

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

Q3 Metric: Report on the latest techniques to detect and understand signaling between plants and microbial communities.

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.

Specific biochemical events lead to particular associations between *Populus* and its microbial partners (microbiome). These interactions are driven by secretion and detection of chemical exudates and small molecule signals, which are produced by both plant and microbes (reviewed

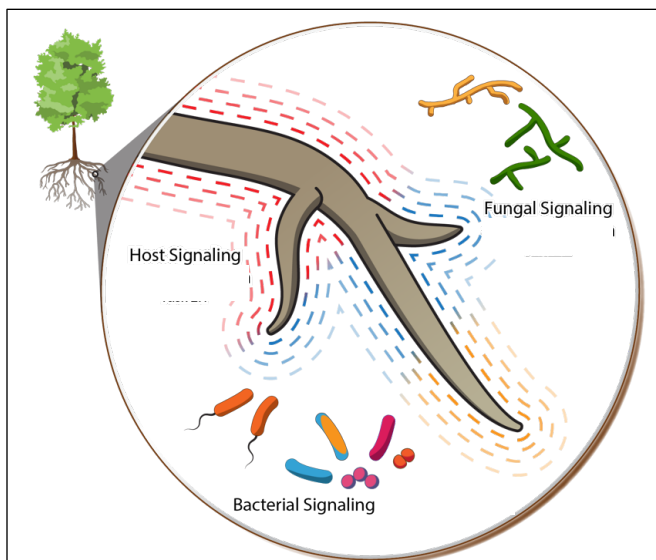


Figure 1. Overview of signaling in the *Populus* rhizosphere. Signals derive from the host plant and its microbial partners. These signals structure the microbial community.

in Leach et al., 2017, Oldroyd, 2013, Venturi and Keel, 2016). The development of ‘omics technologies has greatly improved our understanding of these metabolite cues and signals. The advent of bioinformatics tools to analyze the staggering amount of genome/metagenome sequence data has helped define the metabolic potential of the rhizosphere and analytical chemistry methods allow for untargeted discovery and quantification of these metabolites. Despite these advances in genomics and metabolomics techniques, identifying the signals and their roles in structuring the host microbiome remains largely undefined and is only beginning to be revealed. One of the major goals for the PMI project is to gain a genomics-based understanding of the metabolites and

signals responsible for shaping these plant-microbial interactions (see **Figure 1** overview). In order to define these events at the molecular level, we have developed several tractable bacterial model systems selected from our large inventory of natural isolates with sequenced bacterial genomes (~400), as well as continued research on the model fungal symbiont *Laccaria bicolor* while initiating work on a diverse range of sequenced PMI fungal isolates (~60) from our extensive culture collection, generated from wild and field-grown *Populus*. We also developed technologies for real-time imaging of plant-microbe interactions and temporal sampling of metabolites. Defining these chemical features and the molecular basis for host- and community-directed assembly of the system represent key challenges in understanding the plant-microbiome interface. These advances will help to define the metabolite exchange and signaling events underpinning the molecular, spatial and temporal dynamics of microbial community assembly with the *Populus* host and are likely applicable to understanding other host-microbe systems.

Metabolite exchange between *Populus* and its microbiome

The organization and behavior of plant-microbe communities are dictated by a complex network of physical and chemical interactions between organisms within dynamic environments. These interactions produce spatially and temporally localized micro-environments that change as organisms grow, consume and produce materials, and alter their surroundings. The behavior of certain microbes, and ultimately of the whole biosystem, depends on these local environments. As highlighted in our Q1 Performance Metric Report, *Populus* produces a wide diversity of metabolites that vary based on genotype, age, and environmental conditions (Abraham et al., 2018, Kalluri et al., 2016, Timm et al., 2018, Timm et al., 2016, Tschaplinski et al., 2014). We found that the *Populus* biochemical environment includes soluble sugars, organic acids, amino acids, and fatty acids that serve as substrates for microbial metabolism, as well as secondary

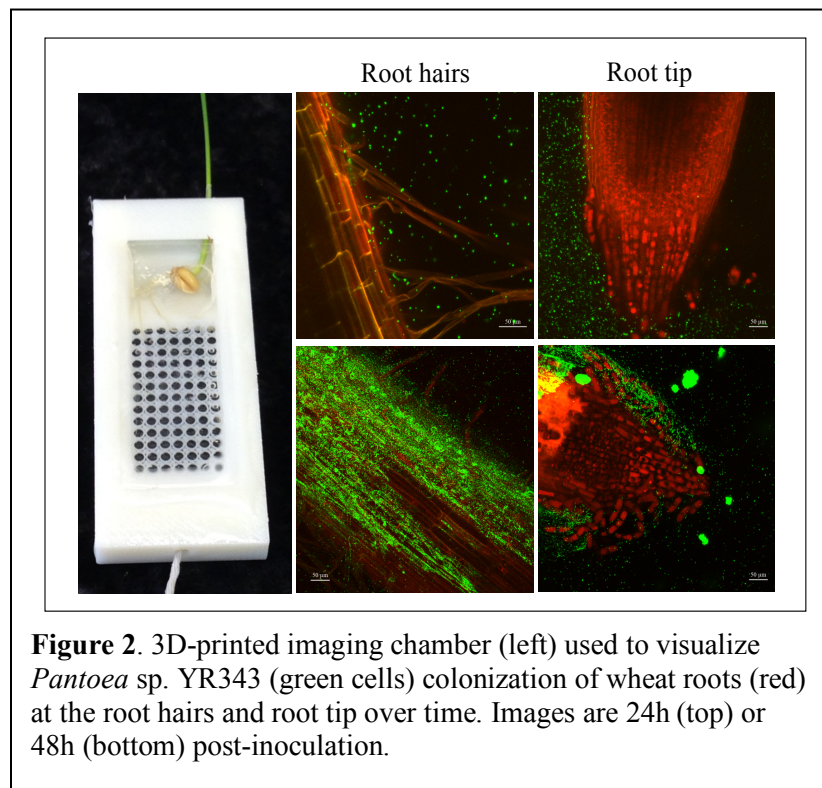


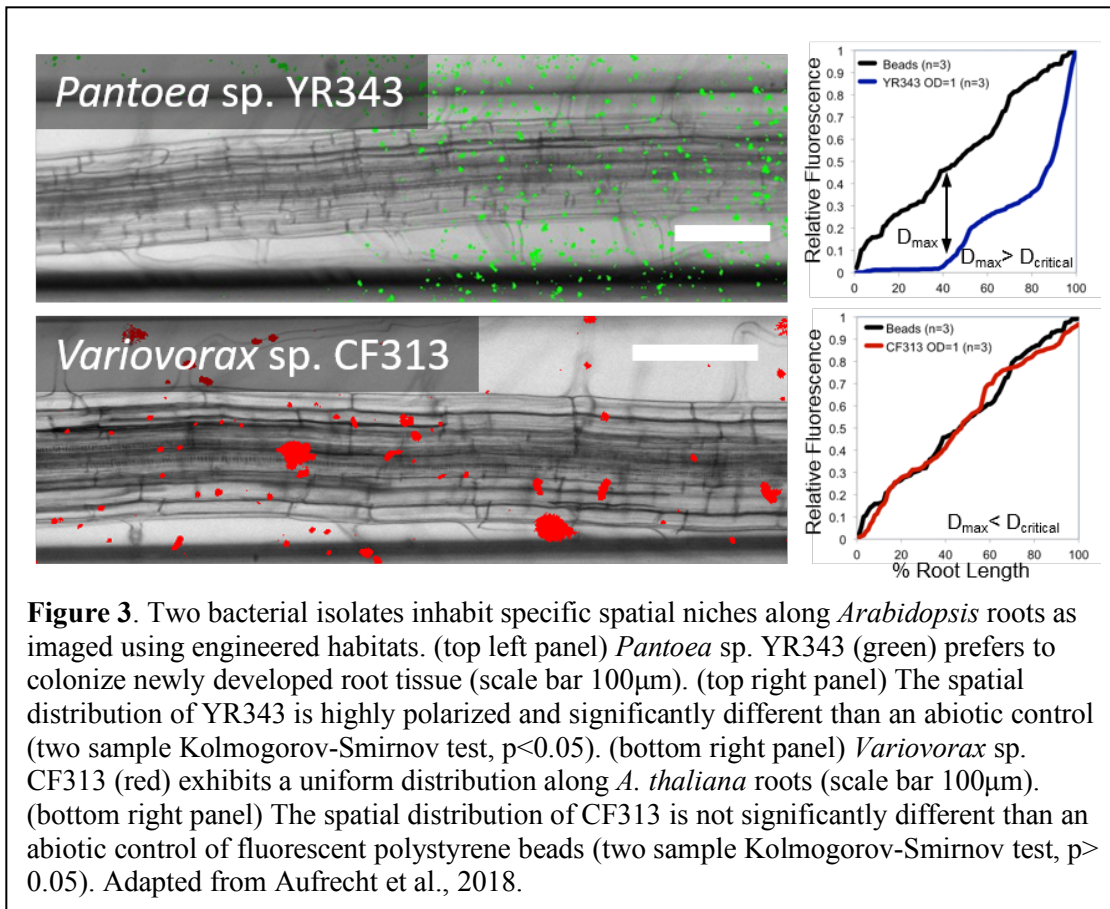
Figure 2. 3D-printed imaging chamber (left) used to visualize *Pantoea* sp. YR343 (green cells) colonization of wheat roots (red) at the root hairs and root tip over time. Images are 24h (top) or 48h (bottom) post-inoculation.

metabolism products such as phenolic glycosides, higher-order salicylates, and flavonoids. These higher order metabolites can be selectively degraded by some bacteria and the resulting byproducts serve as fuel for others, as detailed in our Q1 Performance Metric Report. We also demonstrated that the PMI bacterial and fungal isolates produce and secrete an array of compounds that influence their associations with other microbes and with their *Populus* host (Estenson et al., 2018, Plett et al., 2014, Schaefer et al., 2016). Understanding how the local environment in the rhizosphere influences, and is

influenced, by community composition and function represents a key knowledge gap that we strive to close.

Identifying host metabolite signals and cues, in time and space, to determine their roles in microbial colonization and community organization

Our initial work examined colonization levels of plants grown in several experimental platforms including greenhouse-grown plants and agar-based growth systems. We observed a wide range of colonization efficiencies (from 10^8 to 10^3 cells per gram root tissue) between individual microbes isolated from *Populus* as well as clear plant genotype-specific microbial colonization patterns, e.g., when using a plant line with a downregulated endo-1- β -1,4-glucanase enzyme (*PdKOR1*) distinct differences in bacterial colonization were observed (Kalluri et al., 2016, Veach et al., 2018). This technique is useful for confirming colonization behavior and also highlights the need for technological advancements in order to define niche preferences, temporal behaviors, and patterns of microbe migration when in the proximity of the plant root. In order to address these, and other, crucial questions, we developed two technologies: a 3D-printed imaging chamber (**Figure 2**, US Patent D754,871S) and a plant-on-a-chip microfluidic platform in which we grow and maintain seedlings for 7-10 days (**Figure 3**) (Aufrecht et al., 2018). These engineered habitats have design advantages in that the timing of microbe addition can be controlled and the habitats are compatible with high-resolution imaging. Also, these chambers can be modified to dose or sample the chemical environment with minimal disturbance to the plant, thereby facilitating additional physical sampling and chemical analysis of plants and microbes.

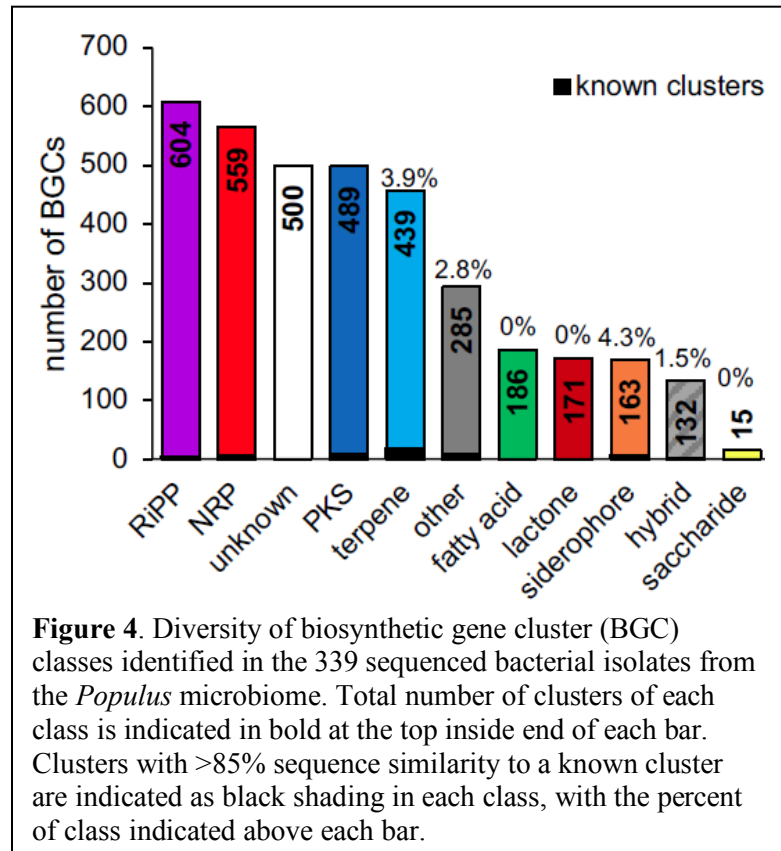


These systems have been used to examine the colonization behavior of bacteria engineered to express fluorescent proteins (to aid in visualization), including *Variovorax*, *Methylibium*, *Rahnella* and *Caulobacter* species, as well as the rhizosphere isolate *Pantoea* sp. YR343, for which we developed genetic tools to utilize this strain as a model of root colonization. We identified several pathways important for its normal colonization including: i) production of the phytohormone indole-3-acetic acid (Estenson et al., 2018), ii) synthesis of exopolysaccharide (EPS), which is important for biofilm formation, and iii) carotenoid synthesis (Bible et al., 2016), which protects against reactive oxygen species. We found that *Pantoea* colonized roots most dramatically after 48 hours, with a preference for root hairs and tips (**Figure 2**). Using a plant-on-a-chip platform, we have quantified *Pantoea* colonization and compared it with that of *Variovorax* sp. CF313, which displays a relatively even distribution along the plant roots (**Figure 3**) (Aufrecht et al., 2018). These approaches provide context for understanding plant-microbe interactions during the early stages of plant development and provide a platform for integrating real time sampling of the local fluid environment to better understand the chemical drivers that shape these colonization patterns.

Inventory of potential microbial natural products

As a starting point for understanding how microbial metabolites could influence bacterial community structure, cell communication, and plant health, we took a top-down approach in studying biosynthetic diversity in the *Populus* microbiome. We evaluated the diversity and uniqueness of biosynthetic gene clusters (BGCs) in 339 sequenced genomes of bacteria isolated from *Populus*, as well as in *Populus* bulk soil, rhizosphere, and endosphere metagenomes (Blair et al., under review). From these

analyses, we identified approximately 3400 individual BGCs (**Figure 4**) including many potential antimicrobials represented by clusters falling into the classes of ribosomally synthesized and post-translationally modified peptides (RiPPs), non-ribosomal peptides (NRPs), and polyketide synthases (PKS); as well as signaling compounds (terpenes, lactones, and siderophores). These BGCs are diverse across natural product type and also distinct from known natural product clusters as only ~1% of all clusters matched a previously characterized BGC, suggesting a great opportunity for the discovery of novel natural products involved in host and/or microbe communication or



microbiome structure and stability. We have optimized and demonstrated a high-resolution mass spectrometry (MS) approach to measure RiPPs, which will aid in the identification and characterization of these products. Current efforts are focused on developing additional analytical tools (MALDI MS-imaging and LC-MS/MS) for detection of natural products in laboratory cultures and *in planta*. While these complex natural product molecules may have profound effects on plant microbiome structure and stability, they are often not expressed ('silent') under laboratory culture conditions. Thus, understanding when and where these BGCs are expressed may provide insight into their functions. Similar bioinformatic analyses to inventory the natural product potential in PMI fungal genomes (*Mortierella* and Russulaceae strains) are ongoing.

Signaling among and between microbes and their plant host

Plant-associated microbes, both beneficial and pathogenic, produce and detect a wide variety of chemical signals that contribute to their ability to colonize plant hosts (Brencic & Winans, 2005, Zeilinger et al., 2016). The inventory of microbial signals is large; some are specific only to a particular species pair of plant and microbe, while other signaling pathways are distributed among microbial groups. The PMI project has focused on microbial signals shared among many microbes with the expectation that our findings will be applicable to other host-microbe systems.

Bacterial quorum sensing signals

One common signaling system distributed among *Populus*-associated bacteria is known as quorum sensing and response (QS). QS is a cell-to-cell communication mechanism that enables cell density-dependent gene regulation in bacteria. Proteobacteria synthesize small diffusible acyl-homoserine lactone (AHL) signals that function with LuxR-type signal receptors to control gene expression, in some cases controlling hundreds of genes. Many plant-associated bacteria use AHL signaling to control traits, including exopolysaccharide production and biofilm production, tissue-destructive enzyme production, antibiotic production, motility, and conjugal plasmid transfer - phenotypes that benefit bacteria when they are present at high cell densities, often while in association with the plant host. Our work has established that AHL-type QS is prevalent among members of the *Populus* microbiome and is enriched in metagenomic DNA libraries from the *Populus* endosphere, relative to rhizosphere and soil libraries (represented in **Figure 4** as the 'lactone' class) (Schaefer et al., 2013, Blair et al., under review). This suggests that QS pathways are important for structuring the *Populus* bacterial microbiome. In addition, many of the AHL synthase genes from PMI α -proteobacterial isolates are members of a recently discovered subfamily of enzymes that utilize coenzyme-A (CoA) substrates for AHL production (Liang et al., under review). We are particularly interested in this subfamily as the CoA-derived AHL signals are likely to be of novel structure. To screen for novel AHL signal production by our PMI isolates, we have developed a bioinformatic and experimental 'road map' (Liao et al., 2018) utilizing an AHL radiotracer assay (Schaefer et al., 2018) followed by treatment with the AHL-degrading enzyme AiiA (Dong et al., 2002).

Characterization of a new *Populus*-bacterial interkingdom signaling system (PipR) Some of the LuxR homologs identified in *Populus*-associated bacteria belong to a widespread, but understudied, subfamily called PipR (OryR) homologs. PipR receptors do not detect AHL signals, instead they have evolved to detect a previously undefined *in planta* signal to control gene expression. These PipR regulators are required for normal plant-microbe interactions in

both pathogenic and symbiotic relationships (reviewed in Patel et al., 2013). We defined a PipR system in the root endophyte *Pseudomonas* sp. GM79 (Schaefer et al., 2016), which can serve as a model for understanding how these plant-responsive PipR systems function (Figure 5). We discovered that the plant signal requires active transport to enter the bacterial cell and that there are two PipR-regulated peptidases that seem to exert a negative feedback loop on the system. This work laid the groundwork and created the tools required for our recent exciting discovery that the GM79 PipR signal is a spontaneously formed degradation product from a well-known plant metabolite (Countinho et al., under review). The

PipR signal is present at extremely low concentrations in *Populus* leaf macerates (~10 pmol/gram leaf tissue, GM79 PipR responds to concentrations as low as 10 pM), which explains why it has been difficult for researchers to identify PipR-type signals. Our discovery sets the stage for new questions as to the role of this signal in bacterial-plant interactions.

Signaling in fungi: the role of lipochitoooligosaccharides (LCOs) and terpenes

Much of our fungal signaling work has focused on protein-based receptors and effectors in the arbuscular mycorrhiza (AM) fungus, *Laccaria bicolor* (highlighted in our Q2 Performance Metric Report). In addition, we are studying how other fungal metabolites mediate mutualistic interactions, including chitin-derived lipochitoooligosaccharides (LCOs) and terpenes. LCOs, which include nodulation (Nod) factors, were first described in rhizobacteria where they act as signaling molecules for initiating nodule formation (Lerouge et al., 1990). Interestingly, mycorrhizal fungi produce similar LCO-type signaling molecules (named Myc-LCOs), which have been demonstrated in arbuscular mycorrhizal fungi (AMF) (Maillet et al., 2011). These important compounds are involved in the molecular cross-talk with plants and mediate the symbiotic accommodation of the microbe. In AM fungi, plant-secreted exudates stimulate hyphal branching (Akiyama et al., 2005) and secretion of specific Myc-LCO molecules. Perception of

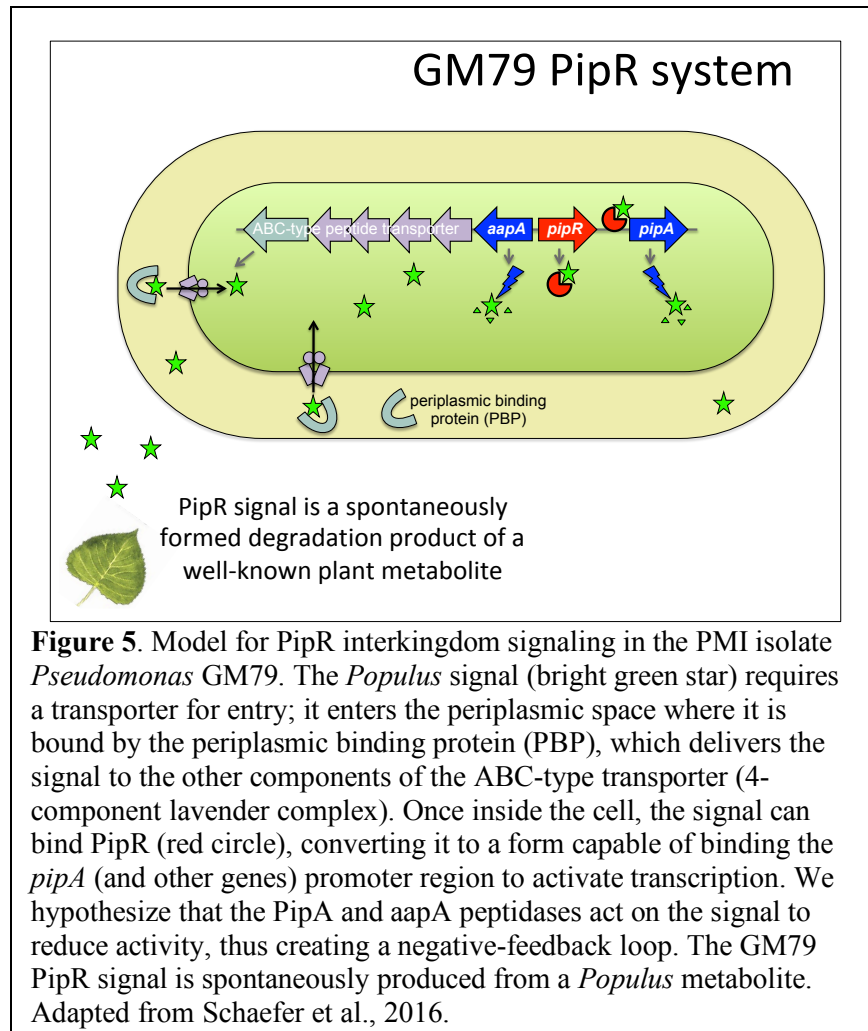


Figure 5. Model for PipR interkingdom signaling in the PMI isolate *Pseudomonas* GM79. The *Populus* signal (bright green star) requires a transporter for entry; it enters the periplasmic space where it is bound by the periplasmic binding protein (PBP), which delivers the signal to the other components of the ABC-type transporter (4-component lavender complex). Once inside the cell, the signal can bind PipR (red circle), converting it to a form capable of binding the *pipA* (and other genes) promoter region to activate transcription. We hypothesize that the PipA and *aapA* peptidases act on the signal to reduce activity, thus creating a negative-feedback loop. The GM79 PipR signal is spontaneously produced from a *Populus* metabolite. Adapted from Schaefer et al., 2016.

these molecules by plant cells leads to a signal cascade that reprograms the root for symbiotic association in AMF (Camps et al., 2015). Despite the functional importance of these molecules in mediating plant-AMF interactions, little is known about the role of similar molecules in ectomycorrhizal fungal (EMF) groups. Based on the report that EMF also produce LCOs (Garcia et al., 2015), we surveyed the genome sequences of diverse EMF and endophytic fungi for LCO gene synthesis

Table 1. Fungal genomes encoding LCO genes identified by sequence similarity searches.

Species Name	Acyltransferase (nodA-like)	Chitin deacetylase (nodB-like)	Chitin synthase (nodC-like)
<i>G. convolens</i> OM19405	142	2	11
<i>H. annosum</i>	87	4	11
<i>L. subvellereus</i> BPL653	103	2	14
<i>L. volemus</i> BPI652	119	5	12
<i>Peniophora</i> sp. CONTA	126	0	16
<i>M. ochricompacta</i> BPL690	100	7	10
<i>R. brevipes</i> BL707	95	4	10
<i>R. compacta</i> BPL669	94	3	14
<i>R. dissimulans</i> BPL704	100	4	10
<i>R. rugulosa</i> BPL654	95	5	10
<i>R. vinacea</i> BPI710	84	4	9
<i>S. hirsutum</i> FP-91666 SS1	108	1	13
<i>L. amethystine</i> LaAM-08-1	90	3	13
<i>L. bicolor</i>	96	2	20
<i>H. cylindrosporum</i> h7	100	7	9
<i>P. adelphus</i> Ve08.2h10	75	0	11
<i>P. ammoniavirescens</i> Pou09.2	85	0	10
<i>P. involutus</i> ATCC 200175	82	0	11
<i>A. muscaria</i> Koide	81	1	10
<i>A. thiersii</i> Skay4041	77	3	10

(Table 1), validated LCO production in several fungal isolates (Russulaceae members) and characterized their effects on the plant host. These results suggest LCO production capabilities are widespread across EMF; however, only a subset of LCOs from mycorrhizal fungi affect *Populus* roots.

Fungi also have the ability to release an array of terpenoids and volatile organic compounds (VOCs) as chemical signals, many of which are still uncharacterized. Typically, the chemical structures of the reported signals correspond to small molecules (100–500 Da). Because of their size and volatility, these molecules play an important role in long-distance interactions in microbial communities and may influence interactions between fungi and/or plant–microbe interactions in the rhizosphere (Bitas et al., 2013). Similar to LCOs, we expect that these molecules can act as intra- and inter- species signals by coordinating gene expression and influencing fungal behaviors such as biofilm formation, virulence, stress tolerance and mutualistic interactions. We have analyzed the genome sequences of diverse EMF and endophytic fungi for terpene synthase genes and validated their production in ten Russulaceae isolates. We expect that defining the function of these fungal metabolites in *Populus* interactions will broaden our understanding of plant-fungal interactions.

Summary

The foundational work aimed at understanding metabolite exchange and signaling in the *Populus* microbiome has resulted in definition of key signaling molecules and has poised the PMI project to continue to make exciting discoveries. We expect that these studies and tools will advance methods for monitoring complex communities and provide fundamental insights into how the

microbiome is organized and proportioned in response to local environmental perturbations. This knowledge could help develop novel strategies for promoting plant performance. Further details on PMI project efforts can be found in our research publications. A complete listing of PMI project publications is available at <https://pmiweb.ornl.gov/portfolio/>

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