Transcriptional regulatory mechanisms linking secondary cell wall biosynthesis and iron homeostasis in *Populus*

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Project Goals: The Quantitative Plant Science Initiative (QPSI) is a capability that aims to bridge the knowledge gap between genes and their functions. A central aspect of our strategy is combining genome-wide experimentation and comparative genomics with molecular-level experimentation. In this way, we leverage the scalability of 'omics data and bioinformatic approaches to capture system-level information, while generating sequence-specific understanding of gene and protein function. By incorporating molecular-level experimentation in our workflow, we are addressing the question of how a protein functions and establishing mechanistic insight into how sequence variation impacts phenotype. This knowledge serves as a touchstone for accurate genome-based computational propagation across sequenced genomes and forms the foundation for robust predictive modeling of plant productivity in diverse environments.

Populus is one of DOE's "flagship" plants and is of special interest as a lignocellulosic biomass feedstock. In *Populus*, the major source of lignocellulosic biomass is secondary cell walls, which are mainly composed of cellulose, hemicellulose, and lignin. Iron (Fe) is an essential micronutrient indispensable for photosynthesis and plant growth. Plants have evolved homeostatic mechanisms in Fe uptake, transportation, utilization, and storage to maintain optimal Fe concentration for normal growth. The availability of some nutrients (e.g., nitrogen) has been found to impact plant secondary cell wall biosynthesis. However, the link between secondary cell wall biosynthesis and Fe bioavailability remains poorly studied. Using the hydroponic growth system in the greenhouse, we discovered that Fe deficiency reduces lignin and secondary cell wall biosynthesis in *Populus* stems. Consistently, our transcriptomic study unveiled significant Fe deficiency-induced gene expression changes of transcription factors and biosynthetic genes involved in secondary cell wall formation. BASIC HELIX-LOOP-HELIX (bHLH) transcription factors play key roles in activating Fe homeostatic mechanisms under Fe deficiency in Arabidopsis. By performing chromatin immunoprecipitation-seq (ChIP-seq) analysis in *Populus* protoplast transient expression system, we found that the *Populus* orthologs of bHLH038 and bHLH121 target master regulators and biosynthetic genes of secondary cell walls, as well as genes involved in Fe homeostasis. It's interesting to observe that the two XYLEM NAC DOMAIN1 (XND1) orthologs in *Populus* are targets of bHLH038 and bHLH121. XND1 is a well-known transcription factor that negatively controls lignin and secondary cell wall biosynthesis. The two XND1 orthologs have one amino acid difference in the C-terminal protein-protein interaction domain and exhibited opposite expression patterns under Fe deficiency, suggesting potentially divergent functions. By screening their interacting proteins using the TurboID-based proximity labeling approach in *Populus*

protoplast transient expression system, we demonstrated that the two XND1 orthologs have different protein-interacting preferences. Both secondary cell wall biosynthesis and Fe homeostasis are tightly controlled by transcriptional regulatory networks. Our results suggest that their regulatory networks have crosstalk to coordinate these two essential biological processes.

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