Defined Tetra-Allelic Gene Disruption of the 4-Coumarate:Coenzyme A ligase 1 Gene by CRISPR/Cas9 in Switchgrass Results in Lignin Reduction and S/G Ratio Alteration

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Project Goals: The BioEnergy Science Center (BESC) focuses on fundamental understanding and elimination of biomass recalcitrance. BESC's approach to improve accessibility to the sugars within biomass involves (1) improved plant cell walls for rapid deconstruction and (2) multi-talented microbes for converting plant biomass into biofuels in a single step [consolidated bioprocessing (CBP)]. Biomass research works with two potential bioenergy crops (switchgrass and *Populus)* to develop improved varieties and to understand cell wall biosynthesis pathways. We test large numbers of natural variants and generate specific modified plants samples. BESC's research in deconstruction and conversion targets CBP manipulating thermophilic anaerobes and their cellulolytic enzymes for improved conversion, yields, and titer. Enabling technologies in biomass characterization, 'omics, and modeling are used to understand chemical and structural changes within biomass and to provide insights into mechanisms.

The production of biofuels from renewable biomass alleviates the dependence on fossil fuels.

Switchgrass *(Panicum virgatum),* a C4 perennial grass species, has been developed into a lignocellulosic feedstock for bioenergy. In order to reduce cell wall recalcitrance and improve bioethanol production, RNAi knock-down technology has been used to suppress genes of interests in switchgrass. In recent years, genome editing methods including zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR associated proteins 9 (Cas9), have been developed to knock-out specific genes by altering the genomic DNA sequences (Bibikova et al., 2003; Boch et al., 2009; Kim and Kim, 2014). We employed the CRISPR/Cas9 system to produce low-lignin switchgrass because the CRISPR/Cas9 system is relatively simple to work with compared to ZFNs and TALENs.

Switchgrass is an outcrossing species with a complex allo-tetraploid genome *(*$2n = 4x = 36$), which causes difficulties in producing homozygote knock-out plants.

Lignin is a major component of secondary cell walls and contributes to the recalcitrance problem during fermentation. In order to develop a CRISPR/Cas9 system in switchgrass, we choose to target 4-Coumarate:coenzyme A ligase (4CL), a key enzyme involved in the early steps of the monolignol biosynthesis. We identified three *4CL* genes, *Pv4CLJ, Pv4CL2* and *Pv4CL3* in switchgrass.

qRT-PCR analysis revealed that *Pv4CLJ* transcripts were more abundant in the internode and the node than in the leaf. *Pv4CL2* transcripts were barely detectable in the three different tissues – internode, node, and leaf. *Pv4CL3* was preferentially expressed in leaf. Internode and node are highly lignified tissues comprised of parenchyma and sclerenchyma cells distributed in the interfascicular region and the vascular sheath.

Therefore, *Pv4CL1* was selected as the main target gene. Specific gRNA was constructed to target Pv4CL1. After introducing the construct into switchgrass calli, forty plants were regenerated. After PCR screening and sequencing, four plants *(Pv4cl1-#25,* #26, #28, and #29) were confirmed to have the tetra allelic mutations simultaneously. The *Pv4cl1* knock-out plants showed reddish stems and reduced cell wall thickness, and had a 20 to 29% reduction in total lignin. The reduction in lignin content in the *Pv4cl1* knock-out plants led to a 9% and a 28% increases in glucose and xylose releases, respectively. This study demonstrated that we have established the CRISPR/Cas9 system in switchgrass, and the system has been successfully used to precisely target the selected *Pv4CL1* gene to create switchgrass knock-out plants with reduced recalcitrance.

References

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