GWAS Studies in *Populus* Reveal Evolution of Two Biosynthetic Enzymes into Transcriptional Regulators Modulating Phosphoenolpyruvate Input and Chorismate Output from the Shikimate Pathway

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Project Goals: The BioEnergy Science Center (BESC) is focused on the fundamental understanding and elimination of biomass recalcitrance. BESC's approach to improve accessibility to the sugars within biomass involves (1) designing plant cell walls for rapid deconstruction and (2) developing multi-talented microbes or converting plant biomass into biofuels in a single step (consolidated bioprocessing). BESC biomass formation and modification research involves working directly with two potential bioenergy crops (switchgrass and *Populus*) to develop varieties that are easier to break down into fermentable sugars. We are testing large numbers of natural variants and generating specific and modified plant samples as well as developing genomics tools for detailed studies into poorly understood cell wall biosynthesis pathways.

Redirecting carbon flow to competing pathways has been proposed as one of the most efficient ways to engineer viable, low lignin cellulosic biofuels feedstocks. To achieve this goal, regulators of carbon flux at key junctions need to be identified and characterized. Using Genome-Wide Association Studies (GWAS), we identified hitherto unknown regulators of carbon flow into and out of the shikimate pathway.

At the entry point to the shikimate pathway, phosphoenolpyruvate (PEP) is the precursor molecule for 3-Deoxy-D-arabinoheptulosonate 7-phosphate (DAHP) biosynthesis. The same molecule is also the precursor for pyruvate biosynthesis in the glycolysis pathway. Similarly, chorismate, the output molecule from the shikimate pathway, is a shared precursor for both downstream phenylpropanoid and tryptophan biosynthesis. As such, pyruvate and tryptophan biosynthesis present valuable sinks to drive carbon flux away from lignin formation. However, not much is known about genetic regulation of carbon flux at both junctions. Here, we used GWAS in *Populus* to identify two transcriptional regulators of carbon flow at these entry and exit junctions. Interestingly, both of these transcriptional regulators were evolved from biosynthetic enzymes in their respective ancestral pathways.

Firstly, we observed that variation in polyglutamine (polyQ) repeat length within a *Populus* locus annotated as 2-hydroxyacid dehydrogenase led to differential activation of marker genes associated with lignin biosynthesis. Analysis of knock-out mutants in *Arabidopsis* revealed that the transcription of genes encoding pyruvate kinase and malate dehydrogenase were significantly

downregulated in the knock-out lines compared to wild type. Since these two genes lie at the entry point of PEP into pyruvate biosynthesis, we evaluated the responses of genes in the competing shikimate pathway for evidence of increased PEP shunt in that direction. There was indeed significant up-regulation of genes associated with shikimate and cell wall biosynthesis in the mutant line. In fact, a total of nine transcription factors with known regulatory activity of secondary cell wall and phenylpropanoid biosynthesis exhibited at least 2-fold upregulation in the mutant line. These results suggested that, in its functional state, this transcriptional regulator represses transcription of genes in the shikimate pathway in favor of pyruvate biosynthesis.

Secondly, we identified a novel isoform of the 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase which catalyzes the sixth step in the shikimate pathway. We observed that this isoform harbored an additional N-terminal helix-turn-helix DNA binding motif and a nuclear localization signal. Naturally-occurring loss-of-function mutations at this locus resulted in increased tryptophan biosynthesis and up to 50% reduction in lignin suggesting that in its functional state, this regulator preferentially drives chorismate towards phenylpropanoid biosynthesis. We confirmed this by evaluating *Populus* overexpression transgenic lines. Consistent with the observations in the natural variants, cell wall biosynthesis master regulators MYB46 and NST1 were significantly upregulated in the transgenic lines overexpressing this EPSP synthase isoform, suggesting that this regulator preferentially allocates chorismate towards the phenylpropanoid pathway. Interestingly, overexpression resulted in increased flavonoid biosynthesis at the expense of lignin formation.

These newly identified transcriptional regulators hold tremendous potential in engineering reduced lignin cellulosic biofuel feedstocks.

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