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Integrating C_4 photosynthesis into C_3 crops to increase yield potential

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The growth rate of the human population is faster than improvements in crop yields. To feed people in the future, multiple strategies are required. One proposed approach is to raise the yield potential of C_3 crops by modifying photosynthesis to the more efficient C_4 pathway. Owing to complex changes associated with C_4 photosynthesis, it is no understatement to define this conversion as one of the Grand Challenges for Biology in the 21st Century. Here we outline the challenges of installing a C_4 system and assess how new approaches and knowledge may help achieve this goal.

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Background to crop yields

The Green Revolution led to large improvements in grain production. However, in recent years, plant breeders have failed to systematically increase yields in line with population [1,2]. It is estimated that world cereal production must increase by 50% by 2030 to meet the projected demand for food [3]. Owing to increases in climate uncertainty, it would be most beneficial if genetic improvements increased yields across a range of environments. Increasing the maximum attainable yield of existing food crops could be part of the solution. It is theoretically possible to increase yield potential by 50% in some species by raising their photosynthetic capacity [2,4–6]. If this proved possible in practice, then it would greatly contribute to food security.

Increasing photosynthetic capacity raises yield potential

Dramatically increasing yield potential is not trivial because the outcome results from complex interactions between contributing components. Yield potential is the product of four factors: (1) total incident solar radiation accrued over the growing season, (2) efficiency of the plant to intercept photosynthetically active radiation (PAR), (3) efficiency with which intercepted PAR is converted into dry matter (radiation use efficiency, RUE) and (4) amount of resources partitioned to the grain (harvest index). During the Green Revolution, light interception and harvest index were maximised. Extending the growing season is undesirable because management practices are tied to cyclical weather patterns that allow production within specific time frames, and canopy production and architecture are thought to be optimised [2,4]. This leaves RUE as a potential source for significant new genetic improvement. Theoretical models predict RUE of C_3 crops would be improved by approximately 50% by using C_4 photosynthesis [2,4]. This led to the suggestion that converting crops from C_3 to C_4 could mitigate the global food crisis [4,7].

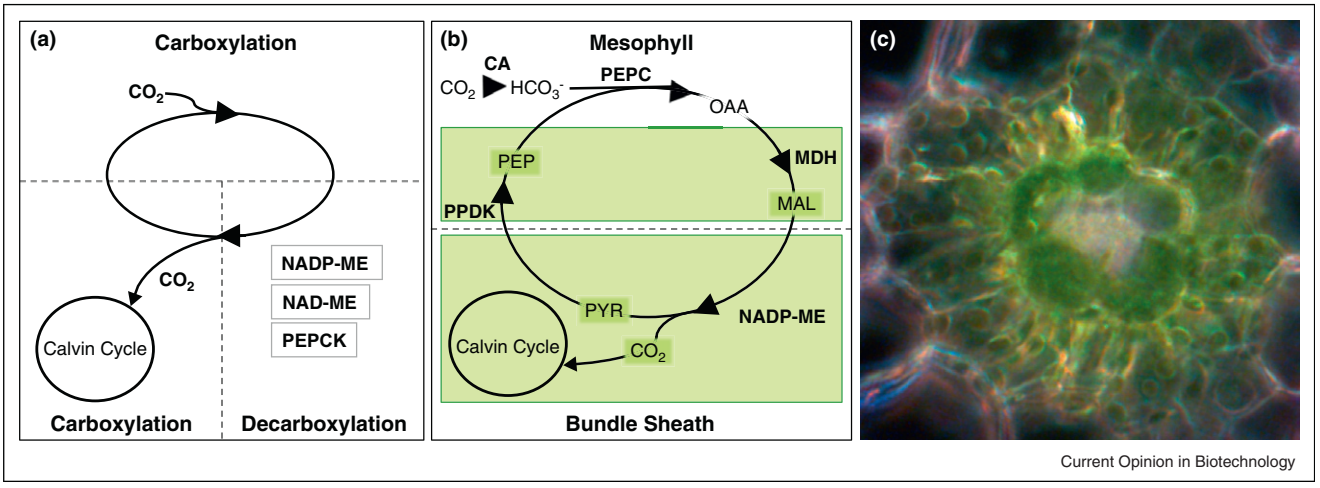
Flavours of C_4 photosynthesis

There are multiple forms of C_4 photosynthesis, but all involve specialised anatomy and biochemistry of leaves. Three major subtypes of biochemistry [8] are superimposed onto at least twenty-five types of leaf anatomy [9] (Figure 1a), and evidence is mounting that these biochemical subtypes are an oversimplification [10,11••, 12•,13•,14]. This diversity leads to the important question of which C_4 flavour should be selected to engineer into C_3 crops. Two main approaches have been undertaken, both of which use NADP-malic enzyme (NADP-ME) biochemistry (Figure 1b) as a basis for converting rice from C_3 to C_4. These are the development of a single-celled C_4 system [15,16] and a two-celled system [17] that would require the development of mesophyll (M) and bundle sheath (BS) cells arranged in classical Kranz anatomy (Figure 1b,c). The latter effort, which is the subject of this review, has been selected by the C_4 Rice Project [18] because it is the type utilised by many of the most productive C_4 crops and is relatively simple. However, it will still be difficult to engineer.

Challenges associated with placing C_4 photosynthesis into C_3 leaves

The complexity of C_4 photosynthesis indicates that its integration into C_3 leaves will be an enormous challenge. Indeed, many domesticated C_3 crops, including rice, belong to genera that are deeply embedded in clades consisting only of C_3 species [19••] and so it can be argued that there is some inherent incompatibility between the

Figure 1



C_4 photosynthesis requires specialised leaf biochemistry and anatomy.
(a) There are three major subtypes of C_4 biochemistry. In each, CO_2 is initially fixed by the cytosolic enzyme phospho*enol*pyruvate carboxylase (PEPC) to form a four carbon molecule that is subsequently decarboxylated by at least one of three enzymes: NADP-malic enzyme (NADP-ME), NAD-malic enzyme (NAD-ME), and/or phospho*enol*pyruvate carboxykinase (PEPCK). While NADP-ME operates in the plastid, NAD-ME and PEPCK function in the mitochondria and cytosol, respectively, requiring diffusion of released CO_2 to the chloroplasts. In all subtypes, a high concentration of CO_2 builds in the vicinity of Ribulose-1,5-Bisphosphate Carboxylase Oxygenase (RuBisCO), favouring its use as a substrate to initiate the Calvin–Benson cycle and dramatically reducing photorespiration. These biochemical reactions may be superimposed onto many different types of leaf cellular anatomy.
(b) The C_4 Rice Project aims to convert rice to a two-celled NADP-ME C_4 photosynthetic system with classical Kranz anatomy. In this system, two distinct photosynthetic cell types, mesophyll (M) and bundle sheath (BS), differentiate to form an interdependent biological unit with a defined spatial arrangement. M and BS cells form concentric circles around the veins, generating a consistent pattern of vein-BS-M-M-BS-vein across the leaf. The C_4 cycle starts in the M cells, where CO_2 is converted to bicarbonate in the cytosol by carbonic anhydrase (CA) and is fixed to phospho*enol*pyruvate (PEP) by PEPC to form oxaloacetate (OAA). OAA moves into the chloroplast where it is converted to malate (MAL) by malate dehydrogenase (MDH). MAL moves from the M cell chloroplast to the BS cell chloroplast where it is decarboxylated by NADP-ME to form pyruvate (PYR) and CO_2. The PYR moves from the BS cell chloroplast to the M cell chloroplast where it is converted to PEP by pyruvate, orthophosphate dikinase (PPDK), thereby completing the C_4 carbon cycle. The CO_2 released in the BS chloroplast is used in the Calvin–Benson cycle.
(c) *Sorghum bicolor* performs two-celled NADP-ME C_4 photosynthesis with classical Kranz anatomy. Shown here is a representative cross section of a *S. bicolor* leaf with the vein (centre) surrounded by a layer of BS and M cells, respectively. The C_4 Rice Project aims to duplicate this anatomical and physiological arrangement in rice.

current genomes of these species and operation of C_4 photosynthesis. Additionally, major gaps in our knowledge of the C_4 leaf must be addressed. No master regulator(s) has been isolated and loci for many of the transporters associated with metabolite fluxes, modifications to cell biology as well as the specialised anatomy of C_4 leaves remain to be identified.

On a more pragmatic note, the number of genes essential to a functional C_4 pathway is large. Existing methods of genetic engineering are probably insufficient for its installation, and the engineering challenge will probably increase as we identify more genes essential to C_4. In the next sections, we propose opportunities that may allow some of these challenges to be overcome.

Opportunities to introduce Kranz(-like) anatomy into C_3 leaves

For a two-celled NADP-ME C_4 leaf to be engineered, a key modification will be the introduction of Kranz(-like) anatomy into C_3 leaves. Classical Kranz anatomy (Figure 1c) is proposed as a target because the most productive C_4 crops have this cellular pattern. Reduction in interveinal distance, larger and/or increased number of chloroplasts within BS cells, specialisation of M and BS chloroplast proteomes, and sufficient plasmodesmata for transport between M and BS cells will be necessary modifications to the C_3 leaf. Although no genes controlling development of Kranz anatomy are known, it is possible to disrupt cell specific functions and patterning in C_4 species. Large-scale screens of *Zea mays* [20–23] yielded mutants in BS and M specific pathways [23,24]. Mutants with large interveinal spaces or altered BS cell development have been identified in *Panicum maximum* [25]. A screen of sorghum [18] yielded lines with significantly increased vein-spacing [17]. Conversely, a screen of rice mutants identified lines with closer vein-spacing relative to wild type [17]. The presence of some flexibility in C_3 and C_4 leaf traits provides hope that Kranz anatomy can be introduced into C_3 leaves.

The compatibility of C_3 leaves with C_4 biochemistry

Some characteristics of C_4 biochemistry are present in C_3 plants. Cells adjacent to veins in tobacco and *Arabidopsis* use C_4 acid decarboxylases to release CO_2 from malate [26,27]. Additionally, some endogenous *Arabidopsis* genes have BS specificity [28]. The ability to accumulate enzymes in a cell-specific manner across diverse C_3 lineages implies a pre-existing regulatory mechanism(s) is recruited during C_4 evolution. Consequently, the specific site of enzyme expression and the amount accumulated may only need modification rather than generation *de novo* when evolving C_4.

The latent ability for C_3 genes to be expressed in a C_4 manner was recently demonstrated [29••]. A region within the coding sequence of *NAD-ME* genes from *A. thaliana* (C_3) and *C. gynandra* (C_4) and *NADP-ME* from maize (C_4) is sufficient for BS specificity in *Cleome gynandra* and maize. Furthermore, some promoters and untranslated regions of cell specific genes from C_4 species can maintain cell specific expression in C_3 species [30–35]. Together, these data indicate first that C_3 leaves can partition gene expression between M and BS cell types, second that coding and non-coding regions can be recruited, and third that a number of promoters can be used as a resource to allow cell-specific accumulation of proteins. Based on the above evidence, we conclude there are no inherent incompatibilities associated with implementing expression of C_4 biochemistry genes in C_3 M and BS cells.

Separate lineages have used very similar paths to generate C_4 photosynthesis

Evidence is emerging that some mechanisms underlying C_4 photosynthesis represent parallel evolution. For example, the same or similar alterations to amino acid sequences of phospho*enol*pyruvate carboxylase (PEPC), NADP-ME, and phospho*enol*pyruvate carboxykinase (PEPCK) [36–38] have been documented in multiple lineages of C_4 grasses. In addition, it appears that cell specific regulation of gene expression in C_4 leaves can be mediated by remarkably similar mechanisms in distant independent lineages of C_4 [29]. Together, these findings indicate that mechanisms underlying the regulation of C_4 photosynthesis are shared by multiple and distantly related lineages. The existence of shared mechanisms, despite significant variation between C_4 lineages, provides hope that installation of C_4 is possible because it implies there are key routes that lead to a C_4 leaf.

Opportunities associated with high-throughput transcriptomics and proteomics

Important shared mechanisms of gene regulation in C_4 are being elucidated with high throughput technologies. Analyses of transcriptomes [11••] and proteomes [39,40•,41,42] of maize along a leaf developmental gradient and/or between BS and M cells have provided insight into regulatory proteins [44,45] putative transcriptional regulators [11••] and putative transporters [14] important to C_4. A proteomics study of distantly related pea (C_3) and maize (C_4) also yielded transporters putatively involved in C_4 [46]. A quantitative comparison of transcript abundance between *C. gynandra* (C_4) and *C. spinosa* (C_3) identified candidate transcription factors and showed that transcripts encoding ribosomal components are reduced in C_4 relative to C_3 [13•,47]. A comparison of transcriptomes from five closely related *Flaverias*, which are C_3, C_4 or C_3–C_4 intermediates, comprehensively quantified the extent to which gene expression differs between C_3 and C_4 leaves and identified transcripts for transporters previously identified as putatively important to C_4 [42,46]. Combining results of these experiments allows selection of candidates for further study.

For example, some chloroplast membrane transporters are more abundant in C_4 species relative to C_3 and have cell specific accumulation in a C_4 context, making them good candidates for C_4 metabolism. In particular, the 2-oxoglutarate/malate translocator (OMT1) is enriched in the M cells of C_4 species. This accumulation pattern is significant because in C_3 leaves, OMT1 exports stromal malate in exchange for oxaloacetate (OAA) [43••]. Therefore, its abundance and M-enriched accumulation in a C_4 context open the possibility that OMT1 was recruited into the C_4 cycle to import OAA, the product of PEPC, into M plastids while exporting malate for transfer to the BS. As such, OMT1 is a good candidate C_4 transporter identified by transcriptomic and proteomic data.

Further comparisons of C_3 and C_4 species should help identify the core C_4 gene set. C_4 photosynthesis is an extraordinary example of convergent evolution found in at least 62 independent lineages [19••,48,49]. This suggests that the evolutionary transition from C_3 to C_4 is relatively simple, occurring via modification of existing genes. The fact that all C_4 genes identified to date are present in C_3 species [50] and the presence of shared regulatory mechanisms across diverse lineages support this notion. The 1000 Plant Transcriptomes Project [51] is using recent advances in sequencing technologies to study in parallel numerous lineages of closely related C_3 and C_4 species. Mining the natural diversity of C_4 plants may make it possible to access the core C_4 gene set, including shared regulatory mechanisms.

Using new models to accelerate understanding

Traditionally, maize and sorghum are C_4 model species. Both are large monocot plants with relatively large genomes and slow generation times. Development of more tractable C_4 model species that are small and rapid cycling, such as *Cleome gynandra* (dicot) [52] and *Setaria viridis* (monocot) [53•], will accelerate identification of important loci by enabling rapid classical forward genetic screens.

Technology development will facilitate the installation of C_4

One problem associated with shifting a plant from C_3 to C_4 is the complexity of gene engineering needed. Even with our current incomplete understanding of C_4 biochemistry, the number of genes that need to be stacked into a C_3 crop is daunting. We estimate that the lower limit is 14 genes, when core components of the pathway and key transporters are counted, but additional genes will almost certainly be necessary. An upper limit of 3582 genes, which express in a C_4-related cluster has been suggested for *Flaveria* species [13]. However, technologies such as zinc-finger nucleases, meganucleases and the ability to engineer mini-chromosomes into plants [54–56] could help overcome this issue. As engineering a C_4 system into crops such as rice is clearly a long-term endeavour, it would be sensible not to allow limitations of current technologies to limit the discovery stage of development.

Summary

Although the above analysis indicates that converting a C_3 crop to C_4 photosynthesis is an extremely challenging undertaking, the economic and societal benefits that would accrue are significant [57]. To achieve this Grand Challenge, we argue that we need to build on the solid platform generated by decades of previous work on the regulation of C_4 gene expression [50], that significant further advances in gene discovery will come from intensified and thoughtful use of mutant screens, and that the ability to direct gene expression in M and BS cells of C_3 leaves will be enhanced by additional analysis of *cis*-elements and *trans*-factors. Furthermore, systems biology studies of rapid-cycling models as well as high throughput sequencing of multiple non-model species will provide new gene candidates for C_4 traits that are currently opaque. By combining genetic resources and new technologies, we are optimistic that the core suite of genes necessary to build and maintain a C_4 plant will be found in a timely manner to alleviate world hunger.

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