Engineering Crassulacean Acid Metabolism (CAM) to Improve Water-use Efficiency of Bioenergy Feedstocks

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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*.

Global warming trends are leading to increased terrestrial soil drying, reduced terrestrial net primary production and carbon sinks, global food security and future biofuel production, and the global expansion of drylands that already cover 42% of the earth's surface. In order to offset these negative effects, an increased reliance upon crassulacean acid metabolism (CAM) crops or the introduction of CAM, a water-wise form of photosynthesis, into C_3 food and bioenergy crops might serve as a useful strategy to improve the water-use efficiency (WUE) of sustainable biomass production systems in the future (1). CAM features inverse stomatal behavior, in which stomata are open at night for CO_2 uptake when evapotranspiration rates are reduced compared with the daytime and closed during all or part of the day, thereby maximizing WUE. CAM also

exploits a temporal separation of nocturnal CO_2 uptake and fixation by phospho*enol*pyruvate carboxylase (PEPC), which leads to the formation of C_4 organic acids that are stored in the vacuole. The subsequent decarboxylation of these organic acids during the day releases CO_2 and concentrates it around ribulose-1-5-bisphosphate carboxylase/oxygenase (RUBISCO), suppressing photorespiration, while resulting in carbohydrate production *via* the C_3 Calvin–Benson cycle.

Detailed functional and integrative 'omics analyses of several CAM model or crop species including *Kalanchoe* (2, phytozome.jgi.doe.gov), *Mesembryanthemum crystallinum*, *Agave* (3), and pineapple (4), have recently defined the basic genetic requirements for CAM. Both *K. fedtschenkoi* and *M. crystallinum* were selected recently as DOE JGI Flagship Genome species. The development of synthetic RNAi-mediated gene silencing strategies targeting multiple genes (5) and CRISPR/Cas9 strategies for precise genome editing (6) are expedient ways to down-regulate, knock-out, or alter the expression of specific gene modules or pathways. Loss-of-function studies of individual enzymes, metabolite transporters, and regulatory proteins or transcription factors are being used to provide critical insights into the basic genetic requirements for CAM. For example, RNAi-mediated gene silencing of specific CAM components, such as mitochondrial NAD-malic enzyme and cytosolic/plastidic pyruvate orthophosphate dikinase revealed not only impaired nocturnal CO_2 uptake, but also reduced circadian clock-controlled phosphorylation of PPC (7). Other studies using RNAi lines of *K. fedstchenkoi* have shown that the route of nocturnal starch degradation is a key point of divergence between C_3 photosynthesis and CAM species. In C_3 species, hydrolytic starch degradation produced glucose and maltose, which is exported from the chloroplast as substrate for the provision of sucrose for growth. In contrast, phosphorolytic starch degradation in CAM species produces substrates such as glucose-6-phosphate, which is exported from the chloroplast for production of PEP in the cytosol (8). Such information is critical for knowing which genes to select when creating synthetic gene circuits to reconstruct CAM carboxylation and decarboxylation subpathways.

Facile gene stacking strategies for the assembly of a large number of transcription units (TUs) with appropriate circadian and drought-inducible expression patterns are necessary for the genetic reconstitution of facultative CAM into host C_3 species (9). We have created a plant-specific position/adaptor/carrier vector system originally designed for engineering mammalian cells (10) that enables the rapid, reliable, and scalable creation of complex gene circuits using the Gibson isothermal assembly process (11). Design and construction of CAM-specific carboxylation and decarboxylation gene circuits containing 9 and 15 genes has been completed and are in the process of being introduced into *Arabidopsis* and Poplar. The gene circuits were designed to include mesophyll-specific, drought-inducible, and appropriately timed circadian expression patterns of the transgenes in order to engage the CAM pathway only during water-deficit stress conditions. Lastly, tissue succulence has been successfully engineering in the C_3 photosynthesis model species *A. thaliana* in order to increased mesophyll cell size for increased malate storage capacity and reduced intercellular air space to limit internal CO_2 diffusion out of the leaf during the day to favor refixation by ribulose 1,5-bisphosphate carboxylase oxygenase (RUBISCO) and thereby increases the capacity to perform CAM. Added benefits of engineered tissue succulence included increased biomass production, increased WUE, and tolerance to water-deficit (drought) and salinity stress. The combination of engineered CAM and tissue succulence is expected to increase the WUE of bioenergy feedstocks and potentially expand their production into more marginal, abandoned, or semi-arid regions.

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