Biological roles of small RNAs expressed during infection of barley by the obligate fungal biotroph, *Blumeria graminis* f. sp. *hordei*

by

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The student author, whose presentation of the scholarship herein was approved by the program of study committee, is 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

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DEDICATION

I dedicate this dissertation to my wife Cathi. Despite the long hours and endless seaming weeks, Cathi was always there for me. Going back to school at my age is a serious challenge, but she inspired me to push beyond me old skill sets and explore new opportunities.

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ABSTRACT

Plants and their pathogens have constantly co-evolving mechanisms that determine infection success. Small RNAs (sRNAs) of 18-30 nucleotides can have a large effect regulating plant defense responses as well as fungal virulence factors. The goal of this project was to understand how sRNAs regulate gene expression both for species of origin, as well as trans-kingdom sRNA communication between barley and *Blumeria graminis* f. sp. *hordei* (*Bgh*), the causal agent of barley powdery mildew. To accomplish this goal we examined *Bgh* sRNA expression over a time course representing the key stages of *Bgh* infection of barley (appressorium formation, penetration of epidermal cells, and development of haustoria) in five barley lines including four fast-neutron derived immune-signaling mutants and their progenitor line CI 16151. The sRNA expression data was complemented by parallel analysis of RNA ends (PARE) analysis that confirms sRNA transcript cut sites with *in vivo* data. In barley, conserved and novel miRNAs were identified with predicted target transcripts enriched in the transcriptional regulation, signaling, and photosynthesis categories. Phasing siRNAs (phasiRNAs) were also identified in barley overlapping with protein coding genes including receptor-like kinases and resistance genes. *Bgh* micro RNA-like RNAs (milRNAs) were identified that are predicted to regulate transcripts encoding effectors, metabolic proteins, and translation-related proteins. A subset of effectors homologous to the AVR_{k1} and AVR_{a10} (EKA) family may be regulated by a sRNA-encoding hairpin that is overlapping and antiparallel to an EKA gene. These genes are heavily regulated by sRNAs, in contrast to most *Bgh* protein-coding genes. Potential trans-kingdom functional sRNAs were identified from both barley and *Bgh*. The predicted *Bgh* trans-kingdom sRNA are highly enriched in transcripts that function in non-

species-specific defenses. The transcript targets encode proteins related to vesicle secretion, cell wall synthesis, protein turnover, transcriptional regulation, ROS response, and fungal cell wall breakdown. The potential barley trans-kingdom sRNAs are predicted to target *Bgh* transcripts including *Bgh*-specific effector proteins, ribosome synthesis/function, core transcription factors, and cell cycle regulators. Overall, these findings indicate that sRNAs are integral in regulation of gene expression during *Bgh* infection of barley leaves.

CHAPTER 1. GENERAL INTRODUCTION

Barley and its Genome

Barley (*Hordeum vulgare* L.) is a member of the grass family Poaceae, in the tribe Triticeae, and genus *Hordeum*. It is the fourth most agriculturally important grass species, following wheat, maize, and rice, and is used mainly for animal feed, malting, and human foods (Office of the Gene Technology Regulator, 2008). The barley genome contains seven chromosomes, and is relatively large with an estimated size of 5.1 Gbp with a high content of repetitive DNA at 80.8% (Mascher *et al.*, 2017). The first draft of the barley genome was assembled using multiple different resources including sequencing multiple BAC libraries, BAC-ends, Illumina DNA-seq genomic reads, SNP marker mapping, RNA-seq, and full-length cDNA alignment (International Barley Sequencing Consortium *et al.*, 2012). The most-recent genome assembly focuses on ordered physical maps of the barley chromosomes enhanced by the Hi-C and Bionano optical mapping techniques (Lieberman-Aiden *et al.*, 2009, Lam *et al.*, 2012). Distal portions of the barley chromosomes were relatively gene-rich and lower in repetitive content, as compared with proximal portions with low gene content and high occurrence of transposable element (TE) families and other repeats. This differential content in repetitive elements helps explain the reduced recombination frequencies seen in proximal portions of barley chromosomes, which can hamper breeding efforts for genes in those chromosomal regions (Mascher *et al.*, 2017).

***Blumeria graminis* f. sp. *hordei* and its Genome**

Powdery mildews are represented by over 400 species that are able to infect virtually all crop plants (Bindschedler *et al.*, 2016). The *Blumeria graminis* group of species infects grasses and is a member of the phylum Ascomycota in the order Erysiphales of the class Leotiomycetes (Spanu, 2014). *Blumeria graminis* f. sp. *hordei* (*Bgh*) is an obligate biotrophic fungus that infects barley exclusively. The life cycle of *Bgh* includes both asexual and sexual stages, but under normal field conditions the asexual reproductive style dominates (Wolfe and McDermott, 1994). The asexual life cycle of *Bgh* starts when conidium lands on a leaf surface and forms a short primary germ tube, followed by a secondary germ tube that elongates and forms an appressorium that penetrates the barley epidermal cell wall. Infection pegs form haustoria that are surrounded by a plant-derived membrane and act both to the suppress plant defenses and to reprogram the cell to provide nutrition to the growing fungus (Bélanger *et al.*, 2002). After 3-5 days, asexual conidiophores grow out the fungal hyphae in stalks with eight conidia for wind distribution, to begin the infection cycle again (Spanu, 2014). The haustorium represents the primary organ for communication between the host and pathogen. It consists of a complex extension of the fungal hypha which invaginates the host cell plasma membrane. The host plasma membrane surrounds the fungal plasma membrane and cell wall, with a small space between them termed the extra-haustorial matrix (EHM). Many fungal effector proteins that contribute to virulence are secreted into the EHM and pass through the host cell plasma membrane by an unknown mechanism (Dörmann *et al.*, 2014).

The *Bgh* genome has been sequenced with an estimated size of 130 Mbp (Spanu *et al.*, 2010). Recently an updated genome sequence was published of the DH14 and RACE1 isolates

(Frantzeskakis *et al.*, 2018). The updated genome assembly includes 7118 genome models, including 805 secretion signal-containing proteins (SPs), representing 11.3% of the annotated genes. This large proportion of genes encoding SPs is directly related to the effector gene content necessary for successful barley infection (Bindschedler *et al.*, 2016). The *Bgh* genome includes 74% repetitive element content, which includes a recent expansion by transposable element (TE) families including long terminal repeats (LTRs) and long interspersed nuclear elements (LINEs) (Frantzeskakis *et al.*, 2018).This relatively high content of TEs has been correlated with lifestyle, as obligate biotrophs have larger genome sizes and higher TE content than other related fungal species (Amselem *et al.*, 2015a).

Plant/Pathogen Interactions

Pathogens possess highly conserved structures called pathogen-associated molecular patterns (PAMPS) such as flagellin or peptidoglycans in bacteria, or glucans or chitin in fungi, which are recognized by plant pattern recognition receptors (PRRs) (Ranf, 2017). PRRs can include both receptor-like kinases (RLKs) and receptor-like proteins (RLPs) that through protein-protein interactions initiate resistance signaling (Tang *et al.*, 2017). Binding of PAMPs to PRRs triggers the PAMP-triggered immunity (PTI) defense response, includes a burst of reactive oxygen species, a build-up of callose, and production of antimicrobial compounds and hydro-lytic enzymes (Giraldo and Valent, 2013, Oliveira-Garcia and Valent, 2015). With non-adapted pathogens, the PTI response will highly suppress pathogen growth and infection. However, some pathogens have evolved effector compounds that act both to suppress the plant PTI defense response and result in effector triggered susceptibility (ETS). Effectors can affect

defense responses in multiple areas including pathogen perception, secretion, transcription, cell wall structure and can come in the form of proteins, metabolites, and small RNAs (sRNAs) (Weiberg *et al.*, 2015, Toruno *et al.*, 2016). To counter ETS, plants have evolved non-membrane bound receptor proteins of the nucleotide-binding, leucine-rich domain (NLR) family. NLR proteins include either an N-terminal coiled coil (CC) domain or a Toll Interleukin-1-like receptor (TIR) domain and a C-terminal hypervariable leucine-rich repeat domain (Sukarta *et al.*, 2016). These NLR proteins respond to the presence or activity of pathogen effectors and trigger a strong defense response called effector triggered immunity (ETI) that can lead to a localized hypersensitive response. (Li *et al.*, 2015).

***Bgh* Effectors**

The *Bgh* genome is predicted to encode two different classes of effector proteins. The first class includes effectors of the EKA type that lack traditional targeting sequences for secretion (Ridout *et al.*, 2006). The second class, candidate secreted effector proteins (CSEPs), were identified using several criteria, including presence of a predicted signal peptide for secretion, smaller size, and lack of homology to other known proteins outside of powdery mildews (Spanu *et al.*, 2010, Pedersen *et al.*, 2012). The two classes of effectors combined, represent around 2000 members, which is a substantial portion of the ~7000 protein-encoding genes (Amselem *et al.*, 2015b, Bourras *et al.*, 2018).

The EKA effector class may include more than 1350 members in the *Bgh* genome (Spanu et al. 2010). The class name EKA comes from its two founding members $AVR_{\underline{K}1}$ and $AVR_{\underline{A}10}$ (Amselem et al. 2015), which were identified as targets of the barley R proteins MLK1 and

MLA10 (Ridout *et al.*, 2006). EKA gene family members are found inside of active Class I LINE retrotransposons (Sacristan *et al.*, 2009, Amselem *et al.*, 2015b). The recent genome expansion of *Bgh*, relative to related ascomycete fungi, has not included an increase in gene copies, but rather an expansion of TE families including LINE and LTR families (Bindschedler *et al.*, 2016). The expansion of the LINE family would allow for rapid evolution of new effects in the EKA class through sequence divergence of EKA family members.

The CSEP effector class currently has 722 members that are grouped into 72 gene families (Spanu *et al.*, 2010, Bindschedler *et al.*, 2011, Pedersen *et al.*, 2012, Kusch *et al.*, 2014, Whigham *et al.*, 2015, Bourras *et al.*, 2018). The RNase-like effector family is highly conserved amongst powdery mildews even outside of grass specific powdery mildew species, and despite its conservation, has an unknown function (Menardo *et al.*, 2017). Out of the hundreds of potential CSEP effector candidates only a small subset has been tested for their relative effect on *Bgh* pathogenicity (Zhang *et al.*, 2012, Pliego *et al.*, 2013, Schmidt *et al.*, 2014, Aguilar *et al.*, 2015, Ahmed *et al.*, 2015, Whigham *et al.*, 2015, Ahmed *et al.*, 2016, Pennington *et al.*, 2016, Spanu *et al.*, 2018). Some CSEPs are expected to contribute quantitatively towards virulence, which may be more difficult to show as infection counts may not show significant differences between HIGS silenced and control samples.

Plant Small RNAs

Small RNAs (sRNAs) in plants are involved in viral and transposable element (TE) defense, as well as the regulation of native gene expression at both the transcriptional and post-transcriptional level. sRNAs generally fall into the two categories micro RNAs (miRNAs)

and short interfering RNAs (siRNAs). The sRNA pathway originated as a defense against invading virus and TE nucleic acids in the last eukaryotic common ancestor (Pumplin and Voinnet, 2013, Tabach *et al.*, 2013). Transcriptional and post-transcriptional control of gene expression by sRNAs may have evolved after the last common ancestor of plants and fungi as the implementation of RNA interference (RNAi) can be quite different between kingdoms (Dang *et al.*, 2011, Borges and Martienssen, 2015). The RNAi pathway is essential for plants, as mutations in Dicer or Argonaute enzymes can result in severe developmental mutations or are embryo lethal (Borges and Martienssen, 2015).

The majority of sRNAs in plants originate from three pathways: miRNA biogenesis, secondary siRNA biogenesis, and heterochromatic siRNAs (hetsiRNAs) (Borges and Martienssen, 2015). Micro RNA genes are transcribed as non-coding transcripts by DNA polymerase II and have 5' caps and poly-A tails to protect from degradation (Iwakawa and Tomari, 2015). The transcripts form fold-over double strand complexes called primary-miRNAs (pri-miRNAs) that are processed by Dicer enzymes into precursor miRNAs (pre-miRNAs) and finally mature miRNAs. The active strand of the miRNA is then bound to an Argonaute enzyme in the RISC complex that guides homology-based cleavage or translational inhibition of target transcripts. Small interfering RNAs in plants can have several origins including viral genomes, transgenes, transposable elements, as well as amplification of transcripts targeted by miRNAs or other siRNAs (Borges and Martienssen, 2015). The siRNAs can act on both the transcriptional level (hetsiRNAs) as well as the post-transcriptional level, and can travel systemically throughout the plant, as in the case of systemic viral resistance.

Both PTI and ETI based immune responses are regulated by sRNAs at the post transcriptional level (Fei *et al.*, 2016). In the study by Navarro et al. (2006), a PTI response by three auxin receptors was shown to be caused by the miRNA miR393 (Navarro *et al.*, 2006). The three auxin receptors TIR1, AFB2, and AFB3 are specifically down-regulated by miR393 by an flg22 trigger, suggesting reduction in growth signaling upon PAMP perception. Conserved miRNAs that regulate the ETI response through expression of *R* genes have been identified in several plants, including *Medicago truncatuala*, soybean, tomato, peach, and apple (Li *et al.*, 2012, Zhu *et al.*, 2012, Arikit *et al.*, 2014, Ma *et al.*, 2014, Fei *et al.*, 2015). These miRNAs mostly target conserved regions in *R* genes including the P-loop, the TIR motifs, and others (Fei *et al.*, 2013). *R* gene regulation by miRNAs has also been shown in barley when the Shen and Wise groups demonstrated that the miR9863 family differentially regulates *Mla* NB-LRR gene in response to pathogen infection (Liu *et al.*, 2014)

Phasing siRNAs (phasiRNAs) are secondary sRNAs that are produced when a miRNA triggers the production of double stranded RNA (dsRNA) from transcript templates, and this template is processively cleaved by Dicer commonly into 21 or 24 bp fragments (Fei *et al.*, 2013). PhasiRNAs were originally described in *Arabidopsis* in the form of trans-acting siRNAs (tasiRNAs) (Deng *et al.*, 2018). The *Arabidopsis* tasiRNAs are encoded by four families of *TRANS ACTING siRNA* (*TAS*) genes including *TAS1*, *TAS2*, *TAS3*, and *TAS4* (Komiya, 2017). *TAS3,* the most highly conserved *TAS* locus is conserved in all land plants and has important functions in plant development related to auxin and auxin response factors (Xia *et al.*, 2017). The broader phasiRNA category describes phased siRNAs that may or may not have activity in *trans* (Fei *et al.*, 2013). PhasiRNAs have been described to have a divergent function between monocots

and dicots. In dicots, phasiRNAs are almost all 21 nt in size and mostly originate from protein coding transcripts (Fei *et al.*, 2013). In monocots, phasiRNA loci are both 21 and 24 nt in size and are expressed in mainly in reproductive tissues from non-coding RNA transcripts (Arikit *et al.*, 2013). This functional divergence between monocot and dicot phasiRNA loci types may have happened before their last common ancestor as both types of phasiRNA types are present in the Gymnosperm Norway Spruce (Xia *et al.*, 2015).

Fungal Small RNAs

Small RNAs in fungi have a wide range of functional diversity and impact on survivability of species. Functional RNAi has been reported in all the major fungal phyla including Ascomycota, Basidiomycota, and Zygomycota (Olson, 2016). Unlike animals and plants, functional RNAi is not required for survival of all fungal species, as some species do not have altered phenotypes with knocked out silencing components or are missing functional RNAi altogether (Kamper *et al.*, 2006, Drinnenberg *et al.*, 2009, Janbon *et al.*, 2010). Processes regulated by fungal RNAi pathways include sexual reproduction (meiotic silencing by unpaired DNA [MSUD]), DNA damage repair (Qde-2 interacting small RNAs [qiRNAs)]), heterochromatin formation and maintenance (Primal RNAs [priRNAs]), viral and TE defense (quelling), and gene expression (exonic-siRNAs [ex-siRNAs]), Dicer-independent siRNAs [disiRNAs] and microRNAs-like RNAs [milRNAs]) (Chang *et al.*, 2012, Villalobos-Escobedo *et al.*, 2016).

To our knowledge, no studies have identified fungal sRNAs predicted to regulate known effector genes. Several reports have identified sRNAs that are differentially regulated between non-infection and infection tissue, for example in *Magnaporthe oryzae* and *Botrytis cinerea*

(Raman *et al.*, 2013, Weiberg *et al.*, 2013). In both cases, the small RNAs that are differentially regulated are predicted to target LTR retrotransposons. In the Oomycota phylum, several *Phytophthora* species have been shown to produce sRNAs that specifically regulate effector gene expression (Vetukuri *et al.*, 2012, Fahlgren *et al.*, 2013, Qutob *et al.*, 2013).

Trans-Kingdom Silencing

Trans-kingdom silencing occurs when sRNAs produced by an organism from one kingdom are taken up and have function in another organism from a different kingdom (Weiberg and Jin, 2015). The mechanisms of trans-kingdom silencing are unclear, but have been observed in multiple systems including human to protozoa, plants to nematodes, fungi to plants, and others (Knip *et al.*, 2014). Trans-kingdom sRNA communication between plants and pathogens have interesting functional implications. When these type of sRNAs are expressed in a plant pathogen and taken up by the host plant they can act by definition as effector molecules (Wang *et al.*, 2015). Since sRNAs do not undergo the same types of surveillance that protein effectors can undergo, they have the potential to silence key defense gene hubs without triggering a defense reaction.

In just such a case, Weiberg et al. (2013) identified three sRNAs that were produced by *B. cinerea* that silenced four genes in *Arabidopsis* involved in pathogen defense. When the *B. cinerea* sRNAs were overexpressed in stable transgenic *Arabidopsis* lines, the plants were phenotypically normal except for enhanced susceptibility to pathogen infection. The production of functional effector sRNAs was dependent on intact Dicer genes (*dcl1* and *dcl2*) in *B. cinerea*, and both single and double mutants were compromised in their pathogenicity. The

targets of the *B. cinerea* trans-kingdom sRNAs are highly conserved and help explain the relatively large host range of the pathogen (Weiberg and Jin, 2015).

In another recent study, the bioactive milRNA *Pst*-milR1 was discovered in *Puccinia striiformis* f. sp. *tritici* (*Pst*) that silences the wheat *pathogenesis-related 2* (*PR2*) gene that encodes a β-1,3-glucanase (Wang *et al.*, 2017). When *Pst*-milR1 was knocked out the resulting *Pst* strain had significantly reduced pathogenicity on wheat and conversely, when the wheat *PR2* gene expression was knocked down by virus induced gene silencing (VIGS) the virus transformed plants had increased susceptibility to *Pst* in incompatible reactions. The demonstration of sRNAs acting as effectors in a plant-pathogen interaction suggests that trans-kingdom sRNA communications may be another layer in the Zig Zag model originally proposed in Jones and Dangl (2006) and updated to Zig Zag Zig by Fei et al. (2016).

If plant fungal pathogens can express active trans-kingdom effector sRNAs, it makes logical sense that plants could express sRNAs that could act as resistance factors, silencing target genes in pathogenic fungi. The potential for anti-fungal sRNAs being expressed in plants has been demonstrated in several studies in barley, wheat, *Arabidopsis*, and other species (Nowara *et al.*, 2010, Wang *et al.*, 2016). The host induced gene silencing (HIGS) technique was developed in barley and wheat where the authors produced sRNAs *in planta* that caused down regulation of the pathogen effector gene AVR_{a10}, and reduced pathogenicity in plants lacking the *R* gene *Mla10*. The success of this technique has been extended to plant pathogen/pest interactions with fungi, oomycetes, and animals (Knip *et al.*, 2014).

The presence of plant expressed sRNAs acting as resistance factors was recently described in cotton, as the miRNAs miR166 and miR159 were taken up by the pathogen

Verticillium dahliae and significantly reduced virulence by silencing transcripts encoding a Ca^{2+}-dependent cysteine protease and an isotrichodermin C-15 hydroxylase (Zhang *et al.*, 2016). The results of these studies demonstrate that trans-kingdom sRNA communication is actively occurring, and represents a new paradigm of plant-pathogen interactions.

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CHAPTER 2. REGULATION OF BARLEY POWDERY MILDEW EFFECTORS THROUGH SMALL RNAS

Modified from a manuscript to be submitted to BMC Genomics

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MH contributed to project development, data analysis and interpretation, and wrote the manuscript with input from RW; ML contributed to statistical analyses; GF performed small RNA sequencing experiments; SM & BM performed PARE sequencing experiments; DN contributed to statistical analyses; RW contributed to project conception, development, and data interpretation.

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Abstract

Background

Fungal small RNAs (sRNAs) are in many ways diverged in function from plants and animals. The expression of sRNAs in fungi has been linked with processes including sexual reproduction, DNA damage repair, viral defense, and regulation of gene expression. Successful barley leaf infection for the obligate biotroph *Blumeria graminis* f. sp. *hordei* (*Bgh*) requires tight regulation of effectors and proteins regulating metabolism, growth, and reproduction. sRNAs can act as post transcriptional regulators in fungi that could control these functions. However, the function of sRNAs can be especially challenging to study in obligate biotrophic plant pathogenic fungi that require the host plant to complete their life cycle. Studying their expression requires co-purification with the host sRNA pool. Therefore, the regulatory impact of sRNAs on *Bgh* gene expression is relatively unknown and unexplored.

Results

Our study explored the sRNA population of *Bgh* infected barley leaves and identified likely gene regulatory targets. To accomplish this goal we examined sRNA expression over a time course representing the key stages of *Bgh* infection of barley (appressorium formation, penetration of epidermal cells, and development of haustoria) in four fast-neutron derived immune-signaling mutants and their progenitor line, CI 16151. The sRNA-sequencing (sRNA-seq) data was analyzed with a custom pipeline to identify 1741 predicted micro RNA-like (milRNA) species. Parallel analysis of RNA ends (PARE) libraries were used to validate target gene prediction and identified target genes enriched in the effector, metabolism, and translation-related functional categories. Several members of the effectors homologous to AVRA1 and AVRA10 (EKA) gene family were identified with natural antisense miRNA-like (nat-miRNA-like) hairpins encoded in the reverse orientation on the opposite genomic strand. This process may result in selective silencing of these loci compared with the overwhelming majority of predicted Bgh genes, that have no observed mapping sRNAs.

Conclusions

Our data suggest that small RNAs from *Bgh* regulate gene expression enriched in several functional categories including metabolism, translation-related, and pathogen effectors. Regulation of effector gene expression though sRNAs is relatively unknown in fungi. PARE-validated targets of predicted *Bgh* milRNAs include both members of the EKA and candidate

secreted effector protein (CSEP) effector families. For some members of the EKA family this regulation involves heavy sRNA production that may lead to broad silencing of the EKA family in a developmentally timed manner.

Keywords

Bgh, barley, small RNA-seq, transposable elements, EKA family, CSEPs, effectors

Background

Obligate biotrophic fungi require a living host throughout their life cycle to successfully reproduce. To do this they must maintain a careful balancing act to suppress plant defenses, obtain nutrients for growth and reproduction, and at the same time keep the host plant alive throughout the infection cycle. Plant defense responses work in multiple layers that overlap, but serve the distinct functions of non-specific immunity, and evolved pathogen defense. The non-specific defense responses are generally triggered by chemical motifs specific to types of pathogens called pathogen associated molecular patterns (PAMPs) such as flagellin for bacteria and chitin for fungi (Ranf, 2017). The PAMP molecules are recognized by pattern recognition receptors (PRRs) that initiate a signaling cascade that induces a multi-faceted defense response called PAMP-triggered immunity (PTI) that can include the production of reactive oxygen species (ROS), a build-up of callose and other cell wall materials, and the production of antimicrobial compounds and hydrolytic enzymes (Giraldo and Valent, 2013, Oliveira-Garcia and Valent, 2015). The PTI response is sufficient to stop pathogen infection for the majority of non-evolved species. However, some pathogens have evolved effector molecules that can suppress the PTI response, thereby creating effector triggered susceptibility (ETS). Filamentous

pathogens produce multiple effector molecules that manipulate host cellular metabolism and defense to create an infection site suitable for growth. Effectors can come in the form of proteins (AVR_{A1} from *Blumeria graminis* f. sp. *hordei)*, sRNAs (Bc-siR3.1 from *Botrytis cinerea*), and metabolites (coronatine from *Pseudomonas syringae*) (Feys *et al.*, 1994, Weiberg *et al.*, 2013, Lu *et al.*, 2016). To overcome ETS, plants have evolved a secondary defense mechanism called effector-triggered immunity (ETI) where nucleotide-binding leucine rich repeat proteins (NLRs) act as receptors that either bind directly to pathogen effectors or recognize effector action on guard proteins and trigger a strong defense response that can include the hypersensitive response (Baggs *et al.*, 2017).

Bgh is an obligate biotrophic pathogen of barley that infects leaf epidermal tissue. The genome of *Bgh* is fairly large at ~130 Mbp, compared with its closest relatives, as is common in fungal biotrophs (Parlange *et al.*, 2011, Raffaele and Kamoun, 2012, Dong *et al.*, 2015). The relative increase in genome size is thought to be mostly due to a proliferation of transposable elements (TEs), as 74% of the *Bgh* genome is made up of repetitive sequences (Spanu *et al.*, 2010, Frantzeskakis *et al.*, 2018). The *Bgh* genome includes several hundred effector genes that encode proteins to both suppress barley defense responses and to create a nutrient sink for fungal growth and reproduction (Kusch *et al.*, 2014, Frantzeskakis *et al.*, 2018). Effector proteins are generally secreted through the haustorial feeding structure, although the mechanism of host uptake is unclear (Giraldo and Valent, 2013, Dormann *et al.*, 2014). Effectors in *Bgh* can be broken into two categories: candidate secreted effector proteins (CSEPs) and effectors homologous to AVR_{k1} and AVR_{a10} (EKA) (Spanu *et al.*, 2010). The EKA effector family is unique in that its members are located inside of an active LINE TE family

(Amselem *et al.*, 2015). The expression of members of both families is required for full *Bgh* virulence (Ridout *et al.*, 2006, Bourras *et al.*, 2018)

Small RNAs (sRNAs) in fungi have been shown to regulate sexual reproduction, DNA damage repair, viral- and transposable element (TE) defense, and regulation of gene expression (Chang *et al.*, 2012, Villalobos-Escobedo *et al.*, 2016). Functional RNA interference (RNAi) has been reported in all the major fungal phyla including Ascomycota, Basidiomycota, and Zygomycota (Olson, 2016). Until recently, no sRNAs had been identified in fungi similar to the canonical plant or animal type miRNAs. However, sRNAs derived in a similar mechanism to miRNAs, called miRNA-like RNAs (milRNAs), were found that are transcribed hairpin genomic sequences in *Neurospora crassa* (Lee *et al.*, 2010). These milRNA sRNAs have been identified in several ascomycete and basidiomycete species and function in developmental and metabolic regulation (Torres-Martinez and Ruiz-Vazquez, 2017). In several filamentous pathogens effector genes have been shown to be regulated by sRNAs. In the oomycete pathogen *Phytophthora sojae* the avirulence factor Avr3a is differentially silenced by small RNAs in a transgenerational fashion, allowing for infection of plants with an *R*-gene recognizing the Avr3a protein (Qutob *et al.*, 2013). In *Phytophthora infestans* sRNAs were identified that target numerous *RxLR* and *Crinkler* effector genes that were differentially accumulated between highly and weakly pathogenic strains (Vetukuri *et al.*, 2012).

Transposable elements make up a large portion of eukaryotic genomes ranging from 44% in humans to 81% in barley (Qin *et al.*, 2015, Mascher *et al.*, 2017). The activity of TEs has both advantages and disadvantages for their host genomes. Active transposable elements can insert directly into coding sequences, knocking out function of these genes. However, TEs have

also directly evolved into functional miRNAs that allow tissue and developmental level regulation of gene expression (Roberts *et al.*, 2014). The phenomenon of TEs evolving into miRNAs has been observed in plants, animals, protists, and fungi (Jiang *et al.*, 2012, Sun *et al.*, 2012, Roberts *et al.*, 2014, Asman *et al.*, 2016).

In this study, we sought to identify sRNAs involved in the regulation of *Bgh* gene expression during parasitism of its barley host. To accomplish this goal we infected seedlings from barley line CI 16151 (containing the *Mla6* powdery mildew resistance gene) and four fast-neutron derived immune-signaling mutants in a time-course experiment representing key stages of *Bgh* development on its barley host: appressorium formation, penetration of epidermal cells, and development of haustoria. RNA extracted from a 48-hour time course of *Bgh*-infected barley leaves was used to make both sRNA-seq and parallel analysis of RNA ends (PARE) libraries to identify *Bgh* sRNAs and identify transcript target sites. Effector targets were highly enriched in the PARE *in vivo* validated targets, along with several other categories including metabolic processes and translational regulation. Several EKA effector family members overlap with predicted sRNA-encoding hairpins, which is correlated with high sRNA mapping density at those genomic locations.

Methods

Fungal and Plant Material

Barley lines CI 16151 (*Mla6*), m18982 (*mla6*), m11526 (*rar3*), m19089 (*bln1*), and m19028 (*mla6 + bln1*) were grown with supplemental lighting under temperature controlled greenhouse conditions. The CI 16151 barley line was created by introgression of the *Mla6* gene into

universal susceptible cv Manchuria (Jørgensen and Moseman, 1972) and is resistant to *Bgh graminis* f. sp. *hordei* (*Bgh*) isolate 5874 (AVR_{A1}, AVR_{A6}, AVR_{A12}). Mutant derivatives of CI 16151 were created through fast- neutron mutagenesis as described previously (Meng *et al.*, 2009a). *Mla6* is a major NLR-type resistance gene, while *Rar3* (*Required for Mla6 resistance 3*) is an unlinked locus required for *Mla6* function. *Blufensin1* (*Bln1*) is a negative regulator of PTI signaling (Meng *et al.*, 2009b) and the *bln1* mutant exhibits enhanced basal defense (Xu *et al.*, 2015). Plants containing the mutant forms of *Mla6* or *Rar3* are susceptible to 5874 infection, unlike the CI 16151 parental line. *Bgh* isolate 5874 was propagated on *Hordeum vulgare* cv. Morex in a growth chamber at 18°C with a 16 hours light, 8 hours dark day/night cycle.

Experimental Design

Planting, stage of seedlings, inoculation, and sampling of leaf tissue were followed as described previously (Caldo *et al.*, 2006, Moscou *et al.*, 2011). Barley tissue used for sRNA libraries was grown in three separate replicates grown in consecutive weeks. Each genotype was planted in 20 × 30–cm trays in sterilized potting soil. Each experimental tray consisted of six rows of 12-15 seedling first leaves, with rows randomly assigned to one of the six harvest times in a split-plot design. Within each replicate the five barley genotypes were infected with a high density of fresh *Bgh* conidiospores (100 / cm^2) and harvested at 0, 16, 20, 24, 32, and 48 hours after inoculation (HAI) for a total of 90 tissue samples.

Small RNA Sequencing and Data Analysis

Total RNA was extracted from *Bgh*-infected barley leaf tissue following the hot (60°C) phenol/guanidine thiocyanate method described previously (Caldo *et al.*, 2004, Caldo *et al.*, 2006). Small RNA libraries were produced using the Illumina TruSeq Small RNA Library kit (Illumina, Inc., San Diego, CA, 92122), as per the manufacturer's protocol. The ninety small RNA Illumina libraries were sequenced on a HiSeq 2500 (Illumina, Inc.) at the Iowa State University DNA Facility in Ames, IA. Reads were quality assessed using the FastQC program version 0.11.3 (Andrews). Reads were quality filtered and adapters were trimmed using Trimmomatic version 0.33 (Bolger *et al.*, 2014). Reads were compared with the Rfam database using the Infernal program version 1.1.2 (Nawrocki *et al.*, 2014) and used to filter tRNAs, rRNAs, snoRNAs and snRNAs from the data. Two programs were used to identify sRNA candidates of interest from *Bgh*: miRDeep-P (version 1.3) and ShortStack (version 2.1.0) (Yang and Li, 2011, Axtell, 2013).

Differential Expression

For each time point, we performed a differential expression (DE) analysis, comparing relative abundance of sRNAs from the different immune mutants compared with CI 16151 (WT). The count datasets were normalized and analyzed by using DESeq2 (Love *et al.*, 2014). We added 0.5 to all counts and rounded them to the nearest integer to fulfill the input data format requirement while applying DESeq2. Reads with 0.9 quantile smaller than a count of 2 are assumed to be expressed at a very low level and were removed from the analysis. The

remaining sRNAs/reads were analyzed for DE. The p-values were adjusted for multiple testing error using Q-value calculations (Nettleton *et al.*, 2006), and sRNAs/reads were filtered for a Q-value of less than 0.05.

PARE Library Sequencing and Data Analysis

Source RNA that was used for sRNA sequencing above was also used for PARE. PARE libraries were prepared as previously described (Zhai *et al.*, 2014) at the Donald Danforth Plant Science Center in St. Louis, MO and sequenced on a HiSeq 2500 (Illumina, Inc.) at the University of Delaware. Reads were quality assessed using the FastQC program version 0.11.3 (Andrews 2010). Reads were quality filtered and adapters were trimmed using Trimmomatic version 0.33 (Bolger *et al.*, 2014). The two PARE analysis programs sPARTA (version 1.21) (Kakrana *et al.*, 2014) and CleaveLand (version 4.4) (Addo-Quaye *et al.*, 2009) were used independently to identify likely sRNA targets using sRNA sequencing data, Ensembl *Bgh* transcriptome (version 32) (Spanu *et al.*, 2010), and PARE sequencing data. PARE validated targets were filtered based on adjusted p-values using a 1% false discovery rate along with a PARE category of less than 2.

Availability of Data and Materials

Small RNA sequencing dataset has been submitted to NCBI GEO under the accession number GSE115992. PARE library sequencing data has been submitted to NCBI under accession number GSE116691. Supplemental Materials for Chapter 2 can be accessed in the zipped folder "Chapter_2_Supplemental_Files" on ProQuest.

Supplemental Material [See Appendix]

Supplemental Table 2.1 DE *Bgh*-mapped read expression details

Supplemental Table 2.2 PARE-validated predicted miRNAs and barley genome mapped sRNAs

Supplemental Table 2.3 PARE-validated transcript target annotations

Supplemental Table 2.4 EKA homolog/hairpin overlap details

Results

Identification of milRNA Candidates from sRNA Data

To identify *Bgh* sRNAs involved in regulation of gene expression, sRNA-seq libraries were produced from barley line CI 16151 and four fast-neutron derived immune-signaling mutants. *Bgh*-inoculated 1st leaves (5 genotypes x 6 time points x 3 biological replications) were harvested from a split-plot design at 0, 16, 20, 24, 32, and 48 HAI for a total of 90 samples. The sequenced libraries contained ~2.8 billion total reads that were filtered and mapped to the *Bgh* genome, as detailed in **Figure 2.1A**. Because there are few, if any, fungal-specific resources for predicting functional sRNAs from sRNA sequencing data, two separate approaches were used to independently identify potentially functional *Bgh* sRNAs. The first approach was to use two plant-specific miRNA prediction programs (ShortStack and miRDeep-P) to predict milRNAs with structural similarities to plant miRNAs from the *Bgh* aligned reads (Yang and Li, 2011, Axtell, 2013). The predictions from these programs will identify milRNA candidates that are similar to plant miRNAs, but will not necessarily identify milRNAs that act according to fungal-specific rules. However, as the rules for *Bgh*-specific milRNAs are not known, the plant rule based programs were used here. The ShortStack and miRDeep-P programs predicted a total of 1,741

milRNAs candidates with plant miRNA-like structural features. The second approach was to filter reads for exact matches to the *Bgh* genome and for at least 10 counts across the 90 libraries. The reads that passed the mapping and count filters were designated *Bgh* genome mapped sRNAs. Of the ~86 million unique reads from the full sRNA-seq dataset, ~955,000 mapped exactly to the *Bgh* genome and had at least ten counts. The size distributions of both the *Bgh* genome mapped sRNAs and the program-predicted sRNAs was concentrated at 20-23 nucleotides (**Figure 2.2A-B**), while the genome mapped sRNAs had much longer tails with a small peak at 49-50 bp with reads mapping to rRNA fragments.

The base composition of the 5' most base of the predicted milRNAs heavily favors uracils in these positions (99.7%) as is common for several predicted fungal milRNAs (**Figure 2.2C-D**) (Lee *et al.*, 2010, Jiang *et al.*, 2012, Lau *et al.*, 2013, Liu *et al.*, 2016).

Candidate milRNAs and Genome Mapped sRNAs are Primarily Differentially Expressed at 48 HAI

We sought to identify *Bgh* sRNAs regulating gene expression in a developmental time course from 0 HAI (undifferentiated conidiospores) to 48 HAI (extensive secondary hyphal growth) during visually asymptomatic stages of barley infection. To identify sRNAs important in *Bgh* development and successful barley infection, milRNA candidates and *Bgh* genome mapped sRNAs were analyzed for differential expression (DE) using the DESeq2 program (Love *et al.*, 2014). Small RNA expression was analyzed at each time point, comparing expression in the four mutant lines individually to expression in wild-type CI 16151. In total, 13311 (14.1%) of the *Bgh*

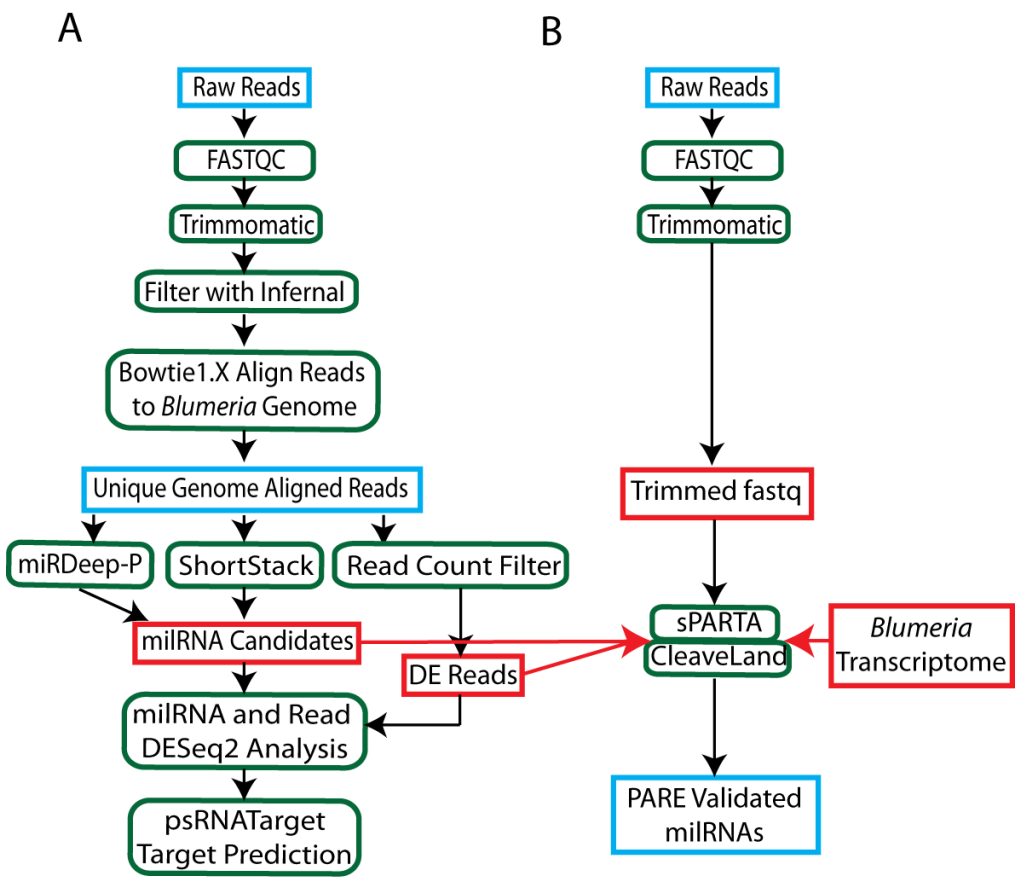


Figure 2.1 Small RNA sequencing and PARE sequencing analysis pipelines.Small RNA-seq Illumina reads were trimmed, filtered, and run through the two plant miRNA identification programs miRDeep-P and ShortStack to identify milRNA candidates and DE sRNA reads. (**B**) Sequencing reads from the PARE libraries were trimmed and filtered and analyzed with the sPARTA and CleaveLand programs (Addo-Quaye *et al.*, 2009, Kakrana *et al.*, 2014). Additional input data was provided from the *Bgh* transcriptome and milRNA candidates plus DE sRNA reads developed from the sRNA sequencing pipeline.

genome mapped sRNAs and 268 (15.4%) of the milRNA candidates were DE in at least one time point as compared with wild type (**Supplemental Table 2.1)**. The vast majority of DE *Bgh* genome mapped sRNAs and milRNA candidates (98.6% and 100%, respectively) were DE only at 48 HAI (**Table 2.1**). The *mla6* mutant had significantly higher number of differentially expressed

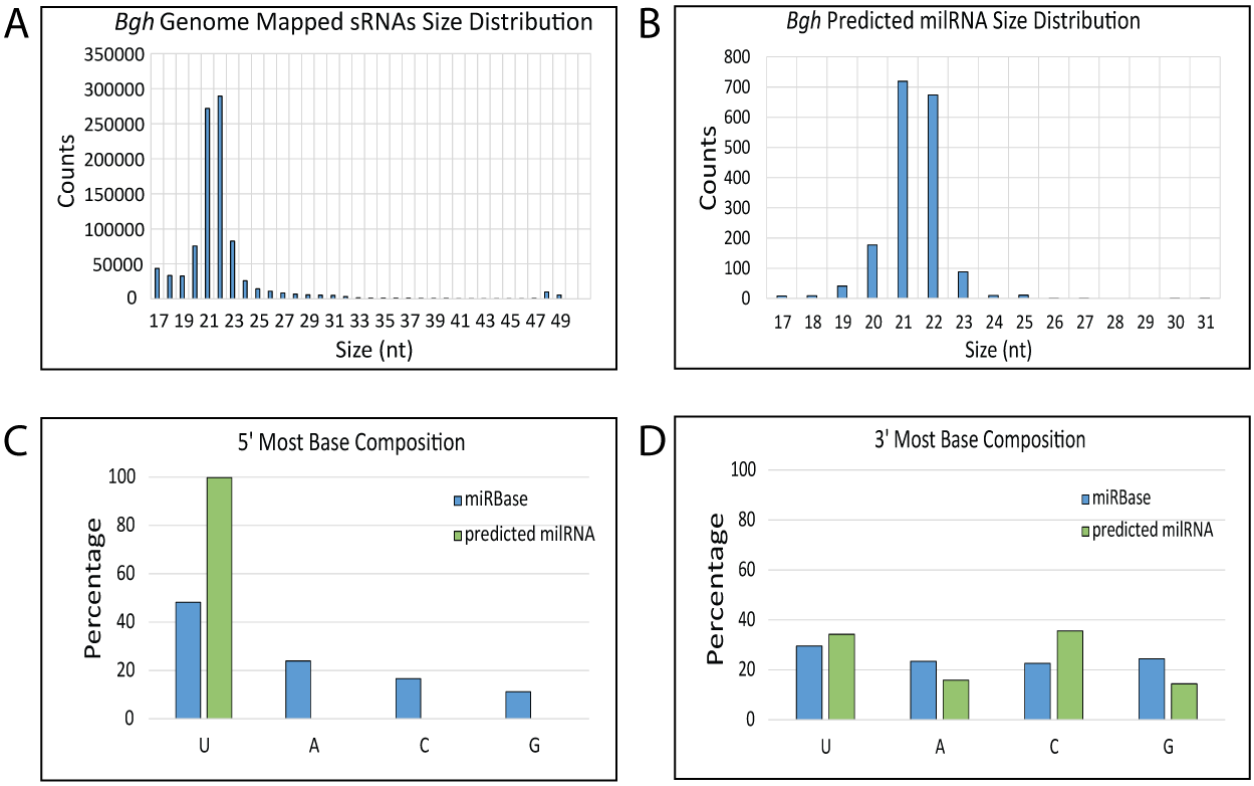


Figure 2.2 Size and base distributions for *Bgh* genome mapped sRNAs and milRNA candidates. (**A**) Size Distribution for sRNAs mapped to the *Bgh* genome. (**B**) Size distribution of *Bgh* milRNA candidates. (**C**) Frequency of 5'-most base of milRNA candidates (green) compared with mirBase (version 21) 5'-most base (blue). (**D**) Frequency of 3'-most base of milRNA candidates (green) compared with mirBase (version 21) 3'-most base (blue).

reads at 48 HAI than any other condition suggesting a large shift in sRNA regulation at that time point. The number of *Bgh* genome mapped sRNAs was not significantly different between compatible and incompatible lines at 48 HAI, suggesting that the peak of DE sRNAs at 48 HAI is unrelated to relative biomass of *Bgh* in incompatible vs. compatible interactions (**Figure 2.3)**. This suggests that *Bgh* is heavily regulating gene expression at the post-transcriptional level in a developmentally timed manner.

In silico *Predictions Suggest milRNA Candidates and DE Genome-Mapped Reads Regulate Effector Gene Expression*

Predicted transcript targets of the DE milRNA candidates and genome mapped sRNAs were identified using the web server-based program psRNATarget (http://plantgrn.noble.org/psRNATarget/). The psRNATarget program models miRNA target sites both by target site complementarity, as well as the accessibility of the target site to the sRNA. The program can also make predictions whether transcript cleavage or translational inhibition are more likely according to their model. Using *in silico* predictions for sRNA targets, without verification can have pitfalls, including high false positive rates of up to 90% (Zhai *et al.*, 2014). However, if used carefully and with these caveats in mind, useful biological data can be extracted from these results. Of the 268 DE milRNA candidates, 78 (29.1%) are predicted to have targets in the Ensembl *Bgh* transcriptome (release 36) by psRNATarget. These predicted transcript targets were compared with the database of predicted *Bgh* transposable elements (TEs) from Amselem et al. (2015a) using BLASTn, and 75 of the 78 (96.2%) had BLASTn e-values of less than 1e-15, indicating the predicted targets have a high level of similarity to known TEs. The DE *Bgh* genome mapped sRNAs were also compared with the Ensembl *Bgh* transcriptome, and 1,315 of 13,311 (9.9%) reads had 702 predicted targets (data not shown). These predicted transcript targets were also compared with the predicted *Bgh* TEs using BLASTn and 274 of 702 (39.0%) transcripts had homology to predicted *Bgh* TEs at an e-value cut-off of 1e-15. The milRNA predicted target transcripts were functionally annotated and categories of interest with

Table 2.1 Number of differentially expressed *Bgh* genome mapped sRNAs as compared to wildtype (CI 16151) at 0 to 48 hours after inoculation

Genotype	Time Point	Positive DE	Negative DE
bln1 (m19089)	0	0	0
mla6 (m18982)	0	1	0
rar3 (m11526)	0	0	0
mla6 + bln1 (m19028)	0	0	0
bln1 (m19089)	16	0	1
mla6 (m18982)	16	0	17
rar3 (m11526)	16	0	5
mla6 + bln1 (m19028)	16	0	2
bln1 (m19089)	20	0	22
mla6 (m18982)	20	15	28
rar3 (m11526)	20	0	40
mla6 + bln1 (m19028)	20	1	13
bln1 (m19089)	24	0	2
mla6 (m18982)	24	2	4
rar3 (m11526)	24	0	0
mla6 + bln1 (m19028)	24	0	2
bln1 (m19089)	32	0	2
mla6 (m18982)	32	3	26
rar3 (m11526)	32	0	0
mla6 + bln1 (m19028)	32	0	4
bln1 (m19089)	48	0	4
mla6 (m18982)[1]	48	8090	997
rar3 (m11526)	48	2433	285
mla6 + bln1 (m19028)	48	1257	55

high percentages include effectors (33.3%), kinase/phosphatase (9.4%), cellular structure and function (5.8%), and metabolism (5.4%) (**Table 2.2**). The relatively high percentage of predicted effector targets (both EKA and CSEP types) may indicate a coordinated control of these

[1] Note that *mla6* at 48 HAI is has significantly more sRNAs than all other conditions (p-value of less than 0.001).

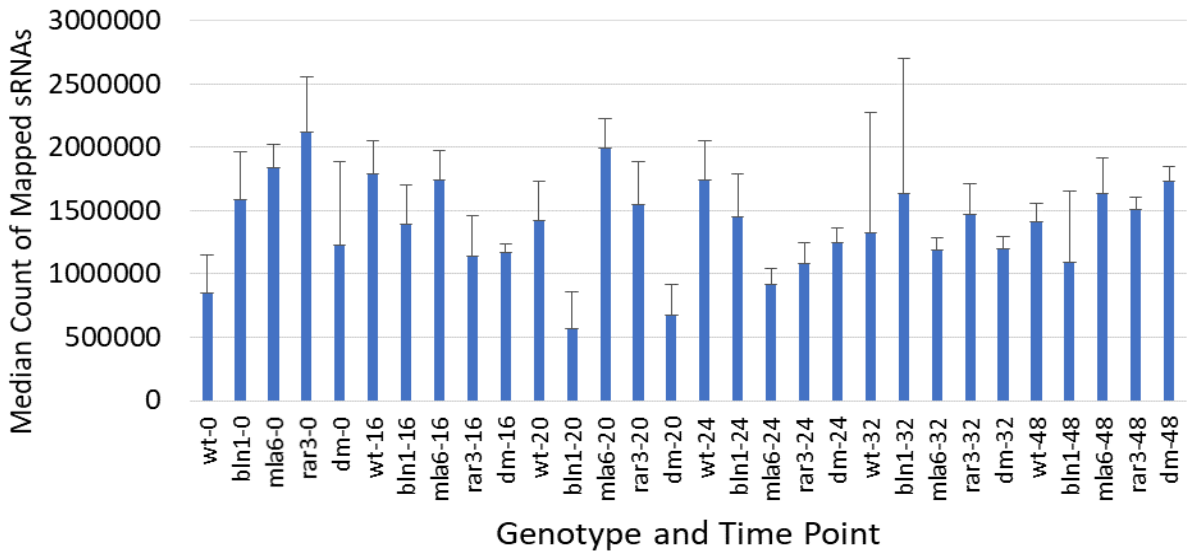


Figure 2.3 Median counts of *Bgh* genome mapped sRNAs for each barley line and time point combination. Reads were mapped to the *Bgh* genome with Bowtie, and median counts from all three replicates for each condition were compared via ANOVA analysis. The null hypothesis was not rejected that the median values are not statistically different with an alpha of 0.05. Standard error bars are shown for each condition.

transcripts at 48 HAI in the *Bgh* strain 5874 in our study. This suggests a developmental transition that may require the function of sRNAs to regulate gene expression during and perhaps after the 48 HAI time point.

PARE-Validated milRNAs and Bgh *Genome-Mapped Reads Target Genes in Effector Function and Metabolic Control*

Traditionally, validating target predictions was carried out one at a time with the 5' RACE technique; however, the PARE method provides a way of surveying transcript cut sites in a high throughput manner *in vivo*(German *et al.*, 2008). The reads in PARE libraries represent a

Table 2.2 Functional annotation of psRNATarget and PARE predicted *Bgh* sRNA transcript targets

Functional Category	psRNATarget Predictions	PARE-Validated Targets
Effector	33.3	19.5
Metabolism	5.4	14.8
Hypothetical/Unknown	17.4	13.4
Translation-related	2.6	12.1
Signaling	11.0	7.4
Transporter	2.3	6.7
Cellular Structure/Function	5.8	6.0
Transcriptional Regulation	5.8	4.0
Protein Folding	0.6	4.0
Vesicle Transport	2.1	3.4
Protein Turnover	2.0	2.7
Energy-related	0.1	2.7
Post Translational Modification	0.6	2.0
Redox Control	0.3	1.3

distribution of cut 5' ends from poly-A containing transcripts. The sequenced PARE libraries in our study contained ~166 million raw reads that were filtered and mapped to the *Bgh* genome as described in **Figure 2.1B**. The two programs sPARTA and CleaveLand were used to analyze the PARE library sequencing data independently and identify sRNA/transcript pairs (Addo-Quaye *et al.*, 2009, Kakrana *et al.*, 2014). The output sRNA/transcript pairs were filtered using an adjusted p-value of less than 0.05 and a PARE category of less than 2 (reads were equal to the maximum for the target transcript).

The results of the filters included a total of 230 pairs, 192 PARE-validated milRNAs and 149 unique *Bgh* transcripts with high likelihood of regulation through transcript cleavage (**Supplemental Table 2.2** [Appendix]). The PARE validated targets were compared with the predicted *Bgh* TEs using BLASTn and 65 of 149 (43.7%) transcripts had homology to predicted

Bgh TEs at an e-value cut-off of 1e-15. Functional annotation of the target transcripts was accomplished using available Ensembl annotations, Interproscan annotation (version 5.15-54-0), and literature review (**Supplemental Table 2.3** [Appendix]). As with the psRNATarget annotations of highly expressed read targets seen in **Table 2.2**, effectors (EKA and CSEP type) are the largest functional category (19.5%) (**Table 2.2**). Other functional categories with higher percentages include metabolism (14.8%), translation-related (12.1%), and signaling (7.4%).

The effector category contains ten CSEP members and twelve members of the EKA family. Several of the predicted CSEP targets, including CSEP0008 (AVR_{a1}) and CSEP0196 (BEC1040), have published functions in *Bgh* pathology (Pliego *et al.*, 2013, Lu *et al.*, 2016). Several of the DE milRNAs regulate effector genes and are upregulated at 48 HAI. This may be related to a change in effector expression associated with a transition in lifestyle from primary infection to reproduction. Homologs of many CSEP and EKA effectors are only found in powdery mildews, and many are undergoing positive selection pressure (Amselem *et al.*, 2015, Bourras *et al.*, 2018). These properties indicate that they are both important to powdery mildew biology and subject to rapid evolution. In *Phytophthora sojae* the avirulence factor Avr3a is silenced by sRNAs, leading to infection of plants carrying the *R*-gene *Rps3a (Qutob et al., 2013)*. In a similar manner, the silencing of effector genes may allow selective escape of barley resistance factors.

Metabolic targets were spread across many facets of primary metabolism, such as amino acids, fatty acids, carbohydrates, and nucleic acids. This broad cross-section of metabolic gene targets indicates that *Bgh* may be controlling long-term metabolic flow with sRNAs in a similar fashion as plants and animals (Hartig *et al.*, 2015, Chien *et al.*, 2017). In one

example of metabolic control a transcript encoding a NAD(+)-dependent glutamate synthase is predicted to be cleaved by seven different sRNAs located at independent loci in the *Bgh* genome. Control of nitrogen metabolism is especially important as *Bgh* lacks enzymes related to the assimilation of nitrate (Spanu *et al.*, 2010). The translation-related category comprises many members that are either components of ribosomes or regulation translation. Control of translation components would allow active gene expression of infection related transcripts without the metabolic cost associated with protein production until they are needed in the infection process. Members of the signaling category include several kinases and calcium signaling-related proteins. Calcium signaling has been shown to be important for successful infection in plant fungal pathogens such as *Magnaporthe oryzae* (Nguyen *et al.*, 2008).

Regulation of EKA Family Members through Embedded PARE-Validated Hairpin RNA

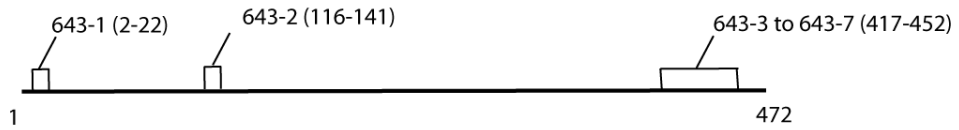
A hairpin forming precursor designated *Bgh*_Cluster_643, identified through the ShortStack program, encodes seven PARE-validated milRNAs that are predicted to target seven different *Bgh* transcripts (**Figure 2.4**). Three of these predicted targets encode effectors including two EKA family members and *CSEP0008*. The *CSEP0008* gene encodes the avirulence protein AVR_{A1} that is recognized by the R-protein MLA1 and was recently identified in *Bgh* using a transcriptome-wide association study (Lu *et al.*, 2016). One of the other *Bgh*_Cluster_643 encoded sRNA targets is the AVR_{a10}-like gene (BGHDH14_bgh06737). The AVR_{A10}-like protein is a member of the EKA effector family and has 861 homologs in the *Bgh* genome at a BLASTn e-value cut-off of 1e-100. The EKA effector family open reading frames are located within an active LINE-type TE, and are spread across the *Bgh* genome (Amselem *et al.*, 2015). Some EKA

family members actively encode peptides, but many are inactive. We identified 20 homologs of the *AVR*$_{a10}$-like gene (BGHDH14_bgh06737) that are encoded in genomic loci overlapping with a homolog of the hairpin precursor *Bgh*_Cluster_643 (BLASTn e-value cut-off of 1e-100) on the opposite strand (**Supplemental Table 3** [Appendix]). Each of these overlapping sequences have exact matching reverse complementary portions with non-overlapping overhangs as shown in **Figure 2.5.** The length of these overlaps and the hairpin nature of the *Bgh*_Cluster_643 homologs suggests a mechanism for control of these EKA family members in a manner similar to natural antisense miRNAs (nat-miRNAs) in plants (Lu *et al.*, 2008). The proposed model for regulation of EKA family members through and opposite-strand encoded hairpin RNA is shown for *Bgh*_Cluster_643 and an *AVR*$_{a10}$-like gene in **Figure 2.6.**

Differential Genic vs Non-Genic sRNA Mapping

We explored the mapping frequency of *Bgh* genome mapped sRNAs both inside and outside of predicted gene models. The supercontigs from the ensembl *Bgh* genome (v32) were divided into genic and non-genic portions, based on the predicted gene models, resulting in 6469 predicted gene segments, and 13311 non-genic segments. The average *Bgh* genome mapping sRNA density was 15.6 read/Kb for genic segments and 1767.6 for non-genic segments. In fact 84.6% of all predicted gene models had no mapped reads, as compared with 14.1% in non-genic segments. In many cases there are regions of high sRNA mapping upstream and downstream of predicted transcripts. There are exceptions to this general trend, as demonstrated in with the *AVR*$_{a10}$-like gene (BGHDH14_bgh06737) and the 20 homologs with predicted overlapping hairpins. These potential EKA family member genes have a predicted mapping density of 4702.7 read/Kb, which can be explained by the presence of the hairpin

A



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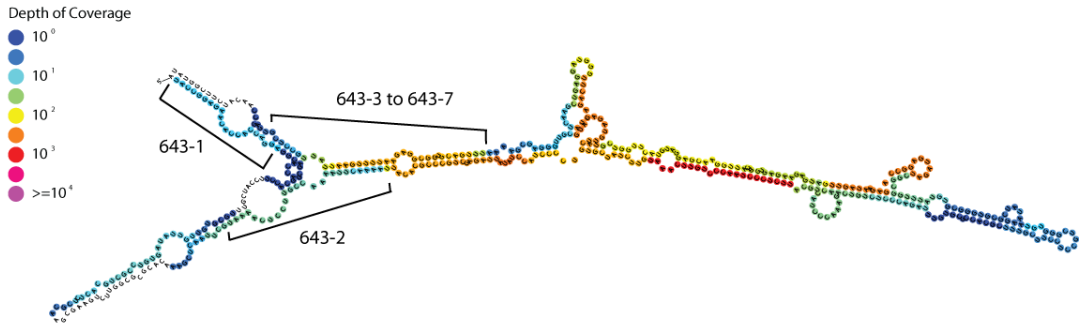

C

milRNA short name	*Bgh* _Cluster_643 location	milRNA target	Predicted cut site	Mismatches/Gaps
643-1	2-22	Zinc finger transcription factor	3646	0
643-2	116-141	AVR_{A10}-like protein	215	0
643-3	417-442	EKA-like_protein	89	7
643-4	424-444	CSEP0008 effector protein (AVR_{A1})	430	5
643-5	424-442	CSEP0008 effector protein (AVR_{A1})	430	4
643-6	424-445	CSEP0008 effector protein (AVR_{A1})	430	5
643-7	434-452	Mitochondrial C1-tetrahydrofolate synthase	2555	5

D

643-1 Transcript Alignment

```
Transcript  3636  TCCTGGTGGTGTTCTCCGGTA  3656
                  |||||||||||||||||||||
643-1       21    TCCTGGTGGTGTTCTCCGGTA  1
```

643-2 Transcript Alignment

```
Transcript  199  ATTTTGAATTTTGGCAAGGAGTTTTA  224
                 ||||||||||||||||||||||||||
643-2       26   ATTTTGAATTTTGGCAAGGAGTTTTA  1
```

643-3 Transcript Alignment

```
Transcript  98  TTTGACGTGGTCGGAGAAGATTTGGA  73
                |||||||||| |||||||  ||
643-3       1   TTTGACGTGG-CGGAGAATTTTGAAT  25
```

643-4 Transcript Alignment

```
Transcript 440  GGGCGTAGTATTTGGAATCA  420
                 |||| || |||| |||| |
643-4      1    TGGCGGAGAATTTTGAATTA  20
```

643-5 Transcript Alignment

```
Transcript  440  GGGCGTAGTATTTGGAAT  423
                  |||| || |||| ||||
643-5       1    TGGCGGAGAATTTTGAAT  18
```

643-6 Transcript Alignment

```
Transcript  440  GGGCGTAGTATTTGGAATCAA  420
                  |||| || |||| |||| |
643-6       1    TGGCGGAGAATTTTGAATTAT  21
```

643-7 Transcript Alignment

```
Transcript  2566  TTCCGAATCCTTGACTCG  2549
                  ||  ||||  ||| ||||
643-7       1     TTTTGAATTATTGCCTCG  18
```

Figure 2.4 *Bgh*_Cluster_643 structure and encoded PARE-validated milRNAs. (**A**) Linear representation of *Bgh*_Cluster_643 with milRNA encoding regions for 643-1 to 643-7 highlighted. (**B**) RNAfold predicted *Bgh*_Cluster_643 structure with sRNA mapping density scale from blue (no coverage) to purple (>=10^4 mapping reads) outputted from the ShortStack program. (**C**) Details of *Bgh*_Cluster_643 predicted milRNAs including name, location on *Bgh*_Cluster_643, predicted transcript target annotation, and number of mismatches/gaps in transcript alignment. (**D**) Alignments of each predicted milRNA to their respective predicted transcript targets with cut sites represented by red arrows.

sequences located on the opposite strand to the EKA gene homologs. As an example, the transcript RNA-seq mapping data, along with sRNA-seq mapping data is shown for *AVR*$_{a10}$-like gene (BGHDH14_bgh06737) and its immediate downstream neighboring gene encoding a lanosterol synthase (BGHDH14_bgh00862) is shown in **Figure 2.7**. The lanosterol synthase encoding gene has 0 mapped sRNA-seq reads, while the *AVR*$_{a10}$-like gene has over 4300 mapped sRNA-seq reads. The functional significance of the sRNA mapping frequencies inside and outside of genic regions is unclear, but one possible explanation is active silencing mechanisms functioning on transposable elements that surround areas of active transcription.

Discussion

In this study we sought to understand how *Bgh* sRNAs affects fungal gene expression during infection of the barley host. To address this question we compared the sRNA expression of *Bgh* isolate 5874 across five barley lines with 6 time points from 0 to 48 HAI and three replications for a total of 90 sRNA sequencing libraries. These libraries contained ~2.8 billion reads that were filtered and mapped to the *Bgh* genome. Two independent approaches were taken to identify potentially biologically important sRNAs. First, plant rules-based miRNA prediction programs were used to predict *Bgh* candidate milRNAs and second, reads were identified that mapped exactly to the *Bgh* genome, had at least ten counts across all libraries, and were DE in at least one line compared to wild type during at least one time point. These two approaches yielded 1741 milRNA candidates and 13,311 DE *Bgh* genome mapped sRNAs. The collection of

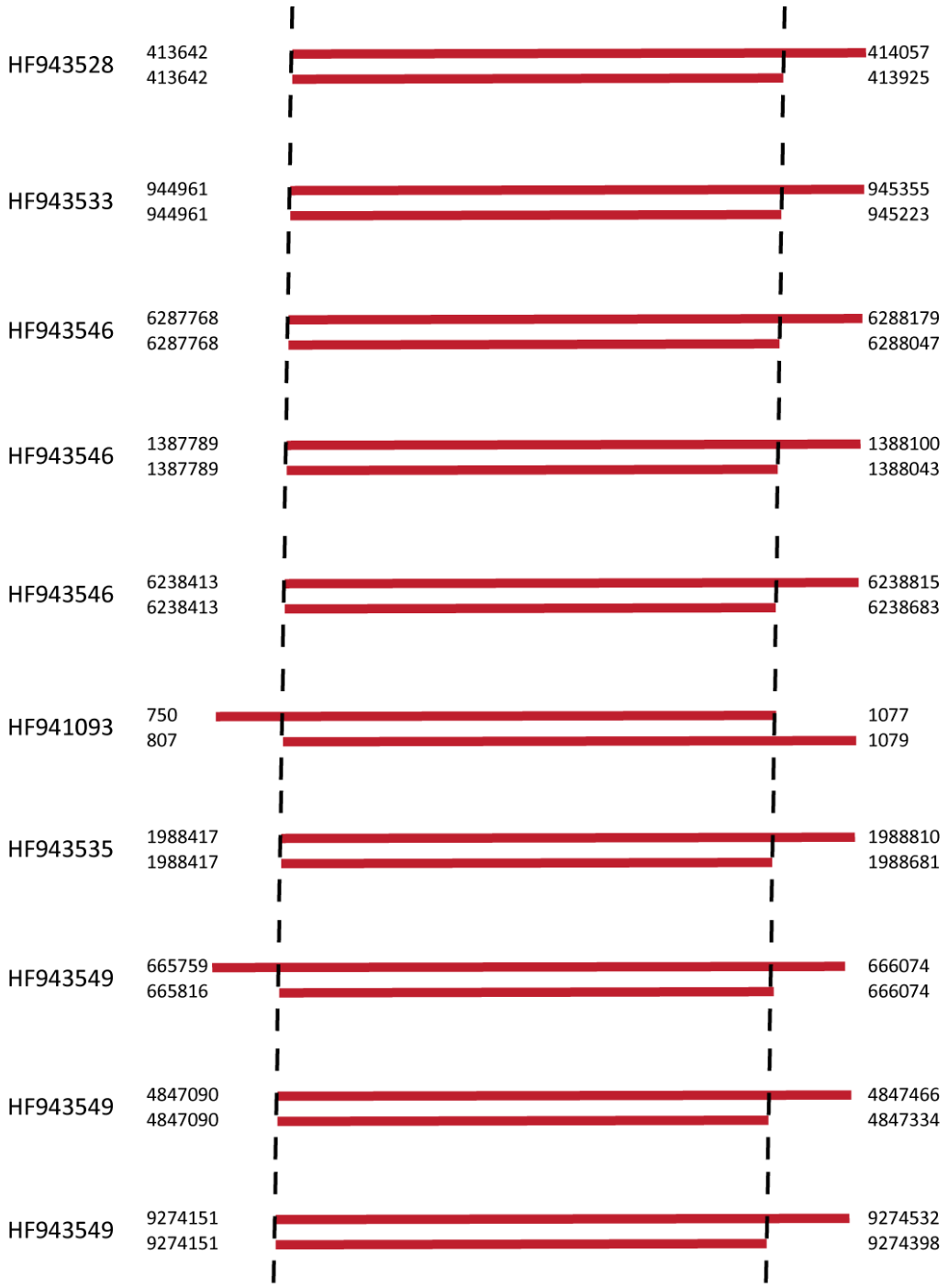

Figure 2.5 Overlapping portions of 20 *AVR*$_{a10}$-like gene homologs with *Bgh*_Cluster_643 homologs. The top line in the diagrams represent the *Bgh*_Cluster_643 homolog-encoding strand, while the lower line represents the *AVR*$_{a10}$-like gene homolog. Exactly overlapping positions are denoted with dashed black lines. *Bgh* supercontigs are listed on the left of each overlap diagram and start and stop portions of the genomic sequences are shown to the left and right of each genomic strand.

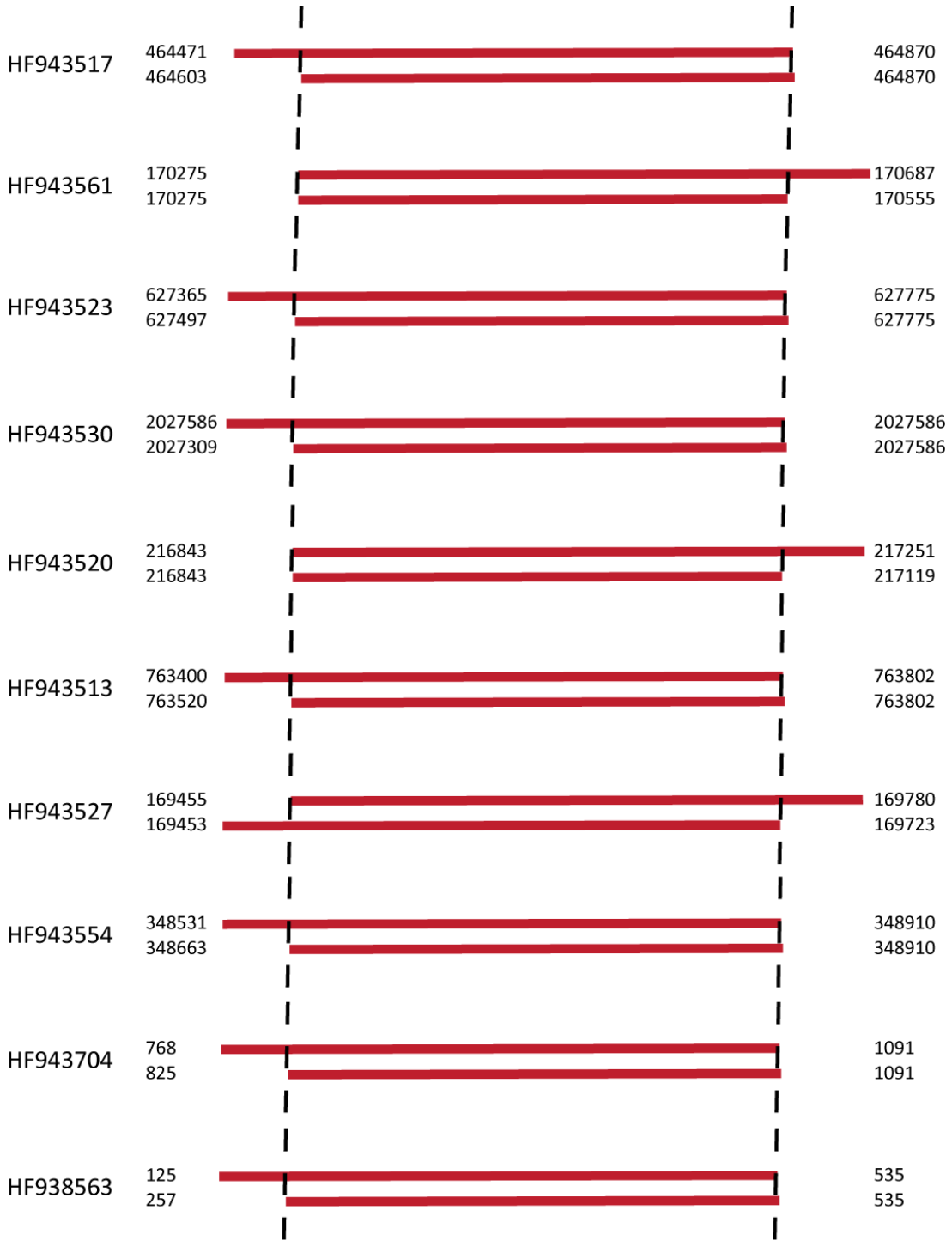

Figure 2.5 (Continued)

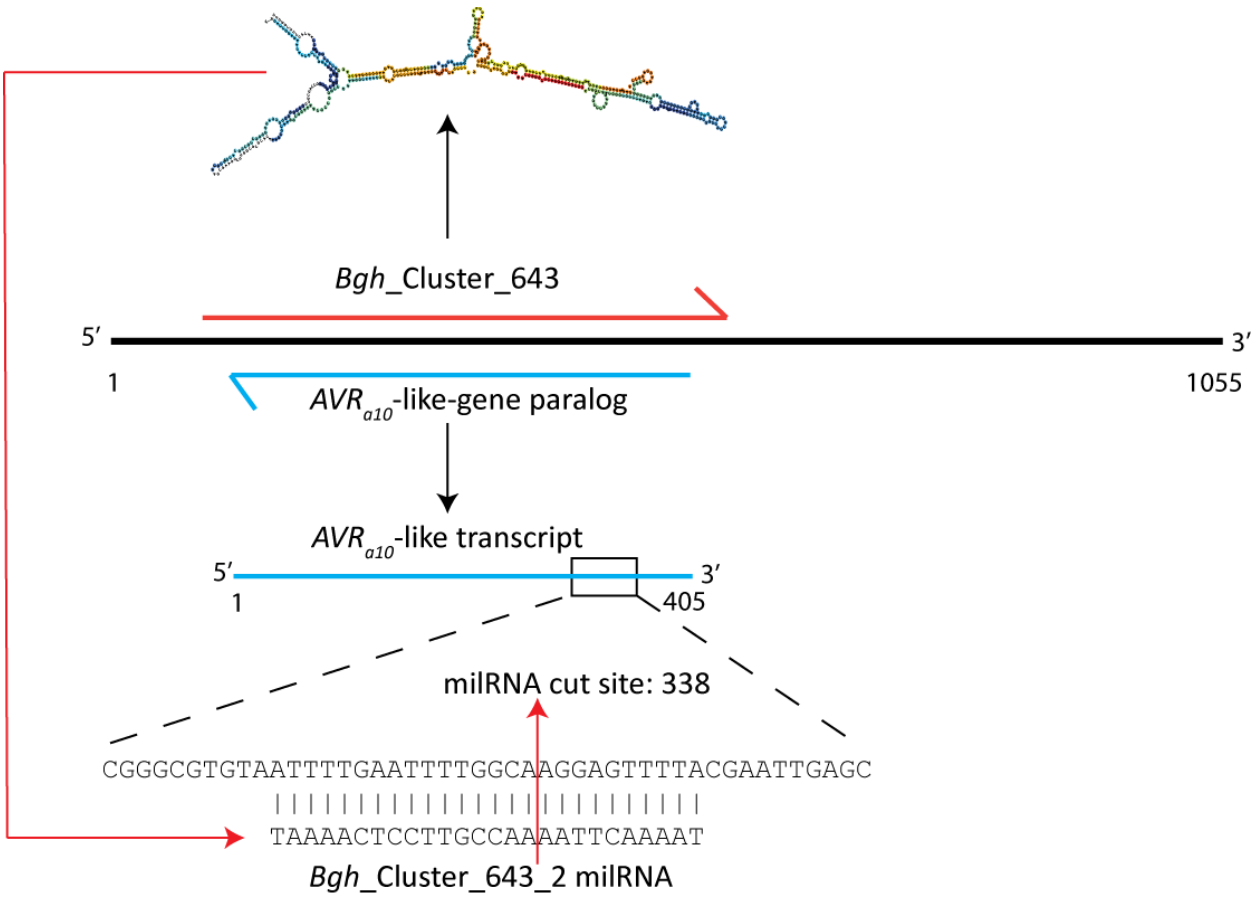

Figure 2.6 *Bgh* genome supercontig HF944340 encodes both a natural antisense siRNA (natsiRNA) transcript as well as a member of the EKA effector gene family. The *Bgh*_Cluster_643 natsiRNA transcript is processed into several milRNAs candidates including *Bgh*_Cluster_643-6. The EKA transcript (BGHDH14_bgh06737) is encoded antiparallel to the hairpin and is transcribed and targeted for transcript cleavage by *Bgh*_Cluster_643-2.

predicted milRNA candidates may not represent the complete and accurate pool of milRNAs from *Bgh* as two plant rule-based programs were used to identify these candidates. However, because of a lack of knowledge of fungal specific rules in the community, the plant rules programs can be used.

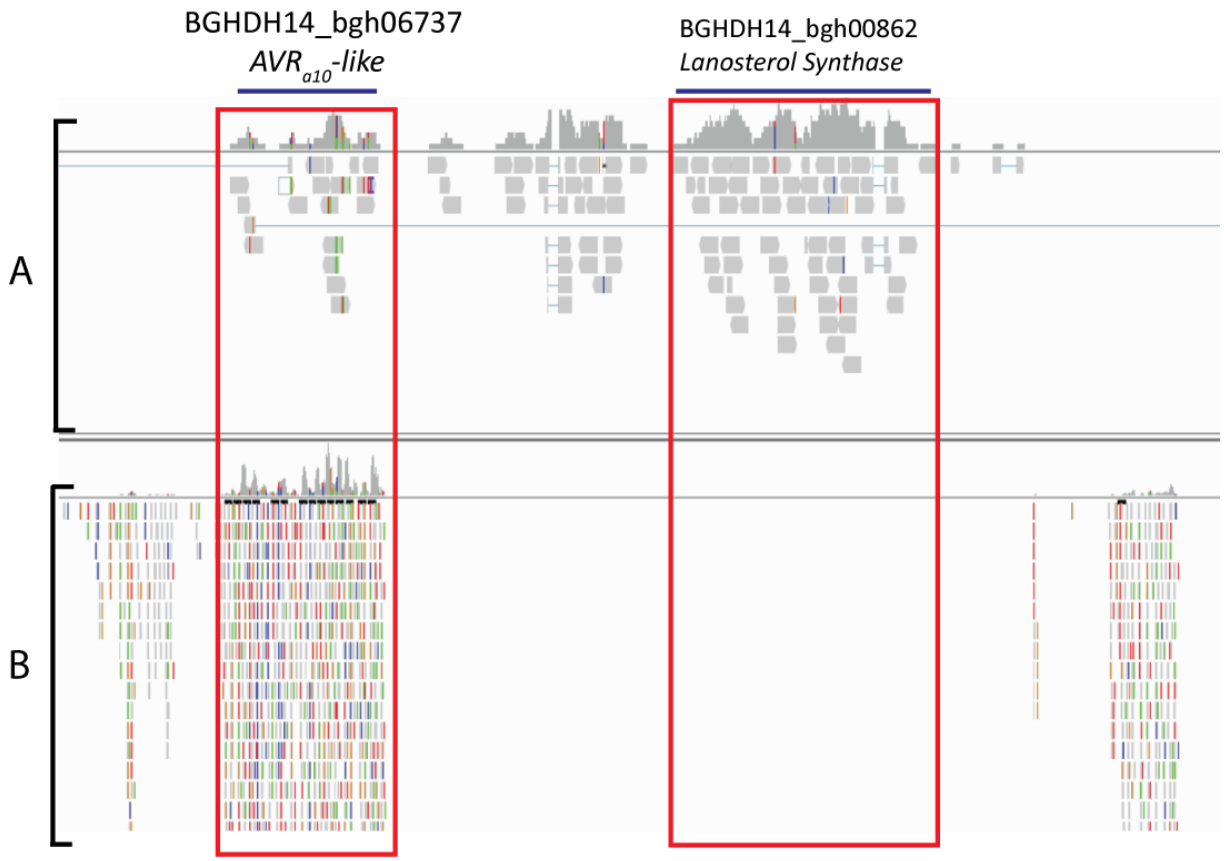

Figure 2.7 Transcript and sRNA sequencing reads mapped to *Bgh* genome positions near BGHDH14_bgh06737 and BGHDH14_bgh00862. The gene transcript models are highlighted with the blue lines, while the transcript and sRNA reads for each gene are highlighted with the red boxes. (**A**) Transcript based RNA-seq reads mapped to the *Bgh* genome. (**B**) sRNA based RNA-seq reads mapped to the *Bgh* genome.

The size distribution of milRNA candidates and *Bgh* genome mapped sRNAs ranged mainly from 21 to 24 nucleotides, with a peak at 21 and 22 nucleotides. The distribution seen in Figure 3A-B is quite similar to some studies with peaks at 22 and 23 nucleotides (Lin *et al.*, 2015), although other studies have a strong peak between 20 and 22 nucleotides (Lau *et al.*, 2013, Chen *et al.*, 2014, Meng *et al.*, 2017). This size distribution may be species or lineage specific as the production of different sizes of small RNAs can fall outside this range as well (Chen *et al.*, 2015, Yang, 2015). The ranges in size distributions for various fungal species reflects the relative lack of conservation of sRNA synthesis pathways between different types of

fungi (Torres-Martinez and Ruiz-Vazquez, 2017). The base distribution of the 5'-most base in many fungal milRNAs has a strong bias towards uracil nucleotides as is seen in this study (Figure 3C) (Lee *et al.*, 2010, Zhou *et al.*, 2012). Plant miRNAs that are loaded into Argonaute1 (AGO1) almost universally have 5' uracils for compatibility with the enzyme binding pocket (Fang and Qi, 2016). A similar mechanism may be conserved in fungi, including *Bgh*. There are two copies of Dicer, two copies of Argonaute, and one copy of RNA-dependent RNA polymerase in the annotated *Bgh* genome, and functional evidence from HIGS experiments demonstrate an active RNAi mechanism in the fungus (Nowara et al., 2010).

Of the 268 DE milRNA candidates and 13311 DE *Bgh* genome mapped sRNAs, we found that 100% and 98.6%, respectively, were only DE at 48 HAI, and only in compatible interactions. This finding is curious, given that transcript DE studies from our group have identified significantly DE transcripts at every time point. This probably means that this huge wave of DE in *Bgh* sRNAs is related to a developmental transition in successful infections (*i.e.*, compatible interactions). This may be an important transition point in the infections, where *Bgh* is moving from nutrient acquisition and defense suppression towards secondary hyphal growth, reproduction, and a new wave of effector expression. This developmental stage change may require a different set of proteins for proper growth, and therefore a specific set of sRNAs is significantly upregulated in expression to quickly reduce target transcript levels.

To complement the sRNA sequencing data, we employed the parallel analysis of RNA ends (PARE) technique to authenticate predicted transcript cleavage sites *in vivo* for both the milRNA candidates and the DE *Bgh* genome mapped sRNAs. The PARE technique validates sRNA transcript targets by sampling transcripts with 5' cut sites in a high throughput manner.

In our analysis, we identified several highly enriched target annotation categories that are directly related to successful barley infection including effectors, metabolic genes, and translation-related genes (**Table 2.2**).

Fungal effector proteins in plant pathogens are vital for both reducing defense responses and nutrient acquisition. *Bgh* isolate DH14 has two effector types, CSEPs and EKAs, that have 722 and ~1350 copies each (Bourras *et al.*, 2018, Frantzeskakis *et al.*, 2018). The combination of these potential effector genes represent ~30% of the predicted genes overall for *Bgh*. *Bgh* effectors are especially important for successful infection of barley, as reducing expression of even a single effector can significantly affect pathogenicity (Zhang *et al.*, 2012, Ahmed *et al.*, 2015, Aguilar *et al.*, 2016). About 20% of all PARE-validated targets in our filtered set were effectors. These potential targets include AVR_{A1} , the cognate avirulence effector to barley Mla1 (Zhou *et al.*, 2001, Lu *et al.*, 2016); *CSEP0196*, an effector that when knocked down with host induced gene silencing (HIGS) results in significant reduction in *Bgh* pathogenicity (Pliego *et al.*, 2013); several additional CSEPs, and a dozen members of the EKA effector family. Differential regulation of these particular CSEP and EKA encoding genes at 48 HAI and after may be important in the transition from survival to reproduction.

Throughout the developmental cycle of *Bgh*, timed expression of metabolic genes is important for both survival and successful infection of barley. Key enzymes in fatty acid, nucleic acid, and amino acid biosynthesis along with nitrogen assimilation and one carbon metabolism are potentially controlled through PARE-validated milRNAs. Control of metabolism through miRNA expression has been shown extensively in plants and animals. Silencing gene expression post-transcriptionally through sRNAs may allow for rapid regulatory changes that immediately

reduce protein biosynthesis levels, as opposed to transcriptional gene silencing. One important example for metabolic control is glutamate synthase which is a key enzyme in nitrogen assimilation. Glutamate synthase is especially important in *Bgh* as many of the other enzymes in nitrogen assimilation are have been lost over evolutionary time (Spanu *et al.*, 2010). The glutamate synthase enzyme was recently shown to be important in *Magnaporthe oryzae* (*M. oryzae*) pathogenesis of rice (Zhou *et al.*, 2017). In the *M. oryzae* glutamate synthase knockouts, both appressorial penetration as well as hyphal spread was significantly reduced. In our study we identified seven separate PARE-validated milRNAs that cleave glutamate synthase transcripts. The nitrogen status of *Bgh* can vary greatly, depending on its infection status of barley. These milRNAs may allow *Bgh* to control the flow of nitrogen depending on its availability.

The translational regulation category represents a fascinating mix of translation initiation factors and ribosomal protein components. It appears that *Bgh* is directly controlling the post transcriptional activity of genes that encode vital components to ribosome structure and activity. PARE-validated milRNA targets include seven members of the ribosomal protein family. Ribosomal biosynthesis is a highly regulated process, and missing components of the pathway will stop ribosome production (Lafontaine, 2015). Under nitrogen-limiting conditions it may be necessary for *Bgh* to block new ribosome production in the conidiaspore while haustorial feeding sites are established. The control of new ribosome production along with limiting nitrogen assimilation machinery may allow for survival of conidiospores during early development when they rely on existing organelles and energy stores, similar to seeds in plants.

One of the milRNA encoding hairpins identified in this study is biologically significant for

three reasons. First, the hairpin *Bgh*_Cluster_643 encodes seven milRNA candidates that are predicted to target eight different *Bgh* genes for cleavage, including three effector proteins. Second, *Bgh*_Cluster_643 is encoded in an antiparallel orientation to one of its encoded milRNA predicted targets: AVR_{a10}-like gene (*BGHDH14_bgh06737*). We have identified 20 additional EKA family members that are highly similar to the *BGHDH14_bgh06737* gene that also encode hairpins highly similar to *Bgh*_Cluster_643. We propose a functional mechanism for these *Bgh*_Cluster_643 hairpin-forming homologs similar to natsiRNAs in plants. Plant natsiRNAs function as independent units of transcription that can both directly regulate the antiparallel *cis* transcripts, as well as other *trans* transcript targets (Ariel *et al.*, 2015). Although we were only able to identify 20 examples matching the EKA family, we believe that other similar examples will be found, especially in TE-related gene families. And third, the 20 genomic positions have significantly higher sRNA mapping density than other predicted genic positions in the genome. We found that the 20 hairpin positions have an average of 4702.7 read/Kb density, compared with the average genic positions of 15.6 read/Kb. This suggests that these positions are highly regulated by sRNAs.

Conclusions

Understanding regulation of gene expression can be especially challenging in obligate biotrophic fungal species, as most cannot be cultured, and therefore examined with traditional genetics techniques (gene knock outs/downs and overexpression). We sought to understand the post transcriptional regulation of *Bgh* genes by carrying out Illumina small RNA-sequencing on a panel of barley lines infected with *Bgh* over a 48 hour time course. We identified 192

PARE-validated milRNAs that target 149 *Bgh* transcripts through transcript cleavage. We propose that *Bgh* is controlling post transcriptional gene expression of effector, metabolic, and translation-related genes through hairpin-encoded milRNAs that are similar in structure to plant and animal pri-miRNAs. Our data suggests that several members of the EKA effector family are regulated in a post transcriptional fashion through natsiRNAs-like hairpins encoded antiparallel to EKA family members. Increasing our knowledge of post-transcriptional gene expression regulation in *Bgh* opens up a deeper understanding of developmentally-timed gene and protein expression patterns, and therefore gives us insight into *Bgh* pathogenicity.

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CHAPTER 3. EXPRESSION OF BARLEY MIRNA AND PHASIRNA IN RESPONSE TO INFECTION BY THE POWDERY MILDEW PATHOGEN

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Short Title

Bgh-induced barley phasiRNA and miRNA expression

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One-Sentence Summary

Expression of barley leaf phasiRNA and miRNA induced by infection with *Blumeria graminis* f. sp. *hordei* .

Author Contributions

MH contributed to project development, data analysis and interpretation, and wrote the manuscript with input from RW; SB contributed to data analysis; PS contributed to data analysis; ML contributed to statistical analyses; GF performed small RNA sequencing experiments; SM performed PARE sequencing experiments; BM contributed to PARE sequencing experiments ; DN contributed to statistical analyses; RW contributed to project conception, development, data interpretation, and preparation of the manuscript.

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Footnotes

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Abstract

Small RNAs (sRNAs) in plants play key roles in regulating defense responses during pathogen infection. Both micro RNAs (miRNAs) and phasing small interfering RNAs (phasiRNAs) directly regulate plant defense responses through post-transcriptional gene silencing. The timing and intensity of pathogen defense responses are carefully controlled as they divert resources from growth and development. We sought to understand how barley leaves respond to infection by the biotrophic pathogen *Blumeria graminis* f. sp. *hordei* (*Bgh*) through miRNA and phasiRNA expression. We identified 21, 22, and 24 nucleotide (nt) phasiRNA loci with significant overlap with protein encoding genes, in contrast to previous studies in grasses, that showed overlap mainly with long non-coding RNAs (lncRNAs). Receptor kinase genes are significantly over-represented as targets of phasiRNAs, which may indicate a novel defense control mechanism in barley. From sRNA sequencing data we also identified 2423 differentially expressed (DE) barley genome mapping sRNAs along with 9 DE predicted miRNAs. Small RNAs with homology to several conserved miRNAs were overexpressed in the ETI-compromised *mla6* mutant, which has reduced defense responses to *Bgh* infection. PARE validation of barley sRNA transcript

targets identified transcripts predominantly involved in transcriptional regulation and signaling. These results indicate both phasiRNA and miRNAs are involved in barley response to *Bgh* infection.

Introduction

Small RNAs (sRNAs) in plants play key roles in regulating development, metabolism, and response to both abiotic and biotic stress (Martinez de Alba *et al.*, 2013). The expression of pathogen response proteins is carefully controlled through sRNAs and other mechanisms to allow full growth potential during non-infection conditions and with a switch to defense during pathogen challenge (Park and Shin, 2015). Plants have an evolved an innate immune system that allows them to prevent infection from many potential pathogens. The plant immune system is triggered by pathogen associated molecular patterns (PAMPs) such as chitin from fungi or flagellin from bacteria. These PAMP molecules are recognized by receptor-like kinases that trigger a signaling cascade initiating PAMP triggered immunity (PTI) (Zipfel, 2014). The PTI response can include an increase in reactive oxygen species, a buildup of wall materials near the site of infection, and production of anti-microbial compounds such as proteases (Kuan *et al.*, 2016). Pathogens in turn, have evolved effector molecules that mitigate the PTI response through multiple mechanisms. Effectors reduce the strength of PTI allowing pathogens to successfully infect the plant, thereby creating effector triggered susceptibility (ETS). As a response to ETS, plants have evolved nucleotide binding leucine-rich repeat (NLR) proteins that either recognize effectors directly or indirectly that trigger the strong defense response effector triggered immunity (ETI) (Vleeshouwers and Oliver, 2014). The NLR proteins, encoded by *R*-

genes either directly bind effector molecules, or perceive effector action against guard proteins (Cui *et al.*, 2015). This binding triggers a strong immune response, commonly associated with a hypersensitive response and localized cell death.

Expression of defense-related genes is tightly controlled by sRNAs including miRNAs and siRNAs (Fei *et al.*, 2016b). Careful control of defense-related gene expression is important for overall plant health, as studies overexpressing *R*-genes show plants with reduced growth rates (Cheng *et al.*, 2011). Micro RNAs are non-coding hairpin forming RNA elements located in the genome that are transcribed by RNA polymerase II. These miRNAs guide sequence-specific transcript cleavage or translational inhibition of target transcripts as part of the RISC complex (Iwakawa and Tomari, 2015). Small interfering RNAs on the other hand are generally produced through the action of RNA-dependent RNA polymerase (RDRP) that produces double stranded RNA from single stranded templates. These double stranded RNAs are processed by Dicer-like (DCL) to produce 20-25 nucleotide siRNAs including heterochromatic siRNAs (hetsiRNAs), natural antisense RNAs (natsiRNAs), and phased siRNAs (phasiRNAs) (Borges and Martienssen, 2015). These siRNA types are involved in gene silencing at the transcriptional- (hetsiRNAs) and post-transcriptional levels (natsiRNAs and phasiRNAs), respectively (Ariel *et al.*, 2015, Holoch and Moazed, 2015).

Several miRNA families are involved in regulating plant responses to pathogen infection (Baldrich and San Segundo, 2016). The targets of these miRNAs are involved in both PTI and ETI responses. The PTI-related pathways regulated through miRNAs include hormone signaling, reactive oxygen species evolution, callose deposition, and others (Kuan *et al.*, 2016). Auxin signaling is carefully controlled during plant development and can be down regulated during

pathogen infection such as with miR393 that downregulates auxin F-box receptors during a PTI response to infection (Navarro *et al.*, 2006). Callose deposition related to PTI response has both positive regulators such as miR160 and negative regulators such as miR398 and miR773 (Baldrich and San Segundo, 2016). The ETI pathway is regulated through miRNA control of *R*-gene expression. Micro RNAs from several species including *Medicago truncatula*, soybean, tomato, potato, and tobacco have been shown to regulate *R*-gene expression (Fei *et al.*, 2016b). The regulation of these *R*-gene-encoded transcript targets through miRNAs does not however, lead to simple transcript cleavage in many cases. Rather the cleaved transcripts are targets for production of phased small interfering RNAs (phasiRNAs). These phasiRNAs can lead to silencing of hundreds of *R*-gene transcripts (Fei *et al.*, 2015).

The occurrence of phasiRNAs was first observed in *Arabidopsis* with a type of phasiRNA called trans-acting small interfering RNAs (tasiRNAs) (Yoshikawa, 2013). Unlike most phasiRNAs, tasiRNAs are usually encoded on long non-coding RNA templates. The miRNA cleaved templates are reverse transcribed into double stranded RNA by RNA dependent RNA polymerase and cleaved into 21 nt phased small RNAs. Four families of *TRANS ACTING siRNA* (*TAS*) genes have been identified in *Arabidopsis* including *TAS1, TAS2, TAS3,* and *TAS4* (Fei *et al.*, 2013). These phasiRNA then act in *trans* against targets including transcripts encoding auxin response factors, pentatricopeptide repeat proteins, and MYB transcription factors (Allen *et al.*, 2005, Axtell *et al.*, 2006, Rajagopalan *et al.*, 2006). *TAS3* is the most highly conserved member of the *TAS* family and is found in plant species ranging from mosses, gymnosperms, to grasses (Borges and Martienssen, 2015). Grasses have a much larger set of tasiRNAs then found in dicots (Arikit *et al.*, 2013). These tasiRNAs are largely encoded on long non-coding transcripts

expressed in reproductive tissues, and are 24 bases in length as opposed to almost all dicot phasiRNAs, which are 21 bases in length. Very few phasing loci have been reported in non-reproductive tissues in monocots with few exceptions (Liu *et al.*, 2014).

We sought to identify barley sRNAs expressed during barley powdery mildew infection, their transcript targets, and phasing loci in leaf tissue. Small RNA populations were isolated and sequenced from barley leaves infected with *Bgh* during a time course infection from 0 to 48 hours after inoculation (HAI) in five barley genotypes represented by the CI 16151 progenitor (harboring the *Mla6* powdery mildew *R*-gene) and four fast-neutron derived immune signaling mutants (Meng *et al.*, 2009b, Xi *et al.*, 2009). Barley miRNA candidates were validated with parallel analysis of RNA ends (PARE) analysis resulting in identification of conserved PTI-related miRNA families as well as novel miRNA candidates predicted to target transcripts involved in transcriptional regulation and signaling. PhasiRNA loci were identified in *Bgh* infected barley leaves that overlap with protein-encoding transcripts encoding a mix of functional categories including signaling, metabolism, transcriptional regulation, and defense.

Results

Identification of Conserved and Novel Barley miRNAs

To identify sRNAs expressed during *Bgh* infection of barley leaves, seedlings from the lines CI 16151 (*Mla6*) [WT], m18982 (*mla6*), m11526 (*rar3*), m19089 (*bln1*), and m19028 (*mla6* + *bln1*) were infected with *Bgh* isolate 5874 over a time course from 0 to 48 hours after inoculation in three separate replicates for a total of 90 samples. Illumina small RNA libraries produced from these samples resulted in ~2.8 billion reads of total sequencing data. The full analysis pipeline

for the sRNA data is shown in **Figure 3.1**. Briefly the reads were filtered for both quality and to remove known RNA motifs including tRNAs, rRNAs, snoRNAs snRNAs and known barley repeats. The filtered reads were taken through two independent methods to identify miRNAs candidates and barley genome mapped sRNAs. To carry this out the plant-rule-specific miRNA prediction programs miRDeep-P and ShortStack were used to predicted miRNA candidates. Independently the filtered reads were filtered for exact barley genome mapping and a minimum count of ten total counts across the 90 sRNA-seq libraries. The miRDeep-P and ShortStack programs identified 1216 and 209 predicted miRNAs, respectively, for a total of 1425. The separate count filter pathway identified 1,980,623 reads that mapped exactly to the barley genome and had a minimum count threshold to identify reads with above background expression levels.

Analysis of the size distributions of both the barley genome mapped sRNAs and the predicted miRNAs revealed size distributions that were incongruous with expected peaks of 21 and 24 bases as seen in other grasses (Nobuta *et al.*, 2008). The unadjusted size distributions had peaks of 22 and 25 base pairs (**Figure 3.2A**), which may be explained by either a library preparation effect or partially degraded sample (Xie *et al.*, 2015).

The 3' most base of the predicted barley miRNAs from our data were >90% Uracils, as opposed to miRBase barley miRNAs with ~20% Uracils (**Figure 3.2B**). Therefore we removed that 3' most base from our reads and the corrected size distributions and base composition as seen in **Figure 3.3**. The adjusted size distributions of both the barley genome mapped sRNAs,

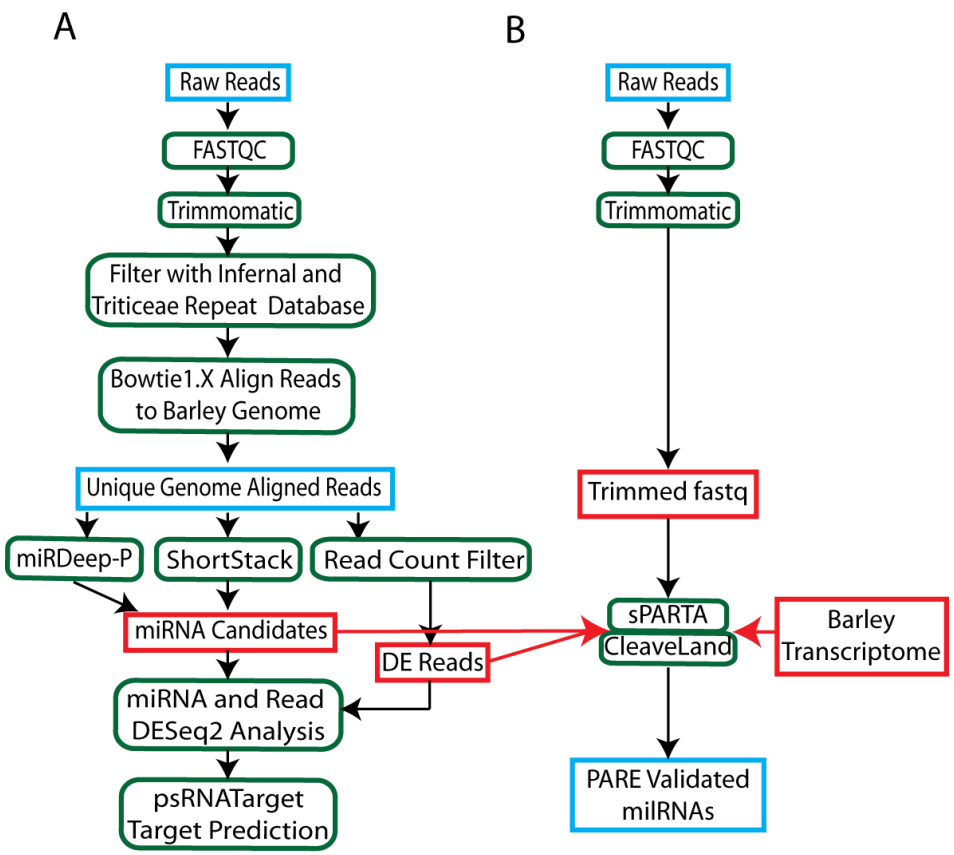
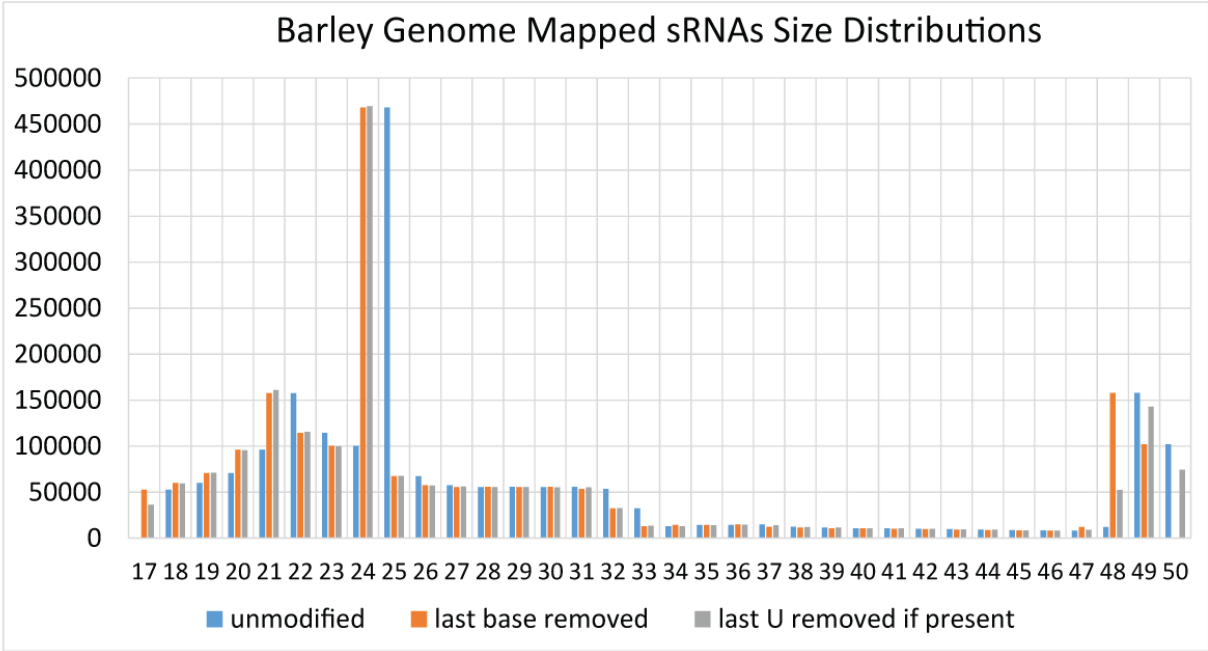


Figure 3.1 Small RNA sequencing and PARE sequencing analysis pipelines.Small RNA-seq Illumina reads were trimmed, filtered, and run through the two plant miRNA identification programs, miRDeep-P and ShortStack, to identify milRNA candidates and DE sRNA reads. (**B**) Sequencing reads from the PARE libraries were trimmed and filtered and analyzed with the sPARTA and CleaveLand programs. Additional input data was provided from the *Bgh* transcriptome and milRNA candidates plus DE sRNA reads developed from the sRNA sequencing pipeline.

as well as the predicted miRNAs have peaks at 21 and 24 bases, as would be expected for plant sRNA distributions (**Figure 3.3A**). The 5' and 3' most base distributions are for the most part similar between the known miRBase barley miRNAs and those predicted in this study, with some exceptions, which may be explained by a larger pool of predicted miRNAs (1425) as

A



B

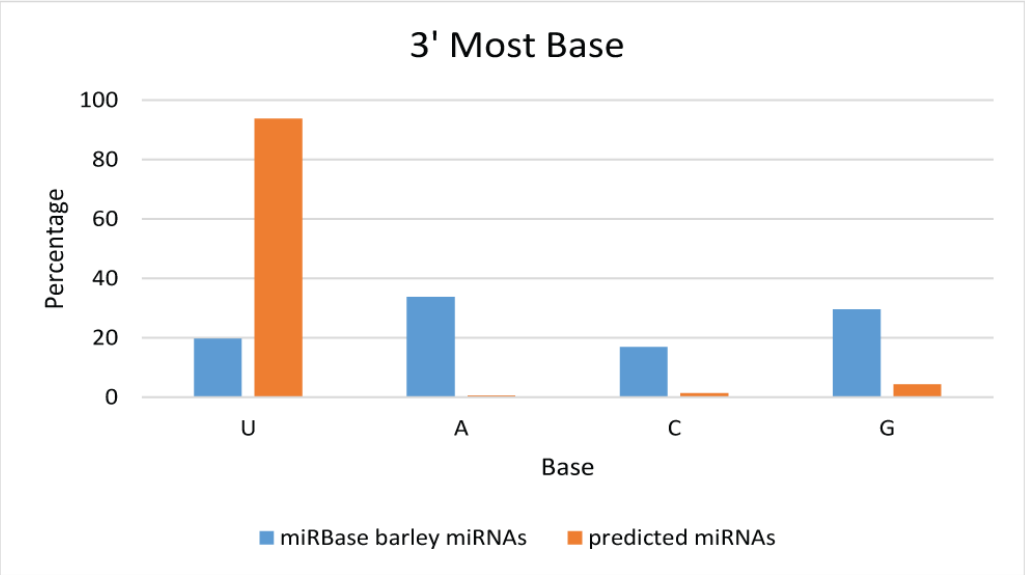


Figure 3.2 Size distributions and 3' most base frequencies for unadjusted barley genome mapped sRNAs and predicted miRNAs. (**A**) Barley genome mapped sRNAs that are unmodified (blue bars), last base removed (orange bars), or last uracil removed if present (grey bars).

opposed to miRBase listed barley miRNAs (71) (**Figure 3.3B** and **C**). In our pool of predicted barley miRNAs, 88 are homologous to 27 conserved plant miRNAs as seen in **Table 3.1.** The pool of miRBase (version 22) recognized barley miRNAs is small (72) compared with *Oryza sativa* (757), however there are many more conserved barley miRNAs published in the current literature (citations in **Table 3.1**). From the conserved miRNAs identified in **Table 3.1**, 17 have been identified as pathogen-responsive miRNAs (Kuan *et al.*, 2016).

Differential Regulation of Reactive Oxygen Species-Related Barley miRNAs

Differential expression (DE) of predicted miRNAs or barley genome mapped sRNAs at each time point were identified by comparing WT to the four mutant lines using the DESeq2 program (Love *et al.*, 2014). Out of 1425 predicted barley miRNAs, there are 730 unique sequences. Of these sequences, 9 (1.2%) are DE during at least one time point (**Table 3.2**). Out of the 9 unique sequences, 4 have homology to miRNA families including miR2120, miR398, and miR528. Both miR398 and miR528 have been linked to control of the reactive oxygen species (ROS) related genes *chloroplast copper/zinc superoxide dismutase 1* (*HvSOD1*) in barley and *L-ascorbate oxidase* (*AO*) in rice (Xu *et al.*, 2014, Wu *et al.*, 2017). The miRNA target site of rice *AO* (XM_015787755.1) from Wu et al. (2017) is located in the 3' UTR, and is not conserved in any barley *AO*, so it is unclear if barley miR528 overexpression in the *mla6* mutant is related to ROS regulation. However, several other studies have indicated that miR528 is involved in

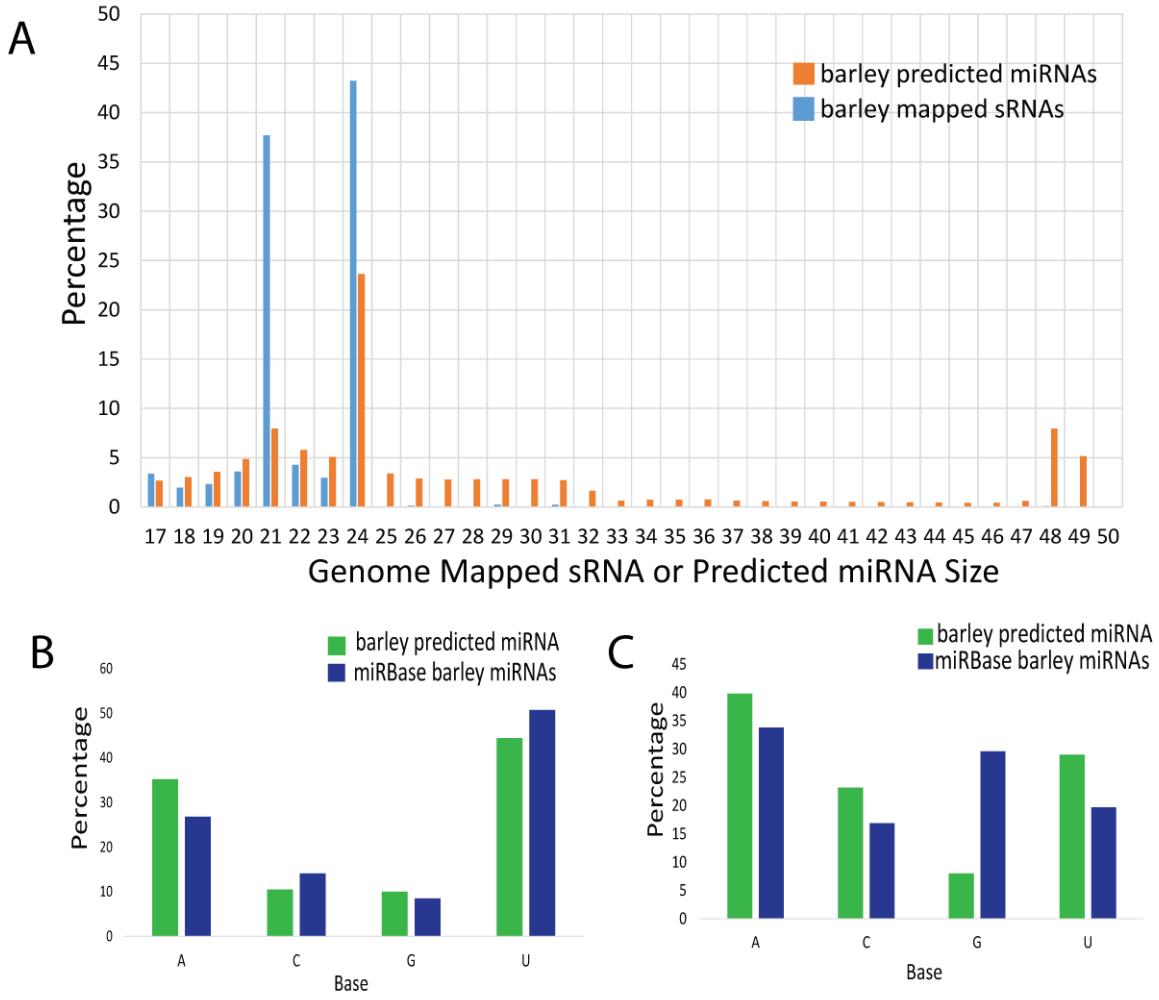


Figure 3.3 Size distribution, 5' end base composition, and 3' base composition of barley predicted miRNAs and barley mapped sRNAs. (**A**) Percentage size distribution of barley predicted miRNAs (orange) compared with barley mapped sRNAs (blue) from 17-50 nucleotides. (**B**) 5' end base composition percentage of barley predicted miRNAs (green) compared with miRBase barley miRNAs (blue). (**C**) 3' end base composition percentage of barley predicted miRNAs (green) compared with miRBase barley miRNAs (blue).

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Table 3.1 Conserved predicted barley miRNAs

Predicted barley miRNA	Predicted miRNA copies	alignment	miRBase (v22) support?	Barley literature support
miR156#	6	Predicted barley miR156 1 UGACAGAAGAGAGUGAGCAC 20 |||||||||||||||||||| osa-miR156 1 UGACAGAAGAGAGUGAGCAC 20	yes	Bai et al. 2017, Colaiacovo et al. 2010, Curaba et al. 2012, Fard et al. 2017, Hackenberg et al. 2012, Hackenberg et al. 2013, Hackenberg et al. 2014, Kantar et al. 2010, Ozhuner et al. 2013, Pacak et al. 2017, Schreiber et al. 2011, Wu et al. 2014
miR159#	4	Predicted barley miR159 1 UUUGGAUUGAAGGGAGCU 18 |||||||||||||||||| hvu-miR159b 1 UUUGGAUUGAAGGGAGCUCUG 21	yes	Bai et al. 2017, Colaiacovo et al. 2010, Curaba et al. 2012, Deng et al. 2015,Fard et al. 2017, Hackenberg et al. 2012, Hackenberg et al. 2013, Hackenberg et al. 2014, Kantar et al. 2010, Ozhuner et al. 2013, Pacak et al. 2017, Schreiber et al. 2011, Wu et al. 2014
miR160#	5	Predicted barley miR160 1 UGCCUGGCUCCCUGUAUGCCA 21 |||||||||||||||||||| osa-miR160 1 UGCCUGGCUCCCUGUAUGCCA 21	no	Bai et al. 2017, Colaiacovo et al. 2010, Fard et al. 2017, Kantar et al. 2010, Kruszka et al. 2014, Ozhuner et al. 2013, Schreiber et al. 2011, Wu et al. 2014
miR164#	5	Predicted barley miR164 1 UGGAGAAGCAGGGCACGUGCA 21 |||||||||||||||||||| osa-miR164 1 UGGAGAAGCAGGGCACGUGCA 21	no	Bai et al. 2017, Colaiacovo et al. 2010, Curaba et al. 2012, Deng et al. 2015, Fard et al. 2017, Hackenberg et al. 2012, Hackenberg et al. 2013, Ozhuner et al. 2013, Schreiber et al. 2011, Wu et al. 2014
miR165/miR166#	11	Predicted barley miR166 1 UCGGACCAGGCUUCAUUCCCC 21 |||||||||||||||||||| hvu-miR166a 1 UCGGACCAGGCUUCAUUCCCC 21	yes	Bai et al. 2017, Curaba et al. 2012, Fard et al. 2017, Ferdous et al. 2017, Hackenberg et al. 2012, Hackenberg et al. 2013, Hackenberg et al. 2014, Kantar et al. 2010, Kruszka et al. 2014, Ozhuner et al. 2013, Schreiber et al. 2011, Wu et al. 2014
miR167#	9	Predicted barley miR167 1 UGAAGCUGCCAGCAUGAUCUGA 22 |||||||||||||||||||||| ccl-miR167a 1 UGAAGCUGCCAGCAUGAUCUGA 22	no	Bai et al. 2017, Curaba et al. 2012, Deng et al. 2015, Fard et al. 2017, Hackenberg et al. 2012, Hackenberg et al. 2013, Kruszka et al. 2014, Pacak et al. 2017, Schreiber et al. 2011, Wu et al. 2014,
miR169#	6	Predicted barley miR169 1 UAGCCAAGAAUGACUUGCCUA 21 |||||||||||||||||||| osa-miR169n 1 UAGCCAAGAAUGACUUGCCUA 21	yes	Colaiacovo et al. 2010, Curaba et al. 2012, Deng et al. 2015, Ferdous et al. 2017, Hackenberg et al. 2012, Hackenberg et al. 2013, Hackenberg et al. 2014, Ozhuner et al. 2013, Pacak et al. 2017, Wu et al. 2014,

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Table 3.1
(Continued)

Predicted barley miRNA	Predicted miRNA copies	alignment	miRBase (v22) support?	Barley literature support
miR170/miR171#	10	Predicted barley miR171 1 UGAUUGAGCCGCGCCAAUAUC 21 ||||||||||||||||||||| osa-miR171 1 UGAUUGAGCCGCGCCAAUAUC 21	yes	Bai et al. 2017, Colaiacovo et al. 2010, Curaba et al. 2012, Deng et al. 2015, Fard et al. 2017, Hackenberg et al. 2012, Hackenberg et al. 2013, Kantar et al. 2010, Ozhuner et al. 2013, Pacak et al. 2017, Schreiber et al. 2011, Wu et al. 2014,
miR172#	2	Predicted barley miR172 1 GCAGCACCACCAAGAUUCACA 21 ||||||||||||||||||||| bdi-miR172a-5p 1 GCAGCACCACCAAGAUUCACA 21	no	Bai et al. 2017, Colaiacovo et al. 2010, Curaba et al. 2012, Deng et al. 2015, Fard et al. 2017, Hackenberg et al. 2012, Hackenberg et al. 2013, Hackenberg et al. 2014, Kantar et al. 2010, Nair et al. 2010, Ozhuner et al. 2013, Schreiber et al. 2011, Wu et al. 2014,
miR319#	4	Predicted barley miR319 2 UUGGACUGAAGGGUGCUCCCU 22 ||||||||||||||||||||| bdi-miR319b-3p 1 UUGGACUGAAGGGUGCUCCCU 21	no	Bai et al. 2017, Deng et al. 2015, Hackenberg et al. 2012, Ozhuner et al. 2013, Pacak et al. 2017, Wu et al. 2014,
miR384/miR394	2	Predicted barley miR384 1 UUGGCAUUCUGUCCACCUCC 20 |||||||||||||||||||| stu-miR384-5p 1 UUGGCAUUCUGUCCACCUCC 20	no	Colaiacovo et al. 2010, Schreiber et al. 2011,
miR390#	2	Predicted barley miR390 1 AAGCUCAGGAGGGAUAGCGCC 21 ||||||||||||||||||||| osa-miR390 1 AAGCUCAGGAGGGAUAGCGCC 21	no	Bai et al. 2017, Colaiacovo et al. 2010, Curaba et al. 2012, Fard et al. 2017, Pacak et al. 2017, Schreiber et al. 2011,
miR393#	2	Predicted barley miR393 1 UCCAAAGGGAUCGCAUUGAUC 21 ||||||||||||||||||||| osa-miR393 1 UCCAAAGGGAUCGCAUUGAUC 21	no	Bai et al. 2017, Colaiacovo et al. 2010, Curaba et al. 2012, Deng et al. 2015, Fard et al. 2017, Ferdous et al. 2017, Hackenberg et al. 2014, Kantar et al. 2010, Pacak et al. 2017, Schreiber et al. 2011,
miR396#	3	Predicted barley miR396 1 GUUCAAUAAAGCUGUGGGAAA 21 ||||||||||||||||||||| zma-miR396b-3p 1 GUUCAAUAAAGCUGUGGGAAA 21	no	Bai et al. 2017, Colaiacovo et al. 2010, Deng et al. 2015, Fard et al. 2017, Ferdous et al. 2017, Hackenberg et al. 2012, Hackenberg et al. 2013, Pacak et al. 2017, Schreiber et al. 2011, Wu et al. 2014,
miR398#	1	Predicted barley miR398 1 UGUGUUCUCAGGUCACCCCUU 21 ||||||||||||||||||||| osa-miR398 1 UGUGUUCUCAGGUCACCCCUU	no	Xu et al. 2014, Wu et al. 2014,
miR399#	2	Predicted barley miR399 1 UGCCAAAGGAGAGUUGCCCUG 21 ||||||||||||||||||||| ath-miR399b 1 UGCCAAAGGAGAGUUGCCCUG 21	yes	Colaiacovo et al. 2010, Hackenberg et al. 2012, Hackenberg et al. 2013, Hackenberg et al. 2014, Kantar et al. 2010, Ozhuner et al. 2013, Schreiber et al. 2011, Wu et al. 2014,

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Table 3.1 (Continued)				
Predicted barley miRNA	**Predicted miRNA copies**	**alignment**	**miRBase (v22) support?**	**Barley literature support**
miR1432#[2]	3	Predicted barley miR1432 1 UCAGGAGAGAUGACACCGACA 21 ||||||||||||||||||||| bdi-miR1432 2 UCAGGAGAGAUGACACCGACA 22	no	Ferdous et al. 2017, Kruszka et al. 2014, Pacak et al. 2017, Schreiber et al. 2011,
miR1436	3	Predicted barley miR1436 1 CAUUAUGGGACGGAGGGAGU 20 |||||||||||||||||||| osa-miR1436 2 CAUUAUGGGACGGAGGGAGU 21	yes	
miR5049	3	Predicted barley miR5049 1 AAUAUGGAUCGGAGGGAGUAC 21 ||||||||||||||||||||| tae-miR5049-3p 1 AAUAUGGAUCGGAGGGAGUAC 21	yes	Fard et al. 2017, Hackenberg et al. 2012, Hackenberg et al. 2013, Hackenberg et al. 2014, Ozhuner et al. 2013, Schreiber et al. 2011, Wu et al. 2014,
miR5071	1	Predicted barley miR5071 1 UCAAGCAUCAUGUCAUGGACA 21 ||||||||||| || |||||| osa-miR5071 1 UCAAGCAUCAUAUCGUGGACA 21	no	Bai et al. 2017, Curaba et al. 2012, Deng et al. 2015, Schreiber et al. 2011,
miR5139	1	Predicted barley miR5139 1 AACCUCGCUCUGAUACCA 18 ||||| |||||||||| rgl-miR5139 2 AACCUGGCUCUGAUACCA 19	no	Hackenberg et al. 2012, Hackenberg et al. 2013, Hackenberg et al. 2014,
miR5205	1	Predicted barley miR5205 1 CUUAUAUUUAGGAACGGAGGGAGU 24 |||||| ||||| |||||||||| mtr-miR5205b 1 CUUAUAAUUAGGGACGGAGGGAGU 24	no	
miR6201	1	Predicted barley miR6201 1 UGACCCUGAGGCACUCAUACCG 22 |||||||||||||||||||||| hvu-miR6201 1 UGACCCUGAGGCACUCAUACCG 22	yes	Pacak et al. 2017,
miR7731	1	Predicted barley miR7731 2 UUCCAAACUCCUGAGCAAAC 21 ||||||| ||||||||||| bdi-miR7731-5p 1 UUCCAAAUUCCUGAGCAAAC 20	no	
miR8175	2	Predicted barley miR8175 1 UCCCCGGCAACGGCGCCA 18 |||||||||||||||||| ath-miR8175 3 UCCCCGGCAACGGCGCCA 20	no	
miR9662	1	Predicted barley miR9662 1 UGAACAUCCCAGAGCCACCGG 21 ||||||||||||||||||||| tae-miR9662b-3p 1 UGAACAUCCCAGAGCCACCGG 21	no	Deng et al. 2015,

[2] #: identified as pathogen-responsive miRNAs (Kuan *et al*., 2016)

regulation of ROS through a copper super oxide dismutase gene and other targets (Liu and Zhang, 2012, Chavez-Hernandez *et al.*, 2015).

Out of 1,980,623 unique reads, 2423 are differentially expressed in at least one time point (**Supplemental Table 3.1** [See appendix]). These include 13 reads that have homology to three conserved miRNA families including miR165/miR166, miR398, and miR528, (**Table 3.2**). Members of the miR165/miR166 family regulate a HD-ZIPIII transcription factor important for plant development, and have been shown to be positively regulated during pathogen infection (Zhao *et al.*, 2012). In barley, the Mla6 R-protein regulates the expression of miR398 which controls ROS levels through *chloroplast copper/zinc superoxide dismutase 1* (*HvSOD1*) gene expression (Xu *et al.*, 2014). Down-regulation of ROS responses controlled by miR398 and miR528 in the susceptible *mla6* mutant would allow for more favorable infection conditions for *Bgh*.

PARE-Validated sRNA Regulation of Transcription Factors and Signaling Proteins

Predicting sRNA transcript cleavage sites based solely on small RNA sequencing data can be challenging. Several *in silico* prediction programs have been developed for this purpose, but are known to have high false positive prediction rates (Zhai *et al.*, 2014). To compensate for the high false positive prediction rate we constructed parallel analysis of RNA ends (PARE) libraries using RNA from our *Bgh* infected barley panel. The PARE technique allows for identification of

Table 3.2 Differentially expressed predicted miRNAs and barley mapped reads with homology to miRBase miRNAs

Predicted miRNA or read	**Sequence**	**miRBase match**	**Number of predicted barley copies**[3]	**DE time points (and log$_2$ fold changes)***[4]	**miRBase blastn overlap**	**Mismatches**
DE predicted miRNA	ACACAAACCGGGACTAAAG	miR2120	9	*mla6* 20 HAI (1.59)	UserSeq 1 acacaaaccgggacuaaag 19 |||| ||||| |||||||| osa-miR2120b-5p 1 acaccaaccgcgacuaaag 19	2
DE predicted miRNA	ATTTTGCTTCGTATGTAGACT	none	17	*mla6* 0 HAI (1.97)	none	NA
DE predicted miRNA	TATTAGTTGACAGAGGGAGTA	none	5	*mla6* 48 HAI (-1.77), *mla6-bln1* 48 HAI (-2.44), *bln1* 48 HAI (-2.40)	none	NA
DE predicted miRNA	AACTAGTACTACTCTAATGTGCCT	none	3	*mla6* 0 HAI (-1.07)	none	NA
DE predicted miRNA	GCTTTCATAGCTCAGTTGGTTAGAGCACCCG	none	1	*bln1* 32 HAI (1.64)	none	NA
DE predicted miRNA	GTGTTCTCAGGTCGCCCCCGC	miR398	2	*mla6* 32 HAI (2.03)	UserSeq 1 guguucucaggucgcccccg 20 |||||||||||||||||||| zma-miR398a-3p 2 guguucucaggucgcccccg 21	1
DE predicted miRNA	TCCTGTGCCTGCCTCTTCCAT	miR528	1	*mla6* 20 HAI (1.97), *mla6* 24 HAI (2.27), *mla6* 32 HAI (2.18)	UserSeq 2 ccugugccugccucuuccau 21 |||||||||||||||||||| zma-miR528a-3p 1 ccugugccugccucuuccau 20	1
DE predicted miRNA	AGAACAGAGAATGGCGATAGACTC	miR398	1	*mla6 0 HAI (1.63),* *mla6* 20 HAI (1.72), *mla6* 24 HAI (1.66), *mla6* 48 HAI (1.93)	UserSeq 1 agaacagagaauggcgauag 20 ||||||||| ||||| | | csi-miR398a-5p 1 agaacagaggguggcguugg 20	4
DE predicted miRNA	AATTTGAACTGTGAAACT	none	1	*mla6* 0 HAI (1.46), *mla6* 20 HAI (1.76), *mla6* 24 HAI (1.56)	none	NA
DE barley mapped sRNA	TTCGGACCAGGCTTCCTTCCC	miR166	NA	*mla6* 48 HAI (1.92)	UserSeq 2 ucggaccaggcuuccuuccc 21 |||||||||||||| ||||| gma-miR166i-3p 1 ucggaccaggcuucauuccc 20	2

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[3] NA: Barley genome mapped sRNAs are multi-mapping

[4] *: DE barley line, timepoint(s), and log$_2$ fold expression change compared with WT

Table 3.2 (Continued)

Predicted miRNA or read	Sequence	miRBase match	Number of predicted barley copies[5]	DE time points (and log_2 fold changes)*[6]	miRBase blastn overlap	Mismatches
DE barley mapped sRNA	TGGGACCAGGCTTCATTCCCC	miR166	NA	*bln1* 20 HAI (-2.24), *rar3* 20 HAI (-1.71)	UserSeq 1 ugggaccaggcuucauucccc 21 | ||||||||||||||||||| tcc-miR166a 1 ucggaccaggcuucauucccc 21	1
DE barley mapped sRNA	TCGGACCAGGGTTCATTCCCC	miR166	NA	*bln1* 48 HAI (-2.31), *mla6* 48 HAI (-1.80)	UserSeq 1 ucggaccaggguucauucccc 21 |||||||||| |||||||||| hvu-miR166b 1 ucggaccaggcuucauucccc 21	1
DE barley mapped sRNA	TCGGACCAGGCTTCATGCCCC	miR165	NA	*bln1* 16 HAI (-2.55) , *bln1* 20 HAI (-2.39), *mla6* 20 HAI (-1.77), *rar3* 20 HAI (-2.14), *bln1* 24 HAI (-2.47), *mla6* 24 HAI (-2.02), *bln1* 48 HAI (-2.41), *mla6* 48 HAI (-1.78)	UserSeq 1 ucggaccaggcuucaugcccc 21 ||||||||||||||| |||| bdi-miR166d-3p 1 ucggaccaggcuucauucccc 21	1
DE barley mapped sRNA	TGTGTTCTCAGGTCGCCCCCG	miR398	NA	*mla6* 24 HAI (1.71), *mla6* 32 HAI (2.57)	UserSeq 1 uguguucucaggucgcccccg 21 ||||||||||||||||||||| zma-miR398a-3p 1 uguguucucaggucgcccccg 21	0
DE barley mapped sRNA	TGGAAGGGGCATGCAGAGGA	miR528	NA	*mla6* 32 HAI (1.86)	UserSeq 1 uggaaggggcaugcagagga 20 ||||||||||||||||||| osa-miR528-5p 1 uggaaggggcaugcagagga 20	0
DE barley mapped sRNA	TTCGGACCAGGCTTCAGTCCC	miR166	NA	*rar3* 48 HAI (-2.10)	UserSeq 2 ucggaccaggcuucaguccc 21 ||||||||||||||| |||| gma-miR166j-3p 1 ucggaccaggcuucauuccc 20	2
DE barley mapped sRNA	TGGAAGGGGCATGCAGAGGAG	miR528	NA	*mla6* 16 HAI (2.20), *mla6* 20 HAI (2.40), *mla6* 24 HAI (2.21), *mla6* 32 HAI (2.09)	UserSeq 1 uggaaggggcaugcagaggag 21 |||||||||||||||||||| osa-miR528-5p 1 uggaaggggcaugcagaggag 21	0
DE barley mapped sRNA	CCTGTGCCTGCCTCTTCCATT	miR528	NA	*mla6* 0 HAI (1.99)	UserSeq 1 ccugugccugccucuuccauu 21 |||||||||||||||||||| zma-miR528a-3p 1 ccugugccugccucuuccauu 21	0

[5] NA: Barley genome mapped sRNAs are multi-mapping

[6] *: DE barley line, timepoint(s), and log_2 fold expression change compared with WT

in vivo sRNA cut sites in a high-throughput next generation sequencing method (Addo-Quaye *et al.*, 2008, German *et al.*, 2008, Gregory *et al.*, 2008). RNA from 0, 16, 20, 24, 32,and 48 HAI was pooled by genotype for a total of five sequenced libraries for the WT, *mla6*, *rar3*, *bln1*, and *mla6 + bln1 Bgh* infected lines. The five libraries averaged around 33 million reads per library for a total of ~166 million reads. These data were processed as shown in **Figure 3.1B**. Briefly the reads were quality trimmed and evaluated separately with the PARE analysis programs sPARTA and CleaveLand (Addo-Quaye *et al.*, 2009, Kakrana *et al.*, 2014).

The PARE analysis programs take three sets of data including barley transcriptome data, candidate sRNAs, and quality-trimmed PARE sequencing data to identify validated sRNA-transcript pairs. Through this process we identified three types of PARE-validated sRNAs (**Supplemental Table 3.2** [See Appendix]). First, we identified 24 conserved miRNAs with known transcript targets. Second, we identified 35 novel miRNAs with PARE-validated cut sites. Lastly, we identified 61 barley mapping DE reads with PARE-validated cut sites. The transcript targets for the PARE-validated sRNAs were functionally annotated using ensembl annotations, blastx comparisons to the nr database, interproscan (v 5.15-54-0), and literature review (**Table 3.3**). Transcriptional regulation, signaling, and energy-related functional categories made up 33.3%, 11.4%, and 6.5% of the functional annotations, respectively. Transcription-related targets included development-related transcription factors (TFs), Auxin response factors, homeobox, MYB, and NAC TFs, as well as transcript splicing factors. Signaling types regulated through sRNAs included calcium, phosphaste (kinases and phosphatases), and phytohormones including JA and auxin.

Table 3.3 PARE-validated transcript target functional annotations

Functional Category	Number	Percentage
transcriptional regulation	41	33.3
hypothetical or unknown	17	13.8
signaling	14	11.4
metabolism	10	8.1
energy-related	8	6.5
cellular structure and function	8	6.5
transporter	5	4.1
defense	5	4.1
cell wall related	5	4.1
vesicular transport	3	2.4
translation-related	3	2.4
redox control	2	1.6
protein turnover	1	0.8
post translational modification	1	0.8

In the energy-related category, photosynthesis related genes are targeted including three isoforms of cytochrome f, four oxidoreductases, and a component of the photosystem antenna complex. Many of these transcriptional regulators, signaling components, and photosynthesis genes may be co-regulated during infection to control growth rates, as defense responses require relatively large energy investments (Göhre *et al.*, 2012).

Barley Leaf Phased siRNAs Regulate Gene Expression

Phased siRNAs (phasiRNAs) in plants are commonly 21 or 24 nucleotide (nt) sRNAs derived from both coding and non-coding transcripts. Monocots primarily produce phasiRNAs in reproductive tissues that regulate non-coding RNA expression (Fei *et al.*, 2013, Komiya, 2017). However, very few studies have reported regulation of gene expression in non-*TAS* loci in monocots with some exceptions (Liu *et al.*, 2014, Zheng *et al.*, 2015). In our study of *Bgh*-

infected barley leaves we identified barley phasiRNA loci with phasing sizes of 21, 22, and 24 nt that overlap with coding transcripts with functional categories enriched in signaling, metabolism, and defense.

To identify barley phasiRNA loci expressed under *Bgh* infection, we mapped sequencing reads from all 90 Illumina sRNA libraries described above to the barley genome with 0 mismatches allowed using bowtie (Langmead *et al.*, 2009). These mapped reads were run through two filters described in (International Brachypodium, 2010) and detailed in Methods. First, the p-value filter was applied to identify loci with a p-value of <0.01. Second, a phasing score was calculated for a 1 Kb region surrounding these loci. These filters were used to identify phasing sites in all 90 libraries individually (individual library phasing), as well as at the genotype level (genotype level phasing). For the individual library phasing, we identified 1650 individual phasiRNA loci with a distribution of phasiRNA sizes with peaks at 21, 22, and 24 nt (**Figure 3.4**). Many predicted phasing loci overlapped and were therefore concatenated, resulting in 101 total phasing loci.

The positions of the concatenated phasiRNA loci were compared to predicted barley protein-encoding genes, miRNA genes, ncRNA-encoding loci, and transposable elements. Of the 101 phasiRNA loci there were no overlaps with predicted miRNAs from this study and the set described in the latest barley genome paper (Mascher *et al.*, 2017). We also found very little overlap (4.9%) between phasiRNA sites and predicted transposable elements (TEs)

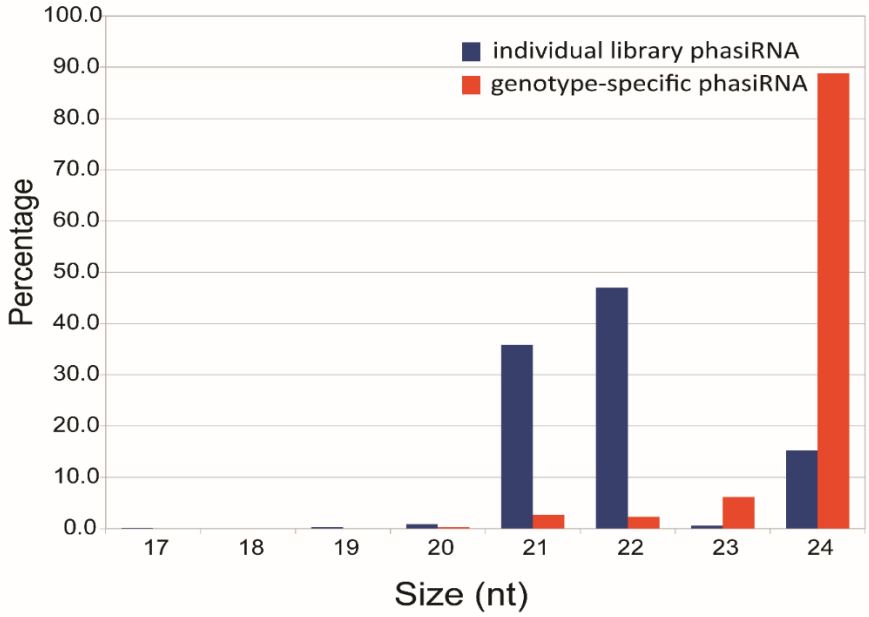

Figure 3.4 PhasiRNA size distributions for individual library phasing (blue) and genotype-specific phasing (orange).

(Mascher *et al.*, 2017) or with the barley ncRNAs from ensembl (v39). We did find 69 (67.6%) phasiRNA loci that overlap within 1 Kb of 46 barley predicted protein coding genes, however. Higher percentage functional categories of the transcripts that overlap with phasiRNA loci include defense, metabolism, and signaling (**Table 3.4**).

Nine transcripts are targeted by phasiRNA identified in at least 10 of the 90 libraries including transcripts encoding three receptor kinases, ubiquitin, a vesicle transport-related SNARE, three metabolic proteins, and a protein related to cytokinin signaling. In addition, three of these transcripts (HORVU6Hr1G081160, HORVU1Hr1G006020, and HORVU1Hr1G069840) have overlapping predicted phasiRNA loci present in at least 30 of the 90 libraries. PhasiRNA loci mapped to these genes in almost all genotypes and timepoints tested, indicating they are likely biologically important to barley leaves during *Bgh* infection. They encode a cysteine-rich

Table 3.4 Individual library phasiRNA transcript target annotations

Functional Category	Number	Percentage
defense	8	17.8
metabolism	8	17.8
signaling	7	15.6
hypothetical or unknown	5	11.1
protein turnover	5	11.1
transcription-related	3	6.7
transporter	3	6.7
vessicle transport	2	4.4
cellular structure and function	1	2.2
energy-related	1	2.2
protein folding	1	2.2
translation-related	1	2.2

receptor-like protein kinase, a LRR receptor kinase SERK-like protein, and the novel plant SNARE 13. Cysteine-rich receptors have been shown to be transcriptionally induced under abiotic and biotic stress conditions (Wrzaczek *et al.*, 2010, Yadeta *et al.*, 2017). HvSERK2 was recently identified as induced during *Bgh* infection and with hydrogen peroxide treatment (Li *et al.*, 2018). SNARE proteins (soluble N-ethylmaleimide-sensitive-factor attachment receptor) are integral in vesicle transport related to plant development and defense (Collins *et al.*, 2003, Ostertag *et al.*, 2013).

Separate from the individual library phasiRNA analysis, the sRNA sequencing data was pooled by genotype to identify genotype-specific phasing. This allows for identification of phasiRNAs expressed at the larger genotype level, and provides insight into their implications for *Bgh* defense. In the genotype-specific phasing analysis we pooled sRNA data by genotype and used a p-value cut off of 0.001 to calculate phasing scores 1 Kb up and downstream of significant sites. This analysis identified 1274 individual phasiRNA loci with a high frequency (88.9%) of 24 nt phasing size (**Figure 3.5**). The overlapping phasiRNAs were concatenated to

form 704 total phasing loci. As was done for the individual library phasing, the concatenated phasiRNA loci were compared to predicted barley protein-encoding genes, miRNA genes, ncRNA-encoding loci, and transposable elements. The concatenated phasing loci did not overlap with miRNA loci from this study or Mascher et al. 2017, nor with barley ncRNAs from ensembl (v39). However, we did uncover 48 out of 701 phasing loci (6.8%) that had overlaps with predicted barley TEs (Mascher *et al.*, 2017). We also found that 225 of the 701 phasiRNA loci (32.1%) overlapped within 1 Kb of 220 barley transcripts. The transcripts overlapped by phasiRNA loci is compared for the five barley genotype pools in **Figure 3.5.** Out of the 220 transcript targets, 161 (73.2%) are uniquely expressed in one genotype pool, with a mix of functional categories including signaling, metabolism, transcription-related, and cellular structure and function (**Table 3.5**). Potentially defense-related transcripts including 8 NLRs and 24 receptor-like kinases are overlapped by phasiRNA loci. Fisher's exact test was applied to show the proportion of receptor-like kinases overlapped by phasiRNA loci is significantly enriched (10.9%) compared with the total receptor-like kinases barley genome (2.6%). This comparison was carried out based on the proportion of Ensembl annotations from predicted phasiRNA transcript overlaps compared to the proportion of total Ensembl annotated barley transcripts that had receptor-like kinase annotations. This suggests that phasiRNA regulation of receptor-like kinases during *Bgh* infection may be an important regulatory feature. One example of the NLR transcripts overlapped by phasiRNA loci, HORVU3Hr1G105020, is of special interest because of its high level of amino acid identity (84%) with *CNL9* from wheat. *CNL9* encodes the CC-NLR protein responsible for *SR35* resistance to Ug99 wheat stem rust (Saintenac *et al.*, 2013). The barley gene HORVU3Hr1G105020 is one of

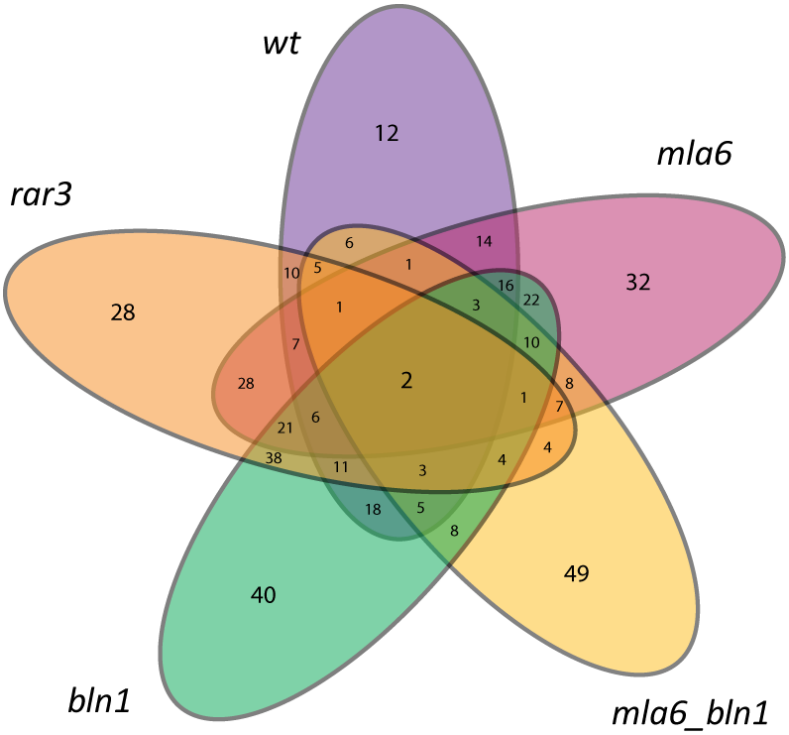

Figure 3.5 Genotype membership distribution for genotype-specific phasiRNA loci. With WT in purple, *mla6* in pink, *mla6_bln1* in yellow, *bln1* in green, and *rar3* in orange.

Table 3.5 Genotype-specific phasiRNA transcript target annotations

Functional Category	Number	Percentage
signaling	41	18.7
metabolism	37	16.9
hypothetical or unknown	36	16.4
transcription-related	24	11.0
cellular structure and function	20	9.1
defense	12	5.5
protein turnover	12	5.5
vessicle transport	9	4.1
energy-related	7	3.2
transporter	7	3.2
cell wall-related	4	1.8
redox control	4	1.8
protein folding	2	0.9
translation-related	2	0.9
post translational modification	1	0.5
stress-related	1	0.5

two potential barley NLRs with a blastx e-value match to *CNL9* of greater than 1e-100. The location of a predicted phasiRNA locus overlapping HORVU3Hr1G105020 coincides with a heavy sRNA expression in the coding region of the NLR-encoding transcript (**Figure 3.6**).

Discussion

Small RNAs including miRNAs and siRNAs have a range of functions regulating gene expression in plants related to growth and development, metabolism, response to abiotic/biotic stress, and many other areas. We sought to understand the effect of infecting barley leaves with barley powdery mildew (*Bgh*) on small RNA populations in wildtype (resistant) and derived immune compromised mutants. Extensive research has been carried out by the barley community examining sRNA expression during development and in response to multiple abiotic stress conditions (Nair *et al.*, 2010, Hackenberg *et al.*, 2013, Kruszka *et al.*, 2014, Fard *et al.*, 2017). However, few studies have sought to examine the expression of the barley small RNAome, in relation to fungal pathogen infection.

Barley PhasiRNA Loci Regulate Diverse Pathways Including Receptor Kinases and Transcription Factors

PhasiRNAs are secondary siRNAs that can silence transcripts in both *cis* and *trans*. They are produced when a RISC-bound miRNA targets a transcript leading to the production of double stranded RNA by RDRP, and cleavage by DCL into phased siRNAs. Most phasiRNA loci in grasses

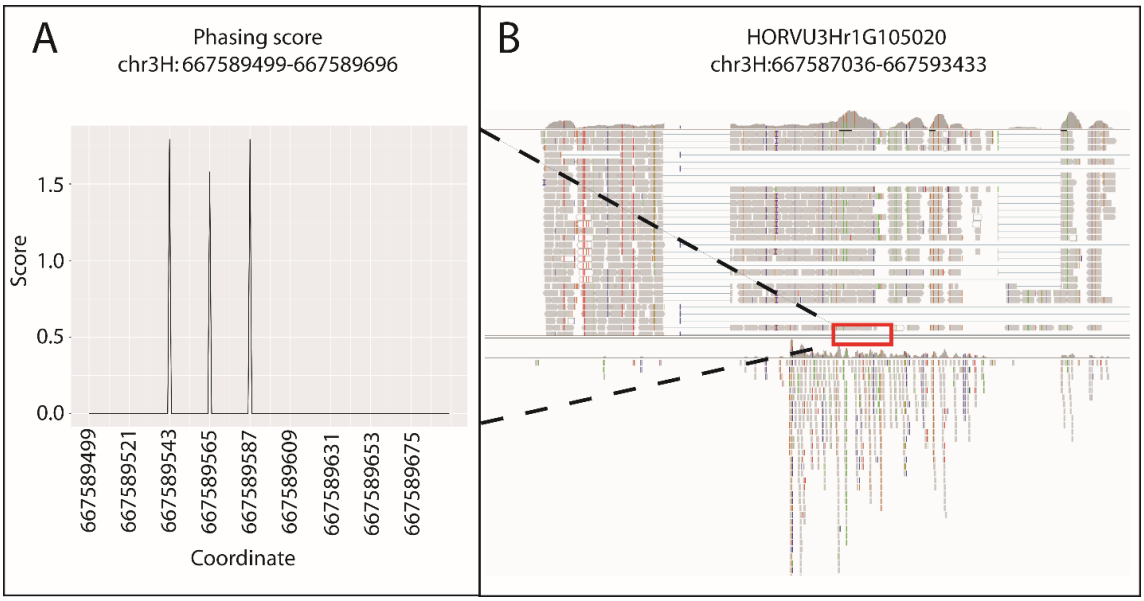

Figure 3.6 PhasiRNA locus phasing score and mapping position relative to barley gene HORVU3Hr1G105020, a NLR gene with homology to wheat *CNL9*. (A) Phasing score diagram on chromosome 3 from 667589499 to 667589696. (B) IGV display of transcript-based RNA-seq reads (top) and small RNA-based RNA-seq reads (bottom) mapping to HORVU3Hr1G105020 with phasiRNA mapping site highlighted with red box.

are associated with silencing ncRNA in reproductive tissues (Komiya, 2017). Two notable exceptions include the *TAS3* tasiRNA locus that regulates auxin response factors and the barley *Mla* resistance gene (Arikit *et al.*, 2013, Liu *et al.*, 2014). The identified phasiRNA loci in *Bgh* infected barley leaves overlap heavily with protein coding transcripts, but do not overlap with known ncRNAs or miRNAs in barley, and very little overlap with TEs. Although there are phasiRNA overlaps with NLR defense genes, the low numbers suggest a lack of a general NLR phasing mechanism as compared with dicots (Park and Shin, 2015). A high number of receptor-like kinase gene targets suggests a different mechanism for defense gene regulation in barley.

Phasing at the individual library level identified phasiRNA loci with sizes including 21, 22, and 24 nt with a distribution of 36%, 47%, and 15%, respectively (**Figure 3.4**). This is quite

different from the phasiRNA size distribution with the genotype level analysis, where 89% were 24 nt in size. The reason for the difference in these size distributions is not clear at this time. The majority of the individual library phasing sites (68%) are located within 1Kb of barley genes. The most highly represented categories include defense, metabolism, and signaling (**Table 4**). These transcript targets include five receptor kinases and six NLR genes, suggesting that defense and pathogen perception proteins are regulated during *Bgh* infection of barley leaves. Phasing loci were identified in at least 30 of the 90 total libraries for three genes: a cysteine-rich receptor-like protein kinase, a LRR receptor kinase SERK-like protein, and the novel plant SNARE 13. The fact that phasiRNA loci mapped to these three genes in almost all genotypes and timepoints in our panel indicates that they are important for barley leaves during *Bgh* infection. The cysteine-rich receptor-like kinase (CRK) family is one of the largest receptor families in plants, and help regulate plant development and response to abiotic and biotic stresses (Bourdais *et al.*, 2015). Several members of this family have been implicated in pathogen responses in grasses including rice *CRK6* and *CRK10*, barley *HvCRK1*, and wheat *TaCRK1* (Rayapuram *et al.*, 2012, Yang *et al.*, 2013b, Chern *et al.*, 2016). Somatic embryogenesis receptor-like kinases (SERKs) are LRR-containing receptor-like kinases that are associated with development and response to pathogen stress (Aan den Toorn *et al.*, 2015). SERK proteins have been implicated in pathogen response in grasses including rice *OsSERK2* and barley *HvCERK2 (Chen et al., 2014, Li et al., 2018)*. SNARE proteins are vital to membrane fusion during vesicular transport, and have functions in development and pathogen defense (LaMontagne and Heese, 2017). There are several SNARE proteins that have been associated with pathogen defense

including *PEN1* from *Arabidopsis*, *OsVAMP714* from rice, and *TaSYP71* from wheat (Collins *et al.*, 2003, Liu *et al.*, 2016, Sugano *et al.*, 2016).

With the genotype-specific phasing data we found that around a third (32%) of the predicted phasiRNA loci were located within 1 Kb of predicted barley protein-encoding transcripts. The genotype-specific phasing analysis identified substantially more transcript overlaps than the individual library phasing (219 vs 45). Functional categories highly represented in the data include signaling, metabolism, and transcription-related at 19%, 17%, and 16%, respectively. In the signaling category receptor-like kinases are significantly over-represented in the genotype-specific phasiRNA targets when compared with the current barley annotated transcriptome, which may indicate a novel mechanism of pathogen defense regulated by phasiRNAs in barley. Several barley receptor-like kinase genes are involved in pathogen response including mutation-induced recessive alleles (*MLO)*, *resistance gene Puccinia graminis 1* (*Rpg1)*, *rat sarcoma homolog binding protein kinase* (*RBK1)*, *somatic embryogenesis receptor-like kinase 2* (*SERK2)*, *LRR/malectin receptor-like kinase* (*LEMK1)*, and *cysteine-rich receptor-like protein kinase 1* (*CRK1*) (Büschges *et al.*, 1997, Brueggeman *et al.*, 2002, Huesmann *et al.*, 2012, Rayapuram *et al.*, 2012, Rajaraman *et al.*, 2016, Li *et al.*, 2018). In dicots, NLR defense genes are regulated heavily by phasiRNAs triggered by miRNAs targeting conserved portions of NLR transcripts (Park and Shin, 2015). For example in a recent study on soybean sRNAs, the authors found 41% of *PHAS* loci overlapped with NLR genes (Arikit *et al.*, 2014). In our genotype-specific phasing data, we found only 4% of the phasiRNA loci overlapped with NLR genes. Our data shows that barley leaves infected with *Bgh* produce phasiRNA potentially regulating the expression of a diverse set of genes affecting metabolism,

transcription, signaling, and defense. Previous studies on phasiRNAs in grasses (besides *TAS3*) have focused almost exclusively on phasiRNAs targeting ncRNAs in reproductive tissues (Johnson *et al.*, 2009, Zhai *et al.*, 2015, Fan *et al.*, 2016, Fei *et al.*, 2016a). The results from our study indicate that barley phasiRNAs overlap extensively with protein-coding transcripts, and that defense response genes including receptor-like kinases are highly regulated by phasiRNAs. Further, we have identified a mix of phasing sizes pools including 21, 22, and 24 nt rather than just 21 nt as found in dicots or 21 and 24 as found in other monocots (Fei *et al.*, 2013). The population of phasiRNAs has not been explored extensively in monocot leaves, and defense mechanisms differentially evolved from dicots are likely and worth exploring further.

PARE-Validated sRNAs Regulate Transcription, Signaling, and Photosynthesis

In silico miRNA target prediction can have success identifying transcript targets. However, many prediction programs produce target lists that are very large, compared with verified *in vivo* targets. Standard practice for many years for the validation of miRNA/transcript cut sites has been the 5' RACE (rapid amplification of cDNA ends) technique (Frohman *et al.*, 1988, Llave *et al.*, 2002). The PARE technique represents a high-throughput version of the 5' RACE technique that allows for the identification of sRNA cut sites with an Illumina sequenced library. We produced PARE libraries from genotype-pooled *Bgh* infected barley leaf RNA to confirm predicted sRNA transcript cut sites *in vivo*. We identified 24 PARE-validated miRNAs, representing eight conserved miRNA families including miR156, miR159, miR160, miR164, hvu-miR165/hvu-miR166, miR169, miR171, and miR396. We further identified 35 novel barley miRNAs and 64 DE barley genome mapped sRNAs with PARE-validated cut sites (**Supplemental**

Table 3.2). The majority of conserved plant miRNAs target transcription factors (Li and Zhang, 2016, Samad *et al.*, 2017), which matches well with our data. The eight conserved miRNA families identified in the PARE data all target transcription factors with roles in development and biotic stress responses (Wang *et al.*, 2005, Allen *et al.*, 2007, Sieber *et al.*, 2007, Wang *et al.*, 2009, Li *et al.*, 2010, Guo *et al.*, 2011, Wang *et al.*, 2011, Sunkar *et al.*, 2012, Curaba *et al.*, 2013, Feng *et al.*, 2014, Soto-Suarez *et al.*, 2017). The transcription-related genes regulated by the PARE-validated sRNAs encode several families of transcription factors including Homeobox, MYB, NAC, ARF, GRAS, bHLH, squamosa promoter-binding-like, and factors related to transcript splicing. These results indicate that transcription factor genes are being regulated at the post transcriptional level during *Bgh* infection. However, significant differences in expression were not found for the miRNAs targeting these gene transcripts in our data. This may mean that the changes in gene expression of transcription-factor genes in the *bln1*, *mla6*, *rar3*, and *mla6* + *bln1* mutant lines are largely not due to differences in expression of regulatory miRNAs.

Additionally, signaling and energy categories were highly represented as regulatory targets of the PARE-validated sRNAs. The signaling transcript targets included proteins involved in phosphate signaling (kinases, receptor-like kinases, and phosphatases), calcium signaling (calmodulin and calcineurin B), and hormone signaling (JA and auxin). Hormone levels are changed as part of the PTI defense response to pathogen challenges in plants (Yang *et al.*, 2013a). For example JA and Auxin function can be downregulated during infection by biotrophic pathogens to reduce growth rates, and promote the effects of SA (Denance *et al.*, 2013). The members of the energy-related category all are directly involved in photosynthesis, including members of the cytochrome f family, NADH-plastoquinone oxidoreductases, and the

CP43 chlorophyll apoprotein. In response to pathogen infections, transcripts encoding photosynthetic machinery are generally downregulated (Bilgin *et al.*, 2010). However, photosystem proteins generally are very stable, which allows an infected plant to divert resources to defense, while maintaining active photosynthesis (Huot *et al.*, 2014).

Differentially Expressed sRNAs Regulate PTI-Related Redox Responses

Differential expression analysis of the predicted miRNAs and barley genome mapped sRNAs identified several conserved miRNA families regulated during *Bgh* infection including miR166/165, miR398, and miR528. The miR166/165 family has diverse roles in development and response to stress through regulation of the Class III homeodomain-leucine zipper (HD-ZIP III) encoding transcripts (Rubio-Somoza and Weigel, 2011, Khraiwesh *et al.*, 2012). Multiple members of the highly conserved miR166/165 family are present in the genome of many plant species with diverse expression patterns (Jung and Park, 2007, Kulcheski *et al.*, 2011, Aravind *et al.*, 2017, Li *et al.*, 2017). The differential expression of members of the miR166/165 family have been associated with multiple stress responses including drought, cold, and pathogen challenge (Xin *et al.*, 2010, Zhao *et al.*, 2012, Zeng *et al.*, 2018, Zhang *et al.*, 2018). We identified 5 different barley genome mapped sRNAs with homology to members of the miR166/165 family that were differentially expressed in at least one time point and barley immune signaling mutant compared with wt. Four out of five barley genome mapped sRNAs had significant decreases in expression relative to wt in at least one condition, while the sixth had a significant increase in expression (**Table 3.2**). In a recent study miR166/165 family member-specific expression was studied and it was discovered that some family members are

strongly upregulated in susceptible lines, whereas others are downregulated (Xin *et al.*, 2010). It is unclear what role downregulation of miR166/165 means for the CI 16151-derived mutants in our study, as both resistant (*bln1*) and susceptible lines (*mla6* and *rar3*) have significant downregulation relative to wt in at least one time point.

miR398 targets two copper superoxide dismutase gene transcripts as well as a cytochrome c oxidase (Jones-Rhoades and Bartel, 2004, Xu *et al.*, 2014). The regulation of miR398 expression has been shown to be important in stress responses including heat, drought, high salt, ABA, and pathogen challenge, amongst others (Zhu *et al.*, 2011). In barley hvu-miR398 targets the *HvSOD1* transcript and is regulated by both *Mla* and *Rom1* in response to *Bgh* infection (Xu *et al.*, 2014). In our study, two predicted miRNAs and one barley genome mapped sRNAs with homology to miR398 were significantly upregulated in the *mla6* barley isoline. These data support the findings of Xu et al. (2014) in that miR398 is upregulated in the *mla6* mutant isoline compared with wt **(Table 3.2**), leading to a suppression of *HvSOD1* expression.

The miRNA miR528 has been experimentally shown to target transcripts encoding L-ascorbate oxidase in rice (Wu *et al.*, 2017), Plastocyanin-like blue copper ion binding protein in sugarcane (Zanca *et al.*, 2010), and the F-box/LRR-repeat protein MAX2 in rice (Ma *et al.*, 2013). The expression of miR528 has been associated with embryo development, metal toxicity, oxidative stress, drought stress, salt stress, and pathogen challenge (Li *et al.*, 2011, Ferreira *et al.*, 2012, Campo *et al.*, 2013, Gupta *et al.*, 2014, Chavez-Hernandez *et al.*, 2015, Yuan *et al.*, 2015, Wu *et al.*, 2017). Similar to miR398, we found one predicted miRNA and four barley genome mapped sRNAs with homology to miR528 to have significantly increased expression in

the *Bgh* susceptible *mla6* mutant. The role of miR528 in Poaceae pathogen defense is unclear as it was upregulated in both resistant and susceptible wheat lines challenged with leaf rust and powdery mildew (Wu *et al.*, 2015, Kumar *et al.*, 2017), but was upregulated only in the susceptible barley *mla6* mutant relative to wt. In our study, however, the expression of miR528 is significantly increased in the susceptible *mla6* barley mutant. This upregulation of miR528 in *mla6* will directly contribute to a reduced ROS response to *Bgh* infection, in a similar described for miR398 (Xu *et al.*, 2014). It is likely that miR528 overexpression has the effect of reducing barley defense capacity against *Bgh* infection.

Known and Novel sRNAs Expressed During* Bgh *Infection

The plant miRNA community is extremely active and identifies thousands of novel miRNAs each year. The miRBase database has represented the central authority on miRNA name certification in the plant community over the last decade (Kozomara and Griffiths-Jones, 2014). However, the number of miRBase-validated barley miRNAs has fallen behind the publications of the barley miRNA community, even for conserved miRNAs (Curaba *et al.*, 2012). In our study, we identified 1425 predicted barley miRNAs expressed during *Bgh* infection using the programs miRDeep-P and ShortStack. These predicted miRNAs include homologs to 27 conserved miRNA families identified in miRBase, including 18 that are not represented by barley entries in miRBase, despite literature support (**Table 3.1**). Of the conserved miRNAs identified from our study, 17 were identified as pathogen infection associated in a recent review (Kuan *et al.*, 2016). Expression of these miRNAs is likely part of a non-species specific defense against fungal

infection, as their expression is highly conserved in multiple plant species in response to fungal infection.

Conclusions

We have identified barley phasiRNAs and miRNAs expressed in response to *Bgh* infection of barley leaves. The multiple phasiRNA loci in this study map within or in close proximity to protein coding genes. This is in direct contrast to many monocot phasiRNA studies that have indicated that the majority of loci map to lncRNAs, especially in reproductive tissues (Arikit *et al.*, 2013). In one notable exception phasiRNAs have been shown to be produced by miR9863 when targeting transcripts of the *R*-gene *Mla1* (Liu *et al.*, 2014). Here we identified phasiRNA loci that overlap with a statistically significant proportion of receptor-like kinases, indicating a different defense mechanism may be active in barley leaves as opposed to heavy *R*-gene phasiRNA overlap in many dicots. In addition, we identified conserved miRNAs, novel miRNA candidates, and barley genome mapped sRNAs that have PARE validated transcript targets in barley. The miRNA target transcripts are enriched in transcription factors, signaling-related proteins, and photosynthesis-related proteins. These results indicate that pathogen infection is causing a strong response in transcriptional regulation related to signaling, photosynthesis, and transcription factor expression which may be directly related to both a PTI and ETI response.

Materials and Methods

Fungal and Plant Material

Barley lines CI 16151 (*Mla6*), m18982 (*mla6*), m11526 (*rar3*), m19089 (*bln1*), and the m19028 double mutant (*mla6* + *bln1*) were grown with supplemental lighting under temperature controlled greenhouse conditions. The CI 16151 barley line was created by introgression of the *Mla6* gene into universal susceptible cv Manchuria and is resistant to *Blumeria graminis* f. sp. *hordei* (*Bgh*) isolate 5874 (AVR_{a1}, AVR_{a6}, AVR_{a12}). Mutant derivatives of CI 16151 were created through fast neutron mutagenesis as described previously (Meng *et al.*, 2009b). *Mla6* is a major NLR-type resistance gene, while *Rar3* (*Required for Mla6 resistance 3*) is an unlinked locus required for *Mla6* function. *Blufensin1* (*Bln1*) is a negative regulator of PTI signaling (Meng *et al.*, 2009a) and the *bln1* mutant exhibits enhanced basal defense (Xu *et al.*, 2015). The mutant forms of *Mla6* or *Rar3* are susceptible to 5874 infection, unlike the CI 16151 parental line. *Bgh* isolate 5874 was propagated on *Hordeum vulgare* cv. Morex in a growth chamber at 18°C with a 16 hours light, 8 hours dark day/night cycle.

Experimental Design

Planting, stage of seedlings, inoculation, and sampling of leaf tissue were followed as described previously (Caldo et al., 2006, Moscou et al., 2011). Barley tissue used for sRNA libraries was grown in three separate replicates grown in consecutive weeks. Each genotype was planted in 20 × 30–cm trays in sterilized potting soil. Each experimental tray consisted of six rows of 12-15 seedling first leaves, with rows randomly assigned to one of the six harvest times in a split-plot design. Within each replicate the five barley genotypes were infected with *Bgh* spores and

harvested at 0, 16, 20, 24, 32, and 48 hours after inoculation (HAI) for a total of 90 tissue samples.

Small RNA Sequencing and Data Analysis

Total RNA was extracted from *Bgh*-infected barley leaf tissue following the hot (60°C) phenol/guanidine thiocyanate method described previously (Caldo et al., 2004, Caldo et al., 2006). Small RNA libraries were made with the Illumina TruSeq Small RNA Library kit (Illumina, Inc., San Diego, CA), as per manufacturers protocol. The ninety small RNA Illumina libraries were sequenced on a HiSeq 2500 (Illumina, Inc.) at the Iowa State University DNA Facility in Ames, IA. Reads were quality assessed using the FastQC program version 0.11.3 (Andrews, 2010). Reads were quality filtered and adapters were trimmed using Trimmomatic version 0.33 (Bolger et al., 2014). Reads were compared with the Rfam database using the Infernal program version 1.1.2 (Nawrocki et al., 2014) and used to filter tRNAs, rRNAs, snoRNAs and snRNAs from the data. The reads were also filtered using the Triticeae Repeat Sequence Database (Wicker, 2008) to remove any known Triticeae-specific repeat sequences. Two programs were used to identify sRNA candidates of interest from barley: miRDeep-P (version 1.3) and ShortStack (version 2.1.0) (Yang and Li, 2011, Axtell, 2013).

Differential Expression

For each time point, we performed a differential expression (DE) analysis, comparing relative abundance of sRNAs/reads from the different mutant genotypes to CI 16151 (WT). The

sRNA/read count datasets were normalized and analyzed by using the DESeq2 program package in R (Love et al., 2014). We added 0.5 count units to all read counts and rounded them to the nearest integer to allow use of the DESeq2 normalization method (Love *et al.*, 2014). Reads with 0.9 quantile smaller than a count of 2 are assumed to be expressed at a very low level and were removed from the analysis. The remaining sRNAs/reads were analyzed for DE. The p-values were adjusted for multiple testing error using Q-value calculations (Nettleton *et al.*, 2006), and sRNAs/reads were filtered for a Q-value of less than 0.05.

PARE Library Sequencing and Data Analysis

Source RNA that was used for sRNA sequencing above was also used for PARE. PARE libraries were prepared as previously described (Zhai et al., 2014) at the Donald Danforth Plant Science Center in St. Louis, MO and sequenced on a HiSeq 2500 (Illumina, Inc.). Reads were quality assessed using the FastQC program version 0.11.3 (Andrews, 2010). Reads were quality filtered and adapters were trimmed using Trimmomatic version 0.33 (Bolger et al., 2014). The two PARE analysis programs sPARTA (version 1.21) and CleaveLand (version 4.4) were used independently to identify likely sRNA targets using sRNA sequencing data, the barley transcriptome (ensembl version 38) (Mascher *et al.*, 2017), and PARE sequencing data. PARE validated targets were filtered based on adjusted p-values using a 1% false discovery rate along with a PARE category of less than 2 (with sPARTA data).

PhasiRNA Analysis

Identification of PHAS loci was completed using methods described previously (Zheng *et al.*, 2014). The sRNA reads were mapped to barley RefSeq1 (Mascher *et al.*, 2017), using bowtie 1 (Langmead *et al.*, 2009). Uniquely mapped reads were chosen for PHAS locus identification. In order to mimic the 3' overhang, an offset of 2 nucleotides was included for sRNAs that were aligned to the antisense strand of the reference. The reference genome was scanned using a nine-cycle sliding window of 189 bp where each cycle was of 21 bp – equal to the length of the sRNA. Windows were reported only when they had at least 10 unique reads, with more than 30% of the reads being 21 nt in length and at least three 21 nt unique reads falling into the phase registers. Windows with overlapping regions were combined into a larger window. P-values for each window was calculated based on the following formula:

$$\text{p-value} = \sum_{x=k}^{m} \frac{\binom{20m}{n-x}\binom{m}{x}}{\binom{21m}{n}}$$

where 'n' represents the total number of unique sRNAs of 21 nt length within the window, 'm' was the number of cycles and 'k' was the maximum number of unique 21nt sRNAs falling into one of the possible phase registers. Windows with a p-value less than 0.001 were considered as positive PHAS loci.

Phasing score was computed using the methods described in de Paoli (De Paoli *et al.*, 2009).

$$\text{Phasing score} = \ln[1 + 10\frac{\sum_{i=1}^{9} P_i}{1+\sum U}]^{k-2}$$

Where 'Pi' was the total number of reads for all 21 nt sRNAs falling into a given phase within a nine-cycle window, 'U' is the total number of reads for all 21 nt sRNAs falling out of the given phase and 'k' is the number of phase cycle positions occupied by at least one 21 nt sRNA within the window.

Accession Numbers

Small RNA sequencing dataset has been submitted to NCBI GEO under the accession number GSE115992. PARE library sequencing data has been submitted to NCBI under accession number GSE116691.

Supplemental Material [See Appendix]

Supplemental Table 3.1 Expression details of DE barley mapped reads

Supplemental Table 3.2 PARE validated predicted miRNAs and barley genome mapped sRNAs

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CHAPTER 4. TRANS-KINGDOM SMALL RNA CROSS-TALK BY BARLEY AND BARLEY POWDERY MILDEW

Modified from manuscript to be submitted to BMC Genomics

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MH contributed to project development, data analysis and interpretation, and wrote the manuscript with input from RW; PS contributed to data analysis; ML contributed to statistical analyses; GF performed small RNA sequencing experiments; SM & BM performed PARE sequencing experiments; DN contributed to statistical analyses; RW contributed to project conception, development, and data interpretation.

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Abstract

Background

Cross-species communication is extremely important between plants and plant pathogens. For obligate biotrophic pathogens such as barley powdery mildew (*Blumeria graminis* f. sp. *hordei* [*Bgh*]), successful completion of their life cycle requires a living host plant. This means that *Bgh* both has to suppress barley's defense responses and extract nutrition from host cells without triggering cell death to complete its life cycle. Plant defense responses include both non-specific, and a species specific mechanisms. The combination of these defenses are potent enough to make pathogen infection the exception, rather than the rule for plants. Recently small RNAs such as plant micro RNAs (miRNAs), fungal micro RNA-like RNAs (milRNAs), and small interfering RNAs (siRNAs) have been identified as potent defense factors and virulence

factors in plants and plant pathogens. These sRNAs can act in a trans-kingdom manner including movement from pathogen into plant and from plant into pathogen to trigger reduced gene expression of sRNA targets.

Results

We sought to identify trans-kingdom sRNAs produced both in barley and *Bgh*. To accomplish this goal we infected seedlings from barley line CI 16151 (containing the *Mla6* powdery mildew resistance gene) and four fast-neutron derived immune-signaling mutants in a time-course experiment representing key stages of *Bgh* infection: appressorium formation, penetration of epidermal cells, and development of haustoria. RNA extracted from these conditions were used to create both sRNA sequencing (sRNA-seq) libraries and parallel analysis of RNA ends (PARE) libraries to identify sRNAs and transcript cleavage sites. Using a custom bioinformatics pipeline these data were used to identify 1742 and 1425 barley miRNAs and *Bgh* milRNA candidates, along with several thousand differentially expressed genome mapped sRNAs. These sRNAs were used along with PARE library data and transcriptome data to identify likely trans-kingdom active sRNAs in both barley and *Bgh*. The potential barley trans-kingdom sRNAs are predicted to target transcripts encoding highly conserved proteins involved in core cellular activities such as ribosome synthesis/function, tRNA modification, core transcription/translation factors, cell cycle regulators, as well as *Bgh*-specific effector proteins. The effector targets include 3 *AVRk1* and *AVRa10*-like (EKA) family members and 4 candidate secreted effector proteins (CSEPs). The predicted *Bgh* trans-kingdom active sRNA are highly enriched in transcripts with functions

related to the non-species-specific defense. The transcript targets encode proteins related to vesicle secretion, cell wall synthesis, protein turnover, transcriptional regulation, ROS response, and fungal cell wall breakdown.

Conclusions

Plants and plant pathogens are constantly evolving new mechanisms of defense and virulence for continued survival. Trans-kingdom regulation of defense and virulence factors through sRNAs represents a novel plant/pathogen communication mechanism of great interest. We identified candidate trans-kingdom sRNAs from both barley and *Bgh* that may act as sRNA effectors or as defense factors. The targets of the *Bgh*-produced trans-kingdom sRNAs indicate a function in re-enforcing the function of protein effectors. The barley-produced trans-kingdom sRNAs target annotations indicate both a non-species and a species-specific defense mechanism.

Keywords: Trans-kingdom, small RNAs, plant pathogen, milRNA, *Blumeria*, barley

Background

Plants encounter pathogenic and non-pathogenic microorganisms on a nearly constant basis. To prevent infection from potential pathogens, plant employ an integrated multi-phasic system including a non-species specific defense response tailored to pathogen type, and a pathogen species specific response. For the first phase plants perceive pathogen-associated molecular patterns (PAMPs) associated with pathogen type such as chitin for fungi and flagellin for bacteria (Zipfel, 2014). These molecules bind to receptor-like kinases to trigger PAMP

triggered immunity (PTI) that can include accumulation of cell wall material, a reactive oxygen species (ROS) response, and accumulation of antimicrobial compounds and hydrolytic enzymes (Dodds and Rathjen, 2010). Successful pathogens have evolved effector molecules that act to compromise the PTI response and lead to effector triggered susceptibility (ETS). To combat pathogen effectors, plants evolved an additional response, designated effector triggered immunity (ETI). ETI is the result of the interaction of resistance (R) proteins, often encoded by NOD-like Receptors (NLRs), and pathogen effector molecules (Cui *et al.*, 2015, Kourelis and van der Hoorn, 2018). This interaction triggers a strong immune response, commonly associated with a hypersensitive response and localized cell death.

Blumeria graminis f. sp. *hordei* (*Bgh*) is an obligate biotrophic fungus of the phylum Ascomycota. Obligate biotrophic fungi complete their life cycle in living hosts, which requires the fungus to both silence defense mechanisms and to extract nutrients from their host species. To accomplish these functions, obligate biotrophs like *Bgh* express effector molecules that act both inside and outside host cells. Effectors actively suppress host defenses and create an environment conducive to fungal growth and reproduction. Effectors can come in the form of proteins metabolites, and as discovered recently, sRNAs.

Gene expression of defense genes in plants and virulence genes in pathogens are often regulated at the post transcriptional level by small RNAs (sRNAs). In most cases sRNAs function within the organism to regulate gene expression in an intra-kingdom fashion. In plants, defense genes related to both PTI and ETI responses are regulated by miRNAs (Kuan *et al.*, 2016). For example, during *Pseudomonas syringae* infection of *Arabidopsis*, miR393 expression significantly increases, leading to downregulation of auxin F-box receptors during PTI response

(Navarro *et al.*, 2006). The downregulation of auxin receptor expression allows a strong salicylic acid response and accumulation of antimicrobial compounds (Robert-Seilaniantz *et al.*, 2011). The expression of *R*-genes regulating the ETI response are also tightly regulated by miRNAs in plants (Park and Shin, 2015). For example, in tomato high expression levels of miR482 and miR2118 and related family members lead to the reduction in *R*-gene expression, except during viral or bacterial infection, where sRNA expression is reduced (Shivaprasad *et al.*, 2012). Some pathogens produce effectors that reduce sRNA expression in plants to increase susceptibility, but can have the opposite effect when sRNAs targeting *R*-gene expression have reduced expression and *R*-gene expression increases (Pumplin and Voinnet, 2013).

Filamentous plant pathogens have been shown to regulate virulence-related genes through sRNA expression. In the oomycete pathogen *Phytophthora sojae* the avirulence factor Avr3a is differentially silenced by small RNAs in a transgenerational fashion, allowing for infection of plants with an *R*-protein recognizing the Avr3a protein (Qutob *et al.*, 2013). In *Phytophthora infestans* sRNAs were identified that target numerous *RxLR* and *Crinkler* effector genes that were differentially accumulated between highly and weakly pathogenic strains (Vetukuri *et al.*, 2012). Small RNAs of the micro RNA-like (milRNA) type were differentially expressed in the plant fungal pathogen *Fusarium oxysporum* f. sp. *niveum* regulating the expression of the two toxins trichothecene and NEP1 (Jiang *et al.*, 2017). Controlling gene expression related to resistance genes and pathogenicity factors is clearly important for determining the outcome of plant/pathogen interactions.

Recent studies have been shown sRNAs to be mobile between two different species in a trans-kingdom manner (Weiberg and Jin, 2015). The mechanisms of trans-kingdom silencing

are unclear, but have been observed in multiple systems including human to protozoa, plants to nematodes, fungi to plants and others (Knip *et al.*, 2014). This movement of sRNAs in host-pathogen interactions clearly has functional implications for gene expression of resistance or pathogenicity factors. For example, a plant pathogen could control gene expression of PTI related genes and reduce defense responses without the action of protein effectors. In fact, active small RNAs expressed in a pathogen that have a trans-kingdom effect on gene expression can be considered small RNA effectors (Wang *et al.*, 2015). In a study by Weiberg et al. (2013), the authors showed that the movement of *Botrytis cinerea* small into *Arabidopsis* and tomato reduced the expression of defense genes including *mitogen activated protein kinase 1* and *2*, *peroxiredoxin*, and a *cell wall-associated kinase* (Weiberg *et al.*, 2013). These genes are highly conserved parts of the PTI pathway in plants and help explain the broad host range of *Botrytis cinerea* (Weiberg and Jin, 2015). Recently, the trans-kingdom sRNA effector mechanism was demonstrated for the monocot pathogen *Puccinia striiformis* f. sp. *tritici* (*Pst*). The milRNA *Pst*-milR1 was shown to target the wheat *pathogenesis-related 2* (*PR2*) gene, leading to significantly reduced resistance in strains carrying the milRNA (Wang *et al.*, 2017).

If fungi can express sRNAs that act as effectors when taken up by plants, it is logical to assume that the same phenomenon should be possible for sRNAs expressed in host plants. The host induced genome silencing (HIGS) technique was developed to test whether double-stranded or antisense fungal constructs expressed in a plant host could move into a fungal pathogen in a trans-kingdom manner (Nowara *et al.*, 2010). Nowara and colleagues (2010) demonstrated that expression of antisense copies of the AVR_{A10} virulence factor in barley and wheat significantly reduced the pathogenicity of *Bgh*. Until recently, it was unclear whether the

plant into fungus sRNA movement occurred in natural systems. However, in a recent study, cotton was shown to export miR159 and miR166 into the plant pathogen *Verticillium dahliae*, causing reduced virulence through silencing of a *Ca^{2+}-dependent cysteine protease* and an *isotrichodermin C-15 hydroxylase* gene (Zhang *et al.*, 2016).

In the current study we sequenced sRNA libraries from *Bgh* infected barley leaves with the goal of identifying trans-kingdom active sRNAs from both barley and *Bgh*. Considering the close plant/host relationship between barley and *Bgh* we expected to find active sRNA communication between the two species. To accomplish this goal we infected seedlings from barley line CI 16151 (containing the *Mla6* powdery mildew resistance gene) and four fast-neutron derived immune-signaling mutants in a time-course experiment representing key stages of *Bgh* development on its barley host: appressorium formation, penetration of epidermal cells, and development of haustoria. Transcript targets of trans-kingdom sRNAs were identified with the PARE technique. The trans-kingdom targets are functionally divergent between barley and *Bgh* expressed sRNAs. Barley trans-kingdom sRNAs are predicted to target vital cellular machinery to directly reduce *Bgh* viability. On the other hand, *Bgh* sRNAs are predicted to target barley gene expression related to defense response, rather than vital cellular machinery.

Methods

Fungal and Plant Material

Barley lines CI 16151 (*Mla6*), m18982 (*mla6*), m11526 (*rar3*), m19089 (*bln1*), and m19028 (*mla6 + bln1*) were grown with supplemental lighting under temperature controlled greenhouse

conditions. The CI 16151 barley line was created by introgression of the *Mla6* gene into universal susceptible cv Manchuria and is resistant to *Blumeria graminis* f. sp. *hordei* (*Bgh*) isolate 5874 (AVR_{A1}, AVR_{A6}, AVR_{A12}). Mutant derivatives of CI 16151 were created through fast neutron mutagenesis as described previously (Meng *et al.*, 2009a). *Mla6* is a major NLR-type resistance gene, while *Rar3* (*Required for Mla6 resistance 3*) is an unlinked locus required for *Mla6* function. *Blufensin1* (*Bln1*) is a negative regulator of PTI signaling (Meng *et al.*, 2009b) and the *bln1* mutant exhibits enhanced basal defense (Xu *et al.*, 2015). The mutant forms of *Mla6* or *Rar3* are susceptible to 5874 infection, unlike the CI 16151 parental line. *Bgh* isolate 5874 was propagated on *Hordeum vulgare* cv. Morex in a growth chamber at 18°C with a 16 hours light, 8 hours dark day/night cycle.

Experimental Design

Planting, stage of seedlings, inoculation, and sampling of leaf tissue were followed as described previously (Caldo *et al.*, 2006, Moscou *et al.*, 2011). Barley tissue used for sRNA libraries was grown in three separate replicates grown in consecutive weeks. Each genotype was planted in 20 × 30–cm trays in sterilized potting soil. Each experimental tray consisted of six rows of 12-15 seedling first leaves, with rows randomly assigned to one of the six harvest times in a split-plot design. Within each replicate the five barley genotypes were infected with fresh *Bgh* conidiospores and harvested at 0, 16, 20, 24, 32, and 48 hours after inoculation (HAI) for a total of 90 tissue samples.

Small RNA Sequencing and Data Analysis

Total RNA was extracted from *Bgh*-infected barley leaves following the hot (60°C) phenol/guanidine thiocyanate method described previously (Caldo *et al.*, 2004, Caldo *et al.*, 2006). Small RNA libraries were made with the Illumina TruSeq Small RNA Library kit (Illumina, Inc., San Diego, CA), as per manufacturers protocol. The ninety small RNA Illumina libraries were sequenced on a HiSeq 2500 (Illumina, Inc.) at the Iowa State University DNA Facility in Ames, IA. Reads were quality assessed using the FastQC program version 0.11.3 (Andrews, 2010). Reads were quality filtered and adapters were trimmed using Trimmomatic version 0.33 (Bolger *et al.*, 2014). Reads were compared with the Rfam database using the Infernal program version 1.1.2 (Nawrocki *et al.*, 2015) and used to filter tRNAs, rRNAs, snoRNAs and snRNAs from the data. The reads were also filtered using the Triticeae Repeat Sequence (TREP) Database (Wicker *et al.*, 2002) to remove any known Triticeae-specific repeat sequences. Two programs were used to identify sRNA candidates of interest from barley: miRDeep-P (version 1.3) and ShortStack (version 2.1.0) (Yang and Li, 2011, Axtell, 2013).

Differential Expression

For each time point, we performed a differential expression (DE) analysis, comparing relative abundance of sRNAs/reads from the different mutants to CI 16151 (WT). The sRNA/read count datasets were normalized and analyzed by using the DESeq2 program package in R (Love *et al.*, 2014). We added 0.5 units to all read counts and rounded them to the nearest integer to allow use of the DESeq2 normalization method. Reads with 0.9 quantile smaller than a count of 2 are assumed to be expressed at a very low level and were removed from the analysis. The

remaining sRNAs/reads were analyzed for DE. The p-values were adjusted for multiple testing error using Q-value calculations (Nettleton *et al.*, 2006), and sRNAs/reads were filtered for a Q-value of less than 0.05.

PARE Library Sequencing and Data Analysis

Source RNA that was used for sRNA sequencing above was also used for PARE. PARE libraries were prepared as previously described (Zhai *et al.*, 2014) at the Donald Danforth Plant Science Center in St. Louis, MO and sequenced on a HiSeq 2500 (Illumina, Inc.). Reads were quality assessed using the FastQC program version 0.11.3 (Andrews, 2010). Reads were quality filtered and adapters were trimmed using Trimmomatic version 0.33 (Bolger *et al.*, 2014). The two PARE analysis programs sPARTA (version 1.21) (Kakrana *et al.*, 2014) and CleaveLand (version 4.4) (Addo-Quaye *et al.*, 2009) were used independently to identify likely sRNA targets using sRNA sequencing data, the barley transcriptome (ensembl version 38) (Mascher *et al.*, 2017) or the *Bgh* transcriptome (ensembl version 32), and PARE sequencing data. PARE validated targets were filtered based on adjusted p-values using a 1% false discovery rate along with a PARE category of less than 2 (with sPARTA data).

Availability of Data and Materials

Small RNA sequencing dataset has been submitted to NCBI under the accession number XXXX. PARE library sequencing data has been submitted to NCBI under accession number XXXX. Supplemental Materials for Chapter 4 can be accessed in the zipped folder "Chapter_4_Supplemental_Files" on ProQuest.

Supplemental Material [See Appendix]

Supplemental Table 4.1 Barley trans-kingdom PARE validated predicted miRNAs and barley genome mapped sRNAs

Supplemental Table 4.2 *Bgh* trans-kingdom PARE validated predicted miRNAs and barley genome mapped sRNAs

Results

Identification of Barley and Bgh *sRNAs*

To identify potential trans-kingdom active small RNAs expressed in *Bgh* infected barley leaves, we developed a panel of sRNA libraries derived from barley line CI 16151 and four fast-neutron derived immune-signaling mutants. *Bgh*-inoculated 1st leaves (5 genotypes x 6 time points x 3 biological replications) were harvested from a split-plot design at 0, 16, 20, 24, 32, and 48 HAI for a total of 90 samples. In total, the 90 libraries contained ~2.8 billion reads, including 86.6 million unique sequences. These raw reads were filtered and analyzed using the custom pipeline shown in **Figure 4.1A**. Briefly, the raw reads were filtered and trimmed for quality followed by a filter to exclude known (non-miRNA) RNA motifs such as snoRNAs, rRNAs, tRNAs, and snRNAs. For the barley pipeline the reads were also filtered with the TREP database to remove known barley genome repeats (Wicker *et al.*, 2002). Following the filtering, the remaining reads were aligned to either the barley or *Bgh* genomes using Bowtie. The set of genome-aligned reads were subjected to two separate and independent pathways. In the first pathway, reads were run through the two plant miRNA rule-specific prediction programs,

ShortStack and miRDeep-P, to identify likely miRNAs/milRNAs. These programs identified 1742 and 1425 barley miRNAs and *Bgh* milRNA candidates, respectively. The relative conservation of the *Bgh* milRNA candidates is unknown as databases of known fungal functional sRNAs are not available at this time. In contrast, the level of conservation in the barley predicted miRNAs was calculated using conserved miRNA families present in the miRBase database (Kozomara and Griffiths-Jones, 2011). In the pool of predicted barley miRNAs, 88 are homologous to 27 conserved plant miRNAs as detailed elsewhere (**Chapter 2 Table 1**). These miRNA homologs include 17 have been identified as pathogen-responsive miRNAs (Kuan *et al.*, 2016).

In the second pathway to identify genome-mapped reads, two stringent requirements were applied to the reads: 1) exact mapping to the genome (no mismatches) and, 2) at least 10 read counts across the 90 libraries. These reads were designated genome mapped sRNAs. These filters reduced the number of candidate reads from 86 million total to 1.98 million barley genome mapped sRNAs and 0.98 million *Bgh* genome mapped sRNAs. These represent a pool of potentially biologically active sRNAs from barley and *Bgh* that have unknown functions.

Predicted miRNA/milRNA Read Size Distributions and Base Compositions

The size distributions of the predicted miRNAs/milRNAs along with genome mapped sRNAs were unexpected, especially in barley. In grasses, the size distribution of leaf sRNAs have peaks at 21 and 24 nucleotides (nt) (Nobuta *et al.*, 2008, Jeong *et al.*, 2011). Both the barley genome mapped sRNAs and predicted barley miRNAs in our study had peaks at 22 and 25 nt (**Figure 4.2A**). We examined the base distributions at both the 5' and 3' ends in predicted barley miRNAs and found a noticeably higher frequency of U bases at the 3' end (94%) as

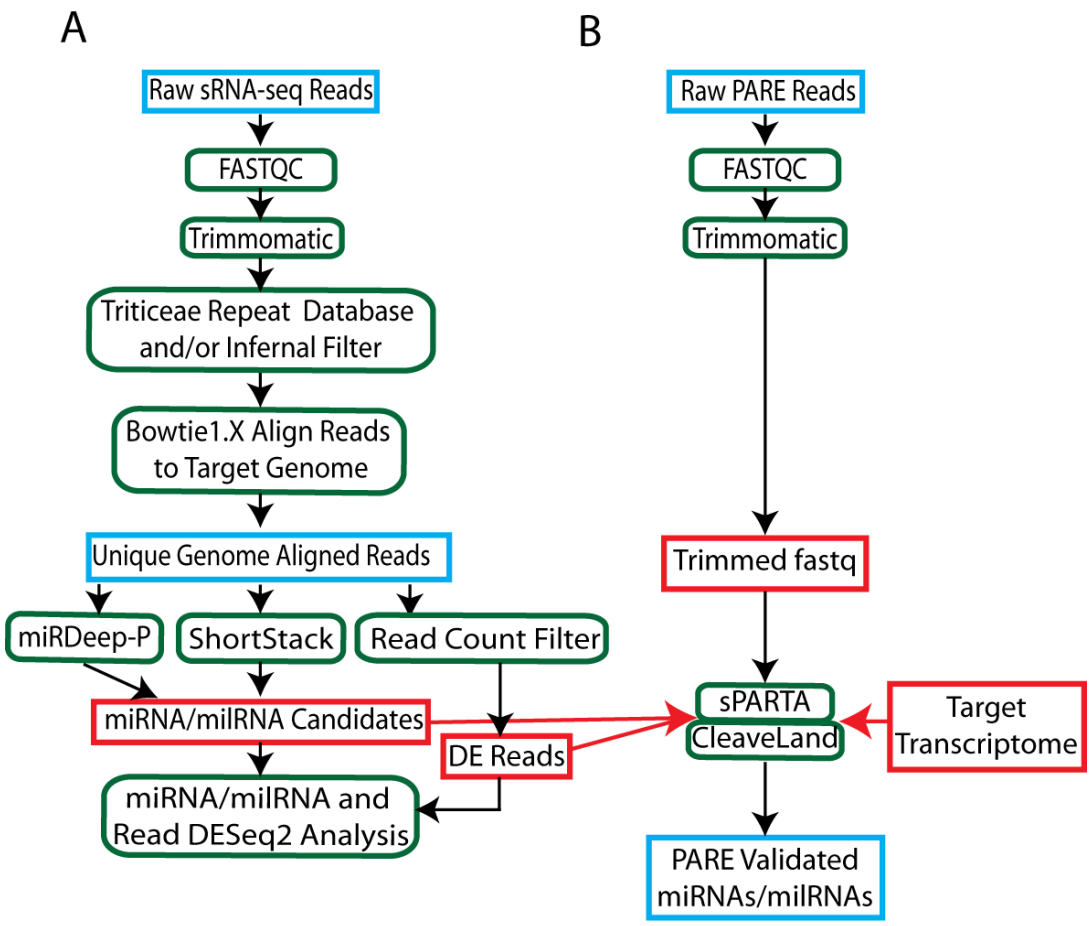

Figure 4.1 Small RNA sequencing and PARE sequencing analysis pipelines. (**A**) Small RNA-seq Illumina reads were trimmed, filtered, and run through the two plant miRNA identification programs miRDeep-P and ShortStack to identify miRNA/milRNA candidates and DE reads. (**B**) Sequencing reads from the PARE libraries were trimmed and filtered and analyzed with the sPARTA and CleaveLand programs. Additional input data was provided from the barley or *Blumeria* transcriptome and miRNA/milRNA candidates plus DE reads developed from the sRNA sequencing pipeline.

compared with known miRBase barley miRNAs (40%) (**Figure 4.2C**). We believe that this effect is either due to a library preparation artifact or due to degraded sRNA samples (Xie *et al.*, 2015). To correct for this effect we tried two approaches: removal of the last base and removal of the last base only if it is a uracil. Both approaches resulted in similar distributions (**Figure 4.2A**), so

we moved forward with last base trimmed data for both barley and *Bgh* sRNAs. The corrected size distribution and base compositions for *Bgh* are shown in (**Chapter 2 Figure 3**).

Differential Expression Analysis of Barley and* Bgh *sRNA Candidates

Differential expression (DE) analysis was carried out on both the predicted miRNAs/milRNAs and genome mapped sRNAs for barley and *Bgh*. The DE analysis compared relative abundance of sRNAs/reads from the different mutant genotypes to WT at each time point. For the barley analysis we identified a total of 9 DE predicted miRNAs and 2423 genome mapped sRNAs including potential members of the miR398 and miR528 stress response-related families that were selectively upregulated in the *mla6* susceptible barley line. In *Bgh* we identified 268 milRNAs and 13311 genome mapped sRNAs that were DE. The pattern of DE was striking in that over 98% of all predicted milRNAs and genome mapped sRNAs were only DE at 48 HAI, indicating a potential role in shifting development and related gene expression at that life cycle time point.

Potential Trans-Kingdom sRNA Candidates Identified Through PARE

In silico prediction of sRNA transcript targets can result in a plethora of predicted targets for each sRNA. However in many cases more than 90% of the predictions can be spurious (Zhai *et al.*, 2014). Considering the time and effort required to check each of these potential targets,

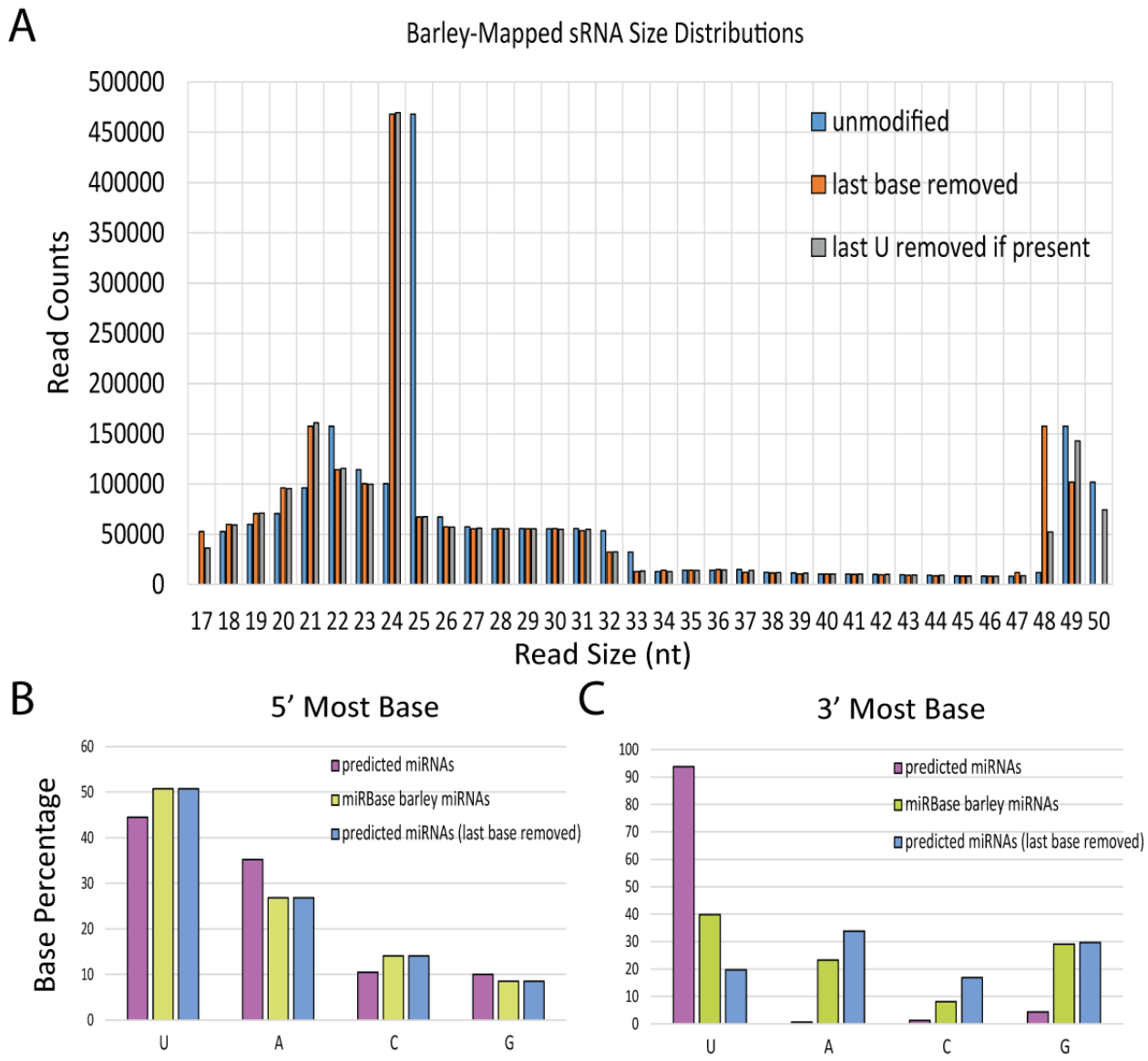

Figure 4.2 Size distributions of barley genome mapped sRNAs and read-end base distributions of barley predicted miRNAs. Barley genome mapped sRNAs size distributions for unmodified reads (blue), last base removed reads (orange) and last U removed reads (if present) (grey). (**B**) 5' most base percentages for unmodified predicted barley miRNAs (purple), miRBase barley miRNAs (yellow), and last base removed predicted barley miRNAs (blue). (**C**) 3' most base percentages for unmodified predicted barley miRNAs (purple), miRBase barley miRNAs (yellow), and last base removed predicted barley miRNAs (blue).

a high throughput alternative has been developed designated parallel analysis of RNA ends (PARE) (Addo-Quaye *et al.*, 2008, German *et al.*, 2008, Gregory *et al.*, 2008). The PARE technique allows for the selective identification of sRNA cut sites in poly-A-containing transcripts by purifying 5' monophosphate RNA ends. The pipeline for processing PARE library data into PARE-validated sRNA-read combinations is shown in **Figure 4.1B**. Briefly raw PARE library reads are quality filtered and trimmed followed by processing by the two PARE analysis programs CleaveLand and sPARTA (Addo-Quaye *et al.*, 2009, Kakrana *et al.*, 2014). The CleaveLand and sPARTA programs require three types of data to function including PARE library reads, a sRNA list, and transcriptome file. Using internal sRNA prediction programs, each program predicts sRNA targets in the given transcriptome and separately maps the PARE reads to the transcriptome data with Bowtie. The overlap between predicted sRNA cut sites and the mapping frequency of PARE reads is compared to determine the statistical likelihood of each predicted cut site, resulting in several criteria for each locus

Barley Trans-Kingdom sRNAs Target Vital Gene Expression to Reduce Pathogen Vitality

To identify potential trans-kingdom barley sRNAs with *Bgh* transcript targets the PARE analysis programs were loaded with three sets of data: 1. the PARE libraries developed from *Bgh* infected barley, 2. barley predicted miRNAs and DE barley genome mapped sRNAs and 3. the *Bgh* transcriptome. The PARE predicted sRNA/transcript pairs were filtered by PARE category (less than 2), adjusted p-value (less than 0.01), and for sPARTA program outputs, PARE abundance (greater than 5). Next these pairs were filtered by removing pairs where the barley sRNA had a PARE validated barley transcript target (adjusted p-value less than 0.05, PARE

category less than 2, and PARE abundance greater than 5). The final filtered list of pairs is shown in **Supplemental Table 4.1** (Appendix) and includes 78 pairs, 71 unique barley sRNAs (predicted miRNAs and barley genome mapped sRNAs), and 51 target transcripts. The transcripts were functionally annotated using ensembl annotations, blastx comparisons to the refseq protein ncbi database, interproscan (v 5.15-54-0), and literature review (**Table 4.1**). The functional categories with higher percentages include cellular structure and function (31.4%), effectors (15.7%), signaling (9.8%), and transcription-related (9.8%) (**Table 4.2**). The predicted *Bgh* transcript targets encode proteins that are vital for cellular function including ribosomal proteins, nuclear transport proteins, cell cycle proteins, chaperones, and transcript splicing factors. Because of the relatively small number of genes in the *Bgh* genome (6470) and the lack of paralogs for many vital genes (Spanu, 2014), it is likely that these sRNAs could have a detrimental effect on *Bgh* growth, especially in combination.

One of the most biologically relevant over-represented functional categories identified were genes that encoded *Bgh* effectors. These can be generally divided into the two categories: candidate secreted effector proteins (CSEPs) and effectors homologous to AVR_{k1} and AVR_{a10} (EKA). The PARE-validated sRNA/transcript pairs identified eight potential effector gene targets including 3 EKA effector class members, 4 CSEPs (*CSEP0254*, *CSEP0263*, *CSEP0304*, and *CSEP0326*) and one unclassified putative effector. *CSEP0254* was recently identified as a significant contributor to *Bgh* virulence and would therefore be a biologically significant target for barley sRNAs (Ahmed *et al.*, 2016).

Bgh *Trans-Kingdom sRNAs Target Defense Genes for Silencing*

To identify barley trans-kingdom sRNAs predicted to target *Bgh* transcripts, the PARE analysis programs were run with all genotype-specific PARE libraries, the predicted *Bgh* sRNAs, and the barley transcriptome. The PARE predicted sRNA/transcript pairs were filtered by PARE category (less than 2), adjusted p-value (less than 0.05), and for sPARTA program outputs, PARE abundance (greater than 5). Next these pairs were filtered by removing entries where the *Bgh* sRNA had a PARE validated *Bgh* transcript target (adjusted p-value less than 0.05, PARE category less than 2, and PARE abundance greater than 5). The final list had a total of 49 pairs, 32 unique *Bgh* sRNAs, and 42 unique barley transcript targets (**Supplemental Table 4.2** [Appendix]). The predicted barley transcript targets were functionally annotated as described above and are shown in **Table 4.3**. While no functional category is dominant over the others in terms of counts, the biological significance of the members of this list are striking (**Table 4.4**). For example, several members of the list have functions directly related to plant and fungal cell walls including cellulose, waxes, and fungal glucans. Cellulose synthase is directly involved in the formation of papillae that help prevent fungal penetration. In a recent study, the barley cellulose synthase gene *HvCslD2* was knocked out resulting in plants with no growth defects, but with reduced resistance to *Bgh* infection (Douchkov *et al.*, 2016). Fungal glucan molecules can serve as potent PTI elicitors in a similar manner to chitin (Fesel and Zuccaro, 2016). Down regulation of barley glucan endo-13-beta-glucosidases may help reduce the small glucan chains that can trigger PTI responses.

Table 4.1 Annotation details for barley sRNA predicted *Blumeria* transcript targets. Annotation details shown from Ensembl, Blastx, Interproscan, and literature based summary functional category.

Transcript Match	Ensembl Annotation	Blastx Annotation	Interpro Annotations Description	Function	Category
BGHDH14_bgh00205	hypothetical protein description	cytoplasmic tRNA 2-thiolation protein-like protein 2 [Cadophora sp. DSE1049]	Cytoplasmic tRNA 2-thiolation protein 2	tRNA modification	cellular structure and function
BGHDH14_bgh00241	hypothetical protein	cell cycle control protein [Marssonina brunnea f. sp. 'multigermtubi' MB_m1]	NA	cell cycle related	cellular structure and function
BGHDH14_bgh00908	Karyopherin-like protein	karyopherin Kap123 [Xylona heveae TC161]	Importin-beta, N-terminal domain	nuclear transport proteins	cellular structure and function
BGHDH14_bgh01050	pescadillo-similar protein	pescadillo [Sclerotinia sclerotiorum 1980 UF-70]	Pescadillo	ribosomal biosynthesis	cellular structure and function
BGHDH14_bgh01105	putative importin subunit alpha-1	karyopherin alpha-1-like protein [Marssonina brunnea f. sp. 'multigermtubi' MB_m1]	Importin-alpha, importin-beta-binding domain	nuclear transport proteins	cellular structure and function
BGHDH14_bgh01273	fungal protein	exonuclease V protein [Rutstroemia sp. NJR-2017a WRK4]	Exonuclease V	nuclease	cellular structure and function
BGHDH14_bgh01755	60S ribosomal protein L26	60S ribosomal protein L26 [Diplocarpon rosae]	Ribosomal protein L26/L24P, eukaryotic/archaeal	ribosomal protein	cellular structure and function
BGHDH14_bgh01959	tRNA modification GTPase 2C mitochondrial	tRNA modification GTPase TrmE [Fusarium oxysporum f. sp. lycopersici 4287]	tRNA modification GTPase MnmE domain 2	tRNA modification	cellular structure and function
BGHDH14_bgh02660	G2/M phase checkpoint control protein Sum2	putative g2 m phase checkpoint control protein [Erysiphe necator]	Lsm14 N-terminal	cell cycle related	cellular structure and function
BGHDH14_bgh02950	peroxin 26	peroxin 26 [Marssonina brunnea f. sp. 'multigermtubi' MB_m1]	L27 domain	peroxisome protein	cellular structure and function
BGHDH14_bgh03764	60S ribosomal protein L39	ribosomal protein L39e, putative [Talaromyces stipitatus ATCC 10500]	Ribosomal protein L39e	ribosomal protein	cellular structure and function
BGHDH14_bgh03977	hypothetical protein	NTF2 and RRM domain-containing protein [Diplocarpon rosae]	Nuclear transport factor 2, eukaryote	mRNA transport factor	cellular structure and function

Table 4.1 (Continued)					
Transcript Match	**Ensembl Annotation**	**Blastx Annotation**	**Interpro Annotations Description**	**Function**	**Category**
BGHDH14_bgh05058	hypothetical protein	putative nap family protein [Erysiphe necator]	Nucleosome assembly protein (NAP)	Nucleosome assembly protein	cellular structure and function
BGHDH14_bgh05263	chromatin remodelling complex ATPase chain ISW1	P-loop containing nucleoside triphosphate hydrolase [Glarea lozoyensis ATCC 20868]	SANT/Myb domain	chromatin remodelling	cellular structure and function
BGHDH14_bgh06915	hypothetical protein	white collar [Marssonina brunnea f. sp. 'multigermtubi' MB_m1]	PAS-associated, C-terminal	light response protein	cellular structure and function
BGHDH14_bgh04063	EKA-like protein	putative eka-like protein [Erysiphe necator]	NA	effector	effector
BGHDH14_bgh04150	EKA-like protein	putative virulence effector, partial [Blumeria graminis f. sp. tritici]	NA	effector	effector
BGHDH14_bgh05751	CSEP0254 putative effector protein	putative secreted effector protein [Blumeria graminis f. sp. tritici 96224]	NA	effector	effector
BGHDH14_bgh06515	CSEP0263 putative effector protein	putative secreted effector protein [Blumeria graminis f. sp. tritici 96224]	NA	effector	effector
BGHDH14_bghG000692000002001	CSEP0304 putative effector protein	putative secreted effector protein [Blumeria graminis f. sp. tritici 96224]	NA	effector	effector
BGHDH14_bghG001225000001001	CSEP0326 putative effector protein	putative secreted effector protein [Blumeria graminis f. sp. tritici 96224]	NA	effector	effector
BGHDH14_bghG003307000001001	EKA-like protein	putative effector protein [Podosphaera xanthii]	Protein of unknown function DUF3129	effector	effector
BGHDH14_bghG006402000005001	putative effector protein	putative secreted effector protein [Blumeria graminis f. sp. tritici 96224]	NA	effector	effector
BGHDH14_bgh02125	hypothetical protein	HCP-like protein [Phialocephala scopiformis]	Sel1-like repeat	unclear	hypothetical or unknown
BGHDH14_bgh06242	hypothetical protein	conserved fungal protein [Diplocarpon rosae]	Protein of unknown function DUF4452	unknown	hypothetical or unknown
BGHDH14_bghG007361000001001	hypothetical protein	no quality matches	NA	unknown	hypothetical or unknown

Table 4.1 (Continued)					
Transcript Match	**Ensembl Annotation**	**Blastx Annotation**	**Interpro Annotations Description**	**Function**	**Category**
BGHDH14_bgh01548	SANT	PHD finger and BAH domain-containing protein (Snt2) [Pochonia chlamydosporia 170]	Zinc finger, PHD-type	pathogenicity related	pathogen factor
BGHDH14_bgh05140	lysophospholipase	lysophospholipase [Phialocephala scopiformis]	Lysophospholipase, catalytic domain	membrane lysis protein	pathogen factor
BGHDH14_bgh00769	Protein disulfide isomerase	disulfide isomeras-like protein [Phialocephala scopiformis]	Thioredoxin domain	disuflide bond formation	post translational modification
BGHDH14_bgh00767	hypothetical protein	heat shock protein 70 [Phialocephala scopiformis]	Heat shock protein 70 family	heat shock protein	protein folding
BGHDH14_bgh01767	putative peptidyl-prolyl cis-trans isomerase	cyclophilin type peptidyl-prolyl cis-trans isomerase/CLD [Marssonina brunnea f. sp. 'multigermtubi' MB_m1]	Cyclophilin-type peptidyl-prolyl cis-trans isomerase domain	protein chaperones	protein folding
BGHDH14_bgh05432	hypothetical	26S proteasome regulatory complex	26S proteasome regulatory complex, non-ATPase subcomplex, Rpn2/Psmd1 subunit	Proteasome-related	protein turnover
BGHDH14_bgh04503	serine/threonine protein kinase domain protein [Blumeria graminis f. sp. hordei DH14]	serine threonine-protein kinase sgk2 [Umbilicaria pustulata]	Protein kinase-like domain	kinase	signaling
BGHDH14_bghG003171000001001	serine/threonine-protein kinase Sgk2	protein kinase [Metarhizium robertsii]	Protein kinase-like domain	kinase	signaling
BGHDH14_bghG005266000001001	serine/threonine protein kinase domain protein	serine/threonine protein kinase Sgk2 [Histoplasma capsulatum H143]		kinase	signaling
BGHDH14_bghG007623000001001	serine/threonine-protein kinase Sgk2, partial	1 protein kinase [Umbilicaria pustulata]	Protein kinase-like domain	kinase	signaling
BGHDH14_bghG009256000001001	serine/threonine-protein kinase Sgk2, partial	serine/threonine protein kinase Sgk2 [Histoplasma capsulatum H143]	Protein kinase-like domain	kinase	signaling
BGHDH14_bgh01259	Transcription initiation factor IIB	transcription initiation factor TFIIB [Pseudogymnoascus verrucosus]	Transcription factor TFIIB	core transcription factor	transcription-related

Table 4.1 (Continued)					
Transcript Match	**Ensembl Annotation**	**Blastx Annotation**	**Interpro Annotations Description**[7]	**Function**	**Category**
BGHDH14_bgh04727	U4/U6.U5 tri-snRNP-associated protein [Blumeria graminis f. sp. hordei DH14]	putative u4 tri-snrnp-associated protein [Erysiphe necator]	Domain of unknown function DUF1777	transcript splicing	transcription-related
BGHDH14_bgh04754	hypothetical protein	DNA-directed RNA polymerase III complex subunit Rpc37 [Marssonina brunnea f. sp. 'multigermtubi' MB_m1]	DNA-directed RNA polymerase III subunit Rpc5	RNA polymerase	transcription-related
BGHDH14_bgh06758	eukaryotic translation initiation factor	putative eukaryotic translation initiation factor 3 subunit C [Phialocephala scopiformis]	Eukaryotic translation initiation factor 3 subunit C	translation initiation factor	translation-related
BGHDH14_bgh02644	Solute	Mitochondrial carrier [Glarea lozoyensis ATCC 20868]	Mitochondrial carrier protein	mitochondrial carrier	transporter
BGHDH14_bgh04773	MFS sugar transporter	putative mfs sugar transporter [Erysiphe necator]	Mso1, N-terminal domain	sugar transporter	transporter
BGHDH14_bgh06370	plasma membrane channel protein Ist2	plasma membrane channel protein [Marssonina brunnea f. sp. 'multigermtubi' MB_m1]	Anoctamin	plasma membrane channel	transporter
BGHDH14_bgh03480	PX domain containing protein/BAR superfamily/vacuolar targeting protein Atg24, putative	Sorting nexin [Blumeria graminis f. sp. tritici 96224]	Phox homologous domain	vessicle transport	vessicle transport
BGHDH14_bgh04897	ADP-ribosylation factor	ADP-ribosylation factor 1 [Marssonina brunnea f. sp. 'multigermtubi' MB_m1]	Small GTP-binding protein domain	vessicle transport	vessicle transport

[7] NA: PFAM annotation unavailable

Table 4.2 Barley sRNA predicted *Blumeria* transcript target annotation category counts and percentages

Category	Number	Percentage
cellular structure and function	16	31.4
effector	8	15.7
signaling	5	9.8
transcription-related	5	9.8
hypothetical or unknown	3	5.9
transporter	3	5.9
metabolism	2	3.9
pathogen factor	2	3.9
protein folding	2	3.9
vesicle transport	2	3.9
post translational modification	1	2.0
protein turnover	1	2.0
translation-related	1	2.0
Total	51	

The defense compound strictosidine and aspartic proteinase CDR1 protein have both been implicated in defense responses in plants. Both genes are predicted targets of *Bgh* trans-kingdom sRNAs. Strictosidine is a precursor for biosynthesis of terpenoid indole alkaloids and is induced by several elicitors including salicylic acid, ethylene, jasmonci acid, and *Alternaria brassicicola* in *Arabidopsis* (Facchini, 2001, Sohani *et al.*, 2009). The aspartic proteinase CDR1 is an apoplastic proteinase that has been linked to systemic acquired resistance in *Arabidopsis* (Xia *et al.*, 2004, Simoes *et al.*, 2007). Silencing of strictosidine synthase and aspartic proteinase CDR1 encoding transcripts through *Bgh* produced trans-kingdom sRNAs could reduce the overall effectiveness of the barley PTI response.

Membrane trafficking is a key component of plant defense responses and is a common target of pathogen effectors (Gu *et al.*, 2017). Creating papilla rapidly to counteract pathogen

penetration attempts requires the full function of the endomembrane system, especially the secretory trafficking pathway (Ellinger *et al.*, 2013). Reticulon and Sec13 are two key components of the endomembrane system related to COPII coat proteins and ER structural integrity (Hwang and Robinson, 2009, Di Sano *et al.*, 2012). Transcripts encoding these proteins are predicted *Bgh* sRNA targets, and reduction in transcript levels for either gene could lead to a less efficient secretory pathway-related PTI responses.

Discussion

Effector proteins produced in plant pathogens act directly to reduce the function of plant defense pathways. Important defense network hubs are more likely to be targeted by pathogen effectors, and because of this are usually guarded by NLR proteins (Mukhtar *et al.*, 2011). The ETI response pathway is a strong defense response that can lead to localized cell death through the hypersensitive response (Cui *et al.*, 2015). An alternative has been discovered that allows pathogens to circumvent the ETI pathway that guards proteins important in pathogen defense: sRNAs. Small RNAs, when imported into plant cells may not trigger defense responses in the same way that avirulence factors can. Rather, they act directly to silence target transcripts thereby reducing expression of a target defense gene without triggering a response. Recent studies have provided examples of sRNA effectors in action in fungal pathogens including *Botrytis cinerea* and *Puccinia striiformis* f. sp. *tritici* (Weiberg *et al.*, 2013, Wang *et al.*, 2017). These sRNAs are important virulence factors for these pathogens, as knocking out these small RNAs leads to significant reductions in pathogenicity.

Table 4.3 Annotation details for *Blumeria* sRNA predicted barley transcript targets. Annotation details shown from Ensembl, Blastx, Interproscan, and literature based summary functional category.

Transcript Match	Ensembl Annotation	Blastx Annotation	Interpro Annotations Description	Function	Category
HORVU1Hr1G026320.6	cellulose synthase 1	probable cellulose synthase A catalytic subunit 1 [UDP-forming] [Brachypodium distachyon]	Cellulose synthase, RING-type zinc finger	cellulose synthase	cell wall-related
HORVU1Hr1G092310.2	Glucan endo-1,3-beta-glucosidase 13	glucan endo-13-beta-glucosidase 13-like isoform X2 [Aegilops tauschii subsp. Tauschii]	GO:0005975	degrade fungal cell wall polysaccharides	cell wall-related
HORVU2Hr1G088860.1	Fatty acid hydroxylase superfamily	protein ECERIFERUM 1-like [Aegilops tauschii subsp. tauschii]	Fatty acid hydroxylase	wax related	cell wall-related
HORVU2Hr1G127260.1	Cytochrome P450 superfamily protein	alkane hydroxylase MAH1-like [Aegilops tauschii subsp. tauschii]	Cytochrome P450	wax biosynthesis	cell wall-related
HORVU5Hr1G052820.1	Glucan endo-1,3-beta-glucosidase 5	glucan endo-13-beta-glucosidase 5 [Aegilops tauschii subsp. Tauschii]	Glycoside hydrolase, family 17	glucan hydrolase	cell wall-related
HORVU4Hr1G039630.1	Elongator complex protein 3	PREDICTED: elongator complex protein 3 [Oryza brachyantha]	Radical SAM, C-terminal extension	histone acetylase	cellular structure and function
HORVU5Hr1G078340.1	strictosidine synthase-like 3	protein STRICTOSIDINE SYNTHASE-LIKE 10-like [Aegilops tauschii subsp. tauschii]	Strictosidine synthase, conserved region	defense protein	defense
HORVU4Hr1G080560.3	TSA: Wollemia nobilis Ref_Wollemi_Transcript_11984_1529 transcribed RNA sequence	protein CHAPERONE-LIKE PROTEIN OF POR1, chloroplastic [Aegilops tauschii subsp. tauschii]	Protein CHAPERONE-LIKE PROTEIN OF POR1-like	chloroplast development and function	Energy-related
HORVU3Hr1G047220.1	ABA-responsive protein	GEM-like protein 5 [Aegilops tauschii subsp. tauschii]	Nuclease-related domain, NERD	ABA responsive protein	hypothetical or unknown
HORVU6Hr1G050440.2	senescence-associated family protein	uncharacterized protein LOC109774684 isoform X7 [Aegilops tauschii subsp. tauschii]	Armadillo-type fold	unclear	hypothetical or unknown
HORVU3Hr1G058440.2	GDSL esterase-lipase	GDSL esterase/lipase At1g28600-like [Aegilops tauschii subsp. tauschii]	GDSL lipase/esterase	lipid related	metabolism

Table 4.3 (Continued)

Transcript Match	Ensembl Annotation	Blastx Annotation	Interpro Annotations Description	Function	Category
HORVU5Hr1G062090.1	Aldehyde dehydrogenase family 7 member A1	aldehyde dehydrogenase family 7 member A1 [Aegilops tauschii subsp. tauschii]	Aldehyde dehydrogenase domain	metabolism	metabolism
HORVU0Hr1G030970.2	Eukaryotic aspartyl protease family protein	aspartic proteinase CDR1-like [Aegilops tauschii subsp. tauschii]	Aspartic peptidase	defense-related protease	protein turnover
HORVU3Hr1G005410.2	E3 ubiquitin-protein ligase	E3 ubiquitin-protein ligase SINA-like 2 isoform X3 [Aegilops tauschii subsp. tauschii]	TRAF-like	Ubiquitin-related	protein turnover
HORVU3Hr1G067470.1	26S protease regulatory subunit 6B homolog	PREDICTED: 26S protease regulatory subunit 6B homolog [Brachypodium distachyon]	ATPase, AAA-type, conserved site	26S protease subunit	protein turnover
HORVU6Hr1G026200.5	polyubiquitin 3	polyubiquitin 11-like [Aegilops tauschii subsp. tauschii]	Ubiquitin domain	protein turnover	protein turnover
HORVU1Hr1G052470.2	Glutathione S-transferase family protein	Glutathione S-transferase [Triticum urartu]	Glutathione S-transferase, N-terminal	glutathione transferase	redox control
HORVU7Hr1G101220.3	Intracellular protease PfpI family protein	DJ-1 protein homolog E [Aegilops tauschii subsp. tauschii]	DJ-1/PfpI	ROS scavenger	redox control
HORVU4Hr1G079250.1	Ras-related protein Rab-18-B	ras-related protein RABC2a-like [Aegilops tauschii subsp. tauschii]	Small GTP-binding protein domain	ABA induced stress tolerance	stress-related
HORVU1Hr1G004860.1	Pentatricopeptide repeat-containing protein	pentatricopeptide repeat-containing protein At4g35130 chloroplastic [Aegilops tauschii subsp. Tauschii]	Pentatricopeptide repeat	Organelle-transcription	transcriptional regulation
HORVU2Hr1G099890.2	Ethylene-responsive transcription factor 4	ethylene-responsive transcription factor 4-like [Aegilops tauschii subsp. tauschii]	AP2/ERF domain	AP2 TF	transcriptional regulation
HORVU3Hr1G018980.6	basic helix-loop-helix (bHLH) DNA-binding family protein	putative transcription factor bHLH041 isoform X1 [Aegilops tauschii subsp. Tauschii]	Myc-type, basic helix-loop-helix (bHLH) domain	bHLH TF	transcriptional regulation
HORVU3Hr1G094860.1	Calmodulin-binding transcription activator 4	calmodulin-binding transcription activator 4-like isoform X1 [Aegilops tauschii subsp. Tauschii]	IQ motif, EF-hand binding site	calcium binding transcriptional activator	transcriptional regulation

Table 4.3 (Continued)

Transcript Match	Ensembl Annotation	Blastx Annotation	Interpro Annotations Description	Function	Category
HORVU6Hr1G072810.6	Homeobox-leucine zipper protein family	homeobox-leucine zipper protein HOX16-like [Aegilops tauschii subsp. Tauschii]	Homeodomain-like	homeodomain TF	transcriptional regulation
HORVU7Hr1G077650.5	PHD finger family protein	PHD finger protein EHD3-like [Aegilops tauschii subsp. tauschii]	Zinc finger, PHD-type	floral development TF	transcriptional regulation
HORVU2Hr1G007760.1	ABC transporter family protein	ABC transporter G family member 28-like [Aegilops tauschii subsp. tauschii]	ABC transporter-like	active transport	transporter
HORVU2Hr1G090960.2	ABC transporter G family member 11	ABC transporter G family member 11-like [Aegilops tauschii subsp. tauschii]	ABC-2 type transporter	active transport	transporter
HORVU4Hr1G050780.2	Ribose import ATP-binding protein RbsA	ABC transporter I family member 6 chloroplastic [Aegilops tauschii subsp. Tauschii]	ABC transporter-like	active transport	transporter
HORVU2Hr1G109500.1	Protein transport protein SEC13 homolog A	protein transport protein SEC13 homolog B-like [Aegilops tauschii subsp. tauschii]	WD40 repeat	vessicle transport	vessicle transport
HORVU7Hr1G075700.2	Reticulon family protein	reticulon-like protein B2 [Aegilops tauschii subsp. tauschii]	Reticulon	vessicle transport	vessicle transport
HORVU7Hr1G081290.2	Reticulon family protein	Reticulon-like protein B2 [Triticum urartu]	Reticulon	vessicle transport	vessicle transport

Table 4.4 *Blumeria* sRNA predicted barley transcript target annotation category counts and percentages

Categories	Count	Percentage
transcriptional regulation	6	18.8
cell wall-related	5	15.6
protein turnover	4	12.5
metabolism	3	9.4
transporter	3	9.4
vesicle transport	3	9.4
hypothetical or unknown	2	6.3
redox control	2	6.3
cellular structure and function	1	3.1
defense	1	3.1
Energy-related	1	3.1
stress-related	1	3.1
Total	32	

Plant PTI defense responses include the production of anti-fungal compounds that are secreted into the apoplast through extracellular vesicles (Samuel *et al.*, 2015). Small RNAs are a recently discovered part of that defense response are an area of active research. The expression of sRNAs with antifungal properties has obvious applications in creating mechanisms for resistance against fungi where strong *R*-genes are not available (Baulcombe, 2015). Recent studies have shown that plant expression of sRNAs targeting fungal gene expression can be a potent antifungal defense strategy (Nowara *et al.*, 2010, Wang *et al.*, 2016). In cotton, the highly conserved miRNAs miR159 and miR166 were shown to silence the expression of virulence factors in *Verticillium dahliae* (Zhang *et al.*, 2016). During pathogen infection miR159 and miR166 expression significantly increases in several species including cotton (Kuan *et al.*, 2016, Zhang *et al.*, 2016). This may mean that these miRNAs have a general role in plant PTI defense responses.

Small RNA Populations in* Bgh *Infected Barley Leaves

To identify sRNAs from barley and *Bgh* that function either as trans-kingdom effectors or resistance factors, we created sRNA libraries from *Bgh* infected barley leaves. We identified 1742 and 1425 barley miRNAs and *Bgh* milRNA candidates, respectively through our bioinformatic pipeline. The final size distributions for barley miRNAs and genome mapped sRNAs had peaks at 21 and 24 nt as expected for grass species after corrections mentioned above (**Figure 2**) (Nobuta *et al.*, 2008). *Bgh* size distributions for predicted milRNAs and genome mapped sRNAs had peaks at 21 and 22 nt. Size distributions for sRNAs in fungi are highly variable between species and even between conditions, but the distribution is similar to other species' (Lee *et al.*, 2010, Zhou *et al.*, 2012, Lau *et al.*, 2013).

Barley Trans-Kingdom sRNAs Target Conserved Pathogen Genes and* Bgh-*Specific Effector Genes

Barley sRNAs were predicted to target *Bgh* transcripts in a trans-kingdom mechanism based on PARE library analysis which allows for identification of sRNA cut sites *in vivo*. The predicted *Bgh* transcripts in many cases encode highly conserved proteins involved in core cellular activities such as ribosome synthesis/function, tRNA modification, core transcription/translation factors, cell cycle regulators, and others (**Table 4.2**). These sRNAs could be produced as part of the PTI defense response to target invading pathogens. The sequences of these sRNAs are divergent from transcripts that encode proteins of similar function in barley, and therefore may have evolved to target likely pathogens. During the PTI response, anti-microbial compounds are exported to the apoplast including proteases, cell wall

degrading enzymes, and others. At this time it is unclear what the mechanism is for trans-kingdom sRNA uptake by either fungi or plants (Baulcombe, 2015), but regardless of the mechanism it may represent a mostly unexplored pathogen defense system.

Several of the predicted *Bgh* transcript targets for barley sRNAs include effector genes. These potential effector targets may mean that barley has evolved both a pathogen-specific sRNA defense, as well as a non-species specific defense as outlined above. Effector genes are highly divergent between fungal species and are under positive selection for rapid evolution to evade detection by host NLR and other defense proteins (Pedersen *et al.*, 2012). Members of both the EKA and CSEP *Bgh* effector families are predicted targets of barley sRNAs. One of the predicted targets of barley sRNAs is *CSEP0254*, which was recently shown to be an important virulence factor in *Bgh* (Ahmed *et al.*, 2016). In a similar fashion to the LRR regions in NLR resistance proteins, young miRNA genes can undergo rapid evolution in terms of both sequence and targets (Debat and Ducasse, 2014). It is possible that the fast evolution of sRNA encoding sequences would allow for new active antifungal sequences that contribute directly to non-species specific resistance (conserved protein targets) and also species-specific resistance (effector targets).

Bgh *Trans-Kingdom sRNAs Act as Effectors Silencing PTI Pathway Members*

The obligate biotrophy of *Bgh* requires it keep its host alive throughout its life cycle. Effectors from *Bgh* have to act to both reduce defense responses and to create positive growth conditions for the fungus. Most effectors studied so far in *Bgh* are secreted proteins. We have identified potentially trans-kingdom active sRNAs that may act as effectors by silencing

defense-related barley genes. The PTI defense pathway functions in multifaceted approach to defend against fungal pathogens by creating both physical barriers against penetration (papilla) and inhospitable environments inside and outside the cell (ROS, proteases, and antifungal compounds). The potential *Bgh* trans-kingdom targets includes genes with annotations related to vesicle secretion, cell wall synthesis, protein turnover, transcriptional regulation, ROS response, and fungal cell wall breakdown (**Table 4.4**). All of these areas are important for a fully functional PTI response to increase resistance against successful *Bgh* penetration and haustorial function. When protein effectors are combined with sRNA effectors that are not detected by R-proteins, a potent combination is made that reduces the defense capacity of barley.

Conclusions

Communication between plants and plant fungal pathogens is a two way street. Defense and virulence compounds are transported into the extra-haustorial matrix (EHM) via vesicle transport and can be taken up again by the other species as well (Dormann *et al.*, 2014). The direct chemical communication between the two species allows for the transport of nucleic acids such as sRNAs (Weiberg and Jin, 2015). These sRNA molecules when produced by the plant, can in turn act as defense compounds that reduce pathogen virulence or when produced by fungal pathogens, as effectors that increase susceptibility of plant hosts to infection (Weiberg *et al.*, 2013, Zhang *et al.*, 2016).

We have identified sRNAs produced in *Bgh* that may act as effectors in barley that reduce PTI defense mechanisms. These sRNAs are predicted to act on several areas in plant

defense including protein turnover, ROS response, vesicle secretion, cell wall synthesis, transcriptional regulation, and fungal cell wall breakdown. If these sRNA effectors can act to enhance the functions of protein effectors, this combination may selectively silence important hubs in the barley defense machinery.

The sRNAs identified in barley act on two levels. First, they target highly conserved proteins involved in core transcription/translation factors, cell cycle regulators, ribosome synthesis/function, tRNA modification, and other areas. Second, they target *Bgh* specific effector proteins. From these two predicted target types it appears that barley has evolved both a non-species specific and a species-specific sRNA defense capability.

Both the predicted barley and *Bgh* trans-kingdom sRNAs should be functionally validated *in planta* to evaluate both their ability to cleave their predicted target(s), as well as their ability to affect *Bgh* virulence. To accomplish this goal sRNAs and their target transcripts can be co-expressed in a heterologous system such as *Nicotiana benthamiana* to evaluate the target transcript protein expression levels when compared with controls. Functional sRNA could then be expressed in barley using either VIGS or HIGS to determine their effect on susceptibility of barley to infection and/or *Bgh* pathogenicity.

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CHAPTER 5. GENERAL CONCLUSIONS

The overall goal of this project was to examine the biological impact of small RNAs (sRNAs) on the regulation of gene expression in barley leaves infected with *Blumeria graminis* f. sp. *hordei* (*Bgh*). This goal was accomplished in three parts that are represented in Chapter 2, 3, and 4. In Chapter 2, I described *Bgh* sRNAs expressed during barley leaf infection. Predicted micro RNA-like RNAs (milRNAs) were identified using sRNA sequencing data, and target transcripts were predicted using parallel analysis of RNA ends (PARE) data. The predicted transcript targets were enriched in the effector, metabolism, and translation-related functional categories. The regulation of effector gene expression has not been studied detail in most obligate biotrophic plant pathogens, and it appears that *Bgh* is regulating effector expression in a post transcriptional mechanism with sRNAs. I also identified several members of the EKA family that appear to be regulated through hairpin-producing RNAs that are encoded antiparallel to the gene. This mechanism may be similar to natural antisense small interfering RNAs (natsiRNAs) from plants and animals, that can encode sRNAs that are functional both in *cis* and in *trans*.

In Chapter 3, I examined barley sRNAs expressed during infection by *Bgh*. In that study, I identified conserved and novel micro RNAs (miRNAs), as well as barley genome mapped sRNAs that are predicted to target transcripts encoding transcription factors and signaling proteins for cleavage. I also identified phasing siRNA (phasiRNA) loci in barley that overlap heavily with protein coding transcripts including defense related genes encoding receptor-like kinases and resistance proteins. Barley appears to be regulating its defense response through the expression of miRNAs and phasiRNAs in a combined defense response that has not been

described in other grasses. Most described grass phasiRNAs have been identified in reproductive tissues targeting long non-coding RNAs (lncRNAs). Here, we describe phasiRNAs potentially involved in the regulation of defense-related genes during pathogen infection.

In Chapter 4, potentially trans-kingdom active sRNAs were identified in both barley and *Bgh*. sRNAs were identified from both barley and Bgh that mapped to their respective genomes, but did not have validated PARE transcript target sites, meaning that they were not regulating gene expression in their genomes of origin. In a fascinating find, these sRNAs had validated cut sites with trans-kingdom targets. The predicted barley-produced trans-kingdom sRNAs had *Bgh* targets that represent two contrasting profiles. Many targets encoded highly conserved proteins involved in core cellular activities such as ribosome synthesis/function, tRNA modification, core transcription/translation factors, and cell cycle regulators. However, some predicted targets encoded effector proteins that are highly species-specific in evolution. This points to the possibility of a barley non-species specific and species specific trans-kingdom sRNA defense. The potential Bgh-encoded trans-kingdom sRNAs appear to be more related in function to effectors. The predicted targets of the *Bgh* trans-kingdom sRNAs include key portions of plant defense responses including vesicle secretion, cell wall synthesis, protein turnover, transcriptional regulation, of reactive oxygen species (ROS) response, and fungal cell wall breakdown. This suggests the *Bgh* trans-kingdom sRNAs may act as effector molecules that complement the function of protein effectors in disabling the pathogen associated molecular patterns triggered immunity (PTI) defenses in barley.

Confirmation of these results can be accomplished via functional studies both in heterologous systems as well as in the barley/*Bgh* pathosystem. As a first step, each sRNA/transcript pair

could be co-expressed in *Nicotiana benthamiana* along with related controls to determine whether each sRNA can cleave its targets in vivo. These experiments could be followed up in barley with the appropriate use of virus-induced gene silencing (VIGS), host-induced gene silencing (HIGS), and or over-expression of artificial miRNAs that encode transcripts or sRNAs of interest to determine their effect on barley susceptibility to *Bgh* infection.

APPENDIX. SUPPLEMENTARY MATERIALS

Supplemental Materials for Chapter 2, 3, and 4 can be accessed in the zipped folders "Chapter2_Supplemental_Files", "Chapter3_Supplemental_Files", and "Chapter4_Supplemental_Files", respectively, on ProQuest. A brief description of each file is available below.

Supplementary Files for Chapter 2

Supplemental Table 2.1 DE *Bgh*-mapped read expression details. Differentially expressed reads are detailed including name, genotype and time point differentially expressed, $\log_2$ fold change, Rfam database membership, and similarity to predicted *Bgh* milRNAs

Supplemental Table 2.2 PARE validated predicted miRNAs and *Bgh* genome mapped sRNAs. This table includes details on both the PARE-validated *Bgh* sRNAs as well as their predicted targets including data on PARE validation, sequences, and sRNA target locations.

Supplemental Table 2.3 PARE validated *Bgh* transcript target annotations. Annotation information for each predicted *Bgh* transcript including ensembl, blastx, interproscan, and literature based categories.

Supplemental Table 2.4 EKA homolog/hairpin overlap details. Mapping locations, direction of transcript and hairpins, as well as information on overlap type.

Supplementary Files for Chapter 3

Supplemental Table 3.1 Expression details of DE barley mapped reads. Differentially expressed barley genome mapped sRNAs details including name, sequence, size, condition DE, and matches to predicted miRNAs and Rfam motifs.

Supplemental Table 3.2 PARE validated predicted miRNAs and barley genome mapped sRNAs. PARE-validated predicted miRNA and barley genome mapped sRNA information including proposed name, mapping location, predicted transcript targets, and annotations

Supplementary Files for Chapter 4

Supplemental Table 4.1 Barley trans-kingdom PARE validated predicted miRNAs and barley genome mapped sRNAs. Barley PARE-validated predicted miRNA and barley genome mapped sRNA information including proposed name, mapping location, predicted transcript targets, and annotations

Supplemental Table 4.2 *Bgh* trans-kingdom PARE validated predicted miRNAs and *Bgh* genome mapped sRNAs. *Bgh* PARE-validated predicted miRNA and *Bgh* genome mapped sRNA information including proposed name, mapping location, predicted transcript targets, and annotations