Progress towards defining a minimal parts list for the biodesign of Crassulacean acid metabolism (CAM): identification, characterization and ground-truthing of candidate CAM genes using the model species *Kalanchoë fedtschenkoi* and *K. laxiflora*

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Project Goals: The long-term goal of this project is to enhance the water-use efficiency (WUE) and adaptability to hotter, drier climates of species that normally perform C_3 photosynthesis by introducing crassulacean acid metabolism (CAM). Photosynthetic performance and WUE will be enhanced in *Arabidopsis* and *Populus* by: 1) defining the genetic basis of key CAM modules in both eudicot and monocot CAM species, 2) characterizing the regulation of 'carboxylation', 'decarboxylation', and 'inverse stomatal control' gene modules of CAM via loss-of-function studies in model CAM species, 3) deploying advanced genome engineering technologies to enable transfer of fully functional CAM modules into C_3 species and 4) analyzing the effects of these transgenic modules on 'stomatal control', CO_2 assimilation and transpiration rates, biomass yield, and WUE in *Arabidopsis* and *Populus*.

CAM plants are characteristic of arid, semi-arid and seasonally dry habitats, suggesting that a key driver for CAM evolution was likely to have been high temperatures and drought-prone conditions. CAM assists plants to achieve enhanced WUE by virtue of a temporal separation of primary and secondary CO_2 fixation in individual photosynthetic leaf mesophyll cells. Stomatal opening and associated primary atmospheric CO_2 fixation occurs during the cooler more humid dark period, thereby keeping transpirational water loss to a minimum. Dark CO_2 fixation is catalyzed by phosphoenolpyruvate carboxylase (PPC) in photosynthetic mesophyll cells. PPC has a much higher affinity for CO_2 than ribulose 1,5-bisphosphate carboxylase oxygenase (RuBisCO), and lacks oxygenase activity. CO_2 fixation by PPC generates oxaloacetate, which is converted to malate by malate dehydrogenase (MDH). Malate is stored in the vacuole as malic acid until dawn when it is decarboxylated by either NAD(P)-malic enzyme, or a combination of MDH and phosphoenolpyruvate carboxykinase, generating an internal supply of CO_2 inside the leaf. High internal [CO_2] is believed to signal stomatal closure in the light, minimizing water loss during the hottest and driest part of the 24 h cycle. The carbon dioxide from malate decarboxylation is refixed by RuBisCO in the Calvin-Benson cycle in the chloroplast, yielding sugars to fuel growth and reproduction. The high concentration of CO_2 that builds up inside the leaf during malate decarboxylation behind closed stomata minimizes the oxygenase activity of RuBisCO, thus preventing the inefficient side reaction of photosynthesis known as photorespiration. Temporal coordination of stomatal opening and primary CO_2 fixation in the dark, and stomatal closure and secondary refixation of CO_2 in the light is achieved through tight coupling of the entire CAM pathway to the central circadian clock. Clock-control allows dawn and dusk to be anticipated and CAM biochemical steps to be optimized to prevent futile cycling.

Forward engineering of CAM into non-CAM crop species in order to enhance their WUE requires a comprehensive knowledge of the minimal set of genes and proteins required for the efficient operation of CAM. Here we describe progress with functional genomics research that aims to define and characterize the complete CAM genetic blueprint from the model species *Kalanchoë fedtschenkoi* and *Kalanchoë laxiflora*. A draft assembly of the 246 Mbp *K. fedtschenkoi* genome has been assembled and annotated and recently further improved through the addition of 100X coverage of PacBio long reads. Quantitative RNA-seq analysis of light/ dark time course samples from C_3 and CAM leaves identified candidate CAM-associated genes that increase in transcript abundance concomitant with CAM. Many of these CAM-induced genes also undergo robust oscillations in transcript abundance over the light/ dark cycle, consistent with a role in the circadian optimization of the pathway. The RNA-seq data has guided the reconstruction of a comprehensive model of CAM for which candidate CAM-recruited genes are allocated to each step in the pathway. This in turn has allowed targeted RNAi gene silencing and over-expression approaches to be applied to each candidate CAM gene through the generation of stable transgenic lines of *K. fedtschenkoi* and *K. laxiflora*. Detailed analysis of CAM-associated phenotypes in the transgenic lines is revealing which genes are essential for efficient CAM, and which genes are dispensable (1). We will present data on the phenotypic characterization of RNAi lines of *K. laxiflora* lacking key CAM genes. Several lines fail to fix atmospheric CO_2 in the dark period, and the phenotypic consequences of this will be described.

In addition, data will be presented from quantitative Illumina RNA-seq analysis of a light/ dark timecourse of transcript levels in epidermal peels (enriched for guard cells) and separated mesophyll tissue from CAM leaves of *K. fedtschenkoi*. This data provides unique insights into the temporal coordination of known guard cell signaling genes including those known to be involved in CO_2-, light-, and ABA-dependent regulation of stomatal aperture. Several of these guard cell-signaling genes display a 6 to 12 h shift in the timing of their transcript abundance peak in CAM leaves of *K. fedtschenkoi* when compared to the temporal regulation of the orthologous transcripts in C_3 leaves of Arabidopsis. These unique discoveries are providing unprecedented insights into the gene regulatory networks that associate with reverse stomatal opening required for CAM.

Finally, an overview will be presented of work on the characterisation of light/ dark- and circadian clock-controlled transcription factors (TFs) that are induced coincident with CAM. These TFs are candidates for controlling key regulatory steps in the circadian coordination and optimization of the daily CAM cycle, potentially linking the central circadian clock to CAM as part of a CAM-specific clock output pathway. Highlights will be presented from RNAi silencing of novel CAM-associated TFs, and progress towards ChIP-seq analysis of TF genomic targets will be summarized.

References

1) Dever LV, Boxall SF, Knerova J, Hartwell J (2015) Transgenic perturbation of the decarboxylation phase of crassulacean acid metabolism alters physiology and metabolism but only has a small effect on growth. Plant Physiol. **167**: 44-59.

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