Immune system of a plant

ABSTRACT

Two light signalling factors, FAR-RED ELONGATED HYPOCOTYL3 (FHY3) and FAR-RED IMPAIRED RESPONSE 1 (FAR1) regulate chlorophyll biosynthesis, seedling growth and modulate plant immunity by controlling HEMB1 expression in *Arabiopsis thaliana.* We show that fhy3 far1 double null mutants display high levels of reactive oxygen species, salicylic acid and high expression of pathogen related genes. We analyse the effects of this constitutively activated immune response on commensal microbial communities through use of a next generation sequencing based approach. We determine that fhy3 far1 mutants contain greater species diversity and a greater resistance against pathogenic bacteria. Fungal pathogens increase in abundance in fhy3 far1 mutants. Taken together, this study demonstrates the important role of FHY3 and FAR1 in commensal microbial community composition as well as the importance of bacterial – fungal relations.

INTRODUCTION

The Microbiome

Microorganisms are an extremely diverse group of organisms; making up an astonishing 60% of the Earths total biomass (Singh, 2009). Soil sustains as many as 4-5×10 ^{30} microbial cells (Singh, 2009), all contributing to soil structure formation, decomposition, and recycling of organic matter into its constituent elements and nutrients. Microorganisms present in the soil adjacent to plant roots are part of the Rhizosphere. (Garbeva, 2004) highlights their pivotal roles in the suppression of plant disease (Badri DV, 2009), promotion of plant growth (Lugtenberg, 2009), development and health (Mendes, 2011). Leaves usually dominate the aerial part of the plant,

representing of the most significant terrestrial habitats for microorganisms: the Phyllosphere (Vorholt JA, 2012). A diverse community of bacteria and fungi inhabit this challenging habitat; with nutrient deficiency and fluctuations in temperature, humidity and UV radiation (Lindow SE, 2003). The microbial communities here are shaped by biotic factors: (Yang CH, 2001) states that species, genotype (van Overbeek L, 2008) and age of plant (Redford AJ, 2009) all have their respective impacts. Abiotic factors also have a profound influence over the communities present within the phyllosphere. Plant location and growth conditions such as soil composition and climate can also have a strong impact due to the physiochemical alterations they impart. (JH, 1999) also notes how plant genotype and phenotype has an impact on community assembly. Although the majority of communities exist on the plant surface, and are therefore epiphytic – some exist within the plant as endophytes. Species present within the phyllosphere tend to assimilate plant derived ammonium, simple carbohydrates and amino acids, which are their primary nitrogen and carbon sources (Thomas R Turner, 2013). Microorganisms' energy metabolism isn't entirely dependent on the plant; some species contain rhodopsin's.

Due to the abundance of processes which play a role in community composition (Weiher E, 2011), phyla with the best adaptations for survival and reproduction tend to predominate communities. These microorganisms can promote plant growth through the production of hormones, or protect plants from pathogenic organisms by producing antibiotic compounds, competing for resources (Berg G, 2009) or induction of systemic resistance (Conrath U, 2006). The use of *Arabidopsis thaliana* as a model organism has

been vital for these studies (Innerebner G, 2011). *A. thaliana* is an annual forb, occurring at temperate regions worldwide in a diverse range of habitats (Elena García, 2013)

In order to analyse microbial communities; a few terms need to be defined. Biodiversity is defined as the range of significantly different types of organisms and their respective relative abundance within a community, encompassing three main levels; genetic variation between species, number of respective species and community or ecological diversity (Harpole, 2010). Two main components make up species diversity: the total number of species present (species richness) and the distribution of individuals amongst said species (evenness). Operational taxonomic units (OTU) or communities provide information on an ecosystem (Mannan, 2013). Species diversity relates to the stability of a community; well organized communities tend to have the greatest stability (Yannarell, 2005). Stresses can cause disturbances in a homeostatic community, thereby disrupting it and leading to changes in species abundances. When characterizing an ecosystem such as *A. thaliana,* one must determine three things: The type of microorganisms present, their roles and how these roles relate to the ecosystems function (Sani, 2011).

Plant Immune Response

The immune system of a plant has a selective effect upon its microbiome. Upon pathogen encounter, a plant will elicit an immune response with the goal of limiting pathogen growth. Biotrophic and hemibiotrophic pathogens (those who obtain nutrients from living host tissue) are repelled by Salicylic acid dependent defence responses. Necrotrophic pathogens (which kill their

host to obtain nutrients) are sensitive to Jasmonic acid (JA) and Ethylene (ET) dependent defence responses (Christine Vogel, 2016). Plants lack specialised immune cells; therefore, their cells must have an ability to sense pathogens and mount an appropriate immune response. Pathogens are detected by pattern recognition receptors (PRR's) which bind to the microbe or pathogen associated molecular patterns (MAMP/PAMP), thereby issuing a layer of basal defence known as PAMP triggered immunity (PTI) to prevent pathogen colonization (Chuanfu An, 2011). In order for pathogens to cause disease, they must inject effectors into plant cells, thereby interfering with PRR complexes or downstream signalling to overcome the PTI. Plants have evolved resistance proteins which recognise effectors directly or indirectly and induce effector triggered immunity (ETI).

This response is far more specific, and is often followed by a hypersensitive response (HR). R proteins, mostly leucine-rich repeat (LRR) domain containing proteins and Nucleotide-binding (NB) proteins are the intracellular receptors which sense pathogen derived molecules (Heidrich K, 2012). Figure 1 shows a summary of these processes. When these proteins are activated, production of salicylic acid occurs. Salicylic acid (SA) is a phenolic phytochrome present in plants. SA holds roles in growth, development, transpiration, photosynthesis and the uptake of ions. It's also vital for the process of endogenous signalling, mediating plant defence against pathogens. Activation of defence signalling pathways causes the generation of mobile signals from the infected tissue, where they can spread to distal tissue. Here they can upregulate expression of pathogenesis related genes and induce systematic acquired resistance (SAR), a long-lasting immunity

against a broad spectrum of pathogens. Salicylic acid mediated immune responses are important factors of both PTI and ETI, essential for the activation of SAR.

NB-LRR mediated disease resistance may only be effective against pathogens grown on living host tissue such as obligate or hemibiotrophic pathogens, but not against nectrotrophs (Dangl, 2006). Downstream of the NB-LRR R proteins, the pathways ENHANCED DISEASE SUSCEPTIILITY1 (EDS1) and its partner PHYTOALEXIN DEFICIENT 4 (PAD4) act in basal resistance and ETI initiated by Toll-like/Interleukin 1 receptor (TIR) type NB-LRR R proteins (Vlot AC, 2009). Both PAD4 and EDS1 amplify SA signalling through a positive feedback loop (Wanqing Wang, 2015). Coiled-coil (CC) type NB-LRR proteins are regulated by NONSPECIFIC DISEASE RESISTANCE 1. When SA levels increase as a result of pathogen challenge, redox changes are induced which cause reduction of NON EXPRESSOR OF PATHOGENESIS-RELATED GENES 1 (NPR1) to a monomeric form which activates defence responsive gene expression by accumulating within the nucleus. This results in plant immunity (Fu ZQ, 2013).

Most bacteria which colonize *A. thaliana* are not pathogenic however still produce MAMPs. It is currently not known how plants are able to tell apart pathogenic and commensal microorganisms, and whether the recognition of these non-pathogenic phyllosphere bacteria triggers plant immune signalling networks downstream of PTI or ETI activation, with knock on effects on community structure. (Christine Vogel, 2016) determined that in response to some non pathogenic species, the detection of MAMPS leads to no change in gene expression. Note that some species of bacteria can induce

transcriptional changes to protect the plants from infections of other species (Judith E. van de Mortel, 2012).

FHY3 FAR1

Plants have developed regulatory mechanisms in order to cope with adverse abiotic and biotic conditions (Bray EA, 2000), however these are a detriment to their growth and development. These regulatory mechanisms activate immune responses and resistance pathways in the case of biotic stress. Constitutive activation of plant immunity would lead to impaired growth and fitness, so in the absence of stress, the immune response must revert the massive transcriptional reprograming, requiring tight genetic control (Tian D, 2003).

Arabidopsis thaliana has to adapt to changes of environmental stimuli, such as light signals or temperature. Light duration, direction, wavelength, and quantity are determined by a battery photoreceptors which monitor incident red (R, 600-700 nm) and far red (FR, 700-750 nm) light wavelengths. This is achieved by switching between R absorbing and FR absorbing modes through biologically inactive Pr and active Pfr forms (PH, 2002). Photo activation of the primary photoreceptor for FR light phyA, causes translocation from the cytoplasm to the nucleus. This translocation allows induction of FR-responsive gene expression required for various photoreceptors. Two pairs of homologous genes are essential for the phyA signalling; FAR1 (far-red-impaired response 1) and FHY3 (far-red elongated hypocotyl 3). (Hudson, 2003) determined that these genes encode mutator like transposase derived transcription factors which directly bind to the promotor region HEMB1, which itself encodes a 5-1minolevulinic acid

dehydratase, ALAD) and activates its expression, thereby regulating both chlorophyll biosynthesis and seedling growth (Tang W, 2012). These regulators small plant specific proteins, which are necessary for the nuclear accumulation of light activated phyA.

(Wanqing Wang, 2015) determined that fhy3 far1 double null mutants display an autoimmune response; accumulating SA and ROS, inducing PR genes and having an increased resistance to pathogen infection. They all displayed a dwarf phenotype, with necrotic lesions developing on their leaves as a result of premature cell death. Wang and his colleagues determined that FHY3 and FAR1 may act as defence-responsive gene repressors; mutants had high abundances of R genes and upregulated levels of PR genes, hinting at a possible link with regulation of NB-LRR mediated SA signalling pathways. Fhy3 far1 mutants increased expression levels of EDS1, PAD4, SID2 and EDS5 – all genes involved in SA pathways. Reduction of HEMB1 in fhy3 far1 lead to a constitutively activated immune response, inducing system acquired resistance. (Wang Q, 2007) hypothesized that FHY3 and FAR1 may negatively regulate SA signalling and plant immunity through regulation of HEMB1 expression – providing a possible linkage between light signalling and plant immunity.

Next Generation Sequencing

Most microbial communities present within nature are yet to be cultured within a laboratory; thereby leaving biomolecules such as nucleic acids, proteins, and lipids as our only source of information. For phylogenetic studies, surveys of the small ribosomal subunits (SSUs) for bacteria and the internal transcribed spacer (ITS) region of fungi are vital. Ribosomal genes

are present in all organisms and contain regions which evolve slowly, coupled with faster evolving regions which permit fine tuning of taxonomic levels, to either family or genera. Note, that there also exists numerous databases for reference sequences and their respective taxonomies, such as SILVA (Pruesse, 2007) and the Ribosomal Database Project. This technique uses multiple primer pairs for each of the marker genes, each associated with its own taxon (William Walters, 2015). SSU rRNA genes are the standard reference sequence for taxonomic classification; calculating similarity between rRNAs. ITS regions are primarily sequenced for fungi due to the higher degree of variation they display as a result of low evolutionary pressure, and clear resolution below genus level (Bellemain, 2010). PCR amplification is performed, cloning and Illumina sequencing of the bacterial 16S rRNA and fungal 18S ITS performed and compared to databases hosted by NCBI to allow a benchmark for assessment of phylogeny (Cole JR, 2009).

Illumina sequencing was chosen due to the low cost and sequencing quality (Gregory B. Gloor, 2010). (Wang Q, 2007) determined that longer sequences are easier to assign to taxonomic groups, in this case, reads of 300bp were determined. Illumina sequencing has two main technologies: HISEQ, which generates more reads but requires a longer time, and MISEQ which provides less reads but at a longer sequence length, reduced time and reduced cost, hence its use in this experiment. The workflow of Illumina has four basic steps; a sequencing library is produced by random fragmentation of DNA/cDNA samples, followed by ligation of 5′ and 3′ adapters. These adapters are amplified through polymerase chain reaction (PCR) and the gel purified. Libraries are loaded onto flow cells, binding to a lawn of surface

bound oligonucleotides which are complementary to the library adapters. Each of these fragments is amplified into distinct clonal clusters by the process of bridge amplification. Single bases are then incorporated into DNA template strands. All the 4 reversible dNTPs are present during sequencing, natural competition reduces incorporation bias, thereby reducing error rates. Data analysis involves alignment of new identified sequence reads with a reference genome (Illumina, 2016).

Predictions

A previous understanding of the microbial communities to be expected on wild type *Arabidopsis thaliana* was vital in order to discern changes in community composition of fhy3 far1 double null mutant plants. Numerous studies have been performed to determine the microbiome of the rhizosphere and phyllosphere, mostly through the use of fingerprinting and clone libraries (Reisberg EE, 2012). *Arabidopsis thaliana* microbial communities have been studied at a genome wide level (Matthew W. Horton, 2014), due to potential ecological and agricultural interest – particularly when it comes to micro biotic resistance.

(Matthew W. Horton, 2014) determined that in wildtype Arabdopsis, the majority of OUT's are from families of Proteobacteria, Bacterioidetes and Actinobacteria. Common genera included *Sphingomonas, Flavobacterium, Rhizobium* and *Pseudomonas.* (J. M. Whipps, 2007) determined that the phylosphere was dominated by Alpharoteobacteria, Gammaproteobacteria and Bacteroidetes. Betaproteobacteria and firmicutes have also been noted to be present at high abundances. Acidobacteria, Actinobacteria and cyanobacteria have all been found in low abundances (J. M. Whipps, 2007).

Fungal OUT's tend to be from Ascomycete classes Dothideomycetes and Sordariomycetes and the basidiomycete class Tremellomycets (Matthew W. Horton, 2014). A study by (Delmotte N, 2009) analysed what bacterial communities are most abundant in naturally occurring *A. thaliana* phyllosphere and discovered Methylobacterium, Sphingomonas and Pseudomonas to be the most prevalent. Commensals belonging to the genus *Sphingomonas* have been linked with protecting plants from pathogens (Innerebner G, 2011). Many of the genera are pathogenic; such as *Epicoccum, Alternaria, Mycospharella, Fusarium* and *Plectspharella..* Interestingly, a lot of these genera are seed transmitted, suggesting a reason for their permanent association with *A. thaliana.*

Microbial communities are largely shaped around host genetics, with changes in genes relating to defence response yielding the greatest changes in microbial communities. Due to the fhy3 far1 double null mutants constitutively activated immune response, one can assume that the plant will have an enhanced resistance against pathogenic organisms.

Materials and Methods

Plant Material, Growth Conditions and Extraction of Phyllospheric Microbes

The fhy3 far1 double null mutant line of *Arabidopsis thaliana* with a Nossen (No-0) ecotype was obtained from the Xing Wang Deng group at Yale university, New Haven, USA (Wang and Deng, 2002). Double mutant plant lines fhy3-4 and far1-2 were produced through 1-Methylsulfonyloxyethane (EMS)-mutagenesis by Hudson et al (1999). Plants displayed a dwarfism

phenotype, necrotic lesions on their leaves and accumulation of both ROS and SA.

Plants were grown in standard controlled environment chambers in white light at a Photon Flux Density of 164 µmol m-2 s-1 in short day conditions which correspond to 8 hours of light and 16 hours of darkness for 4 weeks. Plants were grown on a compost mixture consisting of 6 parts Levington M3 (Scotts, UK), 6 parts John Innes number 3 (Westland, UK), and 1 part (Sinclair, UK).

Phyllospheric microbes were extracted according to the protocol from Zhou et al (1996). The above ground growing parts from at least six plants were pooled for each sample. 100 mg of above ground growing parts of WT and fhy3 far1 mutant plants, 2. 7 ml of DNA extraction buffer and 10 µl of proteinase K (10 mg/ml) were added in falcon tubes. Tubes were shaken horizontally at 225rpm at RT for 30 mins. 0. 3 ml of 5% SDS was added and tubes were incubated at 65°C for 2 h with gentle mixing. The samples were centrifuged at 6, 00 g for 10 min at RT and supernatants were collected. Pellets were extracted two more times after addition of 0. 8 ml of extraction buffer and 20 µl of 5 % SDS. Tubes were vortexed for 10 sec, incubated at 65°C for 10 min and centrifuged. Supernatants from all three cycles of extractions were combined and mixed with equal volumes of chloroform-isoamyl alcohol (24: 1, vol/vol). The aqueous phase was recovered by centrifugation and precipitated with 0. 6 volume of isopropanol at RT for 1 h. The pellet of crude nucleic acids was obtained by centrifugation at 16, 000g for 20 min at RT. The pellet was washed with ice cold 70 % ethanol, dried at 37°C and resuspended in sterile deionized water for a final volume of 500 µl.

DNA extraction buffer contained 100 mM Tris-HCl (pH 8. 0), 100 mM sodium EDTA (pH 8. 0), 100 mM sodium phosphate (pH 8. 0), 1. 5 M NaCl and 1% CTAB.

PCR for High-throughput Sequencing and Sequencing Analysis

PCRs for bacteria and fungi rDNA-related sequences were performed in volumes of 20 µl, with 1 x GoTaq Flexi Buffer, 1. 5 mM MgCl2, 200 µM dNTPs, 0. 2 µM forward primer, 0. 2 µM reverse primer, 1. 25 units of GoTaq Flexi DNA Polymerase, 1 µl colony suspension and distilled water.

To amplify bacterial 16S rDNA and reduced mitochondria- and chloroplast-specific rDNA-amplicons, two PCRs were run. PCR primer pair 63f 63f (5′-CAGGCCTAACACATGCAAGTC-3′) / 1492r (5′-GGCTACCTTGTTACGACTT-3′) used for amplification of bacterial, mitochondria and chloroplast specific rDNA amplicons. The degenerative primer 783r (5′-CTACCVGGGTATCTAATCCBG-3′) is a mix of nine primers (783r-a1 (CTACCAGGGTATCTAATCCTG), 783r-b1 (CTACCGGGGTATCTAATCCCG), 783r-c1 (CTACCCGGGTATCTAATCCGG), and 783r-a2 (CTACCGGGGTATCTAATCCTG), 783r-b2 (CTACCCGGGTATCTAATCCCG), 783r-c2 (CTACCAGGGTATCTAATCCGG), and 783r-a3 (CTACCCGGGTATCTAATCCTG), 783r-b3 (CTACCAGGGTATCTAATCCCG), 783r-c3 (CTACCGGGGTATCTAATCCGG)). The degenerative primer 783r was designed to reduce amplification of chloroplast 16S rDNA (Sakai et al., 2004). For amplification of fungal intergenic spacers, the primer ITS1-F (CTTGGTCATTTAGAGGAAGTAA) and ITS2 (GCTGCGTTCTTCATCGATGC) (White et al., 1990) were used.

Eventually, 200 ng of DNA per sample, consisting of 100 ng DNA from bacteria-specific primer PCR and 100 ng DNA from fungi-specific primer PCR, were sent for high-throughput sequencing using the Illumina MiSeq platform to the Department of Epidemiology and Biostatistics, Institute for Computational Biology, Case Western Reserve University, Ohio, USA.

Data processing

Samples S13 and S15 consisted of sequences from the fhy3 far1 double null mutant whilst samples S14 and S16 belonged to the wild type *Arabidopsis thaliana* . A collective total of 182218 and 496243 sequences were present for fhy3 far1 and wildtype samples respectively. The first 20, 000 sequences of each of the four samples were retrieved from the raw FASTQ data files using the cut feature of NextGen Sequence Workbench (Heracle BioSoft, 2016). FASTQC High Throughput Sequence QC Report v0. 11. 5 (Simon Andrews, 2011-15) was used to analyse sequence quality. FASTQ sequences were converted to FASTA format with FASTQ to FASTA converter from the Galaxy platform (Gordon, 2016). Sequences with a Phred quality score under 20 were trimmed using default parameters of Trim Galore! (Krueger, 2016). Paired end reads were trimmed to discard the leading 8bp barcode. VSearch was used for sample dereplication (Rognes Torbjørn, 2015). Due to the composite nature of the samples (containing both bacterial and fungal reads), a method had to be devised to separate them.

SILVAngs was used to provide data analysis for 16S bacterial amplicon reads through an automatic software pipeline using the SILVA rDNA database (Quast C, 2013). SILVAngs was unable to process the 18S ITS fungal sequences. Through the SILVA output, recognised bacterial sequences were

determined for each sample. Using NextGen Sequence Workbench (Heracle BioSoft, 2016), these recognised bacterial sequences could be marked as contaminants and removed from the raw FASTA sequence data files, thereby leaving the fungal reads. Basic Local Alignment Search Tool from NCBI were used on the FASTA sequences (Altschul, 1990). Parameters were altered so that only the ten most similar alignments were retrieved per sequence.

A pipeline was built using python and local copies of mapping files maintained by GenBank (Dennis A. Benson, 2005): ftp://ftp. ncbi. nih. gov/pub/taxonomy/gi_taxid_nucl. dmp. gzfor corresponding taxonomic ID's for GID's andftp://ftp. ncbi. nih. gov/pub/taxonomy/taxdump. tar. gzfor matching taxonomic ID to scientific names. The pipeline functioned by converting genbank ID's to taxonomic ID and abundance count. The taxanomic ID was then matched to scientific names and defined to a taxonomic hierarchy. Sequences with an abundance under 3 were removed as singletons. Sequences assigned to *A. thaliana* chloroplast 16S rRNA gene or mitochondria were removed.

Statistical analysis

For diversity computation, samples were rarefied to the sample with the lowest sampling effort (3390 for fungal and 4988 for bacterial). Diversity indices, richness estimators, rarefaction curves and eigenvector techniques such as principal component analysis were all performed using PAST 3. 14 (Hammer, 2001). Wilcoxon Signed-Rank test was performed using IBM SPSS Statistics (IBM Corp, 2013). Heatmaps were generated using (Wahlestedt, 2016). Krona plug in was used for abundancy chats (Ondov BD, 2011)

Results

Statistical Analysis of Bacterial Communities

Statistical analysis at a genus level indicated the following. Rarefaction curves showed a lack of sampling depth in fhy3far1. Diversity t tests determined that fhy3 far1 mutants displayed a greater diversity in comparison to wildtype *A. thaliana* , with a Shannon index of 3. 51 and 2. 85 respectively. Dominance values indicate that wild type *A. thaliana* contained select few genera which dominated the sample size. Simpson_1-D indicated that fhy3 far1 mutants possessed the greatest amount of sample diversity, though only marginally (0. 95 and 0. 91 respectively), whilst Evenness was highest in wildtype. Shannon index determined that fhy3 far1 samples had greater alpha diversity, confirmed by a Chao-1 score of 222. 7, indicating greater species richness. Beta diversity was also greater in fhy3 far1. Alpha diversity indices are all displayed in table 1.

Wilcoxon Signed-Rank test was performed with the null hypothesis that wild type and fhy3 far1 samples would contain similar bacterial community composition. The results indicate that the fhy3 far1 plant had 165 species with a higher abundance than in wild type *A. thaliana.* Test statistics indicated that fhy3 far1 contained a statistical difference in microbial abundances ($P < 0.05$).

Principal component analysis at a phylum level revealed that PC 1 (98. 5%) and PC2 (1. 46%) were able to explain 99% of the variation. The result indicated a higher association of Baceroidetes and Acidobacteriales with fhy3 far1, separating it from the wild type which had higher correlation with

Actinobacteria and Firmicutes. At a genus level (figure 2), wild type *A. thaliana* is correlated with *Bacillales, Bacillus, Brevibacterium, Sphingomonas, Rhizobiales* and *Lysobacter.* Genera associated with fhy3 far1 were determined to be *Devosia, Advenella, Chitinophaga, Shinella, Rhizobium, Pricia* and *Pedobacter.*

Discussion

Despite *Arabidopsis thaliana* having been studied for over 20 years in respect to the mechanisms of its immune responses (Kunkel, 1996), it's not until the works of (Joel M. Kniskern, 2007) and (Matthew W. Horton, 2014) that an insight into the natural bacterial and fungal communities of *A. thaliana* was made. The aims of this project were to determine the commensal bacterial and fungal communities of *A. thaliana* and investigate the effect of the fhy3 far1 mutants constitutively activated immune response on said communities. In this study, we characterized the phyllosphere of wild type and fhy3 far1 mutant *Arabidopsis thaliana* using an Illumina sequencing survey of 16S rRNA and 18S ITS genes.

To explain the results observed, we had to examine the effects of a constitutively activated immune response. The fhy3 far1 double null mutant has no way of negatively regulating SA signalling, this is due to the fact that FHY3 and FAR1 negatively regulate both stress and defence responsive genes, some of which are involved in the SA signalling pathway (EDS1, SID2, PAD4 and NDR1) (Wanqing Wang, 2015). This also induced the expression of a large amount of CC-NB-LRR and TIR-NB-LRR type R proteins. Many of these R genes will encode for protein homologs which mediate resistance against specific genera of bacteria and fungi. Some gene products can contain

pathogen growth by indirect means; reinforcing the defensive capabilities of host cell walls and inducing stomatal closure (Jorg Durner, 1997). Alternatively, R gene products which have direct effects are usually antimicrobial metabolites (phytoalexins), papillae formation and induction of JA signalling and HR. Due to ETI being a direct tailored response to specific effectors detected by R proteins, it stands to reason that the activation of R genes will have a more profound effect on pathogenic species producing effectors. ETI commonly leads to an apoptic hypersensitive response, as observed by the necrotic lesions (Jorg Durner, 1997). As non-pathogenic species are unlikely to produce effectors (Toni J. Mohr, 2008), they won't receive an ETI response and therefore may be resistant to the immune response. Alternatively, non-pathogenic species may possess a suite of effector proteins which allow the nonpathogen to overcome some host defence systems (Grennan, 2006). The reactive oxygen species accumulation can be seen as the plants establishment of defence, strengthening host cell walls by cross linking glycoproteins, or act as executioners of pathogens by lipid peroxidation and membrane damage (Miguel Angel Torres, 2006). Alternatively, it may function as a plant signalling molecule, much in the likes of salicylic acid.

Constitutive immune activation reduces abundance of pathogenic bacteria, but not pathogenic fungi.

Interestingly, we discovered that fhy3 far1 *A. thaliana* plants showed a decreased abundance of bacterial species associated with pathogenesis, thereby indicating that the effector triggered immunity response was effective and targeted towards pathogens. We were not able to show a

specificity in plant response to non-pathogenic bacteria, as these too were affected by the ETI, seemingly without discrimination. Numerous reports indicate that the effects of plant defence processes on the microbiome are variable, with SAR being responsible for controlling the populations of some bacteria. (John W. Hein 2008) determined significant differences in rhizopshere bacterial community composition in *A. thaliana* mutants deficient in systemic acquired resistance (SAR), however, direct chemical activation of SAR by (Peter A. H. M. Bakker, 2013) caused little difference in community composition. (Joel M. Kniskern, 2007) analysed the effects of salicylic acid mediated defense induction, simmilarly to what we have tried to show in this experiment, conclusing a change in phyllospheric communities; notable a reduction in deiversity of endophytes, but higher epiphytic diversity, in concordance with our findings.

We also concluded that the mutants constitutively activated immune response had no real effect on pathogenic fungi, in fact- the mutant hosted an increased abundance of pathogenic fungi. This was unusual due to the assumption that ETI would be targeted towards these species. This hints at the possibility that fungal communities are shaped by the bacterial communities present on the plant. It has been noted that SA and SAR do not contribute to resistance to necrotrophic pathogens (Joanna Å i az´niewska, 2010), however some literature contradicts our findings.

Bacterial community diversity is increased in fhy3 far1 *A. thaliana*

Our initial survey of the wild type bacterial communities of *A. thaliana* in samples 14 and samples 16 revealed a disparity in initial composition, however a Wilcoxon Signed Ranks test indicated no statistically significant

difference between the two. 91 different morphotypes were detected and assigned to species on the basis of 16S sequence alignment.

The most abundant species, *Bacillales* and *Bacillus* from the order Bacillalesare unusual in that they have not been previously described in *A. thaliana.* These high abundances are only from Sample 14, and were not observed in Sample 16. This may be a sequencing error or alternatively due to contamination. Bacillus have been described as mutually beneficial rhizobacterium in some plants; providing plants with growth promoting traits (Nathaniel A. Lyngwi, 2016). The Gammaproteobacteria of the genera Pseudomonas were found in a high abundance, a result which coincides with the literature (Matthew W. Horton, 2014) (J. M. Whipps, 2007). (Fumiaki Katagiri, 2002) has noted that *Pseudomonas syringae* is pathogenic to *A. thaliana,* triggering a hypersensitive response (HR) – a rapid associated death of plant cells. The fhy3 far1 mutant showed a severe decrease in abundance; which could be associated to the over expression of *Arabidopsis* R genes: *RPS2, RPM1, RPS4, RPS5* and *PBS1,* which mostly belong to nucleotide binding site-leucine rich repeat classes of R genes (Fumiaki Katagiri, 2002). (Wanqin