Engineering CAM Photosynthetic Machinery into Bioenergy Crops for Biofuels Production in Marginal Environments

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Description/Abstract
CAM provides an excellent opportunity to engineer enhanced photosynthetic performance and water-use efficiency (WUE) into bioenergy crops. Our vision was to understand the enzymatic and regulatory pathways required for CAM in order to engineer CAM photosynthetic machinery into *Arabidopsis* and *Populus*. Major advances were made in understanding the molecular "parts list" necessary for engineering CAM through the sequencing the genomes and transcriptomes of several model and agronomically important CAM species including *Agave*, *Ananas*, Clusia, *Kalanchoe*, *Mesembryanthemum*, and *Opuntia*. Comparison of diel co-expression network analyses in *Agave*, *Ananas*, and *Kalanchoe* with expression patterns in C_3 photosynthesis species revealed the rescheduling of gene expression associated with stomatal opening and closing and led to the identification of many candidate CAM-related genes for further functional testing. Both RNAi-mediated gene silencing and CRISPR/Cas genome editing for gene knockout were used to investigate over 70 CAM-related enzymes, regulatory kinases/phosphatases, and transporters and 25 transcription factors implicated in playing key regulatory roles in CAM. Knockdown of key C_4 cycle enzymes within the carboxylation and decarboxylation modules of CAM not only perturbed CAM itself, but also altered the expression of core circadian clock components demonstrating that loss of CAM function also perturbs the central circadian clock and alters the regulatory patterns of key guard cell signaling genes that are linked with the characteristic inverse pattern of stomatal opening and closing during CAM. An advanced system for joining D.AN fragments from a universal library was developed that automatically maintains open reading frames (ORFs) and does not require linkers, adaptors, regions of sequence homology, amplification (or mutation for domestication) of fragments to function systems for the construction of gene circuits. Alternatively, Gibson assembly was used to create gene circuits consisting of up to 15 unique gene expression cassettes expressing either the core C_4 cycle of CAM comprising carboxylation or decarboxylation modules or both modules together. The expression of each gene cassette was driven by a drought-stress inducible 5' regulatory region or promoter (and cognate 3' terminator region) from *A. thaliana* so that CAM gene expression would be enhanced under conditions of water-deficit stress. These gene circuits were introduced into the C_3 photosynthesis model species *A. thaliana*. Expression of the nocturnal carboxylation module resulted in increased biomass and seed yield arising from net CO_2 fixation at night and malate accumulation typical of a CAM plant along with daytime CO_2 fixation typical of a C_3 photosynthesis plant. To create a leaf anatomy suited for the optimal performance of CAM in a C_3 photosynthesis plant, increased tissue succulence was engineered in *A. thaliana* (and *Nicotiana sylvestris*), which also improved biomass and seed yield and drought attenuation and improved salinity tolerance. The basic CAM Biodesign principles described in this report can be extended to increase the productivity and WUE of food and bioenergy crops and are expected to have broad applicability and ensure sustainable biofuel feedstock production in the face of the global climate crisis.

Project Overview

CAM provides an excellent opportunity to engineer enhanced photosynthetic performance and water-use efficiency (WUE) into bioenergy crops. Our vision was to understand the enzymatic and regulatory pathways required for CAM in order to engineer CAM photosynthetic machinery into *Populus*. Target species included *Arabidopsis* as a simple proof-of-concept test species and *Populus* as a dedicated bioenergy crop. To achieve this long-term goal, four major objectives for enhancing photosynthetic performance and WUE in *Populus* were pursued: 1) Define the genetic basis of key CAM modules in both dicot and monocot CAM species via co-expression modeling using 'omics (transcriptome and metabolome) technologies. 2) Characterize the regulation of 'carboxylation', 'decarboxylation', and 'inverse stomatal control' modules of CAM using comparative genomics, network and molecular dynamics modeling. 3) Deploy advanced genome engineering technologies to enable stacking of a large number of transgenes into a single genomic locus to improve transgene persistence and transfer fully functional 'carboxylation' and 'decarboxylation' modules from CAM species to C_3 species that can accommodate overnight malic acid storage in the vacuole. 4) Analyze the effects of these transgenic modules on 'stomatal control', CO_2 assimilation and transpiration rates, biomass yield, and WUE in *Arabidopsis* and *Populus*. CAM was engineered under the control of drought-inducible promoters with appropriate circadian clock controlled expression in order to maximize photosynthetic productivity by invoking CAM only when prevailing conditions require its operation. The project has broad applicability and impact for expanding and ensuring sustainable biofuel feedstock production.

We outlined the basic requirements, design principles, and approaches and potential design considerations necessary to increase WUE in bioenergy crops in a series of informative review articles (Borland et al., 2014; Borland et al., 2015). Discussion of specific synthetic biology approaches for CAM Biodesign was also summarized (DePaoli et al., 2014). The genetic and epigenetic regulation of drought responsive pathways and genomic resources developed for bioenergy crops within a comparative and evolutionary genomics context was leveraged to provide new insights beyond what is known within individual species and to create new opportunities for studying drought tolerance using the emerging technologies (Yin et al., 2014). The critical importance of water-saving CAM species as bioenergy feedstocks for semi-arid and arid lands was also highlighted (Cushman et al., 2015). A road map for CAM Biodesign highlighted the need for improving WUE in plants and outlined the fundamental paths for achieving the goal of development and impacts of using water-wise bioenergy feedstocks (Yang et al., 2015). Key differences in carbohydrate processing in CAM species relative to C_3 photosynthesis species were explored for informing CAM Biodesign engineering strategies (Borland et al., 2016). Basic and applied research perspectives on genome editing, synthetic biology, and the development and utilization of CAM crops summarized the need for high-throughput phenotyping and functional genomics research for CAM engineering to enhance WUE and drought tolerance in plants (Liu et al., 2018). Comparative genomic and evolutionary genomics approaches led to a deeper understanding of the CAM-related genes and gene functions critical for engineering CAM into crops (Yang et al., 2019). The physiological implications of leaf anatomical traits have also been discussed in terms of water use and leaf hydraulic properties as well as the impacts on CO_2 uptake and carbon gain that are likely to be important determinants for the mode and level of CAM that might be engineered into non-CAM species as a means of improving plant water-use efficiency (Borland et al., 2018). We have further explored the importance of many co-adapted traits of CAM plants including tissue succulence, thick cuticles, epicuticular wax, low stomatal density, high stomatal responsiveness, and shallow rectifier-like roots that limit water loss under conditions of water deficit (Niechayev et al., 2019). In addition to abiotic stress, the availability and assimilation of mineral nutrients such as nitrogen, phosphorus, potassium, and calcium can have a major impact on the expression and regulation of CAM (Pereira and Cushman, 2019). Lastly, various aspects of comparative and functional genomics in both model and non-model species were highlighted in terms of the recent advances that motivate further investigations into gene function and interaction (Yang et al., 2015).

Project Accomplishments:

Aim 1: Define the genetic basis of key CAM modules in both dicot and monocot CAM species via co-expression modeling using 'omics (transcriptome, proteome and metabolome) technologies. The approaches used in this aim included RNA-Seq transcriptome analyses, proteomic analyses, metabolome profiling, genome sequencing, and chromatin immunoprecipitation-deep DNA sequencing (ChIP-Seq).

In order to understand the enzymatic and regulatory requirements for CAM performance, we characterized the genomes of several model and agronomically important CAM species. In order to successfully design and transfer fully functional modules that permit the temporal separation of carboxylation and decarboxylation processes, along with a day/night pattern of stomatal closure/opening, transcriptome data sets were collected that captured temporal and tissue-specific (e.g., epidermal peels, mesophyll) mRNA and protein expression patterns along with concurrent metabolomic profiling. For monocot CAM species, the pineapple (*Ananas comosus*) genome was sequenced in an effort lead by Ray Ming's lab at the University of Illinois along with transcriptome time courses (Ming et al., 2015) and more detailed temporal and special transcriptomic and miRNA dynamics (Wai et al., 2017). Integrated transcriptomic, proteomic, and metabolomic temporal dynamics revealed key differences in the regulation of CAM-related processes between CAM and C_3 photosynthesis species (e.g., *Arabidopsis thaliana*) including the identification of diel co-expression modules and evidence for the rescheduling of genes associated with stomatal opening and closing (Abraham et al., 2016).

For dicot species, the obligate CAM *Kalanchoe fedtschenkoi* genome was sequenced along with detailed diel transcriptomic analyses to reveal rescheduling of mRNA expression patterns of genes involved in nocturnal CO_2 fixation, stomatal movements, heat tolerance, circadian clock control, and carbohydrate metabolism as well as discovery of amino sequence signatures associated with the convergent evolution of CAM (Yang et al., 2017). Genome and transcriptome sequencing for the facultative CAM species, *Mesembryanthemum crystallinum* was completed (Yim et al., in preparation). The *M. crystallinum* transcriptome was leveraged to reveal miRNAs involved in responses to high salinity stress (Chiang et al., 2016). The genome (and transcriptome) of the diploid, prickly pear cactus (*Opuntia* cochenillifera L.), an obligate CAM species was also completed (Yim et al., in preparation). Lastly, RNA-Seq was used to reveal transcript-abundance regulation and photosynthetic physiology in *Portulaca oleracea* indicating that C_4 and CAM coexist within a single *P. oleracea* leaf under mild drought conditions (Ferrari et al., 2019).

In order to improve the confidence of protein-protein interaction predictions, a novel system called protein-protein interaction (PPI) classifiers merger (PPCM) was developed that combines two existing PPI tools using Random Forest algorithms (Yao et al., 2015). The result is increased reliability of PPI prediction and will be particularly useful in non-model species. In order to accelerate BLAST and other sequence analysis tools, Divide and Conquer BLAST (DCBLAST) was developed to perform NCBI BLAST searches within a cluster, grid, or HPC environment by using a query sequence distribution approach (Yim and Cushman, 2017). A proteome classification approach was developed to organize proteins within a proteome based on the distribution of two protein attributes or fingerprints: protein lengths and protein intrinsic disordered contents (Guo et al., 2018). Proteomic analysis in *M. crystallinum* revealed marked temporal changes in the mitochondrial proteome likely related to CAM function (Hong et al., 2019). A complete temporal analysis of protein abundance changes during the C_3 photosynthesis to CAM transition was performed for *M. crystallinum* (Abraham et al., in preparation).

Aim 2: Characterize the regulation of 'carboxylation', 'decarboxylation', and 'inverse stomatal control' modules of CAM using comparative genomics, network and molecular dynamics modeling. The approaches taken in this aim included co-expression and gene regulatory network modeling by computational simulation, validation of CAM module design via loss-of-function knock-down *K. fedtschenkoi* and testing of CAM module design using transient and stable gene expression systems.

Novel approaches for the discovery and creation of co-expression networks were developed (Petereit et al., 2016). A novel tool called petal allows for the creation of co-expression network models of whole-genomics

experiments and has been implemented in R. Its application to several whole-genome experiments has generated novel meaningful results and has allowed for testing new hypothesizes for further biological investigation.

Integrated genomics, gene co-expression networks, comparative genomics and protein structure analyses, along with comparative genomics analyses among analyses among C_3 photosynthesis, C_4 photosynthesis, and CAM species were used to identify the core metabolic components required for CAM in *Agave* (Yin et al., 2018). This investigation showed that the metabolic components required for CAM are shared by non-vascular and vascular plants, but that the regulatory proteins involved in diel re-programming of carbon fixation and metabolite transport appeared more recently and are shared among C_3 photosynthesis, C_4 photosynthesis, and CAM species.

Diel time-course gene expression data using multiple periodicity detection algorithms were used to identify rhythmic gene sets in *K. fedtschenkoi* and *A. thaliana* (Moseley et al., 2018). Clustering of ortholog protein groups by sequence conservation and rhythmic gene expression resulted in five groups of genes with minimal functional overlap. CAM-related orthologs displayed phase shifts between species suggesting several mechanisms by which the circadian clock plays a role in the evolution of CAM. Candidate core CAM genes were identified within a periodic gene network and then a Local Edge Machine (LEM) algorithm was used to infer regulatory relationships between the candidate core clock genes and orthologs of known core clock genes in *K. fedtschenkoi* (Moseley et al., 2019). LEM was also used to identify stomata-related gene targets for *K. fedtschenkoi* core clock genes and to construct a subsequent gene regulatory network to provide new insights into the mechanism of circadian control of CAM-related genes in K. *fedtschenkoi*. A comparative analysis of diel time-course gene expression data was performed between *K. fedtschenkoi* and two C_3 photosynthesis species (i.e., *Arabidopsis thaliana* and *Solanum lycopersicum*) to identify differential gene expression between the dusk and dawn phases of the 24-h cycle (Moseley et al., 2019). A rescheduled catalase gene known to be involved in stomatal movement was identified, suggesting a role for H_2O_2 in CAM-like stomatal movement. A genome-wide expression atlas was created for *K. fedtschenkoi* grown under different light intensities and qualities resulting in the creation of a light-responsive co-expression network (Zhang et al., 2019). These results support the hypothesis that both light intensity and light quality can modulate the CAM pathway through regulation of CAM-related genes in *K. fedtschenkoi*. Similar conditions were also used for gene atlas expression profiling in the facultative CAM species, *M. crystallinum*.

Strategies for knocking down/out up to seven non-homologous genes using multiple synthetic RNAi fragments in C_3 photosynthesis host species were also demonstrated in *Arabidopsis thaliana* (Czarnecki et al., 2016). Such a system might be necessary for rewiring complex sets of genes with diel expression patterns shared between C_3 photosynthesis and CAM species. To expedite the use of CRISPR/Cas genome editing, a genome-wide gRNA database targeting 45,183 protein-coding genes annotated in the *K. fedtschenkoi* genome has been developed (Liu et al., 2019).

Loss-of-function assays have been used to probe the functional contribution of key enzymes and regulatory kinases/phosphatases, and transporters of CAM-related genes in the obligate CAM models *Kalanchoe fedtschenkoi* and *K. laxiflora* for which a reliable, efficient, and robust transformation system is available (Hartwell et al., 2016). Knockdown of either mitochondrial NAD-ME, or cytosolic/plastidic PPDK, dramatically reduced CAM performance, reduced the activity of other CAM enzymes, particularly PEPC, and reduced the circadianly controlled phosphorylation of PEPC by PPCK and disrupted or dampened the circadian rhythmic expression of PEPC phosphorylation and PEPC kinase transcript accumulation, and CAM CO_2 fixation patterns (Dever et al., 2015). Knockdown of NADP-ME did not have the same effect, suggesting that NAD-ME is the major decarboxylation enzyme of CAM in this species. Knockdown of phosphoenolpyruvate carboxylase (PEPC) kinase, which is the dedicated regulatory protein kinase of PEPC, resulted in reduced or no detectable nighttime phosphorylation of PEPC and up to a 66% reduction in nocturnal CO_2 fixation, reduced malate accumulation at dawn and reduced nocturnal starch turnover, and perturbed the expression of many core circadian clock genes suggesting that loss of CAM function also perturbs the central circadian clock (Boxall et al., 2017). Lastly, RNAi

silencing of phosphoenolpyruvate carboxylase 1 in the obligate CAM species *Kalanchoe laxiflora* resulted in a loss of dark period CO_2 fixation and malate accumulation while causing arrhythmic expression of some components of the core circadian clock, while enhancing the expression and rhythmic oscillations of a different subset of genes and adjusting the temporal regulation and relative abundance of guard cell signaling genes (Boxall et al., 2019). These results provided direct evidence that the altered regulatory patterns of key guard cell signaling genes are linked with the characteristic inverse pattern of stomatal opening and closing during CAM. In the obligate CAM species K. *fedtschenkoi*, guard cell anion channel activity was shown to follow KfSLAC1 and KfALMT12 transcript abundance, declining to near-zero by the end of the light period. Furthermore, extracellular Mal suppressed anion currents of *Kalanchoë* guard cells, both in wild-type and RNAi mutants with impaired Mal metabolism. Thus, the diurnal cycle of anion channel gene transcription, rather than the physiological signal of Mal release, is a key factor in the inverted CAM stomatal cycle (Lefoulon et al., 2019).

Analysis of additional CAM-gene loss-of-function mutants using RNAi or overexpression is in progress in stably transformed *K. laxiflora* accession 1982-6028. RNAi constructs were designed and constructed in intron-containing hairpin RNA Gateway-ready binary vector pK7GWIWG2(II), and/ or 35S constitutive over-expression vector pK2GW7 in the case of the full-length over-expression of the coding sequence. The genes targeted included the majority of CAM-associated genes that were induced coincident with CAM in leaf pair 6 relative to leaf pair 1 that performs C_3 photosynthesis. For multi-gene families, each candidate CAM-associated gene family member that showed induction of transcript abundance coincident with CAM in leaf pair 6 was selected and targeted in separate stable transgenic lines of *K. laxiflora*. For example, in the case of malate dehydrogenase (MDH), genes encoding cytosolic-MDH, plastidic NAD-MDH, peroxisomal MDH, and plastidic NADP-MDH were targeted. Publications describing the detailed phenotypic characterization of the ALMT RNAi lines and the KNAT3 RNAi lines are in preparation. In summary, 69 CAM enzymes and transporters, and 12 transcription factors were targeted by RNAi-mediated gene silencing in Kalanchoe. In addition, 13 and 12 HA-tagged transcription factors are being analyzed by ChIP-seq analysis at the University of Liverpool and University of Nevada, respectively.

Aim 3: Deploy advanced genome engineering technologies to enable stacking of a large number of transgenes into a single genomic locus. The approaches taken in this aim included CAM gene module assembly, site-specific stacking of CAM modules in *A. thaliana* and succulence engineering in *A. thaliana*. Success of proof-of-concept experiments have now set the stage to undertake similar engineering in *Populus*.

For assembly a complex gene circuits, an advanced system for joining DNA fragments from a universal library was developed that automatically maintains open reading frames (ORFs) and does not require linkers, adaptors, regions of sequence homology, amplification (or mutation for domestication) of fragments to function (De Paoli et al., 2016). This system will facilitate the assembly of complex multi-gene circuits from different DNA fragments. Similarly, advances in genome editing using CRISPR/Cas approaches were summarized with an emphasis on editing plant genomes and applications for perturbing gene expression through loss or gain of function or spatiotemporal changes, dynamic visualization of genomic loci, characterization of gene modules, gene stacking, transcriptional reprogramming of endogenous genes, and building of synthetic gene circuits (Liu et al., 2016). CRISPR/Cas systems can also be used to functionally characterize non-coding RNAs, which represent attractive targets for functional characterization and acceleration of the domestication of horticultural crops (Liu et al., 2017). Advances in CRISPR/Cas genome editing were applied to CAM species including K. *fedtschenkoi*. For example, knockout of the PHOT2 gene in this species reduced stomatal conductance and CO_2 fixation in the late afternoon, but increased stomatal conductance and CO_2 fixation in the early evening suggesting a role for blue-light signaling in controlling CAM (Liu et al., 2019).

A basic helix-loop-helix (bHLH) transcription factor from wine grape called VvCEB1 was used to engineer increased tissue succulence in the model C_3 photosynthesis plants *A. thaliana* (and *Nicotiana sylvestris*) while also increasing vegetative biomass, reproductive organ size, number, and seed yield through auxin-mediated transcriptional reprogramming (Lim et al., 2018). Examination of diverse *Clusia spp.* with varying degrees of

succulence has shown that leaf anatomical configurations with enlarged and densely packed palisade mesophyll cells, as well as large vacuoles, can accommodate greater nocturnal C_4 carboxylation and organic acid storage capacity than less succulent species (Barrera Zambrano et al., 2014). *A. thaliana* overexpressing VvCEB1 also show a striking improvement in drought attenuation and salinity tolerance (Lim et al., 2019). The mechanistic basis for these improvements included a reduction in stomatal density and apertures combined with a dilution of ion concentrations within leaf tissues. Stress-responsive transcription factors from *M. crystallinum* have been used to engineer improved abiotic stress tolerance in *A. thaliana* (Amin et al., 2019).

Aim 4: Analyze the effects of these transgenic modules on 'stomatal control', CO_2 assimilation and transpiration rates, biomass yield, and WUE in *Arabidopsis* and *Populus*. The approaches taken in this aim included real-time RT-PCR analysis of expression analysis of transgenes, measurement of biochemical parameters, CO_2 assimilation, stomatal conductance and transpiration rates, leaf carbon balance and level/mode of CAM activity, and biomass productivity of transgenic plants. Several high-throughput methods have been developed to the reduce costs and improve the resolution of assessing the circadian rhythms of plants including leaf movements, delayed fluorescence, and infra-red gas exchange analysis (IRGA) (Tindall et al., 2015).

We have laid the foundation for CAM Biodesign by investigating the subcellular localization and phenotypic effects of ectopic overexpression of each of 13 enzymes of the core C_4 carboxylation/ decarboxylation pathway of CAM in the C_3 photosynthesis model *A. thaliana* (Lim et al., 2019). Overexpression of most carboxylation module components resulted in increased stomatal conductance and dawn/dusk titratable acidity (TA) as an indirect measure of organic acid (mainly malate) accumulation in *A. thaliana*. In contrast, overexpression of the decarboxylating malic enzymes reduced stomatal conductance and TA. This comprehensive study provided fundamental insights into the relative functional contributions of each of the individual components of the core C_4-metabolism cycle of CAM and represents a critical first step in laying the foundation for CAM Biodesign.

Multi-site Gateway cloning was combined with Gibson assembly to create gene circuits consisting of up to 15 unique gene expression cassettes expressing either the core C_4 cycle of CAM comprising carboxylation or decarboxylation modules or both modules together. The expression of each gene cassette was driven by a drought-stress inducible 5' regulatory region or promoter (and cognate 3' terminator region) from *A. thaliana* so that CAM gene expression would be enhanced under conditions of water-deficit stress. These gene circuits were introduced into the C_3 photosynthesis model species *A. thaliana*. Expression of the nocturnal carboxylation module resulted in increased biomass and seed yield arising from net CO_2 fixation, increased stomatal conductance, and malate accumulation at night typical of a CAM plant along with daytime CO_2 fixation typical of a C_3 photosynthesis plant (Lim et al., 2020). Detailed analysis of the decarboxylation module and the combined carboxylation/ decarboxylation modules is in progress.

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Bilgi *et al.* Large-scale metabolic profiling of wildtype and CAM/starch-deficient mutant of the facultative CAM species, *Mesembryanthemum crystallinum* during acclimation to drought stress reveals circadian oscillations in key metabolites. *In preparation*.

Boxall, *et al.* RNA-seq analysis of diel time course samples from separated epidermal peels and mesophyll tissue from Crassulacean acid metabolism (CAM) leaves of *Kalanchoë fedtschenkoi* reveals the transcriptome-wide reprogramming of guard cell signaling and mesophyll metabolism underpinning the temporal optimisation of CAM. *In preparation*.

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Yim *et al*. The genome of the common ice plant (*Mesembryanthemum crystallinum* L.) a facultative crassulacean acid metabolism (CAM) species. *In preparation*.

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Patent awarded:
Tuskan GA, Yang X, De Paoli HC. TNT Cloning System. (US Patent No.: 10,017,770 B2; Date of patent: July 10, 2018). This invention was selected as a 2018 R&D 100 Award Finalist.

Patents pending:
Genes for enhancing drought and heat tolerance in plants, and methods of use (2018; Invention Disclosure 201603693, DOE S-138,334).

Sung Don Lim and John C. Cushman (2018) Methods for Engineered Tissue Succulence in Plants. U.S. Patent Application No. US15/977,930, filed on April 11, 2018.

Sung Don Lim, Won Cheol Yim, John C. Cushman (2018) Engineered Crassulacean Acid Metabolism (CAM) Pathways in Plants. U.S. Patent Application No. US62/276,438, filed on July 13, 2018.

Invention disclosures:
Invention disclosure 201804140 Gene for enhancing salt and drought tolerance in plants.
Invention disclosure 201804142 Gene for enhancing photosynthetic performance and biomass production in plants.

Additional data sets generated:
The University of Nevada – Reno has generated Sanger, 454, and Illumina-based RNA-seq data sets for the facultative CAM species *Mesembryanthemum crystallinum* under Short Read Archive (SRA) projects PRJNA245116, PRJNA217685. The Transcriptome Shotgun Assembly (TSA) project has been deposited at DDBJ/EMBL/GenBank under GBLK01000000 - GBLK01024204. The complete *M. crystallinum* genome assembly has been deposited under PRJNA354455.

The Centre for Genomic Research (CGR), University of Liverpool Bioinformatics team has completed the PacBio *de novo* whole genome assembly, HiC scaffolding by Phase Genomics, and annotation of the 17 chromosome-scale scaffolds by the has for diploid *Kalanchoë fedtschenkoi* (Kew/ Glasgow/ Liverpool [KGL] accession/ ecotype). CGR has completed the PacBio *de novo* whole genome assembly and annotation for diploid *K. laxiflora* accession (Oxford Botanic Gardens accession 2014019.4) that is able to set viable seed following self-fertilization. CGR has completed the PacBio de novo whole genome assembly, HiC scaffolding by Phase Genomics generating 17 chromosome-scale scaffolds, and annotation of the scaffolds by the CGR Bioinformatics team, Liverpool for diploid *K. gracilipes* (C_3 species with drought-inducible weak CAM).

CGR has completed the Illumina-based RNA-seq analysis of a light/ dark time course of RNA isolated from CAM leaves (leaf pair 6) of *K. fedtschenkoi* KGL accession sampled at 2 h intervals throughout 24 h under 12-h-light/ 12-h-dark cycles. Epidermal peels, which are enriched for intact stomatal guard cells, were separated from the underlying mesophyll tissue, which had the major veins removed, and RNA isolated for each tissue at each time point (72 RNA samples). CGR has completed the Illumina-based RNA-seq analysis of a light/dark time course of RNA isolated from CAM leaves (leaf pair 6) of *K. fedtschenkoi* KGL wild type accession and CAM mutant RNAi lines rNAD-ME1, rPPDK1 (Dever et al., 2015) and rPPCK1 (Boxall et al., 2017) plus a circadian clock mutant TOC1-overexpressor (unpublished), sampled at 4h intervals throughout 24 h under 12-h-light/ 12-h-dark cycles (90 RNA samples). The same samples used for the above RNA-seq analysis comparing wild type *K. fedtschenkoi* and the four transgenic mutants were also submitted for non-targeted metabolomics analysis performed by Metabolon – project code UNNV-01-14VW.

CGR has completed Illumina-based RNA-seq analysis of a light/ dark time course of RNA isolated from CAM leaves (leaf pair 6) of *K. laxiflora* wild type and CAM mutant RNAi line *PPC1* RNAi line B (*rPPC1-B;* Boxall et al., 2019). Leaf pair 6 from both lines were sampled at 4 h intervals over 24 h under 12-h-light-12-h-dark cycles (36 RNA samples). CGR has completed the Illumina-based RNA-seq analysis of a light/ dark time course of RNA isolated from CAM leaves (leaf pair 6) of *K. laxiflora* wild type and CAM mutant *KNAT3* RNAi (Boxall et al., unpublished). Leaf pair 6 from both lines were sampled at 4 h intervals over 24 h under 12-h-light/ 12-h-dark cycles (36 RNA samples). CGR has completed the Illumina-based RNA-seq analysis of a drought-stress induction of weak CAM experiment on *K. gracilipes*. Leaf pairs 1 & 2 were sampled separately from leaf pairs 3, 4 & 5 from both well-watered control plants and drought-stressed plants. Both developmental stages of leaves were sampled every 4 h over a 12-h-light/ 12-h-dark cycle (72 RNA samples). CGR has completed the Illumina-based RNA-seq analysis of a drought-stress induction of CAM time course on both wild type *Kalanchoë laxiflora* and the *KNAT3* RNAi line. Leaf pair 6 were sampled at 3, 6 and 8 days after water-witholding, with samples collected

at both 2 h before dawn (22:00) and 2 h before dusk (10:00). Gas exchange measurements on equivalent plants demonstrated that wild type developed stronger CAM (especially reduced phase II and IV and extended phase II across the whole of the light) during this short-term drought treatment, and that the *KNAT3* RNAi mutant transitioned from performing C3 photosynthesis when well-watered to CAM following drought-stress (36 RNA samples).

To complement traditional genomic sequencing, the CGR has undertaken methyl-Seq using the NEB-Next Methyl-Seq kit to profile the genome wide methylation patterns of genomic DNA from wild type *K. laxiflora* and the *KNAT3* RNAi mutant both well-watered (day 3 after water with-holding) and drought-stressed (day 8 after water with-holding) with samples collected at 2 h before dawn (22:00). Samples were sequenced for biological duplicates (8 genomic DNA samples were sequenced in total). This sequence data has completed library preps and Illumina sequencing and bioinformatics mapping of the reads to the *K. laxiflora* genome is complete. Comparative analysis of genome-wide DNA methylation patterns between wild type and *KNAT3*-RNAi is currently underway in the Bioinformatics facility at the Liverpool CGR.

Diagenode ChIP-seq analysis of *K. laxiflora* KNAT3 has been completed using a custom polyclonal anti-KNAT3 peptide antibody, plus HA-tag captured chromatin immunoprecipitation for 35S-HA-transcription factor (TF) expressing stable transgenic lines of *K. laxiflora*. The HA-tagged TFs analyzed with ChIP-seq were Bel-like, CYCLING DOF FACTOR2 (CDF2), CAM-INDUCED bZIP (CIB), EPR1, GATA, Homeobox-7 (HB7), LNK3, MYB27, MYB90, MYB439, PLATZ1 and KNAT3.

Additional ChIP-seq analysis of 12 additional HA-tagged TFs from is also in progress at the University of Nevada HA-tag captured chromatin immunoprecipitation for 35S-HA-transcription factor (TF) expressing stable transgenic lines of diploid *K. fedtschenkoi*. The HA-tagged TFs include AP2/ERF72, ANAC012, bZIP29, CRF1, LHY1, MYB59-1, MYB59-2, HD-like, NAC83, NFY-B3, Zn-finger CONSTANS-like 3, and Zn-finger CONSTANS-like 5,