SUMMARY

The international C_4_ rice consortium aims to introduce into rice a high capacity photosynthetic mechanism, the C_4_ pathway, to increase yield. The C_4_ pathway is characterised by a complex combination of biochemical and anatomical specialisation that ensures high CO_2_ partial pressure at RuBisCO sites in bundle sheath (BS) cells. Here we report an update of the progress of the C_4_ rice project. Since its inception in 2008 there has been an exponential growth in synthetic biology and molecular tools. Golden Gate cloning and synthetic promoter systems have facilitated gene building block approaches allowing multiple enzymes and metabolite transporters to be assembled and expressed from single gene constructs. Photosynthetic functionalisation of the BS in rice remains an important step and there has been some success overexpressing transcription factors in the cytokinin signalling network which influence chloroplast volume. The C_4_ rice project has rejuvenated the research interest in C_4_ photosynthesis. Comparative anatomical studies now point to critical features essential for the design. So far little attention has been paid to the energetics. C_4_ photosynthesis has a greater ATP requirement, which is met by increased cyclic electron transport in BS cells. We hypothesise that changes in energy statues may drive this increased capacity for cyclic electron flow without the need for further modification. Although increasing vein density will ultimately be necessary for high efficiency C_4_ rice, our modelling shows that small amounts of C_4_ photosynthesis introduced around existing veins could already provide benefits of increased photosynthesis on the road to C_4_ rice.

Keywords: C_4_ photosynthesis, rice, metabolic engineering, bundle sheath cells, plasmodesmata, photosynthetic electron transfer.
single cell organism *Hydrilla verticillata* (Bowes and Salvucci, 1984; von Caemmerer et al., 2014). Four enzymes of the C₄ photosynthetic pathway were successfully introduced into rice by Miyao and collaborators and although no photosynthetic gains were observed, Miyao et al. (2011) have elegantly summarised the extensive contribution this endeavour made to our understanding of gene expression and regulation of C₄ genes expressed in rice. Current progress on building a two cell C₄ pathway in rice are built on these earlier insights.

The current C₄ rice project (https://c4rice.com/) which aims to introduce Kranz anatomy into rice was first conceived by John Sheehy (Mitchell and Sheehy, 2006) who invited a group of C₄ photosynthesis experts to the international rice research institute (IRRI) in the Philippines to discuss the potential of C₄ rice. Engineering the C₄ pathway into a C₃ plant requires manipulation of both anatomical and biochemical traits and progress of this research has been reviewed a number of times (Hibberd et al., 2008; Hibberd and Covshoff, 2010; Langdale, 2011; Sedelnikova et al., 2018). To introduce Kranz anatomy into rice requires a change of vein spacing patterns so that veins are closer together in the leaf and BS cells need to be ‘functionalised’ for increased photosynthetic capacity, including increased chloroplast content. At this point while there are established candidates for the genes and transcription factors that potentially control vein spacing, the complete transcriptional network remains to be elucidated (for review see Sedelnikova et al., 2018). However, some progress has been made in photosynthetic functionalisation of the rice BS (Wang et al., 2017b) While insertion of C₄ biochemistry in rice faces challenges of engineering high level and cell-specific gene expression (Hibberd and Covshoff, 2010), genes encoding the C₄ pathway enzymes and most of the metabolite transporters have now been identified. Here we review current progress and how they have been enabled by technological advances in cloning techniques and high-light future challenges.

**BUILDING THE BIOCHEMISTRY FOR C₄ RICE**

In 2008, the year the C₄ rice consortium commenced, a remarkable amount of information in regard to the genes encoding the key proteins in the C₄ pathway was already known. The cDNA sequences for maize phosphoenolpyruvate carboxylase (PEPCK), pyruvate orthophosphate dikinase (PPDK), and NADP-malate dehydrogenase (MDH) had all been reported and expressed in the M cells of rice (reviewed in Miyao et al., 2011). The cDNA sequence for NADP-malic enzyme (NADP-ME) had also been reported for both the rice endogenous gene and the maize gene (Drinovich et al., 2001). The genes encoding carbonic anhydrase (CA) in maize had also been cloned by this time, but the precise identity of the gene product located in the cytosol of the M cells in C₄ leaves was not definitively proven (reviewed in DiMario et al., 2016). Therefore, the key genes encoding the entire biochemical pathway of the NADP-ME type C₄ mechanism as shown in Figure 1 were all available at the commencement of the C₄ rice project.

What set the current C₄ rice project apart from previous activities was the desire to engineer a full Kranz two cell-type mechanism in rice (von Caemmerer et al., 2012). This approach requires not only the cDNA sequences for the relevant photosynthetic proteins but high level expression with suitable promoters in the appropriate cell type of rice. For M cell-specific expression, the promoters of genes from C₄ species encoding PEPCK, PPDK, and aspartate aminotransferase (AspAT) had all been tested in C₃ species and lead to M-specific accumulation of the β-glucuronidase (GUS) reporter protein (Hibberd and Covshoff, 2010). The PEPCK promoter from maize had been the most extensively tested in rice and various truncated versions have been shown to produce M-specific expression of the reporter gene GUS in rice (Hibberd and Covshoff, 2010).

High level BS cell-specific expression of proteins such as NADP-ME has proven to be the greatest challenge for establishing a C₄ metabolic pathway in rice. While there are potential anatomical constraints in regard to the photosynthetic competence of the rice BS compartment (Wang et al., 2017b), the paucity of promoters available to drive BS expression in a C₃ plant has been a major obstacle. At the commencement of the C₄ rice project, the promoters of the genes encoding phosphoenolpyruvate carboxykinase (PEPCK), NADP-ME, AspAT, small subunit (Engelmann et al.) of RuBisCO, and the P subunit of glycine decarboxylase (GDCP) from C₄ species had been tested in C₃ plants (Chen et al., 2001; Nomura et al., 2005a; Nomura et al., 2005b; Engelmann et al., 2008). Of these, a version of the *Zoisia japonica* PEPCK promoter (Nomura et al., 2005a) resulted in BS-specific GUS accumulation in rice. The *Flaveria trinervia* GDCP promoter was shown to be BS/basal-specific in Arabidopsis and the promoter of this gene from *Flaveria anomala* (Chen et al., 2001) was vascular-specific in rice. Both the PEPCK and GDCP promoters have subsequently been utilised in the C₄ rice strategy although promoter strength has been an ongoing issue for engineering efforts and it is notable that in the single cell C₄ project (reviewed in Miyao et al., 2011), genomic clones often but not always gave superior expression levels compared with cDNAs driven by their own promoters.

Stacking of genes in transgenic rice in the initial phases of this project required the crossing of homozygous lines harbouring single gene constructs to build a rice plant expressing a complete set of the genes encoding the major enzymes of the C₄ pathway. Such a crossing strategy was a hugely time consuming effort as expression levels and cell-specific expression of the recombinant protein must be checked for each line and a crossing donor identified for each transgene. With segregation of the transgenes...
inserted at different loci in the stacked lines, many hundreds of individuals had to be genotyped at each cross to obtain a single line harbouring the genes encoding the five key photosynthetic enzymes shown in Figure 1. Indeed, this crossing strategy has taken almost 6 years to achieve in indica rice in the current project. However, five genes are not enough for creating C₄ rice and the biochemical pathway is being complemented by a suite of membrane transporters ensuring fast transport of metabolites between cell compartments. Most of the transporters have now been identified and are listed in Figure 1, however there is still some uncertainty about malate import to the BS chloroplast and the export of pyruvate following malate decarboxylation. A recent review of transporters was given by Schuler et al. (2016). Physiological gas exchange techniques exist to allow for the identification of a complete, functioning C₄ pathway. These include measurements of CO₂ compensation points, reduced oxygen sensitivity of CO₂ assimilation and reduced carbon isotope discrimination (Furbank et al., 2009). In the meantime new ¹³C pulse chase labelling techniques have been developed to analyse the paths of carbon during C₃ and C₄ photosynthesis (Arrivault et al., 2009) and confirmed that malate is in fact being formed in our current five gene rice transgenics.

SYNTHETIC BIOLOGY ACCELERATES THE PACE

A consensus definition drafted by a group of European experts more than a decade ago defined synthetic biology as follows: ‘Synthetic biology is the engineering of biology; the synthesis of complex, biologically based (or inspired) systems, which display functions that do not exist in nature. This engineering perspective may be applied at all levels of the hierarchy of biological structures—from individual molecules to whole cells, tissues and organisms. In essence, synthetic biology will enable the design of ‘biological systems’ in a rational and systematic way’ (Serrano, 2007). Synthetic biology has evolved and adopted many of the commonly used terms in mainstream engineering such as ‘switch’, ‘rewire’, ‘design, test and redesign cycle’. Although in the creation of C₄ rice, in which a template or design already existing in nature is being used, the installation of up to 20 genes to completely ‘rewire’ rice metabolism and anatomy surely fits the definition above. A major limitation in the synthetic biology approach however is the cycle time for the design, test and redesign in crops such as rice.

The last 5 years have seen an exponential growth in synthetic biology tools and the cost of gene synthesis has plummeted. This has enabled the C₄ rice consortium to adopt a more rapid cycle of design, test and prototype coupled to the adoption of a rapid Agrobacterium-based rice transformation system in the japonica rice variety ‘Kitaake’. Kitaake is fast flowering, day neutral, small in stature and an established model for functional genomics studies (Li et al., 2017). The obstacle of genetic transformation with a single gene construct at a time and crossing has largely been solved by gene synthesis and Golden Gate cloning or similar ‘gene building block’ approaches (Engler et al., 2014). Gene synthesis allows the ‘domestication’ of coding sequences to remove or insert rare Type IIIS restriction enzyme recognition sites while leaving the amino acid sequence unchanged, thus enabling the assembly of gene modules which can be pasted together, often in a ‘one pot cloning’ approach. Assembly of these modules into a T-DNA suitable for Agrobacterium transformation is therefore greatly accelerated over traditional restriction/ligation approaches (Andreou and Nakayama, 2018). In principle, assembling all the metabolic and transporter components of Figure 1 for rice transformation on a single construct should be readily achievable and this work is currently underway. This approach has so far enabled a 6-year crossing strategy to be reduced to a 6-month single transformation experiment in rice.

In a large multigene overexpression construct, it is not desirable to reuse ‘parts’ multiple times due to the possibility of recombination deletion, post-transcriptional gene silencing or inactivation at the promoter level via methylation (Wassenegger, 2002). Epigenetic promoter silencing is a poorly understood process and can be a major challenge
for metabolic engineering. This also presents a challenge for the design of the gene constructs described above for C₄ rice. For example, expression of just CA, PEPC, MDH and PPDK would ideally require four heterologous M-specific promoter sequences. While this may be possible for the M compartment, it is not for the BS compartment (see above).

Synthetic biology has also provided a potential solution to this paucity of promoters in rice. Brückner et al. (2015) described a system compatible with the Golden Gate cloning which utilises multiple promoters (Synthetic TALE Activated Promoters or STAPs) designed to be orthogonal to the genome of the plant to be transformed, which can be activated by a single Transcription Activator-Like Effector (TALE). This approach provides the opportunity to build multiple transcriptional units driven by different promoters on the same gene construct, trans-activated by a single transcription factor.

FUNCTIONALISATION OF THE BUNDLE SHEATH: AN ANATOMICAL ROADBLOCK?

Figure 2(a) shows fresh transverse sections of a rice (C₃) and Setaria viridis (C₄) leaf imaged with the laser confocal microscope using chlorophyll fluorescence overlaid with cell wall fluorescence. This enables visualisation of the chloroplast contents of BS cells in each species. This image clearly indicates that the BS compartment of the C₃ leaf is tightly packed with chloroplasts, whereas the rice BS compartment is only sparsely populated by chloroplasts and the cells are highly vacuolated. This has previously been pointed out and ‘photosynthetic functionalisation’ of the BS has been proposed as an early step in evolution of C₄ photosynthesis, probably occurring at the C₂ or proto-Kranz stage (Sage, 2004; Wang et al., 2017b). The underlying mechanisms responsible for the proliferation of chloroplasts in BS cells of C₄ leaves are largely unknown. However, recently a transgenic approach has been used in rice to ‘recreate’ this key step in evolution by overexpressing the transcription factors GOLDEN2 (G2) or GOLDEN2-LIKE (GLK) (Wang et al., 2017b) which had been implicated as important in BS cell differentiation in terrestrial plants including Zea mays and more recently in rice (Wang et al., 2013 and references therein). These transcription factors are thought to act in the cytokinin signalling pathway (Wang et al., 2013). Overexpression of these Z. mays transcription factors in rice indeed increased the proportion of vascular cell area occupied by chloroplasts, the mitochondrial population in rice BS cells, and the plasmodesmal connectivity at the BS/M cell interface (Wang et al., 2017b). The BS chloroplast abundance data from this work are summarised in Figure 3. It is evident from these quantitative data that overexpression of this class of transcription factor can increase BS chloroplast content as a proportion of total leaf chloroplasts by approximately five-fold (in the case of G2 expressed from the ubiquitin promoter of Z. mays: UBI-G2). However, another three-fold increase is required to reach levels equivalent to C₂ leaves and potentially another six-fold to reach C₄ levels of chloroplast area in the BS. This can be visualised in Figure 2 which shows a representative image of a leaf transverse (a) and paradermal (b) sections of the BS compartment of rice (Wang et al., 2017b) compared with rice and Setaria leaves. Comparison of rice and C₄ leaf chloroplast distribution is complicated, however, by the change in vein spacing and reduction in M chloroplast numbers seen in C₄ leaves relative to C₃ (Stata et al., 2016).

Alternative approaches are also under investigation for increasing chloroplast abundance/volume in the BS compartment of rice (Wang et al., 2017a). The transcription factor CYTOKININ RESPONSIVE GATA FACTOR 1 (CGA1) has...
also been shown to regulate leaf chloroplast abundance via the cytokinin signalling network (Chiang et al., 2012; Hudson et al., 2013; Wang et al., 2017a) and overexpression of CGA1 in rice resulted in a 30% increase in flag leaf chlorophyll and close to a doubling of chloroplast numbers on a fresh weight basis (Hudson et al., 2013). It has been proposed that regulation of the FtsZ chloroplast division gene by CGA1 is responsible for this phenotype, presenting opportunities for regulating levels of this protein directly or other genes in this pathway controlling chloroplast division (Hudson et al., 2013; Wang et al., 2017a).

It may be that part of the difficulty obtaining high level expression of chloroplast proteins in rice BS cells is the lack of sufficient chloroplast volume to house the recombinant proteins targeted to this compartment. The developmental programme of BS cells in a C3 grass may reflect a more ‘parenchyma-like’ role in temporary sugar storage and the sugar status of the BS cells may not be conducive to photosynthetic functionalisation. The high level expression of sugar effluxers such as the SWEET13 gene family in the BS of C4 plants has been proposed as evidence that sugar status in the BS or C4 grasses may be quite different from that in C3 grasses (Emms et al., 2016). In addition, partitioning of starch almost exclusively into the BS of C4 grasses is a curious and potentially relevant observation (Lunn and Furbank, 1999).

WHAT CAN WE LEARN FROM COMPARATIVE LEAF ANATOMY BETWEEN C3 AND C4 GRASS SPECIES?

It has previously been proposed that an early step in C4 evolution was ‘inflation’ of BS cell size (Sage, 2004; Sage et al., 2012; Christin et al., 2013). However, our results from anatomical measurements performed in 25 grass species, representing different photosynthetic types and seven independent C4 evolutionary origins (Figure 4a), reveal that C4 leaves do not necessarily have larger BS cells, nor do they always have shorter interveinal distances than C3 leaves (Figure 4b). Rather, a C4 leaf has greater BS surface area per leaf area (Sb), fewer interveinal M cells than a C3 leaf and most notably, more plasmodesmata (PD) at the BS/M cell interfaces (Figure 4; Danila et al., 2016; Danila et al., 2018). For a functional C4 rice, these findings mean that increasing PD connections between M and BS, increasing Sb, and reducing M cells between veins of the rice leaves may be essential for the operation of an efficient photosynthetic pathway.

It has long been thought that a key feature of C4 leaf anatomy was increased abundance of the symplastic nanochannels (PDs) that facilitate the rapid exchange of metabolites between M and BS cells during C4 photosynthesis (Hatch and Osmond, 1976). Recent work, however, has provided quantitative data on this parameter (Danila et al., 2016; Danila et al., 2018; Figure 4). Enhancement of the PD connections between M and BS of rice to levels observed in closely related C3 species would require increases of at least five-fold (Danila et al., 2018). While there are few genes known which control PD development and proliferation, it was recently observed that when maize GLK genes were constitutively expressed in rice, increased organelle volume was accompanied by increased M-BS PD density; although the enhancement is only double that of wild type (Wang et al., 2017b). Coordination of chloroplast

![Figure 3](image-url) Per cent of total chloroplast area found in vascular tissue area. The data are taken from Table S5 Wang et al. (2017b). Ubi-G2 and Ubi-Glk1 are Oryza sativa plants in which the transcription factor GOLDEN2 (G2) or GOLDEN2-LIKE1 (GLK1) has been overexpressed from the ubiquitin promoter of Zea mays (Wang et al., 2017b). Other monocots shown are Dichanthelium oligosanthes, a C3 from the PACMAD clade; Steinchisma hians, which operates the C2 photosynthetic pathway; Panicum virgatum, a C4 species with the NAD-ME decarboxylation type; and Setaria viridis, a C4 species with the NADP-ME decarboxylation type.

![Figure 4](image-url) (a) Phylogenetic tree of the C3 and C4 grass species examined generated using sequences from ndhF and rbcL chloroplast genes. Species names are colour-coded according to photosynthetic types. The seven independent evolutionary origins of C4 photosynthesis according to GPWGII (2012) are indicated with green circles at the midpoint of the branches. Note that Panicum milioides, a.k.a. Steinchisma hians, which technically a C2-C3 intermediate (Duvall et al., 2003) was categorised as C4 in GPWGII (2012). Numerical value at internal nodes is the percentage of non-parametric bootstrap replicates that support the bipartition. Scale bar indicates amino acid substitutions per site.

(b) Distribution of leaf trait values among photosynthetic types in (a) excluding the C2-C4 intermediate type, in which only one species was measured. The distribution of eight variables is summarised by boxplots. Box and whiskers represent the 25 to 75 percentile, and the minimum and maximum distribution. Means are denoted by (○). Letters show the statistical ranking using a post-hoc Tukey test among photosynthetic types (different letters indicate differences at P-value < 0.05). BS, bundle sheath; M, mesophyll; PD, plasmodesmata; Sb, bundle sheath area per unit leaf area.

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and PD development has been suggested previously (Brunkard et al., 2013) and cytokinin has also been implicated in the proliferation of PDs in the shoot apical meristem (Ormenese et al., 2006). These findings offer some hope that a single gene or at least an established transcriptional network could provide a master switch for C₄ BS anatomy.

The BS surface area per leaf area (Sₛₐ) is a physiological parameter which has proven to be an important feature of modelling the M-BS interface, including to estimate BS conductance to CO₂ diffusion (first estimates ranged between 0.6 m² m⁻² and 3.1 m² m⁻² (Apel and Peisker, 1978; Brown and Byrd, 1993)). Sₛₐ was obtained by dividing measurements of BS tissue perimeter from micrographs by interveinal distance (Pengelly et al., 2010). Increasing Sₛₐ can be achieved in multiple ways; modification of BS cell size, vasculature size, and the distance between BS. Because BS cell size does not differ substantially between C₃ and C₄ grass species (Figure 4, Danila et al., 2018), reducing the interveinal distance appears to be the most logical path to increase Sₛₐ in rice. Ideally, this would mean upregulation of gene(s) that would promote insertion of additional veins between existing veins of rice, thus reducing interveinal distance and, at the same time, decreasing the number of M cells between veins (Sedelnikova et al., 2018; Hughes et al., 2019).

**PAYING THE ENERGY COST OF C₄ PHOTOSYNTHESIS**

The energy cost of C₄ photosynthesis is significantly higher compared with C₃ as it requires a minimum of two more ATP molecules per one CO₂ fixed (Furbank et al., 1990). While high conductance of the M-BS interface to metabolites is essential for the operation of C₄ photosynthesis, it also allows a proportion of the CO₂ concentrated in the BS to escape back to M (called CO₂ leakage; von Caemmerer and Furbank, 2003). This CO₂ is either refixed or lost to the intercellular spaces in the M, which increases the cost of C₄ photosynthesis (Furbank et al., 1990; von Caemmerer and Furbank, 2003). To sustain higher energy requirements, C₄ plants adapt the photosynthetic electron transfer chains of M and BS cells depending on specific needs of the C₄ subtype they belong to (Munekage and Taniguchi, 2016). As the efforts of the C₄ rice project have targeted NADP-ME as the decarboxylating enzyme, we focus here on specific energy requirements of the NAPD-ME subtype of C₄ photosynthesis.

An early observation on the anatomy of tropical grasses which predated the discovery of C₄ photosynthesis was that chloroplasts in the two cell types of leaves with Kranz anatomy are dimorphic, with the BS chloroplasts often lacking grana stacks (Rhoades and Carvalho, 1944). Subsequently, it was discovered that these grasses such as Z. mays were in fact C₄ plants and specifically used the NADP-ME pathway of C₄ photosynthesis (Edwards et al., 1971; Hatch and Kagawa, 1976). As NADP-ME plants primarily use malate as a C₄ acid diffusing from M to BS cells, NADPH produced in the M chloroplasts is consumed for malate synthesis but it is then produced in the BS upon malate decarboxylation to pyruvate (Figure 1). The net transfer of NADPH from M to BS cells means that there is a reduced requirement for linear electron flow to produce NADPH in the BS chloroplast, which have reduced Photosystem II (PSII) content but have highly developed cyclic electron flow (CEF) machinery around Photosystem I (PSI) (Figure 5). Therefore, the observation that BS chloroplasts of NADP-ME plants are mostly agranal with little or no grana thylakoids functionally reflects the reduced PSII, which would normally be located in the granal stacks (see Munekage and Taniguchi, 2016). However, grana content in BS chloroplasts of NADP-ME plants is rather variable between species (Unno et al., 2005) and also within species in response to environmental conditions (Omoto et al., 2009; Danila et al., 2019) and depending on leaf age (Andersen et al., 1972). It has also been shown that aspartate can be used as a transported C₄ acid in many NADP-ME type C₄ plants, providing some flexibility in the amount of reducing power transferred to the BS from the M and suggesting that PSII content in the BS cells might in fact respond to the NADPH/NADP⁺ ratio or the redox state of the BS cells (see Furbank, 2011). This is supported by over-expression of maize NADP-ME in rice M cells which resulted in agranal chloroplasts, conceivably, due to the increased NADPH/NADP⁺ ratio depleting PSI of electron acceptor and causing over-reduction of the electron transfer chain (Takeuchi et al., 2000). While prolonged reduction of PSII acceptors promotes the formation of reactive oxygen species and causes damage and degradation of PSII (Vass, 2012) and thus also grana, there might be also regulatory mechanisms preventing transcription and de novo assembly of PSII polypeptides and grana formation in over-reduced conditions (Pfannschmidt et al., 1999).

It has been proposed that elevated CEF in BS chloroplasts allows NADP-ME plants to accommodate the extra costs of C₄ photosynthesis by producing ATP without affecting NADPH/NADP⁺ ratio (Furbank et al., 1990). The chloroplast NADPH dehydrogenase-like complex and PROTON GRADIENT REGULATION 5 (PGR5) mediate two different CEF routes (Takabayashi et al., 2005; Munekage, 2016). Interestingly, in C₃ plants, CEF is promoted in conditions causing over-reduction of the electron transport chain (Suorsa, 2015) and therefore CEF might be naturally upregulated in BS cells of C₄ rice in response to high NADPH/NADP⁺ ratio.

At present, it is unknown whether alteration of BS chloroplast electron transport components is a strict requirement for C₄ rice or only ‘fine tuning’. If the required regulatory mechanisms already exist in rice, BS chloroplasts could conceivably adjust PSII and grana content.
according to the redox state of cells in C4 rice (Figure 5). Consequently, just the right amount of PSII will be fully assembled in BS to donate electrons for the CEF and compensate for the shortage of NADPH via linear electron transfer. However, the desired adaptation of electron transport requires a strictly coordinated expression and activity of NADP-ME in rice BS cells and efficient NADPH oxidation by the Calvin cycle to maintain an appropriate NADPH/NADP⁺ balance. It is worth mentioning that the flexibility of PGA reduction between M and BS cells in C4 plants also contributes to the maintenance of NADPH/NADP⁺ balance, however, it is not clear whether this pathway will be immediately available in C4 rice. The composition of thylakoid protein complexes and energy requirements are similar between C3 and C4 M cells (Munekage and Tani-guchi, 2016), but rice BS chloroplasts will require some reorganisation of thylakoid complexes and increased abundance of the NADPH dehydrogenase-like complex (Majeran et al., 2008; Hernandez-Prieto et al., 2019). This fine tuning will be necessary to run an efficient C4 pathway as our recent research shows that the rate of C4 photosynthesis is strongly dependent on the electron transport capacity of both M and BS cells (Ermakova et al., 2019).

PARTIAL C4: MODELLING MIXED C3 AND C4 PHOTOSYNTHESIS ON THE PATH TO C4 RICE

The concept of C4 rice has excited photosynthetic modellers and there are a number of photosynthetic models that have tried to evaluate the efficacy of introducing C4 photosynthesis into rice (Bellasio, 2016; Yin and Struik, 2017; Wang et al., 2017c; Bellasio and Farquhar, 2019). Each of these models has a different focus. Wang et al.
(2017c) developed a 3D reaction diffusion model of BS and connected M cells with anatomy based on a C2 rice leaf in which C4 photosynthesis was integrated with existing C3 photosynthesis. They concluded that the C4 cycle can operate adjacent to C3 photosynthesis in rice M cells, but that the energy partitioning between the C3 and C4 cycle is an important consideration. In their current model every M cell is adjacent to a BS cell so it is built on a rice leaf with altered vein spacing. Bellasio’s models consider C4 photosynthesis as an addition to C2 photosynthesis and also provides a valuable discussion on energy partitioning. Yin and Struik (2017) consider C4 photosynthesis in rice at the canopy and crop level in different environments.

It is common to compare rice with *Z. mays* but it is informative to widen this comparison. The analysis of 25 monocot C3 and C4 leaves shows that all C4 leaves have closer vein spacing than C3 species with at most 2-3 M cells between BS (Figure 4). However there are examples in which C4 photosynthesis is naturally supported around widely spaced veins such as in maize husk tissue, albeit at lower rates with little photosynthetic activity in the interveinal M cells (Langdale et al., 1988; Pengelly et al., 2011). Here, in the context of the introduction of C4 metabolism into rice without altered vein spacing, we have asked the simple question: can we detect a small amount of C4 photosynthesis introduced around existing veins using gas exchange techniques? The modelling here uses the Farquhar et al. (1980) C3 model of photosynthesis combined with the enzyme limited C4 model of photosynthesis described in von Caemmerer (2000) with C3 kinetic constants for RuBisCO. Rice leaves have approximately 7 M cells between veins (Figure 4b; Chatterjee et al., 2016). The modelled curves show that partitioning a small amount of RuBisCO to low capacity C4 photosynthesis around veins (i.e. in 2 out of 7 M cells) lowers the compensation point and increase CO2 assimilation rates at all CO2 partial pressures (Figure 6). Hence this should be easily detected with gas exchange techniques. The modelling approach is very basic and has not considered the added energy requirements in M cells running both C3 and C4 photosynthesis given the low capacity C4 photosynthetic rates considered here. Nevertheless it suggests that even this small addition, which would result in the small amount of RuBisCO in BS being more efficient, could have a physiological benefit.

CONCLUSIONS

Considerable progress has been made in building C4 rice. The initial concept of assembling a tool box of components and a biochemical, anatomical and molecular blueprint based on evolution and 50 years of study has now become one of the largest synthetic biology projects of all time in plant biology. Discovering that our ‘toolbox’ was often lacking and that new knowledge and technologies would continually reshape the strategy and redefine the blueprint should come as no surprise. ‘Functionalisation’ of the BS compartment is the immediate hurdle. For a fully functional C4 cycle to operate in rice across two cell types, the BS must be functionally capable of housing the photosynthetic machinery necessary for C4 acid decarboxylation and CO2 fixation by RuBisCO. It appears that we have the biochemical and molecular-genetic components necessary for C4 function; the means to prototype and fine tune them; a promise that C4 photosynthesis around rice leaf veins may be possible and even beneficial, but there is still some distance yet to be travelled on the road to C4 rice.

ACKNOWLEDGEMENTS

We dedicate this review to the late John Sheehy, the champion of the C4 rice project. The Research was funded by a C4 rice project grant from The Bill & Melinda Gates Foundation to the University of Oxford (2015–2019; OPP1129902) and Australian Research Council Centre of Excellence for Translational Photosynthesis (CE1401000015).

AUTHOR CONTRIBUTIONS

All authors contributed to the writing of this review.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY

The datasets generated in this paper are available from the corresponding author on request.

REFERENCES

Andersen, K.S., Bain, J.M., Bishop, D.G. and Smillie, R.M. (1972) Photosystem II activity in agranal bundle sheath chloroplasts from *Zea mays*. Plant Physiol. 49, 461–466.

Androu, A.I. and Nakayama, N. (2018) Mobius assembly: a versatile golden-gate framework towards universal DNA assembly. PLoS ONE, 13, e0189892.

Apel, P. and Peisker, M. (1978) Influence of high oxygen concentrations on the CO2 compensation concentration in C4 plants. *Kulturpflanze*, 26, 99–103.

Arrivalet, S., Guenther, M., Ivakov, A., Feil, R., Vosloh, D., van Dongen, J.T., Sulpice, R. and Stitt, M. (2009) Use of reverse-phase liquid chromatography, linked to tandem mass spectrometry, to profile the Calvin cycle and other metabolic intermediates in Arabidopsis rosettes at different carbon dioxide concentrations. *Plant J.* 59, 824–839.

Bellasio, C. (2016) A generalized stoichiometric model of C4, C2, C2+ C4, and C3 photosynthetic metabolism. *J. Exp. Bot.* 68, 269–282.

Bellasio, C. and Farquhar, G.D. (2019) A leaf-level biochemical model simulating the introduction of C2 and C4 photosynthesis in C3 rice: gains, losses and metabolite fluxes. *New Phytol.* 223, 150–166.

Bowes, G. and Salvucci, M.E. (1984) *Hydrida*: Inducible C2-type photosynthesis without Kranz anatomy. In *Advances in Photosynthesis Research* (Sybesma, C., ed). The Hague: Martinus Nijhoff/Dr. W. Junk Publishers, pp. 829–832.

Boyd, R.A., Gandin, A. and Cousins, A.B. (2015) Temperature responses of C4 photosynthesis: biochemical analysis of rubisco, phosphoenolpyruvate carboxylase, and carbonic anhydrase in *Setaria viridis*. *Plant Physiol.* 169, 1850–1861.

Brown, R.H. and Bouton, J.H. (1993) Physiology and genetics of interspecific hybrids between photosynthetic types [Review]. *Annu. Rev. Plant Physiol.* 44, 435–456.
Munekage, Y.N. (2016) Light harvesting and chloroplast electron transport in NADP-malic enzyme type C4 plants. Curr. Opin. Plant Biol. 31, 9–15.

Munekage, Y.N. and Taniguchi, Y.Y. (2016) Promotion of cyclic electron transport around photosystem I with the development of C4 photosynthesis. Plant Cell Physiol. 57, 897–903.

Nomura, M., Higuchi, T., Ishida, Y., Ohta, S., Komari, T., Imazumi, N., Miyao-Tokutomi, M., Matsuoka, M. and Tajima, S. (2005a) Differential Expression Pattern of C4 Bundle Sheath Expression Genes in Rice, a C4 Plant. Plant Cell Physiol. 46, 754–761.

Nomura, M., Higuchi, T., Katayama, K., Taniguchi, M., Miyao-Tokutomi, M., Matsuoka, M. and Tajima, S. (2005b) The promoter for C4-type mitochondriald aspartate aminotransferase does not direct bundle sheath-specific expression in transgenic rice plants. Plant Cell Physiol. 46, 743–753.

Omoto, E., Kawasaki, M., and Miyake, H. (2009) Salinity Induces Granal Development in Bundle Sheath Chloroplasts of NADP-Malic Enzyme Type C4 Plants. Plant Prod. Sci. 12, 199–207.

Ormenese, S., Bernier, G. and Perilleux, C. (2006) Cytokinin application to the shoot apical meristem of Sinapis alba enhances secondary plasmodesmata formation. Planta, 224, 1481–1484.

Pengelly, J.J.L., Sirault, X.R.R., Tazoe, Y., Evans, J.R., Furbank, R.T. and von Caemmerer, S. (2001) Kranz anatomy is not essential for terrestrial C4 plant photosynthesis. Nature, 414, 543–546.

Pengelly, J.J.L., Kwasny, S., Bala, S., Evans, J.R., Voznesenskaya, E.V., Koteyeva, N.K., Edwards, G.E., Furbank, R.T. and von Caemmerer, S. (2011) Functional analysis of corn husk photosynthesis. Plant Physiol. 156, 503–513.

Pfannschmidt, T., Nilsson, A. and Allen, J.F. (1999) Photosynthetic control of chloroplast gene expression. Nature, 397, 625.

Rhoades, M.M. and Carvalho, A. (1944) The function and structure of the parenchyma sheath plastids of the maize leaf. Bull. Torrey Botanical Club, 71, 335–346.

Sage, R.F. (2004) The evolution of C4 photosynthesis. New Phytop. 161, 341–370.

Sage, R.F., Sage, T.L. and Kocacinar, F. (2012) Photorespiration and the evolution of C4 photosynthesis, Annu. Rev. Plant Biol. 63, 19–47.

Schuler, M.L., Mantegazza, O. and Weber, A.P.M. (2016) Engineering C4 photosynthesis into C3 chassis in the synthetic biology age. Plant J. 87, 51–65.

Sedelnikova, O.V., Hughes, T.E. and Langdale, J.A. (2018) Understanding the genetic basis of C4 kranz anatomy with a view to engineering C4 crops. Ann. Rev. Genet. 52, 249–270.