Leveraging *Agave* and *Kalanchoë* Genomics Resources to Transfer Crassulacean Acid Metabolism (CAM) Modules into C_3 Species Using Synthetic Biology Approaches

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Project Goals: Crassulacean acid metabolism (CAM) is a specialized mode of photosynthesis that features a temporal CO_2 pump with nocturnal CO_2 uptake, facilitates increased water-use efficiency (WUE), and enables CAM plants to inhabit water-limited semi-arid or seasonally dry environments. CAM provides an excellent opportunity for engineering both enhanced WUE and photosynthetic performance into bioenergy crops. This project has two main goals: 1) to identify the CAM-associated genes and gene networks using systems biology approaches and 2) to engineer CAM gene modules into C_3 species using synthetic biology approaches. The success of the project could allow biomass production on semi-arid, abandoned, or marginal agricultural lands.

CAM-into-C_3 engineering requires multiple CAM-related genes to be manipulated in a modular manner, including: 1) a carboxylation module for CO_2 fixation and nocturnal accumulation of malic acid in the vacuole; 2) a decarboxylation module for release of CO_2 from malate; and 3) a stomatal control module for nocturnal stomatal opening and stomatal closure during the daytime (Yang et al. 2015). We have generated rich genomics resources for two important CAM models *Agave* (Abraham et al. 2016) and *Kalanchoë* (phytozome.jgi.doe.gov). Our comparative analysis of protein sequences and gene expression data have identified CAM-related genes in *Agave* and *Kalanchoë*. To characterize the function of CAM-related genes, the CRISPR/Cas9-based genome-editing technology (Liu et al. 2016) was used to create loss-of-function mutants in *K. fedtschenkoi*. Some of the genes related to carboxylation, decarboxylation, and stomatal movement in *Agave* and *Kalanchoë* have been engineered into three C_3 species as described below.

Engineering of carboxylation module: Genes related to carboxylation in *Agave* and *Kalanchoë*, including β-type carbonic anhydrase (β-CA), phospho*eno*lpyruvate carboxylase (PEPC), PEPC kinase (PPCK), malate dehydrogenase (MDH) and tonoplast aluminium-activated malate transporter (ALMT), were successfully introduced individually into C_3 species including model plants (i.e., *Arabidopsis* and tobacco) and bioenergy crop *Populus*. The transgenic plants were characterized using RT-PCR and western blot analysis to validate the expression of the transgenes. Phenotypical characterization of the transgenic plants is being conducted.

Engineering of decarboxylation module: Genes related to decarboxylation in *Agave* and *Kalanchoë*, including tonoplast dicarboxylate transporter (tDT), pyruvate phosphate dikinase (PPDK) and NADP-dependent malic enzyme (NADP-ME), were successfully introduced into C_3 species including model plants (i.e., *Arabidopsis* and tobacco) and the bioenergy crop *Populus*.

The transgenic plants were characterized using RT-PCR and western blot analysis to validate the expression of the transgenes. Phenotypical characterization of the transgenic plants is being conducted. Also, we made use of transgenic plants (*amiRs*), which show timely increase of malate by ≈2-fold at early night, to reprogram decarboxylation using synthetic biology principles. First, we complemented *amiRs*, which are partially deficient in CO_2 release from malate, with a dark-induced promoter driving a functional NADP-ME enzyme and monitored gas exchange during a 20-hour period. Next, we segregated the functional NADP-ME out and re-evaluated CO_2 release from malate in the reverted line (amiR-r). Our results demonstrate the robustness of a controller to effectively link 3 inputs, which includes an ON-OFF switch, and generate two distinct outputs for diel control of intracellular CO_2 in leaves. This capability has different applications including the making of future CAM-engineered plants inducible under a specific input (e.g., drought).

Engineering of stomatal control module: Two strategies were used to change the stomatal behavior of C_3 plants: 1) transfer of multiple stomatal movement-associated genes from *K. fedtschenkoi* to C_3 plants and 2) synthesizing AND gate genetic circuits based upon the CRISPR-dCas9 system. More than 10 genetic circuits targeting 'CAM-like stomatal movement' were designed, assembled, and transferred into three target C_3 species (i.e., *Arabidopsis*, tobacco, and poplar) via *Agrobacterium*-mediated transformation. Transgenic plants were generated, and identified by PCR, qRT-PCR, and western blot analysis. The effects of these genetic circuits on stomatal movement in the transgenic plants will provide useful information for optimizing the strategy of CAM-into-C_3 engineering. Also, success of this research could facilitate future efforts to engineer other multi-gene traits through synthetic biology approaches.

This material is based upon work supported by the Department of Energy, Office of Science, Genomic Science Program (under award number DE-SC0008834). Oak Ridge National Laboratory is managed by UT-Battelle, LLC for the US Department of Energy (under contract number DE-AC05-00OR22725).

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