Membrane trafficking in resistance gene-mediated defense against the barley powdery mildew fungus

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Membrane trafficking in resistance gene-mediated defense against the barley powdery mildew fungus

by

Priyanka Surana

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Bioinformatics and Computational Biology

Program of Study Committee:
Roger P. Wise, Major Professor
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The student author and the program of study committee are solely responsible for the content of this dissertation. The Graduate College will ensure this dissertation is globally accessible and will not permit alterations after a degree is conferred.

Iowa State University

Ames, Iowa

2017
DEDICATION

I dedicate this dissertation to my parents Raj Kumar and Asha Surana, without whose support I would not have been able to complete this work, and to my sister Pooja Dugar, who is the source of my motivation. I would additionally like to thank my parents for teaching me the importance of education and for providing a wonderful start and a world of opportunity.
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CHAPTER 1. GENERAL INTRODUCTION

This dissertation focuses on membrane trafficking genes identified as associated with plant defense pathways. This research seeks to better understand the complex interactions and response that the barley host plant has to the biotrophic, powdery mildew fungal pathogen.

1.1 Biotrophic Mildews

Powdery mildews are some of the most easily recognizable plant diseases (Glawe, 2008). They are caused by ascomycete fungi belonging to a single order of the Erysiphales. All Erysiphales are obligate biotrophic pathogens. Obligate biotrophic pathogens are only capable of life on a living plant and maintain host cells alive while they complete their life cycle. Biotrophic interactions are those in which the microbial partner infects a plant without causing death of the host cells and tissues. The best-known and well-studied members of this group are the fungi causing rusts and the powdery mildews. Most powdery mildews exclusively colonize the epidermis of aerial structures in host plants. In fact, most of the fungus is epiphytic. Only the intracellular haustoria penetrate the plant. Infection is initiated by ascospores, which germinate to form a hypha that produces a swollen, elongated appressorium.

A variation of this theme is found in powdery mildews of grasses (Blumeria sp.). They first form a primary germ tube with a short, determinate growth whose function is essentially that of surface sensing. The appressorium produces a hyphal peg to
penetrate the epidermal cell wall. This hypha then differentiates a haustorium surrounded by a plant-derived perihaustorial membrane (Dormann et al., 2014). The haustoria are known to be devoted to nutrient uptake. Unlike most other plant pathogenic fungi, which have intercellular hyphae at least to some extent, mildews have essentially no other way of obtaining nutrients (Spanu, 2012).

Haustoria also control host immunity and achieve this by secretion of effector molecules (Panstruga and Dodds, 2009). Once the first haustorium is functional, nutrients are transported to the epiphytic hyphae, which then proliferate rapidly and abundantly, producing secondary appressoria and secondary haustoria to feed the colony. Three to four days after inoculation conidiophores develop and generate masses of conidia by repeated budding. These conidia are dispersed by air currents and can re-infect appropriate hosts. Toward the end of the host's growth season, compatible strains mate and develop chasmothecia (formerly cleistothecia), which can survive inclement conditions. Therefore, reproduction during the disease epidemics may be exclusively asexual. Sexual reproduction occurs immediately prior to the resting (overwintering) season and is responsible for the production of ascospores, which infect hosts at the beginning of the next growth season. Some powdery mildews are highly fastidious, for example *Blumeria* exists as formae speciales, which are capable of infecting only a single species and discriminate between closely related plants (Wyand and Brown, 2003). *Blumeria graminis* f. sp. *hordei* (*Bgh*) exclusively infects barley plants, whereas *B. graminis* f. sp. *tritici* causes disease in wheat.
One of striking features of the mildew genomes is their size (Spanu et al., 2010). The genome sizes for the species analyzed are ~130 Mb in *Bgh*, ~180 Mb in *B. graminis* f. sp. *tritici*, ~151 Mb in *Erysiphe pisi*, and ~160 Mb in *Golovinomyces orontii* (Spanu et al., 2010, Wicker et al., 2013). Their genomes are up to four times larger than those of most closely related ascomycetes. The increase in all mildew genomes can be explained entirely by a massive proliferation of retrotransposons that generate repetitive DNA accounting for at least 64% of the genome in *Bgh*. In the mildews, retrotransposons have caused significant rearrangements of the mating-type loci, which are usually highly conserved in ascomycetes (Brewer et al., 2011).

The increase in mildew genome size is accompanied by a reduction of the number of protein coding genes: Only 5,854 manually curated gene models were reported in *Bgh* (Spanu et al., 2010). These are much fewer than the number of genes usually found in filamentous fungi. The reduction can be attributed mostly to loss of paralogs and to loss of some specific pathways. In addition to the missing genes and pathways, there are some functionally related genes that are reduced. These losses are considered a consequence of a lifestyle that no longer requires these capabilities rather than the cause for the obligate nature of the interactions.

### 1.2 Secreted Effectors

There are two classes of genes that have opposed the trend in gene reduction. These genes encode effectors and effector-like proteins. The first recognized class
of effector genes in the mildews were paralogs of EKA (Effectors homologous to Avr_\text{k1} and Avr_{\text{a10}}) genes (Ridout et al., 2006, Sacristán et al., 2009). The genome sequence revealed that over 1,350 copies of EKA genes exist in the Bgh genome. Silencing AVR_{\text{k1}} affects Bgh haustorial development on susceptible plants (Nowara et al., 2010, Amselem et al., 2015). The second class of putative effectors is a group of genes encoding candidate secreted effector proteins (CSEPs). These proteins are predicted to be secreted but do not have sequence similarity outside the powdery mildews. Originally, 248 CSEPs were described in Bgh, and their salient properties are that they are prevalently upregulated in haustoria (Spanu et al., 2010) and harbor an YxC motif previously identified in candidate mildew and rust effectors in an independent study (Godfrey et al., 2010). Refined analyses of the CSEP complement in Bgh indicated there are close to 500 such genes (Pedersen et al., 2012), that are highly expressed in haustoria, and thus expected to be released from the haustorial membrane into the extrahaustorial matrix. The high level of CSEP expression at an RNA level is consistent with the finding that many of these genes encode proteins specifically associated with infection structures in high-throughput proteomic studies (Bindschedler et al., 2009, Bindschedler et al., 2011). The CSEPs are therefore a lineage specific group of genes that has expanded and diversified within the mildew species, possibly accompanying the evolution of host adaptation (Pedersen et al., 2012, Amselem et al., 2015).

How effector proteins subsequently enter the plant cell is still unclear. In general, CSEPs have very limited sequence similarity and only a few common protein motifs
have been found such as the YxC motif, suggesting they may target different proteins in the host and fulfill different functions (Pedersen et al., 2012). One group of predicted CSEP proteins share structural similarities to ribonucleases, which is why they are suspected to mimic and compete with plant proteins involved in pathogen defense (Bourras et al., 2016). Furthermore, several CSEPs have homology to enzymes that could be involved in processes of plant–pathogen interaction such as cell-wall remodeling or protein degradation (Pliego et al., 2013).

In summary, our understanding of the actual action by CSEPs is very limited.

1.3 Plant Immune Response

Plant pathogenic fungi deliver effector molecules (virulence factors) into the plant cell to enhance microbial fitness. Plants rely on two-tier innate immunity for defense responses (Dangl and Jones, 2001, Chisholm et al., 2006, Jones and Dangl, 2006). These are initiated by cell-surface or intracellular immune receptors upon the detection of pathogen-derived molecules (Zipfel, 2014). The first uses transmembrane pattern recognition receptors (PRRs) that respond to slowly evolving microbial- or pathogen-associated molecular patterns (MAMPs or PAMPs), such as flagellin (Zipfel and Felix, 2005). In response to PAMP-triggered immunity, pathogens release effector proteins. Effectors can interfere with PTI, resulting in effector-triggered susceptibility (ETS).

Effectors can also activate corresponding intracellular nucleotide binding and leucine rich repeat (NLR) protein receptors encoded by most R genes (Dangl and
Jones, 2001, Jones and Dangl, 2006, Maekawa et al., 2011b, Jacob et al., 2013). In effector-triggered immunity (ETI), NLRs detect either the action or the structure of pathogen effectors inside host cells. An effector molecule recognized by an NLR-type disease resistance (R) protein is designated an avirulence (AVR) effector, and these effector variants are typically present only in specific isolates of a pathogen species. ETI is an accelerated and amplified response, resulting in disease resistance and, usually, a hypersensitive cell death response (HR) at the infection site (Jones and Dangl, 2006). HR typically does not extend beyond the infected cell: it may retard pathogen growth in some interactions, particularly those involving haustorial pathogens (such as mildews), but is not always observed, nor required, for ETI.

1.4 NLR protein encoding R genes

R genes encoding NLR-type receptors are frequently members of larger gene families, organized in complex clusters of paralogous genes, and can evolve through tandem and segmental gene duplications, recombination, unequal crossing-over, point mutations, and diversifying selection (Meyers et al., 2005, Jacob et al., 2013). There are several examples of allelic series of NLR-type R genes known in plants (Ellis et al., 1999, Allen et al., 2004, Srichumpa et al., 2005, Bhullar et al., 2010, Seeholzer et al., 2010, Kanzaki et al., 2012). In these cases, multiple distinct recognition specificities evolved in the host population at a single R gene with each allele detecting a corresponding strain-specific AVR in the pathogen population (Dodds et al., 2006, Hall et al., 2009).
One of the powdery mildew R genes, designated *Mla*, has been subject to exceptional functional diversification with large numbers of resistance alleles each detecting a corresponding *Bgh* AVR effector (Seeholzer et al., 2010). The *Mla* ortholog *Sr33* in wheat (Periyannan et al., 2013) and *Sr50* in rye (Mago et al., 2015) confer resistance to the devastating Ug99 stem rust pathogen *Puccinia graminis* f. sp. *tritici* (*Pgt*) even though *Pgt* and *Bgh* belong to different phyla. Barley *Mla* encodes intracellular NLRs with an N-terminal coiled-coil (CC) domain (Zhou et al., 2001), where the protein sequences of 23 characterized alleles exhibit >90% sequence identity. The crystal structure of the CC domain of barley MLA10 indicates that homodimerization is necessary for downstream signaling activity (Maekawa et al., 2011a). Furthermore, *Mla1* confers race-specific disease resistance to *Bgh* isolate carrying the cognate avirulence gene *AVRa1* in transgenic *Arabidopsis thaliana*, suggesting ~150 million years of evolutionary conservation in the underlying immune mechanism (Maekawa et al., 2012). Interestingly, this resistance remains effective in *Arabidopsis* mutants defective in ethylene, jasmonic acid, and salicylic acid signaling, indicating the presence of a hormone independent NLR-mediated defense mechanism.

The activation of NLR proteins is commonly associated with significant transcriptional reprogramming. Several plant NLRs, including barley MLA, have been shown dynamic nucleocytoplasmic distribution, with increased accumulation in the nucleus upon effector-induced activation (Shen et al., 2007, Wirthmueller et al.,
2007, Caplan et al., 2008, Hoser et al., 2013, Inoue et al., 2013, Ma et al., 2013). One study implicates SGT1 (Suppressor of G2 allele of SKP1) for regulating nucleocytoplasmic partitioning. Along with RAR1 (required for MLA12 resistance 1) and HSP90 (heat shock protein 90), SGT1 positively controls the steady state levels of MLA (Azevedo et al., 2002, Bieri et al., 2004). However, MLA lacks a nuclear localization signal (NLS) and thus, it is unknown how the distribution between cytoplasm and nucleus is regulated. Additionally, the endomembrane trafficking system, that may be utilized to translocate NLRs, is emerging as an important arm of the R-gene-mediated defense system that is also targeted by pathogen effectors (Nomura et al., 2006, Nomura et al., 2011, Zhang et al., 2011, Elmore et al., 2012).

Furthermore, several transcription factors function in NLR-mediated resistance (Chang et al., 2013, Inoue et al., 2013, Padmanabhan et al., 2013). For example, in the presence of the cognate powdery mildew effector $AVR_{a10}$, the barley MLA10 translocates into the nucleus and interacts with both WRKY transcriptional repressors and MYB6, a transcriptional activator, to activate defense responses (Shen et al., 2003, Chang et al., 2013).

### 1.5 Transcriptomics

Transcriptomics is the study of the transcriptome – the complete set of RNA transcripts that are produced by the genome, under specific circumstances or in a specific cell – using high-throughput methods, such as microarray analysis and RNA
sequencing (RNA-Seq). Microarrays quantify a set of predetermined sequences, whereas RNA-Seq captures all sequences.

Microarray technology can measure expression only of genes that have corresponding probes on the array, which in most cases are designed only to cover a portion of a gene. Consequently, it is not possible to detect novel transcribed regions or the presence of alternative splice forms of a gene. Both these problems can potentially be tackled using Illumina sequencing data, if enough reads span exon – exon junctions.

Microarrays and RNA-Seq both rely on image analysis in different ways. In microarray, each spot on a chip is a defined oligonucleotide probe, and fluorescence intensity directly detects the abundance of a specific sequence (Affymetrix, Santa Clara, CA). In a high-throughput sequencing flow cell, spots are sequenced one nucleotide at a time, with the color at each round indicating the next nucleotide in the sequence (Illumina Hiseq, San Diego, CA).

A draft sequence of the barley genome was unavailable till 2012 (International Barley Sequencing Consortium et al., 2012), with a new high-quality reference assembly across the pericentromeric space published recently (Mascher et al., 2017). Therefore, for the eQTL study presented in Chapter 1, GeneChips representing 22,840 gene sequences were utilized. The Affymetrix Barley1 GeneChip was designed from 350,000 previously sequenced ESTs (Close et al.,
Since 2012, a transition to RNA-Seq was made possible (Chapter 2) which addressed several limitations of GeneChip technology, especially the inability to interrogate novel transcripts. However, one study comparing the Illumina RNA-Seq and Affymetrix GeneChip in humans detected 81% of genes called significantly differentially expressed from the array data in Illumina sequencing platform as well. Further, the correlation of fold change ratios between the two technologies was quiet high (Spearman correlation = 0.73) (Marioni et al., 2008).

Comparison of transcriptomes allows the identification of genes that are differentially expressed in distinct cell populations, or in response to different treatments. It can also help to infer the functions of previously unannotated genes. To gain an insight into transcription activity during barley – powdery mildew interactions, I utilized gene expression to identify temporal changes associated with different genotypic environments.
CHAPTER 2. INTER-CHROMOSOMAL TRANSFER OF IMMUNE REGULATION DURING INFECTION OF BARLEY WITH THE POWDERY MILDEW PATHOGEN

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2.1 Abstract

Powdery mildew pathogens colonize over 9,500 plant species, causing critical yield loss. The Ascomycete fungus, \textit{Blumeria graminis} f. sp. \textit{hordei} (\textit{Bgh}), causes powdery mildew disease in barley (\textit{Hordeum vulgare} L.). Successful infection begins with penetration of host epidermal cells, culminating in haustorial feeding structures, facilitating delivery of fungal effectors to the plant and exchange of nutrients from host to pathogen. We used expression Quantitative Trait Locus (eQTL) analysis to dissect the temporal control of immunity-associated gene expression in a doubled haploid barley population challenged with \textit{Bgh}. Two highly significant regions possessing \textit{trans} eQTL were identified near the telomeric ends of chromosomes 2HL and 1HS. Within these

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regions reside diverse resistance loci derived from barley landrace, *H. laevigatum* (*MILa*), and *H. vulgare* cv. Algerian (*Mla1*); which associate with the altered expression of 961 and 3,296 genes during fungal penetration of the host and haustorial development, respectively. Regulatory control of transcript levels for 299 of the 961 genes is re-prioritized from *MILa* on 2HL to *Mla1* on 1HS as infection progresses; with 292 of the 299 alternating the allele responsible for higher expression, including Adaptin Protein-2 subunit µ AP2M and Vesicle Associated Membrane Protein VAMP72 subfamily members VAMP721/722. AP2M mediates effector-triggered immunity via endocytosis of plasma membrane receptor components. VAMP721/722 and SNAP33 form a SNARE complex with SYP121 (PEN1), which is engaged in PAMP-triggered immunity via exocytosis. We postulate that genes regulated by alternate chromosomal positions are repurposed as part of conserved immune complex to respond to different pathogen attack scenarios.

**2.2 Introduction**

Powdery mildew fungi infect greater than 9,500 plant species, resulting in more yield loss than any other single type of plant disease (Inuma *et al.*, 2007). Cereal grains, particularly wheat and barley, are among the most important agricultural crops that suffer. Barley powdery mildew, caused by the filamentous Ascomycete fungus, *Blumeria graminis* f. sp. *hordei* (*Bgh*), has been developed into a model system to study the relationship between obligate biotrophs and their host plants. Infection of barley by *Bgh* begins when a conidiospore lands on the leaf surface, germinates and differentiates into an appressorial germ tube. This is followed by penetration of the
epidermal cells and development of haustoria, which facilitate delivery of pathogen effectors to the host plant and uptake of nutrients from the plant to the fungus.

To defend themselves from invading pathogens, such as *Bgh*, plants have evolved innate and induced immune responses. Innate immunity is species non-specific and recognizes conserved pathogen associated molecular patterns (PAMPs) via pattern recognition receptors at the cell surface; this initiates intracellular signaling pathways that lead to PAMP triggered immunity (PTI) (Macho and Zipfel, 2014). Interconnected with PTI is a second layer, designated effector triggered immunity (ETI) (Cui et al., 2015). ETI is considered species-specific and is activated when a plant resistance protein interacts, directly or indirectly, with corresponding pathogen secreted effector proteins, which alter host processes to promote nutrient acquisition and colonization (Bent and Mackey, 2007, Jacob et al., 2013, Cesari et al., 2014). This interaction triggers defense signaling in the cytoplasm or the nucleus, resulting in programmed cell death (Li et al., 2015).

Reaction to *Bgh* is controlled by several loci distributed across the barley genome, termed *Ml* (for *Mildew resistance locus*). Positioned on the short arm of chromosome 1H (Wei et al., 2002), the *Mla* locus harbors over 30 alleles that confer varying levels of resistance when corresponding avirulence effectors are present in the pathogen (Shen et al., 2003, Halterman and Wise, 2004, Seeholzer et al., 2010). *Mla* alleles encode coiled-coil, nucleotide binding, leucine-rich repeat (NLR) proteins that accumulate in the nucleus after recognition of corresponding avirulence (AVR) effector proteins from *Bgh*.
After recognition, MLA dissociates the MYB6 transcriptional activator from the WRKY1 repressor and promotes its binding to corresponding cis-regulators, initiating disease resistance signaling (Chang et al., 2013).

Despite the observation that plant resistance to disease is often associated with single segregating loci, the underlying genetic architecture that contributes to the ultimate phenotype can be comprised of hundreds to thousands of genes (Kliebenstein, 2009a). Expression quantitative trait locus (eQTL) analysis is used to associate gene expression measurements with polymorphisms in a segregating population (Farrall, 2004, Gilad et al., 2008). eQTLs are classified as cis- or trans-acting by comparing their genomic location to the position of the target genes. A cis-regulatory change is inferred when the eQTL coincides with the target gene, whereas a trans-regulatory change is assumed when the eQTL is not near the target gene (Wittkopp, 2005). Regions of the genome that associate with the expression of many genes are referred to as eQTL hotspots (Chesler et al., 2005, Hansen et al., 2008), which tend to act in trans, regulating greater than 1,000 genes in some cases (West et al., 2007, Hansen et al., 2008, Li et al., 2013). Thus, eQTL hotspots can be used to characterize chromosomal positions of major regulators (de Koning and Haley, 2005), including, for example, those involved in disease resistance (Mozhui et al., 2008, Kliebenstein, 2009b, Chen et al., 2010, Moscou et al., 2011b, Samad-Zamini et al., 2017).

We sought to address the temporal regulation of immunity in barley-powdery mildew interactions by eQTL analysis. Two main questions were addressed: (i) which genes
are regulated by \textit{Ml} loci, and (ii) how are these genes regulated. Briefly, genome-wide transcriptome analysis of the barley Q21861 x SM89010 doubled-haploid population identified two major regions containing \textit{trans} eQTL near the telomeric ends of chromosomes (Chr) 2HL and 1HS. Residing within these regions are the resistance loci, \textit{MILa} (connected to intermediate quantitative resistance) and \textit{Mla} (conferring strong qualitative resistance), which associate with expression levels of 961 and 3,296 genes, respectively. Intriguingly, the \textit{MILa} region controlled expression during \textit{Bgh} penetration while the \textit{Mla} region controlled expression during development of fungal haustoria. Moreover, of the 961 genes under transcriptional control of the \textit{MILa} locus, 299 are reallocated to \textit{Mla} as infection progressed. These findings suggest that a conserved immune regulon is activated by disparate resistance loci to achieve immunity in response to multiple infection stages.

\textbf{2.3 Materials and Methods}

\textbf{Plant growth, RNA extraction and GeneChip hybridization}

The 75 Q21861 x SM89010 (QSM) doubled haploid barley lines (Steffenson \textit{et al.}, 1995) were derived from a single F$_1$ plant, and have been maintained via single seed descent (Moscou \textit{et al.}, 2011b). Seedlings were grown in two 98-cone-tainer trays in a climate-controlled greenhouse using a randomized block design. Each tray contained five seedlings/cone of the 75 QSM lines plus four replicates of each parent. Seven days after sowing, both trays were inoculated with \textit{Bgh} isolate 5874 (\textit{AVR$_{a1}$}, \textit{AVR$_{a6}$}, \textit{vir$_{a6}$}, \textit{vir$_{a13}$}, \textit{AVR$_{La}$}) at an average density of 200 conidiospores per cm$^2$. The five seedlings were harvested, pooled and placed in liquid nitrogen for each progeny and parent line at
16 and 32 hours after inoculation (HAI). Total RNA was isolated using a hot (60°C) phenol / guanidine thiocyanate method (Caldo et al., 2004). Labeling, hybridization, washing, and scanning were performed according to standard Affymetrix protocols at the Iowa State University GeneChip Core facility using 22K Barley1 GeneChip expression arrays (Affymetrix part number 900515) (Close et al., 2004).

**Phenotyping**

*Bgh* isolate 5874 was propagated on *H. vulgare* cv. Morex at 18°C while in 16 hours of light and 8 hours of darkness. *H. laevigatum* derived lines were obtained from the USDA-Germplasm Resources Information Network (GRIN). Alleles at the *Mla* locus were amplified via polymerase chain reaction (PCR) using primers Mla_LRR F and R (Table S1) which amplified the LRR region, spanning ~850 nucleotides between positions 1897 and 2725 base pairs relative to the *Mla6* genomic sequence (AJ302293.1). Sequence verification was done by alignment against known *Mla* alleles (Seeholzer et al., 2010) and the alleles in Q21861 and SM89010 were assigned as *Mla1* (AY009939.1) and *Mla8* (GU245940.1) respectively.

To phenotype the QSM population, five 98-cone trays of Q21861, SM89010 and 75 QSM progeny were planted in sterilized potting soil. Each of the five experimental trays (7 rows x 14 columns) consisted of 5 seedlings for each parent (Q21861 and SM89010) and 1 seedling for each QSM progeny. One seedling was planted per cone and genotypes were completely randomized within each of the 5 trays. Seedlings were grown in a 20°C controlled glasshouse to 10 cm prior to the application of treatment.
Seedlings were inoculated with fresh *Bgh* conidiospores and trays were transferred to a growth chamber at 18°C (16 hours light and 8 hours darkness) where the trays were stored side-by-side in randomly assigned positions. Six days after inoculation, all leaves were photographed and scored for infection type (Table S2). The entire procedure was repeated three times in successive weeks.

**Expression QTL Mapping**

eQTL analysis was carried out separately for the 16 and 32 HAI time points. Each of 22840 probe sets was scanned for association with each of 377 non-redundant markers (Moscou *et al.*, 2011b) using data from the 75 QSM lines. For probe set i=1, ..., 22840 and marker j=1, ..., 377, a simple linear regression model was fit to 75 data points with RMA-normalized expression level as the response and an indicator of marker genotype as the explanatory variable. For missing genotypes, observed flanking marker genotypes were used with linear interpolation to impute a continuous genotype value in the interval [0,1]. A t-statistic ($t_{ij}$) for testing the significance of the slope coefficient in the simple linear regression model was computed to quantify the strength of association between probe set i and marker j. For each probeset i, $T_i =$ the maximum value of $|t_{ij}|$ over markers j=1, ..., 377 was computed, and the identity of the marker yielding the maximum value was recorded.

To identify statistically significant associations of expression level with marker genotype, the statistics $T_1$, ..., $T_{22840}$ were first converted to p-values ($p_1$, ..., $p_{22840}$) using a standard permutation approach that accounts for multiple testing across the 377
markers (Churchill and Doerge, 1994). Specifically, the values of $T_1, \ldots, T_{22840}$ were recomputed for each value of 100 permutations, where for each permutation, the 75 expression vectors associated with the 75 QSM lines were randomly assigned to the 75 genotype vectors associated with the 75 QSM lines. This yielded a null distribution of $22840 \times 100$ permutation test statistics. The p-value for the original statistic $T_i$ was given by the proportion of the $22840 \times 100$ permutation test statistics greater than or equal to the value of $T_i$. To account for multiple testing across 22840 probe sets, the 22840 permutation p-values were converted to q-values ($q_1, \ldots, q_{22840}$) (Benjamini and Hochberg, 1995). To control false discovery rate (FDR) at level 0.001, the association between probe set $i$ and its most strongly associated marker was declared significant if and only if its q-value ($q_i$) was no greater than 0.001.

For this experiment, we interrogated the data set for only the most associated markers to identify the primary eQTL that had the greatest impact on gene expression. While other secondary and tertiary eQTL can also exert influence, we focused on changes in the most associated regulatory eQTL, with the hypothesis that it represents the most significant modification in the underlying biology of the gene in question.

Differential Expression Analyses for QSM Progeny Groups

The 75 QSM progeny were grouped by the four $Ml$ gene + allele combinations: 1) $Mla1$, $MILA$; 2) $Mla1$, $mlLa$; 3) $mla1$, $MILA$; 4) $mla1$, $mlLa$; and analyzed using R package limma in Bioconductor (Ritchie et al., 2015). A model was fit with expression value as response, and treatment (genotype * time point) as explanatory variables. P-values
were adjusted for multiple testing using R package qvalue (Storey et al., 2015). Genes that had a q-value of no greater than 0.01 were considered significant.

Comparing QSM Progeny Lines to Q and SM Parents

The gene expression for each of the QSM progeny was compared to the gene-wise median for Q and SM parents combined for the respective time point. A log-fold difference of two between QSM progeny and the parents was considered a sign of differential expression.

Transcript Pattern Analyses of Published Data Sets

We conducted linear model analyses of the normalized signal intensities for each of the 22840 Barley1 probe sets (Close et al., 2004) using packages in BioConductor/R (Gentleman et al., 2004, R Core Team, 2016). RMA normalization and data transformation was done using package affy (Gautier et al., 2004). Linear model analysis and multiple testing adjustments were conducted using package limma (Ritchie et al., 2015) and qvalue (Storey et al., 2015). The model used RMA normalized expression values as the response, and treatment (genotype*timepoint) as the input variable. Contrasts were used to compare transcript levels between treatments after infection with Bgh 5874. Q-values were estimated using the smoother method described in (Storey and Tibshirani, 2003).

Custom R program scripts were used to parse for highly similar patterns of expression among incompatible and compatible interactions up to 16 HAI, followed by significant
(q-value no greater than 0.01) divergence from 16 to 32 HAI. This was used to distinguish two opposing scenarios: (i) down regulation of the transcript in the compatible interaction after 16 hours, as compared to paired time-points in the incompatible interaction (designated *PacMan*), or (ii) up regulation of the transcript in the compatible interaction after 16 hours, as compared to paired time-points in the incompatible interaction (designated *iPacMan*).

**Table 1.** Statistical definitions for *PacMan* and *iPacMan* expression patterns.

<table>
<thead>
<tr>
<th>Time points</th>
<th>0 – 16 HAI</th>
<th>20 – 24 HAI</th>
<th>32 HAI</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>PacMan</em></td>
<td>Not differentially expressed</td>
<td>No constraints</td>
<td>Differentially expressed and downregulated in compatible as compared to incompatible interactions</td>
</tr>
<tr>
<td><em>iPacMan</em></td>
<td>Differentially expressed and upregulated in compatible as compared to incompatible interactions</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**MILa marker design and testing**

The two markers, MLOC_20004 and MLOC_25435 on chromosome 2H, represent the outermost boundaries of a region flanked by recombination breakpoints and implicated to contain *MILa*, at 621 Mb and 624.5 Mb respectively. Using the available barley Morex genome on Ensembl (Assembly 082214v1, INSDC Assembly GCA_000326085.1) (International Barley Sequencing Consortium *et al.*, 2012), 29 high confidence candidates were selected. To further delineate the region that contains *MILa*, markers were generated to identify genes within this region that co-segregated with the *MILa* phenotype. Multiple primer sets were used to amplify candidate genes using standard *Taq* polymerase (Invitrogen, Carlsbad, CA) from cultivars SM89010 (*Mla8, MILa*) and Q21861 (*Mla1, mlLa*). Any primer sets that showed potential as PCR-based markers (presence/absence or band-shift) were utilized on the QSM DH population (Table S3) and *H. laevigatum* derived lines (Table S4). Those that showed no discernible
difference between the two parents were sequenced to design cleaved amplified polymorphic sequence (CAPS) markers.

**Computational prediction of secretion-associated genes/proteins**

To reliably map the *Arabidopsis* proteome to the Barley1 GeneChip probesets, we obtained empirical scores for coverage and identity. Rice (*Oryza sativa* subsp. *japonica*) was selected as the monocot representative as it had the highest number of protein sequences (3,716) in Swiss-Prot (UniProt Consortium, 2015) as compared to barley (350 sequences). The coverage and identity scores were based on a Rice – *Arabidopsis* comparison of protein sequences obtained from Swiss-Prot using blastp in the BLAST+ command line suite (Camacho *et al.*, 2009). This alignment gave an average of 86% coverage and 60% identity among the orthologous sequence pairs.

Next, protein sequences for *Arabidopsis* assembly Araport11 were obtained from TAIR ([www.arabidopsis.org](http://www.arabidopsis.org)) and compared against the Harvest 21 assembly that was used to derive Barley1 GeneChip probesets (Close *et al.*, 2004) using blastx in the BLAST+ command line suite (Camacho *et al.*, 2009). Coverage was calculated using the query as well as the subject and the maximum of the two was used. Sequences that aligned with 86% coverage and 60% identity were selected as candidates for homologs.

**Connecting alternatively regulated genes to biological function**

Of the 344 membrane trafficking encoding genes associated with 2HL at 16 HAI and/or 1HS at 32 HAI (Table S5), we focused on the 37 Barley1 probesets that exhibit
alternate regulation between the two developmental stages. *Arabidopsis* proteins corresponding to these probesets were interrogated for previously established protein-protein interactions in BioGRID (Stark *et al.*, 2006). This resulted in a network of 129 nodes partitioned into 19 communities developed using the igraph package (Csardi and Nepusz, 2006) in R (R Core Team, 2016). These communities had a high modularity of 0.842, which reflects dense connections within communities and sparse connections across communities.

**Data Access**

All MIAME-compliant GeneChip profiling data from the QSM eQTL experiment are available as accession BB96 at the PLEXdb expression resource for plants and plant pathogens, as well as accession GSE68963 at NCBI-GEO. The previously published PLEXdb data set, BB4, is also available as NCBI-GEO accession GSE33396. Supplemental Materials for Chapter 2 can be accessed in the zipped folder “Chapter2_Supplemental_Files” on ProQuest. A brief description of each file is available below.

Table S1 details the PCR primers used in this study. Table S2 shows infection type data for three replications. Table S3 contains the complete QSM genetic map. Table S4 details the genotyping and phenotyping results for *H. laevigatum* and Laevigatum-derived lines. Table S5 features genes associated with membrane trafficking. Table S6 lists genes associated with each marker on the QSM map. Table S7 provides differential expression results for comparison between CI 16151 and CI 16155 (BB4)
and between QSM progeny groups \((Mla1, mlLa)\) and \((mla1, mlLa)\). Table S8 shows the modes of alternate regulation for the genes transferred between 2HL at 16 HAI and 1HS at 32 HAI. Figure S1 shows a subnetwork connected via PEN1 (SYP121) in community A and VAMP721, SNAP33 and KEULE in community B. Figure S2 illustrates a community with the alternatively regulated AP2M as the hub that interacts with the remaining five proteins, including AP2A1.

### 2.3 Results

**Mla1 is epistatic to MiLa**

In order to determine if interactions between \(Mla\) and \(MiLa\) had an effect on infection phenotype (IT), the 75 QSM barley lines (Steffenson *et al.*, 1995) were inoculated with *Bgh* isolate 5874. This isolate harbors both the \(AVRa_{a1}\) and \(AVRa_{La}\) avirulence effectors, and thus, was used to monitor the various infection types segregating in the QSM progeny. Figure 1 illustrates that Q21861 confers an IT of 0 in response to *Bgh* 5874, whereas SM89010 imparts an average IT of 2 with the same isolate. A 1,259 cM transcript derived marker (TDM) map of the QSM population (Moscou *et al.*, 2011b) was used to position \(Mla1\) and \(MiLa\). This map is divided into 377 bins across 7 chromosomes and harbors 1,494 Barley1 GeneChip derived markers (Table S3).
Figure 1. Infection types of Q21861, SM89010 and their recombinant progeny containing different combinations of $Ml$ genes. Seven-day-old seedlings of each genotype were inoculated with $Bgh$ isolate 5874 and photographed six days later. The three leaves shown represent an average infected plant for each genotypic group. An average infection phenotype of $< 2.5$ across three replications was considered resistant (incompatible interaction), whereas a value of $\geq 2.5$ was considered susceptible (compatible interaction). The values are rounded to the nearest 0.5.

Infection phenotyping (Wei et al., 1999) positioned a $Bgh$ resistance specificity at the $Mla$ locus on chromosome 1HS (1H.05), and another on distal end of chromosome 2HL. The second resistance that mapped to 2H.67 co-localized with the position associated with $Mlla$ (Schweizer and Stein, 2011). Infection phenotypes for three replications of each QSM recombinant line are detailed in Table S2.

$Mlla$ is derived from the barley landrace, $H. laevigatum$, but its precise position and sequence are currently unknown. Therefore, to determine whether we were indeed tracking the $Mlla$ locus, we compared infection types and $Mlla$ flanking marker
sequences of SM89010 (the QSM parent line putatively harboring \( \textit{MILa} \)) to \( \textit{H. laevigatum} \) and 10 other Laevigatum-derived accessions (Dros, 1957). SM89010 displayed identical infection type and DNA marker sequences to \( \textit{H. laevigatum} \), as well as the Laevigatum-derived Minerva and Vada. This suggests that the locus mapped is likely \( \textit{MILa} \) (Table S4). \( \textit{Mla} \)-derived sequences were amplified and aligned against known \( \textit{Mla} \) alleles (Seeholzer \textit{et al.}, 2010), confirming that Q21861 possessed \( \textit{Mla1} \), which was consistent with the observed IT of “0” associated with this allele.

Taking epistatic interactions into account, it was conceivable that some of the recombinant progeny in the QSM population would display an IT of 0 in response to \( \textit{Mla1} \), but still contain \( \textit{MILa} \). Indeed, all 38 lines that contained \( \textit{Mla1} \) displayed an IT of 0, whereas the 14 lines that contained \( \textit{MILa} \) only displayed an average IT of 2, and lines that contained neither \( \textit{Mla1} \) nor \( \textit{MILa} \) exhibited an average IT of 3. Genotypes for the double haploid progeny fit a ratio of 1:1:1:1 (\( \chi^2 \) 1:1:1:1 = 2.28; p-value = 0.5164) and a phenotypic ratio of 2 (IT=0):1 (IT=2):1 (IT=3) (\( \chi^2 \) 2:1:1 = 2.17; p-value = 0.3373) as expected for an epistatic interaction.

\textit{trans-eQTL} hotspots are associated with \( \textit{MILa} \) during \( \textit{Bgh} \) penetration and \( \textit{Mla} \) during haustorial development

The QSM population is ideal for identifying regulatory components that reprogram the defense transcriptome of barley in response to inoculation with \( \textit{Bgh} \). Parental and QSM progeny lines were inoculated with \( \textit{Bgh} \) isolate 5874 and harvested at 16 and 32 HAI; time points that are associated with appressorial penetration and early formation of
haustoria, respectively (Caldo et al., 2004, Caldo et al., 2006, Moscou et al., 2011a).

Gene expression for each of the 166 experimental units was estimated using the 22K Barley1 GeneChip (Close et al., 2004) (BB96 in PLEXdb; GSE68963 in NCBI-GEO).

A simple linear regression model was fit to 75 data points with normalized expression level as the response and an indicator of marker genotype as the explanatory variable to identify eQTLs. Significance thresholds were determined by using q-values to control the false discovery rate (Benjamini and Hochberg, 1995). Table S6 lists all 22840 probe sets along with their most associated eQTL marker and its level of significance at 16 and 32 HAI. With the false discovery rate controlled at 0.001, 357 eQTL marker positions (bins) were classified as most significantly associated with at least one probe set (gene) at 16 HAI; five of these eQTL markers were of highest significance to at least one hundred probe sets each. For the same threshold, 371 positions were classified as the top association for at least one probe set at 32 HAI. Likewise, 6 of these 371 were found to be most associated with at least one hundred probe sets. In simulations performed with a multinominal distribution, the probability for an eQTL marker position to be randomly associated with 100 probe sets by chance is essentially 0 with eQTL marker associations controlled at q-value ≤ 0.001. Thus, for the purposes of this manuscript, only loci associated with greater than 100 probe sets were considered eQTL hotspots.

The five eQTL hotspots significant at 16 HAI, corresponding to penetration of epidermal cells by Bgh, map to linked positions on Chr 2HL Bins 63 and 65 – 68 (MILa region) and
associate with a total of 3,103 genes (Table S6). Likewise, the six hotspots significant at 32 HAI, corresponding to formation of Bgh haustorial feeding structures and delivery of secreted effectors into the host, localized to Chr 1HS Bins 3 – 8 (including Mla1) and associate with 5,070 genes. In this report, “2HL” will be the designation for Chr 2H Bins 63 and 65 – 68 and “1HS” refers to Chr 1H Bins 3 – 8. Figure 2 illustrates off-diagonal probe sets, indicative of trans eQTL, including those associated with 2HL at 16 HAI and 1HS at 32 HAI.

![Figure 2. Trans eQTL hotspots localize to chromosomes 2HL and 1HS. Of the 1,494 probe sets that are positioned on the QSM genetic map, (A) illustrates significant off-diagonal probe sets associated with Chr 2HL at 16 HAI with Bgh 5874 (q-value ≤ 0.001), and (B) demonstrates significant off-diagonal probe sets associated with 1HS at 32 HAI. These off-diagonal probe sets on 2HL and 1HS are indicative of trans eQTL hotspots.]

We then wished to examine any changes within each of the four genotypic progeny groups and identify the alleles at 2HL and 1HS eQTL that suppress or upregulate associated genes. To do this, we compared the gene expression for each progeny line against the median of the parents. Figure 3 illustrates the number of up- and down-
regulated genes in each QSM line ordered by genotype. In Figure 3B and 3C at 16 HAI, there is an over-representation of up-regulated genes in QSM lines that possess the functional MlLa gene (SM genotype), i.e., (Mla1, MlLa; purple) and (mla1, MlLa; blue), whereas there is a clear trend of down-regulated genes in QSM progeny lacking the MlLa gene (red and brown). Similarly, in Figure 3E and 3F at 32 HAI, there is an over-representation of up-regulated genes in QSM lines with the functional Mla gene (Q genotype), i.e., (Mla1, MIlA; purple) and (Mla1, mlLa; red), whereas there is a clear trend of down-regulated genes in QSM progeny lacking either R gene (brown). This indicates that the MILa and Mla1 alleles in the two eQTL regions, tend to be associated with upregulation of associated genes rather than suppression.
Figure 3. Number of genes up- and down-regulated in each QSM progeny for 16 and 32 HAI as compared to the gene-wise median for the Q and SM parent lines. At 16 HAI, (A) all genes, (B) genes associated with 2HL, (C) genes associated with 2H.67 (Mla) are plotted for each QSM progeny. At 32 HAI, (D) all genes, (E) genes associated with 1HS, (F) genes associated with 1H.05 (Mla) are represented. The purple, red, blue and brown lines refer to QSM progeny genotype (Mla, Mla), (Mla1, Mla), (Mla1, Mla) and (Mla1, Mla), respectively. Within each group, the barley lines are in numerical order (Table S2). Note that the graphs are plotted on different scales.
Transcript pattern analysis associates eQTL hotspots on chromosomes 2HL and 1HS with compatibility or incompatibility

Next, we integrated the current eQTL results with previous Barley1 GeneChip expression data to examine changes in transcript accumulation across developmental time. Accession BB4 (PLEXdb; GSE33396 in NCBI-GEO) facilitated comparison with independent incompatible and compatible interactions mediated by the Mla1 and Mla13 alleles, respectively, at six different time points (0, 8, 16, 20, 24 and 32 HAI) with Bgh isolate 5874 (AVRa1, AVRLa, avrLa3) (Caldo et al., 2004).

Of particular interest were two classes of transcript accumulation; both were highly similar among incompatible and compatible interactions up to 16 HAI, coinciding with germination of Bgh conidiospores and formation of appressoria, followed by significant divergence from 16 to 32 HAI, during development of the perihaustral membrane between fungal haustoria and host epidermal cells (Caldo et al., 2004). Computational pattern analysis was used to distinguish the two opposing scenarios: The first represents genes down regulated during compatible interactions after 16 HAI, as compared to corresponding time-points in incompatible interactions – this pattern was designated “PacMan” (Table 2, Figure 4A-C). In contrast, the second represents genes up regulated from 16 to 32 HAI during compatible interactions, as compared to the equivalent time-points in incompatible interactions – this pattern was designated “i (inverse) PacMan” (Table 2, Figure 4D-F). The first scenario may represent positive regulators of host immune signaling that are suppressed by the pathogen during
compatible interactions, whereas the second may represent a situation where the pathogen is inducing host factors for disease susceptibility. Of the 3,103 genes associated with the eQTL hotspot cluster on Chr 2HL, 321 and 258 exhibited PacMan and iPacMan expression patterns, respectively. Similarly, of the 5,070 genes associated with 1HS, 404 are associated with a PacMan pattern, whereas 606 displayed an iPacMan pattern (Table 2, Table S6).
Figure 4. Examples of genes exhibiting PacMan or iPacMan patterns of transcript accumulation. The red and black lines represent RMA-normalized expression for barley lines CI 16137 (Mla1) and CI 16155 (Mla13) infected with Bgh isolate 5874 (AVRa1, AVRa6, avr13), respectively. The following genes are associated with various eQTL at 16 HAI, and then are re-purposed to associate with 1H.05 (Mla1) at 32 HAI. (A) Contig3209_s_at (blue copper-binding protein homolog) from 2H.67 (Mla1). (B) HY07P02u_at (anthranilate synthase alpha 2 subunit) from non-MIaChr 2HL hotspot (2H.66). (C) Contig15099_s_at (pathogenesis-related protein 4) from non-2HL hotspot (3H.57). (D) Contig14413_at (auxilin-like protein) from 2H.67 (Mla1). (E) Contig19029_at (bacterial-induced peroxidase precursor) from non-MIa Chr 2HL hotspot (2H.66). (F) baak21d06_s_at (Unknown) from non-2HL hotspot (7H.03).
Table 2. Number of genes associated with each hotspot in the Chr 2HL and 1HS clusters and the number represented by PacMan and iPacMan expression patterns, respectively.

<table>
<thead>
<tr>
<th>Position (Chr.Bin)</th>
<th>Associations(^a)</th>
<th>Expression Pattern(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>PacMan</td>
</tr>
<tr>
<td>2H.63</td>
<td>217</td>
<td>17</td>
</tr>
<tr>
<td>2H.64(^c)</td>
<td>26</td>
<td>0</td>
</tr>
<tr>
<td>2H.65</td>
<td>261</td>
<td>33</td>
</tr>
<tr>
<td>2H.66</td>
<td>1,331</td>
<td>163</td>
</tr>
<tr>
<td>2H.67 (\textit{MlLa})</td>
<td>961</td>
<td>100</td>
</tr>
<tr>
<td>2H.68</td>
<td>333</td>
<td>8</td>
</tr>
<tr>
<td>2HL Total</td>
<td>3,103</td>
<td>321</td>
</tr>
<tr>
<td>1H.03</td>
<td>261</td>
<td>13</td>
</tr>
<tr>
<td>1H.04</td>
<td>276</td>
<td>14</td>
</tr>
<tr>
<td>1H.05 (\textit{Mla1})</td>
<td>3,296</td>
<td>341</td>
</tr>
<tr>
<td>1H.06</td>
<td>783</td>
<td>21</td>
</tr>
<tr>
<td>1H.07</td>
<td>114</td>
<td>7</td>
</tr>
<tr>
<td>1H.08</td>
<td>340</td>
<td>8</td>
</tr>
<tr>
<td>1HS Total</td>
<td>5,070</td>
<td>404</td>
</tr>
</tbody>
</table>

\(^a\)Associated genes are represented at 16 HAI for Chr 2H and 32 HAI for Chr 1H hotspots at a q-value ≤ 0.001. The annotations for these genes are presented in Table S6.

\(^b\)Expression pattern changes across developmental time in the comparison of incompatible (\textit{Mla1}-\textit{AVR}_{a1}) and compatible (\textit{Mla13}-\textit{avr}_{a13}) barley-\textit{Bgh} interactions (Accession BB4 at PLEXdb.org). Described further in “Pattern Analyses of Published Data Sets” under Materials and Methods.

\(^c\)Marker bin not included in analyses as the number of associations is below 100 but shown here for informational purposes.

The genotypes represented by PLEXdb accession BB4 lack \textit{MlLa}, but do possess \textit{Mla1}. Therefore, when inoculated with \textit{Bgh} 5874 (\textit{AVR}_{a1}, \textit{AVR}_{La}, \textit{avr}_{a13}), a comparison of differential gene expression can be made between QSM progeny groups (\textit{Mla1}, \textit{mlLa}; resistant) and (\textit{mla1}, \textit{mlLa}; susceptible) in BB96, and between CI 16151 (\textit{Mla1}, \textit{mlLa}; resistant) and CI 16155 (\textit{Mla13}, \textit{mlLa}; susceptible) from BB4. At 32 HAI, of the 1,712 genes that are differentially expressed between CI 16151 and CI 16155, 1,356 (~80%) are also differentially expressed between (\textit{Mla1}, \textit{mlLa}) and (\textit{mla1}, \textit{mlLa}) in BB96. Of
these 1,356 genes, 559 are upregulated and 797 are downregulated in the Mla1, mlLa QSM progeny group (Table S7).

**Temporal regulation of Immunity**

Up to now, a handful of genes (and their encoded proteins) have been functionally connected to NLR-mediated pathways (Shirasu, 2009, Jacob et al., 2013), but these interactions have not been associated with specific stages of pathogen development. Previous studies have shown that MLA1 interacts with SGT1 (suppressor of the G2 allele of skp1) (Bieri et al., 2004), which binds SKP1, a component of the SCF (Skp1-Cullin-F-box) ubiquitin ligase complex, and facilitates regulation of defense in both yeast and plants (Kitagawa et al., 1999, Azevedo et al., 2002). Along with RAR1 (required for Mla12 resistance 1) and HSP90 (Heat Shock Protein 90), SGT1 positively controls the steady state levels of resistance proteins recognizing viral, bacterial, oomycete or fungal pathogens (Azevedo et al., 2002, Takahashi et al., 2003, Schulze-Lefert, 2004, Shirasu, 2009). Our findings show that SGT1 is associated with 2H.67 (MlLa locus) at 16 HAI, whereas HSP90 is associated with 1H.05 (Mla1) at 32 HAI. RAR1, however, does not associate significantly with either 2HL or 1HS, or any other eQTL marker.

Formerly, HvWRKY10, HvWRKY19, and HvWRKY28 were shown to positively regulate Mla-triggered immunity to Bgh, however, current evidence suggests that these three nuclear-localized WRKY transcription factors do not directly bind MLA (Meng and Wise, 2012). Of these, we show that WRKY19 is regulated (either directly or indirectly) by 2H.67 (MlLa) at 16 HAI, whereas, WRKY10 is associated with 1H.05 (Mla1) at 32 HAI.
Additional WRKYs associated with 2HL at 16 HAI include WRKY20, WRKY33, WRKY34, WRKY41, WRKY46, and WRKY61. From this group, WRKY33, WRKY41 and WRKY46 change association from 2H.65, 2H.66 and 2H.67 (MiLa) at 16 HAI, respectively, to 1H.05 (Mla1) at 32 HAI (Table S6).

Lastly, the previously known defense related proteins, syntaxin (VAMP727), thaumatin and SOD1 display no significant associations at 16 HAI, but were associated with 1H.08, 1H.05 (Mla1) and 1H.06 at 32 HAI, respectively. BLN1, a negative regulator of basal defense (Meng et al., 2009, Xu et al., 2015), was associated with 2H.68 during penetration, but 1H.07 during haustorial growth, implying control by unlinked host loci during pathogen development.

To further extend the temporal analysis of the known NLR-associated proteins above, we also queried the 2HL and 1HS associations for encoded proteins annotated to membrane trafficking, including the secretory pathway, nuclear and vesicle transport, signaling G-proteins, and their known interactors (Stark et al., 2006, Paul et al., 2014, Rutter and Innes, 2017). This identified 356 distinctive probesets encoding several Soluble N-ethylmaleimide-sensitive factor Attachment Protein REceptors (SNAREs), Rab GTPases (part of the Ras superfamily of small GTPases), tethering factors, COat Protein complex (COPs) and Clathrin-Coated Vesicle proteins (CCVs). Of these 356 probesets, 80 were associated with 2HL at 16 HAI, 126 associated with 1HS at 32 HAI, and 38 were associated with both 2HL at 16 HAI and then 1HS at 32 HAI (Table 2, Table S6). Taken together, the current eQTL analysis identifies proteins coupled to
NLR-mediated signaling, but also provides new associations that appear to play important temporal roles during penetration, haustorial growth, or both.

**Alternating temporal control: Prioritizing immune regulation from 2HL (associated with penetration) to 1HS (associated with haustorial development)**

Plant transcriptomes undergo significant reprogramming in response to environmental stimuli, such as cold, salinity, drought, or pathogen stress. A marked example of this reprogramming is the transfer of association with Chr 2HL (Bins 63, 65 – 68) at 16 HAI, and then to Chr 1HS (Bins 3 – 8) at 32 HAI, where a total of 1,470 genes come under alternate regulation post penetration (Figure 5). Of these 1,470 genes, 260 exhibited *PacMan* patterns of transcript accumulation, whereas, 192 showed patterns similar to *iPacMan*. 
Figure 5. Illustration of 16 and 32 HAI eQTL hotspots projected onto the QSM genetic map. Chr 1HS (Mla1 region, red box) and 2HL (MiLa region, blue box) hotspots projected onto the barley QSM genetic map. The bold red and blue lines in each box represent the bins associated with Mla1 and MiLa respectively. Inset on the bottom right lists the transfer of association between hotspots.

Alternate transcriptional control suggests that post-penetration regulators at 1HS supplant those at 2HL. There are four modes of regulation between 2HL at 16 HAI (penetration) and 1HS at 32 HAI (haustoria) as shown in Table 3 and Table S8. We designated these SM$_{2HL,16}$ – Q$_{1HS,32}$, SM$_{2HL,16}$ – SM$_{1HS,32}$, Q$_{2HL,16}$ – Q$_{1HS,32}$, and Q$_{2HL,16}$ – SM$_{1HS,32}$, represented by the quadrants delineated at point (0,0) in Figure 6. Genes depicted in the upper-left-hand quadrant (SM$_{2HL,16}$ – Q$_{1HS,32}$) display higher expression
in recombinant lines where the SM allele is present on 2HL at 16 HAI, and in lines possessing the Q allele on 1HS at 32 HAI. This scenario also represents the presence of an active \textit{MILa} during penetration, in addition to a functioning \textit{Mla1} during haustorial development. Thirty-one percent of the genes in this quadrant are associated with \textit{PacMan} patterns of expression (vs. 0\% \textit{iPacMan}), and include, among other annotations, BLN1, RAB GTPase, and Syntaxin.
Figure 6. Summary of allelic effects between Chr 2HL and *Mla1* (1H.05) show modes of alternate regulation. (A) Representation of the allelic effects between 2HL at 16 HAI and 1HS at 32 HAI. The allelic effects between 1H.05 (*Mla1*) and (B) 2H.63, (C) 2H.65, (D) 2H.66, (E) 2H.67 (*MlLa*), (F) 2H.68, are shown in respective panels. The quadrants delineated by (0,0) in each panel illustrate the four modes of regulatory transfer between hotspots and are labeled along with their number of associated genes.
Table 3. Modes of alternate regulation between 2HL at penetration and 1HS in haustoria and associated expression patterns.

<table>
<thead>
<tr>
<th>Modes of alternate regulation</th>
<th>Number of Genes</th>
<th>Expression Pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>PacMan</td>
</tr>
<tr>
<td>SM2HL,16 – Q1HS,32</td>
<td>836</td>
<td>260</td>
</tr>
<tr>
<td>SM2HL,16 – SM1HS,32</td>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td>Q2HL,16 – Q1HS,32</td>
<td>38</td>
<td>0</td>
</tr>
<tr>
<td>Q2HL,16 – SM1HS,32</td>
<td>584</td>
<td>0</td>
</tr>
</tbody>
</table>

Genes depicted in the lower-left-hand quadrant (SM2HL,16 – SM1HS,32) display higher expression at 16 HAI in recombinant lines where the SM allele is present on 2HL, and at 32 HAI in lines possessing the SM allele on 1HS. This scenario is indicative of the presence of a functional *MlLa*, but is accompanied by the absence of *Mla1*. Genes depicted in the upper-right-hand quadrant (Q2HL,16 – Q1HS,32) display higher expression at 16 HAI in recombinant lines where the Q allele is present on 2HL, and at 32 HAI in lines possessing the Q allele on 1HS. This scenario is indicative of the absence of a functional *MlLa*, but the presence of a functional *Mla1*. Genes in these two quadrants do not have a distinct expression pattern or associated pathway.

Lastly, genes depicted in the lower-right-hand quadrant (Q2HL,16 – SM1HS,32) display higher expression at 16 HAI in recombinant lines where the Q allele is present on 2HL, and at 32 HAI in lines possessing the SM allele on 1HS. Similar to the second quadrant, this scenario is indicative of the absence of a functional *MlLa*, but also the absence of *Mla1*. Thirty-two percent of these genes are associated with transcript accumulation designated as *iPacMan* (vs. 0% *PacMan*). Among other annotations, these genes include nuclear transport factor 2 (NTF2), aquaporin PIP and a calcium-binding EF hand.
family protein. Of the 1,470 genes that show alternate regulation between 2HL at 16 HAI and 1HS at 32 HAI, 1,420 switch the allele responsible for higher expression (SM$_{2HL,16}$ to Q$_{1HS,32}$ or Q$_{2HL,16}$ to SM$_{1HS,32}$) when association is reallocated from 2HL to 1HS.

2.4 Discussion

We used eQTL analysis of the QSM doubled haploid barley population after infection with the fungal Ascomycete pathogen, *B. graminis* f. sp. *hordei*, to interrogate temporal control of plant immunity. Near-synchronous penetration by thousands of *Bgh* conidiospores delivers an ideal stimulus to induce dynamic reprogramming of the leaf transcriptome to response to pathogen attack. Two major clusters of *trans* eQTL hotspots on Chr 2HL and 1HS were identified along with immune response genes associated with them, corresponding to appressorial penetration and development of haustoria, respectively.

Dynamic regulation of immunity

*Trans*-regulatory regions typically influence transcript levels via a protein intermediate and have the potential to interact with many proteins, depending on the milieu present in the cell in a given condition. A single variant may be able to activate transcription in one condition and repress it in another, resulting in a change in direction of the effect, as observed when the two carbon sources, glucose and ethanol, are compared during yeast fermentation and aerobic respiration, respectively (Smith and Kruglyak, 2008). In the current eQTL study, 1,470 genes associated with 2HL during penetration were
repurposed by 1HS at later stages of infection, but more specifically, 299 genes re-
allocated control from the \textit{MILa} locus (2H.67) to 1H.05 (\textit{Mla1}). Finally, an additional 138
genes were identified that change association from other non-Chr 2HL positions at 16
HAI to 1H.05 (\textit{Mla1}) at 32 HAI. Of the total 1,608 (1,470 + 138) genes that undergo
alternate regulation, 271 exhibited \textit{PacMan} patterns of expression, which is indicative of
host immune signaling that is typically suppressed by pathogen effectors (Caldo \textit{et al.},
2004). Another 197 genes displayed a contrasting pattern of transcript accumulation
designated \textit{iPacMan}, which we postulate represent a scenario where the pathogen
induces defense, possibly by recognition of PAMPS by pattern recognition receptors,
but then coopts these genes for its own purposes (Figure 4). While we focused on
transcript accumulation signified by \textit{PacMan} and \textit{iPacMan} patterns, undoubtedly there
are additional changes influenced post-transcriptionally or post-translationally, for
example by small RNAs (Liu \textit{et al.}, 2014, Xu \textit{et al.}, 2014, Fei \textit{et al.}, 2016, Zhang \textit{et al.},
2016). Nonetheless, the population-based eQTL analysis reported here supports the
conclusion that these genes are part of an immune regulon that can be activated or
repressed by disparate resistance loci in response to multiple infection stages.

Alternate control of gene expression suggests that one or more regulators on 1HS
 supersede that exerted by eQTLs on 2HL. There are four modes of alternate
transcriptional control, specifically \textit{SM}_{2HL,16} – \textit{Q}_{1HS,32}, \textit{SM}_{2HL,16} – \textit{SM}_{1HS,32}, \textit{Q}_{2HL,16} – \textit{Q}_{1HS,32},
and \textit{Q}_{2HL,16} – \textit{SM}_{1HS,32} (Table 3). For example, \textit{SM}_{2HL,16} denotes that at 16 HAI
recombinant lines with the SM allele at 2HL (including \textit{MILa} at 2H.67) had higher gene
expression compared to the lines with the Q allele at 2HL. Similarly, \textit{Q}_{1HS,32} implies that
at 32 HAI the lines with the Q allele at 1HS (including Mla at 1H.05) had higher gene expression compared to the lines with the SM allele at 1HS. As illustrated in Figure 6A, allelic effects were interchanged (Q_{2HL,16} to SM_{1HS,32} or SM_{2HL,16} to Q_{1HS,32}) for 1,420 of the 1,470 genes that re-allocated control from 2HL at penetration to 1HS during haustorial development.

One explanation for these correlated phenomena may be multiple linked polymorphisms. *Trans*-regulatory regions are much larger targets for variation than *cis*-regulatory regions (Brem *et al.*, 2002, Smith and Kruglyak, 2008). Multiple mutations could accumulate at loci and, depending on the condition, could compensate differentially. The mean phenotype would be stable over conditions, yet the direction of the effect within a condition could vary across the population, as observed in the phenotypic range for lines lacking a functional Mla1. Another example, which is observed across taxa rather than the population, is suggested by the hotspots on Chr 1HS, including those influenced by Mla1 (1H.05). In this case, the emerging narrative is consistent with multiple polymorphisms that influence disease resistance to diverse pathogens (Seeholzer *et al.*, 2010, Periyannan *et al.*, 2013, Mago *et al.*, 2015, Cesari *et al.*, 2016).

**Membrane trafficking regulated by immune-associated loci**

To further elucidate the underlying mechanisms behind these phenomena, we queried the 2HL and 1HS associations annotated to secretory pathway, vesicle transport and signaling G-proteins. This identified 356 probesets including several Soluble N-
ethylmaleimide-sensitive factor Attachment Protein REceptors (SNAREs), Rab GTPases (part of the Ras superfamily of small GTPases), tethering factors, COat Protein complex (COPs) and Clathrin-Coated Vesicle proteins (CCVs) (Table S5). Rab GTPases regulate vesicle formation, vesicle movement, and membrane fusion (Stenmark and Olkkonen, 2001), while SNARE proteins mediate fusion of vesicles with their target membrane bound compartments (Burri and Lithgow, 2004). These processes make up the route through which cell surface proteins are trafficked from the Golgi to the plasma membrane and are recycled. In plants, membrane vesicles targeted to the cell division plane fuse with one another to form the partitioning membrane, progressing from the center to the periphery of the cell. This is seen in the two types of SNARE complexes formed by KNOLLE that are jointly needed to mediate membrane fusion in cytokinesis. One of these complexes, a KNOLLE – SNAP33 – VAMP721/722 trimer (El Kasmi et al., 2013) (Figure S1), is localized on the plasma membrane and drives membrane fusion of vesicles destined for exocytosis (Collins et al., 2003). PEN1 is engaged in a similar SNARE complex mediating extracellular immunity via exocytosis also with SNAP33 and VAMP721/722 (Kwon et al., 2008) (Figure S1). Intriguingly, the alternate regulation exhibited by VAMP721/722, first by the MlLa locus at penetration, and then by Mla1 during haustorial growth could provide clues as to how its secretion apparatus is co-opted for immune responses to pathogens.

Tethering factors have emerged as key regulators of membrane traffic and organellar architecture (Dubuke and Munson, 2016). The restricted subcellular localization of tethering factors and their ability to interact with RABs and SNAREs suggests that
tethers participate in determining the specificity of membrane fusion. An accepted model of tether function considers them molecular "bridges" that link opposing membranes before SNARE pairing. Tethers are also implicated to function as integration switches that simultaneously transmit information to coordinate distinct processes required for membrane traffic. Additionally, CCVs selectively sort cargo at the cell membrane, trans-Golgi network, and endosomal compartments, whereas COPs transport proteins between the Golgi complex and the rough endoplasmic reticulum. Adaptor protein complexes are vesicle coat components and appear to be involved in cargo selection and vesicle formation. AP-2 is involved in clathrin-dependent endocytosis in which cargo proteins are incorporated into vesicles surrounded by clathrin (CCVs) that are destined for fusion with the early endosome (Happel et al., 2004). The μ-adaptin of AP-2 (AP2M) localizes to the plasma membrane in plants for clathrin coated vesicle formation, similar to AP-2 in animals. AP2M is involved in ETI mediated by plasma membrane–localized disease resistance proteins, possibly by mediating endocytosis of immune receptor components from the plasma membrane (Hatsugai et al., 2016) (Figure S2). However, it is not the coat proteins that determine the target of a transport vesicle but the SNAREs (Sanderfoot and Raikhel, 1999). Together, the coat proteins and SNAREs coordinate the trafficking of cargo between various organelles of the endomembrane system.

Membrane trafficking pathways are involved in immune receptor activation, signal transduction, and execution of multiple defense responses including programmed cell death (Teh and Hofius, 2014). Although mechanisms of pathogenic modulation of
endocytic, secretory, and vacuolar trafficking and their roles in plant–microbe interactions need additional investigation, these pathways appear to function in the rapid responses to environmental stimuli (Inada and Ueda, 2014).

**Shared components in immunity**

In previous studies, we localized a *trans* eQTL hotspot to 2H.16 that associates with an enhancer for adult plant resistance to the obligate Basidiomycete fungus, *Puccinia graminis tritici* race TTKSK, more commonly known as Ug99 stem rust (Moscou *et al.*, 2011b). This hotspot, unlinked to the *Bgh* responsive eQTL described here, overtakes regulatory control of 368 genes from several unlinked loci when plants are challenged with Ug99 (Moscou *et al.*, 2011b). Of these 368 genes, 97 are associated with the current *Bgh*-induced 2HL at 16 HAI, 139 with 1HS at 32 HAI and 57 undergo alternate regulation between 2HL and 1HS (Table S6), indicating substantial conservation of defense components to these two obligate biotrophic fungi.

The *Bgh* susceptible, *Arabidopsis pen2 pad4 sag101* triple mutant transformed with *Mla1* recognizes *Mla1*-incompatible isolates of *Bgh* (Maekawa *et al.*, 2012). In order to compare MLA1-dependent signaling in *Arabidopsis* and barley, the top 100 differentially expressed genes from (Maekawa *et al.*, 2012) were mapped onto Barley1 probesets; 14 of these returned high-confidence alignments (Table S6). Seven of these 14 were associated with 2HL at 16 HAI, six with 1HS at 32 HAI, and four associated with alternate regulation, first by 2HL (including *MILa*) at penetration, and then 1HS (including *Mla1*) during development of haustoria.
Only one MLA1-dependent protein from *Arabidopsis* also aligned with a probeset associated with both *MiLa* and *Mla1* in barley. This was annotated as a putative serine/threonine-protein kinase (PBL2). PBL2 contributes to PTI signaling and defense responses downstream of LRR receptor-like serine/threonine-protein kinase (FLS2) (Zhang et al., 2010). Ligand-activated FLS2 is internalized in a clathrin-dependent manner that converges at ARA7 (Rab GTPase Homolog F2B) endosomes facilitating the responses required for full plant immunity (Mbengue et al., 2016).

**Future prospects**

Plant immune systems exemplify multi-tiered signaling networks comprised of biological molecules interacting in space and time. For example, we show that of the HSP90-RAR1-SGT1 chaperone complex, SGT1 is associated with 2H.67 (*MiLa* locus) at 16 HAI, whereas HSP90 is associated with 1H.05 (*Mla1*) at 32 HAI. We also show that the transcript encoding BLN1, a negative regulator of basal defense, is associated with 2H.68 at penetration, but 1H.07 during haustorial development, inferring specific control by unlinked chromosomal regions in response to different phases of pathogen infection. The regulatory mechanisms of the many examples in this report need further study, and questions also remain whether there is an epigenetic component to these phenomena. It would be intriguing to quantify methylation sites across the QSM population at different infection stages and integrate that with the current eQTL data.
One general implication of our results is that many genetic effects on most traits are likely to be detected without testing for gene–environment interactions, provided that the relevant environmental factors are known and controlled either experimentally or statistically. However, analyses that ignore temporal gene by environment interactions introduce strong biases regarding the types of loci that are detected. Our focus on transcript levels as quantitative traits allowed us to study a very large number of traits simultaneously and to delineate general patterns, as well as to provide detailed examples of loci that show gene by environment interactions, specifically infection of a plant host by an obligate biotrophic pathogen. The quantitative details would undoubtedly differ if different species, environments, and phenotypes were studied. However, some environmental differences (for example, exposure to pathogens) can have a dramatic effect on health. Our detailed studies in a model grain crop provide examples of the types of effects that may be expected in host-parasite interactions, and thereby inform practical study design.

2.5 Acknowledgements

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2.7 Author Contributions

Conceived and designed the experiments: RPW. Performed the experiments: GF, RPW, AC. Analyzed the data: PS, RX, DN, RPW. Contributed reagents/materials/analysis tools: PS, RX, GF, AC. Wrote and edited the paper: PS, RX, GF, AC, DN, RPW.
CHAPTER 3. MEMBRANE TRAFFICKING IN R-GENE-MEDIATED DEFENSE AGAINST BARLEY POWDERY MILDEW

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3.1 Abstract

*Blumeria graminis* f. sp. *hordei* (*Bgh*) is the causal agent of powdery mildew on barley. *Bgh* secretes over 700 proteins that may function to modify host processes for colonization. Differential expression analysis of *Bgh* genes encoding secreted proteins in an isogenic panel of host immune signaling mutants, including a novel mutation *Rar3* (*Required for Mla6 Resistance3*), identified fungal membrane trafficking genes. Infection phenotyping and kinetics show that immune compromised mutants have similar fungal growth, thereby, attributing any differences observed in expression to mutation rather than development. Thus, we hypothesize that powdery mildew genes may be targeting the host membrane trafficking pathway to both internalize effector proteins and suppress plant defense

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signal relay. Next, to address the modulation of host membrane trafficking genes, differential expression analysis identified ADP Ribosylation Factor 1 (ARF1) as well as its activator Brefeldin A-inhibited guanine nucleotide-exchange protein 5 (BIG5) during host penetration by Bgh. ARF1 is a GTP-binding protein, involved in the recruitment of E3 ubiquitin-protein ligase. Further, ARF1 is a candidate interactor for MLA6, and Arf1 silenced plants displayed increased cell death. However, R gene Mla6 and Rar3 are required for hypersensitive response, and the loss of either gene leads to loss of cell death upon infection. Taken together, these results corroborate the pivotal role membrane trafficking plays in R gene-mediated defense against powdery mildew. Specifically, it shows that ARF1 is a negative regulator of cell death and possible interactor of MLA6, whereas RAR3 is required for MLA6-mediated hypersensitive response.

3.2 Introduction

The powdery mildew fungus, *Blumeria graminis*, is an obligate biotrophic pathogen of cereals that has significant impact on food security (Dean et al., 2012). *B. graminis* f. sp. *hordei* (*Bgh*) is the causal agent of powdery mildew on barley (*Hordeum vulgare* L.). Its infection starts when a spore lands on a leaf, germinates, forms an appressorium, and penetrates the host epidermal cell. The penetrating hypha produces a haustorium, a specialized feeding structure for nutrient uptake and secretion of effector proteins (Dodds and Rathjen, 2010).
The 130-Mb Bgh genome (Spanu et al., 2010) encodes 547 candidate effector proteins, designated BECs (Blumeria Effector Candidates) (Bindschedler et al., 2009, Bindschedler et al., 2011, Pliego et al., 2013) or CSEPs (Candidate Secreted Effector Proteins) (Pedersen et al., 2012, Kusch et al., 2014). These are predicted to encode virulence factors that are released into host tissue and/or cells to promote nutrient acquisition and colonization (Selin et al., 2016). To date, ~30 Bgh effector candidates have been probed for their role in fungal pathogenesis, often by Host Induced Gene Silencing (HIGS) or yeast-two-hybrid protein-protein interaction screens (Zhang et al., 2012, Pliego et al., 2013, Schmidt et al., 2014, Aguilar et al., 2015, Ahmed et al., 2015, Amselem et al., 2015, Whigham et al., 2015, Ahmed et al., 2016). Effectors usually either act at the host cell surface (Stergiopoulos and de Wit, 2009) or are secreted into the plant cells to act intracellularly (Ellis and Dodds, 2011).

In response to pathogen attack, plants have intricate systems for the detection of microbial invaders (Boller and Felix 2009). Plant defense is comprised of at least two interdependent components, commonly referred to as pattern triggered immunity (PTI) (also known as basal defense) and effector trigger immunity (ETI). The former is activated upon recognition of highly conserved molecular structures, known as pathogen associated molecular patterns (PAMPs), by surface-localized plant pattern recognition receptors (PRRs) (Jones and Dangl, 2006, Cook et al., 2015). Alternatively, ETI is activated by recognition of pathogen effectors by intracellular or membrane receptors (Jones and Dangl, 2006, de Wit, 2007, Stuart et al., 2013,
Cook *et al.*, 2015). In plants, these receptors are encoded by resistance (*R*) genes, and induce race-specific resistance (Jones and Dangl, 2006, de Wit, 2007, Cook *et al.*, 2015). Adapted pathogens utilize effectors to suppress PTI and ETI, and otherwise manipulate colonized cells to evade host defenses (Jones and Dangl, 2006, de Wit, 2007, Cook *et al.*, 2015).


Plant cells are reliant on membrane trafficking to respond to changes in their environments, such as the absence of nutrients, or presence of predators and pathogens (Wang and Dong, 2011). Membrane trafficking function to deliver newly synthesized proteins from the endoplasmic reticulum to the plasma membrane and
the vacuole, and to internalize extracellular components or plasma membrane-associated proteins for recycling or degradative regulation (Inada and Ueda, 2014). These trafficking pathways play pivotal roles in the rapid responses to environmental stimuli and provide an essential interface between plant hosts and their associated microbial partners. Many plant membrane trafficking factors have been identified for their roles in plant–microbe interactions and as possible targets of microbial effectors. In addition to possessing conserved mechanisms with eukaryotes, plants have also evolved plant-specific membrane trafficking mechanisms (Fujimoto and Ueda, 2012).

We sought to address the temporal regulation of membrane trafficking associated gene expression in barley-powdery mildew interactions. We created an isogenic panel of immune signaling mutants to address three main questions: (i) which *Blumeria* secreted proteins are differentially regulated in response to different compromised genotypes, (ii) which barley membrane trafficking genes are altered in response to pathogen attack, and (iii) how are these genes interacting across genotypes and infection stages.

Expression analysis identified a conserved core of *Bgh* effectors associated with successful infection, as well as those uniquely differentially expressed (DE) in individual immune–compromised mutants. Taken together, these provide insight into how the pathogen may target distinct membrane trafficking pathways to both internalize pathogen effector proteins and suppress plant defense signaling.
Unlike the pathogen, the host exhibited two waves of differential expression, at penetration and during haustorial growth, with partial overlap between them. The larger peak during penetration has a large conserved core among susceptible mutants, including Heat Shock Cognate 70kDa protein (HSC70) and ADP-Ribosylation Factor 1 (ARF1). ARF1 was also identified as a candidate interactor of MLA6 via a yeast-two-hybrid screen. Moreover, Arf1 silenced plants displayed increased cell death, indicating it is being co-opted by *Blumeria* or negatively regulating defense.

### 3.3 Results

**Development of loss-of-function *Bgh* immune signaling mutants**

We developed an isogenic panel of fast-neutron mutants derived from CI 16151, which contains the NLR-type *R* gene, *Mla6* (Moseman, 1972), to interrogate immune signaling in the interaction between barley and the obligate biotrophic fungus, *Bgh*. These mutants are morphologically identical throughout development and enabled the quantitative comparison of PTI and ETI in the host, as well as pathogenesis by the fungus, at the whole plant and molecular level.

**Susceptible genotypes have similar fungal growth**

Figure 1 illustrates the infection phenotypes of CI 16151 and its derived mutants seven days after infection with *Bgh* isolate 5874. CI 16151 (wildtype progenitor) contains the functional *Mla6 R* gene that recognizes the *Bgh AVR*$_{a6}$ effector and is
resistant. The *mla6-m18982* mutant lacks the functional *R* gene, thus, leading to full susceptibility. *Rar3* is a novel locus required for *Mla6* function, including *R*-gene mediated generation of H$_2$O$_2$ and the hypersensitive response (HR). The *rar3-m11526* mutant lacks *Rar3*, and thus exhibits a susceptible phenotype. *Bln1*, a negative regulator of basal defense, is highly induced by attack from *Bgh* and functions in an *R*-gene independent manner (Meng et al., 2009, Xu et al., 2015). The *bln1-m19089* mutant exhibits enhanced basal defense (Meng et al., 2009, Xu et al., 2015), however since *Mla6* is also present, exhibits a resistant phenotype. The (*mla6+bln1*)-*m19028* double mutant is susceptible with accompanying tip wilt, depending on the inoculum density and environmental conditions. Based on the 7-day IT data, the various susceptible mutants do not display significant differences in *Bgh* fungal growth.

<table>
<thead>
<tr>
<th><em>Bgh</em> Isolate</th>
<th>CI 16151 Derived Barley Genotype</th>
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<tr>
<td></td>
<td>Mla6</td>
<td>mla6-m18982</td>
<td>rar3-m11526</td>
<td>bln1-m19089</td>
<td>Double Mutant</td>
</tr>
<tr>
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<td>Mla6</td>
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<td>Mla6</td>
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**Figure 1.** Barley line CI 16151 and fast-neutron-derived mutants infected with *Bgh* isolate 5874. Plants were inoculated with *Bgh* isolate 5874 and scored for infection type (IT) seven days later. The CI 16151 wild type and *bln1-m19089* mutant exhibit an infection type (IT) of 0 (resistant), whereas the three susceptible mutants, *mla6-m18982*, *rar3-m11526* and the double mutant (*mla6 + bln1*)-*m19028* displayed an IT of 4. An IT equal to 0 signifies no visible pathogen development on the leaf surface whereas an IT equal to 4 signifies moderate to high sporulation.
Next, we assayed *Bgh* early infection kinetics to quantitatively determine the temporal nature of compatibility or incompatibility specified by each host-pathogen interaction. The percentage of elongating secondary hyphae (ESH) was used as a measure of successful infection and functional haustoria (Ellingboe, 1972, Wise and Ellingboe, 1983). ESH were quantified for three replications of each genotype at six time points – 16, 20, 24, 28, 32 and 48 hours after inoculation (HAI). A mixed model was fit for pairwise comparison between treatments with ESH as the response (Table S1). As shown in Figure 2, the resistant and susceptible genotypes begin to diverge after 24 HAI. Intriguingly, there were no significant differences found at any given time point between immune compromised genotypes. Coomasie blue staining of spore structures during the time course, however, identified subtle differences between the resistant genotypes. *Blumeria* spores inoculated onto the *bln1* mutant developed a greater number of secondary pegs at penetration as compared to CI 16151.
Figure 2. Percentage elongating secondary hyphae of *Bgh* isolate 5874 measured at six time points on CI 16151 and its derived mutant genotypes. (A) The x-axis represents the time points at which measurements were taken and the y-axis represents the percentage elongating secondary hyphae. The percentage elongating secondary hyphae was calculated as 100 x (Sum of three hyphal indices / Total). Total is sum of spore, appressorium and the three hyphal indices. The dashed and solid lines represent the susceptible and resistant genotypes, respectively. (B) Microscopy images of the four time points (16, 24, 32 and 48 HAI) for each genotype.
**Blumeria** exploits unique paths to infection in immune-compromised hosts

To further ascertain how *Bgh* exploits immune compromised pathways, we performed differential expression analysis on the transcriptome of *Bgh* 5874 after infection of CI 16151 and its derived isogenic mutants (Figure 1). Sequence reads were aligned to the *Bgh* isolate DH14 genome (Spanu *et al.*, 2010) and read counts were measured using RSEM (Li and Dewey, 2011). Differential expression analysis was performed using R/BioConductor (Gentleman *et al.*, 2004, R Core Team, 2016) package DESeq2 (Love *et al.*, 2014) within a given time point with genotype and replication as explanatory variables. The six time points can be grouped into four main infection stages, corresponding to early response (0 HAI), penetration of barley epidermal cells by *Bgh* appressoria (16 – 20 HAI), a transition phase (24 HAI), and development of haustoria (32 – 48 HAI). Results were adjusted for multiple testing with the false discovery rate controlled at 0.05.

To focus on *Bgh* proteins most likely to interact with host factors, we selected all 797 genes that encode secreted proteins, including the entire *Bgh* effector repertoire, which represent a subset of the total 6,252 genes that are expressed. Of these 797 genes, 731 were expressed at a discernible level in at least one of the time points, and follow a similar trend as the 6,252 total. Table 1 displays a summary of the differentially expressed genes for each infection stage in susceptible mutants as compared to the CI 16151 progenitor and Table S2 details the results for each secreted gene. The similarity in *Bgh* infection kinetics among susceptible mutants
implies that variance in expression may be attributed to the \textit{mla6}, \textit{mla6+bln1}, or \textit{rar3} mutations and not to differences in fungal development.

\textbf{Table 1.} \textit{Bgh} transcript accumulation encoding candidate effectors and putative secreted proteins grouped by developmental stages$^a$.

<table>
<thead>
<tr>
<th>Progenitor Genotype</th>
<th>Mutant Genotypes</th>
<th>Differential Expression $^b$</th>
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<tr>
<td></td>
<td></td>
<td>0 HAI Early Response</td>
</tr>
<tr>
<td>CI 16151</td>
<td>\textit{mla6-m18982}</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>\textit{rar3-m11526}</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>\textit{bln1-m19089}</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>(\textit{mla6+bln1}-m19028)</td>
<td>40</td>
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</table>

$^a$The results are based on 731 genes encoding candidate effectors and putative secreted proteins.

$^b$Differential expression results were controlled at a false discovery rate $\leq 0.05$ and an absolute log$_2$ fold change $\geq 1$.

\textbf{Figure 3.} Number of \textit{Blumeria} differentially expressed genes between mutants and wild type progenitor at different infection stages. The number of DE \textit{Bgh} secreted and effector genes (out of 731 detected), as (A) upregulated and (B) downregulated among CI 16151 progenitor and derived isogenic mutants during early response (0 HAI), penetration (16 or 24 HAI), transition (24 HAI), and haustorial development (32 or 48 HAI). Venn diagram comparing (C) upregulated and (D) downregulated genes between susceptible mutants when compared to CI 16151 during haustorial development. Differential expression results were controlled at a false discovery rate $\leq 0.05$ and an absolute log$_2$ fold change $\geq 1$. The \textit{mla6-m18982}, \textit{rar3-m11526}, \textit{bln1-m19089} and double (\textit{mla6+bln1}-m19028) mutants as compared to CI 16151 are represented in red, green, brown and blue, respectively.
Secretory pathway genes are associated with penetration of host cells in \textit{rar3} and \textit{(mla6+bln1)} mutants.

During haustorial development, \textit{Blumeria} genes which act as virulence factors are differentially expressed in all susceptible mutants, as expected. A large overlap among \textit{Bgh} DE genes on susceptible mutants as compared to progenitor suggests a common core set required for successful infection. However, each mutant also has genes exclusively associated with it, signifying back-and-forth communication that may influence the conserved core. It is these uniquely DE genes that provide insight into how the pathogen adapts to different immune-compromised environments.

Figure 3 illustrates an increase in the number of DE genes as the infection progresses. For example, \textit{Bgh} genes that perturb protein synthesis and cell wall structure are significant on the \textit{mla6} mutant. These genes could disrupt essential cell functions and lower physical barriers thereby creating a compatible environment for \textit{Bgh} effectors identified in the conserved set. In the \textit{rar3} and \textit{(mla6+bln1)} mutants, we identify several important secretory pathway genes. This could indicate that the uniquely DE set is targeting the protein transport pathway to internalize the effectors in the core set. Also, these genes could simultaneously be suppressing response to pathogen presence by the secretory pathway.

A conserved core of 353 \textit{Bgh} genes is differentially expressed in all susceptible mutants when compared to the CI 16151 progenitor during development of haustoria (32 – 48 HAI) (Figure 3). These 353 genes include five of the eight CSEPs (Aguilar
et al., 2015) and six of the eight BECs required for early stages of infection (Pliego et al., 2013). Of these 353 genes, 225 are upregulated in all three susceptible mutants and similarly, 127 are downregulated (Figure 3). Genes that are upregulated would be expected to be required for $Bgh$ infection, two such genes are BEC1011 and BEC1054, bona fide effectors that function within the plant cell (Pliego et al., 2013). Another upregulated gene is BEC1019, which significantly reduces fungal colonization when silenced, thereby demonstrating a central role in virulence (Whigham et al., 2015). Significantly, an EKA-like protein, a family related to avirulence genes is downregulated in susceptible mutants (Amselem et al., 2015).

In addition to the conserved core of 353, there are also genes uniquely associated with each mutant that may impact this set. For example, $mla6$ deletion mutants without the functional R protein would lack AVR$_{a6}$ recognition, leading to an ineffective ETI. Therefore, we postulate that the nine $Bgh$ genes DE on $mla6$ plants as compared to the CI 16151 progenitor are targeting NLR mediated defense pathways. Of these nine genes, four are upregulated including three ribonuclease/ribotoxin (RNase) domain containing CSEPs (Figure 3). These RNase-like proteins associated with haustoria are called RALPH effectors, which include many of the cereal powdery mildew AVRs (Spanu, 2017). RALPHs represent about a quarter of the CSEPs and are expressed at very high levels in the infected plant (Pedersen et al., 2012). It has been suggested that host R proteins have evolved to target RALPHs because they are so abundant or because they exert key functions during fungal pathogenesis (Spanu, 2017). Five additional genes are downregulated
including a protein belonging to the GPI mannosyltransferase II (MT II) co-activator (PGA1) family. In yeast, PGA1 encodes an ER-localized transmembrane protein. Yeast pga1 mutants cause accumulation of Gas1 (1,3-beta-glucanosyltransferase), required for proper cell wall assembly and morphogenesis. A downregulated PGA1 could disrupt cell wall structure, thereby reducing the physical barrier to penetration and haustorial formation. Similarly, upregulated RNase domain containing proteins could be inhibiting translation of barley defense genes. Thus, we predict these differentially expressed Bgh genes could be inhibiting the host from mounting an effective immune response.

Similarly, Rar3 is required for Mla6 function, thus, Bgh genes that are uniquely expressed in rar3 plants, as opposed to mla6 or mla6+bln1, may be expected to modulate Rar3-specific functions in NLR signaling. Fourteen genes are differentially expressed as compared to Bgh on CI 16151; nine are upregulated and five are downregulated. Of the nine upregulated genes, three are RNase domain containing CSEPs, two membrane trafficking proteins and two CSEPs involved in cell adhesion. This includes an orthologue of the small secretory pathway protein, kish (KSH1), from Saccharomyces cerevisiae and localizes to the ER and Golgi in Drosophila (Wendler et al., 2010). Another protein has the AP complex mu/sigma subunit domain, and may also localize to the Golgi apparatus. This Adaptor Protein complex domain represents the small sigma and mu subunits of adaptins from AP clathrin adaptor complexes, and the zeta and delta subunits of coatamer (COP) adaptors (Wegmann et al., 2004, Ma et al., 2009). AP complexes connect cargo proteins and
lipids to clathrin at vesicle budding sites, as well as binding accessory proteins that regulate coat assembly and disassembly (Boehm and Bonifacino, 2001). The sigma subunit plays a role in protein sorting in the late-Golgi/trans-Golgi network (TGN) and/or endosomes (Phan et al., 1994). While clathrin mediates endocytic protein transport from ER to Golgi, coatomers (COPI, COPII) reversibly associate with Golgi (non-clathrin-coated) vesicles to mediate protein transport and for budding from Golgi membranes (McMahon and Mills, 2004, Bethune et al., 2006). Upregulation of membrane trafficking proteins indicate a possible role in Blumeria secreted proteins’ transport (Palmer, 2010). Furthermore, Bgh could co-opt this pathway to suppress host defense response to pathogen signals.

Lastly, the mla6+bln1 double mutant is compromised for both ETI and PTI, thus, Bgh genes that are uniquely expressed in these plants, may play a role in modulating both phases of immunity. Thirteen genes were found uniquely DE on double mutant as compared to Bgh on CI 16151. Six genes are upregulated, including a protein from the peptidase S41 family and another protein with the AP complex, mu/sigma subunit domain. The peptidase S41 family protein has the tail-specific ClpP/crotanase-like protease domain. These C-terminal processing proteases have different substrates in different species, including processing of D1 protein of the photosystem II reaction center in higher plants (Oelmuller et al., 1996). The protein with the AP complex is an ortholog of yeast coatmer subunit zeta (RET3) (Cosson et al., 1996, Yamazaki et al., 1997). The zeta subunit may be involved in regulating the coat assembly and, hence, the rate of biosynthetic protein transport due to its
association-dissociation properties with the coatamer complex (Yamazaki et al., 1997). Additionally, seven of the thirteen DE genes were downregulated, including a protein with L-type lectin domain. The lectin family protein is orthologous to yeast endomembrane proteins EMP46 and EMP47. EMP46 and EMP47 are involved in the secretion of glycoproteins and in nucleus architecture and gene silencing (Sato and Nakano, 2002, Teixeira et al., 2002, Sato and Nakano, 2003, Sato and Nakano, 2004). These proteins also interact with coatamer subunit alpha (COP1), coatamer subunit gamma (SEC21) and a component of coat protein complex II (SEC23) (Sato and Nakano, 2002, Sato and Nakano, 2003). The prevalence of membrane trafficking proteins among the uniquely DE Bgh genes on the mla6+bln1 double mutant, further emphasize this pathway as a target for both transporting pathogen proteins and suppressing plant defense signaling (Fujimoto and Ueda, 2012). We postulate that the upregulated Bgh genes are probably required for virulence, whereas those downregulated may be redundant in immune-compromised hosts.

**Two waves of differential expression in the barley host: Response to Bgh penetration and haustorial formation**

Membrane trafficking is pivotal for plant cells to respond to the presence of pathogens, and provides an essential interface between plant hosts and their associated microbial partners. Many plant membrane trafficking factors have been identified for their roles in plant–microbe interactions and as possible targets of microbial effectors. In addition to possessing conserved mechanisms with eukaryotes, plants have also evolved plant-specific membrane trafficking
mechanisms (Fujimoto and Ueda, 2012). Although there is little information regarding the mechanism of pathogenic modulation of plant membrane trafficking thus far, our work has identified many membrane trafficking factors as possible targets of microbial attack.

We used the same 90-sample experiment to interrogate gene expression in the barley host in response to Bgh infection. Barley sequence reads were aligned to the Morex genome (Mascher et al., 2017) and read counts were analyzed for differential expression. By cross-referencing data from Arabidopsis (Stark et al., 2006, Paul et al., 2014, UniProt Consortium, 2015, Rutter and Innes, 2017), we identified 774 barley genes that are associated with membrane trafficking including those in the nuclear transport pathway. Of the repertoire of membrane trafficking candidate genes, Table 2 summarizes those identified as differentially expressed in each infection stage for each of the mutants as compared to the CI 16151 progenitor and Table S3 details the results for each membrane trafficking associated gene.

**Table 2.** Differentially expressed barley membrane trafficking genes between susceptible genotypes and wildtype for each infection stage.

<table>
<thead>
<tr>
<th>Progenitor Genotype</th>
<th>Mutant Genotypes</th>
<th>Differential Expressiona</th>
<th>Early Response</th>
<th>16 or 20 HAI Penetration</th>
<th>24 HAI Transition</th>
<th>32 or 48 HAI Haustoria Growth</th>
</tr>
</thead>
<tbody>
<tr>
<td>CI 16151</td>
<td><em>mla6-m18982</em></td>
<td>5</td>
<td>101</td>
<td>28</td>
<td>57</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>rar3-m11526</em></td>
<td>0</td>
<td>108</td>
<td>19</td>
<td>39</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>bln1-m19089</em></td>
<td>2</td>
<td>16</td>
<td>11</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(<em>mla6</em> + <em>bln1</em>)-m19028</td>
<td>14</td>
<td>124</td>
<td>41</td>
<td>55</td>
<td></td>
</tr>
</tbody>
</table>

aThe results are based on 774 genes encoding membrane trafficking proteins, including those involved in nuclear transport.
bDifferential expression results were controlled at a false discovery rate ≤ 0.001 and an absolute log2 fold change ≥ 1.
Extracellular vesicles (EVs) play a central role in intercellular signaling and are enriched in plants, particularly in response to pathogen infection (Rutter and Innes, 2017). At 0 HAI, when little to no differential expression is expected, we identify five genes DE in \textit{mla6}, two in \textit{bln1} and fourteen in \textit{mla6+bln1} double mutants, but none in the \textit{rar3} mutant as compared to the CI 16151 progenitor. Of the sixteen genes DE in the three mutants, fourteen are associated with EVs, which are known to be highly enriched in proteins involved in biotic and abiotic stress responses (Rutter and Innes, 2017). One example is the barley hypersensitive-induced response protein 3 (HIR3) (Rostoks \textit{et al.}, 2003). Barley HIR3 showed high percentage identity to \textit{Arabidopsis} HIR proteins. AtHIR proteins are physically associated with RPS2, induced by PAMPs, and quantitatively contribute to RPS2-mediated ETI but do not affect basal resistance. \textit{Arabidopsis} RPS2 is a NLR protein, which indirectly recognizes the bacterial effector protein AvrRpt2 and thereby activates ETI (Qi \textit{et al.}, 2011).

Early response genes are primarily associated with extracellular vesicles. However, we also wish to highlight the synergistic effects in the double mutant, lacking both the functional \textit{R} gene \textit{Mla6} and the negative regulator of basal defense \textit{Bln1}. Therefore, we examined genes exclusively DE in the double mutant but not in the individual \textit{mla6} or \textit{bln1} mutants, these ten genes include the barley HIR3 as well as the GTP binding protein, SAR1, a component of the COPII machinery. In response to ER stress, SAR1 interacts with bZIP28, an ER membrane-associated transcription factor (Srivastava \textit{et al.}, 2012). Any such gene could represent the synergistic
effects of the double mutation beyond those seen in the individual mutants of *mla6* and *bln1*. Alternatively, these genes could be illustrating an *mla6* dependent effect (ETI compromised) that is amplified by the *bln1* mutant (PTI compromised). In barley, we postulate early response genes could be PTI associated, sensing the pathogen’s presence, and transmitting signals to ETI associated proteins using membrane trafficking.

**Figure 4.** Number of barley differentially expressed genes between mutants and wild type at different infection stages. The number of DE barley membrane trafficking genes (out of 774 detected), as (A) upregulated and (B) downregulated among isogenic mutants and wild type during early response (0 HAI), penetration (16 or 24 HAI), transition (24 HAI), and haustorial development (32 or 48 HAI). Venn diagram comparing (C) upregulated and (D) downregulated genes during penetration as well as (E) upregulated and (F) downregulated genes during haustorial growth between susceptible mutants when compared to CI 16151 during penetration. Differential expression results were controlled at a false discovery rate ≤ 0.001 and an absolute log₂ fold change ≥ 1. The *mla6-m18982, rar3-m11526, bln1-m19089* and double (*mla6+bln1*)-m19028 mutants as compared to CI 16151 are represented in red, green, brown and blue, respectively.

**Barley ARF1 and BIG5 are downregulated during penetration of host cells**

Unlike a linear trend in *Blumeria* (Figure 3), barley illustrates two waves of differential expression (Figure 4) – the first and the larger peak is observed during
penetration of barley cells by Blumeria (16 – 20 HAI), whereas the second peak is detected during haustorial growth (32 – 48 HAI). During penetration, there is a large overlap in differentially expressed genes among susceptible mutants and the CI 16151 progenitor. In the core set, we identify genes such as HSC70 and ARF1. The genes that associate with each mutant uniquely emphasize the unique paths to defense signaling. For example, BIG5, activator of ARFs is downregulated in the mla6+bln1 double mutant. And, RAE1 which is part of the E3 ubiquitin ligase complex is upregulated in mla6 mutant, whereas, RBG8 is also downregulated. RBG8 is known to be targeted by bacterial effectors and could be regulated by Bgh effectors for its role in innate immune response. Barley genes that are upregulated could indicate genes co-opted by the pathogen, whereas downregulated ones could be suppressed by Bgh.

During penetration, 80 genes are differentially expressed in all susceptible genotypes when compared to the CI 16151 progenitor. These DE genes are expected to represent genes that are required for Mla6 function as well as contingent on it, such as the RAR1 interactor heat shock cognate protein 70 (HSC70). These DE genes also include ADP-ribosylation factors (ARFs), such as the GTP-binding protein involved in protein trafficking (Vernoud et al., 2003). They are required for the ER-to-Golgi transport and for the Golgi-derived transport to the plasma membrane. ARFs are also involved in the recruitment of E3 ubiquitin-protein ligase (COP1) and GRIP-related ARF-binding domain-containing Arabidopsis protein 1 (GDAP1) to membranes (Matheson et al., 2007).
Even though there are 80 genes DE in all susceptible mutants, there are also genes that are uniquely DE in each of these mutants. These genes define the different paths that the immune compromised lines seize to mount a successful defense to \textit{Bgh} but fail. For example, the nine genes that are DE exclusively in the \textit{mla6} mutant may be NLR-mediated associated genes that depend on a functional MLA6 for their function. Of the nine genes, the two upregulated genes are overexpressed in the mutant and may be co-opted by the pathogen to aid penetration. These include the RNA export factor 1 (RAE1), part of the E3 ubiquitin ligase complex and has the WD40 repeat domain. In \textit{Arabidopsis}, several WD40-containing proteins act as key regulators of plant-specific developmental events (Lee \textit{et al.}, 2008).

Likewise, the 21 genes (Figure 4) that are DE in the \textit{mla6+bln1} double mutant but not the individual \textit{mla6} or \textit{rar3} mutants, may be PTI associated genes or the synergistic effect of the double mutant. The downregulated genes include heat shock protein 90 (HSP90), which in complex with RAR1 and SGT1 regulates the levels of MLA6. Brefeldin A-inhibited guanine nucleotide-exchange protein 5 (BIG5) is also downregulated in double mutant as compared to wildtype. BIG5 activates the ARF proteins and plays a role in vesicular protein sorting. It also acts as the major regulator of early endosomal vesicle trafficking but is also involved in the endocytosis process (Tanaka \textit{et al.}, 2009). Additionally, it is a target of hopM1, a conserved \textit{Pseudomonas syringae} virulence protein that directs BIG5 to its own proteasome-mediated degradation (Nomura \textit{et al.}, 2011). BIG5 plays a broad role in
PTI, ETI, as well as salicylic acid-regulated immunity. Proteins such as HSP90 and BIG5 denote the extensive effect Bgh suppression has on plant defense responses during penetration.

**Oxidative stress response emphasized in barley during haustorial growth**

A larger core set of differentially expressed genes, as opposed to those unique in each genotype, is present in barley during penetration and *Blumeria* through all the infection stages. However, during haustorial growth (32 – 48 HAI), this trend is reversed for barley and only 26 genes are DE in all susceptible genotypes as compared to CI 16151 progenitor. This indicates a greater diversion between RNA expression profiles and hence, predicted protein function between immune-compromised genotypes. This core set includes the serine/threonine-protein kinase RIPK, which similar to BLN1, acts as a negative regulator of plant basal defense responses and may play a role in PTI (Liu *et al.*, 2011). During *Pseudomonas syringae* infection, and in response to the bacterial effectors AvrB and AvrRpm1, RIPK phosphorylates the host target RIN4, which subsequently activates RPM1-dependent ETI (Russell *et al.*, 2015). RIPK is also required for the bacterial XopAC/AvrAC ETI against *Xanthomonas campestris* (Guy *et al.*, 2013). RPK1 could be acting in a similar fashion in response to *Blumeria* infection, is expected to be contingent on MLA6 and thereby, may play a crucial role in NLR mediated defense responses.
However, genes involved in oxidative stress response are also frequently observed during haustorial response. For example, rotamase cyclophilin, which is involved in reactive oxygen species production in response to pathogen infection (Kumari et al., 2013), is suppressed in the mla6 mutant. Similarly, a probable glutathione peroxidase that may protect against oxidative stresses is upregulated, whereas glyceraldehyde-3-phosphate dehydrogenase C (GAPC) which is involved in oxidative stress response (Vescovi et al., 2013) in downregulated in the mla6+bln1 double mutant.

These uniquely significant genes define each susceptible mutant. For example, 31 genes that are DE in the mla6 mutant exclusively, of which twelve are upregulated and nineteen are downregulated. The upregulated genes include component of the exocyst complex EXO70A, involved in the docking of exocytic vesicles with fusion sites on the plasma membrane during regulated or polarized secretion (Kulich et al., 2010). Also overexpressed are Ras-related protein homologs H1 and G3. RABH1 is a regulator of membrane traffic from the Golgi apparatus towards the ER, whereas RABG3 is involved in intracellular vesicle trafficking and protein transport (Zelazny et al., 2013, Cui et al., 2014). The downregulated genes feature an E3 ubiquitin-protein ligase PUB22 that negatively regulates PTI (Trujillo et al., 2008). A syntaxin protein that functions in intracellular protein transport, exocytosis, vesicle docking and vesicle fusion is also downregulated. The downregulated genes may represent positive regulators of host immune signaling that are suppressed by the pathogen during compatible interactions.
Similarly, the thirteen genes that are DE in the *rar3* mutant exclusively, may represent NLR-associated defense genes. Of the thirteen genes, nine are upregulated including SNARE-interacting protein KEULE that regulates vesicle trafficking and is involved in vesicle docking during exocytosis (Wu et al., 2013). An ABC transporter G family member is also overexpressed. It is a key factor that controls the extent of cell death in the defense response and is required for limiting invasion by non-adapted powdery mildews (Consonni et al., 2006, Kobae et al., 2006, Stein et al., 2006).

Likewise, the 29 genes that are DE in the *mla6*+*bln1* double mutant but not the individual *mla6* or *rar3* mutants, may be PTI associated genes or the synergistic effect of the double mutant. Of the 29 genes, sixteen are overexpressed including probable glutathione peroxidase that may constitute a protective system against oxidative stresses. Additionally, thirteen genes are downregulated including nuclear transport factor 2 (NTF2) that facilitates protein transport into the nucleus (Zhao et al., 2006). Also, suppressed is the GAPC, which is involved in response to oxidative stress by mediating plant responses to abscisic acid and water deficits.

**Trans-Golgi-Network protein ARF1 is a negative regulator of cell death**

Barley illustrates biphasic differential expression (Figure 4), during penetration of host epidermal cells and during development of haustorial feeding structures. During penetration, the barley E3 ubiquitin ligase genes, such as ARF1 as well as its
activator BIG5 stand out during host membrane trafficking. Furthermore, *Mla6* and *Rar3* modulate several genes involved in oxidative stress response during haustorial growth. Additionally, *Mla6* and *Rar3* were also revealed to mediate resistance by reactive oxygen species production (Figure 1). Both *Mla6* and *Rar3* are required for hypersensitive response, and the loss of either gene leads to loss of cell death upon infection (Figure 1).

*Mla6* triggers a suite of defense responses associated with programed cell death, after detecting pathogen effector proteins. However, the molecular mechanisms that control NLR activation and signaling remain poorly understood (Qi and Innes, 2013). Thus, to further investigate how NLR proteins are activated or induce defense responses, we utilized MLA6 as bait in a yeast-two-hybrid screen against the total proteome of barley and *Blumeria* to identify candidate targets.

We identified seven positive interactors with MLA6 from two independent yeast two-hybrid library screens, from a total of 167.3 million tested interactions. Notable interactors included BIG5 and ARF1. BIG5 activates the ARF proteins and its transcript is suppressed in the *mla6+bln1* double mutant as compared to CI 16151 during penetration. ARF1 is a GTP-binding protein involved in protein trafficking, especially ER-Golgi transport. ARF1 encoding transcripts are downregulated in all susceptible mutants during penetration, indicating possible suppression by *Bgh*. 
Barley stripe mosaic virus (BSMV)-mediated virus-induced gene silencing (BSMV-VIGS) was used to assess the function of Arf1. Each of the two fragments (Arf1\textsubscript{1-253} and Arf1\textsubscript{246-474}) were amplified using two primer sets and inserted into BSMV-VIGS constructs according to (Meng et al., 2009). Seven-day old barley seedlings were bombarded with Arf1 VIGS construct as well as mock and BSMV:00 treatments. Infected leaves were collected and processed to obtain recombinant virions, which were used to mechanically infect seven-day old healthy CI 16151 (Mla\textsubscript{6}) and CI 16137 (Mla\textsubscript{1}) barley plants. Plants were phenotyped 14 days after infection. Figure 5 illustrates that both constructs caused cell death phenotype in CI 16151 and to a lesser degree in CI 16137. Cell death was not observed in the empty vector (BSMV:00) and mock constructs. Independent replicated experiments revealed similar results. Therefore, we conclude that Arf1 negatively regulates cell death.

Figure 5. BSMV-mediated silencing of Arf1. Gene silencing was performed on (A) CI 16151 (Mla\textsubscript{6}) and (B) CI 16137 (Mla\textsubscript{1}) plants and mediated by Barley stripe mosaic virus (BSMV). Seven-day-old plants were treated with phosphate buffer (pH 7.5) (Mock) and viruses BSMV:00, BSMV:Arf1\textsubscript{1-253} and BSMV:Arf1\textsubscript{253-474}. The cell death phenotypes were photographed 14 days after BSMV mechanical infection.
3.4 Discussion

Barley illustrates biphasic differential expression, during penetration of host epidermal cells and during development of haustorial feeding structures. During penetration, the barley E3 ubiquitin ligase pathway genes, such as ARF1 as well as its activator BIG5 are differentially expressed among host membrane trafficking. Further, ARF1 is a candidate interactor for MLA6, and Arf1 silenced plants displayed increased cell death, indicating negative regulation of defense. However, R gene Mla6 and Rar3 are required for hypersensitive response, and the loss of either gene leads to loss of cell death upon infection. Furthermore, Mla6 and Rar3 modulate several genes involved in oxidative stress response during haustorial growth. Taken together, these results corroborate the pivotal role membrane trafficking plays in defense against pathogens. Specifically, it shows that ARF1 is involved in R protein-mediated resistance to powdery mildew, and RAR3 is required for MLA6-mediated hypersensitive response.

ARF1 is a negative regulator of programmed cell death and a candidate interactor of Mla6, which elicits hypersensitive response. ARF1 increases in all mutants from 0 to 20 HAI, however, it significantly decreases post penetration in resistant genotypes but stabilizes for susceptible genotypes (Figure 5). In the mla6 mutant, ARF1 is downregulated at penetration but upregulated during haustorial growth as compared to wild type progenitor (Mla6). Their interaction could indicate that MLA6 is suppressing ARF1 post penetration. Alternatively, ARF1 could be modulating MLA6
during penetration since it is upregulated in genotypes with Mla6 but not much less in mutants lacking Mla6 functionality.

Several studies indicate that secretion may play an important role in NLR-mediated defense. For example, *Arabidopsis* MIN7, an ADP Ribosylation Factor – Guanine nucleotide Exchange Factor (ARF-GEF) protein, was shown to be required for resistance mediated by RPS2 and RPS5, CC-NB-LRR family proteins like MLA (Nomura *et al.*, 2011). ARF-GEF proteins regulate the activity of small GTPases involved in endomembrane trafficking. AtMIN7 is a target of HopM1, a *Pseudomonas syringae* effector that promotes proteasome-dependent degradation of AtMIN7 (Nomura *et al.*, 2006). Activation of RPS2 and RPS5 prevents HopM1-mediated degradation of AtMIN7 (Nomura *et al.*, 2011). RPS2 has also been shown to upregulate production of miR393b, a microRNA that targets at least three different genes likely involved in endomembrane trafficking – MEMB12, a golgi-localized SNARE protein; VPS54, homologous to a yeast protein involved in retrograde transport from late endosomes to the Golgi, and EXO70H3, a subunit of the exocyst complex thought to be required for exocytosis (Zhang *et al.*, 2011). MEMB12 functions as negative regulator of exocytosis, with RPS2 inducing production of a miR393b that inhibits translation of the MEMB12 protein. Lower MEMB12 protein levels then enable an increase in defense protein secretion. Consistent with this model, the Arabidopsis MEMB12 knockout displays enhanced basal resistance in the absence of RPS2 activation (Zhang *et al.*, 2011). These data show
endomembrane trafficking as central to NLR-mediated resistance, most probably to increase secretion of antimicrobial compounds.

The membrane trafficking system is thus emerging as important arm of the NLR-mediated defense system that is also targeted by pathogen effectors. *Phytophthora infestans* secreted RXLR effector AVR1 interacts with SEC5, a subunit of the exocyst complex that is involved in vesicle trafficking. Both, AVR1 and SEC5 suppress CRINKLER2-induced cell death and SEC5 is required for secretion of the pathogenesis-related protein PR1 and callose deposition. Conversely, AVR1 promotes colonization and suppresses callose deposition (Du et al., 2015). This shows another example where a pathogen manipulates and disturbs host vesicle trafficking, an important process for innate immunity.

Differential expression analysis of *Blumeria* candidate secreted genes highlight several fungal membrane trafficking genes encoding orthologues to KSH1, RET3, EMP46/47, as well as AP complex mu/sigma subunit domain protein. Infection phenotyping and kinetics show that immune compromised mutants have similar fungal growth. Therefore, any differences observed in expression between susceptible genotypes can be attributed to mutation rather than development. Therefore, we hypothesize that *Bgh* secreted proteins may be targeting the barley membrane trafficking in a strategy similar to how late blight pathogen modulates potato vesicle trafficking. This could be utilized by pathogens to both internalize effector proteins and suppress plant defense signaling.
Alternatively, *Blumeria* may be outcompeting the plant proteins in host complexes. For instance, chitin preferably binds LysM effector Ecp6 of the tomato leaf mold fungus *Cladosporium fulvum* over the host immune receptor (Sanchez-Vallet *et al.*, 2013). LysM effectors are known to prevent recognition of cell wall-derived chitin by host immune receptors. This lack of chitin detection is through interference with the host immune receptor complex and not chitin sequestration (Sanchez-Vallet *et al.*, 2013). In another instance, LysM effector Slp1 of the rice blast fungus *Magnaporthe oryzae* competes with host chitin elicitor binding protein (CEBiP) for binding of chitin oligosaccharides. Slp1 is required by the rice blast fungus for full virulence and exerts a significant effect on tissue invasion and disease lesion expansion. However, gene silencing of CEBiP makes resistant rice plants susceptible even in the absence of Slp1. Slp1 may sequester chitin oligosaccharides to prevent PAMP-triggered immunity in rice, thereby facilitating rapid spread of the fungus within host tissue (Mentlak *et al.*, 2012). Barley orthologue of CEBiP, HvCEBiP is involved in basal resistance against appressorium-mediated infection and that basal resistance might be triggered by the recognition of chitin oligosaccharides derived from *M. oryzae* (Tanaka *et al.*, 2010).

**Future directions**

The competition described above between rice CEBiP and *Magnaporthe oryzae* effector Slp1 may be occurring between barley and *Bgh. Blumeria* has two Slp1 orthologues, however neither are predicted to be secreted and thus require further
testing. The first question is whether these proteins are mis-predicted and are in fact secreted, and do they indeed bind chitin and does that affect the susceptibility of barley irrespective of presence of chitin receptors. This would illustrate if *Blumeria* uses a similar strategy as the rice blast fungus to evade chitin detection and suppress PTI. Second, one should identify host protein interactors of *Blumeria* differentially expressed membrane trafficking secreted proteins. This would further elucidate how pathogen effectors are transported within the host and these complex binding sites would provide candidate motifs to modify for creating more resistant barley varieties. These findings would have implications in monocots and potential for sustainable agriculture.

3.5 Materials and Methods

**Phenotyping**

*Bgh* isolate 5874 was propagated on *H. vulgare* cv. Morex at 18°C (16 hours of light / 8 hours of darkness) and utilized to characterize the fast-neutron derived mutants.

To verify that *Rar3* segregates independently of *Mla6* and *Rar1*, F2 progeny from two crosses, *rar3-m11526 x mla6-m9472* and *rar1-m100 x rar3-m11526*, were examined 7 days after inoculation (DAI) with *Bgh* isolate 5874. In both cases, the observed ratios were not significantly different than the expected 9 resistant (R) : 7 susceptible (S) ratio, respectively 56R:40S ($X^2 = 0.169, p = 0.68$) and 113R:103S ($X^2 = 1.359, p = 0.24$). In addition, to verify that the susceptible *rar3-m11526* phenotype is a single gene response, 96 CI 16151 x *rar3-m11526* F2s were phenotyped 7 DAI with *Bgh* isolate 5874. The observed phenotypes (78R:18S) were
not significantly different than the expected 3R:1S ratio \((X^2 = 2.0, p = 0.16)\) indicating a single gene response.

**Infection kinetics**

Data was collected on CI 16151 and its isogenic panel of fast-neutron mutants. The plants were grown in a split plot design with genotype as the whole plot and time point as the sub plot. Six days after planting, barley lines were inoculated with \(Bgh\) isolate 5874 and harvested at six time points – 16, 20, 24, 28, 32 and 48 HAI. Five leaves were harvested for each treatment and fixed in ethanol:acetic acid (3:1) for 24 hours. Stained leaves were then incubated for 24 hours in 70% ethanol and subsequently moved to 20% ethanol for storage. For scoring, leaves were submerged in Coomassie blue stain for 10 mins and washed twice with 10% glycerol to visualize \(Bgh\) spores and hyphae. Leaves were scored on both sides, abaxial and adaxial. Each experiment was replicated three times.

Leaves were scored for spore, appressorium, hyphal indices 1, 2 and 3. A spore was defined with no discernible germ tubes, to the presence of a primary germ tube and the beginning of a secondary germ tube. An appressorium was indicated by the growth of a medium sized secondary germ tube up to a fully formed appressorium and infection peg. Hyphal index 1 is defined as a single elongating secondary hyphae (ESH) growing from the secondary germ tube with no extra branches. Hyphal index 2 is defined as an ESH with one branching, whereas
hyphal index 3 is defined by an ESH with two or more branches. Percentage ESH was calculated as follows:

\[
\%\text{ESH} = 100 \times \frac{\sum \text{Hyphal indices}}{\sum \text{Spore} + \text{Appressorium} + \text{Hyphal indices}}
\]

A mixed model was fit for pairwise comparison between treatments with percentage ESH as the response (Table S1) using SAS software (SAS Institute Inc., Cary, North Carolina, USA). Treatment, leaf side and treatment * leaf side were the fixed factors, whereas leaf (treatment), replication and treatment * replication were random factors in a mixed model design. Results were adjusted for multiple testing with the false discovery rate controlled at 0.05.

**Fast-neutron mutagenesis and screening**

Barley line CI 16151 \((Mla6)\) was obtained by the introgression of \(Mla6\) into the universal susceptible cv Manchuria (Moseman, 1972). Fast-neutron mutagenesis was performed as described in (Xi et al 2009; Meng 2009). Briefly, seeds of CI 16151 were treated with fast neutrons at 4 Gy Nf at the International Atomic Energy Agency (Vienna, Austria) and M2 families were screened for mutant segregants following the method of (Wise and Ellingboe 1985). Individuals that produced cell death symptoms or sporulating \(Bgh\) colonies were selected for rescue. Susceptible mutant \(m11506\), containing \(Mla6\) and \(Bln1\) deletions, was backcrossed to CI 16151 and selfed to fix isogenic mutants \(m18982\) \((mla6/mla6, Bln1/Bln1, Rar3/Rar3)\), \(m19089\) \((Mla6/Mla6, bln1/bln1, Rar3/Rar3)\), and \(m19028\) \((mla6/mla6, bln1/bln1, Rar3/Rar3)\). Similarly, a \(Rar3\) mutant, \(11526\) \((Mla6/Mla6, Bln1/Bln1, rar3/rar3)\), was selected from 36 F2 individuals following two backcrosses to CI 16151.
Plant growth, RNA extraction and sequencing

CI 16151 and its derived mutant barley lines were maintained via single seed descent. Genotypes were grown in separate trays (19.5 x 27.5-cm) in a climate-controlled greenhouse using a split plot design. Each tray consisted of six rows of 16 seedlings, with rows randomly assigned to one of six harvest timepoints (0, 16, 20, 24, 32, and 48 HAI). Seven days after sowing, seedling trays were inoculated at 16:00 with a high density of Bgh isolate 5874 (AVR$_{a1}$, AVR$_{a6}$, viir$_{a8}$, AVR$_{a12}$, viir$_{a13}$, AVR$_{La}$) and positioned randomly in a controlled growth chamber (18°C, 8 h darkness, 16 h light). Rows of seedlings were harvested at the assigned time points, pooled, and immediately placed into liquid nitrogen. The entire procedure was replicated three times in successive weeks resulting in 90 experimental units (5 genotypes x 6 timepoints x 3 replications).

Total RNA was isolated from 0.5 g of each pool of seedlings using a hot (60°C) phenol / guanidine thiocyanate method (Caldo et al., 2004). A split-split-plot design was used to run the 90 samples on three Hi-Seq 2500 flow cells. Each replication was run on a separate flow cell with each of the five plant genotypes randomly assigned to each lane and 6 barcodes randomly assigned to the 6 time points within each genotype (lane). RNA-sequencing (RNA-seq) libraries were prepared by the Iowa State University DNA Facility (Ames, Iowa, USA) using the Illumina TruSeq stranded RNA sample preparation kit and were subjected to single-end sequencing (100-bp reads) using the Illumina HiSeq2500 Sequencing System.
Illumina read preprocessing, alignment and expression estimation

The 100 base pair single-end reads obtained from Illumina HiSeq 2000 were preprocessed using FastQC (Andrews, 2015) and Trimmomatic version 0.32 (Bolger 2014). First, quality control checks were performed on raw sequence data using FastQC (Andrews, 2015). Then, the raw reads were processed using Trimmomatic (Bolger et al., 2014). We (i) cut adapters and Illumina-specific sequences from the reads, (ii) perform a sliding window trimming, cutting once the average quality within the window of 4 base pairs fell below a threshold of 32, (iii) cut bases off the start of a read, if below a threshold quality of 36, (iv) cut bases off the end of a read, if below a threshold quality of 36, and (v) drop the read if it is below a length of 50 base pairs. We performed FastQC (Andrews, 2015) check again to ensure that any data quality problems were fixed.

Bowtie2 (Langmead and Salzberg, 2012) indices were built for the reference genome for Barley (Mascher et al., 2017) and Blumeria (Ensembl Fungi Assembly EF 1, INSDC Assembly GCA_000151065.1) (Spanu et al., 2010). The single-end reads were then aligned using the TopHat2 (Kim et al., 2013) with the “-read-realign-edit-dist” parameter set to 0. This forces TopHat2 to map every read in all the mapping steps (transcriptome, genome, and finally splice variants detected by TopHat2), reporting the best possible alignment found in any of these mapping steps. This may greatly increase the mapping accuracy. This was followed by genome guided Cufflinks version 2.2.1 (Trapnell et al., 2010, Trapnell et al., 2012)
with the TopHat2 BAM output file as input. Finally, transcripts sequences were extracted with the gffread utility (part of the Cufflinks software) using the GTF file from Cufflinks as input.

For each of the 90 samples, read count estimation was done using RSEM (Li and Dewey, 2011) with Trimmomatic (Bolger et al., 2014) trimmed reads as input. Transcript references were built for RSEM along with Bowtie2 indices (rsem-prepare-reference) separately for Blumeria and Barley using respective reference genomes. This was done with the “--gtf” option turned on, this means RSEM assumes that reference file contains the sequence of a genome, and will extract transcript reference sequences using the gene annotations specified in that file. Gene and isoform expression was estimated using “rsem-calculate-expression” with the “--bowtie2” option.

**Differential expression analysis**

Duplicated and rRNA genes were discarded from the read count data set. The 90 samples were then grouped by time point (0, 16, 20, 24, 32 and 48 HAI) and analyzed using R package DESeq2 in Bioconductor (Love et al., 2014). Genes with a read count of less than two in 16 or more samples (out of 18 in a group) were discarded. A model was fit with read counts as response, and replication and genotype terms as explanatory variables. P-values were adjusted for multiple testing using Benjamin and Hochberg methodology (Benjamini and Hochberg, 1995). This was done separately for both barley and Blumeria read counts. Genes that had an
absolute log2 fold change of at least one, as well as were differentially expressed at a q-value of no greater than 0.05 for *Blumeria* and 0.001 for barley were considered significant.

**Computational prediction of barley membrane trafficking genes/proteins**

To reliably map the Arabidopsis proteome to the barley transcriptome, we obtained empirical scores for coverage and identity. Rice (*Oryza sativa* subsp. japonica) was selected as the monocot representative as it had the highest number of protein sequences (3,716) in Swiss-Prot (UniProt Consortium, 2015) as compared to barley (350 sequences). The coverage and identity scores were based on a Rice – Arabidopsis comparison of protein sequences obtained from Swiss-Prot using blastp in the BLAST+ command line suite (Camacho *et al.*, 2009). Rice – Arabidopsis sequences were considered the same if they had the same descriptive annotation. This alignment gave an average of 86% coverage and 60% identity among the sequence pairs considered the same.

Next, protein sequences for Arabidopsis assembly Araport11 were obtained from TAIR (www.arabidopsis.org) and compared against the barley Morex proteome (Mascher *et al.*, 2017) using blastp in the BLAST+ command line suite (Camacho *et al.*, 2009). Coverage was calculated using the query as well as the subject and the maximum of the two was used. Sequences that aligned with a coverage of 86% and an identity of 60% were selected. Genes from (Paul *et al.*, 2014, Rutter and Innes,
2017) as well as their multi validated physical interactors (Stark et al., 2006) were selected using this method to identify barley membrane trafficking genes.

**Yeast-two-hybrid screen**

Total RNA was used from the same 90-treatment experiment as described above (Caldo et al., 2004). mRNA was enriched and further purified using Dynabeads® mRNA Purification Kit (ThermoFisher Scientific, Grand Island, NY). Twenty-five micrograms of each mRNA enriched sample were pooled for library construction by HYBRIGENICS SERVICES (Paris, France) using MLA6 as bait to screen a random-primed barley + *Blumeria* cDNA prey library. This was repeated twice for the bait construct containing MLA6\textsubscript{2-225} and the two different libraries described below.

The first library (*Hordeum vulgare* mildew Inoculated RP1) was constructed using cDNA samples extracted from tissues harvested at 0, 16, 24 and 32 hours after *Bgh* inoculation, whereas the second library (*Hordeum vulgare* Inoculated MILDEW-2) was constructed using cDNA samples extracted from tissues harvested at 16, 24, 32 and 48 hours after *Bgh* inoculation. This whole time-frame covers *Bgh* infection stages from the attachment of conidia on barley epidermis, differentiation of appressorium, penetration of host cell, to the development of haustorial complex. The MLA6 protein 2-225 amino acids from the amino terminus, designated as MLA6(225), was fused with DNA binding domain of LexA in the orientation N-LexA-MLA6(225)-C and has been used to screen these two libraries. A total of 167.3 million interactions were tested and 535 positive clones were processed.
Processing yeast two-hybrid results

The bait – prey interactions from the yeast two hybrid screen were ranked from A to F and N/A. We discarded any interactions below B. Next, the 5’ and 3’ sequences of the remaining preys were aligned to the Barley (Mascher et al., 2017) and Blumeria (Ensembl Fungi Assembly EF 1, INSDC Assembly GCA_000151065.1) genomes (Spanu et al., 2010) using NCBI nucleotide BLAST command line application (Altschul et al., 1990, Camacho et al., 2009). Sequences with 95% identity and coverage of 95% were considered a match. All anti-sense alignments were discarded. The 5’ and 3’ prey sequences were second-frame translated using EMBOSS Transeq (Rice et al., 2000) and aligned to barley proteome using NCBI nucleotide BLAST command line application (Altschul et al., 1990, Camacho et al., 2009). Only sequences with 95% identity and coverage of 95% were retained.

Barley stripe mosaic virus (BSMV) induced gene silencing (VIGS)

Two fragments for Arf1 BSMV-VIGS were amplified by PCR using two pairs of primers (72618Vf1: ATATTAATTAATGGGCTCACGTTTACC, 72618Vr1: TATGC GGCCGCTGTTCTGGAAAGTAATGCCTCC; 72618Vf2: ATATTAATTAACAGGTCTCATCTTTGTGT, 72618Vr2: TATGC GGCCGCTGTTCTGGATGTAGCCT). The amplified product were digested by restriction enzymes PacI and NotI and ligated into the BSMV:γ vector digested by PacI and NotI to generate Arf1 VIGS constructs BSMV:Arf1-253 and BSMV:Arf1-256-474, corresponding to the Arf1 coding sequence 1-253 and 256-474.
region. Silencing experiments were performed as described previously (Meng et al., 2009). In brief, biolistic bombardment of barley plants was carried out using a biolistic PDS-1000/He system (Bio-Rad). Gold particles (Bio-Rad) were coated with plasmid BSMV:α, BSMV:β, and BSMV:γ (or the recombinant BSMV:Arf1<sub>1-253</sub> and BSMV:Arf1<sub>256-474</sub> constructs) at a molar ratio of 1:1:1. The mixture was delivered to leaves using 900-psi rupture discs using a Hepta adaptor microcarrier. Eight 7-day-old Black Hull-less barley seedlings (susceptible to BSMV) were used per bombardment. Virus-infected barley was maintained in a growth chamber 10 days (Percival Scientific, Perry, IA, USA) with 16 h of light at 24°C (550 μmol m<sup>−2</sup> s<sup>−1</sup>) and 8 h darkness at 20°C. Black Hull-less plants displaying a BSMV infection phenotype were selected to grind with carborundum (0.05 g; Sigma-Aldrich) in 2 to 5 volumes of 0.05 M phosphate buffer (pH 7.2). Seven-day-old healthy barley seedlings were then infected with the appropriate recombinant virions by rubbing the first leaf with crude virus extract and maintained in a growth chamber. Twelve days after mechanical infection, plants displaying BSMV infection symptom were used for phenotyping.

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3.8 Author Contributions
Conceived and designed the experiments: PS, GF, WX, AC, RPW. Wrote and edited the paper: PS, GF, WX, AC, ML, DN, RPW. Phenotyping and RNA preparation: GF. Bgh infection kinetics: AC. Computational and statistical analyses: PS. Yeast two-hybrid and VIGS: WX.

3.9 Data Access
Supplemental Materials for Chapter 3 can be accessed in the zipped folder “Chapter3_Supplemental_Files” on ProQuest. Table S1 provides infection kinetics data on CI 16151 and its derived mutants at 16, 20, 24, 28, 32 and 48 hours after inoculation with Bgh isolate 5874. Table S2 details differential expression results for candidate secreted Blumeria genes, whereas Table S3 details differential expression results for barley membrane trafficking genes.
CHAPTER 4. GENERAL CONCLUSION

In Chapter 2, I used an expression Quantitative Trait Locus (eQTL) approach to address temporal regulation of immunity in barley – powdery mildew interactions. Two major clusters of trans eQTL on barley chromosomes 2HL and 1HS were identified, which are associated with penetration of host cells and development of haustorial/fungal feeding structures, respectively. Regulatory control of 22% of these genes shifted from 2HL to 1HS as infection progressed. Master regulators responded to pathogen attack by activating genes according to temporal programs. The mechanisms of the regulatory models in this study need further experimental validation and functional analyses. One way to do so would be identify MLA binding sites using ChiP Sequencing. This would help identify the genes directly regulated by MLA not just associated with it. A second option is to identify over-represented binding site sequences in promoters of genes associated with the eQTL clusters and backward engineer the regulatory components of this pathogen induced defense signaling cascade.

In Chapter 3, I used differential expression analysis to address the modulation of host membrane trafficking and secreted pathogen genes. Further, a yeast two-hybrid screen provided ARF1 as a candidate interactor of MLA6 and a negative regulator of cell death. Arf1 is downregulated in susceptible mutants during penetration by Bgh. I also identified Blumeria membrane trafficking pathway genes as differentially expressed in immune-compromised host environments. In the future, a larger yeast two-hybrid screen involving a larger number of barley and Blumeria
proteins in different genotypic backgrounds is required to better understand how pathogen manipulates the host and how the plant responds. For barley, including proteins encoded by Arf1 and other differentially expressed transcripts would be interesting, whereas for Blumeria, including several effectors and membrane trafficking proteins may help understand how it, if at all, targets the host transport pathways.

In conclusion, we have shed some light on barley – powdery mildew interactions especially as regulated by MLA. I have identified genes (1) associated with Mla and (2) modulated due to lack of lack of Mla functionality. We have shown that these candidate genes influence host resistance, for example, Arf1 is a negative regulator of cell death. I have also identified pathogen genes that may target host proteins, however that needs further testing.
REFERENCES


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interactor ROH1 are involved in the localized deposition of seed coat pectin. *New Phytol*, 188, 615-625.


APPEXDIX: SUPPLEMENTAL MATERIALS

Supplemental Materials for Chapter 2 and 3 can be accessed in the zipped folders “Chapter2_Supplemental_Files” and “Chapter3_Supplemental_Files”, respectively, on ProQuest. A brief description of each file is available below.

Supplemental Files For Chapter 2

- Table S1. Details of PCR primers used in this study. The sequences, reaction conditions and expected sizes for PCR primers used in this study for both the MlLa markers and Mla LRR amplification. Enzymes used for the CAPS markers are also included.

- Table S2. Infection type data for three replications of each parent (Q21861 and SM89010) and QSM progeny lines. An infection phenotype of < 2.5 is considered a resistant reaction, while a value of ≥ 2.5 is considered susceptible. Genotypes for the double haploid progeny fit a ratio of 1:1:1:1 ($\chi^2$ 1:1:1:1 = 2.28; p-value = 0.5164) and a phenotypic ratio of 2 ($^{IT=0}$):1 ($^{IT=2}$):1 ($^{IT=3}$) ($\chi^2$ 2:1:1 = 2.17; p-value = 0.34).

- Table S3. Complete QSM genetic map with summary infection type data for Mla1 and MlLa loci. The table shows the recombination events associated with 1,505 markers across 75 QSM recombinant lines. Markers displaying the Q21861 parental phenotype are represented by "A", and those displaying the SM89010 parental phenotype are represented by "B". Three replications of phenotyping with Bgh isolate 5874 genetically mapped the MlLa marker, which interacts with Bgh AVRLa, to 2H.67. Sporulation was scored from 0 (completely
resistant) to 4 (completely susceptible) with 2.5 as a cut-off to identify lines with \textit{Mila}. Three genes (MLOC\_74329, MLOC\_66083, and MLOC\_13479) also reside in 2H.67, which is defined by recombination events at QSM034 and QSM078. Due to the epistatic nature of \textit{Mla1} over \textit{Mila}, the \textit{Mila} genotype cannot be determined via \textit{Bgh} phenotyping in QSM lines with dominant \textit{Mla1}. These lines are therefore labeled with a "-".

- \textbf{Table S4. Genotyping and phenotyping results for H. laevigatum and \textit{Laevigatum}-derived lines.} Laevigatum, Gold and two lines derived from this cross were tested for the markers that co-segregate and flank \textit{Mila}. These were also tested for infection phenotype with \textit{Bgh 5874} and genotyped for \textit{Mla}.

- \textbf{Table S5. Genes associated with known pathways or regulatory components in the interaction between hosts and pathogens.} The 2HL and 1HS associations for encoded proteins annotated to the secretory pathway, vesicle transport, signaling G-proteins and known NLR (\textit{Ml}) interactors. This table also provides the most significant marker at 16 and 32 HAI with significance level, annotation, expression pattern and alternate regulation information on each gene.

- \textbf{Table S6. Genes associated with each marker on the QSM map along with the level of significance.} The table lists all 22,840 probe sets in the Barley1 Affymetrix GeneChip along with the most significant marker at 16 and 32 HAI as well as the significance level in \textit{p}- and \textit{q}-values. This table also provides annotation, expression pattern and alternate regulation information on each gene.
- Table S7. Differential expression results for comparison between CI 16151 (Mla1) and CI 16155 (Mla13) in BB4 and between QSM progeny groups (Mla1, mlLa) and (mla1, mlLa) in BB96. The table lists all 22,840 probe sets in the Barley1 Affymetrix GeneChip along with the significance level in p- and q-values at 16 and 32 HAI.

- Table S8. Allelic effects and modes of alternate regulation for the genes transferred between 2HL at 16 HAI and 1HS at 32 HAI. This table also provides the most significant marker at 16 and 32 HAI with significance level, annotation, expression pattern and alternate regulation information on each gene.

- Figure S1. Two communities (A and B) form a sub-network of proteins annotated to the secretory pathway, vesicle transport, and signaling G-proteins, as well as their known interactions. Community A has 14 nodes, including VTI1B, VPS45, Aquaporin (PIP) and Syntaxin (SYP) proteins. Community B has 15 nodes, including EXO70, VAMP721/722, SNAP33, SYP111 and SEC proteins. Blue, red and black boxes indicate association with 2HL at 16 HAI, 1HS at 32 HAI and neither, respectively. The solid black connecting lines represent multi-validated physical interactions between proteins.

- Figure S2. A community of 6 nodes, including clathrin heavy chain, SNARE-like and adaptin proteins. Blue, red and black boxes indicate regulation by 2HL at 16 HAI, 1HS at 32 HAI and neither, respectively. The solid black lines represent multi-validated physical interactions between proteins.
Supplemental Files For Chapter 3

- **Table S1. Infection kinetics data on CI 16151 and its derived mutants at 16, 20, 24, 28, 32 and 48 hours after inoculation with *Bgh* isolate 5874.** This file provides data on both raw values as well as the analysis results. Values details the raw data collected for the 5 genotypes at 6 time points for 3 replications each. In each replication, data was collected for multiple leaves. For each leaf, the values were recorded for both the abaxial (B) and the adaxial (D) sides. Results provides the pairwise comparison between the treatments (genotype * time point) of interest.

- **Table S2. Differential expression results for candidate secreted *Blumeria* genes.** The table provides adjusted p-values and Log$_2$ fold change values for pairwise comparison for *Blumeria* genes on the CI 16151 derived mutants vs. the wild type progenitor for six time points.

- **Table S3. Differential expression results for barley membrane trafficking genes.** The table provides adjusted p-values and Log$_2$ fold change values for pairwise comparison for barley genes in the CI 16151 derived mutants vs. the wild type progenitor for six time points.