EMSL Intramural Proposal
FY2015 Call

Multi-Omics Evaluation of Tripartite Associations in Red Alder Roots: Integrated Transcriptomics, Proteomics, Metabolomics and Metabolite Imaging of Red Alder Symbiotic Associations in Root Tissue

Kim K. Hixson – EMSL (PI)

Christopher R. Anderton (EMSL), **Rosalie K. Chu** (EMSL), **Alice C.Dohnalkova** (EMSL), **Lye Meng Markillie** (BSD), and **Hugh D. Mitchell** (BSD)

*In collaboration with **Norman G. Lewis, Laurence B. Davin, Barri Herman** – Washington State University, Institute of Biological Chemistry*

Contact: Kim K. Hixson
Senior Research Scientist
Environmental Molecular Sciences Laboratory
Pacific Northwest National Laboratory
3335 Innovation Boulevard, Richland WA 99354
Tel: (509) 371-6338, Fax (509) 371-6546, Email: kim.hixson@pnnl.gov

Background and Impact of Research:

This proposal offers EMSL/PNNL the unique opportunity to conduct the first ever integrated multi-omics and imaging evaluation of the *Alnus rubra* (red alder tree)/*Frankia alni* (bacteria)/*Alpova diplophloeus* (fungus) tripartite rhizosphere. We present the case here that the red alder tree could serve as a good DOE mission related organism, with great potential to produce high yields of biofuel/biochemical precursors from marginal lands1,2. This ability stems from its symbiotic relationships both with nitrogen-fixing/nodule-producing soil bacteria3-5 and also various fungi6, which allow for enhanced essential mineral (e.g., phosphorus) uptake7. While it has been documented that red alder roots can symbiotically associate with several microorganisms, *F. alni* and *A. diplophloeus* interactions are some of the most ubiquitous8 in wild red alder populations.

Listed here are **twelve attributes** out of many which highlight why red alder would make a good potential bioenergy feedstock organism warranting further study: **1)** It would be the first N2 fixing tree to be sequenced and annotated; **2)** Growth rates are similar to Poplar tree but the biomass is ~25% more dense9; **3)** It can grow into very dense groves (i.e., 50,000 trees/acre)10, easily out-competing weeds11; **4)** It can produce a high level of biomass at ~4-33 dry tons per acre annually12; **5)** It contributes substantially to overall soil fertility13; **6)** It can be planted in mixed stands (e.g., with Poplar) providing fixed nitrogen to other plants14; **7)** It does not compete with food crop land2; **8)** Red alder biomass can alternatively be used in the lumber market12; **9)** It is naturally free from most insect and disease problems and can be self-pruning13,15; **10)** It has substantially lower biogenic volatile organic carbon (VOC) emissions16 compared with many other plants (e.g., Poplar, Eucalyptus), with isoprene emissions for red alder =~0.025 µgCg-1h-1 compared to poplar =~110 µgCg-1h-1; **11)** Red alder synthesizes metabolites with medicinal properties17,18; and **12)** *Frankia* synthesizes antimicrobial polyketides19-21.

In 2011, Weyerhaeuser Company was legally forced to sell its Northwest Hardwood Division because the company became a real estate investment trust (REIT). All genetic materials from their red alder/*Frankia* improvement initiative were gifted to Dr. Norman Lewis (WSU) with the contractual agreement that WSU would continue research and propagation of the lines. Accordingly, EMSL now has the unique opportunity to collaborate with Dr. Lewis (PhD advisor to Kim Hixson) and Dr. Barri Herman to obtain preliminary molecular/genetic characterizations of red alder and its symbiotic associations. This study will not only provide a cursory model for the red alder/*Frankia*/*Alpova* rhizosphere, but the data obtained will enable future development of genetically engineered red alder for use in the high yield production of biofuels/biochemicals from marginal lands.

Specific Aims:

1. To obtain a genomic sequence and draft annotation and assembly of *A. rubra* clone 639 (our red alder clone with the most superior growth characteristics), and *A. diplophloeus*.

2. To assemble a differential expression atlas of transcripts, proteins and phosphoproteins found in the red alder root system in specific association with each of its microbial symbionts.

3. Identify potential root exudates and metabolite biosynthesis, mobilization and exchange in red alder root using pulse chase metabolic LC-MS/MS profiling, followed by MALDI FTICR (PNNL) and MALDI metabolite (WSU) imaging of root, stem and leaf sections that associate with *Frankia* and *A. diplophloeus* as has been demonstrated in prior work22.

4. Obtain microscopic imaging to reveal morphological features and ultrastructure of root tissue regions which correspond with the localization of the metabolites identified in Specific Aim 3.

5. Integration and mapping of all omics and imaging data into a model of tripartite red alder/*Frankia*/*A. diplophloeus* symbiosis.

Research/Technical Approach:
Plant growth: Shoots from *A. rubra* clone 639, will be harvested and rooted using sterile sand in controlled greenhouse conditions (WSU). After 2 weeks of growth, viable trees will be externally sterilized and washed in a peroxide solution and then grown in sterilized aeroponic chambers. These systems will be retrofitted with an enclosure to allow for pulsed 15N or 13CO2 gas (for pulse-chase experiments) and filtered atmospheric air flow (for temperature control). After acclimation, plants will be inoculated with 1) sterile control media, 2) *F. alni* (to produce N2 fixing nodules), 3) *A. diplophloeus* spores (to produce ectomycorrhiza) or 4) *F. alni* followed by *A. diplophloeus* to produce nodulation as well as ectomycorrhiza. After nodule and ectomycorrhiza development (~4 week post inoculation), root, root nodule, and ectomycorrhiza samples will be collected for multi-omic and imaging analyses.

Genome Sequencing: We will produce the first genome sequence of red alder (A. rubra) clone 639 and *A. diplophloeus* by submitting representative samples for sequencing to the new Illumina long-read commercial sequencing service, which is especially tailored for new organism genome mapping and multi-organism communities sequencing. The genome will be annotated collaboratively by Hixson (EMSL) and post-docs in the Lewis Lab (WSU).

Sampling: Three biological replicates or pooled replicates of representative samples of (non-inoculated) roots, root nodules, ectomycorrhiza, and nodules with ectomycorrhiza will be harvested and sectioned using the Cryostar NX70 (EMSL), for imaging (microscopy and metabolite). Specific known anatomic regions identified clearly by microscopy (i.e., periderm, vascular tissue, uninfected cortex, infected cortex, Hartig net, and mantle) of each root, root nodule (Figure 1A) and/or root ectomycorrhiza (Figure1B) will be subjected to electron microscopy and FTICR MALDI imaging.

Electron Microscopy: Un-inoculated roots, root nodules and/or ectomycorrhiza will be selectively dissected, and prepared for scanning electron or helium ion microscopy (SEM or HeIM)(EMSL) and transmission electron microscopy (TEM)(EMSL), to provide ultrastructure characterization.

Metabolomics & Metabolic Imaging: We will initially perform LC-MS/MS (as is routine in the Lewis lab at WSU) on the root exudates by passing water used to wash the roots through a 0.45 μm filter to remove microorganisms and then through a C18 SPE tube to concentrate and extract the metabolic exudates. In addition, whole cell metabolites will be extracted from pulverized root tissue after undergoing pulse chase analysis with plants that have been exposed to 13CO2 and 15N2, with analysis of 5 time points (T=0, T=30 min, T=2 hr, T= 8 hr, and T=32 hr) post-exposure, and will be analyzed using a XEVO-G2 qTof mass spectrometer (WSU) operating in tandem with a UPLC system. Special attention will be paid to the analysis of phytohormones, sugars, amino acids, and flavonoids as well as their conjugates as such metabolites are known to be important in hormone synthesis and/or are known to be exchanged between microorganisms and plant roots in other nitrogen fixing root associations23. Identified metabolites of interest from the LC-MS/MS analysis, will undergo MS imaging within the major anatomical features of each root sample described above in the sampling section by using the 15T MALDI FTICR MS (EMSL) which is capable of high mass resolution (100 μm) and mass accuracy chemical maps providing validation in the identity and spatial information of each metabolite of interest.

Transcriptomics: Collected root structures of interest will be pooled into 3 samples each and will be pulverized in liquid nitrogen. We will extract RNA, with ribosomal RNA depletion and poly A-based purification to generate cDNA libraries from each sample for RNA-seq using the 5500XL SOLiD sequencing technology (EMSL). In the event that the genome sequencing efforts described above are not feasible, we will use the Trinity software package for de novo assembly of transcript data, which will allow us to do comparative transcriptomic profiling of red alder in the treated groups as well as gene expression profiling of *A. diplophloeus*. Since the *Frankia* genome is currently available, mapping bacterial reads using the LifeScope mapping analysis tool will be straight-forward. Once sample mapping is complete, we will use the edgeR software package, available through Bioconductor, to identify differentially expressed genes between the various root samples.

Proteomics/Phosphoproteomics: A portion of material pulverized for transcriptomics will also be used for proteomics characterization. From the frozen powder, proteins will be extracted, digested with trypsin, multiplexed with 8plex iTRAQ labeling, fractionated into 24 fractions using an off-line basic (pH 10) separation, followed by global proteome analysis using an orthogonal low pH reversed phase C-18 separation of each fraction followed by analysis using an Orbitrap Velos (EMSL) in HCD mode. The phosphoproteome will be extracted (8 fractions total) from the fractionated and concatenated global fractions using magnetic Ni-agarose beads. The phoshoproteome will then be analyzed similarly to the global proteome. All differential peptide and protein abundance changes will be analyzed using the EMSL data management system and in-house developed proteome software tools.

Multi-Omics Data Analysis & Integration: Statistically significant features in the metabolomics, transcriptomics and proteomics datasets will be normalized and p-values and false discovery rates (fdr) will be calculated. Pathway Studio, Mapman, and Kegg pathway maps and analysis tools will be used to provide visual maps of cell signaling and metabolic pathways as they relate to each dataset. The transcript data may reveal splicing variants, alternative transcriptional start sites, single nucleotide polymorphisms, and the array of potential proteins which may be expected to be present. Proteomics data will reveal what actual enzymes/proteins are present and their relative abundances. Post-translational modifications (e.g., phosphorylations) on proteins may reveal enzyme activation or deactivation. Poor correlations between proteins and transcripts abundances may be indicative of post-transcriptional or translational regulation. Metabolic contributions from red alder, *F. alni* and *A. diplophloeus* will be delineated and correlated to the metabolite and structural localizations determined by the imaging data. These data will then be correlated to the other omics datasets (i.e., RNA-Seq, proteomics) to construct an overall model of metabolite localization, combined plant and microbe metabolism and hormone signaling found between red alder, *F. alni* and *A. diplophloeus.* Datasets will additionally be compared and contrasted to other previously characterized bacteria/fungi/root studies and all data and interpretations will be published and made publically available.

Our proposed research fits seamlessly with the EMSL Biosystem Dynamics and Design (BDD) Science Theme, as it sets out to characterize the tripartite relationship between a plant, fungi and bacteria; a system that works in concert to produce a rapidly growing, sustainable feedstock, for DOE mission related biofuel and biochemical production. This proposed research also addresses BER's mission "…to understand how genomic information is translated to functional capabilities, enabling more confident redesign of microbes and plants for sustainable biofuel production…" as described in the recently DOE published "Research for Sustainable Bioenergy Workshop Report"24.

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