Final Report DOE: DE-SC0006645 "Defining determinants and dynamics of cellulose microfibril biosynthesis, assembly and degradation".

Abstract:

The central paradigm for converting plant biomass into soluble sugars for subsequent conversion to transportation fuels involves the enzymatic depolymerization of lignocellulosic plant cell walls by microbial enzymes. Despite decades of intensive research, this is still a relatively inefficient process, due largely to the recalcitrance and enormous complexity of the substrate. A major obstacle is still insufficient understanding of the detailed structure and biosynthesis of major wall components, including cellulose. For example, although cellulose is generally depicted as rigid, insoluble, uniformly crystalline microfibrils that are resistant to enzymatic degradation, the *in vivo* structures of plant cellulose microfibrils are surprisingly complex. Crystallinity is frequently disrupted, for example by dislocations and areas containing chain ends, resulting in "amorphous" disordered regions. Importantly, microfibril structure and the relative proportions of crystalline and non-crystalline disordered surface regions vary substantially and yet the molecular mechanisms by which plants regulate microfibril crystallinity, and other aspects of microfibril architecture, are still entirely unknown. This obviously has a profound effect on susceptibility to enzymatic hydrolysis and so this is a critical area of research in order to characterize and optimize cellulosic biomass degradation.

The entire field of *cell wall assembly*, as distinct from *polysaccharide biosynthesis*, and the degree to which they are coupled, are relatively unexplored, despite the great potential for major advances in addressing the hurdle of biomass recalcitrance. Our overarching hypothesis was that identification of the molecular machinery that determine microfibril polymerization, deposition and structure will allow the design of more effective degradative systems, and the generation of cellulosic materials with enhanced and predictable bioconversion characteristics.

Our experimental framework had been based on the idea that the most effective way to address this long standing and highly complex question is to adopt a broad 'systems approach'. Accordingly, we assembled a multi-disciplinary collaborative team with collective expertise in plant biology and molecular genetics, polymer structure and chemistry, enzyme biochemistry and biochemical engineering. We used a spectrum of cutting edge technologies, including plant functional genomics, chemical genetics, live cell imaging, advanced microscopy, high energy X-ray spectroscopy and nanotechnology, to study the molecular determinants of cellulose microfibril structure. Importantly, this research effort was closely coupled with an analytical pipeline to characterize the effects of altering microfibril architecture on bioconversion potential, with the goal of generating predictive models to help guide the identification, development and implementation of new feedstocks. This project therefore spanned core basic science and applied research, in line with the goals of the program.

Over the course of the project, accomplishments included:

- Establishing platforms through reverse and forward genetics to identify and manipulate candidate genes that influence cellulose microfibril synthesis and structure in a model C3 grass, *Brachypodium distachyon* and a model C4 grass *Setaria viridis.*

- Identifying and characterizing the effects of a number of cellulose biosynthesis inhibitors (CBIs), and particularly those that target monocots with the aim of generating resistance loci.

- Developing protocols for the use of high energy X-ray diffraction (XRD) to study the structure and organization of cellulose microfibrils in plant walls, notably those in Arabidopsis and Brachypodium.

- Using the chemical and genetic based inhibition strategies to develop new mechanistic models of cellulose microfibril crystallization, and of how altering microfibril architecture influences digestibility.

Project Progress:

- **Development of reverse genetics resources for bioenergy feedstock research development in *Brachypodium distachyon* and *Setaria viridis*.**

Over the course of this program, we developed EMS-mutagenized populations of a model C3 grass, *Brachypodium distachyon* and an NMU-mutagenized population of a model C4 grass *Setaria viridis*. We developed a TILLING resource for *B. distachyon* that enables the detection of single nucleotide changes following deep sequencing of pools of mutagenized individuals. A population of approximately 3,840 M2 families was propagated and DNA isolated from a single plant for each family. These DNA samples have been purified and arrayed in pools of 8 samples/well in 5 x 96 well plates. PCR amplifications have been performed on each well (480 reactions) using primers that amplify two target genes of interest. These PCR products have been purified, DNA sheared and Illumina libraries are now being constructed with indexing primers. These libraries have been sequenced and the sequences analyzed with scripts developed in the Brutnell lab to identify rare transition mutations in DNA pools. We have screened for glycosyl hydrolase 9 (GH9) genes and the cellulose synthase (CESA) genes. We also developed a TILLING by sequencing resource for *S. viridis*. During the course of this project, a new genome editing technology using CRISPR/Cas9 was developed that enables precise and targeted edits. To exploit this technology in Setaria, we developed constructs and strategies to create small indels and deletions in candidate genes. We successfully applied this technology to create edits in several genes and have thus established a platform for genome engineering in Setaria.

- Chemical genetics: A number of cellulose biosynthesis inhibitors (CBIs) have been identified that target monocots with the aim of generating resistance loci. A monocot active CBI has been needed, as isoxaben and quinoxyphen (Harris et al., 2012) lose a significant activity rate when applied to monocots. We have published a review on chemical genetic dissection of cellulose synthesis (Brabham and DeBolt, 2012: Frontiers in Plant Science) and characterize the CBIs by groupings. The CBI that targets monocots at the picomole range is indaziflam, a recently released compound from Bayer Crop Science. The characterization of CBI action has been completed and it appears to cause increased CESA density but reduced movement in the membrane, indicative of reduced polymerization rates. A manuscript describing these studies has been drafted and is ready for submission. Utilizing the Brachypodium TILLING populations may be approachable, but the saturation level of the screen is not as promising with fewer seed. Therefore, we complement the inherent risk of screening in Brachypodium with Arabidopsis, assuming that the target is identical across taxa. Indeed, the action mechanism was found to be very consistent between monocot and dicot species. The overarching goal has been to identify resistant mutants that display structural variants in cellulose, similar to that which was reported in the last update for quinoxyphen.

Using a mutant identified from the Brachypodium TILLING population described above, we targeted the exon found adjacent to the *BdCESA1* glycosyltransferase QXXRW motif by SCAM PRing, a high throughput means of Targeting Induced Local Lesion IN Genomes (TILLING). This identified the *Bdcesa1*^{S830N} allele, which is a novel amino acid substitution in the *CESA* family. Based on prior data from Arabidopsis mutations in *rsw1* we investigated the

phenotypes of the *Bdcesa1*^{S830N} allele as it pertains to cell expansion and gross morphogenic phenotypes. Notably, we found only a subset of tissues displayed aberrant expansion unlike the Arabidopsis *rsw1* allele, which affected tissues with primary cell walls. We propose that differences in the relative importance of members of the CESA gene family exist in grasses compared with Arabidopsis and that gene homology alone will not be sufficient to predict phenotype.

- Significant efforts have also been made to provide a translational framework to identify regulatory proteins for CESA function. Promoter deletion experiments and *cis*-regulatory candidates have been isolated for genetic characterization and will be pursued in the coming year.

- New sets of high energy X-ray diffraction (XRD) data (using Cornell's synchrotron) have been generated for a set of Arabidopsis mutant and wild type lines (incorporating spatial and developmental variables) as well as Brachypodium wild type stems

Products Delivered:

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- DeBolt, S. (2012) Chemical genetic dissection of cellulose biosynthesis*. Abstracts of papers of the American Chemical Society* 243: 295.

- Brabham, C. and DeBolt, S. (2013) Chemical genetics to examine cellulose biosynthesis. *Frontiers in Plant Science* 3: 309.

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- Petti, C., Shearere, A., Tateno, M., Ruwaya, M., Nokes, S., Brutnell, T. and DeBolt, S., I2013) Comparative feedstock analysis in *Setaria viridis* L. as a model for C4 bioenergy grasses and panicoid crop species. *Frontiers in Plant Science* 4: 181.

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- Petti, C., Hirano, K., Stork, J. and DeBolt, S. (2015) Mapping of a cellulose-deficient mutant named *dwarf1-1* in *Sorghum bicolor* to the green Revolution gene gibberellin20-oxidase reveals

a positive regulatory association between gibberellin and cellulose biosynthesis. *Plant Physiology* 169: 705-716.

- Griffiths, J., Sola, K., Kushwaha, R., Lam, P., Taeno, M., Young, R., Voiniciuc, C., Dean,m G., Mansfild, S.D., DeBolt, S. and Haughn, G.W. (2015) Unidirectional movement of cellulose synthase complexes in arabidopsis seed coat epidermal cells deposit cellulose involved in mucilage extrusion, adherence, and ray formation. *Plant Physiology* 168: 502-520.

- Tateno, M., Brabham, C. and DeBolt, S. (2016) Cellulose biosynthesis inhibitors - a multifunctional toolbox. *Journal of Experimental Botany* 67: 533-542.