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 C₃ 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 Behrensmeyer, 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 Behrensmeyer, 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 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
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 comparisons are made between plants of similar growth form. 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, Byrt 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 (Pearcy 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,
Cassava 12 (16)
Peanuts 11 (18)
Apples 10 (20)
Grapes 9 (31)
Cotton, lint 7 (34)
Potatoes 5 (38)
Soybeans 3 (47)
Wheat 2 (83) Sugar cane 6 (35)
Rice, paddy 1 (136) Maize 4 (40)
Beans (all types) Proso millet (Panicum miliaceum)
Barley Foxtail millet (Setaria italica)
Cauliflower and broccoli Amaranth (Amaranthus)
Carrots and turnips Fonio (Digitaria exilis)
Cabbage Tef (Eragrostis tef)
Berries (all types) Finger millet (Echinochloa coracana)
Banana and plantain (musas) Pearl millet (Pennisetum glaucum)
Yams
Taro
Sweet potato
Sunflower
Sugar beet
Safflower
Rape seed
Pumpkins, squashes, and gourds
Plantains
Peas (all types)
Onions
Papaya
Peas (all types)
Plantains
Pumpkins, squashes, and gourds
Rape seed
Safflower
Spinach
Sugar beet
Sunflower
Sweet potato
Taro
Yams

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

<table>
<thead>
<tr>
<th>C₃ crop</th>
<th>Global rank (US$ billion)</th>
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<tbody>
<tr>
<td>Rice, paddy</td>
<td>1 (136)</td>
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<td>Potatoes</td>
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<td>Cotton, lint</td>
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<td>Tomatoes</td>
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<td>Grapes</td>
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<tr>
<td>Cassava</td>
<td>12 (16)</td>
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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).

<table>
<thead>
<tr>
<th>C₃ crops</th>
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<tr>
<td>Asparagus</td>
<td>Sorghum (Sorghum bicolor)</td>
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<tr>
<td>Banana and plantain (musas)</td>
<td>Pearl millet (Pennisetum glaucum)</td>
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<td>Barley</td>
<td>Foxtail millet (Setaria italica)</td>
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<tr>
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<td>Carrots and turnips</td>
<td>Forio (Digitaria exilis)</td>
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<td>Cauliflower and broccoli</td>
<td>Amaranth (Amaranthus spp.)</td>
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<td>Chilies and peppers</td>
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<td>Citrus (all types)</td>
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<td>Coconut</td>
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<td>Coffee</td>
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<td>Lettuce</td>
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<td>Lentils</td>
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<td>Mango and guava</td>
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<td>Melons (all types)</td>
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<td>Oats</td>
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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 Tschauer, 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 C_4 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 C_4 enzymes will be important for successfully introducing functional proteins into C_3 plants. It is not sufficient simply to introduce the structural gene into a C_3 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 C_4 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 C_4 engineering but have not received as much attention as PEPC. CA, in particular, is not commonly thought of as being a major C_4 enzyme, yet the enhancement of the mesophyll-specific form, and loss of BS forms, are essential for the function of the C_4 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 (Burrell and Hatch, 1988; Hatch and Burrell, 1990). The example of CA highlights the special challenge inherent in all engineering projects—one has to consider the whole system if the basic nature was able to evolve C_4 photosynthesis with such intermediates that can be studied to better understand how nature was able to evolve C_4 photosynthesis with such frequency, as well as providing C_4 engineers with additional species to genetically dissect in their hunt for the critical genes related to C_4 traits.

Molecular phylogenetics has been of great value to the study of C_4 evolution. It has delineated many of the independent lineages of C_4 plants (Kellogg, 1999; Christin et al., 2011b), and recently has been used to estimate the timing of the various C_4 origins. In two seminal contributions, Christin et al. (2008) and Vicentini et al. (2008) estimated that the earliest C_4 grass lineages date to the late-Oligocene period, ~30 million years ago. These origins corresponded to a period when atmospheric CO_2 levels dropped to the low levels of recent geological time, supporting the hypothesis that low atmospheric CO_2 facilitated the origin of the C_4 pathway (Ehleringer et al., 1991). In this issue, Christin et al. (2011a) follow up the grass studies with an evaluation of the timing of C_4 origins in the eudicots. As with the grasses, the origin of C_4 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 C_4 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, C_4-specific genes should be easier to identify.

Physiological perspectives

While bioengineering is often focused on molecular issues, the complexity of C_4 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 C₃ forms late in the evolution of the C₄ pathway (Sage, 2004; Aubry et al., 2011). Three distinct C₄ decarboxylating enzymes are known, and these form the basis for subdividing the C₄ 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 C₄ photosynthesis.

Whole plant approaches continue to provide insights into the sequence of changes during the evolution of C₄ photosynthesis. Pinto et al. (2011) in this issue demonstrate that C₃–C₄ intermediate species lack WUE and NUE enhancements relative to their C₃ congeners, indicating that major improvements in resource use efficiency come late in the evolutionary transition to C₄ photosynthesis. Organelle distribution is important in C₄ species, particularly in the BS, and thus may have to be modified from the ancestral C₃ condition in any engineering effort. However, it is currently not understood how organelle location is controlled in C₄ species. Maai et al. (2011) demonstrate that chloroplast position is variable in the mesophyll of C₄ 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 C₄ 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 C₃ species. How best to identify these critical developmental controls is still unresolved. Kajala et al. (2011) describe how the C₄ 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 C₄ species provides many opportunities for discovering Kranz anatomy genes, since there are many variations of Kranz anatomy in the 62 known C₄ lineages. For example, in Sueada, a genus with four independent C₄ lineages, there are four anatomical versions, including the two known single-celled C₄ variations (Edwards and Vosnesenskaya, 2011). Kotyevea 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 C₄ engineering is a high-profile programme with great potential payoff, there is much to be gained from improving the performance of existing C₄ crops, which account for ~30% of the grains produced by humanity (Brown, 1999). A need to better understand and improve C₄ 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 C₄ 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 CO₂ reduces drought impacts on C₄ photosynthesis. Rising CO₂ will still benefit C₄ plants, despite their reduced photosynthetic response to CO₂ enrichment relative to C₃ plants (Markelz et al., 2011). In particular, optimizing C₄ species for high CO₂ environments could produce varieties with very high WUE and NUE. Because C₄ species are often grown in hot, water-limited environments, impacts of future CO₂ 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, C₄ performance could also be improved by enhancing photosynthesis in low light, and improving allocation of carbohydrate to the economic product. While C₄ plants are commonly described as requiring high light, many routinely experience shade (Sage and Pearcy, 2000). For example, all of the productive C₄ 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 C₄ light use efficiency in the shade. In C₄ plants, leakage of CO₂ 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

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<th>Table 3. Seven requirements for a CO₂-concentrating system in C₄ plants (Leegood, 2002)</th>
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<td>1. A CO₂ capture system centred around PEP carboxylase</td>
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<td>2. Coupling of photosynthetic energy to the C₄ metabolic pump</td>
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<td>3. An intermediate metabolite pool to hold and transport captured CO₂</td>
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<td>4. A mechanism to release CO₂ from the intermediate metabolite pool</td>
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<td>5. A compartment in which to concentrate the released CO₂ around Rubisco</td>
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<td>6. A means to reduce leakage of CO₂ from the site of CO₂ elevation</td>
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<td>7. Modification of Rubisco to operate in a high CO₂ environment</td>
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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 C4 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 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 C4 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 plasmodematal 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 C4 cycle module, where research on the C4 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; 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 C4 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 C4 leaf. Although not shown in Fig. 3, similar layers of understanding are being developed for the other major C4 cycle enzymes (see, for example the articles in this issue by Ludwig for CA, Maier et al. for the decarboxylating enzymes, and Chastain for PPDFK). 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 C4 pathway in a C3 crop.

**Conclusion: the future of C4 research**

The coming decade holds much promise for C4 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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