Highlights (for review)

- Quantification of the degree to which transcript abundance differs between C_3 and C_4 leaves
- Identification of novel components of C_4 metabolism
- Intersection with mathematical models to explain evolution of the complex C_4 phenotype
- Indication that C_4 photosynthesis is underpinned by both convergent and parallel evolution of structural genes and also regulators

Insights into C_4 metabolism from comparative deep sequencing

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Abstract

C_4 photosynthesis suppresses the oxygenation activity of Ribulose Bisphosphate Carboxylase Oxygenase and so limits photorespiration. Although highly complex, it is estimated to have evolved in sixty-six plant lineages, with the vast majority lacking sequenced genomes. Transcriptomics has recently initiated assessments of the degree to which transcript abundance differs between C_3 and C_4 leaves, identified novel components of C_4 metabolism, and also led to mathematical models explaining the repeated evolution of this complex phenotype. Evidence is accumulating that this complex and convergent phenotype is partly underpinned by parallel evolution of structural genes, but also regulatory elements in both *cis* and *trans*. Furthermore, it appears that initial events associated with acquisition of C_4 traits likely represent evolutionary exaptations related to non-photosynthetic processes.

Introduction

C_3 plants inherited a carbon fixation system developed by the photosynthetic bacteria, with primary carbon fixation being catalysed by the enzyme Ribulose Bisphosphate Carboxylase Oxygenase (RuBisCO). The oxygenase activity of RuBisCO generates the toxic intermediate phosphogylcollate, and although this can be detoxified and carbon partially recovered by the photorespiratory pathway, energy is expended in the process. As the oxygenase function of RuBisCO increases with ambient temperature, it is thought that in tropical and sub-tropical habitats, significant selection pressure led to the convergent evolution of carbon concentrating mechanisms [1]. Phylogeny indicates that land plants have repeatedly evolved either temporal (Crassulacean Acid Metabolism) or spatial carbon concentrating mechanisms (C_4 photosynthesis) [2].

Although highly complex, the C_4 pathway is estimated to have evolved in at least sixty-six lineages of plants [3]. Initial analysis of clades that contain C_3 and C_4 species but also 'C_3-C_4' intermediates identified the most common early traits likely associated C_4 photosynthesis, and this led to the development of models that depict the evolution of this complex phenotype along a relatively linear path of trait acquisition [4]. More recently, probabalistic modelling within a Bayesian framework identified flexibility in when C_4 component traits evolve, but also found four major paths likely associated with acquisition of these traits [5]. Despite this flexibility in the acquisition of C_4 component traits, the core C_4 metabolic machinery has converged upon a similar architecture in all C_4 lineages. For example, in all C_4 species, HCO_3^- is initially fixed by phospho*enol*pyruvate carboxylase (PEPC) (Figure 1), which has a higher affinity for HCO_3^- than RuBisCO does for CO_2 [6]. C_4 acids then diffuse down a concentration gradient into insulated cellular, or sub-cellular [7] compartments where C_4 acid decarboxylases increase the local concentration of CO_2 around RuBisCO, thereby reducing its oxygenation activity. In most C_4 species, an altered arrangement of cells within the leaf known as Kranz anatomy facilitates the compartmentation of carboxylation and decarboxylation (Figure 1A). There are three basic biochemical pathways defined by the predominant C_4 acid decarboxylase that releases CO_2 around RuBisCO, but there are also at least 25 forms of Kranz anatomy documented (Figure 1A and 1B).

Progress in understanding C_4 leaf anatomy has recently been critically assessed [8]. Here we focus on how deep sequencing is influencing our understanding of C_4 biochemistry and argue that combined with allied technologies it is opening up a new era of C_4 research. These approaches are helpful for at least three reasons. First, many years of mutant screens, biochemistry and molecular biology have so far failed to unlock many of the molecular components that regulate or induce the C_4 system [9,10], sequencing offers the opportunity to identify candidate genes for these traits. Second, the C_4 pathway should correctly be viewed as a system. Deep sequencing now makes it possible to move from analysis of individual genes and their gene products, to assessing the simultaneous behaviours of both the system and its components. Third, computational advances that have been driven by deep sequencing datasets provide the opportunity to study the natural diversity of all C_4 lineages, rather than being limited to well-studied 'model' species for

which genome sequence is available. With this as background, we now assess how deep sequencing has influenced the understanding of core components of the C_4 cycle, the *trans*-factors likely responsible for their compartmentation between mesophyll (M) and bundle sheath (BS) cells of the C_4 leaf, and also the evolutionary processes that have governed the transition from the ancestral C_3 photosynthetic system to the derived C_4 metabolic pathway.

Defining mRNAs associated with C_4 photosynthesis

Approximately forty genes encoding core C_4 cycle enzymes and components of the Calvin-Benson-Bassham cycle (CBB) have long been known to be involved in C_4 metabolism. RNA-seq has been used to report mRNA signatures associated with the 'NAD-ME', 'NADP-ME' or 'PEPCK' biochemical sub-types [11–13], and along with theoretical and modelling approaches, has provided clear evidence that often two of the decarboxylases operate in parallel, with their relative contributions varying depending on conditions [14–17]. However, our understanding of what changes leaf anatomy such that contact between tissues involved in carbon assimilation and reduction is increased (Figure 1A), and also what sets up and then maintains the patterns of gene expression required for the C_4 cycle are rudimentary. These factors are important, as an understanding of C_4 genetics has implications for strategies being adopted to engineer the pathway into C_3 crop species, dictating whether efforts should be focused on alterations to individual genes, transcriptional regulators or hormone metabolism and signalling. Deep sequencing has allowed estimates of the extent to which global patterns of mRNA abundance differ between C_3 and C_4 leaves. This approach was initiated in the *Cleomaceae,* which in addition to containing C_3 and C_4 species, is phylogenetically the closest-C_4-containing clade to C_3 *Arabidopsis thaliana* [18]. 603 genes showed differential mRNA abundance in C_4 compared with C_3 leaves [12]. Furthermore, in addition to confirmation that mRNAs encoding core C_4 and CBB cycles were up and down-regulated respectively, previously unidentified characteristics of the C_4 leaf as well as new components of the C_4 cycle were reported. For example, reduced abundance of mRNAs encoding ribosomal sub-units in C_4 compared with C_3 leaves was reported [12], while *BASS2,* which was subsequently shown to encode the long-sought-after pyruvate transporter associated with C_4 photosynthesis was up-regulated [19]. Subsequent analysis has led to increased numbers of genes being linked to the C_4 cycle [13] and Table 1. The highest reported differences in transcript abundance between C_3 and C_4 tissues are derived from *Eleocharis*, a species that is able to switch from C_3 to C_4 depending on whether it is aquatic or terrestrial (Table 1). However, a proportion of the mRNAs reported to be differentially abundant in C_4 compared with C_3 *Eleocharis* are likely associated with the different light and temperature conditions caused by the aquatic to terrestrial switch [20].

Comparison of estimates of the number of changes associated with each of the three biochemical sub-types (Figure 1) led to suggestions that establishment of the PEPCK C_4 sub-type requires the fewest changes, in part because of reduced requirements for alterations in photosystem accumulation between mesophyll and bundle sheath cells [11]. An overview of statistics from these studies (Table 1) shows that as sequencing depths have increased there has been an increase in the predicted number of differentially expressed genes, likely due to better quantification of low abundance transcripts. However, as no annotated genomes were available for these species, the data are based either on cross-species mapping of reads, or gene models created by *de novo* transcriptome assembly [21–23]. Both of these approaches introduce inaccuracy compared with direct read mapping to a well-annotated genome. It is important to note that the absolute number of differentially expressed genes detected through congeneric comparisons

is clearly dependent on the phylogenetic distance, statistical cut-offs, quality of transcriptome assemblies and number of species sampled (Table 1). As the number of independent C_4 lineages that are assessed with RNA-seq increase, estimates of the conserved alterations to mRNA abundance will become more reliable. However, it is clear from the current estimates which range from hundreds to thousands of genes showing differential expression in C_4 compared with C_3 leaves, research needs to focus on identification of key transcription factors and signalling events that underlie these patterns of gene expression.

Compartmentation of gene expression between cell-types of the C_4 leaf

As with analysis of any organ or tissue, the C_4 leaf is composed of multiple distinct cell types, and the specialisation of M and BS cells in C_4 leaves (Figure 1) is considered a hallmark of the C_4 pathway. The first publications on global mRNA populations of M and BS cells of C_4 leaves were conducted on maize and supported existing knowledge of genes known to be differentially expressed between these cell types [24,25]. Analysis of two independent C_4 lineages from the grasses indicated that the absolute abundance of mRNAs in M and BS cells of grasses that evolved C_4 photosynthesis independently was statistically more convergent than other differentially expressed genes [26]. This implies that strong selection pressures acted on genes associated with the C_4 pathway to generate very similar expression in separate C_4 lineages. As the M and BS transcriptomes of more C_4 species become available this quantitative convergence could be used to generate a predictive framework that allows unknown components of C_4 photosynthesis to be identified. Although it has long been clear that transcriptional, post-transcriptional and post-translational processes all play a part in generating the C_4 metabolic system [9], omics approaches are now initiating non-biased and systems level quantification of their importance. For example, quantitative proteomics and transcriptomics indicated that the ratio of each cognate protein to its mRNA varies during C_4 leaf development, and that the ratio is often highest where protein function is most relevant [27]. Taken together, these findings start to provide an oversight of the extent of post-transcriptional and post-translational regulation in the C_4 leaf.

Transcriptomic datasets derived from M and BS cells of C_4 leaves highlight an area of ignorance, namely the mRNA populations associated with these two cell types in leaves of ancestral C_3 plants. Without this information it has not been possible to define how much patterns of gene expression have altered in M and BS cells of C_4 compared with those cells in C_3 leaves. A major hurdle was our inability to isolate M and BS cells from C_3 leaves, however immunopurification of ribosomes from specific cell types [28] has initiated our understanding of the BS in C_3 *Arabidopsis thaliana*. Although it was previously known that veinal cells of C_3 plants possessed characteristics of C_4 photosynthesis [30,31], ribosome tagging and deep sequencing of associated mRNAs indicated that components of the C_4 cycle are also preferentially expressed in the C_3 BS [29]. This work also highlighted a role for the C_3 BS in sulphur metabolism, a characteristic that had previously been reported of the C_4 BS [32]. Thus, as more C_3 lineages are sampled, we will develop a much clearer understanding of the extent to which metabolic characteristics currently associated with C_4

photosynthesis are actually ancestral and present in either M or BS cells of C_3 leaves. We therefore conclude that technologies are in place to significantly improve our understanding of M and BS cells in both C_3 and C_4 plants. Data from these approaches are being used to formulate models that relate to the molecular drivers associated with the repeated evolution of this complex trait, and it is this that will be explored in the next section.

Insights into the molecular drivers of C_4 metabolism

It has been clear for some time that prior to their recruitment into C_4 photosynthesis, the major proteins of C_4 photosynthesis typically accumulate at relatively low levels in a constitutive manner in C_3 leaves [33]. Through comparison with a gene expression atlas of closely related species, it is now proposed that expression of orthologues to C_4 genes show a variety of expression patterns, and peak in various tissues, in the C_3 ancestral system [34]. Deep sequencing data has also now provided the insight into the extent to which genes of the C_4 cycle become co-regulated with photosynthesis genes in leaves of both C_4 monotyledons and dicotyledons [23,35]. Overall, these data imply that during the evolution of C_4 photosynthesis, genes of the C_4 cycle are co-opted into the gene regulatory networks that govern photosynthesis gene expression in the ancestral C_3 state [23,34].

The identification of transcription factors responsible for these alterations in expression of genes encoding components of the C_4 cycle is an area where significant progress still needs to be made. However, comparative transcriptomics has now identified candidate regulators for the C_4 cycle in maize [24,25,35–37], *Setaria* [26,38], *Flaveria* [13] and *Gynandropsis gynandra* (formerly known as *Cleome gynandra*) [23,34]. Interestingly, independent lineages of C_4 plants appear to have up-regulated homologous transcriptional regulators in either M or BS cells. This has been reported for two independent lineages of C_4 grasses [26] but also for the C_4 dicotyledon *G. gynandropsis* and the C_4 monocotyledon maize [23]. These data indicated that M or BS preferential expression is not only associated with parallel evolution of regulatory DNA [39] and histone marks [40], but also the recruitment of transcription factors [23,26].

Another striking finding facilitated by deep sequencing has been quantification of the extent to which specific members of multi-gene families are recruited into the C_4 pathway. This was initially reported after phylogenetic reconstructions of individual genes such as *PEPC* [41], but the extent of this process was not clear. Transcriptomics has now quantified this phenomenon in *Alloteropsis*, which contains C_3 and C_4 subspecies [42]. In maize and *Setaria*, which represent two independent lineages of C_4 grass, 87% of C_4 cycle proteins that are up-regulated in C_4 leaves are syntenic orthologues, indicating that the same ancestral gene has repeatedly been recruited into the pathway [26]. Again, the mechanism behind this phenomenon is not clear, but it is possible that these orthologues are repeatedly used into the C_4 pathway because they are part of pre-existing gene regulatory networks that are recruited into C_4 photosynthesis. These data further emphasize that the highly complex C_4 photosynthesis trait is underpinned by a mixture of both convergent and parallel evolution [39,42].

The combination of deep sequencing and metabolic flux modelling has demonstrated the power of an integrated approach, and lead to an enticing hypothesis concerning the repeated evolution of C_4 photosynthesis. Comparing C_3, C_3-C_4 and C_4 species in *Flaveria*, RNA-seq data coupled to metabolic modelling predicted that loss of the full photorespiratory pathway in the M cells of C_3 plants, which is the most common biochemical alteration thought to initiate C_4 evolution [2], leads to a nitrogen imbalance between M and BS cells [43] (Figure 2). The most parsimonious alterations to central metabolism that corrects this imbalance in the leaf is to induce, and compartment, the key components of the C_4 cycle into either M or BS cells (Figure 2). These data strongly imply that the metabolic remodelling during these early stages of C_4 evolution represent an evolutionary exaptation that was initially not related to photosynthetic efficiency *per se*. Thus, it now appears that metabolic and also morphological alterations to C_3 leaves were both unrelated to photosynthesis [5,42,44]. Later in the evolutionary process it is thought that each alteration to the C_4 cycle leads to a steady increase in photosynthetic performance [45], and this is then followed by evolutionary fine-tuning mediated by amino acid substitutions that modify allosteric regulation of these proteins for the C_4 leaf [46]. In the future, deep sequencing will also allow us to determine whether parallel changes to amino acids are associated with parallel or convergent evolution to the nucleotides encoding them. Moving ahead, perhaps a similar combined modelling, sequencing and hormone approach is required to make progress in understanding the molecular basis of Kranz anatomy.

Summary

The use of deep sequencing in C_4 research is in its infancy, and so far is mostly limited to RNA-seq. It is also true that the initial phase has identified many genes that could be important for C_4 photosynthesis, but for which functional analysis has not yet been undertaken. However, it is clear that use of deep sequencing has initiated an unbiased and objective study of C_4 photosynthesis in species that previously lacked any transcriptomic or genomic resources. As outlined above, deep sequencing and improved computational pipelines for data analyses have started to provide significant new insight. This includes defining core components of the C_4 cycle, identifying variations in C_4 metabolism both within and between species, and also providing inference into evolutionary mechanisms associated with the polyphyletic appearance of this highly complex system.

Acknowledgements

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325 **Figure Legends**

326 **Figure 1:** Schematics illustrating variation in leaf anatomy and C_4 biochemical cycles of C_4 leaves. **A.**
327 Diagrams representing transverse sections through a C_3 leaf, and four anatomical variations in Kranz
328 anatomy. Images are based on those reported by [47]. B. The three main cycles that have classically been
329 used to define the three biochemcial sub-types of C_4 photosynthesis. AlaAT = Alanine aminotransferase,
330 AspAT = Aspartate aminotransferase, CA= Carbonic anhydrase, PEPC = Phospho*enol*pyruvate carboxylase,
331 PEPCK = Phospho*enol*pyruvate carboxykinase, NADP-MDH = NADP-dependent malate dehydrogenase,
332 NADP-ME = NADP-dependent malic enzyme, NAD-ME = NAD-dependent malic enzyme, PPDK =
333 Pyruvate,orthophosphate dikinase, CBB = Calvin Benson Bassham cycle, Ala = alanine, Asp = aspartate, Mal
334 = malate, OAA = oxaloacetic acid, Pyr = Pyruvate, PEP = phospho*enol*pyruvate.

335

336 **Figure 2: Impacts of deep sequencing on understanding C_4 metabolism**. Representation of model
337 predicting initial events associated with the evolution of C_3-C_4 intermediacy (based on [43]). Loss of
338 photorespiration in the mesophyll cells would lead to lead to an imbalance in nitrogen metabolism
339 between mesophyll and bundle sheath cells, and accumulation of ammonia (yellow circle) in the bundle
340 sheath. Upregulation of a C_4-like pathway rebalances this nitrogen imbalance. The three panels represent
341 photorespiration (C_2 cycle) operating in both mesophyll and bundle sheath cells of a C_3 leaf (A), the C_2 cycle
342 being lost in the mesophyll cells of C_3-C_4 intermediate species, and the subsequent development of a C_4-like
343 cycle (B), and finally complete implementation of the C_4 cycle (C). Abbreviations as in Figure 1, as well as
344 Glu = glutamate, Gly= glycine, 2-OG = 2-oxoglutarate, Ser = serine. Dashed lines indicate low metabolic flux.
345 Red circles represent carbon atoms while yellow circles represent amine groups.

Figure in original format
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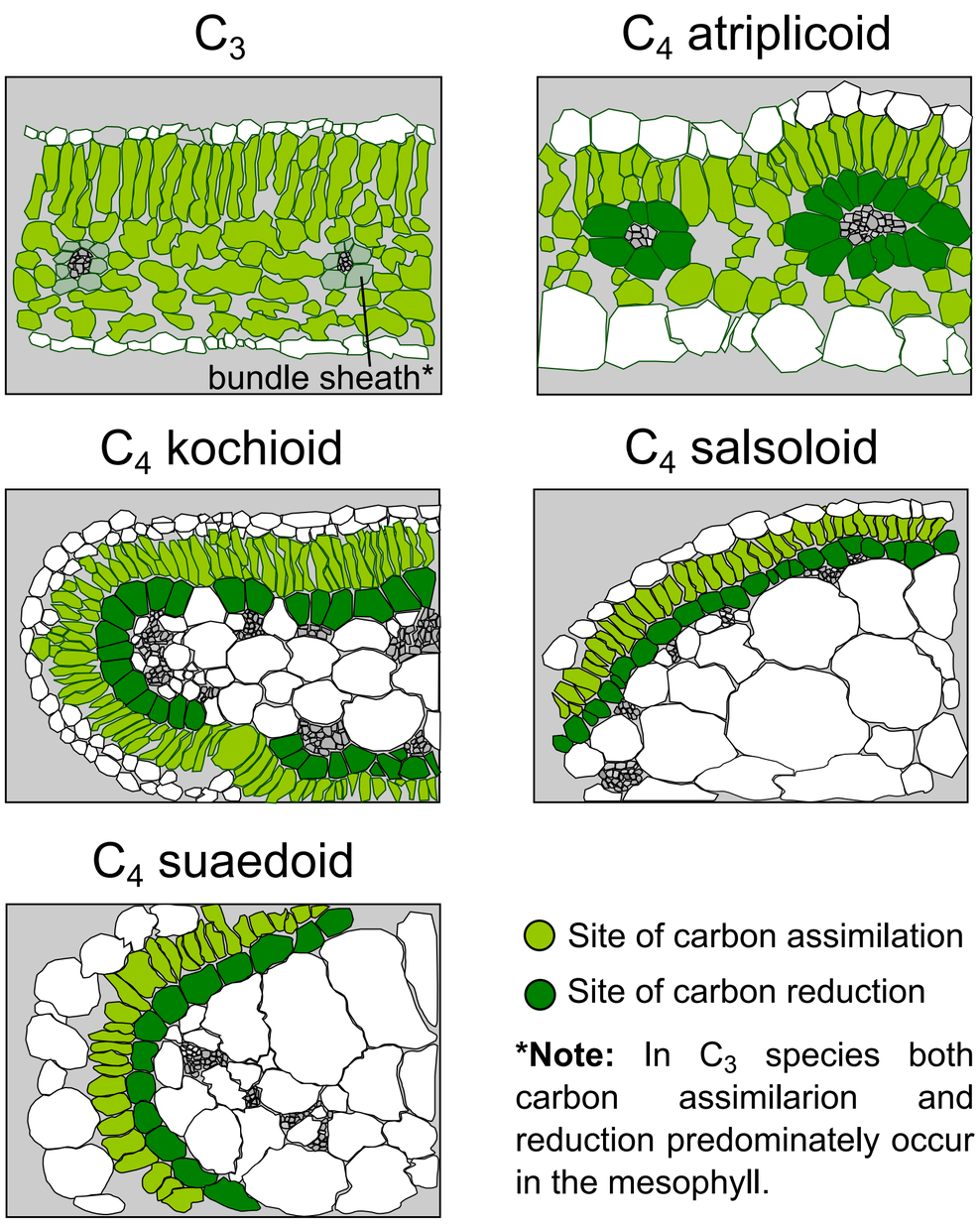
A



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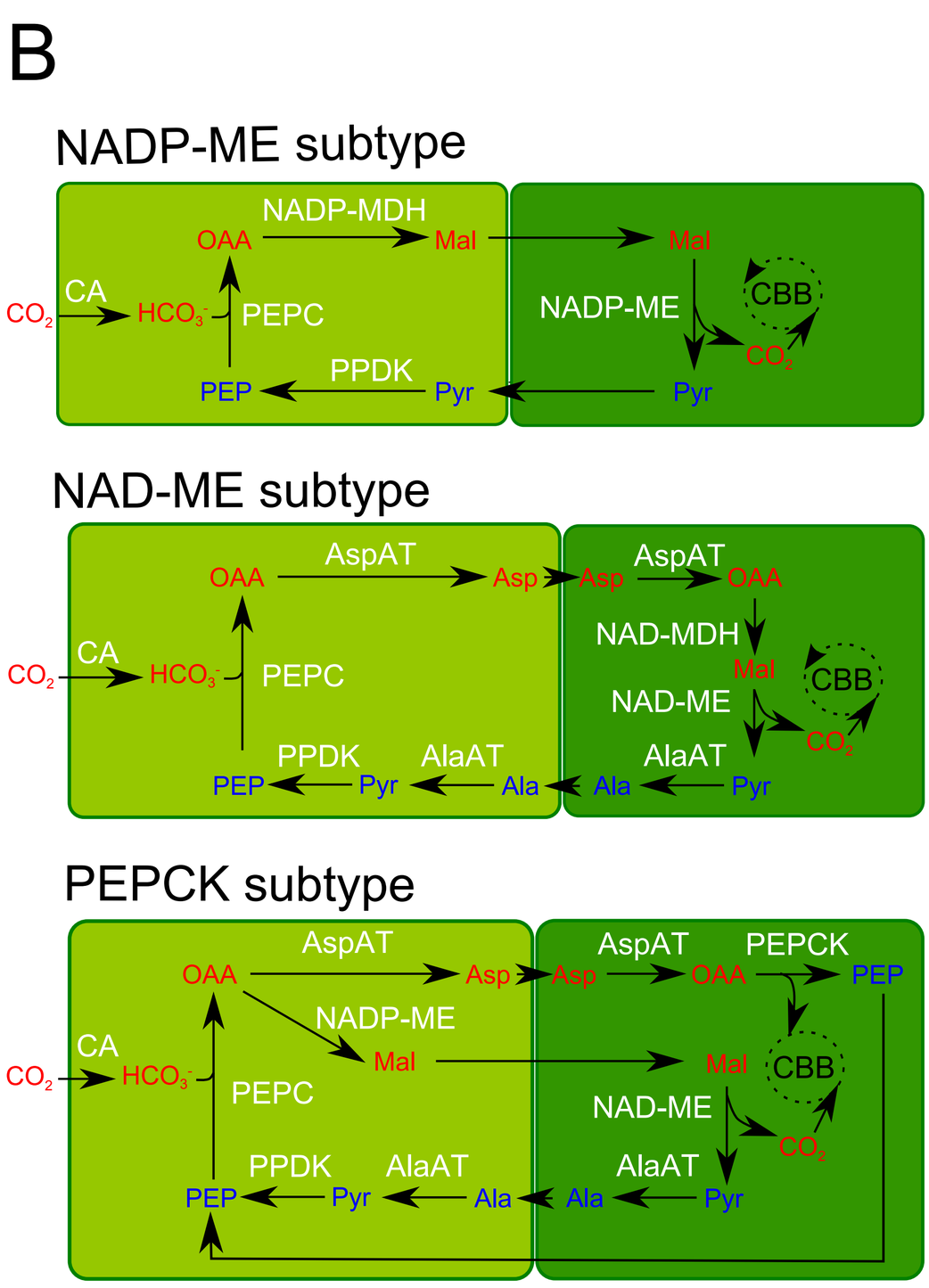

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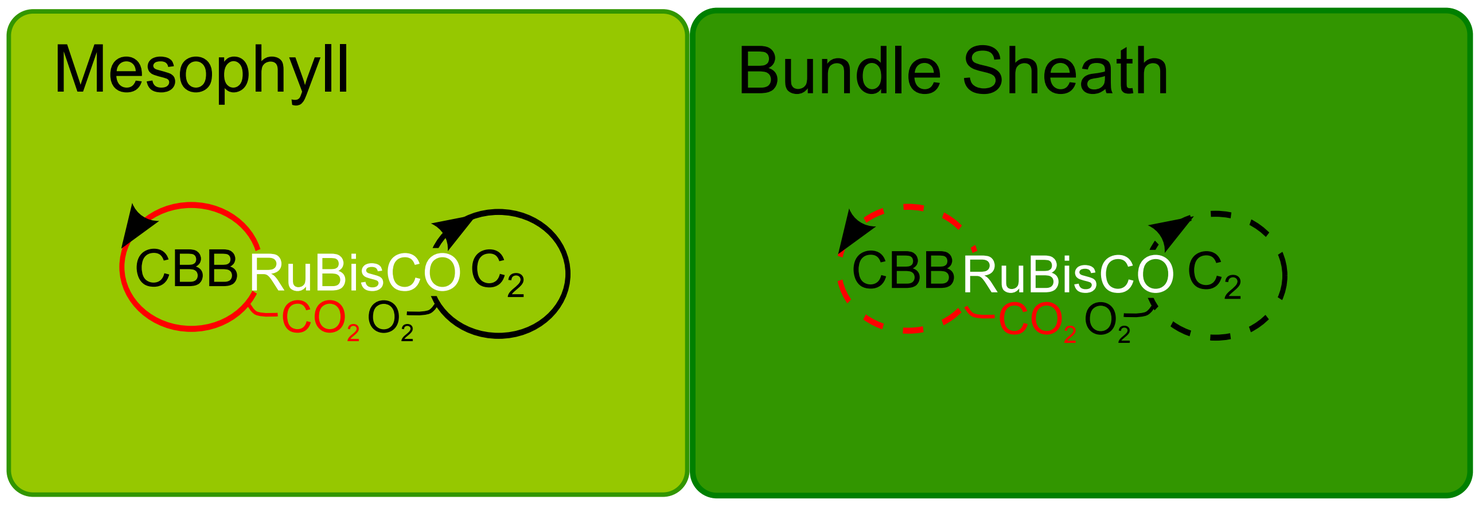
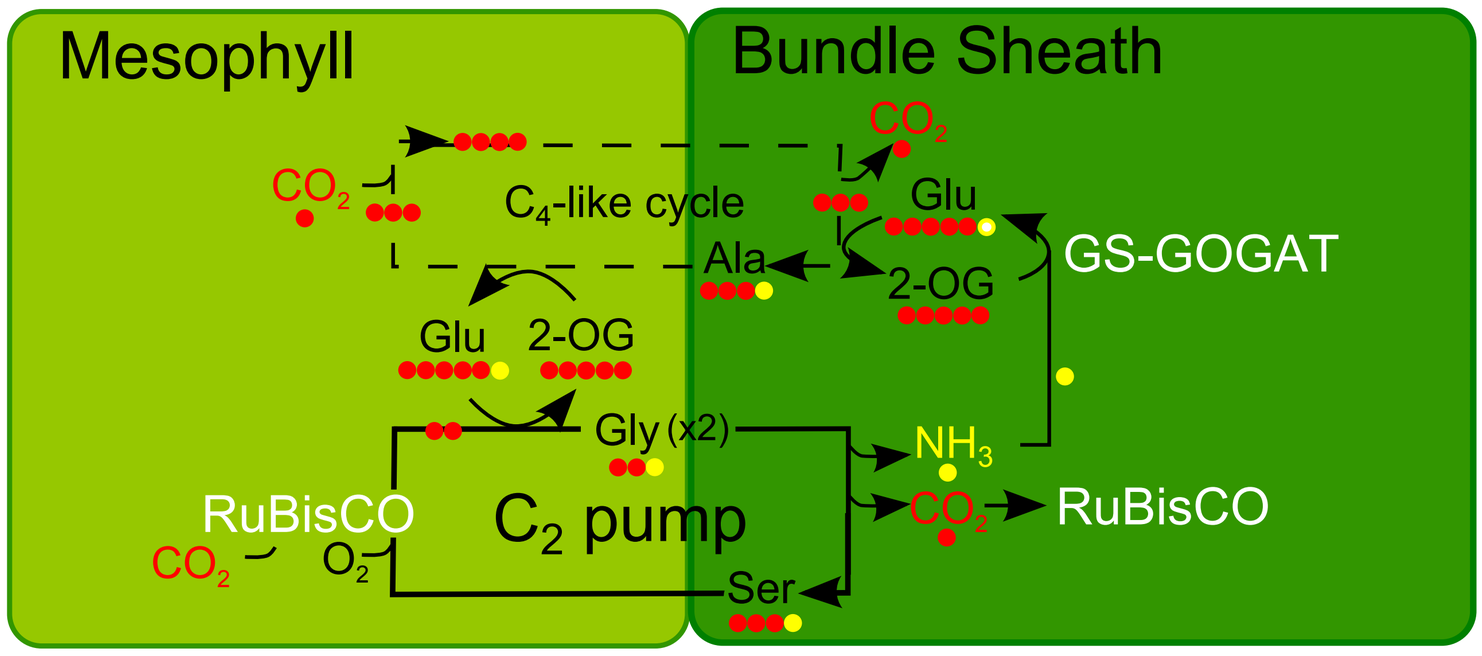
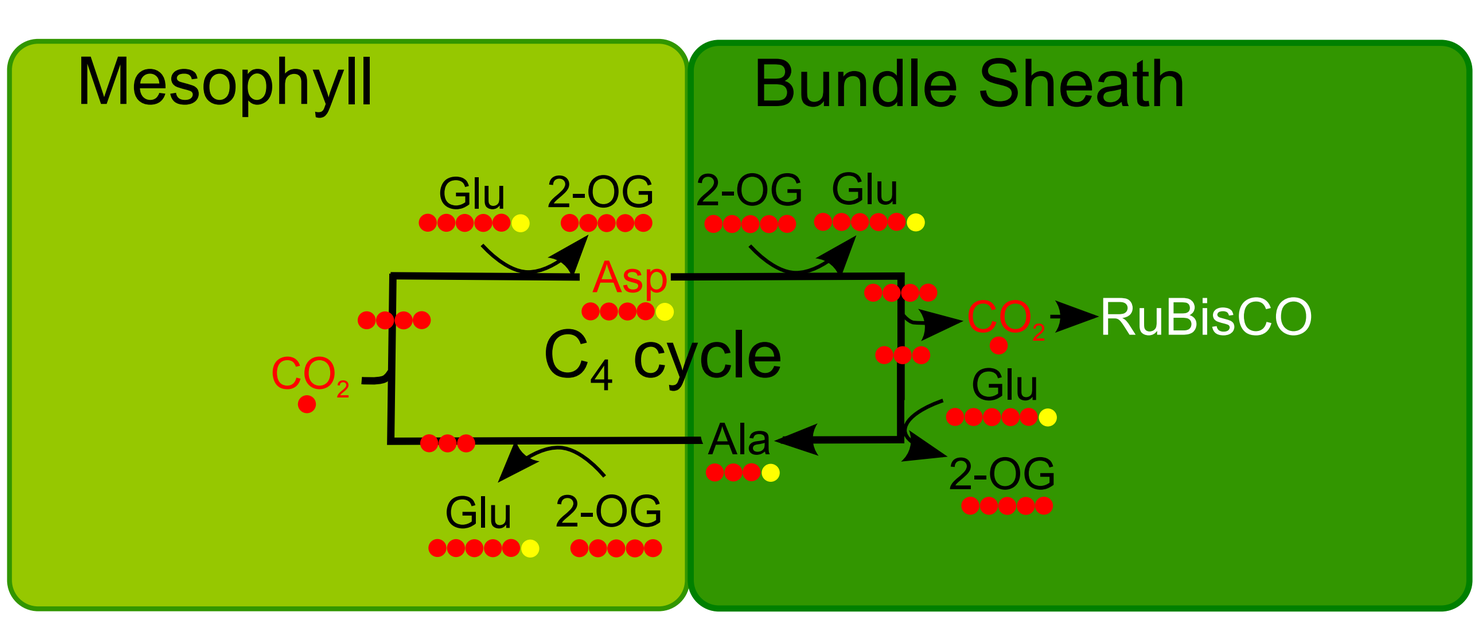


B



C



Table

	Bräutigam et al. (2011) [12]	Gowik et al. (2011) [13]	Bräutigam et al. (2014) [11]	Chen et al. (2014) [20]
Total number of DE transcripts	603	3582	1168	8848
Transcripts more abundant in C_3	258	1418	792	4184
Transcripts more abundant in C_4	345	2164	376	4664
% Transcriptome DE	1.4	NA*	6.1	13.5

Table 1: Comparisons of transcript abundance in closely related C_3 versus C_4 photosynthetic tissues. The total number of transcripts annotated as being differentially expressed (DE) in each study is listed, along with the numbers up or down regulated. Data expressed as percent of the total transcriptome are also reported for each study. Bräutigam *et al.* 2011 assessed C_4 *Gynandropsis gynandra* versus C_3 *Tareneya hassleriana.* Gowik *et al.* 2011 assessed C_4 *Flaveria bidentis* and *Flaveria trinervia* as well as C_3-C_4 *Flaveria ramosissima* and C_3 *Flaveria pringlei* and *Flaveria robusta.* Bräutigam *et al.* 2014 assessed *Panicum maximum* and *Dicanthelium clandestinum*. Chen *et al.* 2014 assessed C_4 and C_3 culms of *Eleocharis baldwinii.* *NA: the values for DE transcripts were based on multispecies comparisons which prohibits expressing the number of DE transcripts as a percentage of transcriptome.