Finding the genes to build C₄ Rice

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Rice, a C₃ crop, is a staple food for more than half of the world's population, with most consumers living in developing countries. Engineering C₄ photosynthetic traits into rice is increasingly suggested as a way to meet the 50% yield increase that is predicted to be needed by 2050. Advances in genome-wide deep-sequencing, gene discovery and genome editing platforms have brought the possibility of engineering a C₃ to C₄ conversion closer than ever before. Because C₄ plants have evolved independently multiple times from C₃ origins, it is likely that key genes and gene regulatory networks that regulate C₄ were recruited from C₃ ancestors. In the last five years there have been over 20 comparative transcriptomic studies published that aimed to identify these recruited C₄ genes and regulatory mechanisms. Here we present an overview of what we have learned so far and preview the efforts still needed to provide a practical blueprint for building C₄ rice.

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Introduction

Photosynthesis underpins agricultural productivity. Most crop species carry out C₃ photosynthesis but the highest yielding crops are often those such as maize, sorghum and sugarcane that carry out C₄ photosynthesis. It has been proposed that if a C₄ photosynthetic system can be introduced into C₃ plants such as rice, yields may be increased by 50-60%, with concomitant increases in nitrogen and water use efficiencies [1,2].

In C₃ plants, photosynthetic reactions occur primarily within the mesophyll cells where ribulose-1, 5-bisphosphate carboxylase/oxygenase (Rubisco) fixes CO₂ in the Calvin cycle [3]. In hot dry environments, however, this fixation reaction is competitively inhibited by O₂, leading to the energetically wasteful process of photorespiration. C₄ plants evolved to overcome this inefficiency, through the development of mechanisms that concentrate CO₂ around Rubisco [1,4]. In the C₄ pathway, CO₂ is first fixed into a four-carbon compound by the O₂-insensitive phosphoenolpyruvate (PEP) carboxylase. The four-carbon compound is then shuttled to a separate compartment (either within the same cell or in another cell) where it is decarboxylated to release CO₂ for fixation by Rubisco in the Calvin cycle. Decarboxylation is carried out by one or more of three enzymes – NAD-malic enzyme (NAD-ME), NADP-ME, phosphoenolpyruvate carboxykinase (PEP-CK), depending on the biochemical subtype [5].

C₄ photosynthesis: one cell, two cells or inducible

C₄ photosynthesis evolved in the context of three types of leaf anatomy. In single-cell C₄ photosynthesis, biochemically and ultrastructurally distinct chloroplasts are partitioned in distinct cytoplasmic compartments within a single chlorenchyma cell-type [6]. In contrast, in plants with Kranz anatomy, photosynthetic reactions are partitioned between dimorphic chloroplasts in morphologically distinct vascular sheath and mesophyll (M) cell-types. A number of vascular sheath types are found but they are commonly referred to as the bundle sheath (BS). A typical monocot C₄ plant (Figure 1a) exhibits increased vein (V) density relative to C₃ monocots, with a consistent, highly optimized V-BS-M-M-BS-V layout in which only two M cells are present between each pair of veins, and all veins run parallel to the proximodistal leaf axis (Figure 1b) [1]. This

layout ensures an efficient C₄ reaction, because every M cell is in direct contact with an adjacent BS cell, and each BS cell is directly in contact with a vein (Figure 1b,c). C₄ dicot leaves also possess high vein density and closely associated BS and M cells but in these leaves the vascular system is reticulate [7]. The third context is inducible C₄ photosynthesis, where metabolism and chloroplasts are partitioned into dimorphic cells but only under certain environmental conditions. A good example of environmental regulation is seen in the amphibious plant *Eleocharis vivipara*. When submerged, the plant uses C₃-like photosynthesis, but acquires C₄ traits in leaves that grow out of the water or when growing as a terrestrial plant. Interestingly, the development of C₄ anatomy and compartmentalization of C₄ enzymes in distinct cell types can be induced by exogenously applied abscisic acid (ABA) in the submerged part of the plant [8].

Figure 1. Schematic representation of C₄ leaf development in maize leaves. (a) Maize leaves develop from the shoot apical meristem (SAM) with opposite phyllotaxy (180° between successive primordia). Primordia are initiated at time intervals known as plastochrons (P), such that the youngest primordium (P1) is closest to the SAM, and older primordia (P2, P3 etc.) are consecutively further away. The first leaf to be produced from the SAM (and hence the oldest) is L1, and subsequent leaves are L2, L3 etc. Each leaf thus has a 'P' number to denote relative developmental stage and an 'L' number to denote age. Leaf blades are separated from leaf sheath tissue by the ligule, which is established around P5. In the example shown, L5 is at P7 (and hence L6 is at P6 and L4 is at P8). **(b)** Schematic diagram of transverse sections of the middle of L3 and L5 leaf blades from (a) showing V-BS-M-M-BS-V layout and differentiation of BS and M chloroplasts. Note chloroplasts are not developed in L5 as it has not yet been exposed to light, whereas large BS and M chloroplasts are seen in the light-exposed L3. **(c)** Schematic images of transverse leaf sections showing the development of Kranz anatomy in leaf primordia. In the images of P2-P6 primordia, the ground meristem layer and BS/M cells derived from it are coloured grey, while other surrounding cells are coloured yellow. Red arrows point to initiating or early developing intermediate veins originating from the ground meristem. Vascular tissues are coloured in blue. The middle of the P6 primordium is developmentally equivalent to the base of the L5 blade in that vascular patterning is established but chloroplasts are etiolated. The middle of the L5 blade exhibits immature greening chloroplasts (depicted as light green cells). The tip of the L5 blade is equivalent to the middle of the L3 blade with mature chloroplasts in both cell types (depicted as dark green cells).

Gene discovery for C₄ rice

The current strategy for C₄ rice is aimed at generating C₄ photosynthesis with Kranz anatomy. This will require an understanding of how vein spacing is regulated, how BS and M cells are specified around veins, how functionally dimorphic chloroplasts develop, and how C₄ metabolism is compartmentalized, regulated and optimized between the two cell-types [9,10,11]. In recent years a number of comparative transcriptomic studies have been reported that provide genome-wide expression profiles in C₃ versus C₄ plants, in C₃ versus C₄ tissues, at different stages of C₄ development and in BS versus M cells (Table 1). A synthesis of the data provides candidate C₄ regulators but also highlights areas where more information is needed.

Table 1. Summary of deep transcriptomic analyses and strategies for C₄ gene discovery.

Comparison	Publication
C ₃ versus C ₄ species	Gowik et al 2011 (C ₃ /C ₄ <i>Flaveria</i>) [12**], Mallmann et al 2014 (C ₃ /C ₄ <i>Flaveria</i>) [13**], Bräutigam et al 2011 (C ₃ /C ₄ <i>Gynandropsis</i>) [14**], Külahoglu et al 2014 (C ₃ /C ₄ <i>Gynandropsis</i>) [15**], Christin et al 2013 (C ₃ /C ₄ <i>Alloteropsis</i>) [16], Covshoff et al 2015 (rice/ <i>Echinochloa</i>) [17], Wang et al 2014 (rice/maize) [18**], Ding et al 2015 (rice/maize, <i>setaria</i> , <i>sorghum</i>) [19], Bräutigam et al 2014 (<i>Dichanthelium/Megathyrsus</i>) [21]
C ₃ versus C ₄ tissue	Chen et al 2014 (C ₃ /C ₄ form of <i>Eleocharis</i>) [25], Li et al 2015 (C ₃ /C ₄ organ of <i>Haloxylon</i>) [26]
Different stages of development	Wang et al 2013 (maize leaf blade/husk sheath) [27**], Liu et al 2013 (maize) [28**], Li et al 2010 (maize) [32**], Pick et al 2011 (maize) [33], Aubry et al 2014 (<i>Gynandropsis gynandra</i>) [34**], Tausta et al 2014 (maize) [35**], Mattiello et al 2015 (sugarcane) [39], Külahoglu et al 2014 (C ₃ /C ₄ <i>Gynandropsis</i>) [15**], Wang et al 2014 (maize/rice) [18**]
BS versus M cells	Li et al 2010 (maize) [32**], Aubry et al 2014 (<i>Gynandropsis gynandra</i>) [34**], Tausta et al 2014 (maize) [35**], Chang et al 2012 (maize) [36], John et al 2014 (<i>Setaria</i>) [37**]

C₃ versus C₄ species

Transcriptomes of C₃ and C₄ species have been compared both between closely related species e.g. within the genus *Flaveria* [12**,13**], between *Tarenaya hassleriana* (previously *Cleome spinosa*) (C₃) and *Gynandropsis gynandra* (previously *Cleome gynandra*) (C₄) [14**,15**] and between C₃ and C₄ *Alloteropsis* [16]; and between distantly related species such as rice (C₃) and

Echinochloa glabrescens (C₄) [17], or rice and C₄ species maize, setaria and sorghum [18**,19]. In many of these studies, the expected expression profiles of C₄ pathway enzymes were used to set bioinformatics filters aimed at identifying novel C₄-associated genes. This approach was particularly successful in terms of identifying transporter proteins that are up-regulated in C₄ species and may thus be involved in transporting C₄ pathway intermediates between BS and M cells [12**,14**,20]. Proteins identified included three plastidic transport proteins, putative bile acid:sodium symporters, a putative proton:sodium antiporter, two mitochondrial dicarboxylate carriers, and one plasma membrane intrinsic protein [12**,14**]. Although, functional validation of transporter activity will be required, these proteins need to be considered in the context of C₄ rice. Comparative transcriptome analysis of NAD-ME, NADP-ME, and PEP-CK C₄ photosynthetic subtypes and a C₃ species *Dichanthelium*, have also been reported and showed that sucrose and starch synthesis, as well as the prevention of leakage of C₄ cycle intermediates to other metabolic pathways, are critical components of C₄ metabolism [21].

In addition to elevated levels of transcripts encoding transporter proteins, comparisons in both *Gynandropsis* and *Flaveria* revealed that one-third to one-half of the genes that contribute to ATP production by cyclic electron flow were up-regulated in C₄ species. These include photosystem I components, the cytochrome b₆/f complex, and proteins mediating cyclic electron flow itself [12**,14**]. NDH-H transcripts, which encode a subunit of the NADH dehydrogenase-like complex, were markedly increased in BS cells of C₄-like and C₄ *Flaveria* species that develop agranal chloroplasts [22]. These observations are perhaps not surprising given the higher energetic requirement of the C₄ cycle (5 ATP per CO₂ molecule fixed as opposed to 3 in the C₃ cycle), but they highlight the need to consider basic metabolic processes in addition to C₄-specific traits in any engineering efforts. Notably, levels of plastid-localized Sigma70-like transcription factors were also up-regulated significantly in C₄ species of both genera, as compared with the corresponding C₃ species. It is possible that this difference relates directly to the observed difference in photosynthetic electron transfer chain complexes and thus that manipulation of plastidic sigma factor activity could be used to increase cyclic electron flow in C₃ species.

A more refined comparison of C₃ rice and C₄ maize leaves established a ‘unified developmental model’ to identify new structural and regulatory components of the C₄ pathway [18**]. Because rice and maize leaves have similar developmental trajectories (maturing from the tip to base), it was possible to align corresponding developmental stages in continuous segments from leaves of the two species. Combining metabolic profiles with gene expression patterns along the leaf gradients enabled putative regulatory genes and conserved *cis*-elements to be identified. For example, a comparison of promoter sequences in maize and rice clusters enriched for photosynthesis-related genes identified putative regulatory elements that are specific to maize. Subsequent cross-reference to maize BS and M transcriptomes identified a putative *cis* element (RGCGR; R = A/G) that is over-represented in genes that are preferentially expressed in M cells, and a conserved motif that is the core component of Dof transcription factors (WAAAG; W = T/A) that is enriched in genes that are preferentially expressed in BS cells.

Comparative analyses of C₃ and C₄ species have also provided insight into potential evolutionary trajectories in the transition from C₃ to C₄. By examining sequence and expression differences between C₄ cycle genes and putative C₃ orthologs, it was concluded that there was a series of small-scale changes that led to a modular recruitment of existing genes into the photosynthetic pathway [15**]. This observation is significant as it argues against a general C₄ master regulator and thus against a potential flip switch for C₄ rice. As such, it is important to consider the sequence in which changes need to be introduced. In this regard, recent work on closely related C₃, C₃-C₄, and C₄ *Flaveria* species, that combined transcriptome data with flux balance analysis, developed a mechanistic model for C₄ evolution. The model postulates that a misbalance in nitrogen metabolism between BS and M cells is a prerequisite for the evolution of the C₄ pathway, and that this misbalance is created through compartmentalization of the photorespiratory pathway in BS cells. [13**]. This suggests restriction of photorespiration to the BS cells as a clear first step for C₄ engineering [23,24].

C₃ versus C₄ tissues

Some plant species are capable of performing both C₃ and C₄ type photosynthesis, either in different environmental conditions or in distinct organs. Transcriptomic studies of such species have provided valuable information about the C₄ pathway. For example, transcript profiles of the amphibious species *Eleocharis baldwinii* revealed that genes encoding many transporter proteins were dramatically up-regulated in the induced C₄ tissue, and indicated that ABA, auxin signaling and DNA methylation play critical roles in the induction of C₄ photosynthesis [25]. Comparative analysis between C₃ cotyledons and C₄ assimilating shoots of *Haloxylon ammodendron* revealed putative Kranz regulators that were up-regulated in C₄ assimilating shoots, and notably many of these putative regulators were also identified from a comparison of maize foliar (Kranz) and husk (non-Kranz) tissues [26,27**].

Different stages of development

C₄ leaf anatomy is established during early leaf development. In maize, characteristic Kranz patterns are observed by P5 (Figure 1a, c). Regulators that initiate Kranz development must therefore be active prior to P5. A study of transcriptional dynamics in maize embryonic leaves during seed germination attempted to identify such regulators and indicated that Kranz development may be mediated by modular and stage specific relocation of plant hormones via the action of hormone receptors and transporter proteins [28**]. Another study compared transcript profiles in developing primordia of Kranz (foliar) and non-Kranz (husk) maize leaves. In this case, both spatial and temporal expression patterns were used to identify cohorts of genes that may regulate procambium initiation, vascular differentiation or BS/M cell-type specification [27**]. Notably, up-regulation of maize SCARECROW (SCR) and SHORTROOT (SHR) homologs during vascular development highlighted a role for the SHR/SCR pathway in patterning Kranz anatomy, as previously inferred from the observation of altered Kranz development in maize *scr* mutants [10,29,30]. Further study showed that significantly more genes have been subjected to regulatory neofunctionalization in foliar leaves than in husk leaves, and that both leaf-types have undergone selection for distinct functional roles [31].

Whereas studies of developing foliar and husk primordia have revealed candidate regulators of C₄ leaf anatomy, other studies in maize have focused on understanding the regulation of light-induced chloroplast development and the sink-source transition in C₄ leaves [18**,32**,33]. These studies exploited the maturation gradient in leaves, examining transcriptional profiles in sequential leaf sections (with the least developed tissue at the base and the most developed at the tip). Cluster analysis revealed the transcriptional dynamics of C₄ photosynthetic development, and amongst other things was able to profile the cell wall biosynthesis, cellular metabolism, transcription factors, and alternative splicing. Analogous datasets were generated for the C₄ dicot *G. gynandra* [15**,34**]. Importantly, comparison with the maize dataset revealed that despite the wide evolutionary separation and independent origins of C₄ in the two species, homologous transcription factors appear to have been co-opted to regulate gene expression for C₄ photosynthesis. Further comparison with *T. hassleriana* during leaf ontogeny identified a set of 37 genes that were consistent with the existence of modular recruitments, including co-option of the root SCR/SHR regulatory network into developing leaves.

BS versus M cells

Transcriptome profiles of purified BS and M cell-types are available for maize (32**,35**,36), setaria (37**), and *G. gynandra* (34**). These datasets are a valuable resource for understanding BS- and M-specific traits, and have been used to identify potential pyruvate transporters and transcription factors that are preferentially expressed in one or other of the cell-types. More importantly, however, cross-species comparisons have enabled an assessment of cell-specific traits that have converged in independent lineages of C₄ plants. Specifically, homologous transcriptional regulators were found to be up-regulated in either M or BS cells of both maize and setaria which have separate origins of C₄ within the monocots [37**]. Similar patterns were seen with maize and *G. gynandra*, which evolved C₄ independently in the monocot and dicot lineages [34**]. For example, *GLK1* and *GLK2* transcription factors are preferentially expressed in M and BS cells respectively of both maize and setaria; and presumably as a consequence, many of the downstream targets were also found to be differentially expressed [37**]. Although these datasets have shed light on differences between BS and M traits in C₄ plants, there is currently limited ability

to interrogate differences between expression profiles in C₄ BS versus C₃ BS cells, and similarly between M cells, because of difficulties in separating the two cell-types in C₃ plants. The single report of expression profiles in C₃ BS and M cells was a study in rice that was based on microarrays rather than RNA-seq data [38]. Comparison of the microarray data with RNA-seq data from *G. gynandra* identified rice homologues of 50 genes that are expressed preferentially in the C₄ BS, but of these 50, only 5 were preferentially expressed in the BS cells of rice. For M cells, 25 homologues were detected but none were preferentially expressed in rice M cells [34**]. Generation of genome wide transcriptomes of C₃ BS and M cells would allow a more comprehensive assessment of cell-type specific traits that are essential for elaboration of the C₄ pathway.

Summary and perspectives

Recent transcriptome studies have compared expression profiles in a range of C₃ versus C₄ contexts, along leaf developmental gradients, and in separated BS and M cells. These approaches have identified metabolite transporters and transcription factors that are potentially useful for engineering C₄ rice. However, more is needed:

1. Additional datasets are required, including more temporal, spatial, conditional and cross-species comparisons. In particular, comparisons need to be made between separated cell-types of C₃ and C₄ species. The technical difficulties of isolating BS and M cells from C₃ plants have recently been overcome, at least in Arabidopsis [40], paving the way for similar approaches in rice. Comparisons of early differentiating, and dark/light shifted C₄ BS and M cells will further help dissect the processes underpinning the establishment of Kranz anatomy and dimorphic chloroplasts.
2. Many open questions remain including how transcript profiles relate to epigenetic signatures and to protein function and how cyclic electron flow underpins the C₄ pathway. Further 'omics' approaches are needed to provide information on genome-wide histone and transcription factor footprints, proteomes and metabolomes that are associated with the C₄ pathway.
3. Ultimately, gene function will need to be determined: both in terms of necessity for development in a C₄ species, and sufficiency in terms of engineering C₄ rice. Functional validation of existing candidate regulators is ongoing with necessity being tested by knockdown experiments in maize and setaria, and sufficiency by overexpression in rice. The next steps toward engineering C₄ rice will require coordinated interpretation of these experiments alongside an understanding of the related gene regulatory networks.

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