Comparative transcriptome analysis of emerging young and mature leaves of *Bienertia sinuspersici*, a single-cell C4 plant (#107273)

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Comparative transcriptome analysis of emerging young and mature leaves of *Bienertia sinuspersici,* a single-cell C4 plant

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Background**:** Efficient carbon capture by plants is crucial to meet the increasing demands for food, fiber, feed, and fuel worldwide. One potential strategy to improve the photosynthetic performance of plants is the conversion of C3-type crops to C4-type crops, enabling them to perform photosynthesis at higher temperatures and with less water. C4-type crops, such as corn, possess a distinct Kranz anatomy, where photosynthesis occurs in two distinct cell types. Remarkably, *Bienertia sinuspersici* is one of the four known land plant species that perform C4 photosynthesis within a single cell. This unique single-cell C4 anatomy is characterized by dimorphic chloroplasts and corresponding intracellular biochemistry. Because young, emergent *Bienertia* leaves first exhibit C3 anatomy and then differentiate into the C4 anatomy as the leaves mature, *Bienertia* represents an excellent system to explore the basis for a C3 to C4 transition.

Methods**:** To gain insight into the genes and pathways associated with the C3 to C4 transition between young and emergent *Bienertia sinuspersici* leaves, a comparative transcriptome analysis was conducted in which global gene expression and gene ontologies were compared between the two stages.

Results**:** In the emergent leaf, differentially expressed genes and enrichment of ontologies associated with the cell cycle, cellular developmental patterning, and transcriptional regulatory mechanisms were observed. The mature leaf displayed enrichment of processes associated with photosynthesis, chloroplast components, translational components, and post-translational modifications. Additionally, several transcription factors such as auxin response factor (ARF), basic helix-loop-helix (bHLH), GATA, homeodomain (HD), MYB, NAC, squamosa promoter-binding protein-like (SPL), and zinc finger (ZF) family were differentially expressed in the emergent leaf. These data expand our insights into the molecular basis of *Bienertia*'s unique cellular compartmentalization, chloroplast dimorphism, and single-cell C4 biochemistry and provide information that will be useful in the ongoing efforts to transform C3-type crops into C-4 type.

Comparative Transcriptome Analysis of Emerging Young and Mature Leaves of *Bienertia sinuspersici*, a Single-Cell C4 Plant

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Abstract

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Introduction

The process of photosynthesis enables the conversion of light energy into chemical energy. An understanding of photosynthesis in the context of biochemistry, structure, and function has led to several advancements in agriculture (Sharma et al., 2016a, 2016b; Pyc et al., 2017; Oleszek et al., 2019; Carrino et al., 2020; van Vliet et al., 2020; Veiga et al., 2020; Liu et al., 2021). The predominant form of photosynthesis is represented by the C3 type, where the first product of carbon fixation is a 3-carbon organic molecule fixed by the ubiquitous enzyme Rubisco. C3 photosynthesis is vulnerable to higher temperatures and water limitations, and Rubisco prefers oxygen (O_2) over carbon dioxide (CO_2) at higher temperatures, thereby reducing photosynthetic efficiency (Yamori et al., 2014).

Plants have also evolved Crassulacean acid metabolism (CAM) and C4-type photosynthesis to concentrate CO_2 around Rubisco and reduce the competition from O_2 by [illegible]pturing CO_2 during the night or in biochemically different type of cells, respectively (Edwards and Ogburn, 2012; Edwards, 2019). In C4 plants, the 4-carbon molecule that is the first product is shuttled to a physically separate location to concentrate CO_2 around Rubisco to achieve higher photosynthetic efficiency, especially under heat and drought conditions. The primary example of C4 photosynthesis is maize, wherein the leaves possess the canonical “Kranz anatomy”. The generation of C4 organic molecules takes place in the mesophyll cells, and the C3 biochemical processes occur in the bundle sheath cells. Interestingly, plants that carry out C4 photosynthesis without the Kranz anatomy have also been reported (Freitag and Stichler, 2000; Voznesenskaya et al., 2001, 2002).

The single-cell C4 (SCC4) photosynthesis-performing land plant species are represented by the genera *Bienertia*, and a lone species in its sister clade, *Suaeda* (Chenopodiaceae) (Freitag and Stichler, 2002; Voznesenskaya et al., 2002; Voznesenskaya et al., 20[illegible] Akhani et al., 2005). Species in the *Suaeda* genus, which can be found from North America to the Persian Gulf, employ both C3 and C4 photosynthetic types (Fisher et al., 1997). Within these two novel C4 genera, *Suaeda aralocaspica*, *Bienertia cycloptera, Bienertia sinuspersici*, and *Bienertia kavirense* are the only known terrestrial species to perform single-cell C4 photosynthesis. SCC4 is characterized by dimorphic chloroplasts that develop in single chlorenchyma cells (Voznesenskaya et al., 2002; Muhaidat et al., 2007; Edwards and Voznesenskaya, 2011; Langdale, 2011).

The dimorphic plastids of SCC4 plants are physiologically different and morphologically distinct (reviewed in Sharpe and Offermann, 2013). There are two forms of cell morphology in the SCC4 species. In the first one, exemplified by *Suaeda aralocaspica*, dimorphic chloroplasts are localized to the distal and proximal poles of the cell in relation to the vascular tissue. In the second, exemplified by *Bienertia* species, dimorphic chloroplasts are located in a densely packed cytoplasmic compartment localized in the center of the cell, as well as in the cytoplasmic layer lying adjacent to the plasma membrane. These two chloroplast types are compartmentalized from each other by a large vacuole and are connected to each other via cytoplasmic strands traversing the vacuole (Freitag and Stichler, 2000, 2002; Voznesenskaya et al., 2002). Both *Suaeda* and *Bienertia* are NAD-ME [illegible]chemical C4 photosynthetic types, which was confirmed by using $\delta^{13}C$, titratable acid, CO_2 compensation, and enzymatic activity experiments (Sharpe and Offermann, 2014). In *Bienertia* species, the chlorenchyma cells that house the SCC4 process

develop from an undifferentiated state in emergent leaves (e.g., no dimorphic chloroplasts or intracellular compartmentalization), with full differentiation only manifesting in mature leaves (Offermann et al., 2015). The anatomy, protein distribution, and photosynthetic physiology of SCC4 structural types has been elucidated previously (Sharpe and Offermann, 2014; Offermann et al., 2015; Erlinghaeuser et al., 2016; Uzilday et al., 2023), with few molecular exceptions (Park et al., 2010). In this study, we performed a comparative transcriptome analysis of emergent and mature leaves of SCC4 *Bienertia sinuspersici* with the aim to understand the genetic basis of how the SCC4 anatomy, morphology, and underlying biochemistry emerges as the leaf develops.

Materials & Methods

Plant material: *Bienertia sinuspersici* plants were maintained in 10-gallon citrus pots in growth chambers under a 14-hour light/10-hour dark cycle with a stepwise light regime increasing to 525 PPFM at full light and an 18°C (dark) to 35°C (light) temperature regime. Plants were watered once a week and were fertilized with Peters 20-21-5 in between waterings. Within two hours after light initiation, whole, fully expanded, mature leaves and newly emerging young leaves (approximately 0.2 mm in size) were harvested from three separate *B. sinuspersici* plants, combined as a pooled sample, and immediately flash frozen in liquid nitrogen. Flash frozen leaf tissue was ground into a fine powder with a liquid nitrogen cooled mortar and pestle. Approximately 100 mg of frozen powder was transferred to a liquid nitrogen-frozen 2 mL Eppendorf tube and stored at -80°C until RNA was extracted.

RNA extraction: Total RNA was extracted using an acid guanidinium thiocyanate phenol chloroform extraction method similar to that described by Chomczynski and Sacchi (1987). Briefly, 1 mL of 0.8 M guanidinium thiocyanate, 0.4 M ammonium thiocyanate, 0.1 M sodium acetate pH 5.0, 5% w/v glycerol, and 38% v/v water saturated phenol were added to approximately 100 mg powdered tissue, shaken to evenly mix sample, and incubated at room temperature for 5 minutes. 200 µL chloroform was added and shaken vigorously until the entire sample became uniformly cloudy before incubation at room temperature for 3 minutes. Samples were then centrifuged at 17,000 x g at 4°C for 15 minutes, and the aqueous phase was removed and transferred to a clean 1.5 mL Eppendorf tube. 600 µL 2-propanol was added, rocked 5 to 6 times, and incubated at room temperature for 10 minutes. Samples were centrifuged 17,000 x g at 4°C for 10 minutes, and the supernatant was discarded. 1 mL 75% DEPC-treated water mixed with ethanol was added to pellet, vortexed for 10 seconds, and centrifuged 9,500 x g at 4°C for 5 minutes. Pellets were suspended in RNase free water and incubated at 37°C with RNase free DNase I for 30 minutes; the DNase I was inactivated at 65°C for 10 minutes. 450 µL buffer RLC from the Qiagen (Valencia, CA) RNeasy Plant Mini Kit was added to the digestion, processed in accordance with the manufacturer's recommendations, and eluted in 50 µL RNase free water. Extracted RNA was quality checked either with the Bio-Rad (Hercules, CA) Experion system using the Experion RNA High Sens Analysis kit or the Agilent (Santa Clara, CA) 2100 Bioanalyzer system using the RNA Nano Chip and Plant RNA Nano Assay Class.

Illumina Sequencing: The Illumina Hi Seq 2000 sequencing platform was used to sequence 2x100 PE reads from the cDNA libraries generated from the above RNA extractions at Michigan State University's Research Technology Support Facility. cDNA and final sequencing library

molecules were generated with Illumina's TruSeq RNA Sample Preparation v2 kit and instructions with minor modifications. Modifications to the published protocol include a decrease in the mRNA fragmentation incubation time from 8 minutes to 30 seconds to create the final library proper molecule size range. Additionally, Aline Biosciences' (Woburn, MA) DNA SizeSelector-I bead- based size selection system was utilized to target final library molecules for a mean size of 450 base pairs. All libraries were then quantified on a Life Technologies (Carlsbad, CA) Qubit Fluorometer and qualified on an Agilent (Santa Clara, CA) 2100 Bioanalyzer (Dr. Jeff Landgraf personal communication).

454 Sequencing: cDNA libraries were constructed from the RNA extractions using the SMARTer™ PCR cDNA Synthesis Kit from ClonTech (Mountain View, Ca.) according to the manufacturer's instructions. cDNA quality and size distribution were verified via 1% TAE gels and the Bio-Rad (Hercules, CA) Experion system. cDNA libraries were processed to attach the Rapid Library Multiplex Identification (RL MID) Adapters according to the manufacturer's protocol. Libraries were then quality checked for size distribution with Agilent's (Santa Clara, Ca.) 2100 Bioanalyzer, quantified via fluorometry, pooled, and then sequenced on Roche Applied Science's (Indianapolis, IN) Genome Sequencer FLX System with GS FLX Titanium technology.

Sequencing Data QC and Assembly: The raw reads generated by both sequencing platforms were checked for quality, and all low-quality, adaptor sequences, and contaminating reads were removed. Sequence read information from Roche's GS FLX Standard Flowgram Format (sff) files representing 70,867 Mature dataset reads and 54,462 Emergent leaf dataset reads, and Illumina HiSeq 2000 2x100 PE fastq files representing 178,716,218 Mature dataset reads and 218,726,388 Emergent leaf dataset reads, were used as input for the CLC Bio Genomics Workbench v6 and were assembled according to methods previously described by our research group (Hewitt et al., 2020, 2021; Sharpe et al., 2020). All developmental read datasets were processed with the CLC Create Sequencing QC Report tool to assess read quality. The CLC Trim Sequence process was used to trim the 454 read datasets for a Phred value of 15; and the Illumina reads were trimmed for a Phred score of 30, and the first twelve 5' bases were removed due to GC ratio variability. All read datasets were trimmed of ambiguous bases. Illumina reads were then processed through the CLC Merge Overlapping Pairs tool, and all reads were *de novo* assembled to produce contiguous sequences (contigs). Trimmed reads used for assembly were mapped back to the assembled contigs, mapped reads were used to update the contigs, and contigs with no mapped reads were ignored. Consensus contig sequences were extracted as a multi-fasta file. The individual mature and emergent leaf read datasets, from the original non-trimmed reads, were mapped back to the assembled contigs to generate individual developmental sample reads counts for each contig; read counts were then normalized with the Reads Per Kilobase per Million reads (RPKM) method (Mortazavi et al., 2008).

Functional Annotation: The OmicsBox Functional Annotation module relies on the results generated by a blastx alignment of an *in silico* nucleotide translation, Gene Ontology InterproScan using the InterPro protein families and domains database (Blum et al., 2021) (BioBam Bioinformatics, 2019). Contig sequences were identified by alignment with blastx through the Blast2GO feature (Götz et al., 2008), as well as local, stand-alone blastx alignments against the NCBI nr database (ver. 2.2.27+) (Altschul et al., 1997). Reciprocal blastn alignments

were obtained through CLC Main Workbench (ver 6) against a local stand-alone NCBI nt database (ver. 2.2.27+) (Altschul et al., 1997). Gene ontology (GO) annotation, enzyme code annotation, and the EMBL-EBI InterProScan annotation of predicted protein signatures were all annotated through OmicsBox's Blast2GO feature (Götz et al., 2008). The BLAST-annotated RNAseq datasets from the emergent and mature leaves of *Bienertia sinuspersici* were analyzed for GO enrichment by Fisher's exact test.

Differential Expression Analysis: Pairwise differential expression analyses were conducted for the emergent versus mature samples in the OmicsBox Transcriptomics Module using NOISeq-sim to compare the treatments at each time point. NOISeq-sim infers significant differential expression without experimental replicates. For absent replicates, NOISeq-sim uses a multinomial distribution to model technical replicate read counts (Tarazona et al. 2011, 2013, 2015). Default parameters were used to simulate 5 replications with a set variability of 0.02 in each replication. Genes with a NOIseq probability of greater than 0.9 and a |log2| fold change expression values greater than 1.0 for at least one treatment and time point were considered to be differentially expressed. We have previously used this approach, and these cutoff values to establish thresholds for differential expression (Hewitt et al. 2021).

GO Enrichment Analysis: GO enrichment analysis using Fisher's exact test was conducted in OmicsBox to identify the cellular components, molecular functions, and biological processes that were enriched in each of the two developmental stages (FDR-corrected p-value <0.01). Lists of the differentially expressed, functionally annotated genes were generated for the emergent and mature *Bienertia.* These lists served as the treatment datasets for enrichment analyses, and the master annotated transcriptome was used as the reference dataset. Prior to conducting enrichment analysis, the Go-Slim feature was used to reduce the number of GO terms present in the annotated reference transcriptome to overarching functions and processes displaying greatest enrichment.

Results & Discussion

Transcriptome Assembly

A total of 116,257 contigs were assembled from 141,504,502 trimmed reads with an N50 of 792 bases. All contigs greater than 200 nucleotides in length and with average read depth coverage of five times or greater were used in the generation of a robust and comprehensive transcriptome assembly that yielded a total of 72,524 expressed transcripts. Of these, 72,252 transcripts were mapped with at least a 1x coverage from the mature developmental stage read dataset (mature dataset) and 72,320 were mapped with at least a 1x coverage from the young emerging leaf developmental stage read dataset (emergent dataset). Using the functional annotation Blast2GO feature of the OmicsBox Functional Annotation module, top blast hit descriptions were assigned to 26,100 contigs, and a subset of 24,732 contigs were annotated with corresponding gene ontologies (Supplementary File 1).

Differentially Expressed Genes and Gene Ontology Enrichment Analysis

Assignment of gene ontologies (GOs) to annotated datasets, enabled identification and classification of biological processes, molecular functions, and cellular components that are overrepresented (or underrepresented) in treatment datasets, mature and emergent tissue, versus a

reference dataset of total transcripts (Consortium, 2000). Of the genes that were assigned GO annotations, 1,000 functionally annotated genes were identified to be differentially expressed in the emergent leaf tissues and 612 in the mature leaf tissue (Supplementary File 1). We utilized the OmicsBox Functional Analysis Module employing the Fisher's exact test to compare the ontologies associated with differentially expressed genes in the emergent leaf tissues to those associated with differentially expressed genes in the mature leaf tissues, for which complete results can be found in the Supplementary File 2. An FDR-corrected p-value of 0.01 was used as the cutoff for determining statistical significance and to reduce the GO assignations to the most specific terms. A total of 388 GOs associated with biological processes (bpGOs), 92 GOs associated with molecular functions (mfGOs), and 59 GOs associated with cellular components (ccGOs) were overrepresented in the emergent tissues; 9 bpGOs, 19 mfGOs, and 14 ccGOs were overrepresented in the mature tissues (Supplementary File 2). To further simplify the dataset and identify the more inclusive ontologies representative of each developmental stage, we used the OmicsBox GO-slim tool to create a reduced list of enriched ontologies. The simplified ontology list was comprised of 75 bpGOs, 21 mfGOs, and 18 ccGOs in the emergent tissue, and 5 bpGOs, 1 mfGO, and 1 ccGO in the mature tissue (Table 1, Supplementary File 2).

Results of the GO enrichment analyses suggest that transcriptomic activity of the emergent tissue is dominated by regulation of components associated with the cell cycle and cellular developmental patterning while overrepresented GOs in the mature tissue primarily related to photosynthetic processes and cellular energetics. Differentially expressed genes associated with these enriched pathways are discussed in further detail in the following sections.

Genes Associated with the Cell Cycle

It is well established that leaf cells in an emerging meristematic stage actively divide and then, as they mature, stop dividing and begin to elongate (Gonzalez et al., 2012). During the maturation stage, leaf area increases as cells undergo cell wall modification and begin to enlarge, and the consequent increase in peripheral vasculature, production of cellular contents, and cytoplasmic membrane production results in an increase of the total cytoplasmic area. During cell enlargement, cellular content and the movement of organelles are orchestrated to their most efficient functional location as the cells mature. In *Bienertia* the largest group of enriched GO terms identified in the emergent tissue are associated with the cell cycle processes. This result is similar with previously reported abundance pattern of genes in the developing leaves of *Bienertia sinuspersici* (Lara et al., 2006; Voznesenskaya et al., 2008; Offermann et al., 2011; Koteyeva et al., 2014, 2016; Sharpe and Offermann, 2014).

One of the earliest processes in cellular biogenesis is cellular duplication. During the cell cycle, cyclins (CYCs), cyclin dependent kinases (CDKs), serine threonine protein kinases, and condensin complexes work in concert to govern phase transitions of the plant cell. Analysis of the emergent vs mature *Bienertia* leaf tissue transcriptome revealed differential expression of genes corresponding to various components of the cell cycle, particularly genes associated with cellular duplication. Transcripts corresponding to *CDKB1-1*, along with *CYCA1*, *CYCA2*, *CYCA3-1*, *CYCD2*, *CYCD3*, *CYCA1-like*, and *CYCS13-6* were among those that were significantly upregulated in the emergent versus the mature tissue (Table 2); all of these are implicated in regulation of the cell cycle, and they are known to accumulate during the mitotic synthesis phase (S), the second gap (G2) phase, and the mitosis (M) phase in *Arabidopsis* (Boudolf et al., 2004, 2009; Inzé and De Veylder, 2006). Only one cyclin-associated transcript,

CDKF4, was differentially expressed in mature tissue. For the emergent tissue, significantly increased expression of transcripts corresponding to serine threonine protein kinases aurora-1, aurora-2, aurora-3 (*Aur1*, *Aur2*, *Aur3*), condensin complex subunits 1, 2, and 3 (*SMC1*, *SMC2*, and *SMC3*), and mitotic spindle checkpoint proteins mitotic arrest deficiency 2 and 3 (*Mad2* and *Mad3*) was observed. Aur and SMC proteins are important mediators of cellular mitosis, with crucial roles in G2/M transition, chromosome binding, and kinetochore separation (Collette et al., 2011; Willems et al., 2018).

Genes Involved in Cellular Developmental Patterning

Cell division and structural development are intertwined, and a portion of this interactivity is related to cytoskeletal dynamics. Composed of actin and microtubules, cytoskeletal components contribute to the structural stability of the cell, anchor proteins and the mitotic spindle cytoplasmic streaming action (Smertenko et al., 2018). Actin is mainly involved in cytoplasmic streaming while microtubules are involved in cell wall assembly.

The dimorphic chloroplasts of *Bienertia* species have been shown to interact with microtubules and actin as the chlorenchyma cell develops (Voznesenskaya et al., 2005; Chuong et al., 2006). The differential distribution of organelles, resulting from local anchoring and limited local movement, can be attributed to the chloroplast light avoidance reaction (Kasahara et al., 2002; Suetsugu et al., 2015). Microtubule-associated proteins (MAPs) interact with microtubules composed of alpha and beta-tubulin heterodimers. The MAPs belong to two main groups; non-motor MAPs, which regulate the dynamics and stability of microtubules, and MAPs with motor ability, which are the interacting molecules between the microtubules and the cellular components traversing the cell (Parrotta et al., 2014).

In addition to CDK/CYC, Aur, and SMC, the developmental patterning of leaf tissue can be monitored via gene expression activity of leucine-rich repeats-receptor-like protein kinases (LRR-RLK) family members. Members of the LRR-RLK family detect and transduce signals that initiate responses in the development of shoot organs. One clade of the LRR-RLK family members, ERECTA and the ERECTA-like genes (ERL), have been shown to localize and function in the shoot apical meristem (SAM) and the organ primordia. Previous work has shown that ERECTA mRNA is expressed at low levels in the SAM, with expression increasing in the developing vegetative organs and decreasing in the mature organ (Yokoyama et al., 1998). The *Bienertia* emergent leaf tissue displayed significantly higher expression levels of transcripts corresponding to ERECTA and ERL genes compared to the mature tissue (Table 2). Not all LRR-RLKs are developmentally expressed to induce actively dividing cells, such as those found in emergent tissues; alternatively, some act in maturing tissues to suppress cell division or in expanding tissue to generate function-specific cell types when necessary. Two such LRR-RLKs that were significantly differentially expressed at the mature leaf stage were PHLOEM INTERCALATED WITH XYLEM 2-like (PXL2-like) and wall-associated receptor kinase CLAVATA1 (CLV1). The PXL2-like gene has been shown to be involved in phloem and xylem development (Etchells et al., 2013). CLAVATA1 has been shown to facilitate phyllotaxis formation of leaf primordia, as well as suppression of undifferentiated cells at the shoot meristem, leading to the commitment of organ development (Clark et al., 1993). Two major gene families involved in receptor and transduction components of cell signaling pathways, the MAPKs and Wall-Associated Kinase-Like (WAKL), were significantly upregulated in the *Bienertia* mature leaf tissue (Verica et al., 2003; Sharma et al., 2020) (Table 2). Only one WAKL gene family member, WAKL20, was significantly expressed in the emergent tissue. WAKL20

has previously been implicated in the negative regulation of heat tolerance in *Capsicum annuum* L., and may play a similar role in mitigation of heat stress in mature *Bienertia* leaves (Wang et al., 2019). A substantially higher representation of WAKL gene family members was observed in the mature tissue, with WAKL2, WAKL4, WAKL8, and WAKL9 displaying significantly elevated expression at this developmental stage.

Genes Associated with Photosynthetic Development

Photosystem I (PSI) complex subunits, PSI-E (*PsaE*)—which facilitates binding of ferredoxin and FNR and also plays a role in cyclic electron transport (Jensen et al., 2007)—and PSI-K (*PsaK*)—which modulates organization and binding of light-harvesting complexes on the core antenna of PSI (Jensen et al., 2000, 2007)—were significantly upregulated in the emergent versus the mature *Bienertia* leaf tissues. In addition, transcripts corresponding to Photosystem II (PSII) protein D1 (*PsbA*)—which forms the reaction core of PSII as a heterodimer with the D2 protein—and light-harvesting complex B6 protein (*Lhcb6*)— a minor chlorophyll-binding antenna protein that plays a photoprotective role in alleviation of oxidative stress in PSII (Chen et al., 2018)—were upregulated in the emergent tissue.

The observed differential abundance of photosynthetic transcripts between emergent and mature leaf tissues provides insights into the progression of development of photosynthetic functionality in *Bienertia*. At the emergent stage, *Bienertia* leaves are in the process of establishing their photosynthetic capacity, evidenced by upregulation of key photosynthetic transcripts. Transition to the mature stage is accompanied by downregulation of these transcripts, as the mature leaves have already established their optimized photosynthetic system. Overall, the differential abundance of photosynthetic transcripts and patterns observed between emergent and mature leaf tissues are consistent with developing versus developed leaf.

Enriched Gene Ontologies in Transcription Factors of Photosynthetic Development

Developmental regulation is largely modulated by transcription factors (TFs). The dataset was analyzed for significantly expressed transcription factor families (Table 1). In general, a greater level of transcription factor gene expression activity was observed in the emergent tissue than in the mature tissue. Specifically, we found a higher abundance of auxin response factor (ARF), basic helix-loop-helix (bHLH), GATA, homeodomain (HD), MYB, NAC, squamosa promoter-binding protein-like (SPL), and zinc finger (ZF) family transcription factors in emergent tissue, while the mature tissue exhibited an enrichment of ethylene-responsive transcription factors (ERFs). The higher diversity and abundance of differentially expressed TF families in emergent tissue suggests coordinated TF involvement in regulating gene expression associated with early leaf anatomical and photosynthetic development, in particular, regulation of processes such as cell differentiation, growth, and establishment of leaf structures, and photosynthetic anatomy and physiology characteristic of *Bienertia*. ARF and MYB TF families are known to be involved in signaling of hormones, including auxin and gibberellin; the increased abundance of these TFs in emergent tissue implies their roles in coordinating hormonal responses necessary for leaf growth and development. NAC and SPL TF families have been implicated in stress responses and regulation of metabolic processes; the higher abundance of these TFs in emergent tissue suggests their involvement in early stress perception and adaptation mechanisms, ensuring the survival and proper development of the emergent leaves. In mature tissues, the enrichment of ERF transcription factors may indicate increased transcriptional

regulation of stress response and senescence, in addition to hormone-mediated cross talk and cell signaling.

The results gleaned from this study indicate that the developmental program to establish the SCC4 type of photosynthesis is most likely initiated at the emergent stage. What is not clear is which of these processes ultimately contribute to the SCC4 phenotype. An emerging hypothesis is that altered energy metabolism plays a role in the establishment of SCC4 photosynthesis in *Bienertia* (Han et al., 2023). This study performed transcriptome analysis at three different developmental stages along with a comparative gene expression profiling of *B. sinuspersici* and *Suaeda aralocaspica*, *Amaranthus hypochondriacus*, and *Arabidopsis thaliana*. However, besides energy flow, to understand the identity of the genes and their respective roles crucial for the entire SCC4 developmental program, there is a need to analyze additional developmental stages of the leaf along with the development of functional genomics approaches for SCC4 plants.

C4 species, overall, are better adapted to warm and dry climates with the climate trending towards warmer and more arid cycles, the future ability of C3 crops to incorporate C4 attributes will play a greater role in ensuring a food secure future (Jon E. Keeley and Philip W. Rundel, 2003; von Caemmerer, 2013; Taylor et al., 2014; Eckardt et al., 2023). A quest to understand the genetic determinants of Kranz anatomy to ultimately have the knowledge to convert C3 plants to adopt C4 photosynthesis is currently underway (Pradhan et al., 2022; Rebecca and Hirasawa, 2022). While several genes have been identified that contribute to the spatial separation of carbon fixation by Rubisco in C4 plants, research continues to identify the various genetic networks. In the same context, understanding the molecular basis underlying the SCC4 developmental program in *Bienertia* will be critical to have an expanded tool box to guide the development of climate-resilient crops (Eckardt et al., 2023).

Conclusions

Comparative transcriptome analysis of emergent and mature leaves provides an insight into the genetic components involved during the development of emergent and mature leaves in SCC4 species *Bienertia sinuspersici*. Our findings highlight the significance of cell cycle-related genes, such as cyclins and cyclin-dependent kinases, in driving cellular duplication and division during early leaf development. The enrichment of genes associated with photosystem components, light-harvesting proteins, and cellular energetics in the mature leaf tissue indicates its photosynthetically active state. The establishment of SCC4 anatomy and biochemistry is most likely a result of the orchestration of various genetic and cellular events working in concert during leaf development. The differential expression of certain transcription factors related to cell cycle progression, cellular patterning and differentiation, and microtubule-associated proteins (MAPs) and actin-related proteins implicated in cellular organization, structural stability, and the regulation of organelle movement are noteworthy. The spatio-temporal expression of these genes most likely contributes to establishing the unique leaf architecture and functionality observed in *Bienertia*. C4 photosynthesis, especially SCC4 photosynthesis, when engineered into C3 crop species, will increase the arable land area, providing increased production potential and food security.

Acknowledgments

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Table 1(on next page)

Results of GO enrichment analysis using GO-slim.

Differentially expressed (DE) genes were determined to be those with a log2(fold change) > 1 in mature vs emergent leaves, and the DE gene lists for emergent and mature leaves were used as the test sets for enrichment analysis. Results are shown by GO Category: BP = Biological process; MF = Molecular Function; CC = Cellular Component. Darkness of boxes indicates degree of enrichment, and vertical lines denote ontologies that were significantly enriched ($p<0.05$).

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GO Term	GO Name	GO Category	Emergent	Mature
GO:0015979	photosynthesis	BP		
GO:0006091	generation of precursor metabolites and energy	BP		
GO:0019725	cellular homeostasis	BP		
GO:0042592	homeostatic process	BP		
GO:0050896	response to stimulus	BP		
GO:0042221	response to chemical	BP		
GO:0009607	response to biotic stimulus	BP		
GO:0006950	response to stress	BP		
GO:0009719	response to endogenous stimulus	BP		
GO:0005975	carbohydrate metabolic process	BP		
GO:0007165	signal transduction	BP		
GO:0050794	regulation of cellular process	BP		
GO:0051716	cellular response to stimulus	BP		
GO:0023052	signaling	BP		
GO:0007154	cell communication	BP		
GO:0006810	transport	BP		
GO:0051179	localization	BP		
GO:0051234	establishment of localization	BP		
GO:0009605	response to external stimulus	BP		
GO:0009628	response to abiotic stimulus	BP		
GO:0019748	secondary metabolic process	BP		
GO:0050789	regulation of biological process	BP		
GO:0008219	cell death	BP		
GO:0012501	programmed cell death	BP		
GO:0007049	cell cycle	BP		
GO:0009987	cellular process	BP		
GO:0048856	anatomical structure deve	BP		
GO:0032502	developmental process	BP		
GO:0008152	metabolic process	BP		
GO:0009791	post-embryonic development	BP		
GO:0016043	cellular component organization	BP		
GO:0071840	cellular component organization or biogenesis	BP		
GO:0032501	multicellular organismal process	BP		
GO:0007275	multicellular organism development	BP		
GO:0003006	developmental process involved in reproduction	BP		
GO:0048608	reproductive structure development	BP		
GO:0048731	system development	BP		
GO:0061458	reproductive system development	BP		
GO:0009908	flower development	BP		
GO:0048367	shoot system development	BP		
GO:0090567	reproductive shoot system development	BP		
GO:0000003	reproduction	BP		
GO:0022414	reproductive process	BP		
GO:0065007	biological regulation	BP		
GO:0006325	chromatin organization	BP		
GO:0006338	chromatin remodeling	BP		
GO:0010468	regulation of gene expression	BP		
GO:0019222	regulation of metabolic process	BP		
GO:0040029	epigenetic regulation of gene expression	BP		
GO:0043933	protein-containing complex organization	BP		
GO:0060255	regulation of macromolecule metabolic process	BP		
GO:0071824	protein-DNA complex organization	BP		
GO:0071704	organic substance metabolic process	BP		
GO:0044238	primary metabolic process	BP		
GO:0006259	DNA metabolic process	BP		
GO:0090304	nucleic acid metabolic process	BP		
GO:0036211	protein modification process	BP		
GO:0043412	macromolecule modification	BP		
GO:0040007	growth	BP		
GO:0006629	lipid metabolic process	BP		
GO:0010467	gene expression	BP		
GO:0043170	macromolecule metabolic process	BP		
GO:0006807	nitrogen compound metabolic process	BP		
GO:0019538	protein metabolic process	BP		
GO:1901564	organonitrogen compound metabolic process	BP		
GO:0016049	cell growth	BP		
GO:0006139	nucleobase-containing compound metabolic process	BP		
GO:0006725	cellular aromatic compound metabolic process	BP		
GO:0046483	heterocycle metabolic process	BP		
GO:1901360	organic cyclic compound metabolic process	BP		
GO:0034641	cellular nitrogen compound metabolic process	BP		
GO:0044237	cellular metabolic process	BP		
GO:0009058	biosynthetic process	BP		
GO:0009056	catabolic process	BP		
GO:0065009	regulation of molecular function	BP		
GO:0030154	cell differentiation	BP		
GO:0048869	cellular developmental process	BP		
GO:0009314	response to radiation	BP		
GO:0009416	response to light stimulus	BP		

GO Term	GO Name	GO Category	Emergent	Mature
GO:0030246	carbohydrate binding	MF		
GO:0003824	catalytic activity	MF		
GO:0003700	DNA-binding transcription factor activity	MF		
GO:0140110	transcription regulator activity	MF		
GO:0003677	DNA binding	MF		
GO:0005488	binding	MF		
GO:0005515	protein binding	MF		
GO:0003774	cytoskeletal motor activity	MF		
GO:0097159	organic cyclic compound binding	MF		
GO:1901363	heterocyclic compound binding	MF		
GO:0016740	transferase activity	MF		
GO:0003676	nucleic acid binding	MF		
GO:0016787	hydrolase activity	MF		
GO:0036094	small molecule binding	MF		
GO:0000166	nucleotide binding	MF		
GO:1901265	nucleoside phosphate binding	MF		
GO:0030234	enzyme regulator activity	MF		
GO:0098772	molecular function regulator activity	MF		
GO:0003682	chromatin binding	MF		
GO:0044877	protein-containing complex binding	MF		
GO:0016301	kinase activity	MF		
GO:0016772	transferase activity, transferring phosphorus-containing groups	MF		

GO Term	GO Name	GO Category	Emergent	Mature
GO:0009579	thylakoid	CC		
GO:0005576	extracellula	CC		
GO:0030312	external encapsulating structure	CC		
GO:0005618	cell wall	CC		
GO:0043228	non-membrane-bounded organelle	CC		
GO:0043232	intracellular non-membrane-bounded organelle	CC		
GO:0016020	membrane	CC		
GO:0110165	cellular anatomical entity	CC		
GO:0005622	intracellular anatomical structure	CC		
GO:0043226	organelle	CC		
GO:0043229	intracellular organelle	CC		
GO:0005634	nucleus	CC		
GO:0043227	membrane-bounded organelle	CC		
GO:0043231	intracellular membrane-bounded organelle	CC		
GO:0005856	cytoskeleton	CC		
GO:0005737	cytoplasm	CC		
GO:0071944	cell periphery	CC		
GO:0005886	plasma membrane	CC		
GO:0005773	vacuole	CC		

Table 2 (on next page)

Differentially expressed genes associated with cell cycle, developmental patterning, and photosynthetic processes in emergent (E) and mature (M) *Bienertia sinuspersici* leaf tissue.

The log2FC (E/M) expression values that were significantly upregulated, value >1, in the young tissues are colored green, and those that were significantly upregulated, value of < -1, in the mature tissues are colored yellow.

Table 2. Differentially expressed genes associated with cell cycle, developmental patterning, and photosynthetic processes in emergent (E) and mature (M) *Bienertia sinuspersici* leaf tissue. The log2FC (E/M) expression values that were significantly upregulated, value >1, in the young tissues are colored green, and those that were significantly upregulated, value of < -1, in the mature tissues are colored yellow.

Contig #	Gene Name	Associated process	Emergent RPKM	Mature RPKM	Log2FC(E/M)
26433	Aur1	Cell cycle	28.76	4.01	2.50
26432	Aur1	Cell cycle	27.28	3.91	2.54
41110	Aur2	Cell cycle	47.22	7.13	2.42
24169	Aur3	Cell cycle	11.01	1.67	2.41
14854	CDKB1-1	Cell cycle	47.13	5.78	2.72
15194	CDKF4	Cell cycle	0.08	9.43	-7.25
8415	CYC1-like	Cell cycle	30.89	4.05	2.62
35088	CYCA1	Cell cycle	26.2	3.67	2.53
39391	CYCA2	Cell cycle	25.48	3.84	2.42
14582	CYCA3-1	Cell cycle	10	1.82	2.16
41850	CYCB	Cell cycle	47.18	7.57	2.33
49181	CYCB1	Cell cycle	23.15	3.93	2.25
37875	CYCD2	Cell cycle	18.38	2.92	2.35
10418	CYCD3	Cell cycle	141.66	19.25	2.57
41309	CYCS13-6	Cell cycle	46.52	7.58	2.31
60415	CYCS13-6	Cell cycle	23.01	3.82	2.28
19390	Mad2	Cell cycle	25.11	2.99	2.76
34358	Mad3	Cell cycle	8.36	1.36	2.31
3636	MAPKKK 1-like	Cell cycle	1.12	4.1	-2.18
13131	SMC1	Cell cycle	26.45	5.32	2.01
24311	SMC2	Cell cycle	16.77	3.22	2.07
16156	SMC3	Cell cycle	18.26	2.87	2.36
16157	SMC3	Cell cycle	10.73	2.04	2.09
19543	CLV1	Developmental patterning	2.39	8	-2.05
25901	ERECTA	Developmental patterning	87.81	11.52	2.62
3912	ERECTA	Developmental patterning	67.72	8.02	2.77
24473	PXL2-like	Developmental patterning	3.46	13.97	-2.32
30868	WAKL2-like	Developmental patterning	2.51	11.46	-2.50
53995	WAKL20	Developmental patterning	7.19	1.29	2.17
11102	WAKL4	Developmental patterning	2.85	11.77	-2.35
28646	WAKL8-like	Developmental patterning	1.25	4.75	-2.23
28645	WAKL9-like	Developmental patterning	1.2	5.36	-2.46
3756	Carbonic anhydrase	Photosynthesis	27.68	146.62	-2.71
169	LCHB	Photosynthesis	2975.1	9136.22	-1.92
34	LHCB-like	Photosynthesis	1505.8	5130.86	-2.07
3123	LHCB6	Photosynthesis	180.73	474.78	-1.70
5116	LIL3	Photosynthesis	206.24	43.94	1.92
11856	PsaC	Photosynthesis	1.52	5.28	-2.11
198	PsaE	Photosynthesis	311.53	1295.95	-2.36
266	PsaH	Photosynthesis	1129.81	3158.12	-1.79
721	PsaI	Photosynthesis	753.25	2097.68	-1.78
599	PsaK	Photosynthesis	674.62	2059.64	-1.92
771	PsbY	Photosynthesis	222.42	578.99	-1.69
18279	PSII D1	Photosynthesis	3.17	14.73	-2.52
819	RIP1 precursor	Photosynthesis	26.32	430.38	-4.34

Table 3 (on next page)

Differentially expressed transcription factor-encoding genes in emergent (E) and mature (M) *Bienertia sinuspersici* leaf tissue.

The log2FC (E/M) expression values that were significantly upregulated, value >1, in the young tissues are colored green, and those that were significantly upregulated, value < -1, in the mature tissues are colored yellow.

Table 3. Differentially expressed, transcription factor-encoding genes in emergent (E) and mature (M) *Bienertia sinuspersici* leaf tissue. The log2FC (E/M) expression values that were significantly upregulated, value >1, in the young tissues are colored green, and those that were significantly upregulated, value < -1, in the mature tissues are colored yellow.

Contig #	Gene Name	TF Class	Emergent RPKM	Mature RPKM	Log2FC(E/M)
32838	AP2 domain-containing transcription factor	AP2	11.89	1.57	2.61
87	Auxin response factor 3-like	ARF	22.68	3.29	2.48
16663	Auxin response factor 4	ARF	30.09	5.02	2.28
33726	Auxin response factor 4-like	ARF	16.12	2.75	2.24
56357	b3 domain-containing protein	B3	6.63	0.8	2.74
37973	bHLH domain-containing protein	bHLH	10.96	2.16	2.03
3634	Transcription factor bHLH 145-like	bHLH	43.85	6.04	2.56
31753	Transcription factor bHLH66	bHLH	6.2	1.17	2.10
47610	Transcription factor bHLH93	bHLH	22.78	4.51	2.03
13021	Transcription factor bHLH96-like	bHLH	51.6	11.51	1.86
21892	Transcription factor ORG2-like	bHLH	9.4	1.61	2.24
27143	Transcription factor SPEECHLESS-like	bHLH	13.74	1.04	3.42
72889	Ethylene-responsive transcription factor ERF017-like	ERF	25.84	0.71	4.88
83348	Ethylene-responsive transcription factor	ERF	12.51	1.62	2.64
57854	GATA transcription factor	GATA	12.49	2.16	2.23
54258	GATA transcription factor 18-like	GATA	4.76	0.81	2.25
13232	GRAS transcription factor	GRAS	11.15	0.88	3.36
34385	GRF domain class transcription factor	GRF	26.77	4.53	2.26
49851	Homeobox-leucine zipper protein ATHB-40-like	HD	15.76	1	3.67
25421	Homeobox-leucine zipper protein ATHB-6-like	HD	9.3	1.74	2.11
22718	Homeobox-leucine zipper protein HDG2-like	HD	51.85	9.66	2.12
20552	Homeobox-leucine zipper protein ROC3-like	HD	18.95	3.1	2.31
27094	Wuschel-related homeobox 1-like	HD	4.84	0.74	2.41
25331	Heat stress transcription factor B-4	HSF	7.28	1.14	2.37
57231	MYB transcription factor	MYB	5.78	1.02	2.20
32314	MYB transcription factor r2r3	MYB	6.44	1.13	2.21
17730	MYB-related protein 3r-1-like	MYB	29.21	4.41	2.42
16556	MYB-related protein 3r-1-like	MYB	32.22	5.36	2.28
17731	MYB-related protein 3r-1-like	MYB	11.04	1.9	2.23
40625	MYBJ6 transcription factor	MYB	20.44	3.47	2.25
17270	Transcription factor ICE1	MYC	59	13.07	1.87
49741	NAC domain containing protein ipr003441	NAC	14	1.33	3.09
24842	NAC domain-containing protein 8-like	NAC	4.88	0.76	2.38
23556	SQUAMOSA promoter binding protein	SPL	23.77	1.53	3.65
36093	SQUAMOSA promoter binding protein	SPL	31.84	4.88	2.40
10750	SQUAMOSA promoter binding-like protein 12-like	SPL	89.29	18.25	1.98
19993	SQUAMOSA promoter binding-like protein 16-like	SPL	14.65	2.5	2.25
36043	SQUAMOSA promoter-binding-like protein 8	SPL	22.67	2.75	2.74
30755	Trihelix transcription factor	TTF	22.65	4.1	2.16
43513	C2H2 zinc finger protein	ZF	27.98	3.01	2.91
27533	ZF CCCH domain-containing protein	ZF	9.97	1.37	2.56
53388	ZF CCCH domain-containing protein 31	ZF	5.69	0.33	3.80
28812	ZF CCCH domain-containing protein 66-like	ZF	31.56	6.68	1.93
11427	ZF protein nutcracker-like	ZF	13.45	2.3	2.24
41587	ZF-HD homeobox protein at4g24660-like	ZF	22.94	4.01	2.21
25703	ZF-HD homeobox protein at4g24660-like	ZF	10.85	2.11	2.06
40970	ZF-HD homeobox protein at4g24660-like	ZF	79.33	17.74	1.85
40810	AP2 domain-containing transcription factor	AP2	0.83	4.39	-2.71
16662	Auxin response factor 4	ARF	2.81	15.17	-2.74
13125	Transcription factor UNE10-like	bHLH	12.75	37.22	-1.85
16393	bZIP transcription factor	bZip	28.02	67.59	-1.58
43088	DOF zinc finger transcription factor	DOF	2.7	11.56	-2.40
46784	Ethylene responsive transcription factor ERF2	ERF	2.22	7.92	-2.14
26140	Ethylene responsive transcription factor ERF1A	ERF	2.63	10.13	-2.25
10322	Dehydration-responsive element binding protein	ERF	22.94	96.89	-2.38
28362	Ethylene responsive transcription factor ERF1b-like	ERF	1.18	6.07	-2.66
52853	Ethylene responsive transcription factor ERF098-like	ERF	0.28	4.12	-4.17
25984	GRAS transcription factor	GRAS	1.67	7.21	-2.42
21136	Homeobox-leucine zipper protein ATHB-12-like	HD	4.73	15.42	-2.01
25288	Homeobox-leucine zipper protein ATHB-52	HD	0.06	3.75	-6.21
29142	Heat stress transcription factor	HSF	1.39	6.17	-2.46
11797	Telomere repeat binding factor 1	MYB	10.18	36.07	-2.13
9320	Myb-related protein 1	MYB	1.82	8.71	-2.57
27346	Two-component response regulator ARR2	MYB	0.3	4.73	-4.30
776	CONSTANS protein	ZF	188.15	483.34	-1.67
4333	COL domain class transcription factor	ZF	111.27	300.94	-1.74