New Insights on the Lignin Pathway in Grasses

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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 lignin biosynthetic pathway was thought for many years to be a highly conserved metabolic process across all plant species. This assumption was based on studies on dicotyledonous plants, mainly the model *Arabidopsis thaliana.* Recent studies have shown that monocots and dicots differ in their patterns of lignification, and that these differences can be found even among species within the monocot or dicot clades.

We previously studied the lignin pathway in the leading biomass crop switchgrass *(Panicum virgatum),* and found that the effects of downregulation of some of the genes involved in the earlier steps of the pathway were not consistent with the current understanding of the monolignol pathway (Shen et al., 2013). Importantly, switchgrass plants downregulated in cinnamate 4-hydroxylase (C4H), coumaroyl shikimate 3'-hydroxyalse (C3'H) and caffeoyl CoA 3-0-methyltransferase (CCoAOMT), showed lignin phenotypes inconsistent with the previous literature on dicot plants (Reddy et al., 2005, Schilmiller et al., 2009, Wagner et al., 2011). Also, the second HCT step in the shikimate shunt described *inArabidopsis* and tobacco (Hoffmann et al., 2003, 2004) is unlikely to occur in switchgrass (Escamilla-Trevino et al., 2014). Finally, a recent study (Ha et al., 2016) shows that caffeoyl shikimate esterase (CSE) is critical for normal lignification in *Medicago truncatula* (dicot), poplar *(Populus deltoides,* dicot) and switchgrass *(Panicum virgatum,* monocot), but *Brachypodium distachyon* and corn *(Zea mays)* do not possess orthologs of CSE. Moreover, preliminary results on enzymatic activities and kinetics of recombinant enzymes in *Brachypodium* and switchgrass suggest that the route caffeate →ferulate →feruloyl CoA could function during monolignol biosynthesis.

To study more precisely the early steps of the lignin pathway in grasses, we used the model *B. distachyon,* a diploid monocot with a small genome size and ease of transformation. *Brachypodium* has eight phenylalanine ammonia-lyase *(PAL)* genes, one of which encodes a bifunctional PTAL (phenylalanine/tyrosine ammonia-lyase) (Barros et al., 2016), three *C4H* genes, two hydroxycinnamoyl CoA: shikimate/quinate hydroxycinnamoyl transferases *(HCT)* genes, and one *C3'H* gene. We generated knockdown lines showing strong downregulation for these gene families *(PAL* 43%, *TAL* 80%, *C4H70%, HCT92%,* and *C3'H90%)* and reduced lignin content *(PAL* 19%, *TAL* 25%, *C4H* 12%, *HCT* 9% and *C3'H* 17%). Metabolite profiling analysis of these lines showed significant changes in the levels of some lignin pathway intermediates, particularly a large accumulation of 3-0-coumaroylshikimic acid in the C3′H line, suggesting that the ester route to lignin is functional in *Brachypodium*. However, an alternative route to caffeate must exist, as *Brachypodium* possess only weak extractable esterase activity with caffeoyl shikimate and has no CSE orthologs (Ha et al., 2015). This may involve the activity of a putative 4-coumarate 3-hydroxylase.

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