sage RF.** 2011b. Complex evolutionary transitions and the significance of C₃–C₄ intermediate forms of photosynthesis in Molluginaceae. *Evolution* **65**, 643–660.
- Dauber J, Jones MB, Stout JC.** 2010. The impact of biomass crop cultivation on temperate biodiversity. *Global Change Biology Bioenergy* **2**, 289–309.
- Dawe D, Pandey S, Nelson A.** 2010. Emerging trends and spatial patterns of rice production. In: Pandey S, Byerlee D, Dawe D, Dobermann A, Mohanty S, Rozelle S, Hardy B, eds. *Rice in the global economy. Strategic research and policy issues for food security*. Los Banos, Philippines: International Rice Research Institute, 15–35.
- Dever LV, Bailey KJ, Leegood LC, Lea PJ.** 1997. Control of photosynthesis in *Amaranthus edulis* mutants with reduced amounts of PEP carboxylase. *Australian Journal of Plant Physiology* **24**, 469–476.
- de Menocal PB.** 2011. Climate and human evolution. *Science* **331**, 540–542.
- Eagles CF, Wilson D.** 1982. Photosynthetic efficiency and plant productivity. In: Rechcigl M, ed. *Handbook of agricultural productivity*. Boca Raton, FL: CRC Press, 213–247.
- Edwards EJ, Osborne CP, Strömberg CAE, Smith SA, the C Grasses Consortium.** 2010. The origins of C₄ grasslands: integrating evolutionary and ecosystem science. *Science* **328**, 587–591.
- Edwards GE, Voznesenskya EV.** 2011. C₄ photosynthesis: Kranz forms and single-cell C₄ in terrestrial plants. In: Raghavendra AS, Sage RF, eds. *C₄ photosynthesis and related CO₂ concentrating mechanisms*. Dordrecht, The Netherlands: Springer, 29–61.
- Ehleringer JR, Pearcy RW.** 1983. Variation in quantum yield for CO₂ uptake among C₃ and C₄ plants. *Plant Physiology* **73**, 555–559.
- Ehleringer JR, Sage RF, Flanagan LB, Pearcy RW.** 1991. Climate change and the origin of C₄ photosynthesis. *Trends in Ecology and Evolution* **6**, 95–99.
- Evans JR, Vogelmann TC, von Caemmerer S.** 2007. Balancing light capture with disturbed metabolic demand during C₄ photosynthesis. In: Sheehy JE, Mitchell PL, Hardy B, eds. *Charting new pathways to C₄ rice*. Los Banos, Philippines: International Rice Research Institute, 127–143.
- FAO.** 2008. The state of food and agriculture 2008: biofuels, prospects, risks and opportunities. Rome: Food and Agricultural Organization of the United Nations. <http://www.fao.org/docrep/011/i0100e/i0100e00.htm> (accessed May 24, 2011).
- Field CB, Campbell JE, Lobell DB.** 2008. Biomass energy: the scale of the potential resource. *Trends in Ecology and Evolution* **23**, 65–72.
- Furbank RT.** 2011. Evolution of the C₄ photosynthetic mechanism: are there really three C₄ acid decarboxylation types? *Journal of Experimental Botany* **62**, 3103–3108.
- Furbank RT, Chitty JA, Jenkins CLD, Taylor WC, Trevanion SJ, von Caemmerer S, Ashton AR.** 1997. Genetic manipulation of key photosynthetic enzymes in the C₄ plant *Flaveria bidentis*. *Australian Journal of Plant Physiology* **24**, 477–485.
- Ghannoum O, Evans JR, von Caemmerer S.** 2011. Nitrogen and water use efficiency in C₄ plants. In: Raghavendra AS, Sage RF, eds. *C₄ photosynthesis and related CO₂ concentrating mechanisms*. Dordrecht, The Netherlands: Springer, 129–146.
- Gowik U, Westhoff P.** 2011. C₄-phosphoenolpyruvate carboxylase. In: Raghavendra AS, Sage RF, eds. *C₄ photosynthesis and related CO₂ concentrating mechanisms*. Dordrecht, The Netherlands: Springer, 257–275.
- Gregory DJ, Haeefele SM, Buresh RJ, Singh U.** 2010. Fertilizer use, markets and management. In: Pandey S, Byerlee D, Dawe D, Dobermann A, Mohanty S, Rozelle S, Hardy B, eds. *Rice in the global economy. Strategic research and policy issues for food security*. Los Banos, Philippines: International Rice Research Institute, 231–263.
- Hatch MD, Burnell JN.** 1990. Carbonic anhydrase activity in leaves and its role in the first step of C₄ photosynthesis. *Plant Physiology* **93**, 825–828.
- Hibberd JM, Covshoff S.** 2010. The regulation of gene expression required for C₄ photosynthesis. *Annual Review of Plant Biology* **61**, 181–207.
- Hibberd JM, Sheehy JE, Langdale JA.** 2008. Using C₄ photosynthesis to increase the yield of rice—rationale and feasibility. *Current Opinion in Plant Biology* **11**, 228–231.
- Jones MB.** 2011. C₄ species as energy crops. In: Raghavendra AS, Sage RF, eds. *C₄ photosynthesis and related CO₂ concentrating mechanisms*. Dordrecht, The Netherlands: Springer, 379–397.
- Kajala K, Covshoff S, Karki S, et al.** 2011. Strategies for engineering a two-celled C₄ photosynthetic pathway into rice. *Journal of Experimental Botany* **62**, 3001–3010.
- Kanai R, Edwards GE.** 1999. The biochemistry of C₄ photosynthesis. In: Sage RF, Monson RK, eds. *C₄ plant biology*. San Diego, CA: Academic Press, 49–87.
- Kapralov MV, Kubien DS, Andersson I, Filatov DA.** 2010. Changes in Rubisco kinetics during the evolution of C₄ photosynthesis in *Flaveria* (Asteraceae) are associated with positive selection on genes encoding the enzyme. *Molecular Biology and Evolution* **28**, 1491–1503.
- Kellogg EA.** 1999. Phylogenetic aspects of the evolution of C₄ photosynthesis. In: Sage RF, Monson RK, eds. *C₄ plant biology*. San Diego, CA: Academic Press, 411–444.
- Kocacinar F, Mckown AD, Sage TL, Sage RF.** 2008. Photosynthetic pathway influences xylem structure and function in *Flaveria* (Asteraceae). *Plant, Cell and Environment* **31**, 1363–1376.
- Kocacinar F, Sage RF.** 2003. Photosynthetic pathway alters xylem structure and hydraulic function in herbaceous plants. *Plant, Cell and Environment* **26**, 2015–2026.

- Kocacinar F, Sage RF.** 2004. Photosynthetic pathway alters hydraulic structure and function in woody plants. *Oecologia* **139**, 214–223.
- Koteyeva N, Voznesenskaya E, Berry J, Chuong S, Franceschi V, Edwards GE.** 2011. Development of structural and biochemical characteristics of C₄ photosynthesis in two types of Kranz anatomy in genus *Suaeda* (family Chenopodiaceae). *Journal of Experimental Botany* **62**, 3197–3212.
- Kubien DS, von Caemmerer S, Furbank RT, Sage RF.** 2003. C₄ plants at low temperature. A study using transgenic plants with reduced amounts of Rubisco. *Plant Physiology* **132**, 1577–1585.
- Leegood RC.** 2002. C₄ photosynthesis: principles of CO₂ concentration and prospects for its introduction into C₃ plants. *Journal of Experimental Botany* **53**, 581–590.
- Li P, Brutnell TP.** 2011. *Setaria viridis* and *Setaria italica*, model genetic systems for the Panicoid grasses. *Journal of Experimental Botany* **62**, 3031–3037.
- Long SP.** 1999. Environmental responses. In: Sage RF, Monson RK, eds. *C₄ plant biology*. San Diego, CA: Academic Press, 215–249.
- Loomis RS.** 1983. Productivity of agricultural systems. In: Lange OL, Nobel PS, Osmond CB, Ziegler H, eds. *Encyclopedia of plant physiology new series volume 12D: physiological plant ecology IV, ecosystem processes: mineral cycling, productivity and man's influence*. Berlin: Springer-Verlag, 151–172.
- Lopez M, Araus J, Van Heerden PDR, Foyer C.** 2011. Enhancing drought tolerance in C₄ crops. *Journal of Experimental Botany* **62**, 3135–3153.
- Ludwig M.** 2011. The molecular evolution of carbonic anhydrase in *Flaveria*. *Journal of Experimental Botany* **62**, 3071–3081.
- Maai E, Shimada S, Yamada M, Sugiyama T, Miyake H, Taniguchi M.** 2011. The avoidance and aggregative movements of mesophyll chloroplasts in C₄ monocots in response to blue light and abscisic acid. *Journal of Experimental Botany* **62**, 3213–3221.
- Maier A, Zell MB, Maurino VG.** 2011. Malate decarboxylases: evolution and roles of NAD(P)-ME isoforms in species performing C₄ and C₃ photosynthesis. *Journal of Experimental Botany* **62**, 3061–3069.
- Markelz RJ, Strellner RS, Leakey A.** 2011. Impairment of C₄ photosynthesis by drought is exacerbated by limiting nitrogen and ameliorated by elevated CO₂ in maize. *Journal of Experimental Botany* **62**, 3235–3246.
- Marshall DM, Muhaidat R, Brown NJ, Liu Z, Stanley S, Griffiths H, Sage RF, Hibberd JM.** 2007. *Cleome*, a genus closely related to *Arabidopsis*, contains species spanning a developmental progression from C₃ to C₄ photosynthesis. *The Plant Journal* **51**, 886–896.
- Matsuoka M, Furbank RT, Fukayama H, Miyao M.** 2001. Molecular engineering of C₄ photosynthesis. *Annual Review of Plant Physiology and Plant Molecular Biology* **52**, 297–314.
- McKown AD, Dengler NG.** 2009. Shifts in leaf vein density through accelerated vein formation in C₄ *Flaveria* (Asteraceae). *Annals of Botany* **104**, 1085–1098.
- Mitchell P, Sheehy JE.** 2006. Supercharging rice photosynthesis to increase yield. *New Phytologist* **171**, 688–171.
- Miyao M, Masumoto C, Miyazawa S-I, Fukayama H.** 2011. Lessons from engineering a single-cell C₄ photosynthetic pathway into rice. *Journal of Experimental Botany* **62**, 3021–3029.
- Monteith JL.** 1978. Reassessment of maximum growth rates for C₃ and C₄ crops. *Experimental Agriculture* **14**, 1–5.
- Nelson T.** 2011. The grass leaf developmental gradient as a platform for a systems understanding of the anatomical specialization of C₄ leaves. *Journal of Experimental Botany* **62**, 3039–3048.
- Osborne CP.** 2011. The geologic history of C₄ plants. In: Raghavendra AS, Sage RF, eds. *C₄ photosynthesis and related CO₂ concentrating mechanisms*. Dordrecht, The Netherlands: Springer, 339–357.
- Pearcy RW, Ehleringer J.** 1984. Comparative ecophysiology of C₃ and C₄ plants. *Plant, Cell and Environment* **7**, 1–13.
- Peterhansel C.** 2011. Best practice procedures for the establishment of a C₄ cycle in transgenic C₃ plants. *Journal of Experimental Botany* **62**, 3011–3019.
- Pinto H, Tissue DT, Ghannoum O.** 2011. *Panicum milioides* (C₃–C₄) does not have improved water or nitrogen economies relative to C₃ and C₄ congeners exposed to industrial-age climate change. *Journal of Experimental Botany* **62**, 3223–3234.
- Plant Cultures.** 2011 Online data base exploring plants and people. Kew Royal Botanical Gardens, Richmond, UK: <http://www.plantcultures.org/index.html>.
- Robertson GP, Vitousek PM.** 2009. Nitrogen in agriculture: balancing the cost of an essential resource. *Annual Review of Environment and Resources* **34**, 97–125.
- Sage RF.** 1999. Why C₄ photosynthesis? In: Sage RF, Monson RK, eds. *C₄ plant biology*. San Diego, CA: Academic Press, 3–16.
- Sage RF.** 2002. Variation in the *k*_{cat} of Rubisco in C₃ and C₄ plants and some implications for photosynthetic performance at high and low temperature. *Journal of Experimental Botany* **53**, 609–620.
- Sage RF.** 2004. The evolution of C₄ photosynthesis. *New Phytologist* **161**, 341–370.
- Sage RF, Christin PA, Edwards EA.** 2011a. The lineages of C₄ photosynthesis on planet Earth. *Journal of Experimental Botany* **62**, 3155–3169.
- Sage RF, Kocacinar F, Kubien DS.** 2011b. C₄ photosynthesis and temperature. In: Raghavendra AS, Sage RF, eds. *C₄ photosynthesis and related CO₂ concentrating mechanisms*. Dordrecht, The Netherlands: Springer, 161–195.
- Sage RF, Kubien DS.** 2003. *Quo vadis C₄?* An ecophysiological perspective on global change and the future of C₄ plants. *Photosynthesis Research* **77**, 209–225.
- Sage RF, Pearcy RW.** 1987a. The nitrogen use efficiency of C₃ and C₄ plants. I. Leaf nitrogen, growth and biomass partitioning in *Chenopodium album* L. and *Amaranthus retroflexus* L. *Plant Physiology* **84**, 954–958.
- Sage RF, Pearcy RW.** 1987b. The nitrogen use efficiency of C₃ and C₄ plants. II. Leaf nitrogen effects on the gas exchange characteristics of *Chenopodium album* L. and *Amaranthus retroflexus* L. *Plant Physiology* **84**, 959–963.

- Sage RF, Pearcy RW.** 2000. The physiological ecology of C_4 photosynthesis. In: Leegood RC, Sharkey TD, von Caemmerer S, eds. *Photosynthesis: physiology and metabolism*. Dordrecht, The Netherlands: Kluwer Academic, 497–532.
- Sage RF, Pearcy RW, Seemann JR.** 1987. The nitrogen use efficiency of C_3 and C_4 plants. III. Leaf nitrogen effects on the activity of carboxylating enzymes in *Chenopodium album* L. and *Amaranthus retroflexus* L. *Plant Physiology* **85**, 355–359.
- Sage RF, Wedin DA, Li M.** 1999. The biogeography of C_4 photosynthesis. In: Sage RF, Monson RK, eds. *C_4 plant biology*. San Diego, CA: Academic Press, 313–373.
- Sage TL, Sage RF, Vogan PJ, Rahman B, Johnson D, Oakley J, Heckel MC.** 2011c. The occurrence of C_2 photosynthesis in *Euphorbia* subgenus *Chamaesyce* (*Euphorbiaceae*). *Journal of Experimental Botany* **62**, 3183–3195.
- Seemann JR, Badger MR, Berry JA.** 1984. Variations in the specific activity of ribulose-1,5-bisphosphate carboxylase between species utilizing differing photosynthetic pathways. *Plant Physiology* **74**, 791–794.
- Sheehy JE, Ferrer AB, Mitchell PL, Elmido-Mabilangen A, Pablico P, Dionora MJA.** 2007a. How the rice crop works and why it needs a new engine. In: Sheehy JE, Mitchell PL, Hardy B, eds. *Charting new pathways to C_4 rice*. Los Banos, Philippines: International Rice Research Institute, 3–26.
- Sheehy JE, Mitchell PL, Hardy B, eds.** 2000. *Redesigning rice photosynthesis to increase yields*. Los Banos, Philippines: International Rice Research Institute.
- Sheehy JE, Mitchell PL, Hardy B, eds.** 2007b. *Charting new pathways to C_4 rice*. Los Banos, Philippines: International Rice Research Institute.
- Snaydon RW.** 1991. The productivity of C_3 and C_4 plants: a reassessment. *Functional Ecology* **5**, 321–330.
- Still CJ, Berry JA, Collatz GJ, Defries RS.** 2003. Global distribution of C_3 and C_4 vegetation: carbon cycle implications. *Global Biogeochemical Cycles* **17**, 1006.
- Taylor SH, Hulme SP, Rees M, Ripley BS, Woodward FI, Osborne CP.** 2010. Ecophysiological traits in C_3 and C_4 grasses: a phylogenetically controlled screening experiment. *New Phytologist* **185**, 780–791.
- Tholen D, Zhu X-G.** 2011. The mechanistic basis of internal conductance—a theoretical analysis of mesophyll cell photosynthesis and CO_2 diffusion. *Plant Physiology* (in press).
- Tilman D, Socolow R, Foley JA, et al.** 2009. Beneficial biofuels—the food, energy, and environmental trilemma. *Science* **325**, 270–271.
- Ubierna N, Sun W, Cousins AB.** 2011. The efficiency of C_4 photosynthesis under low light conditions: assumptions and calculations with CO_2 isotope discrimination. *Journal of Experimental Botany* **62**, 3119–3134.
- Van der Merwe NJ, Tschauer H.** 1999. C_4 plants and the development of human societies. In: Sage RF, Monson RK, eds. *C_4 plant biology*. San Diego, CA: Academic Press, 509–549.
- Vicentini A, Barber JC, Aliscioni SS, Giussani LM, Kellogg EA.** 2008. The age of the grasses and clusters of origins of C_4 photosynthesis. *Global Change Biology* **14**, 2963–2977.
- Vitousek PM, Aber JD, Howarth RW, Likens GE, Matson PA, Schindler DW, Schlesinger WH, Tilman DC.** 1997. Human alteration of the global nitrogen cycle: sources and consequences. *Ecological Applications* **7**, 737–750.
- Weise SE, van Wijk KJ, Sharkey TD.** 2011. The role of transitory starch in C_3 , CAM, and C_4 metabolism and opportunities for engineering leaf starch accumulation. *Journal of Experimental Botany* **62**, 3109–3118.
- Westhoff P, Gowik G.** 2010. Evolution of C_4 photosynthesis — looking for the master switch. *Plant Physiology* **154**, 598–601.
- Zhu X-G, de Sturler, Long SP.** 2007. Optimizing the distribution of resources between enzymes of carbon metabolism can dramatically increase photosynthetic rate: a numerical simulation using an evolutionary algorithm. *Plant Physiology* **145**, 513–526.
- Zhu X-G, Long SP, Ort DR.** 2010. Improving photosynthetic efficiency for greater yield. *Annual Review of Plant Biology* **61**, 235–261.