In planta colocalization and induction of gene expression in wood-degrading fungi

Aims

Wood-degrading fungi make energy from nature's most abundant yet most recalcitrant plant tissue.[9] Their mechanisms have obvious bioenergy relevance, and the brown rot fungi we study accomplish this without removing large amounts of lignin and without exo-acting cellulases. Aided by a Special Call, Quiet Wing proposal, we have begun working at EMSL using Helium Ion Microscopy (HIM) and single-molecule fluorescence in situ hybridization (smFISH) to colocalize dynamics *in planta*. This is already bearing fruit, with smFISH images for a *Nature Communications* submission. We would like to deepen the effort by integrating Quiet Wing microscopy and transcriptomics capabilities at EMSL.

We have two current DOE BER bioenergy-focused efforts probing spatial dynamics in brown rot fungi. These fungi have long been theorized to deploy oxidative radical-based 'pretreatments' prior to secreting cellulases into the wood cell wall,[1,2,3] but mechanisms are putative, in part due to limitations studying *in planta*.[4] Since 2010, we have used coarse (mm-scale) spatial differentiation of wood physiochemistry and mRNA via qPCR to show evidence that reactions are indeed partitioned, perhaps segregated inside wood cell walls.[5] Our goal at EMSL is to test two long-standing hypotheses *in planta* that we are addressing using cutting-edge EMSL instrumentation and technical collaboration. **Aim 1)** Test the **Shield hypothesis -** Reaction partitioning between fungal hyphae and wood cell walls is enabled by leaving a thin tertiary wall layer (S3) intact. **Aim 2)** Test the **Differential hypothesis -** Hyphal tips express oxidative-enabled pathways, specifically Fenton reaction iron-reduction genes, ahead of cellulolytic enzyme expression.

Mission

The biology of brown rot fungi is of interest for industrial biomass processing and bioenergy efforts because they consolidate pretreatments with enzymatic saccharification to efficiently degrade lignocellulose.[6] Wood-degrading fungi also recycle Earth's largest pool of biotic carbon.[7] Defining their metabolic mechanisms therefore dovetails with the energy, environment, and basic research missions of DOE, with a particularly tight fit in BER as evidenced by their ongoing support of this research.

Background

Among the fungi able to deconstruct lignocellulose, the 'brown rot' fungal mechanisms have excellent potential in biofuels applications (Fig 1). A key hindrance to harnessing these fungal mechanisms has been their spatial complexity (Fig 2). Our DOE project has shown that brown rot hyphae penetrating wood are not metabolically uniform, [5] challenging the constitutive pathway paradigm developed from studies in artificial media.[8,14] We have strong evidence that these fungi partition oxidative reaction pathways from enzymatic cellulolysis, and this has been supported by results from smFISH at EMSL (Fig 3). If this reaction consolidation could be tapped for biomass processing, it could reduce costs associated with the stand-alone pretreatment step. [9] With this in mind, our goal is to understand how brown rot fungi partition these incompatible reactions in discrete space, and to add a new, targeted effort when our current EMSL effort ends in September, matching global gene expression to provide spatial overlays *in planta*.

At EMSL, we can test our hypotheses with resolution above what is possible in our facilities. Relevant to the 'Shield' hypothesis, pores in brown-rotted wood cell walls have twice been deemed too small (max 38Å) to permit ingress of cellulolytic enzymes.[10,11] This is difficult to accept given that the porosity of sound wood is 10-15 Å and that density loss exceeded 60% in these studies. One plausible explanation is that the thin S3 layer separating fungi from the S2 cell wall layer is left intact as a protective barrier, segregating non-selective oxidative reactions from enzymes and hyphae in lumens.[12] Testing this has been limited by resolution limits. Coupling HIM with transmission electron microscopy (TEM) can provide resolution (>3.5Å) suitable for tracking S3 integrity over during brown rot, using the model fungus *Postia placenta* on gamma-sterilized pine. Our EMSL attempts have shown clear potential in accomplishing this, but the decay stage and sample preparation need a longer time series and optimization, respectively.

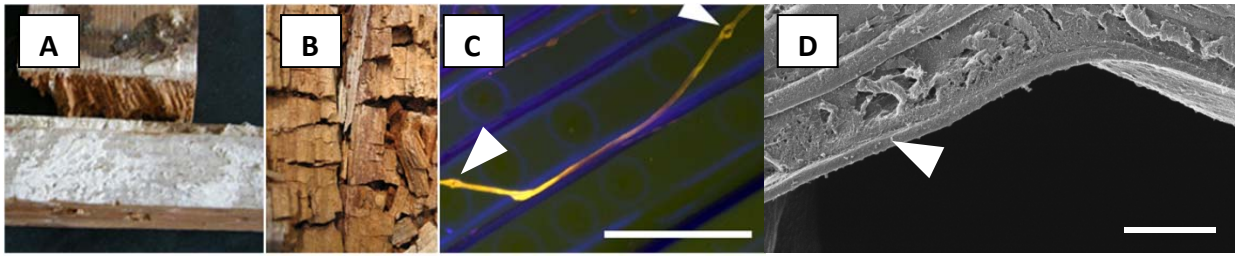

Fig 1 - Brown rot fungi such as sequenced/annotated *Postia placenta*[13] (A) release sugars and (B) leave lignin-rich wood residues. Chitin-stained hyphae (C) in pine show looped septa (arrows) and growth in lumens (scale bar 50 µm). Cross-grain fracture and HIM at EMSL (D) resolved the S3 layer (scale bar 2 µm), distinguishing it (arrow) from other S layers. Image A, DOE. B-D, Schilling & Shutthandandan.

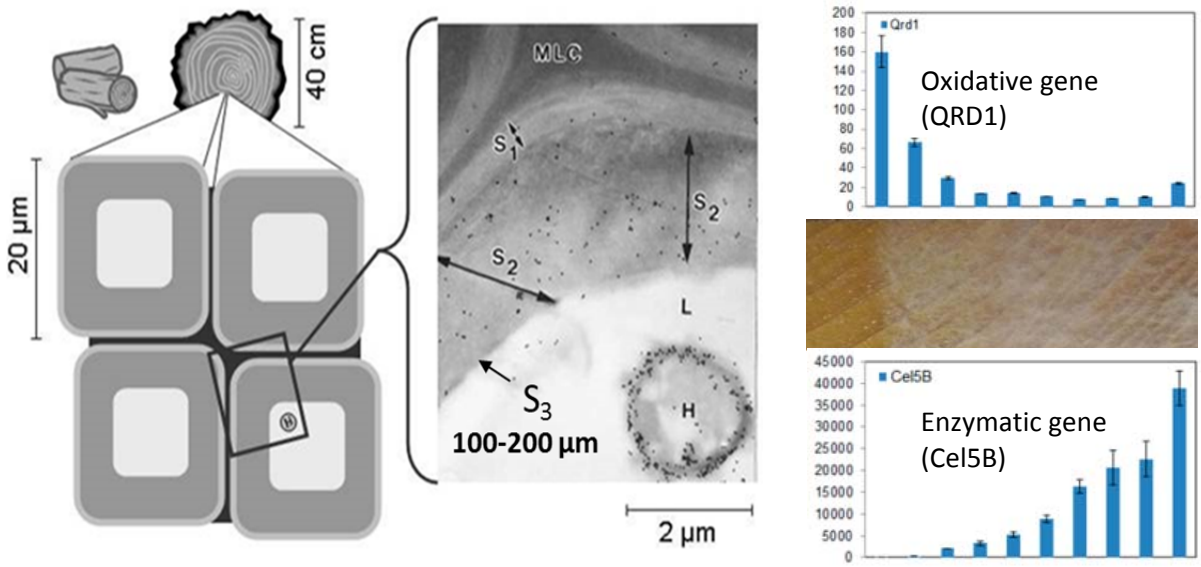

Fig 2 - Resolution context (*left*) for fungi (H) in wood cells. Fungal hyphae (H) inside wood lumens (L) degrade components in wood cell walls extracellularly, but it is unclear if decay is uniform across the first (S1), second (S2, thickest), and tertiary layer (S3). Differential expression (*right graphic*) between the hyphal front (arrow) and behind is evident when co-localizing gene expression. Shown is quinone reductase (QRD1) and endoglucanase (Cel5B). The QRD involved in oxidative Fenton chemistry is up-regulated near the front, while the cellulase enzyme is up-regulated behind, here using qPCR (Zhang, J.).

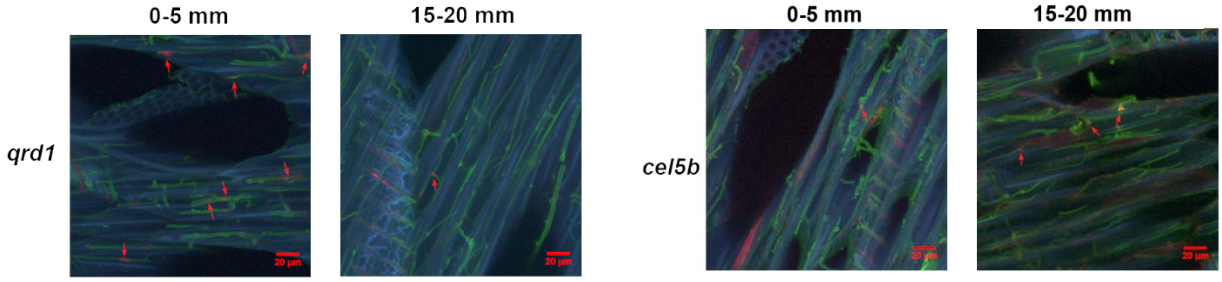

Fig 3 - Using smFISH for finer scale expression analysis (scale bars 20 µm, with expression in red against autofluorescent wood (blue) and chitin-stained fungi (green)) succeeded but needs method development. This will allow us to gauge differential expression at a hyphal scale, unlike coarse mycelial-scale work. It is a high-feasibility effort, and can be logically complemented using RNAseq in the same samples.

Approach

Specific Aim 1: Probe the thin (<200 nm) tertiary wood cell wall layer (S3) segregating fungal hyphae from the thick (5-7 µm) S2 layer, using Helium Ion Microscopy (HIM) and TEM.

Specific Aim 2: Track differential expression of target wood-degrading genes *in planta* and as a function of carbon source, coupling Super Resolution Fluorescence microscopy for smFISH with RNAseq.

Logistics

There are several ways we plan to leverage efforts in our interaction at PNNL within EMSL. First, our most recent BER grant includes collaboration with Dr. Ellen Panisko, which provides an opportunity to leverage travel funds and synergize efforts. Second, sample preparation will not be from scratch, rather we will harness a sample set being developed in our larger effort. Third, we have already developed FISH probes and target genes of interest. Combined, this will help keep tool-use at EMSL on track in a timely manner, and it will build on a network of researchers in a shared effort, broadening its impact.

Techniques

Aim 1: Probe the thin (<200 nm) tertiary wood cell wall layer (S3) segregating fungal hyphae from the thick (5-7 µm) S2 layer, using Helium Ion Microscopy (HIM) and TEM.

Sample # = 20 (4 time points, 2 fungi, 2 tome types + 4 TEM samples) 0 – 60% density loss in pine

The S3 layer in wood cells exists at the interface of the wood cell wall and the empty space of the lumen, visualized as the inner surface of a tube. To use HIM to inspect this layer in context with the other secondary wall layers requires sectioning approaches with minimal artifacts. A freeze-fracture technique was successful at EMSL, with the image in Fig 1 (D) showing HIM resolution potential. Our February EMSL work, however, suggested a need for cleaner sectioning, with a likely pore diameter of <10 nm. We propose using a time series of *P. placenta*- and *Gloeophyllum trabeum*-decayed red pine (gamma-sterilized *Pinus resinosa,* in-hand), mimicking the density range from earlier porosity studies (0-60%) using two distinct brown rot clade representatives.[10,11] This wood decay series will be harvested using pure fungal cultures on a wetted vermiculite:peat:soil mixture, as standard in the Schilling Lab.[15] For freeze-fracture in LiN, samples are dehydrated through an ice-cold ethanol series to 100% ethanol, fractured, critical point dried as for SEM, and then carbon-coated. This sample preparation[16] is done at the University of Minnesota prior to imaging at EMSL, and sectioning may be better done with more decayed material, infiltrated with cryo media for cryo-sectioning rather than fracture, but this will be developed and optimized. The HIM system was able to resolve the S3 layer from the S2 layer as a line of interface, as shown in the Figure (D) where microfibril angle changes orientation.[17] The Rutherford Backscattering Spectrometry (RBS) capability of this scope can then be trained across distinct secondary cell wall layers to attempt to probe elements such as iron (Fe). The oxidative step of brown rot is Fenton-driven and Fe-dependent, and distinguishing concentrations relative to the cell wall layer would be valuable if elemental analyses/concentrations are amenable.[18]

With the goal of HIM to reveal both cross-sectional and inner surface S3 layer features, a logical complement is to use TEM to probe the cross-sectional features, particularly with potential to calculate porosity, outright. Similar has been done in the Himmel lab at NREL using 3-D TEM in pretreated corn stover, but this has not been done in a brown rot-degraded wood sample.[10,11] It could end a long-standing question about porosity, and we further have an immune-labeled endoglucanase probe that could be used specifically in *P. placenta* to match apparent porosity with actual enzyme ingress, microscopically. This would require osmium fixation and embedding after an acetone-dehydration series, along with ultramicrotoming, although thicker samples may be helpful for rotational imaging and depth. For this and the HIM, sample prep optimization is important, along with having some 'hands' to do it. This was clearly most feasible by having member(s) of the Schilling lab there in Richland to work iteratively, at least to start, and is clearly boosted by the capabilities and technical support at EMSL.

Aim 2: Track differential expression of target wood-degrading genes *in planta* and as a function of carbon source, coupling Super Resolution Fluorescence microscopy for smFISH with RNAseq.

FISH Sample # = 15-21 (3 decay stages in wood x 5-7 gene target combinations per wood sample)
RNAseq Sample # = 6-10 (3 carbon sources x 2 time points + controls)

Through our wood wafer design forcing hyphal cells directionally colonize and degrade wood sections, our q-PCR results indicate that newly-formed hyphal fronts express more oxidative genes while older hyphae express enzymatic genes during wood decay. We need to verify, however, these differential locations of oxidative and cellulolytic mRNA transcripts *in planta* along developing and (importantly) branching hyphae with directional growth using high-resolution smFISH. With single-molecule FISH, we are tracking gene transcriptions along single hyphae extending back from the hyphal tip to explore whether the partitioned expression relates to hyphal development. On the other hand, the influences of carbon source utilization on gene expression will be detected through genome-wide RNA sequencing to further explain the potential spatiotemporal gene regulation mechanisms, something we have done for our *Nature Communications* submission research.

For FISH analyses, two samples representing initial and late decay stages would be extracted from wood wafers colonized by *P. placenta,* overlaying expression of 5-7 target genes with putative function in lignocellulose oxidation and saccharification. At the University of Minnesota, samples have been cryo-sectioned into 40-60 µm thin and fixed with 4% paraformaldehyde, then mRNA of three genes *qrd1* (quinone reductase), aox1 (alcohol oxidase) and *cel5b* (endoglucanase) hybridized with 200-300 nt digoxin-labeled RNA probes. Hybridization signals were amplified last time with the Tyramide Signal Amplification method. To colocalize the hyphae relative to mRNA and wood, the Wheat Germ Agglutinin, Alexa Fluor 488 Conjugate dye was used to stain chitin in fungal cell walls. Images of the prepared slides stored in ProLong Gold Antifade Mountant were acquired with the EMSL's confocal fluorescence microscopy, but weak FISH signals made optimization a logical next collaborative step at EMSL, particularly for multi-probe overlays. We would use lysing enzymes to partially digest the fungal cell wall and make use of the short-length RNA probe (50 nt) to improve probe penetration. Multiple strategies will be adopted for single gene to enhance the hybridization signals, using EMSL's stochastic optical reconstruction microscope (STORM) in tandem with the structured illumination fluorescence microscope (SIM) for super resolution fluorescence microscopy. These, along with image analyses and technical help at EMSL, will not only allow us to explain the colocalization of expression at the resolution of single mRNA transcripts in wood, it will also provide a valuable tool broadly for *in planta* research.

Considering the possible influences of carbon source utilization on gene regulation, we also propose exploring the effects of carbon sources on gene expression through RNA sequencing. We have done this using the qPCR approach, and by matching expression induction with the location in the wafers of each carbon source test (e.g. cellobiose, glucose). Polysaccharide cellulose and two simpler sugars cellobiose and glucose will be applied to feed *P. placenta* and *G. trabeum*. Then, total RNA will be extracted in University of Minnesota using our standard Trizol isolation. The cDNA library preparation and sequencing with 5500xl SOLiD System-Applied Biosystems will be performed in EMSL, as we did in February. The data storage and analyses will also being performed with the help of PNNL's staff, server and software, but with the help of Gerry Presley who is being training in bioinformatics at Minnesota. Combined with the smFISH results, we expect to be able to better explain the spatiotemporal regulation mechanisms in these brown rot fungal systems with such clear value in the bioenergy sciences.

APPENDIX 1 – REFERENCES

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APPENDIX 3 – EMSL RESOURCES

Resource	# of Samples	Estimated date of sample shipment to EMSL	Units Requested (specify unit)	Project Team Expertise per Resource	EMSL Support Requested
Quiet Wing					
Helium Ion Microscope (HIM)	16	September 2015 (PI at PNNL)	80 hours	PI via SEM and plan a sample time series in cryo-medium for fracturing in Quiet Wing at EMSL.	Sample fracturing, HIM imaging, and element and pore analyses (Lea group)
Transmission Electron Microscope (TEM)	4	February 2016 (PI at PNNL)	40 hours	PI via TEM, with sample prep iterative with EMSL.	Sample prep guidance, and 3-D TEM analyses (Lea group)
EMSL Transcriptomics					
Structured illumination fluorescence microscope (SIM)	15-21	September 2015 (Zhang at PNNL)	120 hours	Participant probe development for target RNA transcripts, and wood decay time series production.	Assist in probe development, operation, and analysis (Orr group)
Stochastic optical reconstruction microscope (STORM)	15-21(same samples as SIM)	February 2016 (Zhang at PNNL)	120 hours	Participant probe development for target RNA transcripts, and wood decay time series production.	Assist in probe development, operation, and analysis (Orr group)
RNAseq	6-10	February 2016	120 hours	mRNA extraction from wood is already optimized, so it is machine time. Bioinformatics planned in Minnesota.	RNAseq machine time and technician support (Orr group)
Total	50-62	2015-2017	480 hours		