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

John C. Cushman[1]* (jcushman@unr.edu), Rebecca Albion[1], Jungmin Ha[1], Sung Don Lim[1], Bernard W. M. Wone[1], Won Cheol Yim[1], Travis Garcia[1], Pradeep K. Yerramsetty[1], Juli Petereit[2], Richard Tillett[2], Karen A. Schlauch[2], Xiaohan Yang[3], Anne M. Borland[3,4], Alistair Leverett[4], Henrique C. De Paoli[3], Rongbin Hu[3], Degao Liu[3], Kaitlin Palla[3,5], Robert C. Moseley[3,5], Timothy J. Tschaplinski[3], David J. Weston[3], Gerald A. Tuskan[3], Jay Chen[3], James Hartwell[6], Louisa V. Dever[6], Susanna F. Boxall[6], Jade Waller[6], Jack Davies[6], Phaitun Bupphada[6], Nirja Kadu[6], Hong Guo[7], Hao-Bo Guo[7], Paul Abraham[8], and Robert Hettich[8].

[1]Department of Biochemistry and Molecular Biology, University of Nevada, Reno. [2]Nevada Center for Bioinformatics, University of Nevada, Reno; [3]Biosciences Division, Oak Ridge National Laboratory, Oak Ridge, TN; [4]School of Biology, Newcastle University, Newcastle upon Tyne; [5]Bredesen Center, University of Tennessee, Knoxville, TN; [6]Department of Plant Sciences, Institute of Integrative Biology, University of Liverpool; [7]Department of Biochemistry & Cellular and Molecular Biology, University of Tennessee, Knoxville; [8]Chemical Sciences Division, Oak Ridge National Laboratory, Oak Ridge, TN.

http://cambiodesign.org

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*.

In order to meet the grand challenges of overcoming the negative effects of global climate change on crop productivity, an increased reliance on crassulacean acid metabolism (CAM) crops might serve as a useful component of an integrated scheme to develop sustainable agroecosystems for dryland reclamation to provide both bioenergy feedstocks and ecosystem services (1, 2). However, traditional food and bioenergy crops with greater heat and drought durability and greater water-use efficiency (WUE) will also be crucial for sustainable biomass production systems in the future. Thus, one approach to increase crop WUE is to move the water-wise photosynthetic machinery of CAM into C_3 food and bioenergy crops (2, 3). 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 features 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.

Comparative transcriptomic and genomic sequencing projects were used to identify CAM-related genes by examining developmentally regulated or water-deficit stress-inducible gene expression patterns from obligate and facultative CAM species, respectively (3). Clues to the transcriptional control of CAM expression networks are being derived from the analysis of candidate *cis*-regulatory elements and cognate transcription factors controlling circadian and mesophyll expression patterns of CAM genes. For example, yeast one-hybrid screens using candidate consensus *cis*-regulatory elements and proximal 5' regulator regions of the phospho*enol*pyruvate carboxylase (*Ppc*) gene as bait sequences have been completed. A collection of candidate transcription factors including AP2 domain, homeobox-leucine zipper domain, Myb-like, C2H2-type zinc finger, and bZIP family transcription factors have been identified to date.

In addition, 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 silencing of mitochondrial NAD-malic enzyme and cytosolic/plastidic pyruvate orthophosphate dikinase not only impaired nocturnal CO_2 uptake, but also reduced the circadian clock-controlled phosphorylation of PPC (4). 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 and CAM. Data have been obtained which indicate that phosphorolytic degradation of starch produces substrate for production of PEP, while the hydrolytic production and nocturnal export of glucose from the chloroplast primarily directs substrate towards provision of sucrose for growth. Such information is critical for the selection of genes and gene networks for reconstructing and validating of carboxylation and decarboxylation modules.

Tissue succulence engineering in C_3 photosynthesis plants may be a key anatomical attribute for enhancing the efficient operation of engineered CAM in C_3 photosynthesis species. Thus, increased tissue succulence has been accomplished in the C_3 photosynthesis model species, *Arabidopsis thaliana*, in order to increased mesophyll cell size for increased malate storage capacity in the vacuole and reduced intercellular air space (IAS) to limit the diffusion of CO_2 out of the leaf during the day for refixation by ribulose 1,5-bisphosphate carboxylase oxygenase (RUBISCO). For CAM Biodesign, strategies for stacking of multi-gene circuits with appropriate circadian and drought-inducible expression patterns for reconstitution of CAM in *A. thaliana* have been developed using the Gibson assembly process. Mesophyll-specific expression patterns of transgenes were verified by promoter::GUS-LUC reporter constructs and will be tested for circadian expression patterns. Subcellular localization of targeted enzymes and transporters of the carboxylation and decarboxylation modules were verified by GFP-fusion protein localization studies in stably transformed *A. thaliana* lines. To facilitate the construction of gene circuits for CAM biodesign, a novel platform was developed for high-throughput assembly of DNA parts (5). Cutting-edge genome editing tools are being applied to functional genomics research and CAM engineering (6). Design and construction of plant-specific vector systems for Gibson isothermal assembly of gene circuits containing 9 and 15 genes CAM-specific genes has been completed. Lastly, phenotypic testing of various *Populus* varieties with respect to transformability, leaf anatomy, stomatal responsiveness to CO_2, and non-structural carbohydrate resources for nocturnal CO_2 fixation has revealed potentially suitable candidates for CAM engineering.

References

1) Cushman JC, Davis SC, Yang X, Borland AM. (2015) Development and use of bioenergy feedstocks for semi-arid and arid lands. J. Exp. Bot. 66:4177–4193.
2) Yang X, Cushman JC, Borland AM, Edwards EJ, Wullschleger SD, Tuskan GA, Owen NA, Griffiths H, Smith JAC, De Paoli HC, Weston DJ, Cottingham R, Hartwell J, Davis SC, Silvera K, Ming R, Schlauch KA, Abraham P, Stewart JR, Guo H-B, Albion RA, Ha J, Lim SD, Wone BWM, Yim WC, Garcia T, Mayer JA, Petereit J, Nair SS, Casey E, Hettich RL, Ceusters J, Ranjan P, Palla KJ, Yin H, Reyes-García C, Andrade JL, Freschi L, Beltran JD, Dever LV, Boxall SF, Waller J, Davies J, Bupphada P, Kadu N, Winter K, Sage RF, Aguilar CN, Schmutz J, Jenkins J, Holtum, JAC. (2015) A roadmap for research on crassulacean acid metabolism to enhance sustainable food and bioenergy production in a hotter, drier world. New Phytologist. 207*:* 491–504.
3) Borland AM, Hartwell J, Weston D, Schlauch KA, Tschaplinski TJ, Tuskan GA, Yang X, Cushman JC. (2014) Engineering Crassulacean acid metabolism to improve water-use efficiency. Trends Plant Sci. 19: 327–338.
4) 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.
5) De Paoli HC, Tuskan GA, Yang X (2016) An innovative platform for quick and flexible joining of assorted DNA fragments. Scientific Reports. 6: 19278.
6) Liu D, Hu R, Palla KJ, Tuskan GA, Yang X (2016) Advances and perspectives on the use of CRISPR/Cas9 systems in plant genomics research. Current Opinion in Plant Biology. *In press.*

This material is based upon work supported by the U.S. 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 U.S. Department of Energy under Contract Number DE–AC05–00OR22725.