Populus RNAi Transgenes Targeting the Leaf-Spot and Stem-Canker Pathogen *Sphaerulina musiva* Lack Non-Target Effects on Foliar Endophyte Communities in a Field Trial

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Background

The problem

- Biotechnologies involving gene silencing as a crop protection strategy against fungal pathogens may have non-target effects on beneficial fungi, some of which are known to mediate important diseases in *Populus*
- Gene-silencing is thought to be specific if designed well, however, many endophyte species lack genetic resources to inform non-target predictions
- To our knowledge, a community level study investigating non-target impacts of gene silencing technology on fungal endophytes has never been done

Host-induced gene silencing (HIGS)

- HIGS is a transgenic crop protection strategy whereby a host makes its own RNA-based pesticides to confer protection against specific pathogen species
- HIGS plants produce double-stranded RNA (dsRNA) complementary to specific genes of the pathogen, but not to host genes
- This mechanism requires dsRNA transfer/uptake from host to pathogen, as well as native RNA interference (RNAi) pathways in host and pathogen
- Suitable gene targets for HIGS are those that are essential for pathogen growth and or virulence

Biological question

- Do RNAi transgenes specifically targeting essential genes in the leaf spot and stem canker pathogen *Sphaerulina musiva*, affect the composition of non-target fungi in leaves as compared to control trees?

Hypothesis

- HIGS will have a negligible effect on non-target fungal communities

Sphaerulina disease in Populus

- Hybrid poplars are an important short rotation feedstock for paper, energy, and wood products
- Commercial varieties (especially *P. trichocarpa* crosses) are susceptible to the fungal pathogen *Sphaerulina musiva*, a major cause of severe leaf spot and stem canker that can limit productivity by 63% [1,2] (Fig.1)
- Breeding for resistance is currently considered the best way to manage disease but can take several years to produce a resistant cultivar
- A transgenic approach utilizing native RNA interference pathways in the host and pathogen might provide a more rapid method of deploying disease resistant cultivars

Figure 1. Leaf spot and stem canker symptoms (juvenile and mature tree) caused by *S. musiva*

Results

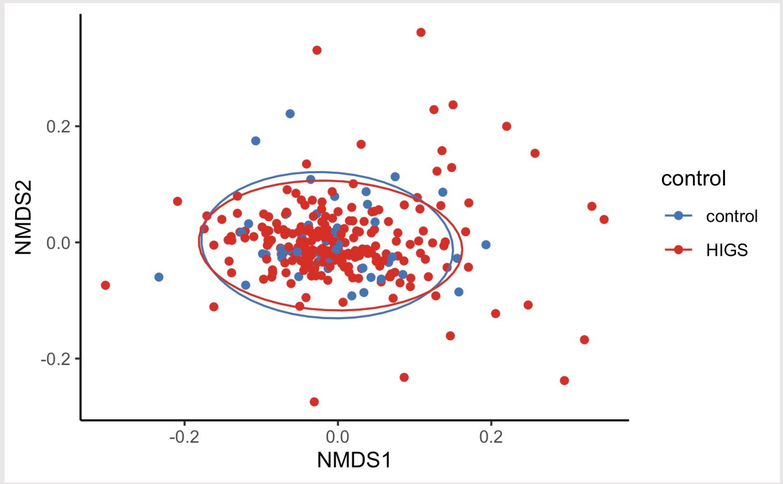

Figure 2. A. NMS ordination of sample units in OTU space (328 OTUs). Points represent fungal endophyte communities of leaves taken from individual trees (270). Distance between points show dissimilarity of communities among sample units. Red indicates samples that use host-induced gene silencing (HIGS) to target *S. musiva* while blue indicates all control genotypes including *wild type*, non-transgenic escapes, and non-specific *gus*-dsRNA lines. Ellipses show 95% confidence around group centroids. Fungal community compositions did not differ between *S. musiva* targeting HIGS samples and controls (PERMANOVA *pseudo-F*=0.865, *p*=0.488) suggesting that crop protection against specific fungi via HIGS was highly specific. No difference in multivariate group dispersions was found (PERMDISP, F=0.220, p=0.615).

Library prep

- Leaf discs washed to remove debris and surface microbes pre-DNA extraction
- Dual-indexed fungal ITS2 rDNA libraries were amplified using a two-stage PCR approach with a peptide nucleic acid clamp to block host amplification
- Stage 2 amplicons were normalized to 25-30 ng prior to pooling
- Sequenced using Illumina MiSeq 2x250bp at Center for Quantitative Life Sciences, Oregon State University

Data analysis

- Average of 9.5K reads per sample; samples containing <250 reads removed
- Operational taxonomic units (OTUs) were generated by clustering amplicon sequence variants at 97% similarity; 328 OTUs x 270 samples
- Sample OTU counts relativized by total number of reads in each sample
- Non-metric multidimensional scaling (NMDS) of Bray-Curtis dissimilarities
- One factor PERMANOVA to test null hypothesis of no difference in community composition between HIGS and control samples
- PERMDISP to test for differences in multivariate dispersions

HIGS transgenic poplars

- Transformation background: *S. musiva* susceptible *Populus trichocarpa* (SLMB-28-1, syn T61)
- Gene targets for knock-down are homologs of effective fungal gene targets in published HIGS studies [3,4]
- Knockdown of *cyp51* is expected to inhibit growth by silencing an enzyme in the ergosterol biosynthesis pathway—a critical cell membrane component
- RNAi against fungal dicer genes has been shown to reduce fungal growth and is thought to work by inhibiting developmental gene regulation [4]

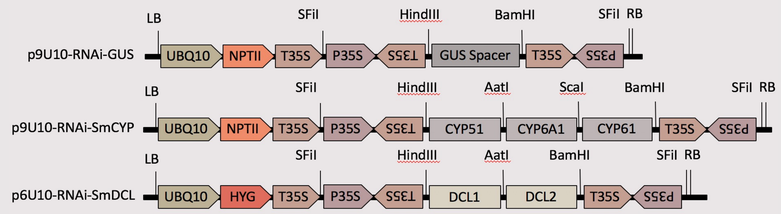

Figure 3. HIGS constructs transformed into *Populus* hybrid T61 using *Agrobacterium*-mediated transformation. p9U10-RNAi- serves as a negative RNAi control as the dsRNA produced has no gene target in *Populus* or *S. musiva.* The stacked RNAi-inducing fragments in the *cyp* and *dcl* constructs target all predicted *S. musiva* paralogs. Expression of dsRNA among transgenic lines was verified by RT-qPCR.

Field trial

- HIGS lines were multiplied in tissue culture, acclimated to soil, grown up in a greenhouse, and hardened off in a lathhouse prior to field planting
- 270 trees sampled: 39 independent *S. musiva* targeting transgenic events, plus 14 transgenic and non-transgenic control genotypes replicated $3 \leq n \geq 12$
- Leaves were sampled after one season of growth in late September of 2021 prior to senescence.
- *S. musiva* was not present at this field site, limiting study to non-target effects only

Figure 4. The sample unit was one tree, represented by the aggregation of twelve leaf discs—four discs from each of three leaves harvested at leaves 5-7 down from meristem.

Conclusions and next steps

- HIGS against *S. musiva* did not perturb non-target fungal communities
- A second year of field data will be collected in 2022 to see if non-target effects of HIGS remain null

Literature cited

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