Investigating the *cis*-Regulatory Basis of C3 and C4 Photosynthesis in Grasses at Single-Cell Resolution

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Abstract:

While considerable knowledge exists about the enzymes pivotal for C4 photosynthesis, much less is known about *cis*-regulation important for specifying their expression in distinct cell types. Here, we use single-cell-indexed ATAC-seq to identify cell-type-specific accessible chromatin regions (ACRs) associated with C4 enzymes for five different grass species. This study spans four C4 species, covering three distinct photosynthetic subtypes: *Zea mays* and *Sorghum bicolor* (NADP-ME), *Panicum miliaceum* (NAD-ME), *Urochloa fusca* (PEPCK), along with the C3 outgroup *Oryza sativa*. We studied the *cis*-regulatory landscape of enzymes essential across all C4 species and those unique to C4 subtypes, measuring cell-type-specific biases for C4 enzymes using chromatin accessibility data. Integrating these data with phylogenetic trees revealed diverse co-option of gene family members between species, showcasing the various paths of C4 evolution. We mapped cell-type-specific ACRs surrounding each C4 gene, discovering that, on average, these genes have two to three cell-type-specific ACRs located beyond the core promoter. These results provide a detailed genomic map of potential regulatory sequences operating on key C4 genes. Examining the evolutionary history of these cell-type-specific ACRs revealed a spectrum of conserved and novel ACRs, even among closely related species, indicating ongoing evolution of *cis*-regulation at these C4 loci. This work illuminates the dynamic and complex nature of CRE evolution in C4 photosynthesis, particularly highlighting the intricate *cis*-regulatory evolution of key loci. Our findings offer a valuable resource for future investigations, potentially aiding in the optimization of C3 crop performance under changing climatic conditions.

Introduction:

Photosynthesis is one of the most critical chemical reactions on the planet whereby CO2 is metabolized into glucose. Plants have evolved numerous variations of photosynthesis. The most common type of photosynthesis uses the enzyme ribulose 1,5-biphosphate carboxylase oxygenase (RuBisCO) in combination with CO2 to generate phosphoglyceric acid. This three-carbon compound is then used in a redox reaction within the Calvin Benson cycle, where glucose is made. The production of this three-carbon compound is what gives this type of photosynthesis, C3, its name. However, although widely evolved and found in many crop plants, C3 photosynthesis struggles to perform in hot and arid conditions. In non-ideal conditions, O2 can competitively bind the RuBisCO active site, causing the formation of a toxic intermediate, and reducing the efficiency of photosynthesis and plant performance (1). Due to increasing temperature caused by anthropogenic climate change, this reduction in photosynthetic capacity for key crop plants poses a major problem (2). However, other types of photosynthesis have evolved in hotter conditions and offer a model to potentially alter key C3 crop plants to be more efficient.

The C4 photosynthetic pathway is an example of a modified style of photosynthesis that is able to perform in hot conditions. In brief, C4 works by sequestering key photosynthetic enzymes into two different compartments in the leaf made up of different cell types. These two cell types/compartments are bundle sheath (BS) cells, which in C4 plants generally form a concentric ring around the vasculature, and mesophyll (MS) cells, which make up large portions of the non-vascularized leaf internal cells (3). In the MS, CO2 is imported, and converted to bicarbonate (HCO3-) by the enzyme carbonic anhydrase (CA). Bicarbonate is then converted to a four-carbon molecule oxaloacetate (OAA) by the O2-insensitive phosphoenolpyruvate carboxylase (PEPC). This OAA molecule made of a four-carbon compound (where C4 derives its name) is finally converted to a stable metabolite, malate. Malate is then transported to the BS where it undergoes a decarboxylation process, by one of three different types of decarboxylases, NAD-dependent malic enzyme (NAD-ME), NADP-dependent malic enzyme (NADP-ME), or phosphoenolpyruvate carboxykinase (PEPCK). This decarboxylation reaction releases a CO2 molecule that enters into the Calvin Benson cycle. The generation and processing of intermediate molecules in cellular compartments allows for concentrated levels of CO2 to interact with RuBisCO, reducing the inefficiencies mentioned above. Current C4 crops such as maize (*Zea mays*), sorghum (*Sorghum bicolor*), pearl millet (*Cenchrus americanus)*, and finger millet (*Setaria italica*) excel in their ability to operate in adverse conditions.

Although the evolution of C4 photosynthesis is a complex process, there is tantalizing evidence that engineering C3 crops to do C4 photosynthesis might be possible. One piece of evidence that points to this is that C4 photosynthesis has evolved independently 61 times in different lineages of plants (4). These results indicate that most plant lineages have the genetic material capable of evolving into C4 photosynthesizers. The *Poaceae* lineage of grassees exemplifies this, as C4 photosynthesis has evolved independently at least 18 times (5). Interestingly, all of these species use the same core C4 enzymes and steps, but many use different decarboxylation enzymes as mentioned above (6–8). Furthering this hypothesis is the fact that many C4 related genes originally evolved from either C3 photosynthetic genes or key enzymes critical in core metabolism (9,10). For instance, PEPC is a key metabolism enzyme in

the glycolytic pathways of the Krebs Cycle, with some copies being important in guard cell metabolism (11–13). Instead of novel gene content being the main driver of C4 photosynthesis, it's more likely due to the correct timing and compartmentalization of key enzymes into specific cell types. This raises the question, how is gene expression of these key C4 enzymes regulated? Moreover, as C4 has evolved multiple times convergently, have similar regulatory networks and paradigms been co-opted to alter when and where these key genes are expressed?

Cis-regulatory elements (CREs) are key players in gene regulation, as they both fine tune expression and provide cell-type specificity (14–17). In brief, these regions operate as binding sites for transcription factors that modulate molecular phenotypes. Previous work has shown that CREs could be key players in the transition to C4 photosynthesis. This was demonstrated by taking C4 genes from *Z. mays* and transforming them into *Oryza sativa,* a C3 species (18,19), which revealed that CREs from *Z. mays* genes were able to drive cell-type-specific expression in MS in *O. sativa* (18,19). Additional analyses have implicated CREs as drivers in the evolution of C4 photosynthesis. In the genus of plants *Flaveria,* which contains both C4 and C3 plants, one key difference in C4 plants was a specific CRE driving gene expression in MS cells. This 41 bp motif named *Mesophyll expression module 1* is critical for cell-type-specific expression of *PEPC* in MS cells, a critical first step in the C4 pathway (14,20). Finally, four conserved non-coding sequences were identified to be critical in MS-specific expression of *PEPC* in monocots (21). These findings show that CREs are important genetic elements that plants use for the evolution of C4 photosynthesis.

Although some CREs critical for cell-type-specific expression of key photosynthetic genes have been identified, they've been restricted to those nearby the transcriptional start sites. This is due, in part, to the challenge of identifying CREs genome wide, as well as limitations in the isolation of BS and MS cells which is labor intensive and challenging. However, a recent study used a multi-omic approach in *Z. mays* BS and MS cells and found CREs genome-wide that might be critical in the cell-type-specific regulation of genes (22). One example is the identification of a potential distal CRE upstream of *SULFATE TRANSPORTER4* (*ZmSFP4)*, a BS-specific sulfate transporter (22). These results highlight the complexity of *cis* regulation and the importance of identifying all CREs for a gene, not just those nearby the transcriptional start site. During the evolution of C4 photosynthesis, it's unclear whether these CREs have been pre-established during evolution and co-opted for C4 photosynthesis or if they evolved independently numerous times. Understanding the ways in which *cis* regulation evolves to control timing and cell-type-specific expression of C4 photosynthesis genes would greatly assist efforts in engineering C3 plants to be more C4 like.

To investigate the role of CREs and their potential contribution in controlling key C4 genes, we used single-cell indexed Assay for Transposase Accessible Chromatin sequencing (sciATAC-seq) to identify cell-type-specific CREs from five grass species representing diverse C4 subtypes, as well as an additional C3 outgroup. We investigated the cell-type specificity of both the core C4 enzymes, and those which are unique to each photosynthetic subtype. Further, we identify CREs of C4 genes, and find previously unknown cell-type-specific CREs that might be critical in C4 gene expression. We find that some of these regulatory regions appear not just conserved in a single C4 subtype, but in all of the C4 species we studied. Finally, we leverage

these data to find transcription factor binding motifs enriched in MS and BS cell types and use these motifs to catalog these regulatory loci.

Results:

Identification and Annotation of Cell Types in Diverse Species:

To investigate CREs in BS and MS cells potentially important in C4 photosynthesis, we generated replicated sciATAC-seq libraries for four different C4 species, comprising three different C4 subtypes NADP-ME (*Z. mays, S. bicolor*), NAD-ME (*Panicum miliaceum)*, and PEPCK (*Urochloa fusca),* and a C3 outlier species (*O. sativa*) (**Figure 1A**). Libraries were filtered for high-quality cells by first pseudo-bulking the sciATAC-seq libraries, and identifying accessible chromatin regions (ACRs). Using these ACRs, per nuclei quality metrics were then calculated such as Fragment of Reads in Peaks, transcriptional start site enrichment, and total integration events per nucleus **(Methods)**. Nuclei found to have a high proportion of organellar reads were also removed, with values being adjusted on a per library basis **(Methods)**. Clustering of cells was done on genomic bins, and with additional cells removed that had a high correlation with *in-silico* generated doublets, and clusters were removed that were skewed towards one replicate by greater than 75% **(Methods).** After filtering on per nucleus quality metrics, we identified 16,060 nuclei in *Z. mays*, 15,301 nuclei in *S. bicolor*, 7,081 nuclei in *P. miliaceum*, 19,110 nuclei in *U. fusca,* and 5,952 nuclei in *O. sativa* (**Supplemental Figure 1-5**).

Due to variation in genome size and content, cell-type annotation for each dataset was done independently using each species reference genomes (**Figure 1B**). We used multiple approaches to annotate cell types. Orthologs of key marker genes from *Z. mays* and *O. sativa* were identified using a phylogenetics based approach **(Methods)**. This allowed for the identification of marker genes for specific cell types in a cross species context. To gauge gene activity of these marker genes, gene body chromatin accessibility was used as a proxy for expression (**Figure 1D**) (16,23). Cell-type annotation was done manually taking into consideration marker gene chromatin accessibility, marker enrichment in clusters, as well as ontological relationships between cell types. Due to the lack of marker genes for many cell types in plants, as well as the challenge of annotating a broad sample of species, we reduced resolution of our annotation across our datasets to ensure accurate comparisons between variable species (**Figure 1B**).

Deeper exploration of the list of marker genes from *Z. mays* showed conservation of gene body chromatin accessibility in markers for certain cell types. As expected, for the C4 plants, *RIBULOSE BISPHOSPHATE CARBOXYLASE SMALL SUBUNIT1 (SSU1) and RIBULOSE BISPHOSPHATE CARBOXYLASE SMALL SUBUNIT2* (*SSU2*) were enriched in BS cells compared to MS cells **(Figure 1C)**, a pattern that was not found in *O. sativa.* As expected, *PEPC1* showed MS-specific chromatin accessibility in all of the C4 species sampled **(Figure 1D)**. Additionally, we found conservation of marker genes like *SUCROSE TRANSPORTER 1* (*SUT1*) in companion cells and sieve elements, and *GLOSSY1* (*GL1*) in epidermis cells, indicating that these historically described marker genes are likely important in this diverse set of species. This analysis provides a first examination of marker genes' chromatin accessibility across a diverse sample of plant species at cell-type resolution.

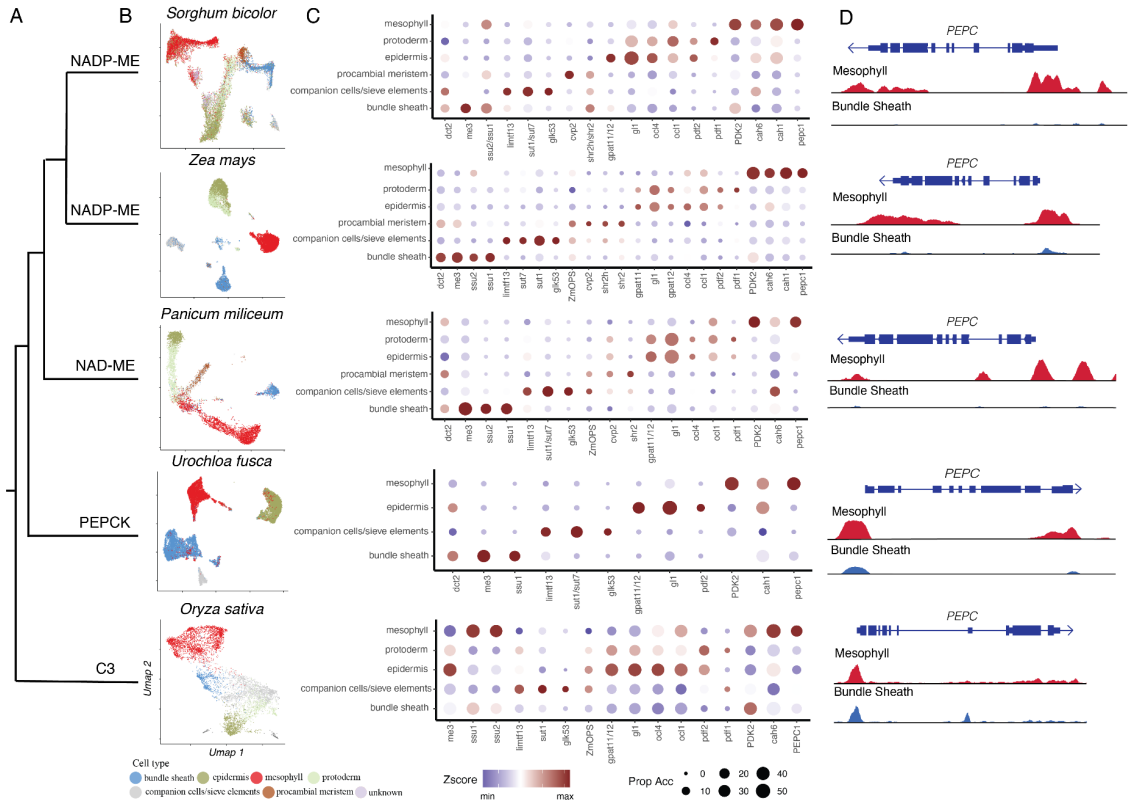

Figure 1: Annotation of cell types in diverse grass species at single-cell resolution **A)** A phylogeny indicating the relationship of various C3 and C4 photosynthesizers sampled. In this sample, two NADP-ME subtypes are represented, one NAD-ME subtype, a PEPCK subtype, as well as a C3 species. **B)** UMAP embedding showing the annotation for each species. A cell type legend is below. **C)** Dotplots for various marker genes used to annotate each species. The y-axis represents cell types, and the x-axis is a list marker genes used to annotate different cell types. The size of each circle is proportional to the number of cells within that cell type that showed chromatin accessibility of the marker. Color is z-score transformed values across clusters of gene chromatin accessibility across the clusters. **D)** Screenshots of the *PEPC* locus for all sampled species. For each screenshot, the top track shows the protein coding, the red track is chromatin accessibility of MS cells, and the blue track is the chromatin accessibility of the BS cells.

Chromatin Accessibility of Core C4 Enzymes Shows Similar Cell-Type Bias, but Differing Evolutionary Origins:

We measured the chromatin accessibility bias of the C4-associated enzymes. Due to the diverse nature of the plants sampled, and the C4 photosynthetic subtypes, we separated

enzymes into core- and subtype-specific groups. This list comprised nine core C4 enzymes, and nine variable enzymes. These enzymes were assigned to one of these two groups based on if they are found in all C4 subtypes (core) or are specific to only one or two subtypes (variable). One example of a core enzyme is carbonic anhydrase, which is used to generate bicarbonate from CO2, as well as for the regeneration of phosphoenolpyruvate from oxaloacetate in the BS cells by means of PEPCK (**Figure 2A).** The list of gene families that we considered as core or variable is found in (**Supplementary Table 1**).

To investigate the cell-type bias of these enzymes, we used chromatin accessibility of the gene (gene body as well as 500 bp upstream of the transcriptional start site) (**Figure 2B)**. Cell-type bias was calculated as the log2 fold change of BS/MS chromatin accessibility. To identify core C4 enzymes across these species, we used Orthofinder, and named and numbered the enzyme models based off of their relatedness to *Z. mays* copies of known core C4 genes (24). Using only cell-type-specific chromatin accessibility data, we observed expected cell-type bias with many of the MS-specific core C4 genes showing MS-specific bias, as compared to BS (**Figure 2C)**. For instance PEPCK, which regenerates PEP from OAA in BS cells, always showed a BS-specific bias (**Figure 2 A and C)**. Additionally PEPC, which conconverts bicarbonate to OAA in MS cells, showed MS-specific bias for all species sampled, except the C3 outgroup *O. sativa* (**Figure 2 A and C)**. These results highlight the quality of the data and the cell-type annotations for these single-cell datasets.

When analyzing these data in tandem with the phylogenetic trees, we noticed that some of the key enzymes showed different cell-type specificity based on their evolutionary origin **(Supplementary 6A)**. For instance, for carbonic anhydrase in *P. miliaceum,* the orthologs that showed the largest bias between MS and BS cell types were not the copies that were the most evolutionary closely related to the *Z. mays* and *S. bicolor* cell-type-specific copies (Here *PmCA1* and *PmCA2*). Rather, a copy found in a separate clade (*PmCA3*) showed the most MS-specific bias (**Figure 2C)**. This indicates that during the evolution of C4, different sets of carbonic anhydrases were likely co-opted for C4. One challenge using chromatin accessibility in this context, however, is the fact that neighboring gene models can occlude cell-type-specific signals. For instance, in the *S. bicolor* copy of *SSU1* (here *RBCS 1)*, a BS-specific gene has a neighboring gene model directly upstream which shares a promoter region making measurement of the cell-type-specific bias of some loci challenging, potentially skewing signals **(Supplementary 6B)**.

One unexpected result from this analysis was the lack of cell-type-specific bias for *MALATE PHOSPHATE ANTIPORT 1 (DIC1),* also known as *DICARBOXYLATE/TRICARBOXYLATE TRANSPORTER 2* (*DCT2*) in *Z. mays*. It has been previously reported that *DIC1* had BS-specific expression bias in *Z. mays* as well as in *P. miliaceum* (25–27). However, there is not a clear signal based on the chromatin accessibility data. This could indicate that some ACRs harbor multiple CREs active in different cell types that are not obvious in chromatin accessibility data or that the cell-type-specificity observed is not due to *cis*-regulation, possibly involving post-transcriptional processes (**Figure 2C)**. Lastly, as expected, there was very little bias in the C3 outgroup (*O. sativa*). In total, 12/13 of the core C4 enzymes showed cell-type-specific bias in *Z. mays*, 7/12 in *S. bicolor*, 16/21 in *P. miliaceum* , 11/13 in *U. fusca*, and finally 0/16 in *O. sativa*. These data demonstrate that

chromatin-accessibility data can be leveraged to investigate the cell-type regulation of C4 genes while also taking into consideration their evolutionary relationships in a cross species context.

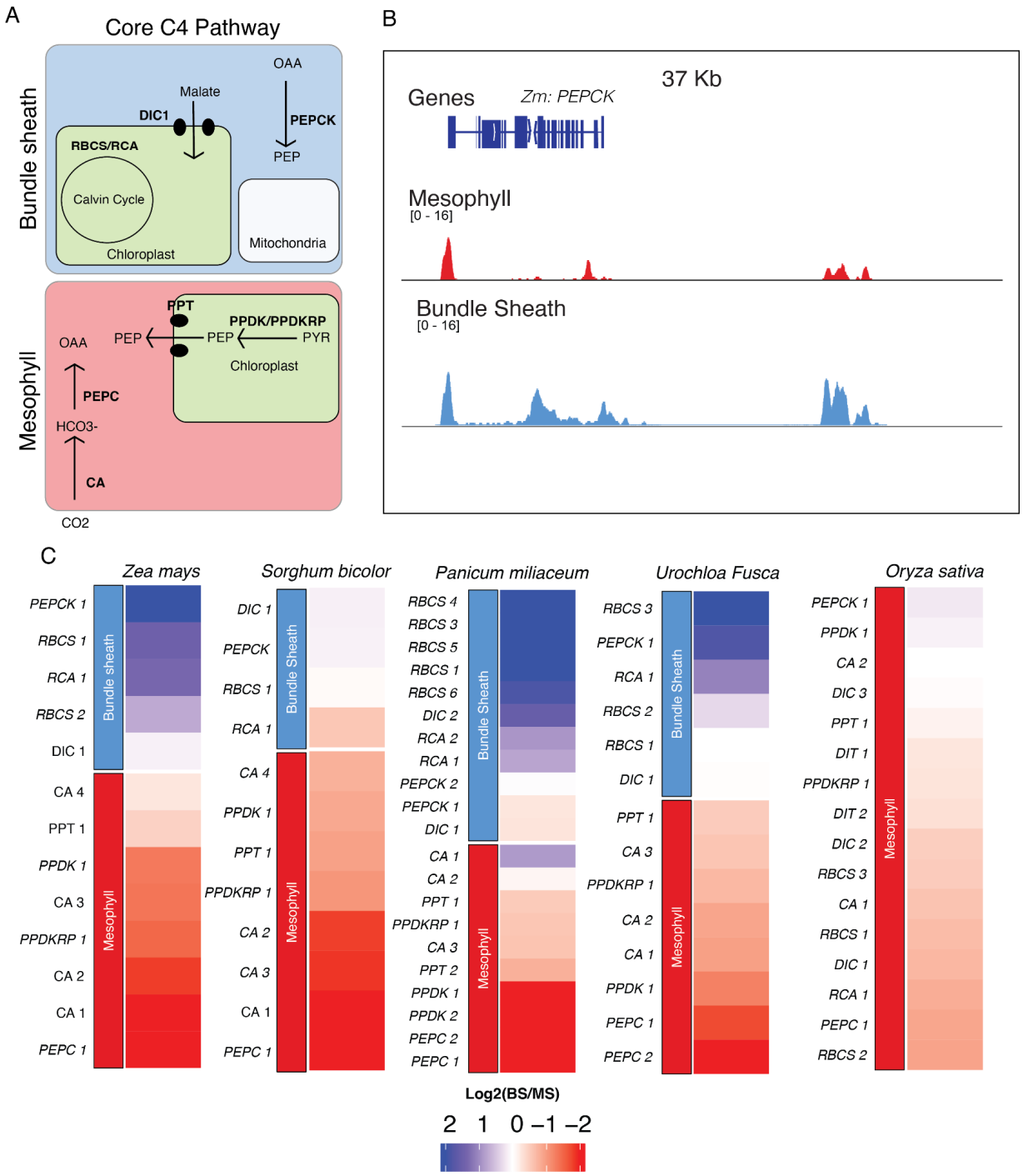

Figure 2: Cell-type chromatin-accessibility bias for core enzymes in C4 and C3 species. **A**) Schematic of the core C4 enzymatic pathway. The red and blue squares represent MS and BS cells, respectively. Enzymes are labeled in bold, and transporters are denoted by shapes. Intermediate molecules are indicated by non-bolded text. **B)** Screenshot of *PEPCK* in *Z. mays*. Blue tracks correspond to BS chromatin accessibility and red tracks show MS chromatin accessibility. Tracks are equally scaled to facilitate comparison. **C)** Heatmaps of chromatin accessibility bias of the core C4 enzymes. Values within each heatmap correspond to

Log2(BS/MS). Blue indicates increased BS chromatin accessibility and red indicates increased MS chromatin accessibility. Each species column and subtype was clustered independently, and genes were assigned as being MS- or BS-specific (top/bottom of heatmap) based on literature. Enzyme copies were distinguished phylogenetically.

Key C4 Subtype Enzymes Show Potential Convergent Evolution in Cell-type-specific Bias:

We investigated the variable enzymes that give each subtype of C4 its unique properties by focusing on two species (*S. bicolor* and *Z. mays*) from the *NADP-ME* subtype **(Figure 3A)**. As expected, chromatin accessibility bias was observed for enzymes previously reported as having cell-type-specific expression patterns, similarly to the core C4 enzyme set (22,28). Reassuringly, one of the most biased enzymes identified was *NADP-ME*, the key enzyme of the redox step in *NADP-ME* subtypes. More specifically, of the multiple copies of *NADP-ME* that exist in *Z. mays,* we observed the expected cell-type bias for the known BS-specific copy, *ME3,* a key factor in C4 (here *NADP-ME1* in *Z. mays*) **(Figure 3B)**. We noticed in *S. bicolor*, the BS-specific *NADP-ME* and the MS-specific *NADP-malate dehydrogenase* (*NADP-MDH*) gene copies are recent tandem duplications, each maintaining their respective cell-type specific chromatin accessibility (**Figure 3B and C**, **Supplemental 7**). The malate transporters *DICARBOXYLIC ACID TRANSPORTER1/2 (DIT1/2)* also demonstrated their expected cell-type-specific bias with *DIT1* being MS specific and *DIT2* being BS specific in both species (**Figure 3B and C)**. However, upon further inspection of the phylogenies of the *DITs* in *S. bicolor,* we noticed a pattern where the most BS-biased copy, *Sb DIT2 (*Sobic.004G035500*)*, was phylogenetically more closely related to the *Z. mays DIT1*. These results indicate that over evolutionary time, even members of the same C4 photosynthetic subtype, which likely share a C4 ancestor, use different loci to achieve cell-type-specific expression. This highlights that C4 evolution is an ongoing process.

NAD-ME subtypes in *P. miliaceum* are interesting, as the intermediate molecule being passed between MS and BS doesn't take the form of malate, but instead aspartate, alanine, and oxaloacetate (**Figure 3D)**. The key redox enzymes, NAD-ME and the NAD-Dependent malate dehydrogenase (NAD-MDH), all show BS-biased chromatin accessibility (**Figure 3E and F)**. Interestingly, of the three copies of *NAD-MDH* analyzed, only two showed bias for BS. Next, we evaluated the key enzymes associated with the generation of critical intermediate metabolites, Aspartate aminotransferase (AspAT), as well as Alanine aminotransferase (AlaAT). Specific copies of AspAT have cell-type-specific expression patterns, with the MS-specific copy being transported to the cytosol and the BS-specific copy being transported to the mitochondria (**Figure 3E and F)** (29–31)**.** Of the four copies of AspAT present, two showed significant MS-specific bias, whereas the other two copies didn't show significant deviation towards BS (**Figure 3E)**. This possibly indicates differing levels of regulation for the AspAT copies that did not show the expected BS bias, or missing copies of AspAT that we have not investigated. Within AlaAT, however, we identified two copies showing MS-specific bias, and one copy showing BS-specific bias. Additionally, somewhat unexpectedly is that we didn't observe bias for sodium bile acid symporters (*BASS*) and sodium:hydrogen antiporters (*NHD*), which are important in the transport of pyruvate into the chloroplast of MS cells (**Figure 3E).** Although two copies of the *BASS* genes were MS biased, only a single copy of *NHD* was slightly MS biased.

However, we do observe slight cell-type-specific chromatin accessibility bias for *DIT1/DIT2*. This is somewhat surprising, as *DIT1/DIT2* haven't been implicated in transport found in NAD-MD subtypes. This highlights the flexible nature of *P. miliaceum* in terms of its C4 photosynthetic style, as it has been implicated as being a flexible C4 subtype, able to do some of the metabolite shuttling by NADP-MEs (8,32,33). This is further supported by the chromatin accessibility patterns of *NADP-MDH* copies found in *P. miliaceum.* The potential flexibility of *P. miliaceum* in its style of C4 makes it an extremely interesting species to study, especially when considering that it doesn't share common C4 ancestry with *Z. mays* or *S. bicolor.* This lack of evolutionary relationship makes the analysis of the more distantly related species *U. fusca* all the more valuable. This set of observations point to the complicated nature of some of these C4 photosynthetic subtypes. While the obvious subtype-specific enzymes show expected chromatin-accessibility bias, others do not.

Using the PEPCK subtype in *U. fusca,* we evaluated cell-type bias of enzymes that operate as an intermediate between NAD-ME and NADP-ME subtypes (**Figure 3G).** Copies of *NAD-ME* and *PEPCK* showed significant BS bias (**Figure 3H and I)**. Additionally, *NADP-MDH* was significantly biased towards MS, reflecting its critical role in the regeneration of malate from pyruvate. We also observed one copy of *BASS,* which was heavily MS biased, as well as a copy of the sole copy of *NHD* being highly MS biased (**Figure 3G)** (34). Within the *BASS* family, based on the phylogenies, it appears one clade of *BASS* genes was co-opted to be MS specific, whereas the other clade remained somewhat BS specific. This potentially indicates that this clade that has been co-opted was potentially poised for co-option into the C4 cycle at the last shared ancestor of *P. miliaceum* and *U. fusca.* Additionally, we also find one MS-biased and one BS-biased version of AlaAT; something that has been previously hypothesized based on biochemical information, but was unknown (35). Finally, when evaluating genes in the C3 outgroup *O. sativa,* we only observed significant chromatin accessibility bias for three of the 14 enzymes. This is expected given the overall lack of enzymatic bias seen in C3 species (**Figure 3K)**. Interestingly though, we did find a single instance where one copy of AspAT is BS specific, suggesting that this copy of AspAT might slowly be co-opted into being more BS specific than MS (**Figure 3K)**. Even more interesting is the slight BS-specific bias of NADMDH, a BS-specific enzyme in the NAD-ME subtypes. These results show a series of complex evolutionary relationships where many different genes can be co-opted into the C4 pathway, and highlights the myriad ways in which C4 evolution occurs.

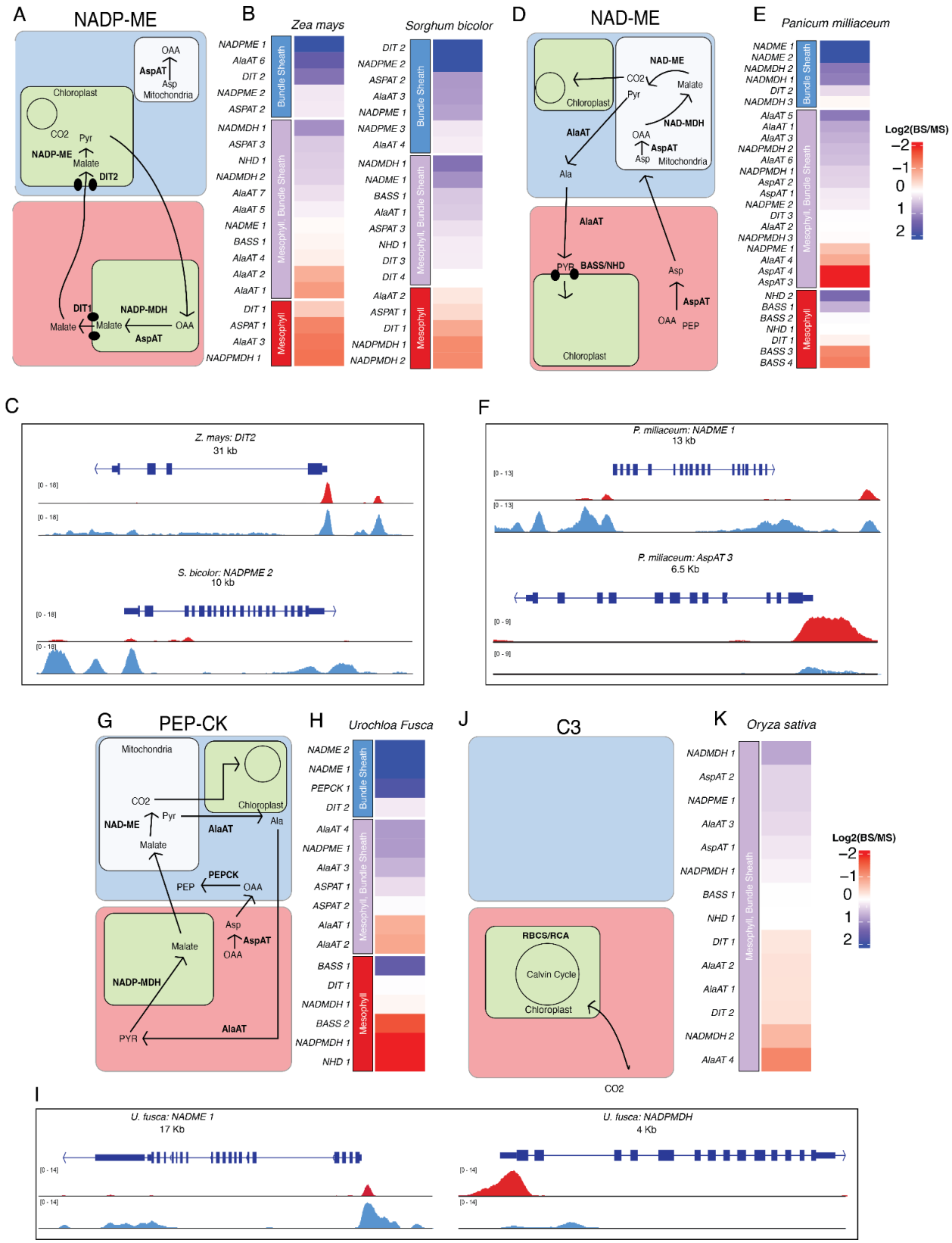

Figure 3: Cell-type chromatin accessibility bias for variable C4 genes associated with C4 subtypes. **A/D/G/J)** Schematic of C4 enzymatic pathways for various C4 subtypes. The red and

blue squares represent MS and BS cells. Enzymes are labeled in bold, and transporters are denoted by shapes. Intermediate molecules are indicated by non-bolded text. For clarity, core enzymes have been removed. **B/E/H/K)** Heatmaps of chromatin accessibility bias in C4 subtype enzymes. Values within the heatmap correspond to Log2(BS/MS). Blue indicates increased BS-chromatin accessibility and red indicates increased MS-chromatin accessibility. Genes were labeled as being BS specific (blue) BS/MS specific (purple) or MS specific (red) based on previous literature. **C/F/I)** Screenshot of various C4 sub-type enzymes and their chromatin accessibility profiles around the TSS. Blue tracks correspond to BS chromatin accessibility and red tracks show MS chromatin accessibility. Tracks are equally scaled to facilitate comparison.

Cell-type-specific Accessible Chromatin Regions of Both Core- and Subtype-Specific Enzymes:

Although measuring the gene body chromatin accessibility of C4 enzymes is valuable, it doesn't inform us about the cell-type-specific *cis*-regulatory environment controlling these genes, as we only included 500 bp upstream in this initial analysis. To identify all potential CREs important for regulation of these genes, we identified cell-type-specific ACRs using a modified entropy metric **(Methods).** In short, cell-type-specific ACRs are those which are unique to either a single cell-type or two or three cell-types. This is in contrast to broadly accessible ACRs which are accessible in many different cell-types. For each C4 enzyme, in both the core and the non-core set, we identified ACRs around them. We only considered ACRs to be potential regulators of a locus based on distance, with assigned ACRs needing to be less than 200 kb away from the target enzyme, and requiring that no other gene intervenes between the ACR and enzyme in question. In total, across all variable and core enzymes and taking into consideration only C4 species, we find that on average, gene models have between 2-3 cell-type-specific ACRs, with an additional 2-3 broadly-accessible ACRs **(Figure 4A)**.

For all of the C4 subtypes, the key redox enzymes all showed BS cell-type-specific ACRs, potentially identifying critical CREs for proper cell-type-specific expression. For instance, in *Z. mays, NADP-ME1* had five BS-specific ACRs, in *S. bicolor, NADP-ME2* had five BS-specific ACRs, in *P. miliaceum, NAD-ME1* had four BS-specific ACRs, and in *U. fusca, PEPCK,* had three BS-specific ACRs **(Figure 4 A and C)**. Additionally, of the MS-specific enzymes, we consistently observed numerous cell-type-specific ACRs around the carbonic anhydrase family. On average, there were 3.5 MS-specific ACRs for each copy of carbonic anhydrase across all of the species. This likely reflects the fact that carbonic anhydrase is critical in the initial steps of C4, and also important in CO2 sensing (36). We also noticed an intriguing pattern where enzymes expressed in one cell type had cell-type-specific ACRs of the other cell type. For instance, around *SSU2,* a BS-specific enzyme, we found a series of MS-specific ACRs **(Figure 4D)**. On average, we found 2.5 BS-specific ACRs around *SSU* and 1.5 MS-specific ACRs. This contrasting pattern was observed in key photosynthetic enzymes in all of the C4 subtypes. This likely indicates that some of these ACRs contain CREs that negatively regulate *SSU* in MS, as cell-type-specific CRE usage has been implicated as being an important driver in proper compartmentalization (37,38). The identification of ACRs around key C4 enzymes provides a detailed map about potential *cis*-regulators of these loci, which

provides the basis for future investigation into the direct function of each of these ACRs and how they might be altering transcription in multiple different ways. These results show that there are likely multiple ACRs important to cell-type specificity of these enzymes.

Traditionally, the field has focused on *cis*-regulation within a set distance from the transcriptional start site, often 1-2 kb, which is thought to generally encompass the promoter (39). However, we observed abundant distal cell-type-specific ACRs for many of these key genes **(Figure 4B)**. For instance, the average distance of an ACR to its C4 enzyme is 10,080 bp (*Z. mays*), 3,017 bp (*S.bicolor*), 4,260 bp (*P. miliaceum*), 2,358 bp (*U. fusca*), and 4,730 bp (*O. sativa*). Indicating that the *cis*-regulatory space for these enzymes is far greater than previously analyzed, where a majority of the focus in the literature is on putative promoters. The genome of *Z. mays* emphasizes this point, as the subtype-specific enzyme *NADP-ME* has three cell-type-specific BS ACRs distal to the transcriptional start site, with the furthest being 34,336 bp away **(Figure 4C)**. Interestingly, we found some enzyme/ACR pairs with opposite cell-type-specificity (*i.e.* BS-specific enzyme, MS-specific ACR). Many of these ACRs were distally located. For example, in *Z. mays,* the MS-specific ACR of *RBCS* was 36,171 bp upstream **(Figure 4D)**. When investigating ACRs around promoters, we were struck at how often cell-type-specific ACRs occurred outside of the bounds of previously analyzed promoters. For example, in *PEPC* in *P. miliaceum*, a recent analysis demonstrated that a series of conserved non-coding sequences found between species were able to drive MS expression (21). When we looked at chromatin accessibility data of the promoter fragment which was cloned from *PEPC*, we identified many MS-specific ACRs within the cloned fragment, but an additional one upstream. This results shows the advantage of using scATAC-seq data to identify candidate CREs for certain genes, removing the guesswork of cloning fragments to investigate and providing a detailed cell-type-specific regulatory map of the locus (**Figure 4E)**. ScATAC-seq greatly improves the search space of the active CREs potentially driving cell-type-specific gene expression.

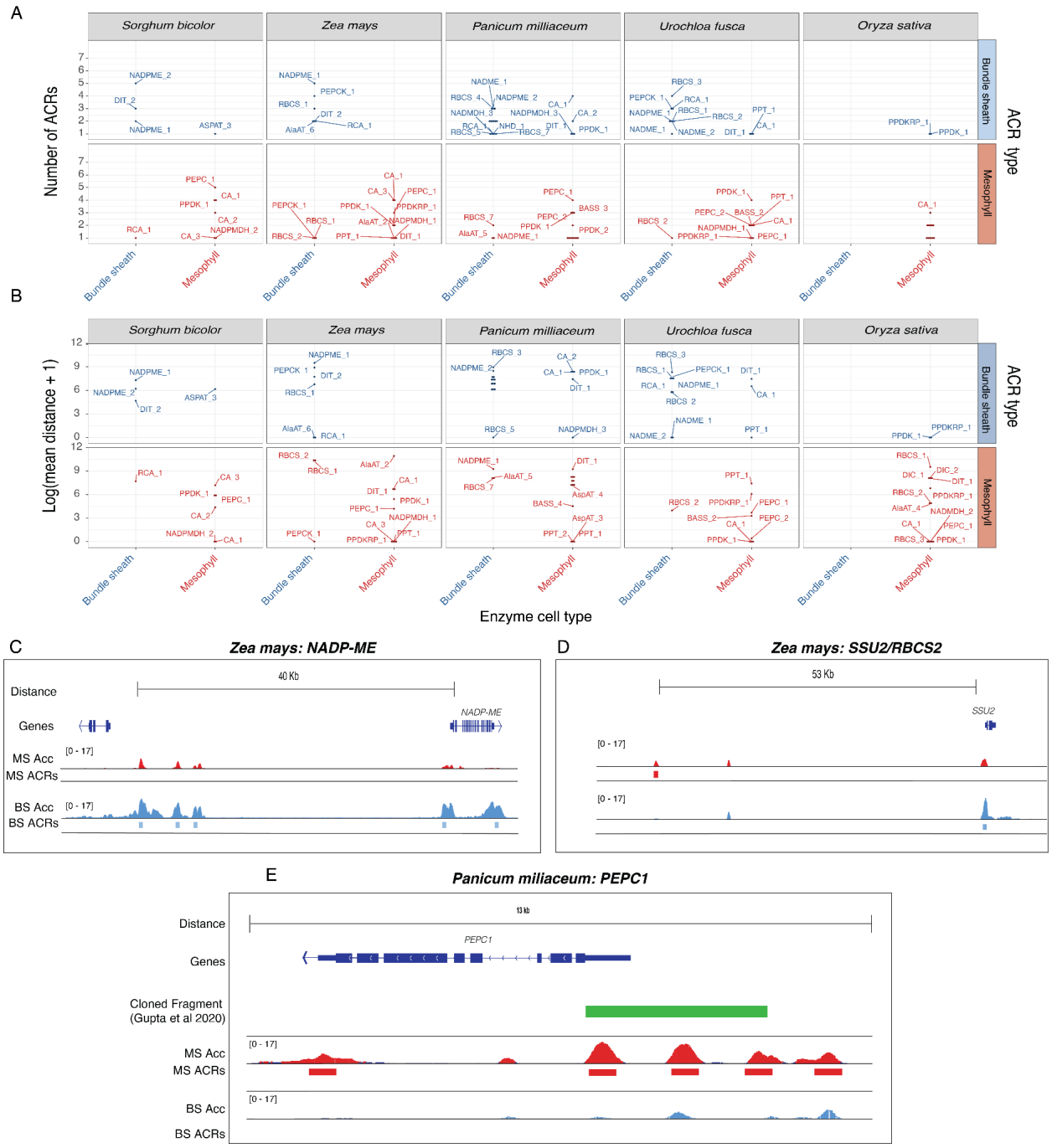

Figure 4: Investigating the number and distance of cell-type-specific ACRs around C4 enzymes across subtypes. **A)** Dot plots showing the number of cell-type-specific ACRs around each enzyme. The x-axis indicates which cell type these enzymes are found in. The y-axis is counts of ACRs. The graph is further subdivided with the top panel being broad ACRs, middle panel BS-specific ACRs, and the bottom being MS-specific ACRs. Enzymes are labeled. **B)** Dotplots showing the mean distance of cell-type-specific ACRs to their closest C4 enzyme. The x-axis indicates which cell type these enzymes are found in. The x-axis is the genomic distance to the C4 enzyme in question. If an enzyme had multiple cell-type-specific ACRs, the distance was

averaged (mean). **C)** Screenshot of *NADP-ME1* in *Z. mays*. Blue tracks correspond to BS chromatin accessibility and red tracks show MS chromatin accessibility. Tracks are equally scaled to facilitate comparison. **D)** Screenshot of *SSU2* in *Z. mays*. Blue tracks correspond to BS chromatin accessibility and red tracks show MS chromatin accessibility. Tracks are equally scaled to facilitate comparison. **E)** Screenshot of *PEPC1* in *P. miliaceum.* The green fragment represents the cloned promoter from Gupta et al 2020, which was identified by minimap2 alignment. Blue tracks correspond to BS chromatin accessibility and red tracks show MS chromatin accessibility. Tracks are equally scaled to facilitate comparisons.

The Evolutionary Relationships of ACRs Associated with C4 Genes is Complex and Variable:

Next, we explored the evolutionary histories of these ACRs. Due to the fact that the C4 subtypes come from different radiation events, (with *Z. mays* and *S. bicolor* likely sharing a C4 ancestor and *U. fusca* and *P. miliaceum* sharing a different C4 ancestor), we were curious to evaluate if a majority of the ACR space around these genes were either novel, or shared among these species. We implemented a pairwise sequence based approach by identifying sequence conservation of ACRs between the study species using BLAST (**Methods)**. The majority of important C4 genes have both novel, and conserved ACRs. For example, PPDK, a MS-specific enzyme, shares ~25% of its ACRs across all species examined including the *O. sativa* C3 outgroup (**Figure 5A**). Interestingly, RUBISCO ACTIVASE (RCA), a critical enzyme in photosynthesis which removes inhibitory molecules from the RuBisCO active site, had novel ACRs in all of the C4 species examined, whereas *RCA* in the C3 species *O. sativa* shared ACRs with all of the C4 species. This might indicate that each of the C4 species gained regulatory sequences at *RCA* or that *O. sativa* might have lost them (**Figure 5A**). Focusing on NADP-ME revealed notable divergence in its associated ACRs, even among closely related species. For example, in *Z. mays*, two out of seven ACRs linked to *NADP-ME1* were unique, lacking counterparts in other species (**Figure 5A**). This is particularly striking given that *S. bicolor*, belonging to the same C4 subtype, diverged from *Z. mays* only 13 million years ago. Similarly, in *S. bicolor*, the BS-specific *NADP-ME2* variant exhibited two out of five unique ACRs. This pattern underscores the rapid and distinct evolutionary trajectories of ACRs in C4 plants. A full list of gene families, and gene models, and their relative conservation is found in **Supplemental figure 9A.** Using this same approach to study all of the core class of C4 enzymes did not reveal a generalizable pattern associated with gain or loss of ACRs around C4 genes (**Supplemental figure 9D)**. Our findings not only confirm the dynamic evolution of *cis*-regulatory sequences in C4 enzymes but also align with existing research that highlights rapid *cis*-regulatory changes among closely related species (39,40).

While investigating the ACRs around the C4 genes is interesting, understanding how cell-type specificity is achieved across C4 subtypes is needed for future efforts to engineer C4 photosynthesis. When looking at just the cell-type specific ACRs around key C4 loci, we find a similar pattern where there is a mix of both conserved and novel ACRs. For example, we discovered that some of the MS-specific ACRs associated with *PPDK* and *PEPC* are highly conserved in all of the studied species. Interestingly, the MS-specific ACRs around *PEPC* were

only found in the C4 species, and not in the C3 outgroup, *O.sativa* (**Figure 5B**). This indicates that some of the CREs that allow *PEPC* expression in MS likely evolved after the split between the most recent common ancestors. We also observed that *NADP-ME* possessed numerous BS-specific ACRs that were conserved in all species, including *O. sativa* (**Figure 5B**). Considering the fact that proper compartmentalization of *NADP-ME* in BS cells is only critical in two of the four C4 subtypes, this was surprising. However, in both *S. bicolor* and *Z. mays*, there were novel BS-specific ACRs associated with each key *NADP-ME*. In *Z. mays,* one out of the five BS-specific ACRs was novel to *Z. mays*, and in *S. bicolor* two out of the five were novel to *S. bicolor.* Upon inspection of all the *NADP-ME* loci in genome browsers, we were struck by the complexities and shuffling that occurred at these BS cell-type-specific ACRs (**Figure 5C**). These results highlight that extensive *cis*-regulatory evolution is occurring in each of these species, and in particular on a cell-type-specific level. Additionally, this may point to the fact that the novel BS-specific ACRs found in *S.bicolor* and *Z. mays* may be more important for proper BS-specific expression than the conserved regulatory elements.

Although binary classification of ACRs was useful to decipher larger scale patterns between key enzymes, we next tested if larger segments of sequence were conserved around some C4 genes as compared to others. We profiled the relative amount of conserved sequence at each of these ACRs, as alignment of sequence between species gives greater resolution as to which ACRs might be important. One interesting observation from this analysis was the fact that the cell-type-specific ACRs around *PEPCK* appear to be novel between *Z. mays* and *U. fusca* (**Figure 5D**). This suggests that these regulatory loci emerged independently, and yet are still likely important in cell-type-specific expression of *PEPCK*. Additionally, around the *NAD-ME* loci in *P. miliaceum*, we found diverse evolutionary histories with both copies *NAD-ME1* and *NAD-ME2* having both conserved and novel BS-specific ACRs (one out of four ACRs were novel for *NAD-ME1*, and zero out of the two were conserved for *NAD-ME2*) (**Figure 5D**). The ACRs from *NADP-ME1* are conserved in *U. fusca*, whereas all three BS-specific ACRs are conserved in relation to *P. miliaceum.* Pointing to the fact that the ACRs have likely maintained their cell-type specificity, and are likely critical drivers in the correct expression of *NAD-ME* loci. These results highlight the dynamic evolution of cell-type-specific ACRs around key C4 loci, and that even closely related subtypes have evolved novel ACRs potentially critical in terms of proper gene expression, as well as compartmentalization.

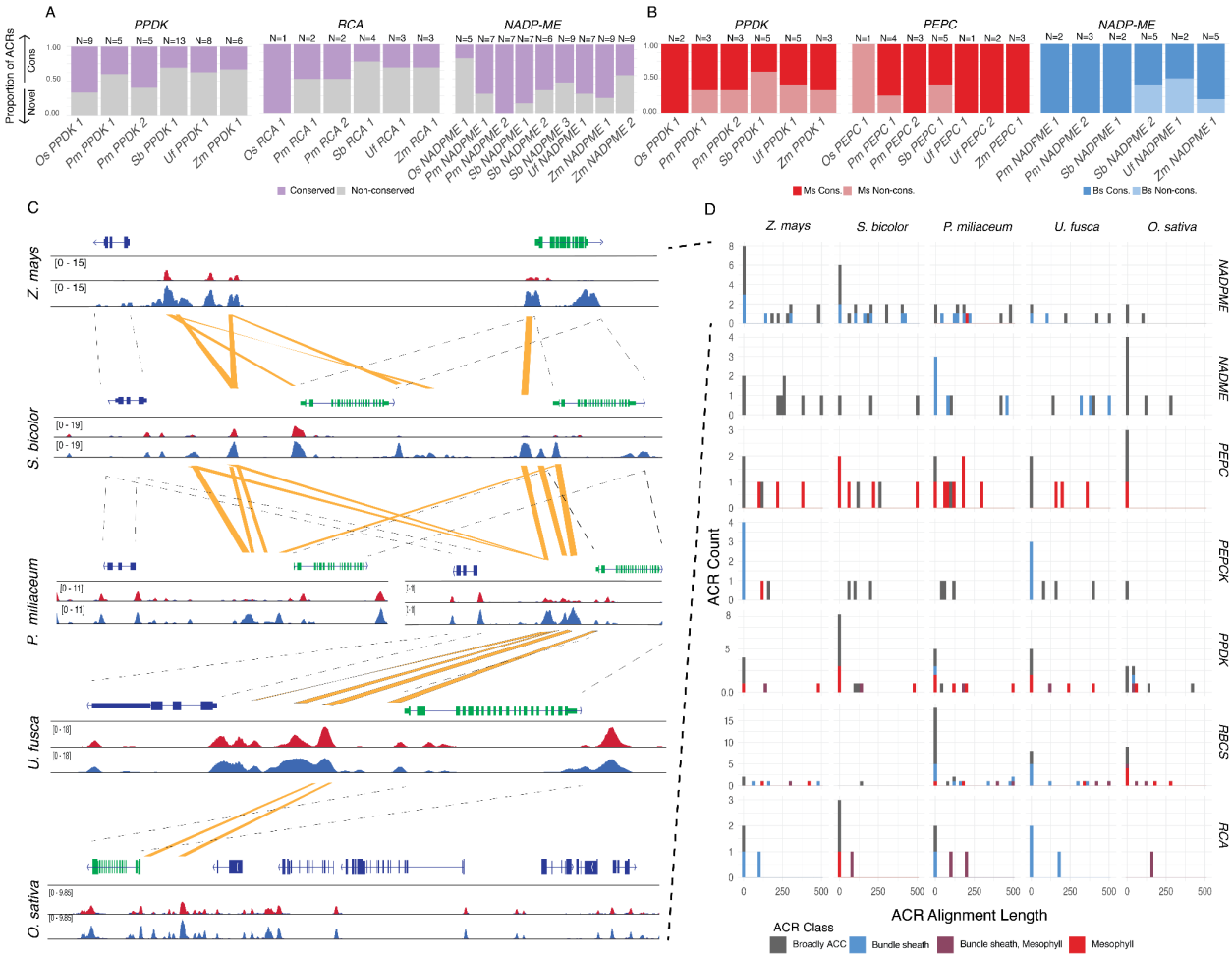

Figure 5: The evolutionary relationships of *cis*-regulatory regions around C4 genes is complex, being composed of both novel and conserved ACRs. **A)** The proportion of all ACRs that are conserved or novel for the following gene families *PPDK*, *RCA*, and *NADP-ME*. Purple bars represent ACRs that have any sequence aligned to them from a different species, and gray represents ACRs where sequence are not alignable. The number of ACRs in each locus is labeled at the top of each column. **B)** The proportion of cell-type-specific ACRs that are conserved and novel for the following gene families, *PPDK*, *PEPC*, and *NADP-ME*. Red bars only consider MS-specific ACRs, and blue bars only consider BS-specific ACRs. **C)** Screenshot of the conservation of BS-specific ACRs around *NADP-ME* across species. From top to bottom the species are *Z. mays, S. bicolor, P. miliaceum, U. fusca, and O. sativa. NADP-ME* is annotated in green for all species. Dashed bars between gene models represent the same gene model, and yellow bars are conserved ACRs. Browser tracks are blue for BS, and red for MS. Browser tracks are scaled within each species to allow for direct comparisons. **D)** The length of ACRs that are conserved in a cross species context. Rows represent gene families, and columns represent species. Each histogram is the number of ACRs within the loci of that gene family. The x-axis is the length of the ACR that is conserved and the x-axis is the count. ACRs are color coded according to the legend.

Discussion:

Understanding the evolution of *cis*-regulation associated with C4 photosynthesis has been a long standing goal in the field of plant biology. In this study, we demonstrated the utility of single-cell ATAC-seq data to investigate many aspects of the evolution of C4 photosynthesis. By identifying cell-type-specific chromatin accessibility from four C4 species composed of three different C4 subtypes, as well as a single C3 outgroup, we were able to compare and contrast key genes and their ACRs which define and distinguish C4 photosynthesis. We have shown that by using gene-body chromatin accessibility data, we can measure cell-type-specific bias of both core, and subtype-specific C4 enzymes. When taken into consideration with the gene family trees of many of these enzymes, we show diverse co-option of enzymes into the C4 pathway. Additionally, we identify cell-type-specific ACRs surrounding these key C4 enzymes. We find numerous cell-type-specific ACRs surrounding key C4 enzymes, many of which fall outside of the core promoter region. Finally, we find that around all of the C4 enzymes there is a mix of both conserved and novel cell-type-specific ACRs indicating that regulatory evolution of these regions is ongoing. However, we do find interesting ACRs which are highly conserved over evolutionary time across the C4 species examined, indicating that these may be critical sequences in cell-type-specific gene regulation.

Investigation of the CREs driving cell-type-specific expression of C4 genes is challenging. This often requires evaluation using transgenic plants, which limits the number of CREs that can be tested. This has greatly hampered efforts at understanding how *cis*-regulation of C4 genes evolves, whether by co-option of existing CREs or emergence of new ones. Our results show the complex nature of CRE evolution of C4 genes, including those specific to C4 subtypes. While we observe conservation of ACRs around many C4 genes, we do see interesting examples where the subtype specific enzymes have evolved novel ACRs (*NAD-ME*'s in *P. miliaceum*, and *PEPCK* in *U. fusca*). These results support that there is likely a combination of both co-opting pre-existing CREs, as well as evolving new ones to facilitate proper expression and cell-type-specification of genes. Another recent single-cell genomic study of the evolution of CREs important for photosynthesis using a comparison between *O. sativa* and *S. bicolor* reached similar conclusions (41). They frequently found different ACRs and TF motifs in promoters of orthologous C4 genes (41). Future efforts to assay these candidate CREs using reporter assays, transgenesis and genome editing will be required. Fortunately, these high-resolution maps of cell-type-specific ACRs of these key genes/species provide a strong foundation to build upon.

Although these studies provide a blueprint for the study of key candidate CREs associated with C4 enzymes, profiling cell-type-specific chromatin accessibility of additional species would be greatly beneficial. Although *O. sativa* is an invaluable outgroup for this study, additional more closely related C3 species might make these comparisons simpler, and add additional resolution. For instance the C3 grass species *Dichanthelium oligosanthes* is more closely related to *U. fusca* and *P. miliaceum* and has a recently completed reference genome (42). Adding additional species would enable greater resolution in the comparison of

cell-type-specific ACRs, as the genetic distance between the species we examined and *O. sativa* make identification of conserved and novel ACRs challenging. As an example, the ACRs associated with *NAD-ME's* in *P. miliaceum* might be co-opted instead of novel, however, based on our sampling, we cannot say.

Genome editing analysis of many of these ACRs would significantly advance which ACRs, and more specifically which CREs within the ACRs are most important for cell-type-specific expression (17). However, currently generating genome edits in monocots is challenging, time consuming and expensive. Fortunately, improvements to transgenesis are constantly improving making achieving these goals more likely in the future (43). It's also important to consider that mutational analysis of CREs is not straightforward, often requiring numerous editing events of the *cis*-regulatory landscape of each gene. Previous studies have shown that deletions of many CREs produce variable molecular and morphological phenotypes, further complicating our understanding of the *cis*-reglatory code (44–46). And finally, many species, including *P. miliaceum* and *U. fusca* have to date never been transformed. This highlights the need to continually improve transgenesis methods to help facilitate the molecular dissection of CRE. In conclusion, this study provides a comprehensive map of cell-type-specific ACRs around key C4 genes, which reveals the dynamic evolution and diversity of *cis*-regulation of C4 genes.

Acknowledgments:

This research was funded by awards from the National Science Foundation (IOS-2134912 and IOS-1856627) to RJS. APM and JPM were supported by the National Institutes of Health (K99GM144742) and (T32GM142623), respectively.

Methods:

Plant Growth Conditions and Sampling:

Seedlings of all five plant species, including maize (*Zea mays* B73), sorghum (*Sorghum bicolor* BTx623), proso millet (*Panicum miliaceum* L. CGRIS 00000390), and browntop signalgrass (*Urochloa fusca* LBJWC-52), along with the C3 plant rice (*Oryza sativa* Nipponbare), were grown under the conditions of 12:12 Light/Dark cycles at 30°C Light/22°C Dark and at 50% humidity. The sampling of the C4 species was timed to coincide with a specific developmental stage, identified when the ligule of the third leaf became visible, marking the third leaf unfolding, yet prior to the appearance of the fourth leaf. For the C3 species, rice, 18-day-old leaves were used to correspond with the equivalent stage of the C4 species.

Library Preparation:

Nuclei isolation for the experiments was conducted using fresh seedlings of both the C4 and C3 species at their respective developmental stages. The methodology for nuclei extraction, encompassing the buffer composition and the subsequent steps, was used with procedures

outlined for single-nucleus combinatorial indexing with transposed-based ATAC-seq library construction, as detailed in a prior study (47).

Genomes:
The *Z. mays* genome version 5 was downloaded from MaizeGDB (48,49). The *O. sativa* genome was downloaded from rice.uga.edu. The *S. bicolor* version v5.1 was downloaded and used from Phytozome version 13, as well as the *U. fusca* genome version 1.1 (50). Finally the *P. miliaceum* genome was downloaded from NCBI, bioproject number PRJNA431363 (33).

Read Alignment and Mapping of Tn5 Insertions:
Reads were aligned using BWA (version 0.7.17) (51). Reads were filtered using samtools (version 1.16.1) for mapping quality of >10 for *Z. mays* , *S. bicolor* , *U. fusca*, and *O. sativa*. *P. miliaceum* required a greater lower threshold of 30 given its recent whole genome duplication event increasing the rate of multi-mapping reads (52). Duplicate reads were removed using picard tools (version 2.25.0) (53). Single-base pair Tn5 integration events were mapped using the python script `makeTn5bed.py` found in the GitHub utils directory (Repo accessible upon publication of this manuscript). Finally, for each barcode only unique Tn5 integrations sites were used for analysis. So if a nuclei had the same identical fragments multiple times, only a single event was considered.

Isolating High-Quality Cells:
Cells were filtered using Socrates (16). In short, Fragment of Reads in Peaks (FRiP) scores were calculated for each cell by pseudo bulking the libraries and identifying peaks. For each individual cell, FRiP was calculated by intersecting Tn5 integration events with peaks. Cells with a FRiP score greater than 0.2 were used. Additionally, TSS enrichment was calculated by looking at the number of Tn5 integrations around TSS. Cells that had a TSS enrichment greater than 0.15 were used. Finally, cells were compared to a random sample of low quality cells which did not pass filtering, representing the “background” of cells, and correlation was calculated between passing cells and background cells using the corr package in R. Cells which had a correlation lower than 0.3 percent as compared to background cells were used for further analysis.

UMAP embeddings were then calculated for each species utilizing genomic bins (54). For each dataset, bins of 500 bp were calculated. To reduce the size of features to cluster on, bins had to show accessible chromatin in at least 0.005% of total cells (roughly 50~100 cells in each species). Additionally, bins that were broadly accessible across greater than 10% of cells in the given dataset were also discarded to remove regions of the genome which were constitutively accessible and wouldn’t facilitate clustering. Finally, regions of the genome which were associated with either blacklist (16), or genes which were known to be related to cell cycle and circadian rhythms were removed. The final resulting matrix, which represented cell barcodes X genomic regions (here bins), were then put through the term-frequency inverse-document-frequency (TF-IDF) algorithm to identify genomic regions more descriptive of the entire dataset (23). The resulting matrix was then input into Singular Value Decomposition, and clustering was then done on the remaining features with the number of principal components (PCs) equaling 50, and any PC with a correlation to read depth greater than 0.5

removed (55) (23). Clustering was done using the Louvain clustering algorithm in order to bin cells into similar groups based off of the PCs calculated above (56). Using the UMAP embeddings, doublets were removed using the software Scrublet as implemented in Socrates software (57). At random, 5,000 cells were used to generate *in-silico* doublets, and cells which were scored as being likely doublets were removed. Adaptive thresholds were set on a per library basis. The doublet rate from Scrublet was compared against a mixed library where genotypes of *Z. mays* were mixed Mo17 and B73, and genotype doublets were identified. We found that Scrublet, on average, removed more cells in a conservative fashion than the birthday problem and genotype doublets identified, so we utilized the Scrublet doublet scores to be conservative.

Identification of Putative Orthologs:
To annotate species with less marker gene information, we identified putative orthologs or marker genes using OrthoFinder (version 2.5.4) (24). For each species, the primary protein sequence of the transcript was used as input to Orthofinder. In the resulting orthofinder outputs, the script “find_markers.orthofinder.py” was used to parse the resulting phylogenies and return back putative orthologs.

Annotation of Cell Types:
Cell types were annotated by calculating gene chromatin accessibility for marker genes in each genome on a per cell basis. These values were then visualized on the UMAP embedding, and clusters with numerous marker genes associated with the same cell-type were used as evidence. Additionally, for each louvain cluster, enrichment of marker genes was calculated by comparing the cluster average as compared to a random shuffle of random cells. The top five most enriched markers were used in tandem with the UMAPs to ascertain cell-type identity. We also tested the statistical significance of the marker gene using Presto, a modified wilcoxon rank-sum test in order to identify the most unique marker gene in each cluster (58). Additionally, for specific clusters showing mixed signals from marker genes, sub-clustering was done by isolating the cluster in question, and then re-clustering these cells on a new UMAP manifold. The same steps were done in order to visualize marker genes, as well as test this enrichment, and statistical significance. In order to ensure that we were comparing similar cell-types, we also took an orthogonal approach where we compared the gene accessibility of the top 2000 most variable orthologs between our species. A linear model was generated for each comparison where the number of Tn5 integrations per library were taken into consideration, and the species was one-hot-encoded. The resulting residuals were used to generate the cell-type correlations.

Peak Identification:
To identify peaks, cells of the same annotation type were pseudo bulked in a replicate aware fashion. Within each replicate MACS2 (version 2.2.9.1) was run with parameters “--nomodel --keep-dup auto --extsize 150 --shift -75 --qvalue .05” and variable genome size flag ‘-g’ (59). Summits for each peak identified in each replicate were extended by 250 bp in either direction. Only peaks which overlapped between replicates were used. To merge peaks from various cell types and select peak boundaries, the p-value associated with each peak in each cell type was compared by calculating the chromatin accessibility score for each peak per million, with those

peaks with the highest accessibility score being selected as the representative peak. Additionally, bigwigs were generated for each cell type by normalizing each dataset to the number of reads/per million scaling factor. Implementation of this algorithm is found in the script call_scACRs.py for ease of use and replication in other experiments.

Identifying Cell-type-specific ACRs:
To identify cell-type specific ACRs, a modified bootstrapping method was used which drew inspiration from the modified entropy metrics found in (60). On a per ACR basis, Tn5 integrations per cell-type were summed and counts per million (CPM) normalized. These values were then converted to probability by using the following equation (below, equation 1) where *pi* is the CPM value for the focal cell-type and *qi* is the total sum of all CPMs. From this probability statement, a modified shannon entropy metric was calculated, followed by a metric of specificity *Hp*. For robust cell-type-specific ACR identification, the real annotation dataset was bootstrapped 5000 times, taking a sample of 250 cells from each cell type annotation, and calculating both entropy and specificity scores. This was done to attempt to get a robust signal of specificity, which takes into consideration their variation in cell quality present in each cell-type annotation. To generate the null distribution of specificity scores, individual cell annotations were scrambled to generate an equal number of null cell-type classifications. For each null value, the entropy and specificity score were calculated. Finally, to calculate a p-value a non-parametric approach was used to identify how many of the real bootstraps fell outside of the null distribution using a one tailed test. ACRs which had a p-value of <0.001 were considered to be significant. ACRs were finally classified by the number of cell types they were specific to. ACRs specific to greater than three were classified as broadly accessible, less than or equal to three as cell type restricted, and a single cell-type as cell-type specific.

1) $$p_i = \frac{q_i}{\sum q_i}$$

2) $$H_p = -\sum_t p_t \log_2(p_t)$$

3) $$Q_{pt} = H_p - \log_2(p_t)$$

gene_family	Type
AlaAT	variable
AspAT	variable

CA	core
DIC	core
DIT	variable
NADMDH	variable
NADME	variable
NADPMDH	variable
NADPME	variable
NHD	variable
PEPCK	core
PEPC	core
PPDKRP	core
PPDK	core
PPT	core
RBCS	core
RCA	core
BASS	variable

Supplementary Table 1: C4-related gene families and their classification as either core or variable dependent on their importance in C4 photosynthesis.

1. Bowes G, Ogren WL, Hageman RH. Phosphoglycolate production catalyzed by ribulose diphosphate carboxylase. Biochem Biophys Res Commun. 1971 Nov 5;45(3):716–22.
2. Wheeler T, von Braun J. Climate Change Impacts on Global Food Security. Science. 2013 Aug 2;341(6145):508–13.
3. Hatch MD. C4 photosynthesis: a unique elend of modified biochemistry, anatomy and ultrastructure. Biochim Biophys Acta BBA - Rev Bioenerg. 1987 Jan 1;895(2):81–106.
4. Sage RF. A portrait of the C_4 photosynthetic family on the 50th anniversary of its discovery: species number, evolutionary lineages, and Hall of Fame. J Exp Bot. 2016 Jul;67(14):4039–56.
5. Sage RF, Christin PA, Edwards EJ. The C4 plant lineages of planet Earth. J Exp Bot. 2011 May 1;62(9):3155–69.
6. Gowik U, Westhoff P. The Path from C3 to C4 Photosynthesis. Plant Physiol. 2011 Jan 1;155(1):56–63.
7. Grass Phylogeny Working Group II. New grass phylogeny resolves deep evolutionary relationships and discovers C4 origins. New Phytol. 2012 Jan;193(2):304–12.
8. Rao X, Dixon RA. The Differences between NAD-ME and NADP-ME Subtypes of C4

Photosynthesis: More than Decarboxylating Enzymes. Front Plant Sci [Internet]. 2016 [cited 2023 May 22];7. Available from: https://www.frontiersin.org/articles/10.3389/fpls.2016.01525
9. Kajala K, Brown NJ, Williams BP, Borrill P, Taylor LE, Hibberd JM. Multiple Arabidopsis genes primed for recruitment into C4 photosynthesis. Plant J. 2012;69(1):47–56.
10. Sheen J. C4 Gene Expression. Annu Rev Plant Physiol Plant Mol Biol. 1999;50(1):187–217.
11. Chollet R, Vidal J, O'Leary MH. PHOSPHO *ENOL* PYRUVATE CARBOXYLASE: A Ubiquitous, Highly Regulated Enzyme in Plants. Annu Rev Plant Physiol Plant Mol Biol. 1996 Jun;47(1):273–98.
12. O'Leary MH. Phosphoenolpyruvate Carboxylase: An Enzymologist's View. Annu Rev Plant Physiol. 1982 Jun;33(1):297–315.
13. Outlaw Jr William H. Kinetic Properties of Guard-Cell Phosphoenolpyruvate Carboxylase. Biochem Physiol Pflanz. 1990 Jan 1;186(5):317–25.
14. Gowik U, Burscheidt J, Akyildiz M, Schlue U, Koczor M, Streubel M, et al. cis-Regulatory Elements for Mesophyll-Specific Gene Expression in the C4 Plant Flaveria trinervia, the Promoter of the C4 Phosphoenolpyruvate Carboxylase Gene[W]. Plant Cell. 2004 May 12;16(5):1077–90.
15. Kim ED, Dorrity MW, Fitzgerald BA, Seo H, Sepuru KM, Queitsch C, et al. Dynamic chromatin accessibility deploys heterotypic cis/trans-acting factors driving stomatal cell-fate commitment. Nat Plants. 2022 Dec;8(12):1453–66.
16. Marand AP, Chen Z, Gallavotti A, Schmitz RJ. A cis-regulatory atlas in maize at single-cell resolution. Cell. 2021 May 27;184(11):3041-3055.e21.
17. Meng F, Zhao H, Zhu B, Zhang T, Yang M, Li Y, et al. Genomic editing of intronic enhancers unveils their role in fine-tuning tissue-specific gene expression in Arabidopsis thaliana. Plant Cell. 2021 Mar 25;33(6):1997–2014.
18. Matsuoka M, Kyozuka J, Shimamoto K, Kano-Murakami Y. The promoters of two carboxylases in a C4 plant (maize) direct cell-specific, light-regulated expression in a C3 plant (rice). Plant J. 1994;6(3):311–9.
19. Matsuoka M, Tada Y, Fujimura T, Kano-Murakami Y. Tissue-specific light-regulated expression directed by the promoter of a C4 gene, maize pyruvate,orthophosphate dikinase, in a C3 plant, rice. Proc Natl Acad Sci. 1993 Oct 15;90(20):9586–90.
20. Gowik U, Schulze S, Saladié M, Rolland V, Tanz SK, Westhoff P, et al. A MEM1-like motif directs mesophyll cell-specific expression of the gene encoding the C4 carbonic anhydrase in Flaveria. J Exp Bot. 2017 Jan 1;68(2):311.
21. Gupta SD, Levey M, Schulze S, Karki S, Emmerling J, Streubel M, et al. The C4Ppc promoters of many C4 grass species share a common regulatory mechanism for gene expression in the mesophyll cell. Plant J. 2020;101(1):204–16.
22. Dai X, Tu X, Du B, Dong P, Sun S, Wang X, et al. Chromatin and regulatory differentiation between bundle sheath and mesophyll cells in maize. Plant J [Internet]. 2022 Jan 14 [cited 2022 Jan 14];n/a(n/a). Available from: https://onlinelibrary.wiley.com/doi/abs/10.1111/tpj.15586
23. Cusanovich DA, Hill AJ, Aghamirzaie D, Daza RM, Pliner HA, Berletch JB, et al. A Single-Cell Atlas of In Vivo Mammalian Chromatin Accessibility. Cell. 2018 Aug;174(5):1309-1324.e18.
24. Emms DM, Kelly S. OrthoFinder: phylogenetic orthology inference for comparative genomics. Genome Biol. 2019 Nov 14;20(1):238.
25. Taniguchi M, Sugiyama T. The Expression of 2-Oxoglutarate/Malate Translocator in the Bundle-Sheath Mitochondria of Panicum miliaceum, a NAD-Malic Enzyme-Type C4 Plant, Is Regulated by Light and Development. Plant Physiol. 1997 May 1;114(1):285–93.
26. Taniguchi Y, Nagasaki J, Kawasaki M, Miyake H, Sugiyama T, Taniguchi M. Differentiation of Dicarboxylate Transporters in Mesophyll and Bundle Sheath Chloroplasts of Maize. Plant Cell Physiol. 2004 Feb 15;45(2):187–200.

27. Tausta SL, Li P, Si Y, Gandotra N, Liu P, Sun Q, et al. Developmental dynamics of Kranz cell transcriptional specificity in maize leaf reveals early onset of C4-related processes. J Exp Bot. 2014 Jul;65(13):3543–55.
28. Borba AR, Reyna-Llorens I, Dickinson PJ, Steed G, Gouveia P, Górska AM, et al. Compartmentation of photosynthesis gene expression in C4 maize depends on time of day. Plant Physiol. 2023 Aug 9;kiad447.
29. Nomura M, Higuchi T, Katayama K, Taniguchi M, Miyao-Tokutomi M, Matsuoka M, et al. The Promoter for C4-type Mitochondrial Aspartate Aminotransferase Does not Direct Bundle Sheath-specific Expression in Transgenic Rice Plants. Plant Cell Physiol. 2005 May 1;46(5):743–53.
30. Taniguchi M, Kobe A, Kato M, Sugiyama T. Aspartate Aminotransferase Isozymes in Panicum miliaceum L, an NAD-Malic Enzyme-Type C4 Plant: Comparison of Enzymatic-Properties, Primary Structures, and Expression Patterns. Arch Biochem Biophys. 1995 Apr 20;318(2):295–306.
31. Taniguchi M, Sawaki H, Sasakawa H, Hase T, Sugiyama T. Cloning and sequence analysis of cDNA encoding aspartate aminotransferase isozymes from Panicum miliaceum L., a C4 plant. Eur J Biochem. 1992;204(2):611–20.
32. Wang Y, Bräutigam A, Weber APM, Zhu XG. Three distinct biochemical subtypes of C4 photosynthesis? A modelling analysis. J Exp Bot. 2014 Jul 1;65(13):3567–78.
33. Zou C, Li L, Miki D, Li D, Tang Q, Xiao L, et al. The genome of broomcorn millet. Nat Commun. 2019 Jan 25;10(1):436.
34. Washburn JD, Strable J, Dickinson P, Kothapalli SS, Brose JM, Covshoff S, et al. Distinct C4 sub-types and C3 bundle sheath isolation in the Paniceae grasses. Plant Direct. 2021;5(12):e373.
35. Son D, Jo J, Sugiyama T. Purification and characterization of alanine aminotransferase from Panicum miliaceum leaves. Arch Biochem Biophys. 1991 Aug 15;289(1):262–6.
36. Engineer C, Hashimoto-Sugimoto M, Negi J, Israelsson-Nordstrom M, Azoulay-Shemer T, Rappel WJ, et al. CO2 sensing and CO2 regulation of stomatal conductance: advances and open questions. Trends Plant Sci. 2016 Jan;21(1):16–30.
37. Bansal KC, Viret JF, Haley J, Khan BM, Schantz R, Bogorad L. Transient expression from cab-m1 and rbcS-m3 promoter sequences is different in mesophyll and bundle sheath cells in maize leaves. Proc Natl Acad Sci. 1992 Apr 15;89(8):3654–8.
38. Viret JF, Mabrouk Y, Bogorad L. Transcriptional photoregulation of cell-type-preferred expression of maize rbcS-m3: 3’ and 5’ sequences are involved. Proc Natl Acad Sci. 1994 Aug 30;91(18):8577–81.
39. Lu Z, Marand AP, Ricci WA, Ethridge CL, Zhang X, Schmitz RJ. The prevalence, evolution and chromatin signatures of plant regulatory elements. Nat Plants. 2019 Dec;5(12):1250–9.
40. Maher KA, Bajic M, Kajala K, Reynoso M, Pauluzzi G, West DA, et al. Profiling of Accessible Chromatin Regions across Multiple Plant Species and Cell Types Reveals Common Gene Regulatory Principles and New Control Modules. Plant Cell. 2018 Jan;30(1):15–36.
41. Swift J, Luginbuehl LH, Schreier T, Donald RM, Lee T, Nery J, et al. Single nuclei sequencing reveals C4 photosynthesis is based on rewiring of ancestral cell identity networks [Internet]. bioRxiv; 2023 [cited 2023 Oct 31]. p. 2023.10.26.562893. Available from: https://www.biorxiv.org/content/10.1101/2023.10.26.562893v1
42. Studer AJ, Schnable JC, Weissmann S, Kolbe AR, McKain MR, Shao Y, et al. The draft genome of the C3 panicoid grass species Dichanthelium oligosanthes. Genome Biol. 2016 Oct 28;17(1):223.
43. Chen Z, Debernardi JM, Dubcovsky J, Gallavotti A. The combination of morphogenic regulators BABY BOOM and GRF-GIF improves maize transformation efficiency [Internet]. Plant Biology; 2022 Sep [cited 2024 Jan 2]. Available from:

http://biorxiv.org/lookup/doi/10.1101/2022.09.02.506370
44. Ciren D, Zebell S, Lippman ZB. Extreme restructuring of cis-regulatory regions controlling a deeply conserved plant stem cell regulator [Internet]. bioRxiv; 2023 [cited 2024 Jan 2]. p. 2023.12.20.572550. Available from: https://www.biorxiv.org/content/10.1101/2023.12.20.572550v1
45. Liu L, Gallagher J, Arevalo ED, Chen R, Skopelitis T, Wu Q, et al. Enhancing grain-yield-related traits by CRISPR–Cas9 promoter editing of maize CLE genes. Nat Plants. 2021 Mar;7(3):287–94.
46. Rodríguez-Leal D, Lemmon ZH, Man J, Bartlett ME, Lippman ZB. Engineering Quantitative Trait Variation for Crop Improvement by Genome Editing. Cell. 2017 Oct;171(2):470-480.e8.
47. Tu X, Marand AP, Schmitz RJ, Zhong S. A combinatorial indexing strategy for low-cost epigenomic profiling of plant single cells. Plant Commun. 2022 Jul 11;3(4):100308.
48. Hufford MB, Seetharam AS, Woodhouse MR, Chougule KM, Ou S, Liu J, et al. De novo assembly, annotation, and comparative analysis of 26 diverse maize genomes. Science. 2021 Aug 6;373(6555):655–62.
49. Liu J, Seetharam AS, Chougule K, Ou S, Swentowsky KW, Gent JI, et al. Gapless assembly of maize chromosomes using long-read technologies. Genome Biol. 2020 May 20;21(1):121.
50. Goodstein DM, Shu S, Howson R, Neupane R, Hayes RD, Fazo J, et al. Phytozome: a comparative platform for green plant genomics. Nucleic Acids Res. 2012 Jan;40(Database issue):D1178–86.
51. Li H, Durbin R. Fast and accurate short read alignment with Burrows–Wheeler transform. Bioinformatics. 2009 Jul 15;25(14):1754–60.
52. Danecek P, Bonfield JK, Liddle J, Marshall J, Ohan V, Pollard MO, et al. Twelve years of SAMtools and BCFtools. GigaScience. 2021 Feb 1;10(2):giab008.
53. Picard Tools - By Broad Institute [Internet]. [cited 2024 Jan 4]. Available from: http://broadinstitute.github.io/picard/
54. McInnes L, Healy J, Melville J. UMAP: Uniform Manifold Approximation and Projection for Dimension Reduction [Internet]. arXiv; 2020 [cited 2022 Dec 19]. Available from: http://arxiv.org/abs/1802.03426
55. Stewart GW. On the Early History of the Singular Value Decomposition. SIAM Rev. 1993;35(4):551–66.
56. Blondel VD, Guillaume JL, Lambiotte R, Lefebvre E. Fast unfolding of communities in large networks. J Stat Mech Theory Exp. 2008 Oct;2008(10):P10008.
57. Wolock SL, Lopez R, Klein AM. Scrublet: Computational Identification of Cell Doublets in Single-Cell Transcriptomic Data. Cell Syst. 2019 Apr;8(4):281-291.e9.
58. Korsunsky I, Nathan A, Millard N, Raychaudhuri S. Presto scales Wilcoxon and auROC analyses to millions of observations [Internet]. Bioinformatics; 2019 May [cited 2023 Dec 28]. Available from: http://biorxiv.org/lookup/doi/10.1101/653253
59. Zhang Y, Liu T, Meyer CA, Eeckhoute J, Johnson DS, Bernstein BE, et al. Model-based analysis of ChIP-Seq (MACS). Genome Biol. 2008;9(9):R137.
60. Zhang K, Hocker JD, Miller M, Hou X, Chiou J, Poirion OB, et al. A single-cell atlas of chromatin accessibility in the human genome. Cell. 2021 Nov 24;184(24):5985-6001.e19.