PMI Performance Metric for FY18: Using genomics-based techniques, develop an approach to explore the functioning of plant-microbe interactions.

Q1 Metric: Report on the latest "omics" techniques used to understand the functioning of microbial communities in association with plants.

Introduction

The Plant-Microbe Interfaces (PMI) project is a Scientific Focus Area directed towards understanding the dynamic interface that exists between plants, microbes and their environment. Project efforts are focused on characterizing and interpreting systems comprising the poplar tree (*Populus*) and its microbial community, in the context of favorable plant microbe interactions. We seek to define the relationships among these organisms in natural settings, dissect the molecular signals and gene-level responses of the organisms using natural and model systems, and rebuild the complexity of these systems using sequence characterized plants and microbes. *Populus* is an ideal host system for examining interfaces between plants and microbes and a leading candidate for bioenergy production. It is a dominant perennial component of many North American temperate forests and among only a few plant species that host both endo- and ectomycorrhizal fungal associates. Numerous other types of microorganisms can be found within, or closely associated with, various *Populus* tissues, and these organisms may range from highly beneficial to pathogenic with respect to effect on host fitness. Ultimately, an improved fundamental understanding of plant-microbe interfaces will enable the use of indigenous or engineered systems to address challenges as diverse as bioenergy production, environmental remediation, and carbon cycling and sequestration.

"Omics" (a shorthand term used to refer to techniques involving genomics, transcriptomics, proteomics, and/or metabolomics, applied either individually or in combination) approaches are central to PMI efforts aimed at understanding the functioning of microbial communities in association with *Populus*. As described below, these diverse techniques have been central to defining the composition and functional contributions of the *Populus* associated microbiome across, host genotypes, tissue types, chemotypes, and environmental conditions.

rRNA gene-enabled microbial community ecology of *Populus*-microbe associations

Over the course of the project, we have used successive generations of amplicon sequencing techniques to characterize soil and plant associated archaeal, bacterial, and fungal communities as part of the PMI project. As technology has advanced, efforts have transitioned from clone-library based Sanger protocols, to 454 pyrosequencing, and now to Illumina-based technologies. The current protocols take advantage of custom mixtures of pooled primers to enhance microbial phylogenetic coverage (Shakya et al. 2013) and customized peptide nucleic acid blockers to decrease plant sequence competition from nuclear and organellar DNA in our sequencing runs (Lundberg et al, 2014). These modifications have improved the overall quality of data generated within these studies so that they now enable robust, repeatable and highly-replicated study designs incorporating hundereds of samples sequenced in parallel.

Environmental drivers of microbial community structure

Across environments and research studies, we have demonstrated that microbial communities vary across geographic locations, soil types, and seasons. Within a plant host, we have found that soil origin plays a large role in structuring archaeal, bacterial, and fungal communities both in the rhizosphere and in root endophyte communities within the plants (Bonito et al. 2014, Shakya 2013). Additionally, fungal root symbionts are likely constrained more by dispersal and

biogeography than by plant host availability (Shakya et al. 2013; Bonito et al. 2014). Not surprisingly, within mature *Populus deltoides* stands, we demonstrated that season explained a significant proportion of microbial community variation (Shakya et al. 2013) with the dominance of different microbial taxa clearly shifting from spring to fall in both soils and root endospheres. For example, *Pseudomonas* sp. dominate as early colonizers of plant roots as they flush in the spring; however, over the course of the growing season *Actinobacteria* increase and dominate root endospheres in the fall.

<u>Host control in shaping microbial communities</u>

Studies conducted in PMI have shown that microbial communities, and to a greater extent bacterial communities, vary significantly in the endosphere of different plant species (Bonito et al. 2014). Within *Populus*, we have shown that although soil and environmental drivers tend to be stronger determinants of community composition, plant host genotype often played a role in structuring these communities in wild populations (Shakya et al 2013, Bonito et al. 2014). However, recently completed studies under controlled greenhouse and common garden conditions are showing that host genotypic variability in plant higher-order salicylate chemistry, or specific alteration of plant cell wall characteristics via RNAi knockdowns, are altered in microbial community composition and diversity. For example, gas chromatography-mass spectrometry (GC-MS)-based metabolomic analyses were used to identify *Populus trichocarpa* clones in a GWAS population with unique chemotypes, including clones with very high or very low concentrations of higher-order salicylates, such as salicortin, salicin, and tremuloidin, as well as clones with a unique variant pathway that allows them to accumulate populin instead of, or in addition to, tremuloidin. Whereas these chemotype classes were initially established in leaves of field-grown plants at Clatskanie, OR, our subsequent metabolomic analyses confirmed a high correlation (R^2=0.93) in the ranking of a clone's salicortin concentration in roots of *P. trichocarpa* growing at a different site (Corvallis, OR), which allowed the selection of 12 clones of variant higher-order chemotypes for subsequent greenhouse studies. This study confirmed these chemotypes and also showed that they are altering rhizospheric microbial communities strongly. Finally, another recently completed study is demonstrating for the

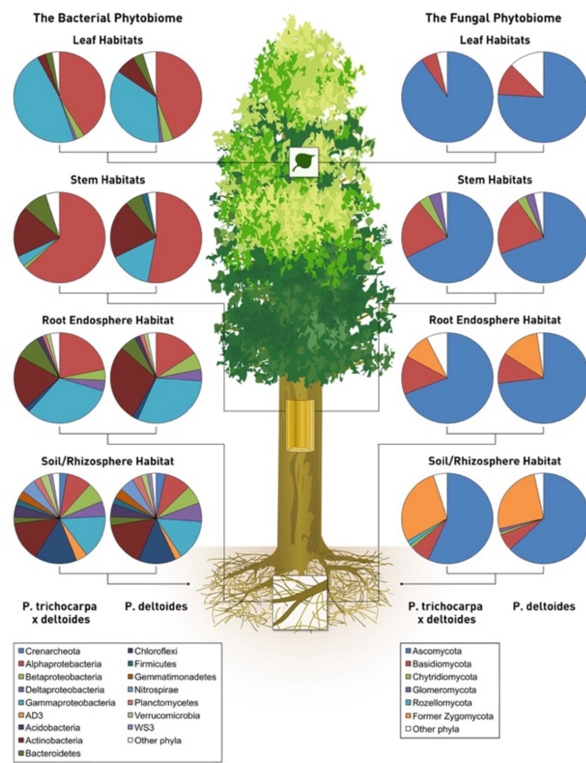


Figure 1. Summary of tissue level microbial community composition across the major habitat zones of *Populus deltoides* versus hybrid (*trichocarpa* x *deltoides*) trees.

first time that there is consistent subset of the core microbiome of *Populus* that responds across different stressors including water-drought stress, toxic soil metal stressors, and shading/energy stress.

Host tissue-level variation in microbial communities

Within *Populus*, we have found that microbial communities vary significantly across the different plant tissues we have measured (**Figure 1**). Aboveground habitats are less diverse than belowground habitats, with the highest diversity seen in soil environments. Belowground, our studies have shown that the root endosphere microbial communities are not simply a subset of rhizosphere microbes, but instead are a diverse independent assemblage of microorganisms (Gottel et al 2011) that are fairly similar in gross composition, even across plant phylogenetic divides, to those of *Arabidopsis* (Lundeberg et al. 2012; Levy et al. 2017). Within each of these plant habitats, variation in microbial communities may demonstrate the unique niche each of these habitats provides and additionally might correlate with different functional profiles. For example, a recent study characterizing the microbial community inhabiting *Populus* heartwood

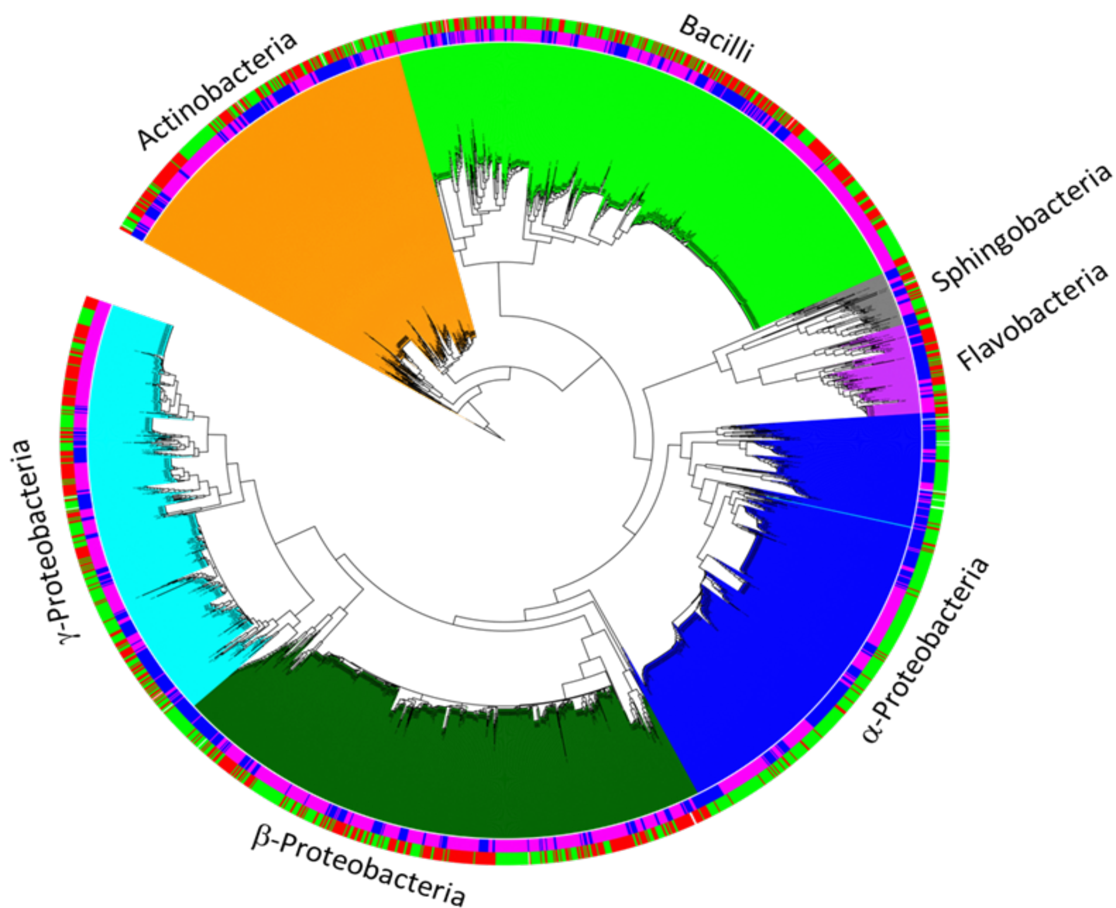


Figure 2. Neighbor-Joining analysis of >2,400 partial 16S rRNA genes generated from isolates of endophytic and rhizospheric bacteria (green and red bars respectively on outer ring) of *Populus deltoides* in the Eastern US and *Populus trichocarpa* in the Western US (blue and magenta bars respectively on outer ring). Seven classes of bacteria are well represented by multiple genera, some with many isolates (with identical 16S) from both host species.

found an abundance of methanogens, demonstrating that this habitat offers an anoxic environment for these organisms.

Characterization of common bacterial root rhizosphere and endosphere associates of *Populus*
rRNA enabled studies have provided valuable insight into microbial community structure and how these communities vary with biotic and abiotic drivers. Increasing evidence, from our work and others, suggests that the varied functional attributes of *Populus* are a result of both its genetic potential and the microbial species that interface with it. Over the past 9 years, we have isolated an unprecedented collection of *Populus*-associated microbes, including >3800 bacterial (**Figure 2**) and >1800 fungal strains (Bonito et al. 2016). These pure culture isolates along with our axenic *Populus* mesocosm system have afforded us the opportunity to investigate host recolonization and phenotyping. Several strains have proven to be robust recolonizers and demonstrate plant growth-promoting effects (Weston et al., 2012; Doktycz et al. 2015) and a new beneficial fungal species *Atractiella rhizophila* has been described from *Populus* roots (Bonito et al. 2017; Velez et al. 2017). These isolates have the ability to benefit the host plant through plant growth promotion, nutrient acquisition and stress tolerance. Many have rRNA sequences that correspond to predominant contributors in our community surveys of *Populus*. In addition, with the assistance from DOE Joint Genome Institute, we have sequenced the genomes of >400 bacterial and 30 fungal strains isolated from *Populus* roots. Mining of these genomic resources, coupled with the development of genetic systems for a number of these microbes, has allowed us to identify potential mechanisms of host recognition, recolonization, and nutrient acquisition.

Genomic identification of functions that influence bacterial associations with host
Plant-microbe interaction assays have led to observations of phenotypic effects on host plants including immune responses (Weston et al. 2012), mycorrhizal helper effects (Labbe et al. 2014), plant growth and root architecture effects (Bible et al. 2016). Our collection of well-characterized bacterial isolates provides an ideal resource for applying state-of-the-art omics techniques in gaining a molecular-based understanding of the physiological and functional consequences of plant-bacteria interactions. To date, genome sequences for strains of Actinobacteria (51), Proteobacteria (257), Bacteriodetes (52) and Firmicutes (39) have been collected. These isolates and genome sequences (Brown et al. 2012, Brown et al. 2012, Klingman et al. 2015, Utturkar et al. 2014, Levy et al. 2017) have allowed us to make significant progress towards gaining a molecular level understanding of plant colonization and of material exchange and signaling between the plant and microbial components. Initial comparative genomic analyses, metabolic reconstructions and phenotypic characterizations have revealed diverse functional capabilities among these microbes, including nitrogen fixation, the production of siderophores, phytohormones, quorum sensing signals, numerous natural products and diverse carbon metabolism (Levy et al. 2017, Jun et al. 2016, Timm et al. 2015, Schaefer et al. 2013)

Shotgun metagenomics and single cell genomics techniques adapted for the *Populus* Root-Rhizosphere-Soil interface
As with rRNA gene analysis, shotgun metagenomics based analyses of the plant associated microbiome presents great challenges due to the host genetic background which can overwhelm microbial sequences in the analyses. To overcome this challenge, the PMI project developed a protocol for sequential separation and enrichment of microbial cells from root material using a combination of differential and density gradient centrifugation. This enrichment technique generates material suitable for both flow cytometry based separation of live cells for single cell

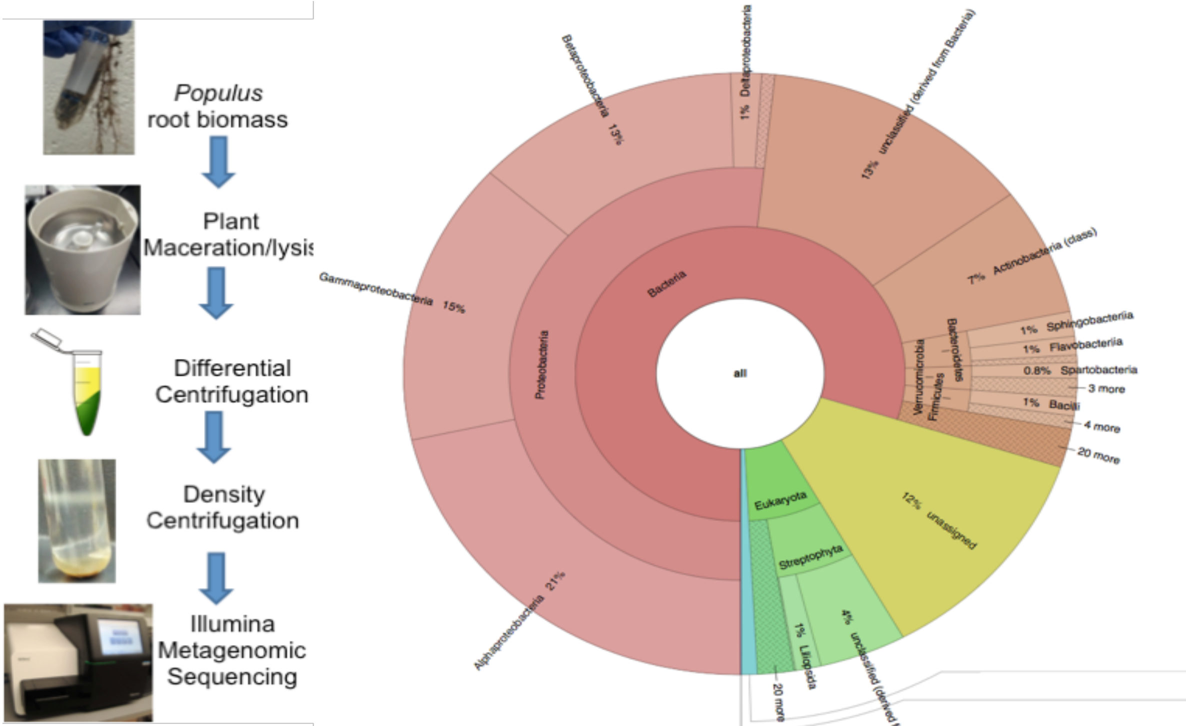


Figure 3. At left is an overview of a protocol developed that involves combined differential and density gradient centrifugation which enables consistent reduction of host background DNA "contamination" (within green wedge of eukaryotes) in shotgun metagenomes to <5%.

genomics (Utturkar et al. 2016) as well as DNA extraction and shotgun metagenomics that consistently contain less than 5% plant host derived reads (**Figure 3**).

We have applied these techniques to prepare libraries for 30 metagenomes sequenced in collaboration with the Joint Genome Institute comparing *Populus deltoides* and hybrid root endosphere, rhizospheres and bulk soils from a plantation in Blount County, TN. The resulting metagenomes show functional and composition changes not only between habitat (soil, rhizosphere, root endosphere) consistent with prior rRNA based studies, but also across host genotype within the endosphere habitat for *P. deltoides* versus *P. trichocarpa* x *P. deltoides* hybrids. Currently we are using similar approaches to advance techniques for metatranscriptomics suitable for the *Populus* host environment.

Single cell genomics and targeted enrichments of endosphere and rhizosphere organisms

A large fraction of microbial taxa from the *Populus* microbiome remains uncultured, including at high taxonomic levels (class, phylum). Using the same density gradient fractionation technique described above for metagenomes (**Figure 3**), we were able to generate Single Cell Genomes (SAGs) for several bacteria that have eluded our cultivation efforts, including members of the *Acidobacteria*, *Armatimonadetes* (former OP10), *Verrucomicrobia*, and *Planctomycetes* from the *Populus* rhizosphere and endosphere (Utturkar et al 2016) and are currently expanding these efforts towards new target taxa. However, new approaches are needed to aid in the cultivation and/or genomic characterization of representatives from the *Populus* "dark microbiome" to obtain a more complete understanding of the microbial community potential and its role in plant-microbe interactions. This is especially true in the rhizosphere surrounding *Populus* roots.

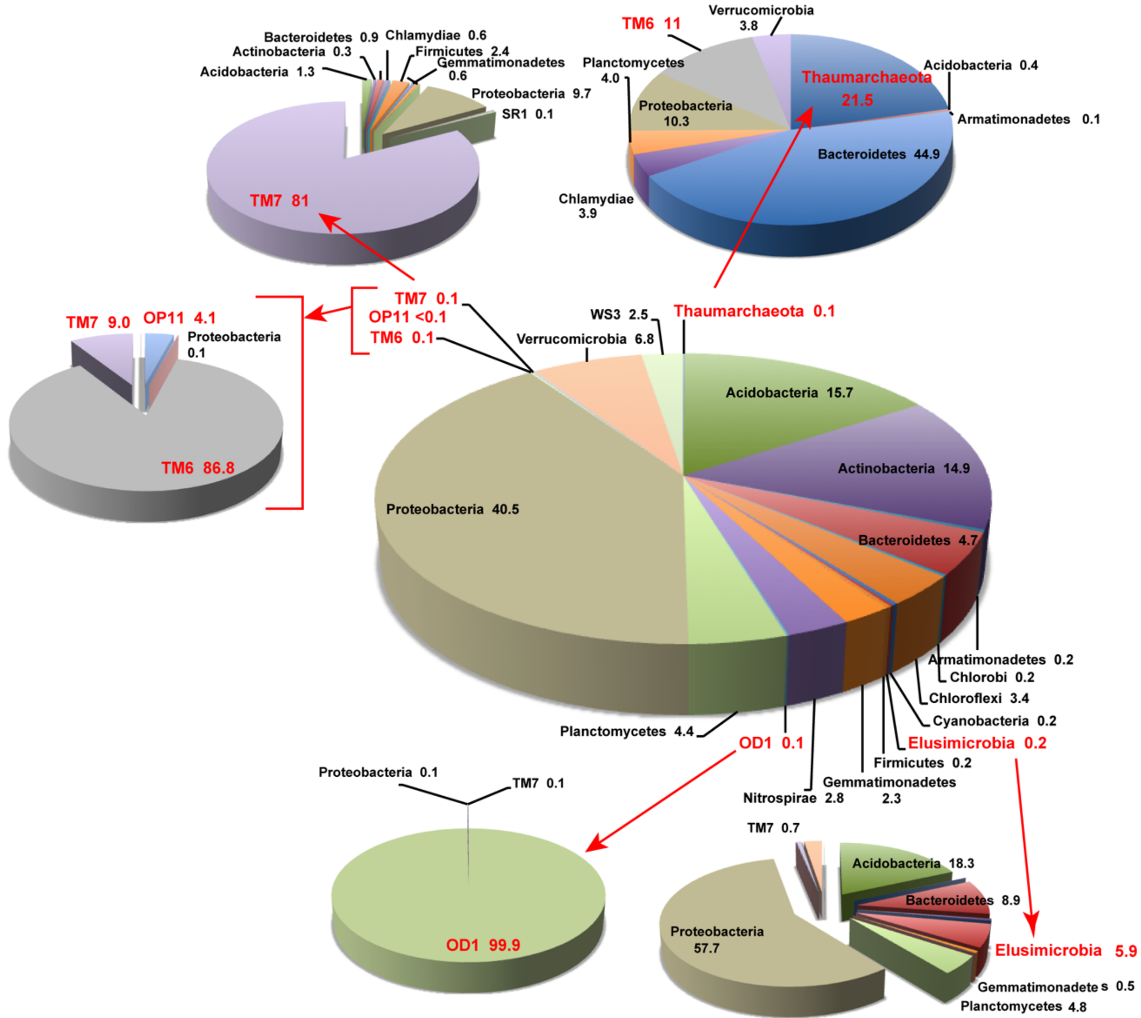


Figure 4. Selective enrichment of uncultured microbial taxa from soil and rhizosphere samples. The central pie-chart illustrates the general microbial diversity (phylum level) of a *Populus* rhizosphere sample, some of the very low diversity taxa being in red. The satellite pie charts show the microbial diversity of selected populations, enriched following flow cytometry sorting, with the target uncultured groups in red. The values are percentage abundance based on rRNA amplicon data.

Using a combination of selective filtering, DNA content and cellular fingerprinting using flow cytometry cell sorting, we performed microbiome fractionation from soil and *Populus* rhizosphere samples obtained from the Pacific Northwest common gardens, UTK and ORNL plantations and natural forest trees. Populations of 1,000-100,000 microbial cells were retrieved and characterized by rRNA gene sequencing on a MiSeq platform. We successfully developed procedures for enriching (10 to >100 fold) for some of the yet uncultured microbiota members, including *Thaumarchaeota*, TM6, TM7, OD1, *Elusimicrobia* and others (**Figure 4**). The DNAs of these enriched cell populations can be sequenced as low diversity metagenomes and will provide much deeper coverage for individual genomes of those uncultured bacteria and archaea. They will also enable investigations into the genomic diversity across these organism groups across geographic and plant genotypic space. Importantly, as the fractionation is non-destructive

on the microbial cells, this approach opens the door for microbial isolation and characterization in pure culture of those taxa or in association with other species and with *Populus*.

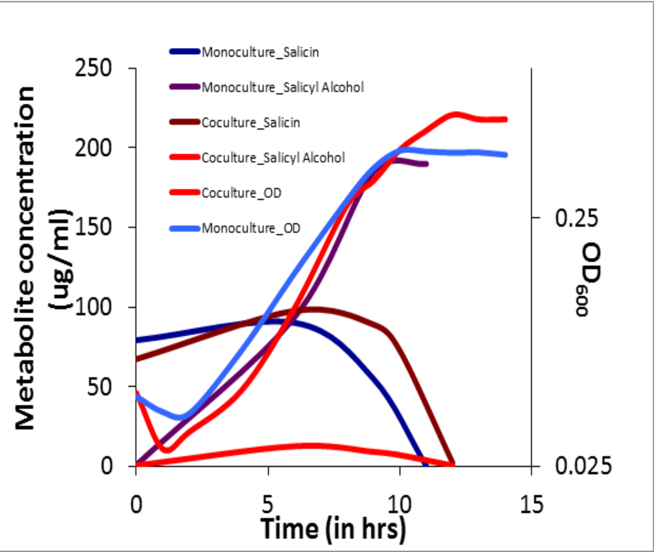


Figure 5. Growth characteristics of mono- vs. co-cultures of *Rahnella* sp. OV744 and *Pseudomonas* sp. GM16 grown on salicin. Metabolite measurements reveal a spike in salicyl alcohol in mono-culture compared to co-culture on salicin, suggesting cross-feeding of salicyl alcohol.

Employment of integrated omics to characterize the mechanism of salicin metabolism by a co-culture of *Populus* microbiome isolates

One of the defining characteristics of *Populus* is the production of secondary metabolites known as higher-order salicylates (HOS), which are involved in host defense and signaling mechanisms. Two interesting bacterial strains, *Rahnella* sp. OV744 and *Pseudomonas* sp. GM16, are involved in the degradation of simple phenolic compounds such as salicin and salicyl alcohol respectively through uncharacterized mechanisms. Using metabolomics, proteomics and qPCR analyses for mono- and co-cultures of *Rahnella* sp. OV744 and *Pseudomonas* sp. GM16, we tested the hypothesis that in a co-culture of salicin-containing medium, OV744 would metabolize salicin to salicyl alcohol, which would then be used as a carbon source by GM16 (which cannot grow on salicin). When monitoring metabolites (GC-MS) and growth of each strain (qPCR) in co-culture on salicin, salicyl alcohol production was diminished compared to that of an OV744 monoculture in salicin (**Figure 5**). For determining whether the proteins detected in monocultures were also the same players in co-culture, a proteomics study was performed comparing OV744 and GM16 in glucose monocultures and salicin co-culture. When OV744 protein expression from co-culture was compared to those in glucose monoculture, the predicted proteins in the salicin metabolism pathway were significantly more abundant. Similar results were observed for GM16. Most of the proteins were detected in co-culture, and when compared to glucose monoculture, several proteins involved in the salicyl alcohol pathway were highly abundant. Only PMI19_03944 (3-oxoadipate enol-lactonase) could not be detected.

These omics experiments suggest that OV744 grows on salicin by degrading it to glucose 6-phosphate and salicyl alcohol. Salicyl alcohol is then secreted and subsequently taken up and utilized by GM16 for its growth. Further, these multi-omics approaches demonstrate that the individual pathways for salicin and salicyl alcohol metabolism are present in OV744 and GM16 respectively. This establishes that in salicin co-culture, these two strains utilize both of these pathways to cross-feed salicyl alcohol. The mechanism for microbial transformation of salicin, and potentially other more complex HOS, demonstrates the intricate interactions that occur within the plant microbiome.

QTL and GWAS mapping of host traits associated with key microbial associations in *Populus*

A key hypothesis driving the development and deployment of unprecedented genomic resources in *Populus* was that the plant host plays a critical role in selecting its associated microbial

symbionts. As described above in microbial community profiling, we leverage the observations that there were significant differences in microbial community profiles across different host genotypes grown in the same environment. This differentiation was especially strong across *Populus* species, *P. trichocarpa* and *P. deltoides*. Labbe et al. (2011) had also demonstrated that *Laccaria bicolor* exhibited preferential colonization of *P. trichocarpa* (T) than *P. deltoides* (D). With these observations in mind, we sought to establish robust genomic resources for interspecific TxD QTL mapping pedigrees 52-124 and 54B to facilitate segregation analysis of colonization efficiency. To that end, whole-genome resequencing to a minimum 30× depth for parental genotypes 93-968 (*P. trichocarpa*), ILL-101 (*P. deltoides*) and D124 (*P. deltoides*) was performed by JGI. Pairwise comparison among these genotypes and stringent curation yielded 5,390 single nucleotide polymorphisms (SNPs) with known genomic positions. These were subsequently used to design the *Populus* Illumina 5K bead array (Muchero et al. 2015).

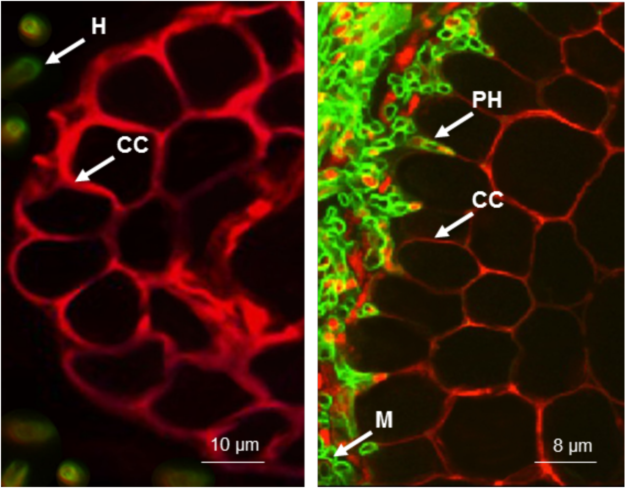


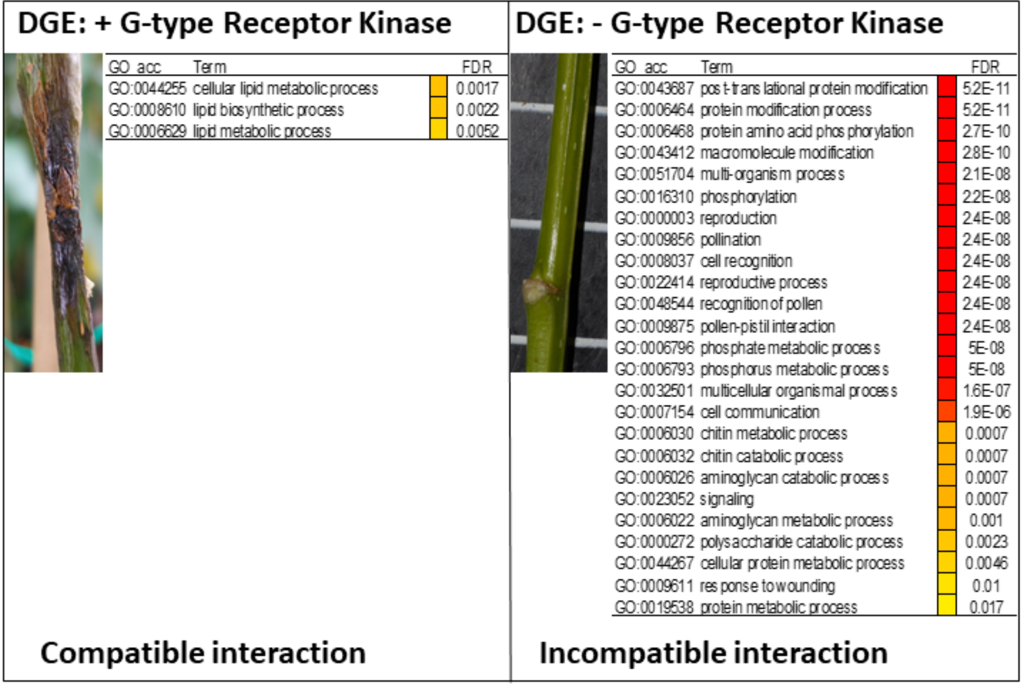
Figure 6: Microscopic observation of a transversal section of wild type plant Col-0 (left) and *35S:PtLecRLK1* transgenic line (right) co-cultivated with *L. bicolor*. Propidium iodide (red) was used to stain root cell walls and UVitex 2B (green) was used to stain fungal cell walls. H, hyphae. CC, cortical root cell. PH, penetrating hyphae. M, mycelium.

We used the bead array to genotype 712 individuals from the 52-124 pedigree and 299 individuals from the 54B pedigree and this exercise yielded a high density genetic map for 52-125 consisting of 3,568 markers and an improved 54B map with an additional 677 SNPs. Both genetic maps allowed us to finely map and identify candidate genes underlying a major QTL associated with *L. bicolor* colonization previously identified in the 54B pedigree by Labbe et al. (2011). This analysis identified a whole-gene deletion in *P. deltoides* involving a G-type lectin-like receptor kinase (PtLecRLK1) which segregated consistently with *L. bicolor* colonization efficiency across the pedigree. The presence of the functional *P. trichocarpa* copy conferred approximately 2X as much colonization efficiency compared to *P. deltoides* deletion variants. The role of the G-type lectin-like receptor kinase in mediating colonization by *L. bicolor* was firmly established upon heterologous expression in *Arabidopsis* which transformed the previous non-host into a host for the fungal symbiont (**Figure 6**). We also demonstrated that this PtLecLRK1 functions by suppressing the host-defense pathways to allow compatible interactions compared to wild-type.

Following the success in harnessing species-specific interactions to map host determinants of *Laccaria* ECM colonization efficiency, we sought to explore sub-species-specificity, large-scale genomics and transcriptional profiling to elucidate the genetic basis of host-microbe interactions. To achieve this, we chose the *P. trichocarpa-Sphaerulina musiva* (formerly *Septoria*) pathosystem as a model to explore to potential of high-resolution genome-wide association mapping studies (GWAS) as a tool to identify host features mediating colonization by microbes. *Septoria* is major pathogen threatening the species range of non-adapted *P. trichocarpa* in the Pacific Northwest region of North America and a key limiting factor for growth hybrid

genotypes in plantations in the Eastern US. In this effort, we leveraged the whole-genome resequencing of 1,084 *P. trichocarpa* natural variants to a minimum 15× depth by JGI which yielded approximately 48 million SNPs representing a nucleotide variant every 10 bp across the genome. This unprecedented marker coverage across the genome coupled with the rapid linkage disequilibrium decay and a robust phenotyping platform allowed us to perform highly-resolved GWAS mapping of *P. trichocarpa* susceptibility to *Septoria* across the species range.

Consistent with observations from the *L. bicolor* study above, we identified another G-type lectin-like receptor kinase that was significantly associated with susceptibility to *Septoria*. To validate this finding, we used RNAseq analysis on susceptible and resistant genotypes to



GO acc	Term	FDR
GO:0044255	cellular lipid metabolic process	0.0017
GO:0008610	lipid biosynthetic process	0.0022
GO:0006629	lipid metabolic process	0.0052

GO acc	Term	FDR
GO:0043687	post-translational protein modification	5.2E-11
GO:0006464	protein modification process	5.2E-11
GO:0006468	protein amino acid phosphorylation	2.7E-10
GO:0043412	macromolecule modification	2.8E-10
GO:0051704	multi-organism process	2.1E-08
GO:0016310	phosphorylation	2.2E-08
GO:0000003	reproduction	2.4E-08
GO:0009856	pollination	2.4E-08
GO:0008037	cell recognition	2.4E-08
GO:0022414	reproductive process	2.4E-08
GO:0048544	recognition of pollen	2.4E-08
GO:0009875	pollen-pistil interaction	2.4E-08
GO:0006796	phosphate metabolic process	5E-08
GO:0006793	phosphorus metabolic process	5E-08
GO:0032501	multicellular organismal process	1.6E-07
GO:0007154	cell communication	1.9E-06
GO:0006030	chitin metabolic process	0.0007
GO:0006032	chitin catabolic process	0.0007
GO:0006026	aminoglycan catabolic process	0.0007
GO:0023052	signaling	0.0007
GO:0006022	aminoglycan metabolic process	0.001
GO:0000272	polysaccharide catabolic process	0.0023
GO:0044267	cellular protein metabolic process	0.0046
GO:0009611	response to wounding	0.01
GO:0019538	protein metabolic process	0.017

Figure 7. Go annotation of differentially expressed genes (DGE) in the presence of a compatible interaction (left) versus an incompatible (right) interaction PtLecLRK in response to inoculation with *Septoria*.

establish implications of the presence of the functional versus loss-of-function PtLecRLK on transcriptional responses to *Septoria*. In line with previous observations, presence of the functional PtLecRLK variant resulted in major suppression of transcriptional response in response to inoculation with *Septoria*. In this condition, only 76 genes exhibiting differential expression. In contract, loss-of-function of the PtLecRLK resulted in > 4,600 exhibiting differential expression at 24-hours post inoculation and included the whole suite of defense responses from signaling to secondary metabolite biosynthesis and cell wall re-organization (**Figure 7**). The functional variant occurs in more than 99% of surveyed genotypes, suggesting that the species range is extremely vulnerable to this pathogen.

Expansion of MS-based metabolomic and proteomic methodologies

Current protocols for proteomic and metabolomic measurements, while very powerful, are still somewhat laborious and limited in performance, thus reducing their broad application and measurement throughput. The availability of new instrumental platforms, such as the ThermoScientific QExactive MS systems for higher performance and enhanced measurement

speeds can be combined with optimization of new sample preparation protocols to dramatically enhance the impact of omics measurements for plant-microbe research.

Current global proteome measurements provide an unprecedented measurement depth, but are typically fairly low in throughput. For example, a deep multi-dimensional LC/LC-MS/MS measurement might require more than 20 hours of MS measurement time. Thus, there is a strong need for development of methods to dramatically increase measurement throughput while maintaining deep measurement depth. To this end, we have demonstrated a new proteome measurement scheme based on a high-performance LC-MS/MS instrument (QExactive Orbitrap mass spectrometer). This instrument provides measurement speeds of up to 12 Hz, enhanced dynamic range measurement, mass accuracies in the low ppm range, and mass resolutions exceeding 100,000 (FWHM). This provides the ability to execute multi-dimensional LC/LC-MS/MS experiments on a higher throughput fashion (up to 5-6 complete runs per day), with equal or superior performance in terms of mass accuracy and measurement depth. By utilizing autosamplers for automated sample injection campaigns, we have been able to dramatically improve the precision and reproducibility of proteome measurement, which greatly enhances differential proteome investigations. These advances increase proteome measurement throughput by 6-10X overall, thereby opening new avenues for the larger sample campaigns expected for both microbial and plant studies.

To extend the range of experimental information possible for metabolomic studies, we have undertaken implementation of LC-MS measurement capabilities for a range of semi-volatile and non-volatile metabolites to complement our existing GC-MS capabilities. To this end, we have implemented a high performance reverse phase LC-MS/MS approach (based on QExactive Orbitrap MS) for bacterial and plant metabolomic studies. As above, this instrument is configured with an autosampler/high performance LC system to permit fairly large-scale metabolomic measurement campaigns with high mass accuracy, deep dynamic range, and tandem mass spectrometry fragmentation.

Summary

The PMI SFA project at ORNL applies genomics, transcriptomics, proteomics, and metabolomics either individually or in combination to understanding the functioning of the microbiome associated with *Populus*. These omics tools are implemented in order to understand the complex multiscale system represented by *Populus* and its microbiome. These omics tools are central to PMI efforts and are further described in many of the PMI project's publications (a complete listing can be found at http://pmiweb.ornl.gov/index.php/publication-list/).

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