NitFix: Engineering Root Nodule Symbiosis in *Populus* sp.

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Project Goals: The collaborative project "Phylogenomic discovery and engineering of nitrogen fixation into the bioenergy woody crop poplar" is focused on identifying the genomic novelties that enable the symbiotic relationship between nodulating plants and Nitrogen-fixing bacteria to support genetically engineering this capability into bioenergy crops. The third aim of this project is engineering nodulation. Root nodules are specialized organs in legumes and actinorhizal plants that provide a proper environment for nitrogen-fixation bacteria such as rhizobia and *Frankia*. We aim to engineer root nodules in lineages of plants that do not form them to improve their associations with nitrogen-fixing bacteria. *Populus* sp. is a model plant unable to develop root nodules and is closely related to legumes and actinorhizal lineages. Genetics in model legumes identified genes such as *NIN* and *LHK1* that are essential and sometimes sufficient to induce nodule development. In this project, we aim to test in *Populus* sp. if these genes are sufficient to develop root nodules. In the future, more candidate genes coming from the Aim 1 of the DOE-funded project will be tested.

Engineering symbiotic nitrogen fixation in crops that do not associate with rhizobia or *Frankia* has been a long-term goal of the scientific community. *Populus* sp. is an essential bioenergy crop and presents natural characteristics that make it a unique platform for such an ambitious engineering project: (1) *Populus* sp. is phylogenetically very close to legumes and actinorhizal lineages. It contains all the known genes required for root nodule symbioses, suggesting that nodules evolved by co-opting pre-existing mechanisms such as lateral root development and symbiotic associations with arbuscular mycorrhizal fungi. (2) *Populus* sp. is easy to transform and to regenerate. The Ané lab has optimized transformation methods for the *Populus tremula x P. alba* hybrid (INRA 717-1B4) using *Agrobacterium rhizogenes* that allow us to generate transgenic roots in 2-3 weeks and full transgenic plants out of these transgenic roots in 2-3 months.

Genetic studied in model legumes, *Medicago truncatula* and *Lotus japonicus*, identified many genes required for nodule organogenesis and colonization by rhizobia. A large fraction of these genes is also involved in lateral root development and associations with arbuscular mycorrhizal fungi suggesting that legume nodulation evolved by recruiting genes from these processes. This observation is not entirely surprising because lateral roots and nodules are both "root lateral organs". Rhizobia and arbuscular mycorrhizal fungi are hosted inside of plant cells (endosymbiosis). Some of these genes are even sufficient to induce nodule development. For instance, overexpression of the NIN (1) transcription factor or the constitutive active LHK1 cytokinin receptor (*snf-2*) led to nodule organogenesis in *L. japonicus* in the absence of rhizobia (2).

In the first year of our DOE-funded project, we identified *Populus* sp. homologs of legume *NIN* and *LHK1*. *Populus* sp. has undergone a significant expansion of the *NIN* family, with seven full-length members and one pseudogene. In the near future, we will test more candidate genes coming from comparative phylogenomic approaches from Aim 1 of our DOE project. In our initial experiments, we expressed the constitutive active *L. japonicus* LHK1 cytokinin receptor (*snf-2*) either constitutively throughout the root or

specifically in the root cortex. We also overexpressed individual *Populus* sp. *NIN* genes in the hybrid INRA 717-1B4 roots.

We observed the surprising development of "root lateral organs" on *Populus* sp. roots expressing *snf-2* and *PtNIN2a*, *b* and *c*. It is too early at this stage to say if these structures are nodules or lateral roots, but some of them present the round morphology typical of root nodules. Further analysis of these structures using microscopy will determine the tissue of origin (cortex or pericycle) and their type of vasculature (central like lateral roots and actinorhizal nodules or peripheral like legume nodules).

Populus sp., like legumes and the vast majority of land plants, associates with arbuscular mycorrhizal fungi. A hallmark of the response of legumes to rhizobia and arbuscular mycorrhizal fungi are oscillations in nuclear and perinuclear calcium concentration, called "calcium spiking". In the first year of this project, we demonstrated that *Populus* sp. responds to some rhizobia by eliciting "calcium spiking" just like legumes. This calcium spiking was also triggered in *Populus* sp. by the application of lipo-chitooligosaccharides (LCOs) produced by rhizobia and arbuscular mycorrhizal fungi. We have initiated RNA-seq and ATAC-seq experiments to test the response *Populus* sp. to various LCOs.

In the next years, we continue testing more genes and promoters for their ability to induce root nodules in *Populus* sp. We will determine if the structures observed are analogous to legume nodules. We will continue investigating the possibility of getting nodules induced and colonized by rhizobia by manipulating the LCO signaling pathway.

References

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