

Title: Single-cell discovery of nodulation regulators in *Medicago* and evaluation of the functional role of homologs in *Populus*

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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 N-fixing bacteria to support genetically engineering this capability into bioenergy crops. As part of this effort, we are identifying new nodulation regulators using single-cell transcriptome analysis of *Medicago* roots treated with rhizobia. In parallel, we are characterizing the function of *Populus* homologs of known nodulation genes, such as the transcription factor Nodule INception (NIN).

Legumes can establish a symbiotic relationship with nitrogen-fixing rhizobia by developing nodules, a modified lateral root organ. Nodule development initiates with anticlinal divisions in a subset of pericycle cells after root exposure to lipochito-oligosaccharides secreted by the bacteria. To discover new regulators of the early differentiation of pericycle cells during nodule formation, we isolated and sequenced the transcriptome of *Medicago truncatula* single nuclei derived from roots, 24 hours after inoculation with rhizobia. Clusters of cells were generated and annotated based on expression enrichment for known marker genes (Fig. 1). Clusters representing all the cell types expected in roots were detected. Next, we re-grouped the pericycle cells at higher resolution and detected two distinct sub-types of cells. Further analysis indicated that one of these clusters involved cells responding to the rhizobia. By following the developmental trajectory of pericycle cells transitioning from a steady to a response-activated state, we identified a homolog of the Arabidopsis STY family proteins as highly differentially expressed. STY proteins are positive regulators of auxin biosynthesis, as regulators of the pericycle activation during nodule initiation. As expected, the RNAi down-regulation of STY resulted in a significant decrease in the number of nodules generated in transgenic *Medicago* plants. While the combined activation of auxin and cytokinin had been previously recognized, several of the specific genetic component activated early in the pericycle differentiation have remained largely unknown. These genetic triggers may be necessary to introduce nodule development in non-legume crops. They are currently being tested for their potential role in inducing the formation of nodule-like structure in *Populus*.

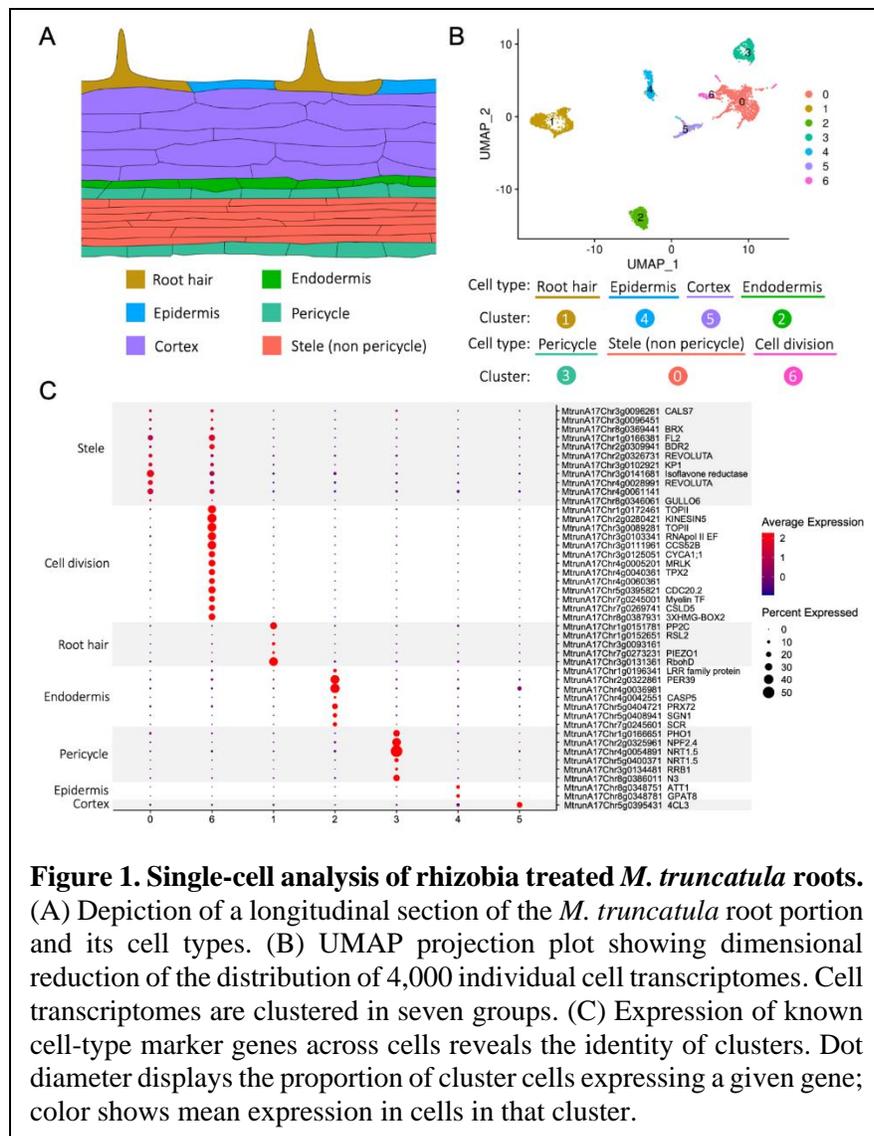


Figure 1. Single-cell analysis of rhizobia treated *M. truncatula* roots. (A) Depiction of a longitudinal section of the *M. truncatula* root portion and its cell types. (B) UMAP projection plot showing dimensional reduction of the distribution of 4,000 individual cell transcriptomes. Cell transcriptomes are clustered in seven groups. (C) Expression of known cell-type marker genes across cells reveals the identity of clusters. Dot diameter displays the proportion of cluster cells expressing a given gene; color shows mean expression in cells in that cluster.

In addition to discovering new nodulation regulators, we are exploring the role in *Populus* of genes previously shown to regulate this process in legumes. The transcription factor Nodule INception (NIN) is critical for establishing root nodule symbioses in legumes and other plant groups within the nitrogen-fixing clade (NFC). Plants within the NFC that have lost the ability to develop nodules strongly correlate with a loss of NIN. Interestingly, NIN homologs are present in plants outside the NFC, suggesting its role in other developmental processes. We discovered that *Populus* sp. contains eight copies of NIN falling into three distinct sub-families.

Lipo-chitoooligosaccharides (LCOs) produced by rhizobia and a wide range of fungi, including mycorrhizal ones, can

trigger root nodule symbiosis as well as lateral root formation. We observed that LCOs induced all three members of the PtNIN2 sub-family, with PtNIN2b showing a rapid induction within 15 minutes of treatment. We further observed that PtNIN2b promotes lateral root formation. Constitutive expression of PtNIN2b overcame the inhibition of lateral root development by cytokinin under high nitrate. Finally, we found that the LCO-induced, NIN-dependent lateral root formation is conserved in legumes. Our study suggests that the primitive function of NIN was to modify root architecture in response to symbiotic signals, which later evolved into more specialized functions in root nodule symbiosis.

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