RNAi-mediated disease resistance against *Sphaerulina musiva* in *Populus*

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Sphaerulina disease in *Populus*

Hybrid poplars are an important short rotation feedstock for paper, energy, and wood products. Most commercial varieties 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*.

RNA interference (RNAi)

RNAi is a gene silencing pathway present in most eukaryotes. This system of targeted mRNA destruction is triggered by double stranded RNA (dsRNA) and serves two known functions, 1) to protect cells from dsRNA viruses, and 2) to regulate host gene expression. Once detected in the cell, dsRNA are processed into small interfering RNA (siRNA) about 20-25 bp in length, that then complex with a nuclease and are guided to mRNA with complementary sequences. Only mRNA with high sequence similarity to the siRNA are degraded, thus the mechanism of RNAi is sequence specific.

By knowing a gene's sequence, and by adding a segment of that gene to plants in a way that produces dsRNA, RNAi can be used in the lab to knock down expression of specific transcripts, aiding the study of gene function. Discovery of the RNAi pathway has led to new plant-biotech applications in which dsRNA-producing transgenes can be used to modify host traits, or to interfere with gene expression in fungi that naturally exchange dsRNA with plants [3].

Host induced gene silencing (HIGS)

HIGS is a transgenic method of producing disease resistant crops whereby a host makes its own RNA-based pesticides that confer protection against specific pathogen species. Transgenes are added to the host that produce dsRNA complementary to specific genes of the pathogen. Suitable gene targets for HIGS are those that are essential for pathogen growth and or virulence. Host derived dsRNAs are naturally taken up by invading fungal cells. HIGS dsRNA are recognized by the pathogen's native RNAi machinery resulting in targeted degradation/silencing of mRNA with sequences complementary to the dsRNA **(Fig. 2)**.

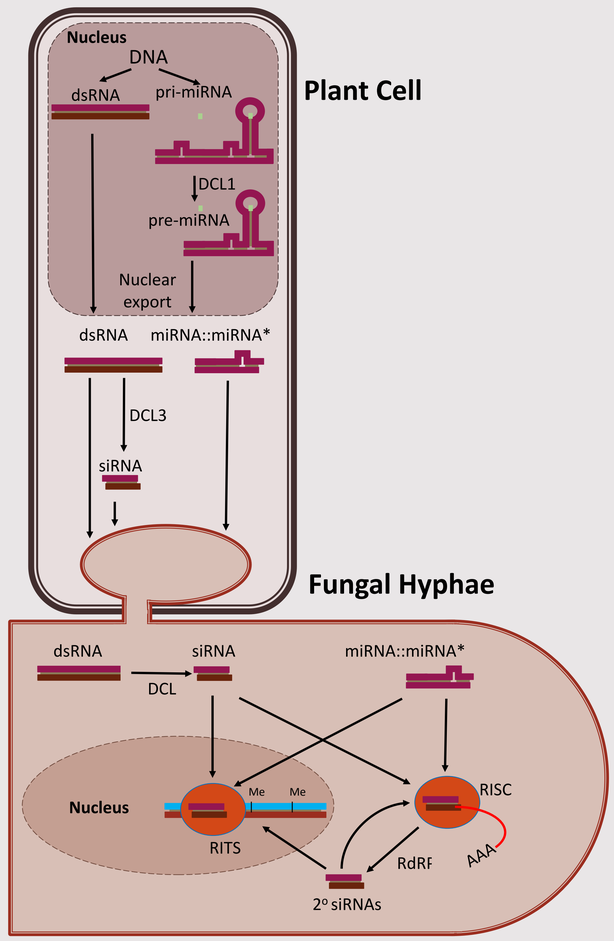

Figure 2. Overview of molecular interactions occurring during HIGS against a fungal pathogen. Nagle et al (2018).

In vitro proof of principle

Many fungi are known to take up dsRNA from their environment which can then induce RNAi [4]. To test whether *S. musiva* was one of these fungi, and to seek evidence for dsRNA suppression of specific genes, we attempted to knock down the fluorescent protein in a marked strain of *S. musiva* by culturing in broth with *in vitro* transcribed dsRNA complementary to the fluorescent protein mRNA **(Fig. 3)**.

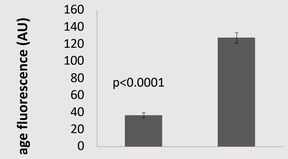

Figure 3. A red fluorescent protein (AsRED) marked *S. musiva* cultured in broth with dsRNAs targeting the AsRED gene showed a 71% reduction in fluorescence compared to a water treated control 41 hours post treatment.

Screening HIGS target genes

Next we demonstrated that the effects of dsRNAs targeting genes necessary for fungal growth could also be observed in broth culture as above. Using optical density of broth cultures as a proxy for fungal growth, the effects of various dsRNA treatments on *S. musiva* growth were observed **(Fig. 4)**. Selected target genes were homologs of genes demonstrated in previous studies as effective HIGS targets against fungi in other pathosystems [4,5]. Knockdown of *cyp51* is expected to inhibit growth by silencing a key enzyme in the ergosterol biosynthesis pathway—a critical cell wall component. RNAi against fungal dicer genes has also been shown to reduce fungal growth and is thought to work by inhibiting developmental gene regulation [5].

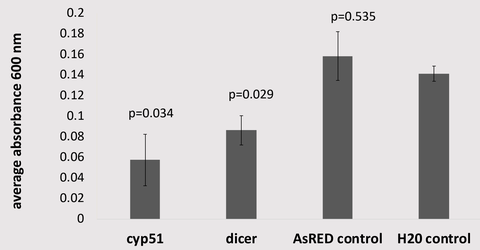

Figure 4. Growth of *S. musiva* hyphae five days post treatment with 20 ng/µl dsRNA. Percent reductions relative to the water treatment for *cyp51* and *dicer* treated wells were 59% and 39%, respectively. The *AsRED* dsRNA treatment controls for RNA toxicity as silencing of the *AsRED* gene should not affect growth.

Generation of HIGS transgenics

Constructs targeting the same genes that caused growth reductions *in vitro* have been transformed into *Populus* **(Fig. 5)**. Transgenic lines will be screened for high transgene expression (qPCR) and by a disease resistance assay in magenta boxes. **(Fig 6)**.

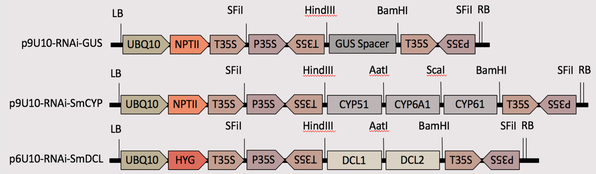

Figure 5. HIGS constructs transformed into *Populus* hybrid T61 using *Agrobacterium*-mediated transformation. p9U10-RNAi- serves as a negative RNAi control as the dsRNA produced has no gene target in *Populus* or *S. musiva.* The stacked RNAi-inducing fragments in the *cyp* and *dcl* constructs target all known homologs.

Figure 6. Magenta box disease resistance assay**.** Susceptible hybrids develops symptoms after inoculation with *S. musiva* while the resistant hybrids show fewer symptoms.

Non-target effects?

The effects of HIGS are thought to be specific if the gene targets are unique to the targeted pathogen. We aim to test this assumption by looking for non-target effects on the poplar microbiome using a shotgun ITS metabarcoding approach on disease resistant lines grown in a field trial.

Conclusions

- *In vitro* studies with synthesized dsRNA provide strong evidence that *S. musiva* has the capacity for targeted gene suppression by RNAi.
- We are producing transgenic plants that produce dsRNA against fungal cell wall and dicer genes to test if specific disease resistance can be generated in *Populus*.

Literature cited

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