

CHARACTERIZATION OF PROGRAMMED CELL DEATH RESPONSES INVOLVED
IN DISEASE RESISTANCE/SUSCEPTIBILITY RESPONSES IN BARLEY

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ABSTRACT

The plant innate immune system relies on regulated programmed cell death (PCD) responses which provide resistance against biotrophic fungal pathogens and are utilized by necrotrophic pathogens. The gene-for-gene interactions leading to resistance against biotrophs has been co-evolving over millions of years and provides many targets for necrotrophic pathogens to subvert the plants PCD for their benefit. Two PCD pathways involved in plant immunity were characterized. The first, *nec3*, appears to control runaway PCD in response to infection by many pathogens who mainly elicit pathogen or damage associated molecular pattern (PAMP or DAMP) triggered immunity. The *nec3* mutant was mapped to chromosome 6H and a pool of candidate genes were identified by RNAseq. The second, *Rcs5*, is a dominant susceptibility gene or a putative necrotrophic effector triggered susceptibility (NETS) target that confers susceptibility to *Cochliobolus sativus*. The candidate *Rcs5* genes are three Wall Associated Kinases (WAKs) found on chromosome 7H (1).

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DEDICATION

I dedicate this to my loving family, Barb, Ron and Haley Sager; my husband Francisco

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LITERATURE REVIEW

Barley

Historical records and molecular evidence has determined barley was domesticated approximately 10,000 years ago and among the grasses is one of the most important crops. Barley was originally cultivated for human consumption, but today its primary use is animal feed (~60%). However, because of the high value added from profits from alcoholic beverages barley is extremely important in the malting industry which is primarily used for brewing beer and liquor distillation (30%). Barley is also utilized for other products for human consumption (~10%) such as soups, bread, tea and various other food items (Ullrich 2011). According to FAOstat.fao.org the average area of barley harvested in the United States over the five-year span from 2009-2012 was approximately 1.1 million Hectares. On average this acreage produced approximately four million tonnes per year with an average of 38,000 Hg/Ha. However, the US is not among the top five barley producing countries; over the last five years the top producers of barley are the Russian Federation with approximately 14.0 million tones /year, France (11.0), Germany (10.0), Australia (8.2) and Canada (8.0) (FAOstat.fao.org).

In the United States barley production has dramatically declined in the past decades due to more profitable animal feed crops such as corn but should maintain its current acreage due to its importance in the brewing and distilling industries. The recent expansion of craft brewing industries has increased the demand for quality malt barley as more malt is used per barrel of beer produced as compared to the commercial beer brewing industry, which relies on adjunct in the brewing process (brewersassociation.org).

Barley has been a part of the human diet for tens of thousands of years. At present barley as a food source is generally more prevalent in less developed countries because it is able to grow well in many conditions including drought, however, more developed countries are starting to supplement food with and use barley as a main ingredient. This resurgence in barley use as a food source is strongly tied to its nutritional benefits as barley was recently determined to be a heart healthy food. In a study conducted by Talati et. al., a meta-analysis of eight randomized controlled studies found barley has been shown to significantly reduce cholesterol, low-density lipoproteins (LDLs) and triglycerides; however there was little effect on high-density lipoproteins. Barley provides the body with soluble fiber and beta-glucans, which have in the past been shown to reduce cholesterol levels. Other grasses including oats have similar beta-glucan levels (Talati 2009).

The spatial and temporal occurrence of barley's domestication is still unknown, however recent research has given us valuable clues to its domestic origin(s). It is agreed that intense domestication of crops including barley occurred some 10,000 years ago and today there are three current hypotheses as to the origins of domesticated barley. The first includes a single domestication in the Fertile Crescent, Isreal/Jordon (Badr 2000), while the second is a double domestication in both the East - Pakistan, Iran, Turkmenistan and the West - the Fertile Crescent (Morrell 2006, Ullrich 2011). The third hypothesis is a multiple domestication events, or a co-evolutionary domesticated origin followed by conscious efforts to harvest certain crops specific to the needs and tastes of the people in the region (Rindos 1980, Jones 2013). Evidence for a third domestication has been demonstrated by performing genetic analysis of Ethiopian barley, which has been shown to be distinct compared to the Eastern and Western cultivars and landraces (Saisho 2007). This Ethiopian barley indicates a third domestication event, however

further analysis of European and Asian barley varieties and landraces will provide a better indication of the probable number of barley origins. If a single domestication event gave rise to cultivated barley then it would be expected that cultivated genotypes contain the same loci for various domestication traits such as seed shattering, row type and seed size. However, this is not what has been found, as an example in studying barley's *vrs1* gene, responsible for row type, two main haplotype groups were found. These data indicate at least two domestication events. Tanno et. al. found evidence for multiple origins of six-row barley derived from two-row barley with two major events. One major event as they designated Type I is widely distributed with accessions found in Turkmenistan. The second major event designated Type II was only found in the Mediterranean region, North Africa and Morocco (Tanno 2002). In light of the recent data it should be understood that domesticated barley likely originated in multiple regions where *H. vulgare* spp *spontaneum* is presently found providing us with the vast diversity of domesticated barley we see today.

The use of sequencing technology to sequence entire genomes is a somewhat recent advancement of which nearly two hundred genomes have been sequenced including Maize, *Arabidopsis*, rice and more recently barley has been partially sequenced, assembled and annotated using a hierarchical approach (IBGSC 2012). The haploid genome of barley is approximately 5.1Gb. Physical and genetic maps were used and further developed providing over 95% of the genome represented in a physical map. The barley genome has a highly repetitive nature much like other cereal groups that have been sequenced such as maize, which has repetitive elements encompassing 85% of the genome (Schnable 2009). The barley genome contains approximately 84% repetitive elements, 99% of which consist of long terminal repeat retrotransposons (IBGSC 2012). Retrotransposons are DNA sequences with RNA intermediates

that amplify themselves in an organism's genomes. These mobile genetic elements can be considered chromosomal parasites as they can interfere with gene expression both positively and negatively (Schnable 2009). Additionally, some siRNA species have been identified that have been shown to reduce the ability of retrotransposon amplification (Hamilton 2002). Over millions of years these retrotransposons have expanded many plant genomes, thus representing a large proportion of the genome leaving both dense and sparse clustering of encoded genes or gene rich and gene poor regions of the genome. The duplication events seen throughout evolutionary history have increased the rate at which transposons are able to multiply in the genome. Repetitive elements include transposons, retrotransposons both LTR and non-LTR, SINEs (short interspersed elements) and MITEs (miniature inverted-repeat transposable elements). LTRs tend to be the most abundant among repetitive elements in the genome (Schnable 2009; IBGSC 2012). However, of most concern to breeders and pathologists are genes; both their structure and the function of the proteins encoded.

As part of the International Barley Genome Sequencing Consortium Illumina sequencing technologies were used to perform RNA sequencing on samples taken from multiple tissues during various periods of the plants life cycle in order to determine when and where genes are being expressed. The data indicates the presence of 79,379 transcript clusters and 26,159 high confidence genes, however the real number of genes is probably not much larger due to highly reliable sequencing methods. High confidence genes were identified through the use of synteny maps and were deemed high confidence if they agreed with at least one of the four reference genomes; rice, sorghum, *Brachypodium* and *Arabidopsis*. Researchers also found multiple RNA transcripts for genes that were spliced in more than one manner and have unique transcriptionally active regions demonstrating the mechanisms of post-transcriptional regulation and processing as

an important aspect of gene regulation within the barley genome (IBGSC 2012). In regards to disease resistance, the sequencing study identified 191 genes with the presence of an N-terminal nucleotide-binding site (NBS) and a C-terminal leucine rich region (NBS-LRR), which are typically clustered at the distal ends of chromosomes. The vast majority of disease resistance genes that have been cloned (>100) from a wide taxonomy of plants were shown to be NBS-LRR protein encoding genes (Brueggeman 2008, Joshi 2013, Wang 2013, Zhang 2014). The only known function for this class of genes in plants is involvement in disease resistance and in two systems the typical NBS-LRR biotroph resistance genes confer dominant susceptibility to necrotrophic pathogens (Lorang 2007, Faris 2012). The NBS-LRR genes typically contain N-terminal domains that fall into one of two classes, the Toll interleukin-1 receptor-like domain (TIR) or the coil-coil (CC) motifs (Hammond-Kosack 1997). Compared to the four reference genomes mentioned earlier there was an expansion of NBS-LRR genes in barley (IBGSC 2012). Although the vast majority of disease resistant genes have been found to be NBS-LRRs, some NBS-LRR R-proteins have been shown to interact directly with pathogen Avr proteins and the LRR is the critical component determining pathogen specificity (Dodds 2006). NBS-LRR genes also function to guard the effector targets detecting modification by pathogen Avr proteins (Jones 2006).

The second major class of *R*-genes contain serine/threonine protein kinase (S/TPK) domains. This class of *R*-genes includes the tomato *Pto* gene, and the *Arabidopsis PBS1* gene, which confer resistance to the bacterial pathogen *Pseudomonas syringae* (Martin 1993, Swiderski 2001), the rice *Xa21* and *Xa26* genes that confer resistance to the bacterial pathogen *Xanthomonas oryzae* (Song 1995; Sun 2004), the barley *Rpg1* and *Rpg5* genes, which confer resistance to the fungal pathogen *Puccinia graminis* (Brueggeman 2002; Brueggeman 2008) and

the wheat *Yr36* gene conferring resistance to *Puccinia striiformis* (Fu 2009). *Rpg1* has a novel *R*-gene structure containing two tandem S/TPK domains while the other S/TPK *R*-genes contain a single protein kinase domain connected to other protein domains characteristic of *R*-genes. For example this combination of domains containing S/TPK is observed in the LRR-S/TPK structures of *Xa21* and *Xa26* (Song 1995; 2004), NBS-LRR-S/TPK structure of *Rpg5* (Brueggeman 2008) and the novel structure of *Yr36* with an S/TPK domain attached to a lipid binding domain (Fu 2009). Other S/TPK kinases were also originally identified as resistance genes in other crops but have more recently been described as effector target proteins guarded by NBS-LRR *R*-genes. One example is PBS1, a protein found in *Arabidopsis* that works directly with an NBS-LRR called RPS5 to induce the hypersensitive response after manipulation by the pathogen *Pseudomonas syringae*'s avirulence protein, AvrPphB (Swiderski 2001). Tomato takes a similar approach in its response to this particular pathogen as in the AvrPto/Pto system. Pto a putative *R* gene that interacts with Pti1 (Pto-Interacting1) a S/TPK, by phosphorylation. Pti1 is hypothesized to be guarded by the NBS-LRR protein PRF, which is responsible for inducing the hypersensitive response and thus resistance in the tomato system (Zhou 1995). This phosphorylation and interaction is required for host resistance. A single mutation in the activation domain can eliminate the interaction and lead to susceptibility to tomato speck caused by *P. syringae* (Sessa 2000). These are among the many examples of both biotrophic and necrotrophic resistance determined by the presence of a particular NBS-LRR guards and S/TPK guardee proteins. What is important to note is that these are all signaling molecules that have a direct link to the immune response in the plant. However, it is the NBS-LRR proteins and their interaction or guarding of the S/TPK genes that is setting the stage for understanding the complexity of host/pathogen resistance and susceptibility pathways.

The typical NBS-LRR disease resistance genes follow Flor's gene-for-gene model (Flor 1971) and confer resistance to biotrophic pathogens as no typical NBS-LRR R-gene has yet been shown to confer resistance against a necrotroph. In fact, recent research has changed the disease resistance paradigm and mounting evidence supports the inverse gene-for-gene model where necrotrophic effectors (NEs) target susceptibility genes, which are typical NBS-LRR type resistance genes that induce programmed cell death (PCD) resistance responses that the necrotrophs take advantage of to cause disease (Friesen 2007, Faris 2010). This manifests itself genetically as recessive resistance or dominant susceptibility and has been coined NETS for necrotrophic effector triggered susceptibility (Liu 2014). The NETS model was established for the *Pyrenophora teres*-barley and *Stagonospora nodorum*-wheat pathosystems which is associated with other necrotrophic-host pathosystems such as *Cochliobolus sativus* and its putative susceptibility gene *rcs5*, which is reported here. *Arabidopsis* produces an NBS-LRR gene (*LOVI*) that is associated with disease susceptibility to the pathogen *Cochliobolus victoriae* (Lorang 2007), a pathogen closely related to *C. sativus* the causal agent of spot blotch. *C. victoriae* was found to produce a toxin called Victorin that interacts with a 100 kD victorin binding protein encoded by a single dominant gene produced by the host oat (Wolpert 1994). This interaction is a susceptibility reaction that causes host PCD and senescence like responses (Navarre 1999). These observations provide new insights into resistance and susceptibility mechanisms in different crop systems as we still see a gene-for-gene interaction, but the outcome is opposite based on the necrotrophic nature of the pathogen.

The necrotroph *Stagonospora nodorum* produces the NEs SnToxA and SnTox2, which interact with specific products produced by the host (Friesen 2007). Of particular interest to the research contained in this thesis is the receptor for ToxA called *Tsn1*, which has a unique

combination of protein domains as it contains an NBS-LRR with a Serine/Threonine Protein Kinase domain signaling component that leads to susceptibility in a dominant manner. However, the researchers were able to show that the two host-pathogen proteins do not directly interact (Faris 2010). This was the first NBS-LRR-S/TPK gene found to induce susceptibility in a dominant manner.

Rcs5

Another example of an S/TPK domain containing protein is the resistance gene *OsWAK1*, a wall associated kinase (WAK) which was shown to confer resistance to the disease rice blast through a so far unknown mechanism. Expression of this gene was found under infection and certain stress conditions but not under normal growth conditions suggesting that it is induced by biotic and abiotic stresses (Li 2009). These constitutively expressed WAK genes are proving to be the receptors for Damage Associated Molecular Patterns (DAMPs) as they have also been shown to distinguish between fragmented and whole pectin molecules (Brutus 2010, Kohorn 2012). This early defense response is similar to PAMP Triggered Immunity (PTI) which may be effective in stopping early infection responses but also may be targeted by necrotrophic pathogens to subvert resistance responses and induce PCD for their own proliferation. The *HvWAK1* gene of barley, like other WAK genes in other plant families, is highly divergent among barley lines. Interestingly, but somewhat expected, sequences were more divergent for the extracellular receptor of *HvWAK1* whereas the inner kinase domain was more conserved (Kaur 2013). This is likely due to the innate variable function of the protein, which detects a range of environmental stimuli including abiotic and biotic stresses that induce cellular damage and transfer these signals to the cytoplasm leading to appropriate cellular responses. The

research contained in this thesis has determined that one or possibly more WAK genes on barley chromosome seven appears to behave as a dominant susceptibility gene, previously referred to as the spot blotch resistance gene *Rcs5*.

Spot blotch, a foliar disease of barley and less commonly in wheat is caused by *Cochliobolus sativus* (S. Ito & Kurib.) Drechsler ex Dastur [teleom.] *Bipolaris sorokinana* (Sacc.) Shoemaker [anam.]. Spot blotch can cause over a 33% yield loss of barley in the conditions that are conducive to disease (Steffenson 1999). *C. sativus* has a polycyclic life style which contributes to an increased infection rate and an increase in inoculum for the following season. Since *C. sativus* also causes blackpoint, an infection of seed it is important to keep healthy soil and clear debris as the pathogen can travel from field to field in this manner. Good farming practices including timely fungicide applications can curb the intensity of the disease, however, genetic resistance is both an economically and environmentally superior option. The barley variety NDB112, a six-row breeding line developed at North Dakota State University, has been used since the early 1960s as a source of spot blotch resistance, with the major resistance given the designation of *Rcs5*. This source of resistance has sufficed for over 40 years however *C. sativus* isolates virulent on this resistance source have been identified indicating the need for further research to abate future epidemics and develop a progressive understanding of the pathosystem.

The recently published genome of *C. sativus* is approximately 34Mb with 12,250 predicted genes with 6% containing repetitive sequences (Condon 2013). Between the two strains sequenced, ND90Pr and ND93-1, there were 60,448 SNPs identified. When compared to *C. heterotrophus* there were 1,981,616 SNPs demonstrating the trend - among strains of the same species of *Cochliobolus*, 3-5% of the DNA differs, while a more dramatic 25% of the DNA

differs between different *Cochliobolus* species. These data were found following sequencing of two *C. sativus*, five *C. heterotrophus*, one *C. victoriae*, one *C. miyabeanus* and one *C. carbonum* isolates. The outlier species used was *Setosphaeria turcica*, although it is closely related to the *Cochliobolus* genus and causes Northern corn leaf blight of sorghum and maize (Condon 2013). The data helped confirm the previously determined age of the fungi to be <20 MYA (Ohm 2012). The millions of years that these host and pathogens have been able to interact and co-evolve offer very interesting resistance, susceptibility and other immune system pathways to study and compare such as the interaction seen with the knockout of the immune system gene *nec3*.

nec3

Lesion mimic mutants (LMM) of barley cultivars have shown interesting phenotypes in regards to responses to pathogen interaction and have been proven to be useful in the investigation of programmed cell death (PCD), the hypersensitive response and other pathways fundamental to the survival of the plant. These mutants are further classified into initiation and propagation mutants. With initiation mutants the necrosis is localized; in propagation mutants the necrosis is uncontrolled (Walbot 1983). To date LMMs have been found in *Arabidopsis*, barley, maize, rice and wheat, which have helped to uncover areas of certain pathways involved in plant defense signaling (Lorrain 2003, Moeder 2008). The *nec3* mutants are propagation mutants as the gene is likely to regulate PCD initiated by pathogen perception. In order to identify *nec3* we developed a mapping population to localize the gene and performed RNAseq to identify candidate genes.

Allelism testing is a common test cross to determine if two lines or genotypes contain the same gene, or allele contributing to a specific phenotype. In the case of the *nec3* mutants we observe recessive inheritance, thus the F₁ generation after crossing the independent mutants are expected to display the mutant phenotype in all progeny if they are allelic. The loss of function of the protein encoded by the mutated gene is the reason mutants have recessive inheritance. When the cross between two independent mutants with a similar phenotype do not display the phenotype in the F₁ generation and the F₂ generation segregate in a 3:1 ratio, it is understood the genes are not allelic as one of the cultivars has a functional copy at each independent locus. When allelic mutants were identified the location of the gene responsible for the phenotype can be mapped in a single mutant line, however you would expect that once the gene is identified the mutations in each independent mutant event should be unique. Genetic mapping is employed to narrow down the location of the gene and consists of making a cross with a genotype that has high polymorphism with the background line with the mutated gene. In my research we attempted to identify the *nec3* gene, which was previously described (Lundqvist 1997, Rostoks 2003, Keisa 2010, 2012) and reported to be on the short arm of chromosome 6. After developing a mapping population from the cross between the *gamma1/nec3* mutant in the cultivar Bowman background (two-rowed barley variety developed by the NDSU barley breeding program) with the cv Quest (six-rowed barley variety developed by the University of Minnesota barley breeding program) the F₂ generation was used to genetically map the mutation and delimit the region harboring the *nec3* gene. The homozygous F₂ mutants were utilized because the phenotype of interest is only observable in a homozygous recessive state, thus we know the genotype at the *nec3* locus should be homozygous Bowman like. The homozygous mutant individuals identified from a small population of F₂ progeny were genotyped at the *nec3* locus with a series of markers

with a goal of identifying flanking markers distal and proximal to the gene of interest. The *nec3* mapping data provided a good framework for narrowing down candidate, differentially expressed genes identified by RNAseq experiments.

The Ion Torrent, a next generation sequencer that harnesses the chemical properties of DNA replication (Ion Total RNA-Seq Kit v2), can be used for many applications including genotype-by-sequencing and RNAseq experiments, which is the focus of one chapter in this thesis. Following mutagenesis by gamma irradiation, fast neutron irradiation and x-ray mutagenesis, genes are disrupted to varying degrees ranging from SNPs to the full elimination of a gene. X-ray and gamma irradiation, are quite similar in their wavelengths and ability to displace small fragments of DNA. Since gamma rays (<0.01nm) are a higher frequency than x-rays (0.01nm-1nm) they result in displacement of smaller fragments of DNA after the endogenous DNA end repair mechanisms repair the break (Mattson/NASA 2013). Based on wavelength size, gamma rays make single to ten base pair deletions, whereas x-rays make ten to hundred base pair deletions. These mutations can be detected using RNAseq technologies because the wild type should produce mRNA for the disrupted gene while the mutant should produce at reduced levels or not at all. Depending on the size and location of the deletion there might be a partial mRNA transcript that could be detected by RNAseq. The RNAseq experiments in this thesis were analyzed under the assumption that differential expression of the mRNA transcript could be detected with the mutant gene being significantly down regulated in the mutant as compared to the wildtype control. Previous Affymetrix transcriptome analysis of the *nec3* mutant did not yield the mutant gene but provided some evidence as to what genes are over or under expressed when compared to the wild type (Keisa 2010). Their research identified the *nec3* gene as likely being related to stress responses to drought and cold weather. Recent

research has found some proteins to be biologically multifunctional, that is they provide a function for both development and disease resistance or susceptibility among many other combinations.

Plant immune mechanisms and the signaling pathways elicited in response to pathogens are slowly being elucidated. Immune responses to pathogens tend to be complex and diverse as pathogens evolve diverse effector suites to manipulate the host for their benefit; studying these complicated pathways will allow a higher understanding of the plant immune system and allow for the separation of pathogen manipulation and host responses. This research project aims to fill a knowledge gap in understanding programmed cell death responses elicited by necrotrophic fungal pathogens. Based on phenotypic observation it is hypothesized that the *nec3* gene is a negative regulator of programmed cell death. Interestingly the *nec3* gene is only employed when the mutant barley plant interacts with certain pathogens. The extreme response to these pathogens may provide another link for the connection between the PTI (general response) and ETI (specific response) pathways, which have recently shown to be intimately linked. Additionally, the *rsc5* gene appears to be a dominant susceptibility factor rather than the previously hypothesized dominant resistance factor, which elicits a PCD resistance response that is hijacked during the necrotrophic phase of the *Cochliobolus sativus* infection process.

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CHAPTER ONE. CHARACTERIZATION OF THE *NEC3* GENE; A PUTATIVE NEGATIVE REGULATOR OF PROGRAMMED CELL DEATH

Abstract

The elucidation of programmed cell death (PCD) pathways triggered upon pathogen recognition that lead to the hypersensitive response (HR) is an important gap in knowledge that must be filled in order to fully understand plant disease resistance and susceptibility mechanisms. We identified a gamma irradiated barley cultivar (cv.) Bowman mutant, that when prompted by a wide range of pathogens, including both fungi and bacteria, elicited a runaway PCD phenotype. This mutant barley line, gamma1, has a distinct large tan to orange necrotic lesion phenotype, consistent with the previously characterized spontaneous disease lesion mimic mutants (LMM) designated as *nec3*. Four independent *nec3* mutants (FN362, FN363, *nec3.d*, and *nec3.e*) were shown to be allelic to gamma1, and like gamma1, only exhibit the runaway PCD phenotype upon specific pathogen challenge. Genetic analysis of F₂ progeny generated by crossing gamma1 and the cultivar Quest indicated that this recessive mutation maps to a region delimited to approximately 5.8 cM at the centromeric region of barley chromosome 6H between the flanking markers GBM1053 and GBM1423, and co-segregates with the marker GBM1212. This region corresponds to the previous low resolution mapping of the *nec3* gene. Transcript analysis of gamma1, under non-infection conditions, by RNAseq identified a list of candidate genes expressed in the wild type (Bowman) but not in the mutant (gamma1) that are located within the delimited chromosome 6H region. A second set of candidate genes have also been identified based on RNAseq analysis following inoculation with the ascomycete fungus *Cochliobolus sativus*, which induces the *nec3* PCD phenotype. Allele analysis of the candidate gamma1/*nec3* genes from the five independent mutants will help identify the *nec3* gene, which will provide an

important piece of information regarding the mechanisms utilized by plants to negatively regulate PCD in plant innate immune responses.

Introduction

In the plant innate immune system the hypersensitive response (HR), also considered a form of programmed cell death (PCD), protects the plant from pathogens in their biotrophic phase by rapidly isolating the invader at the initial point of recognition, effectively stopping further colonization (Glazebrook 2005). The rapid PCD response involved in the microbe associated molecular pattern (MAMP) also referred to as pathogen associated molecular pattern (PAMP) or damage associated molecular pattern (DAMP) triggered immunity, MTI, PTI and DTI respectively, are early defense response elicited by host-parasite molecular interactions. The immune response is triggered by microbial molecular patterns in MTI, pathogen molecular patterns in PTI and damage associated compounds and molecular patterns in DTI. These responses are typically associated with a rapid, precisely regulated PCD response that is generally limited to the first infected cell and/or adjacent cells (Orozco-Cárdenas 2001). The HR, generally associated with effector triggered immunity (ETI) responses, tends to have a higher amplitude of PCD and typically results in more wide spread necrosis, yet it is still believed to be tightly regulated (Jones 2006). The ETI response is limited to sequestering the pathogen; yet the resulting necrosis can be visible, producing a light green/yellow halo surrounding the infection site which is typically indicative of a resistance gene or gene-for-gene mediated resistant reaction. For plants to have effective innate immune responses against biotrophic pathogens they must be able to recognize the invader and elicit rapid PCD while strictly limiting the response to the point of infection in order to effectively kill the pathogen yet

maintain self-integrity. If PCD was left unregulated, after sequestering the pathogen, it could be detrimental to the plants fitness by decreasing the amount of photosynthetic potential of the leaf and in extreme cases, results in the death of the entire leaf or plant. Due to the overlapping nature of many of the genes involved in disease resistance/susceptibility in typical plant processes such as senescence, growth, seed development and cellular maintenance, elucidating the specific function of genes involved in PCD and the specific pathways in which they are involved is challenging (Greenburg 1993, Foyer 2005, Nagakami 2005, Druka 2011, Lacroix 2012). Thus, induced mutants in these conserved pathways can successfully narrow in on genes that result in specific phenotypes, and the genes underlying these pathways through forward genetics. The mutant phenotypes are generally due to the loss of gene function as deletions within genes or promoter regions can lead to lack of protein translation, aberrant truncated non-functional proteins or structural changes to functional domains.

Although induced mutants have been available for close to 100 years (Stadler 1928), in the past couple decades multiple necrotic mutants have been created in various crops, including barley, by exposure to both chemical and physical mutagens (Lorrain 2003, Greenburg 1993, Penmetsa 2000, Druka 2011). In 2001, Li et al., developed fast neutron radiation treatment in order to perform reverse genetics on *Arabidopsis*. They were able to demonstrate that fast neutron irradiation had a similar effect on rice showing that this method can be used on a wide genera of plants for functional studies. It was determined that the fragment size range of fast neutron deletions is approximately 0.8 to 12 kb in length (Li 2001). With forward genetics mutagenized seed are planted, genotyped, and following the gene being discovered via DNA sequencing, the plants are then phenotyped to determine the function of the gene. Similarly X-ray and gamma mutagenesis can be used on a wide variety of plant species and provide similar

results as demonstrated by this dissertation and other works (Stadler 1928, Gregory 1955, Novak 1990, Reski 1994, Predieri 2001, Arunyanart 2002). Although physical mutagenesis is a quick and easy procedure, not every research laboratory has access or funds to utilize this method. As an alternative, chemical mutagenesis is a common method of mutagenesis as it is easier to perform in a typical molecular laboratory. The most frequently used methods of chemical mutagenesis are sodium azide and ethylmethanesulfonate (EMS). Both of the chemical treatments require soaking of seed for a specified amount of time followed by washing steps to control the amount of mutagenesis (Mba 2010). As with radioactive mutagenesis, the identification of mutants must be in the M_2 generation because the mutated gene typically must be in a homozygous state in order to have two non-functional alleles; mutagenesis by any mutagen typically results in a recessive loss of function. Therefore one copy of the wild type functional allele will typically result in sufficient expression of the functional protein resulting in the wild type phenotype. Regardless of the mutational procedure utilized, the point of developing mutants is to identify phenotypes or genotypes of interest so as to utilize forward or reverse genetics approaches to identify the mutated gene and its effect on phenotypes of interest.

One group of induced mutants classified as the necrotic or lesion mimic mutant (LMM) provide a valuable resource to identify genes involved in different developmental processes as well as responses to environmental stimuli including both biotic and abiotic stresses (Lorrain 2003). Thus far two LMM have been cloned from barley, *Hvnec1* and *Hvmlo*, which have been shown to be associated with the formation of spontaneous lesions under non-infection conditions. The *Hvnec1* gene was shown to encode a cyclic-gated ion channel protein, which is the same protein configuration as the homologous Arabidopsis *HLMI* gene that was previously cloned and characterized (Lundqvist 1997, Rostoks 2003, 2006). The *HLMI* gene has both

molecular and phenotypic attributes similar to *nec1* including increased pathogen-related (PR) protein expression, spontaneous necrotic lesions, initial tip necrosis and increased susceptibility to certain pathogens (Balague 2003, Stintzi 1993). The other LMM, *mlo*, is also an induced mutant that has increased resistance to the ascomycete *Erysiphe graminis* f. sp. *hordei*. The *mlo* gene has shown durability in the field. *Mlo* also appears to be a highly conserved gene that is found in pea, *Arabidopsis* and tomatoes (Wolter 1993,). The non-mutated version of the gene *Mlo* has been found to be a seven-transmembrane protein (Acevedo-Garcia 2014). These mutants have been extremely helpful in understanding disease resistance, plant physiology and evolutionary relationships.

A previous attempt to identify the *nec3* gene utilizing gene expression analysis with the Affymetrix Barely1 GeneChip assay was unsuccessful (Kesia 2010). Although the *nec3* gene was not identified it was observed that the *nec3* mutants, FN362 and FN363, had differential regulation of abiotic stress related genes as compared to the wild type. They found that genes differentially expressed upon drought and cold stress were also differentially expressed in the *nec3* mutants as compared to the wild type with the differential expression ranging from 0.4-365 fold change. There were also significant changes in both disease related genes and genes that modify the cell wall. The highest fold change difference in disease related genes was seen in a gene involved in lipid transfer, pathogen defense and cell wall modifying enzymes (Keisa 2010).

PCD pathways in plants are still relatively unknown thus the pathogen induced necrosis and eventual tissue collapse caused by PCD pathways in the *nec3* mutant lines upon pathogen challenge may provide an important gene to begin probing these responses. Recent research has shown that there are likely similar, yet distinct and possibly interconnected pathways that lead to P/DAMP triggered PCD and/or ETI HR (Thomma 2011). The current view of the plant innate

immune system is that there are distinctions between PCD pathways triggered by PAMPS leading to PTI and PCD triggered by the interaction of an effector with an R-gene product from the host leading to HR, the hallmark of ETI. Slowly the dichotomous view of PAMPS, effectors, PTI and ETI responses has become outdated as examples of PAMPS being specific to certain plant species and effectors being general elicitors to conserved plant genes are coming to light (Thomma 2011). Some examples of narrow range PAMPs are the bacterial PAMPs Ef-tu and cold shock proteins that are only recognized by Solanaceae and Brassicaceae plants. These proteins are ubiquitous in bacteria as they are required for pathogen fitness, growth and reproduction but are only recognized by the two genus' previously listed (Zipfel 2006). One example of a broad range protein originally characterized as a race-specific effector is *Avr4*, which binds chitin and increases the virulence of the pathogen, *Cladosporium fulvum* in tomato (Joosten 1994). Initially this effector was not found in any other species. Additional research demonstrated that *Avr4* recognition also occurs in other *Solanum* species, not just tomato (Lauge 2000). Interestingly, it was found that the *Avr4* chitin-binding domain is also found in *Mycosphaerella fijiensis* (Mf-Avr4), a pathogen of banana that causes Black Sigatoka. *Cercospora* species also contain *Avr4* homologs (Stergiopoulos 2010). Both *Mycosphaerella* and *Cercospora* are phylogenetically related to *Cladosporium*.

My research aimed to further delimit the *nec3* region, characterize the phenotype on barley Chromosome 6 previously identified by Rostoks 2006, Keisa 2010 and Keisa 2012 and provide a list of possible candidate genes following RNAseq using the Ion Torrent.

Materials and Methods

Plant Material

Five independent irradiation induced mutants, gamma1, FN362, FN363, nec3.d and nec3.e, were generated in four separate genetic backgrounds (Druka 2011, Keisa 2010) with three different radiation sources (Table 1.1). Four of the five mutants display the characteristic tan-orange expanding necrotic lesion phenotype indicative of the *nec3* mutant (Frankowiak 1997).

Table 1.1. *nec3* independent mutants: origin and mutagenesis type used in this study.

Mutant	Parent	Mutagenesis	Origin
gamma 1	Bowman	Gamma Irradiation	Gamma irradiated Bowman isolated by R. Brueggeman.
nec3.d	Proctor	X-ray	X-ray mutant Proctor (PI 280420) isolated by G. Fischbeck and H. Häuser (GSHO 2065)
nec3.e	Villa	X-ray	X-ray mutant Villa (PI 399506) isolated by G. Fischbeck and H. Häuser (GSHO 2066)
FN362	Steptoe	Fast Neutron	Fast Neutron Steptoe mutant identified by A Kleinhofs
FN363	Steptoe	Fast Neutron	Fast Neutron Steptoe mutant identified by A Kleinhofs

Source: (Falk 1980, Frankowiak 1997)

The genetic backgrounds of the nec3.d and nec3.e mutants are Proctor and Villa respectively, while the mutant lines used in this study, GSHO 2065 (nec3.d) and GSHO 2066 (nec3.e) were back-crossed with cv. Bowman four and three times respectively. Both nec3.d and nec3.e mutant backcrossed lines were obtained from the USDA-ARS National Small Grains Collection (NSGC) in Aberdeen Idaho. The nec3.d and nec3.e mutants are deposited as genetic stocks of Hordeum (GSHO) and were given the designations GSHO 2065 and GSHO 2066, respectively. The two fast neutron induced mutants FN362 and FN363 that had previously been

described and shown to be allelic to the *nec3* locus (Kesia 2010) were generated in the cv. Steptoe background using two doses of fast neutron, 3.5 Gy and 4.0 Gy, respectively, and were provided by Dr. Andris Kleinhofs, Washington State University. The gamma1 mutant was initially identified by R. Brueggeman from gamma irradiated cv. Bowman in an attempt to identify Bowman lines with mutations in the gene shown to confer dominant susceptibility to *Cochlibolus sativus* isolate 90Pr (Zhong 2014). This mutant population was developed by gamma irradiating approximately 0.25 kg of Bowman seed with 20 kRADs at the Washington State University nuclear reactor facility in Pullman, WA in 2011. The M₁ seed was advanced to the M₂ generation and bulked for greenhouse screening for *C. sativus* isolate 90Pr resistant mutants. To identify resistant mutants 2,000 M₂ seedlings from bulked seed were screened with *C. sativus* isolate 90Pr and an individual seedling was identified with a pathogen induced necrosis phenotype that was consistent with the phenotype observed for the *nec3* mutants. This M₂ individual was emasculated and crossed with the genetically different six-rowed malting cv. Quest that was released from the University of Minnesota barley-breeding program in 2011 (Smith 2010). The homozygous F₂ mutant progeny showing the *nec3* phenotype were utilized to map the pathogen induced necrosis phenotype to determine if the gamma1 mutation was located at the *nec3* locus near the centromeric region of barley chromosome 6H.

nec3 Phenotypic Observations

In order to validate these mutants as LMMs an isolation box experiment was conducted over the course of a month to observe the phenotype of the gamma1 mutant and Bowman wild type, independent of pathogen challenge. In a large plexiglass isolation box approximately 2'x1'x1' (H/W/L) with holes on two sides to allow air flow yet packed with cheesecloth to protect the plants from pathogen spores and insects the mutant and wild type barley lines were

planted and observed for the *nec3* phenotype. The experimental plants were grown under sterile conditions (inside the isolation chamber: 16 hour photoperiod at 80°C due to greenhouse effect of the plexiglass) and normal greenhouse conditions (outside the isolation chamber: 16 hour photoperiod at 70°C) and phenotypes observed and recorded every day for two weeks. Two plants were sown per pot and two pots were grown inside and outside of the isolation chamber for a total of 4 plants of each genotype inside and 4 outside the isolation chamber. Plants were watered with autoclaved distilled water as needed.

Phenotypic observations were also gathered in the field during the summers of 2012 and 2013 from seed to adult.

Following *nec3* phenotypic observation of Bowman and gamma1 plants grown in the greenhouse, affected leaves were removed, surface sterilized for one minute in 10% bleach and rinsed with sterile water then plated on water agar. Plates were put under a growth light for 16 hours/day for three to five days allowing the pathogen or potential saprophyte to grow. Leaves were then observed and documented using a camera dissecting microscope. This experiment was performed so as to observe or not observe growth within the necrotic lesions. Additionally following heading and seed production, heads from F₁ and F₂ *nec3* allelic crosses were removed and documented. This study did not observe root growth or root infection.

In order to further understand what elicits the phenotype, inoculations were performed with *Cochliobolus sativus* (Spot Blotch), *Blumeria graminis* (Powdery Mildew), *Xanthamonas translucens* pv *undulosa* (Bacterial Leaf Streak), *Pyrenophthora teres* f. sp. *teres* (Spot Form Net Blotch) and *maculate* (Net Form Net Blotch) and *Puccinia graminis* f. sp. *tritici* races MCCF and QCCJ (Stem Rusts). *Cochliobolus sativus* infections were conducted by growing ten gamma1 plants (*nec3*) and ten Bowman plants in a growth chamber with a 14 hour photoperiod

at 22.5°C and 10 hours dark at 19.1°C. After ten days of growth, *C. sativus* isolate ND85F inoculum, which was plated from a plug derived from an infected plant, was re-suspended at a concentration of 7,000 spores/mL in distilled water and spiked with 10µl of Tween20 in order to reduce spore clustering. The spore suspension was then put into a 25mL atomizer and plants were infected using 7psi of pressure. They were then placed in a mist chamber in the dark and misted for 30s every 12 minutes for 16 hours. After inoculation plants were placed back into the growth chamber and were observed and documented every day for symptom development.

Blumeria graminis infection was not as easily achieved as the pathogen is unable to grow on a plate, however, the spores from newly infected barley leaves provided sufficient inoculum. Seedling gamma1 plants were rubbed with infected *B. graminis* leaves and kept in greenhouse conditions (16 hour photoperiod at 70°C) until symptoms developed. The plants were continually observed on a daily basis, however, pictures were only taken one week after inoculation. The bacteria *Xanthomonas translucens* pv *undulosa* strain BLS-LB10 collected from a wheat plant in Lisbon, North Dakota was grown on WBA plates for 3 days at 28°C and resuspended in water at an optical density of 0.2 (Duveiller 1997, Adhikari 2012). Both Bowman and gamma1 plants were grown as previously described for *C. sativus* infection. Plants were infiltrated using a 5mL syringe with *Xanthomonas translucens* and observed and documented one week after infiltration. *Pyrenophthora teres* f. *teres* and *maculata* were plated on V8PDA and grown in the dark for 3 days at room temperature, then 7 days at room temperature in the light. Spores suspensions were prepared at 5,000 spores/mL. Infections with stem rust, *Puccinia graminis* f. *tritici* races MCCF and QCCJ were performed on gamma1 and Bowman plants grown in the growth chamber as previously described. The inoculations were performed when the first leaf was fully expanded which was between four to five days post emergence. The

plants were separately inoculated using an atomizer set at 28kPa with *P. graminis* f. *tritici* races MCCF and QCCJ each using 12mg of spores/1ml of Soltrol 170 oil for each race. Post inoculation plants were placed in a dark mist chamber with the settings of 30s of misting every 12 minutes. Plants were left in the mist chamber for 18-20 hours and air dried in light for 2 hours prior to being placed into the growth chamber: 14 hrs light 22.5C, 10 hrs dark 19.1C. The infection types were documented 12 dai.

nec3 Map Development

The M₂ gamma1 x Quest population was advanced to the F₂ generation and 200 F₂ individuals were evaluated for the gamma1/*nec3* necrosis phenotype following inoculation with *C. sativus* isolate ND85F as previously described. Isolate ND85F was previously shown to induce the necrosis phenotype on gamma1 M₃ individuals. Because most mutant phenotypes are recessive in nature and the phenotype is only observed in the homozygous recessive mutant individuals, the F₂ progeny from the gamma1 x Quest cross allows for the identification of homozygous gamma1 individuals.

To genetically position the *gamma1* mutation and determine if it is a mutant allele of *nec3*, several markers from the Chromosome 6 *nec3* locus were used to genotype the homozygous mutant individuals. Genomic DNA was extracted from the parental lines Quest and gamma1, and the 34 identified homozygous gamma1 mutant F₂ progeny, representing 68 recombinant gametes. A crude DNA extraction method from the tertiary leaves was performed by homogenizing an ~ 4 cm leaf section with 400ul of Extraction Buffer (A total of 50mL containing 10mL 1M Tris HCL, 2.5mL 5M NaCl, 2.5mL 0.5M EDTA, 2 mL 10% SDS and 32.5mL sterile ddH₂O). After homogenization 200ul of chloroform was added and the tube was vortex for 10s and centrifuged at 16.1rcf for 10 minutes. 300ul of the supernatant was then

transferred to a new a tube and 200ul of chilled 100% isoproponal was added to precipitate the DNA. The tube was then chilled at 4°C for 10 minutes. To pellet the DNA the tube was centrifuged at 16.1rcf for 10 minutes and the supernatant was removed. The pellet was carefully rinsed with 75% EtOH and set to dry in a biosafety hood until all of the EtOH has evaporated. The DNA pellets were resuspended in 50ul of sterile H₂O. Two single nucleotide polymorphism (SNP) markers, and five microsatellite marker designated Bmag and GBM where amplified and directly sequenced by Genscript® using Sanger sequencing or visualized via gel electrophoresis depending on the polymorphism present (Table 1.2). The markers that were sequenced were: GBM1053, GBM1423, SNP10539 and SNP10817 (Figures 1.1 & 1.2). Polymorphisms utilizing CAPs markers were developed from sequencing GBM1053 and GBM1423 and used BsiHKAI and StyI restriction enzymes from New England Biolabs respectively following the manufacturer's standard procedures (Figure 1.1, A1,A2,A4). The polymorphisms found are show in Figure 1.1. The remaining markers GBM1212, Bmag0807 and Bmag0173 and separated on a 12.5% polyacrylamide gel (Amresco) (GBM1212) and on a 1.5% agarose gel (Bmag0807 and Bmag0173) (A3). The primer sequences for the markers and amplification parameters are provided (Tables 1.2 & 1.3).

Table 1.2. Primers used for *nec3* map development.

GBM1053* CAPS - BsiHKAI	Forward CACGAGCTAGCCCACCAT
	Reverse GAAGACTTGGGTCTCTGTGGA
GBM1212* Co-segregating	Forward TGTTGCAAGAAGCAAGGATG
	Reverse GCGCTTACTCTCTCGTCGTC
GBM1423* CAPS - StyI	Forward ACAAATCCCCAAGCCAATCT
	Reverse CTTGCCTGTCAACGTCTTCA
Bmag0807	Forward GGATATAAGGGTCCATAGCA
	Reverse AATTACATCAAATAGGCTCCA
Bmag0173	Forward CTTTTTGTGGTGACGG
	Reverse ATAATGGCGGGAGAGACA
SNP10539	Forward GATCCTGACGATCTTGAGCTTCCTG
	Reverse GAATCAAGAGGCACAACTTCAAGCG
SNP10817	Forward GCAGAAACACACACCCTTCC
	Reverse GATTGTTATCGCTTCAGCTGC

*Top flanking marker is GBM1053, which is 4.4cM from co-segregating marker GBM1212. The lower flanking marker is GBM1423, which is 1.4cM from GBM1212.

Table 1.3. Amplification parameters and analysis of critical markers used for *nec3* mapping.

Marker	bp	Gel/Sequence	Amplification Parameters
GBM1053	590	Sequence CAPS-BsiHKAI	3 min at 94 deg C; 45 cycles with 30 sec at 94 deg C, 30 sec at 60 deg C (touchdown of 0.5 deg C / cycle for initial 10 cycles - final annealing of 55 deg C for remaining 35 cycles), 30 sec at 72 deg C; and a final extension step of 5 min at 72 deg C; hold 4 deg C
GBM1212	150	Gel- Polyacrylamide	
GBM1423	700	Sequence CAPS-StyI	
Bmag0807	120	Gel-1.5% Agarose	3 min at 94 deg C; 1 min 94 deg C, 1 min 57 deg C, 1 min 72 deg C; repeat 40X; 10min 72 deg C; hold 4 deg C
Bmag0173	160	Gel-1.5% Agarose	
SNP10539	180	Sequence	1 min at 95 deg C; 1 min 94 deg C, 1 min 62 deg C, 1 min 72 deg C; repeat 40X; 5 min 72 deg C; hold 4 deg C
SNP10817	170	Sequence	

```

GBM1053

gamma1 CCTCATCGACGACGTCGTCGCAGAGATACTCCTCCGCCTCCCGCCGGATGAGCCCG
      ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Quest  CCTCATCGACGACGTCGTCGCAGAGATACTCCTCCGCCTCCCGCCGGATGAGCCCG

gamma1 AGCATCTCTCCGGCCGCCCTCGTCTGCAAGCCCTGGCTCCGCGTCATCTGGACCCCG
      ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Quest  AGCACCTCTCCGGCCGCCCTCGTCTGCAAGCCCTGGCTCCGCGTCATCTGGACCCCG

gamma1 CCTTCGCGCCGGTACCGCGCTTCCACGGCGCCCTCCGCTGCTCGGCCTCCTCCACA
      ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Quest  CCTTCGCGCCGGTACCGCGCTTCCACGGCGCCCTCCGCTGCTCGGCCTCCTCCACA

gamma1 GGCCCAAGTCTTCC
      ::::::::::::::::::::
Quest  GGCCCAAGTCTTCC

BsiHKAI
5'...GWGCWC...3'
3'...CWCGWG...5'

GBM1423

gamma1 CTCCTCCTCTGTTCTCCCCCGCGCCTAAGGTACGGGATTCCTTCCCGTCTCCCG
      ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Quest  CTCCTCCTCTGTTCTCCCCCGCGCCCAAGGTACGGGATTCCTTCCCGTCTCCCG

gamma1 TACCTTCGATCCACGTTTCCGTTGACTGGAGGATTATTGCCGTCGAATTTGGCCCG
      ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Quest  TACCTTCGATCCACGTTTCCGTTGACTGGAGGATTATTGCCGTCGAATTTGGCCCG

gamma1 CTTCTGCCACGATCTGATGTTGCGGGAACGAAACTTGTGTCCCGATCGATCGCTCGTGT
      ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Quest  CTTCTGCCACGATCTGATGTTGCGGGAACGAAACCTGTTGTCCCGATCGATCGCTCGTGT

StyI
5'...CCWWWG...3'
3'...GGWWCC...5'

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Figure 1.1. CAPS markers used to develop the *nec3* map: GBM1053, GBM1423. Both GBM1053 and GBM1423 utilize SNPs for restriction sites that allow phenotyping between the *nec3* mutant and Quest.

```

Snp10817

gamma1 CGGGTAGCTTGACCACACAACATTGGAAGTGTTGTAAACTTCAAAGAGTTGTGATACT
      ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Quest  CGGGTAGCTTGACCACACAACATTGGAAGTGTGTCAACTTCAAAGAGTTGTGATACT

gamma1 TGAACTCAAAAACTGTTCAGCAGCTGAAGCGATAACAATC
      ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Quest  TGAACTCAAAAACTGTTCAGCAGCTGAAGCGATAACAATC

Snp10539

gamma1 CACCCGCCCTGGGGTTGTCCCGCACCGATCGAACCTAGGGCTCTGTGGCTCTTGGAAC
      ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Quest  CACCCGCCCTGGGGTTGTCCAGCACCGATCGAACCTAGGGCTCTGTGGCTCTTGGAAC

gamma1 GTGAGTCATCAGACCACGCTTGAAGTTGTGCCTCTTGATT
      ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Quest  GTGAGTCATCAGACCACGCTTGAAGTTGTGCCTCTTGATT

```

Figure 1.2. SNP markers used for *nec3* map development: SNP10539, SNP10817. The SNPs highlighted in red were sequenced from the critical recombinants and utilized as markers to delimit the *nec3* region.

RNAseq

Bowman wild type (BWT) and gamma1 (*nec3*) were grown in a growth chamber with settings: 14 hours light at 22.5° C and 10 hours dark at 19.1° C for ten days. Total RNA was extracted using the RNeasy Qiagen mini kit (#74104) from the secondary leaf of six gamma 1 plants and seven BWT plants totaling 54 ug and 43 ug, respectively. The mRNA was isolated from the total RNA, using Ambion's Poly(A)Purist Kit (#AM1916), yielding ~200 ng BWT and 100 ng gamma1 purified mRNA. Due to the lower than optimum mRNA concentrations, the RNaseIII treatment, using the Ion Total RNA-Seq Kit v2, was allowed to proceed for 2.5 minutes amended from the manufacturer's protocol, which suggests 3 minutes. The Ion Total RNA-Seq Kit v2 was used to prepare the extracted mRNA for sequencing on the Ion Torrent following the recommended procedures. Both the gamma1 and BWT RNAseq libraries were sequenced using separate version 2 318 Chips with 200 bp chemistry. The sequences were assembled using CLC-bio software version 7 with the barley Unigene36 EST set downloaded from the Institut für Pflanzengenetik und Kulturpflanzenforschung (IPK) website (<http://webblast.ipk-gatersleben.de/barley/>).

RNAseq was also performed on RNA extracted from plants that were challenged with *C. sativus* to identify candidate genes that may only be induced under pathogen challenge. Bowman and gamma1 plants were grown in the same growth chamber conditions as previously described for the non-induced RNAseq experiment, however, at ten days (the two leaf stage) the plants were inoculated with *Cochliobolus sativus* ND85F with approximately 5,000 spores/mL using an atomizer at 7psi. Samples were taken at 24 and 96 hours post inoculation. Downstream processing for the samples mirrored the non-induction assay as previously described. These data are not available in this publication.

Candidate Gene Elimination

Following alignment with the CLC-bio software version 7 the sequences were complemented with their corresponding Unigene code (U36_*****). The Unigene set used for the alignment was Unigene36 found on IPK (<http://www.ipk-gatersleben.de/en/bioinformatics-tools/sequence-data/>). These codes did not provide information on chromosome location so Blastn searches were performed on the IPK website against the Morex database. The cv. Morex genome sequence was used because the data provided is more robust and contigs are anchored to the genetic and physical maps. The chromosomal locations were determined by IPK in collaboration with the International Barley Sequencing Consortium (IBSC) by population sequencing of 90 Morex x Barke individuals. They were then aligned with IBSC WGS contigs totaling 5.1 million SNPs providing substantial coverage of the genome; the previously assembled contigs were aligned with the SNPs (EnsemblPlants). For both non-induced and induced phenotype experiments the total number of reads and reads per 1,000bp/million reads (RPKM) were used to identify probable candidate genes. Blastx was performed on the candidate genes in order to determine the putative function of the genes found to be within 40-60cM of centromeric region of chromosome 6 based on the Blastn Morex data previously described. The Unigenes considered candidates were those that had zero reads in gamma1 and those that are significantly down regulated in gamma1 compared to Bowman wild type. The annotated full-length gene sequences were acquired from IPK and were used to develop primers to amplify the exons from the candidate genes. Using the Mini-Prep technique developed by Dr. Brueggeman and associates, DNA was extracted from Bowman, Quest, Steptoe, Morex, Villa, Proctor, gamma1, nec3.d, nec3.e, FN362 and FN363 seed embryos. The mini-prep method was performed as follows: tissue was homogenized in a tube using 400ul of extraction Buffer (for

50mL total – 10mL 1M TrisHCL, 2.5mL 5M NaCl, 2.5mL 0.5M EDTA, 2.5mL 10% SDS and 32.5mL distilled water). Following homogenization 200ul chloroform was added to the tube and vortexed for 10s. The tube was then centrifuged at 16.1rcf for 10 minutes. 300ul of supernatant was transferred to a new tube and 300ul of 100% Isopropanol was added and mixed well. The tubes were incubated at room temperature for 10 minutes or 5 minutes at 4° C. Finally the tube was centrifuged at 16.1rcf for 10 minutes, the supernatant was carefully removed while the DNA pellet remained in the bottom of the tube. The pellet was rinsed with 75% EtOH, air dried and resuspended in DNase and RNase free sterile water (Ambion).

All genes that produced amplicons across all mutant lines and background genotypes were eliminated as candidate genes due to the expectation that at least one of the five independent mutants should have detectable polymorphisms due to DNA deletions. Thus, the deletion of DNA from the mutated gene should not produce an amplicon or produce an amplicon with a visible polymorphism expected to show a smaller sized DNA fragment. However, if the deletion is a small such as a single nucleotide polymorphism (SNP) it can only be determined via sequencing or polyacrylamide gel electrophoresis. Due to the large number of candidate genes only electrophoresis gels were analyzed here.

Allelism Crosses

Allelism tests were performed by crossing different combinations of the independent *nec3* mutant lines with *gamma1* which shared a similar *nec3* mutant phenotype. The putative *nec3* mutant, *gamma1*, was crossed in the field successfully with all lines except FN363. The allelism testing was performed by emasculating the seed head to eliminate the possibility of self-fertilization. This was done by removing the anthers from the female plant and allowing the remaining ovaries to mature for two to three days. Once the ovaries began to swell and were

reproductively viable the anthers were carefully removed following incubation by heating the head by gently blowing warm air onto the top of the newly clipped florets. The anthers were then carefully placed inside the emasculated florets on top of the ovary so as to induce fertilization and seed production. Each cross was covered with a bag to reduce the risk of foreign pollen landing on a viable ovary due to an open floret. The F₁ and F₂s of this cross were planted and phenotyped in the field as the *nec3* phenotype is consistently displayed under field conditions.

Infiltrations

C. sativus isolate ND85F was grown on V8-PDA at room temperature for one week under 18 hours of light per day from a plug that was made following plating infected leaves. After 1 week of growth on the plate, 6mL of sterile dH₂O was added to the plate and spores were gently removed with a rubber policeman. Following re-suspension of spores, 1mL was taken from the plate and 75-100mL of Fries media was inoculated (Liu 2004). Fries Media: for 1 Liter, 5 grams Ammonium Tartrate, 1 gram Ammonium Nitrate, 0.5g Magnesium Sulfate, 1.3 grams Potassium Phosphate (KH₂PO₄), 4.6 grams Potassium Phosphate (K₂HPO₄ + 3 H₂O), 30 grams Sucrose, 1 gram Yeast Extract, 1 Liter H₂O, 2mL Trace Element Solution (in 250mL H₂O add 41.75 mg of Lithium Chloride, 26.75mg Copper Chloride, 8.5mg Molybdic Acid, 18mg Manganese Chloride, 20mg Cobalt Chloride, and store at 4°C). This growth technique introduces 10-20,000 spores depending on how well the fungi grew from the plug. The flasks were then incubated at 26° C in the dark while shaking at 100 rpm for three days. They were removed and stored for four days in the dark at room temperature. After seven days of growth in Fries media the exudates were filtered with miracloth and concentrated using a 15mL Microsep Advance Centrifugal Device with a 3kD cut off. The exudates were concentrated 8x from 4mL to 0.5mL. Pronase (Sigma) (a cocktail of protein degrading enzymes) was used to treat the

exudates in an attempt to determine if the elicitor was a protein. A syringe without a needle was used to infiltrate the leaves of Steptoe, Bowman, gamma1, Quest, FN362, FN363, nec3.d, and nec3.e with 1:1 ratios of the concentrated exudates + Fries Media, concentrated exudates + MOPs buffer, concentrated exudates + MOPs + Pronase, and Fries Media + MOPs + Pronase, according to the specifications in Liu 2004. Leaves were scored at 4 and 7 days post infiltration. The first symptoms appear at approximately 3 days depending on the volume of the infiltration and the concentration of the exudates. Pronase treatments were initially performed using a 1mg/mL concentration, based on the preliminary results Pronase concentrations were increased to 2mg/mL and 5mg/mL and the amount of time the treatment were incubated was also increased from 4 hours to overnight.

Infiltrations were also attempted on Steptoe, Bowman, FN362 and gamma1 with trigalacturonic acid a component of the plant cell wall characterized as a DAMP (10mg/mL, 1mg/mL, and 0.1mg/mL) and FLG22 a subunit of bacterial flagella categorized as a MAMP at 1mg/mL. Infiltrations were performed at the two-leaf stage with the third leaf just emerging. The plants were kept in the growth chamber for the duration of the experiment with a 14 hour photoperiod at 22.5° C and 10 hours of dark at 19.1° C. Plants were observed every day and pictures were taken if a reaction was seen seven days after infiltration.

Results

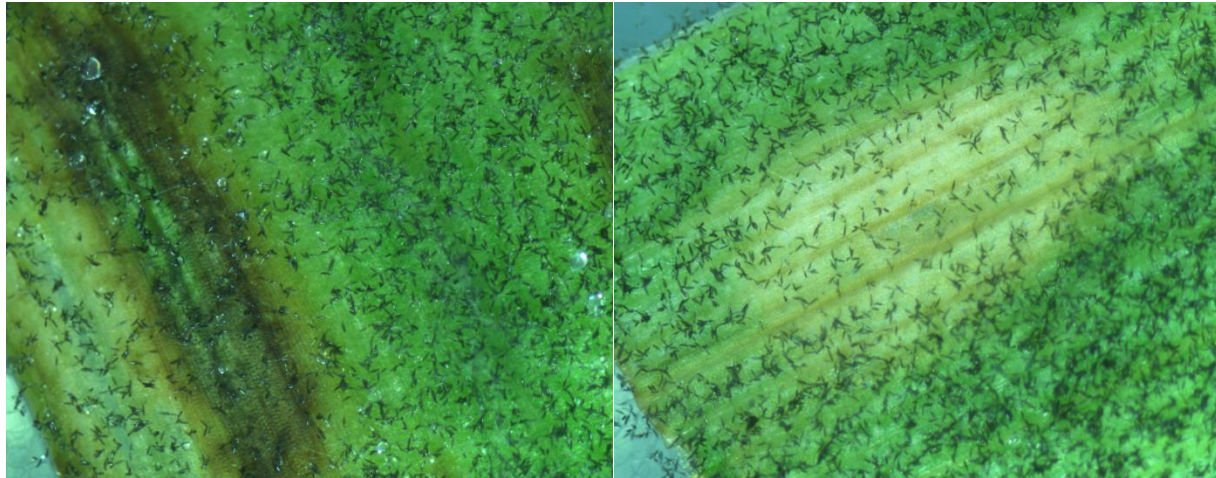
nec3 Phenotypic Observations

The isolation box experiment allowed us to demonstrate that the *nec3* phenotype is not a LMM as previously described (Frankowiak 1997, Keisa 2006), but is only observed after infection by a pathogen or infiltration with culture filtrate exudates from pathogen known to elicit the phenotype upon infection. The observed phenotype is hypothesized to be the plant eliciting PCD via a resistance response elicited by several pathogens that are regulated by the wild type *Nec3* gene, yet the *nec3* mutation allows the PCD response to runaway unregulated. The phenotype was not observed when the *nec3* plants were grown in pathogen free growth chamber or greenhouse. These data led to the observation that the *nec3* mutant is not a spontaneous lesion mimic mutant but rather is a pathogen induced runaway PCD (PIRP) mutant. Following leaf surface sterilization of gamma1 and Bowman plants grown in the greenhouse, there was a noticeable difference between phenotypes when viewed under a microscope from infection from *C. sativus* (Figures 1.3 and 1.4). Both magnifications validated the phenotype that was observed on a macro level, the wild type produces the typical dark necrotic lesions seen following the induction of PCD while the mutant produces a tan lesion with no clear margins that expands at a faster rate compared to the wild type (Figure 1.5). *Cochliobolus sativus* however is not the only pathogen that is able to produce the *nec3* phenotype.

Inoculation with *Blumeria graminis*, an ascomycete biotrophic pathogen of barley, elicited the *nec3* phenotype. (Figure 1.6). The same phenotype is also observed after infection from *Xanthomonas translucens* pv *undulosa*, a parasitic bacteria, and *Pyrenophora teres* f. sp. *teres* and *maculata*, necrotrophic ascomycetes (Figures 1.7 and 1.8, respectively). Alternatively,

the phenotype is not observed following infection with *Puccinia graminis* f. *tritici* races MCCF and QCCJ, the stem rust basidiomycete biotrophic pathogen (Figure 1.9).

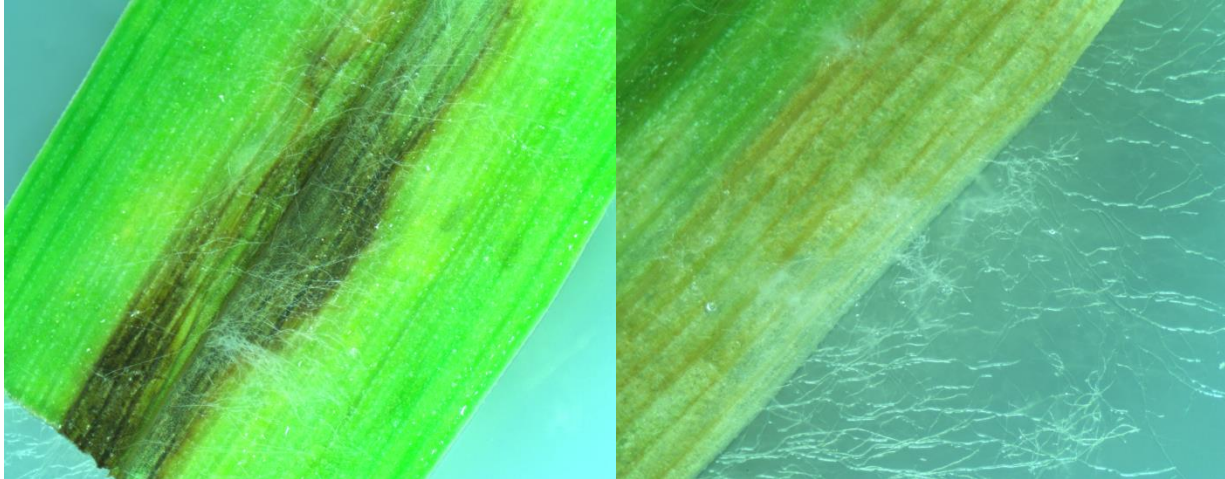
In addition heads from allelic crosses were harvested and documented (Figure 1.10). None of the heads were able to produce full seed. This could be due to a number of factors discussed in the next section.



Bowman

gamma 1

Figure 1.3. Magnification (5x) of leaf surface of Bowman and gamma1 post surface sterilization and plating on water agar of tertiary leaves (1). The wild type is presenting a typical hypersensitive response by producing phenolics (dark brown area) while the gamma1 plants displays a bleached out appearance following pathogen infection. The pathogen in this figure is *Cochliobolus sativus*. The structure being observed is exterior mycelia with an asexual spore above the leaf surface.



Bowman

gamma 1

Figure 1.4. Magnification (5x) of leaf surface of Bowman and gamma 1 post surface sterilization and plating on water agar of tertiary leaves (2). The fungal structure observed growing over the leaf onto the plate is mycelia of *C. sativus*. Again we are able to observe a dark phenolic brown in response to infection in the wild-type however the mutant displays the bleached out appearance.

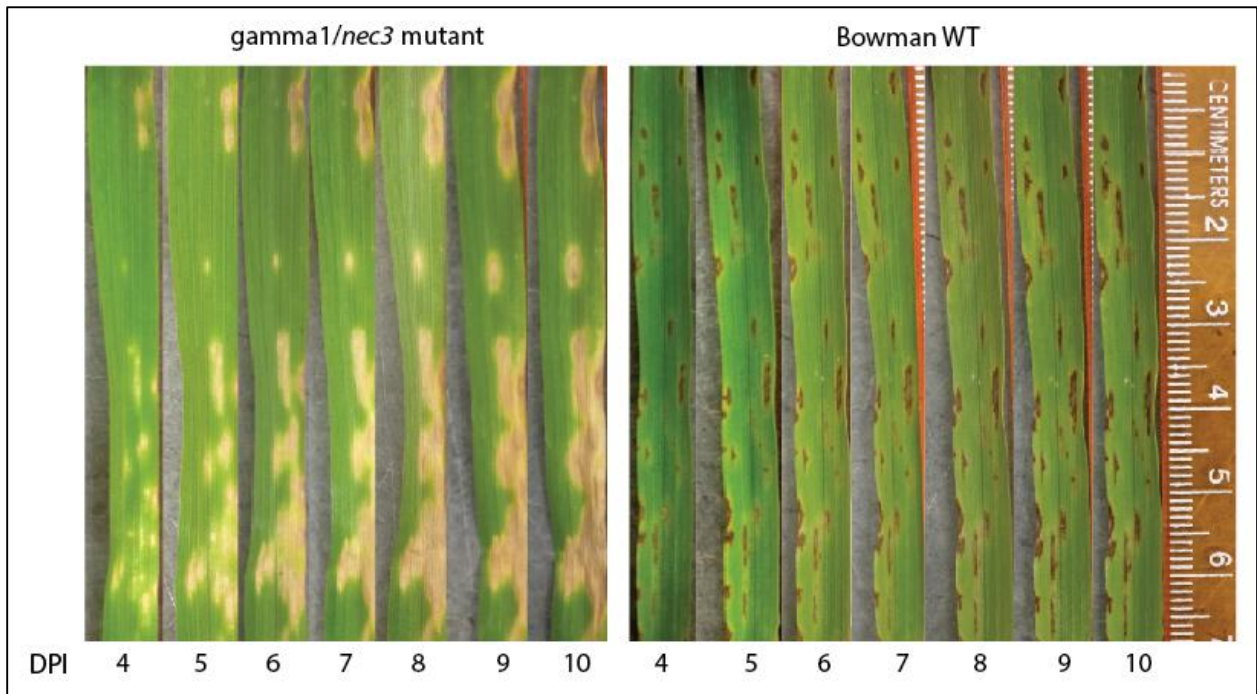


Figure 1.5. Time-course experiment of gamma1 and Bowman inoculation with the pathogen *C. sativus*. Pictures were taken every day from 4-10 days after inoculation. The gamma1 image displays the runaway tan necrotic phenotype whereas the wild type the typical resistance necrotic phenotype.



Figure 1.6. *nec3* phenotype observed in greenhouse after powdery mildew infection; *Blumeria graminis*. The image shows the typical runaway PCD *nec3* phenotype.



Neg Bowman gamma1

Figure 1.7. Infiltration with *Xanthomonas translucens* pv *undulosa*. Following 7 days of infection the Bowman phenotype observed displays very little reaction whereas the *nec3* mutant shows the runaway necrotic phenotype. The phenotype is not as prominent as that seen with the necrotrophic ascomycetes, but the phenotype is still present.

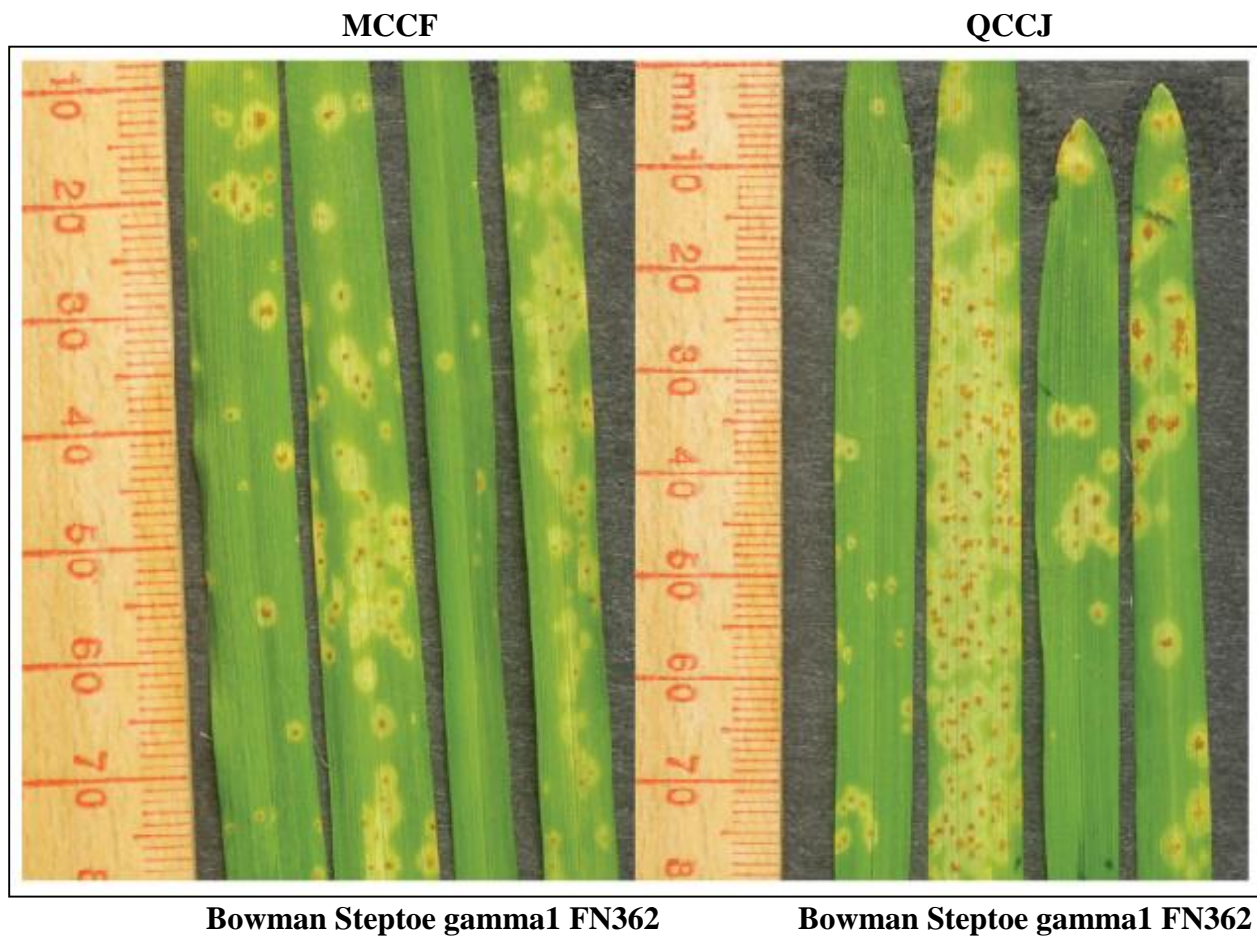


Figure 1.8. Inoculation of Bowman, Steptoe, gamma1 and FN362 with stem rust races MCCF and QCCJ. This figure shows the lack of the typical necrotic response from the *nec3* mutant gamma1 following 7 days of infection. The text above the image indicates what race was used and the text below the image indicates the variety inoculated.



Figure 1.9. Inoculation of Bowman and gamma1 with *Pyrenophthora teres* f. sp. *teres* (Ptt) and *Pyrenophthora teres* f. sp. *maculta* (Ptm). This figure shows the gamma1 phenotype is observed after inoculation with both net form net blotch and spot form net blotch pathogens. Photos courtesy of Dr. Timothy Friesens lab at the USDA in Fargo, ND.



Figure 1.10. Incomplete seed head formation of *nec3* mutants from allelism test crosses picked at random. From left to right: F₁ gamma1/*nec3*.d, F₁ gamma1/*nec3*.e, F₂ g1/*nec3*.d, F₂ gamma1/*nec3*.e, F₂ gamma1/*nec3*.d, and F₂ gamma1/*nec3*.d. FN362 cross not shown but the results match these shown. This image shows the negative effects of the mutation on seed formation.

nec3 Map Development

Using the homozygous mutant F₂ progeny from the cross between gamma1 and Quest a genetic map was developed. A 3:1, wild type to mutant phenotype, segregation ratio was expected for the F₂ progeny. Unfortunately, due to different mutation in the background of the gamma1 mutant selected for the necrosis phenotype that was not expressed in the M₂, fifty-three of the two-hundred F₂ plants died of a lethal chlorophyll mutation which segregated in a recessive 3:1 single gene manner ($\chi^2 = 0.32$). These chlorophyll mutants produce fewer chloroplasts and generally do not reach the adult plant stages. Fortunately this mutation was not linked to the gamma1 mutation and of the 146 plants that survived, 34 had the gamma1/*nec3*

phenotype, which fit the expected 3:1 segregation ratio ($\chi^2 = 0.17$), indicating a single mutant gene segregating in a recessive manner.

The co-segregating marker for the *nec3* gene, GBM1212, is found at approximately 49.07cM on chromosome 6 and the proximal flanking marker, GBM1423, is positioned at 49.22cM according to the cv Morex contig sequences on the IPK barley genome database. The flanking marker GBM1423 was positioned ~ 1.4 cM proximal of GBM1212 on our gamma1 X Quest genetic map (Figure 1.11, A2). The cv Morex contig (43256) containing the GBM1053 sequence was not anchored to a position in the IPK genome database, but mapped ~ 4.4 cM distal of GBM1212 in our mapping population (Figure 1.11, A1). Of the 34 recombinants tested, the recombinant designated gamma1.3 initially appeared to be a double recombinant however after replanting the recombinant was homozygous for gamma1 (A5). Synteny analysis is a powerful tool for the identification of markers for high resolution mapping in barley, however, this region appears to be out of synteny with *Arabidopsis* and *Brachypodium*. This could be due to translocations and expansion followed by divergence since the split from the progenitor species (Thiel 2009). The proximal non-flanking marker (SNP10539) was the only barley marker sequence that matched with a *Brachypodium* contig, which was Bradi3g03270.1.

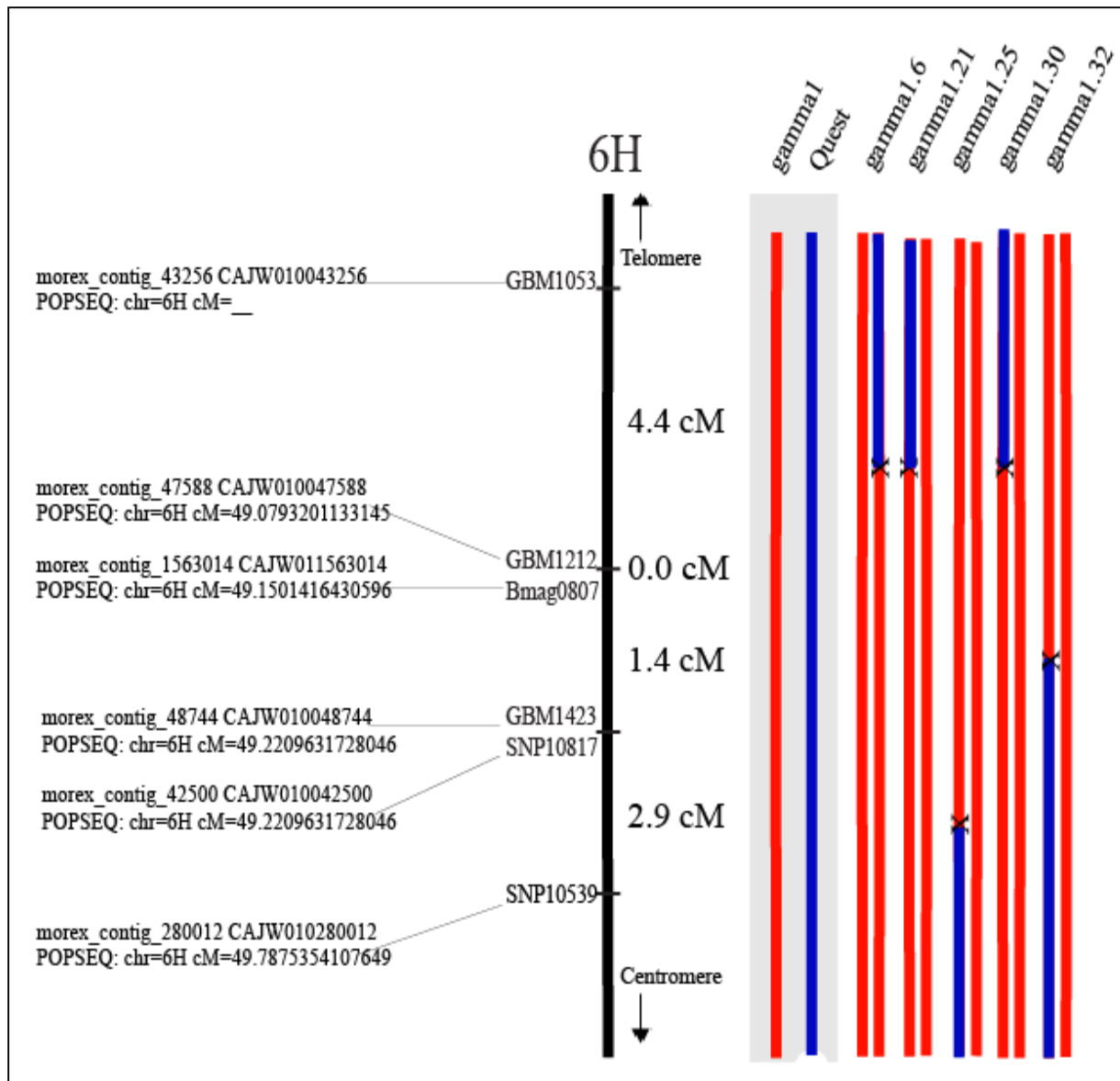


Figure 1.11. Genetic and Physical map of the *nec3* region. On the left is the genetic recombination map developed from screening the 34 gamma1 F₂ recombinants from the cross between gamma1 and Quest. The red line segments indicate a gamma1 genotype while the blue line segments indicate a Quest genotype. The sideways 'X' indicates a region where recombination occurred during karyogamy. The distance genetic distance, based on recombination, is given just right of the 6H chromosome with the markers that were used for map development; GBM1053, GBM1212, Bmag0807, GBM1423, SNP10817 and SNP10539. The relative physical location of the markers on the genetic map is displayed on the far left which were derived from blastn Morex searches on the IPK database.

RNAseq and Candidate Gene Identification

For the non-inoculated gamma1 and Bowman plants, RNAseq was conducted using the Ion Torrent with a separate 318 chip for each RNAseq library; Bowman wild type and gamma1. The RNAseq were not replicated. The Bowman chip had 4.9 million reads while gamma1 had 4.3 million reads. The transcript analyses from total reads determined that 6,136 genes were expressed in the Bowman wild type but absent in the gamma1 mutant. Of those, 100 were analyzed by entering the Unigene code associated with the gene into IPK blastn database search against the cultivar Morex because it has more anchored contigs providing a genetic distance unlike the Bowman database. Of these data 24 of the 100 reside on chromosome 6 while 13 of the 24 reside within the region delimited by the flanking markers GBM1053 and GBM1423 (Figure 1.11). Following another analysis of the RNAseq data using CLC-biosoftware, the data was aligned to only chromosome 6 giving 385 genes that were expressed in Bowman but not in gamma1. This is a good estimate of the number of genes that would have been found if all 6,136 genes were analyzed.

Because the different sources of irradiation used to cause the independent *nec3* mutations generate single nucleotide to tens of thousands of base pair deletions in genomic DNA it was expected that the *nec3* gene would not amplify or produce a smaller amplicon in at least one of the five independent mutants. Therefore gene specific primers were designed from the predicted gene structure of five of our top candidate genes (Table 1.4). The top five candidate genes included HBP1a, which is a bZIP transcription factor that interacts with Histone 1. HBP1a has been shown to be involved in disease resistance and is a regulator of various environmental stresses (Lee 2006). The PB1 protein is a conserved protein found in plants, fungi and animals. PB1 is involved in the MAPK system, and is an integral part of defense response and auxin

signaling which is related to the third candidate gene, the Auxin response factor 5 (Sumimoto 2007, Guifoyle 2015). The Auxin response factor 5 is a response factor that interacts physically with PB1 ubiquitin associated protein and initiates DNA binding which in turn induces further auxin response proteins (Guifoyle 2015). The fifth candidate gene was a Formin protein that is involved with cellular integrity as formins belong to a larger class of an actin nucleator family. These genes assemble actin fragments, which is important in defense responses by actin cytoskeleton dynamics which form the network for trafficking components involved in defense responses including the creation of a physical barrier between pathogen and host (Yi 2005). The primers were designed to produce amplicons for each of the candidate genes spanning the predicted exons (Table 1.4; Figure 1.12). However, these five candidate genes with known function relate to disease resistance or physiological plant defenses have been eliminated due to amplification of the candidate genes across all the lines that contain the gene and no polymorphism was detected. The full list of candidate genes will be analyzed and prioritized by putative functional domains and their hypothesized roles in PCD pathways.

The first twenty genes expressed in Bowman but showing no transcription in gamma1 were identified (Table 1.5) and compared to the transcriptome analysis of the *nec3* mutants FN362 and FN363 by Keisa et al in 2010 to determine if genes with similar function were being differentially regulated. It was found that there is some overlap in protein function however due to the lack of corresponding codes between the Unigene21 assembly and the Unigene36 assembly.

Table 1.4. Primers for candidate gene eliminated from the non-infection assay.

Gene	Primer	Sequence 5' to 3'
Zn+ Finger	ZnF1	GATGAGGTACGGAGGCTCACC
	ZnR1	CTACTCTCATGTTTCACTGGTAGCC
	ZnF2	GAGAACTCTGAAGCCAGCGCG
	ZnR2	GAAGAAGCATCTGTGCGAGTG
HBP1a	G1F1	GGAGCTTCCATCTCTTCTTCCTCG
	G1R1	TTCGAAAACCTACCAGCAGGGCCTC
	G1F2	GTTCCGGCGACGGCCGCTTCTCTG
	G1R2	GCAAGGCATCAACATTCTTGAGCC
	G1F3	GTCGCGAGCACCTAATATGATAC
	G1R3	CCCTGAGCTCCCTGAAAGACATG
	G1F4	GAGCAACCTCCAGCTACTACCTCTG
	G1R4	CAGAGAAGCGGCCGTCGCCGGAAC
	G1F5	TGTGCAGGGTGTGCATCCATTTC
	G1R5	GATCCAACAGGGTATTTCTTCCG
	G1F6	CAGCATGGACTGCATTACTGAAGG
	G1R6	CCCATATTCAAGCCGAGGCAGACATC
	G1F7	CATCTGATCCTACCTCTGTTGATG
	G1R7	CCTTCAGTAATGCAGTCCATGCTG
	G1F8	CGGAAGGAAATACCCTGTTGGATC
	G1R8	CGCCTTAGTTTTCTATTCTCTG
	G1F9	GATGTCTGCCTCGGCTTGAATATG
	Auxin Response Factor 5	AuxF1
AuxR1		GAGTAGACTGTAGAGCGCTGTGG
AuxF2		GCTCTGCATGTTTCCAATGCCT
AuxR2		GCAAATAGCTTCTTGCCGCTCAC
AuxF3		GTGAGCGGCAAGAAGCTATTTGC
AuxR3		CAGTTATCGTACCCATGTACCTG
AuxF4		GGATGCTTGCTACAGTTCT
AuxR4		CAAATTCTGGTCTGCAGTTGATG
AuxF5		CATCAACTGCAGGACCAGAATTG
AuxR5		CATCTGAGCAGTAGAGTGTGG
AuxF6		CCACACTCTACTGCTCAGATG
AuxR6		GTGCACCACACAACATGTAC
AuxF7		GAGATGGTCACACATAGGCACATAG
AuxR7		TCATCTGCACATCCTATATGCACC

Table 1.4. Primers for candidate gene eliminated from the non-infection assay (continued).

Formin-like Protein	FormF1	CAGGACCAGCAGTTCTTGCTC
	FormR1	GGTGACGATCCTGCATGAAATTG
	FormF2	CAATTCATGCAGGATCGTCACC
	FormR2	CTTGAATGTCCTCTACACTGAC
	FormF3	GTCAGTGTAGAGGACATTCAAG
	FormR3	CTTGCTTCGCGGACACCTTCAGATC
	FormF4	GATCTGAAGGTGTCCGCGAAGCAAG
	FormR4	CATCAGATACTAACGTAGAGGTGTG
Ubiquitin Assoc.	PB1F1	CGCTCGCTGTACGCACAGAAATC
PB1	PB1R1	CATGCGCACAACCTGTGTGATCAATC
	PB1F2	GGATCATTATGATGTCCTCATGG
	PB1R2	GACAGGTGAACTCTCAGCATGCC
	PB1F3	GGCATGCTGAGAGTTCACCTGTC
	PB1R3	CTGATGGCAGAAACAGAAGATGTC
	PB1F4	GGCCACGCTACAAGTCTAATGTG
	PB1R4	GAATCTGCACAACCATAACCAACTC
	PB1F5	GAGTTGGTATGGTTGTGCAGATTC
	PB1R5	CTAGATCATACATCCACGTACAG
	PB1F6	CTGTACGTGGATGTATGATCTAG
	PB1R6	CATCAGTATCAGCGACATTCAG
	PB1F7	CTGAATGTGCTGATACTGATG
	PB1R7	GCACATAGGTCTTCCACTCACAATAG

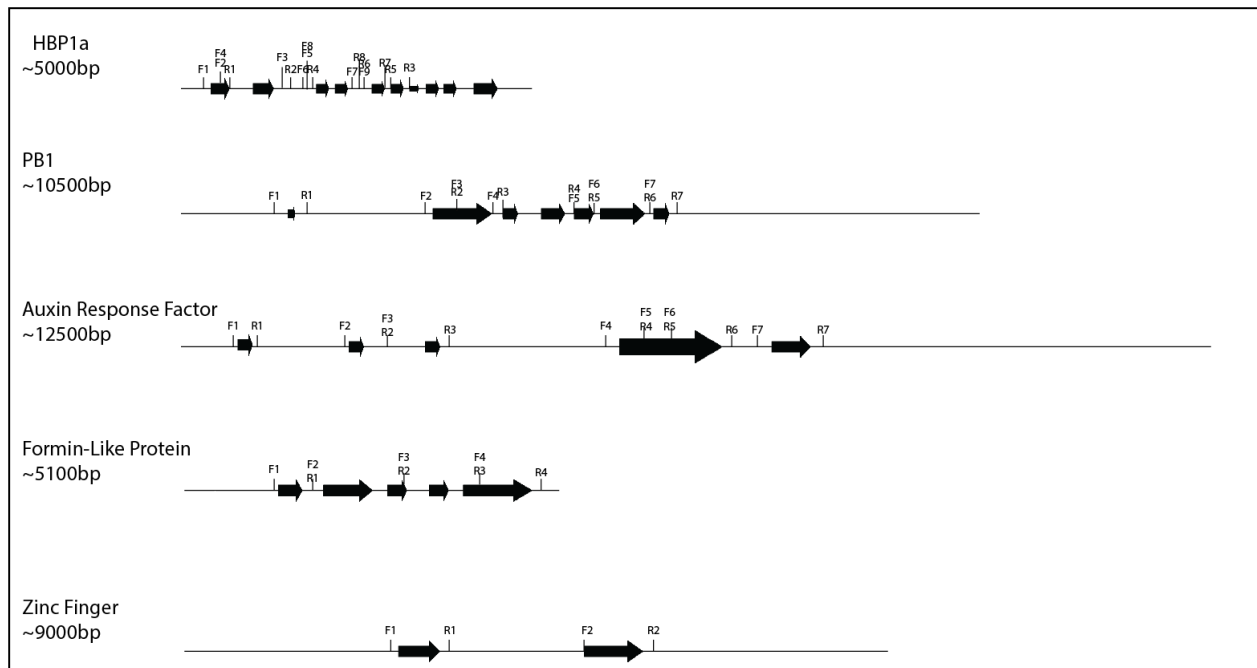


Figure 1.12. Gene structure of the candidate genes eliminated from non-inoculated plants. All of the genes were amplified across mutant lines. The first gene listed is HBP1, second is PB1, third is Auxin Response factor, fourth is Formin-like protein, and the final gene eliminated is the zinc finger protein. The arrows indicate the exons and all genes are presented in a 5' to 3' orientation. The tick marks on each gene are the locations of the primers presented in Table 1.6.

Table 1.5. The results of genome wide RNAseq data from non-inoculated plants.

Putative Function	Chromosomal Location	Feature ID	Bowman Reads*
<u>IPK</u>		<u>UNIGENE</u>	
unknown, f-box protein like, isocitrate lyase, gtpase binding	chr 4,5,2	U36_13636	66
unknown, phd finger transcription factor like, anthocyanidin -o- glucosyltransferase like *** ¹	chr 4,5,2,7	U36_31235	49
HB1a**	chr 6HS	U36_52766	21
MFS-type transporter, sugar transporter, letm1-like protein	chr 2,7	U36_20815	20
RuBisCO; Ribulose bisphosphate carboxylase/oxygenase activase B chloroplastic	chr 4,2,5,7	U36_26165	16
Fidgetin-like protein (seven microtubules), aaa-type ATPase-like protein, ribonuclease h protein, Vps4_C superfamily, zf-RVT superfamily (Zn binding reverse transcriptase)	chr 5	U36_2187	14
Beta-D-glucan exohydrolase, isoenzyme ExoII, beta-xylosidase, lysosomal beta-glucosidase	chr 5,1	U36_49626	13
F-box protein At-B-like protein	chr 7,4	U36_64235	13
Upf0481 protein, 2889793 protein, 2614809 protein, 928409 heavy meromyosin-like	chr 5	U36_68476	13
unknown protein	chr 6HL	U36_19168	12
alkaline and neutral invertase (catalyzes hydrolysis of sucrose), beta-fructofuranosidase	chr 3	U36_23319	12
shock protein src2, ribonuclease h-protein, protein kinase receptor type precursor, probably inactive LRR receptor like protein kinase, fructose 1,6-bisphosphatase class 3 *** ²	chr 7	U36_23547	12
transducin wd-40 repeat-containing protein, homeobox transcripton factor hox7-like protein, phototropin family protein kinase, general transcription factor 3C polypeptide 2	chr 1,3	U36_27183	12
predicted protein	chr 1	U36_27713	12
proline rich protein, RuBisCO- Ribulose bisphosphate carboxylase oxygenase small subunit, small subunit precursor *** ³	chr 2	U36_32347	12
ran-binding protein 1 homolog c-like, charged multivesicular body protein 5-like	chr 2,3	U36_36953	12
iron ion binding protein, 2OG-F3II_Oxy superfamily - N terminal region- non-haem dioxygenase, isopenicillin N synthase, flavanone-3-hydroxylase, oxidase reductase *** ⁴	chr 3	U36_390	12

Table 1.5. The results of genome wide RNAseq data from non-inoculated plants (continued).

Putative Function	Chromosomal Location	Feature ID	Bowman Reads*
Probable lrr receptor-like s/t kinase at1g63430-like, u-box domain-containing protein 35-like *** ⁵	chr 2,4	U36_45926	12
amino-acid acetyltransferase-like, acetylglutamate kinase chloroplastic-like, NAT superfamily, ArgB kinase	chr 7	U36_53471	12
oxysterol binding domain family 2A-like, 1C-like	chr 7	U36_17526	11
8-amino-7-oxononanoate synthase pyridoxal 5'-phosphate binding pocket, serine palmitoyltransferase 2-like, aminopelargonate synthetase related, aspartate aminotransferase (AAT) superfamily fold type 1 of pyridoxal phosphate (PLP)-dependent enzyme	chr 1,3	U36_2462	11

*Bowman Reads: The number of times the sequence was read by the Ion Torrent. Gamma1 reads were all zero for this analysis.

**HBP1a is the only candidate gene on this list because the Bowman reads (21X) for the other candidate genes found on chromosome 6H ranged on the low end from 5-7 which is below the Bowman read value.

***Genes that were found in the Keisa 2010 *nec3* Transcriptome analysis publication to be differentially expressed in FN362/FN363.

***¹ HarvEST Assembly 21 Unigene_33510, Unigene_39248, Unigene_12799 are all glucotransferase-like.

***² HarvEST Assembly 21 Unigene_4024, Unigene_6843 are a protein kinase domain and an LRR transmembrane protein kinase respectively.

***³ HarvEST Assembly 21 Unigene_10206, Unigene_4725, Unigene_22092, Unigene_68 are proline rich proteins.

***⁴ HarvEST Assembly 21 Unigene_18035 is a flavonal-sulfotransferase protein.

***⁵ HarvEST Assembly 21 Unigene_6843 is an LRR transmembrane protein

A subsequent RNAseq experiment was also performed on seedlings that had been inoculated with *C. sativus* isolate ND85F. Of the two time points collected, 24 and 96 hours post inoculation (hpi), only 96 hpi was used for RNAseq analysis. This time points was chosen based on the observation that the *nec3* phenotype is visible 2-3 days post inoculation. As with the non-inoculated plants the mutant and wild type RNAseq libraries were sequenced on separate 318 chips on the Ion Torrent PGM®. These data are currently being analyzed and this work is not reported in this thesis.

Allelism Crosses

Allelism crosses were completed in the field to determine if the four confirmed *nec3* mutants, FN362, FN363, *nec3.d* and *nec3.e* (Kesia 2010), are allelic to our newly identified *gamma1* mutant (Figure 1.13). Crosses were attempted between *gamma1* and the other four mutants. All crosses produced seed except for the FN363 cross which was previously shown to be allelic with FN362 (Keisa 2010). Because of the head sterility (Figure 1.10) resulting in low numbers of seed produced from the homozygous necrotic plants used for crossing very few F₁ individuals were planted from each cross (2-5 F₁ individuals). However, 100% of the F₁ plants displayed the *nec3* phenotype following *C. sativus* inoculation in the greenhouse (Figure 1.14). The F₂ generation was planted in the field for easy phenotyping to validate the cross with FN362, which has a Steptoe background containing the recessive six-row head type, whereas Bowman has the dominant two-row head type. The six-row phenotype was segregating at the F₂ generation validating that this was a true cross. The *nec3* pathogen elicited runaway PCD phenotype is distinct from the wild type Bowman, which shows small pinpoint necrotic flecks indicative of a typical resistance reaction to the leaf spot diseases such as spot blotch caused by *Cochliobolus sativus*. Spot blotch was probably the pathogen eliciting the response in the field nursery as a spot blotch inoculated nursery was growing adjacent to the field plot containing the mutants and F₂ progeny. The *nec3* phenotype as seen in the F₁ generation following growth in the greenhouse (Figure 1.14) and the F₂ generation following inoculation with *C. sativus* (Figure 1.15) are significant as it shows that *gamma1* is fifth *nec3* mutant line.

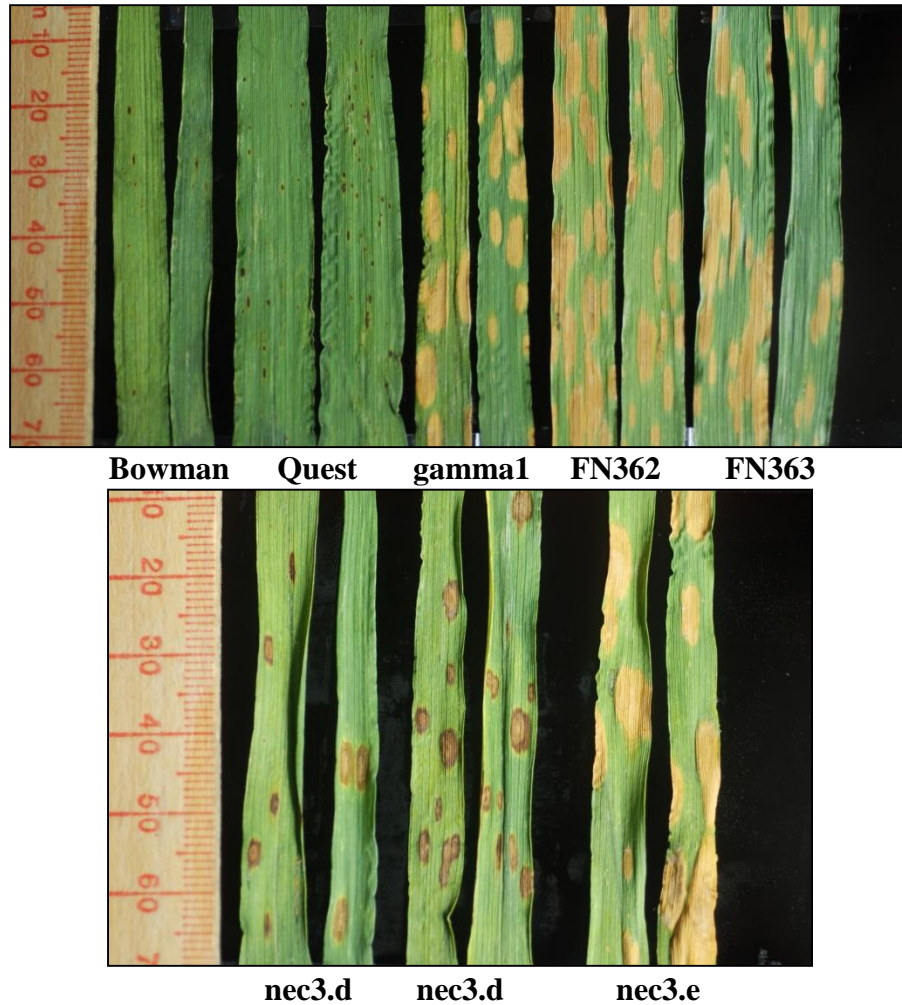


Figure 1.13. Phenotype of the mutants *gamma1*, FN362, FN363, *nec3.d* and *nec3.e* with Bowman and Quest. The leaves were gathered from the field in 2013 at the adult stage and documented. The runaway necrosis typical of *nec3* is visible in all mutants following pathogen infection. The *nec3.d* mutant produces a different yet allelic phenotype in response to the pathogen. *nec3.d* is pictured twice because we received seed from two different institutions, Washington State University and the National Small Grains Collection.

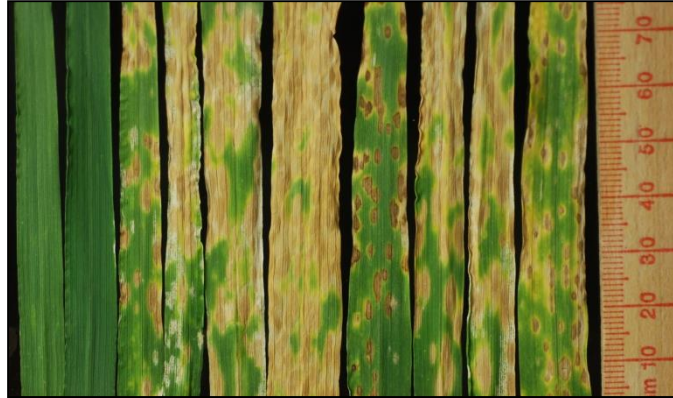


Bowman Steptoe Morex Quest Villa Proctor

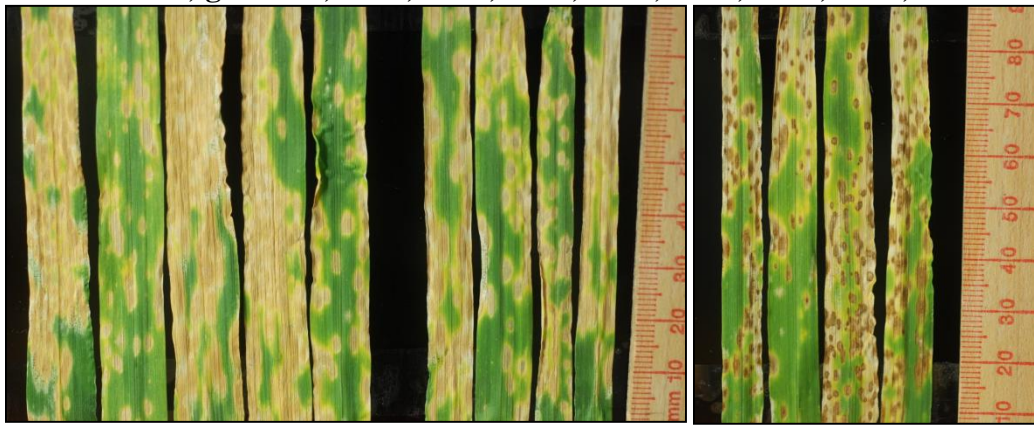


**Gamma1/nec3.d; gamma1/nec3.d; gamma1/FN362; gamma1/nec3.e; gamma1/nec3.e;
nec3.d/gamma1; nec3.d/gamma1**

Figure 1.14. F₁ progeny and the parents of allelic test crosses conducted in the field in 2013 planted in the greenhouse in 2014 and documented after infection with *C. sativus*. The plants were advanced to F₂ (Figure 1.15) and assessed for head type and *nec3* phenotype.



Bowman; gamma1; 3323; 3324; 3325; 3326; 3327; 3328; 3329; 3330



3331; 3332; 3333; 3334; 3335; 3336; 3337; 3338; 3339; 3340; 3341; 3342; 3343; 3344

Figure 1.15. F₂ progeny of allelic test crosses post *C. sativus* inoculation. Ten seed from each head were analyzed in the F₂ progeny however only one of the ten was documented. The F₂ progeny of all allelism crosses demonstrate the unique *nec3* from four independent mutations as a result of different mutagenic procedures. The *nec3.d* mutants although allelic display a different necrosis phenotype. **3323** – F₁ gamma1/*nec3.d*; **3324** – F₁ gamma1/*nec3.e*; **3325** – F₁ gamma1/*nec3.e*; **3326** – F₁ gamma1/FN362. **3327-3330** & **3341-3344** F₂ gamma1 and *nec3.d* crosses. **3331-3334** F₂ gamma1 and FN362 cross. **3335-3340** F₂ gamma1 and *nec3.e* cross.

Infiltrations

Infiltrations on the mutants and wild types showed differential reactions after *C. sativus* exudates from culture filtrates were injected into the secondary leaves of the mutant and wild type plants (Figure 1.16). The reaction between the mutants and wild type are quite prominent with the wild type having the typical *Cochliobolus sativus* infection appearance with the dark margin typical of phenolics production whereas the $\gamma 1/nec3$ mutants show the typical runaway PCD phenotype with the lack of defined margins around the lesions and a bleached out appearance. The *Cochliobolus sativus* culture filtrates that elicit the *nec3* phenotype were treated with Pronase prior to infiltrations to determine if a secreted protein or a chemical metabolite is inducing the *nec3* runaway PCD. The Pronase treated culture filtrates still induced the *nec3* phenotype (Figure 1.16).

Trigalacturonic acid infiltrations of Bowman, $\gamma 1$, Steptoe and FN362 at three concentrations, 10mg/mL, 1.0mg/mL and 0.1mg/mL yielded no reaction from wild type nor mutants $\gamma 1$ and FN362 up to a week after infiltration (data not provided). FLG22 infiltrations were also conducted on $\gamma 1$, Bowman, Steptoe and FN362 at 1mg/mL. FLG22 infiltrations also did not elicit the PCD phenotype in either wild type or the mutants (Figure 1.17).

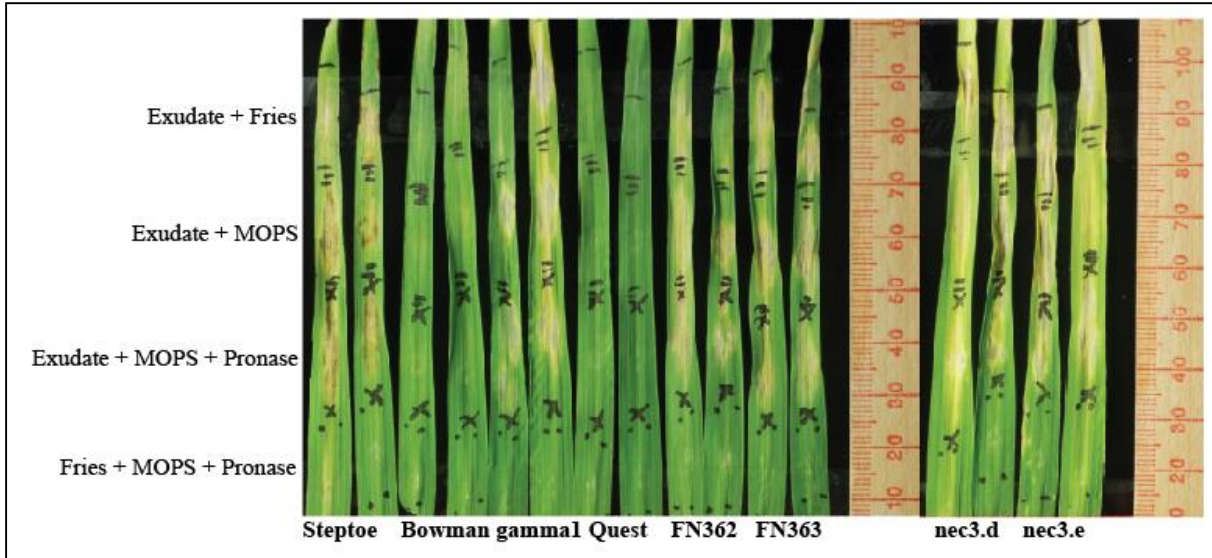


Figure 1.16. Infiltrations with *C. sativus* exudates and Pronase treatment. Infiltrations were performed at the two leaf stage with the third just emerging were rated and documented 4 and 7 days after infiltration.

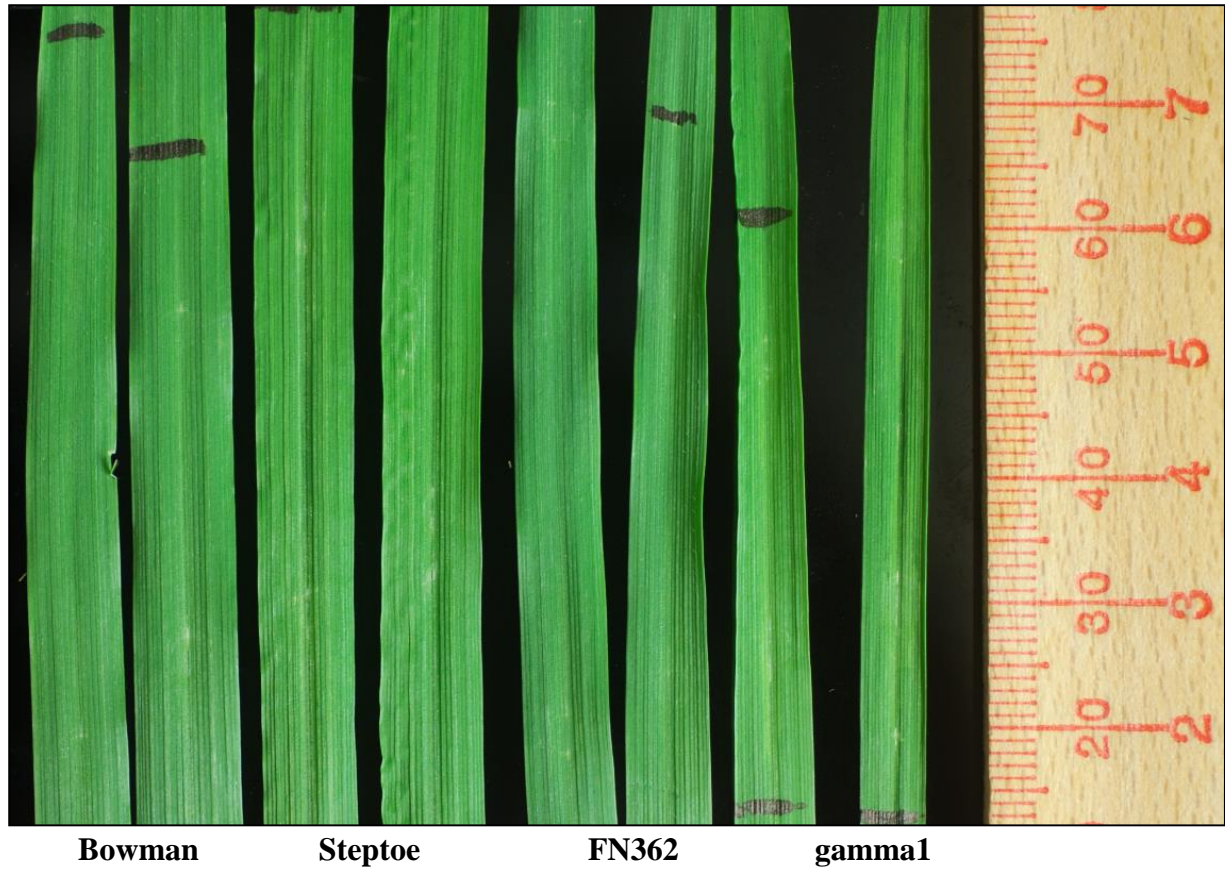


Figure 1.17. FLG22 infiltrations (1mg/mL) of Bowman, Steptoe, FN362 and gamma1. Secondary leaves were infiltrated and were rated and documented 7 days after infiltration. No *nec3* phenotype was observable 7 dai.

Discussion

In order to understand disease resistance/susceptibility mechanisms it is important to further our knowledge of the role that PCD pathways play in the outcome of plant immune responses. This knowledge could be used to protect crops from a wide range of pathogens if we had more knowledge of these important pathways. The NEC3 protein and other regulatory molecule are involved in signaling pathways leading to PCD as well as negative regulation of these pathways. We hypothesize that NEC3 is involved in the early tightly regulated PTI elicited PCD, but may not play a role in the regulation of the higher amplitude PCD known as the hypersensitive response as the result of ETI. We are basing this hypothesis on the observation that the runaway PCD is observed following infection by various ascomycete pathogens including *C. sativus*, *P. teres* f. *teres*, *P. teres* f. *maculata* and *B. graminis*, as well as the bacterial pathogen *X. translucens*, which infect the host cells directly and cause cellular damage that may elicit an early DAMP or PAMP triggered immunity response. However, the compatible and incompatible Basidiomycete pathogens *Puccinia graminis*, which elicit a putative ETI strong PCD response manifested as a post haustorial hypersensitive response, did not set this runaway PCD phenotype into motion. It appears that this runaway PCD response is not due to increased susceptibility to these pathogens because the expansion of the lesions is independent of the living pathogen within them, as culture exudates set the phenotype into motion independent of the presence of the pathogen. However, the phenotype may leave the host more susceptible to the necrotrophic pathogens due to uncontrolled PCD providing nutrients to the fungus.

Originally the *nec3* mutants were classified as LMMs (Fischbeck 1976), but this research has provided evidence that the *nec3* phenotype is elicited by a diverse taxonomy of pathogens, possibly through the detection of conserved effectors secreted by the pathogen or conserved

essential molecular patterns. Many LMMs share the same characteristics that are seen in the *nec3* mutants including expanding necrotic lesions, non-controlled PCD and stunted growth with fewer seeds borne (Balague 2003, Lorrain 2003). However, due to the lack of phenotype in the absence of pathogen challenge we propose that the *nec3* mutants are not LMMs, but are pathogen induced runaway PCD (PIRP) mutant. It is likely that the lesion mimic mutants and *nec3* share similar PCD pathways, however, we hypothesize that a broad range of pathogens with a specific lifestyle elicit an early PAMP or DAMP PCD pathway that is inhibited or controlled by the *nec3* gene or pathway. Thus, the *nec3* mutants result in a runaway PCD phenotype at the point of pathogen challenge or with infiltration using some culture filtrates.

The pathogens tested to date that induce the typical *nec3* phenotype use infection processes that cause damage to the host cell. For instance *C. sativus*, *P. teres* and *B. graminis* infect epidermal cells at the leaf surface by creating an appresoria and penetration peg that punches through the cell wall and plasma membrane in order to initiate the infection processes (Acharya 2011, O'Connell 2006, Schmidt 2014). This invasive infection process is far from incognito and sets off an early defense alarm known as PTI or DTI resulting in a signaling cascade. The PTI/DTI signaling responses activate transcription factors that induce a very succinct and tightly regulated programmed cell death response that is limited to one or a few cells at the point of pathogen introgression. The induction of PTI leads to the production of PR proteins, which are general defense response proteins whose purpose are two fold; to rapidly arrest the pathogen's infection process and induce systemic acquired resistance (SAR) (Borad 2008). Although *X. translucens* enters the host through the stomata initially, they also damage the host cells by the use of the type three secretion system to secrete effectors into mesophyll (Beattie 1999). These bacteria colonize the leaf surface by first increasing in number in regions

closest to the stomata and other open or weaker areas of the leaf. During colony increase, an exudate EPS (extracellular polysaccharides), provides protection as well as a transport medium for the enzymes that increases plant cell permeability. This cellular leakiness allows further bacterial division and eventual leaf collapse if there are inadequate defenses in the plants genetic arsenal.

When the *nec3* mutant plants were infected with avirulent and virulent races of *P. graminis*, races MCCF and QCCJ respectively, on the *Rpg1* containing barley line Bowman, the background genotype of gamma1, there were no observable differences between the wild type Bowman and gamma1/*nec3* mutant phenotypes; the wild type cv Bowman is incompatible with *P. graminis* race MCCF and compatible with race QCCJ. Histology data with race MCCF on *Rpg1* containing barley lines similar to Bowman show a post haustorial HR response suggesting an ETI triggered PCD (Zurn 2015). This is interesting because the defense response is the induction of PCD to control the biotroph, but it appears that the PCD pathway for this R-gene mediated resistance response is different than those observed in the *nec3* pathway.

Due to the rather rapid *nec3* phenotypic induction following infection, it was originally hypothesized that the *nec3* gene is constitutively expressed. To test this RNAseq was performed on plant tissue that had not been exposed to any pathogen. The results, following assembly against the barley EST Unigene_36 set, found 6,136 genes across the genome that were differentially expressed compared to the wild type; that is expression in the wild type and little to no expression in the gamma1/*nec3* mutant. Of those about 24 that were found on chromosome 6 13 genes found between the flanking markers GBM1053 and GBM1423. Multiple genes were eliminated in the region following amplification and visualization via gel electrophoresis of the genes. This assay did not yield the *Nec3* gene, but because the phenotype is only observed

following pathogen infection an RNAseq assay was performed following induction with the pathogen *C. sativus* and yielded an expanded set of candidate genes. This new set of candidate genes are currently being analyzed.

Based on data gathered during the experiments we have developed a new working hypothesis that will lead to the identification and cloning of the *Nec3* gene. Following infection from a pathogen that produce DAMPS or possibly PAMPs as a result of cellular disruption or that cause cellular disruption, the PTI pathways are induced resulting in the tightly regulated PCD to rapidly arrest the pathogen at the point on entry. However, because of the uncontrolled spread of the PCD we hypothesis that the *nec3* gene is a negative regulator of this specific pathway and the lack of this functional protein or transcription factor leads to the inability of the plant to regulate this important PCD pathway. It is possible that the *nec3* gene is a conserved (non-specific) gene downstream of the pathogen recognition or host-parasite recognition since it is observed following infection from a wide variety of pathogens. Many biological pathways need to be kept in check through both positive and negative regulation following environmental and developmental cues in order to maintain proper cellular and organismal health (Latchman 2010). If a response or pathway is left unhindered, although it is initially beneficial and necessary for the organism, it can lead to detrimental effects. In the case of a mutated *nec3* gene we see a non-regulated PCD response that affects the photosynthetic potential of the plant, normal growth and seed development. The *nec3* mutants rarely produce a full head when in the presence of a pathogenic challenge, rather on average 10-50% of the seeds are aborted presumably due to the runaway PCD (Figure 1.10). This indicates that this regulatory factor is not specific to the leaves but is also utilized for defense in other tissues including the

reproductive structures. The *nec3* roots were not tested in this study but it would be interesting to determine if *C. sativus*, which also causes the root rot disease would induce runaway PCD.

The hypothesis that the *nec3* gene is involved in a DAMP recognition and response pathway appears to agree with the infiltration data gathered as proteins and other molecules produced by pathogens, both intra- and extra-cellularly, can induce the production of DAMPs followed by an immune response. The most common DAMPs are cell wall components, eATP, eDNA, and other endogenous molecules disrupted by pathogen interaction. This particular series of events is seen across all multicellular life studied including algae, fungi, fish, insects mammals, and plants (Heil 2014). Since we are not privy to the proteins and other molecules produced and released during fungal growth and reproduction in Fries Media it is unclear exactly what may be eliciting the PCD pathway, however the typical *nec3* reaction was observed following infiltration with these exudates and the Pronase treatment did not eliminate the phenotype. Due to the wide range of endogenous elicitors it may be secondary metabolites or non-proteinaceous toxins produced by the pathogen that may lead to DAMP release and ultimate recognition and eventually the PCD pathway, thus rendering the Pronase treatment ineffective.

The *Nec3* gene could also potentially play a role as virulence target of necrotrophic effectors, which would benefit the pathogen by stopping the suppression of PCD in order to facilitate the colonization of the host. As demonstrated from the mutation of the *nec3* gene a runaway PCD response elicited by the wide range of pathogens that elicit putative early response PCD makes the *nec3* gene a valuable gene to study as it could provide a marker gene to differentiate between PTI and ETI elicited PCD pathways.

The main goal of this research is to clone the *Nec3* gene, however a finer map needs to be developed in order to fully take advantage of the candidate genes derived from the RNAseq

induction assay following 48 and 96 hours of infection. Further characterization of the mutant gene should include screening with a wider range of pathogens across Kingdom and Phyla with varying lifestyles. Since *Nec3* appears to be involved in a conserved PCD pathway that affects both leaves and seed production it would also be interesting to observe root growth and PCD under both infected and non-infected conditions.

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CHAPTER TWO. FINE MAPPING AND IDENTIFICATION OF CANDIDATE SPOT BLOTCH DOMINANT SUSCEPTIBILITY RCS5 GENES IN BARLEY

Abstract

The *Rcs5* gene initially identified from unimproved line CI-7117-77 was shown to confer dominant resistance to *Cochliobolus sativus*. Previous research put considerable effort into positional cloning of *Rcs5* delimiting the gene to a small genetic region on barley chromosome 7H co-segregating with markers at position 23.79 cM based on the barley cultivar Morex genome sequence (IPK). The *Rcs5* region has been genetically delimited by the flanking markers BF257002, which is ~ 0.23 cM distal and ABC167 ~ 0.13 cM proximal of *Rcs5* and this region was physically spanned by two cv. Morex BAC clones 805o02 and 370p04. The sequencing and annotation of these BACs revealed five candidate genes. Four of the candidate *Rcs5* genes (*HvWAK2*, *HvWAK3*, *HvWAK4*, *HvWAK5*) are predicted to encode two different families based on sequence similarity of Wall Associated Kinases (WAKs) and the fifth candidate gene encoded a truncated putative non-functional imperfect Leucine Rich Repeat gene fragment, thus was eliminated. To determine which candidate gene is *Rcs5*, virus induced gene silencing (VIGS) was used to specifically post transcriptionally silence *HvWAK2* and non-specifically silence the *HvWAK345* family of genes. The antisense cDNA fragments were inserted into the barley stripe mosaic virus (BSMV)-VIGS gamma positive stranded RNA genome using the infectious clone pSL38.1. One of the BSMV-VIGS constructs was specific to the *HvWAK2* gene and the second was predicted to silence the *HvWAK345* gene family complex due to their conserved gene homology. Post BSMV-VIGS infection on the barley lines Steptoe (*Rcs5*-), and Morex (*Rcs5*+) determined that *HvWAK2* is not the *Rcs5* gene as no shift from avirulence to virulence or virulence to avirulence was observed on Morex or Steptoe, respectively.

Surprisingly, it was determined that the remaining WAK genes in the region may not be dominant resistance genes but in Steptoe may confer additive susceptibility to *C. sativus*. This was determined by a shift from virulence to avirulence in the Steptoe genotype. Thus, *rsc5* may represent a recessive resistance gene or more appropriately a dominant susceptibility target in the susceptible genotype Steptoe.

Introduction

Cochliobolus sativus (Ito. And Kurib.) Drechsl. ex Dastur (anamorph: *Bipolaris sorokiniana* (Sacc) Shoem.) is among the most prevalent pathogens causing foliar spot disease and infecting barley roots worldwide. Yield losses in barley can range from 30-70% depending on environmental factors, host susceptibility/resistance and pathogen pathotypes (0,1 and 2) (faostat.org, Veljavec-Gratian 1997). In order to manage the pathogen at both the seedling and adult plant stage chemical seed treatment, fungicide use, residue burning, and reduced tillage, can be employed, however, the most cost effective control is the use of resistant cultivars (Mehta 1998). QTL analysis on bi-parental mapping populations has identified 6 resistance genes (*Rcs1-6*) in the primary barley germplasm pool that confer resistance to the *C. sativus* (Bilgic 2006). The most effective resistance source providing both seedling and adult plant resistance is *Rcs5* (Bilgic 2005). The original source of the *Rcs5* gene was the barley line CI0017-07, which was the source of *Rcs5* resistance in cultivar NDB112, which is also the parent of many *Rcs5* resistant cultivars including Morex, Manker, Cree, Dickson and Robust (Wilcoxson 1990). The pathogen *C. sativus* also infects the cereal crop wheat dramatically reducing yield in areas that tend to have a warmer growing season (Acharya 2011). Thus, characterization of resistance or susceptibility genes to *C. sativus* in the relatively simple diploid barley genome may also provide

insight into resistance in the more complex genome of hexaploid bread wheat, and orthologs of the spot blotch resistance genes may exist in the evolutionarily related cereal grass genomes.

Cochliobolus sativus is primarily a necrotrophic pathogen that promotes cell death, presumably through the elicitation of host induced programmed cell death (PCD) pathways or through the production of phytotoxic compounds in order to complete its infection cycle. *C. sativus* is considered a hemibiotroph which means it has a relatively short biotrophic phase in the beginning of the infection process (Kumar 2002), which then switches to a necrotrophic phase. Recently it has been hypothesized that most necrotrophs may need to have a biotrophic phase in order to effectively establish infection (Mendgen 2002), thus the delineation between a necrotroph and a hemi-biotroph is somewhat blurry.

Necrotrophs or hemibiotrophs are believed to utilize a three pronged approach or set of immune ‘arsenals’; 1) those that evade early detection, 2) armor to protect themselves against early defense responses once recognized, and 3) those that take advantage of the hosts innate immune responses leading to programmed cell death (Wen 2012). The pathogen initially colonizes the host apoplast undetected in a biotrophic phase until it switches its infection strategy and aggressively makes its presence known through the production of necrotrophic effectors (NE), which are recognized by host immunity receptors. This recognition sets the host’s defense responses into motion, although these PCD responses are effective defenses against a biotroph, they are setting up an environment conducive to the necrotroph’s needs where it can finish its life cycle culminating in sporulation from the necrotic lesions produced. Kelley et al (2010) identified the effector, SNE1, produced by *Phytophthora infestans* that controls the timing of PCD induced by multiple divergent pathways in tomato that both allows the biotrophic phase to develop as well as maintain control over the timing of PCD as the antagonistic protein PiNPP1.1

produced by the fungus induces PCD (Kelley 2010, Lee 2010). SNE1 was found to only be produced during the biotrophic phase whereas PiNPP1.1 is only expressed during the necrotrophic phase. Thus, necrotrophic or hemibiotrophic fungi not only produce NE effectors to induce PCD but they also appear to have mechanisms to control PCD for a more precise regulation, particular to the phase of the lifestyle, dictating which route is of priority; that is PCD or no-PCD. Hemibiotrophs, although spending a short period in the biotrophic phase, spend the majority of their lifecycle colonizing dead necrotic tissue, thus, the elicitation of PCD is essential to complete their life cycle. As mentioned earlier the mechanisms of the pathogen used to take advantage of the host's immune system is to target host immunity receptors giving the pathogen several targets whether plasma membrane associated receptors (Chisolm 2006) or cytoplasmic receptors. Although to date the receptors targeted by necrotrophic effectors have been in the class of NB-LRR cytoplasmic receptors including *lov1* and *tsn1* (Sweat 2008), there has been recent data suggesting that some NE are probably targeting membrane bound receptors in the related *Pyrenophora teres* pathosystem (Richards 2015, Brueggeman personal communication). One of the membrane bound immune receptors that fits these criteria are the Wall Associated Kinases (WAKs) which have been shown to have an N-terminal elongation growth factor domain, a transmembrane domain and a C-terminal serine/threonine protein kinase domain (Kohorn 2011).

WAKs are relatively large proteins that span the plasma membrane and can serve as a sensor of extra cellular stimuli whose signal can be transduced to the cytoplasmic signaling cascades because of its exterior domain and cytoplasmically localized kinase domain (Figure 2.1). Interestingly WAKS are found in all plants and are required for many cellular functions including growth, elongation, seed development, and programmed cell death (Lally 2001). These

genes tend to be clustered in barley and other plants (Wagner 2001). WAKs are composed of an elongation growth factor, Serine/Threonine Kinase domain, and a transmembrane domain (Figure 2.1).

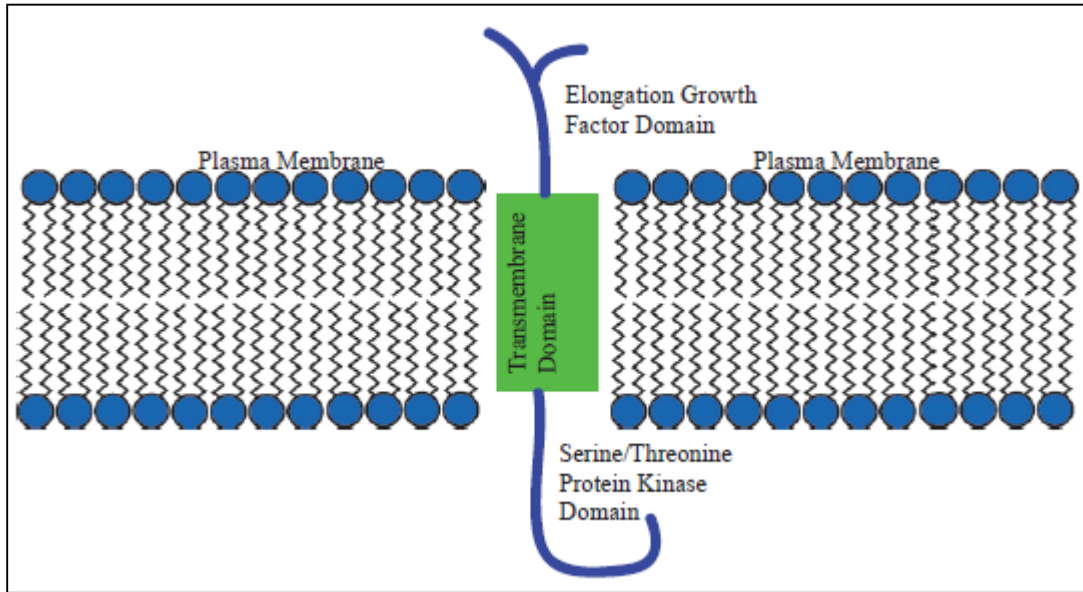


Figure 2.1. Simplistic model of a Wall Associated Kinase. These domains are found in the candidate WAK genes *HvWAKs* 3-5. The extracellular domain consists of an elongation growth factor that is connected to a transmembrane domain which sends the signal to an intracellular serine/threonine protein kinase. This kinase sets off a cascade of molecular steps leading to cell wall growth, disease susceptibility/resistance and other unknown pathways.

This predicted protein structure was observed for the candidate *Rcs5* WAKs. The predicted protein structure suggests that the transmembrane domain connects the extracellular elongation growth factor (EGF) domain and the cytoplasmic Serine/Threonine Kinase signaling domain such that extracellular or apoplast environmental signals perceived by the putative receptor domain imbedded in the cell wall transfers the message across the plasma membrane possibly inducing the MAPK pathway or other yet to be determined pathways which may elicit ethylene and/or jasmonic acid defense signaling hormones as well as other pathogen resistance (PR) proteins to protect the cell from pathogen infection (Anderson 2001, Kohorn 2012). It has

only been over the past decade that WAKs have been shown to be involved in disease resistance, however, WAK genes have yet to be identified as a necrotrophic effector target inducing PCD and dominant susceptibility to a necrotroph or hemi-biotroph. The focus of this research is the validation of the previously delimited *Rcs5* candidate genes, which includes four WAK genes that fall into two families identified by a positional cloning strategy (Drader 2010).

This research attempted to utilize virus induced gene silencing (VIGS) to eliminate or validate the candidate WAK genes as *Rcs5* by post transcriptional gene silencing (PTGS). In order to utilize VIGS for PTGS a genetically engineered virus that is infectious to barley and other grasses, but optimally causes minor phenotypic changes, has an antisense fragment of a candidate gene inserted into the cDNA infectious clone. The target gene is expected to have a specific function with a well-characterized phenotypic outcome. The *E. coli* propagated VIGS construct is engineered to contain a positive stranded cDNA of the virus positive stranded genomic RNA (gRNA) with a 200-300 bp antisense fragment of the target gene inserted outside of an essential open reading frame coding for viral proteins essential for viral replication. The positive stranded virus genomic RNAs are in vitro transcribed from the linearized cDNA, utilizing DNA dependent RNA polymerase along with the other virus genomic RNAs independently transcribed from other infectious clones in the case of partite virus genomes. The virus gRNAs are mixed and mechanically infected into the host. The virus replication within the host produces double stranded RNAs through replication intermediates as well as through hybridization with endogenous mRNAs inducing the virus defense mechanism DICER protein complex in the plant, which produces 22-25 mer nucleotide from the double stranded RNAs that are loaded onto the RNA induced silencing complex (RISC). RISC then uses the single stranded antisense RNA oligos as template to seek out the endogenous mRNA which it degrades

effectively silencing the host genes (Waterhouse 2003). For *Rcs5* the mechanically transmitted, tripartite, positive-sense Barley Stripe Mosaic Virus (BSMV) was used. Infection of barley with BSMV leads to yellowing of leaves in a stripe pattern as the virus moves throughout the infected leaves (Holzberg 2002).

In general, since 1951 when Flor proposed the gene-for-gene model to explain host parasite genetic interactions, disease resistance reactions have been observed that fit this model yet many that do not fit the model have been characterized (Flor 1971). The gene-for-gene model functionally suggests that one dominant resistance protein in the host interacts directly with a pathogen Avr protein eliciting the resistance response. However, the characterization of many host-parasite interactions at the functional level has determined that this model does not fit the majority of interactions. But, at the genetic level most interactions can still be distilled down to single genetic interactions. These single gene-for-gene interactions have also been found in necrotrophic pathosystems. However, the necrotrophs have evolved a means of deceiving the host into eliciting its own PCD immunity pathways that usually result in a sequestration of a biotrophic pathogen in dead necrotic tissue effectively killing the invader. However, when the necrotroph lets its presence be known the host immune system elicited PCD only provides the necrotroph with an environment conducive to complete its infection cycle resulting in more disease. Thus, these necrotrophic effector triggered susceptibility targets, typical R-genes against a biotroph, functionally behave as susceptibility targets and genetically look like recessive resistance genes. This is based on interactions observed that fit a dominant susceptibility ratio following crossing, that is 3 susceptible to 1 resistant, whereas the gene-for-gene model dictates a dominant resistant gene with 3 resistant to 1 susceptible. This model has been termed the inverse gene-for-gene or Necrotrophic Effector Triggered Susceptibility model (Liu 2014). In

this case the recessive gene, allowing resistance, is likely a non-functional protein that leads to no host-pathogen interaction thus no PCD stifling the hemibiotrophic lifestyle of *C. sativus* and other necrotrophic pathogens.

Materials and Methods

VIGS knockout vector development

In order to perform virus induced gene silencing (VIGS) of the candidate *Rcs5* genes, a Barley Strip Mosaic Virus (BSMV) gamma RNA infectious clone pSL38.1 was engineered to introduce double stranded RNA into the host to induce post transcriptional gene silencing utilizing the endogenous plant DICER/RISC RNA silencing mechanism. The pSL38.1 vector has the full length BSMV gamma gRNA cloned into an *E. coli* vector backbone with a high copy origin of replication and ampicillin resistance. The BSMV gamma cDNA cloned into pSL38.1 contains tandem NotI and PacI restriction sites at the 3 prime terminus of the gamma orf B gene. These restriction sites allow for the insertion of a specific cDNA fragment of genes targeted for silencing (Figure 2.2).

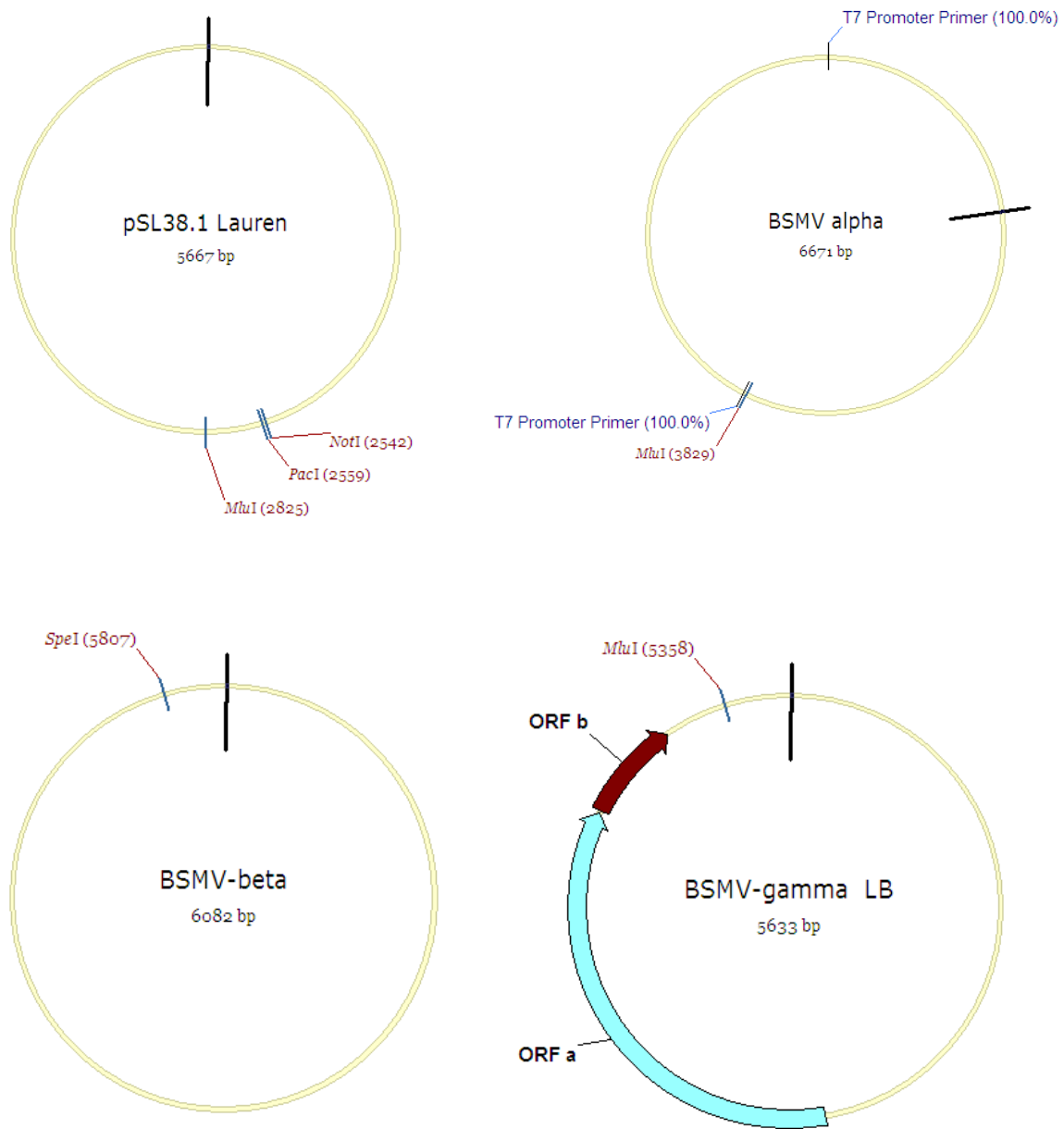


Figure 2.2. BSMV-VIGS tri-partite infectious clones (alpha, beta, and gamma) used to in vitro transcribe the tripartite BSMV genomic RNAs for post transcriptional gene silencing of *HvWAK2* and *HvWAK345*. The MluI and SpeI site is a linearization site that is required prior to in-vitro transcription. The pSL38.1 vector, containing a modified BSMV gamma genome strand utilizes the NotI and PacI site to directionally insert the gene specific antisense cDNA fragments for targeted gene silencing.

The BSMV-VIGS antisense (as) *HvWAK2* and *asHvWAK345* (BSMV-*asHvWAK2* and BSMV-*asHvWAK345*) constructs were engineered by first inoculating 2mL of LB media supplemented with 100ug/ml of ampicillin, with a monoclonal *E.coli* colony (*E. coli* strain DH10 alpha) containing the empty pSL38.1 vector, and a monoclonal colony containing the BSMV gamma1 vector synthesized by Genscript with a sense oriented cDNA fragment from the *HvWAK3* gene cloned into the PacI and NotI restriction sites designated BSMV-ctg37. The BSMV-ctg37 construct contains the infectious cDNA clone of BSMV gamma1, however the vector used has the PacI and NotI sites, 5' to 3' respectively, at the C-terminus of the orf B gene, which is the opposite orientation of the sites compared to the pSL38.1 vector. The BSMV-ctg37 construct was kindly provided by Dr. Andris Kleinhofs at Washington State University. The cultures of pSL38.1 and BSMV-ctg37 were grown at 37° C for 16 hours while shaking at 150rpm. DNA was extracted using a modified alkaline lysis Mini-Prep method (Brueggeman 2002). Using a 2mL tube the broth containing the bacteria was centrifuged at maximum speed for 15 minutes. The supernatant is then removed and cellular lysis is performed on the pelleted bacteria. Using 400ul of solution 1 (1mL 2M glucose, 1.6mL 0.5M EDTA, 1mL Tris pH 8.0, 36.4mL H2O) which had been chilled the bacteria was resuspended with the force of the pipettor. 400ul of solution 2 (45.5mL H2O, 2mL 5N NaOH, 2.5mL 20% SDS) was then added to the resuspended bacteria in order to lyse the cells. The tube was then inverted five times over the course of one minute. 400ul of solution 3 (14.7g KAoc, 5.7mL glacial Acetic Acid, bring volume to 44mL with H2O) was added to neutralize the lysis solution and stabilize the extracted DNA. The tube was then centrifuged again at max speed for 5 minutes pelleting bacterial debris. The supernatant was then removed and the DNA was precipitated out of solution by adding 1/10 volumes of 3M NaOAc, 1ul of glycogen, and 2.5 volumes of 95% ethanol, followed by mixing

by inversion. The solution was placed in -20°C for 15 minutes followed by centrifugation at max speed for 20 minutes, supernatant was poured off and DNA pellet washed with 75% ethanol. After pouring off and removing residual ethanol the DNA was allowed to dry at room temperature and resuspend in 30ul of sterile H_2O . Following DNA extraction a 10ul double digest (0.5ul NotI, 0.5ul PacI, 2ul buffer, 2ul H_2O , 5ul DNA) using NotI and PacI was performed on both DNAs in order to remove the 17 bp spacer from the pSL38.1 vector for directional cloning and to liberate the 202 bp *HvWAK2* fragment from BSMV-ctg19 for antisense cloning into pSL38.1. The restriction digestions were performed following the manufacturers (New England Biolabs) standard procedure (Figure 2). The 10ul reactions were loaded on a 1.5% agarose electrophoresis gel and DNA separated at 160V for approximately 40 minutes in order to fully separate and visualize the 202 bp insert and ~5.6 kb plasmid vector pSL38.1. The 202bp insert for ctg37 and the linearized pSL38.1 vector containing compatible NotI and PacI sticky ends were extracted from the gel and purified using the E.Z.N.A.[®] gel extraction kit (Omega Biotek, Cat.No.D2500-02). Following quantification on the Qubit[®] 2.0 fluorometer (Life Technologies) a 10ul (3xMM- 1ul T4ligase, 3ul buffer, 26ul H_2O) 3:1 (insert:vector) molar end ratio (~1.3ng insert: 12ng vector) ligation reactions were performed at 4°C for approximately 18 hours using T4 DNA Ligase (Life Technologies, Cat.No.45224-017). 5ul of the ligation reaction was transformed into chemically competent DH10B *E.coli* cells (Life Technologies) and plated on LB agar + ampicillin [10mg/mL] and grown overnight at 37°C . The colonies were screened for the BSMV-ctg37 insert by performing plasmid DNA extractions (as previously described) and plasmid DNAs were double digested using both NotI and PacI enzymes to visualize the ~5.6 kb pSL38.1 vector and expected 202 bp insert on a 1% agarose gel. The plasmids containing the expected insert following digestion were sequenced. Sanger

sequencing was performed by Genscript using the pSL38.1 specific primer Gamma-F7.2 (CCATCCTGGATGCAAGCATAGTTAGC). The construct with the expected antisense fragment was designated BSMV-*asWAK345* (5'-

TTGCAGGAGAGGCAACTCACGGAGAAGAGCGACGTTTACAGCTTTGGCGTTGTGCT
GCTGGAGTTGATCACGGGCAAGACAGCCATCTACCACGACGGCCCAAGGAAGGCA
AGAGCCTTGCGTCGTCCTTCTGCTCGCGATGAAGGATGGAAGCCTTGATGCCATCC
TGGATGCAAGCATAGTTAGCGCCGGGATGGAGA-3').

Similar methodology was used to produce the BSMV-*asWAK2* construct. Primers were developed from the ctg19 sequence kindly provided by Andris Kleinhofs at Washington State University. The primer set was developed to amplify a 134 bp fragment from the *HvWAK2* gene designated HvWAK2-F3 and -R3. The fragment at position +2453 to +2587 relative to the predicted ATG start methionine codon was used to produce the amplicon from Steptoe genomic DNA (**F3**: ATATTAATTAAGGCATAGAGACGCCCCGATGATCAG; **R3**: ATA
GCGGCCGCAAGCTCAGATGCCGCATAGTCAGAC). The primers amplified the 134bp insert sequence [5'-

TTACATTTGAACCTTCCACCAGAATGCCCAACTAATTTACCGTACTGGTAGAAGTAT
AGAGTATCCAAGTAAATGTTGTCATGGGATTCGAGTGTGTTTTGTGTTTCGTCGTGG
AGTTTTGCCATGACAGTACA-3']. In order to insert the fragment in the antisense orientation the forward primer contains a 5' terminus antisense PacI adaptor sequence (TTAATTAA) and a 5' terminus of the reverse primer contains a NotI adaptor sequence (GCGGCCGC). The pSL38.1 plasmid vector contains the cloning site in the NotI then PacI orientation thus ligation on the double digested amplicon results in the cloning of an antisense cDNA transcript fragment. Following amplification a double digest using NotI/PacI was performed with the purpose of

introducing the sticky ends that will ligate with the same ends on the pSL38.1 linearized vector. The ligation and downstream procedures used were the same as previously described for the BSMV-*asHvWAK345* construct.

BSMV-VIGS Construct Processing

The BSMV virus is a tripartite RNA virus requiring three positive stranded gRNAs containing the complement of seven virus encoded proteins for a viable virus infection process. However, the β RNA has a mutated coat protein to inhibit the virus from producing a mature virion. Each of the gRNA chromosomes was previously cloned into circular *E. coli* DNA plasmids designated alpha, beta and gamma (Figure 2.2). In order to amplify the plasmids they are transformed into competent *E. coli* cells. Transformations are performed by thawing competent cells, in this case *E. coli* DH5 α cells were used. The cells are then gently homogenized on ice with a pipettor. In a fresh tube that has been cooled on ice, 50ul of the *E. coli* cells were aliquoted and 1-10ng of DNA was added while being mixed gently with a pipettor. Cells were then placed on ice for 30 minutes. The cells were then heat shocked by placing the tubes in a water bath at 42°C for 20 seconds. After heat shock treatment the cells were placed in ice for 2 minutes and 950ul of pre-warmed media (LB) was added to the transformed cells. The cells were then incubated at 37°C for one hour shaking at 225rpm to induce the first round of replication. Aliquots of 20ul and 200ul were spread onto LB plates amended with Ampicillin using a sterilized glass spreader. The plates were then allowed to dry in a sterile hood and sealed with parafilm. The plates were placed into a 37°C incubator inverted so as to decrease the chance of contamination from the condensation for 16-18 hours. The resulting colonies were inoculated into 2ml of sterile LB media amended with 10ug/ml of ampicillin. DNA from all constructs, Alpha, Beta, MCS, and BSMV-*asHvWAK1* and BSMV-*asWAK234*

were extracted using the Mini-Prep method previously described. The extracted DNA was treated with RNase at 1mg/mL at room temperature for 2 hours. Following RNase treatment, Proteinase K treatment was performed to remove the RNase as the residual RNase will rapidly degrade the in-vitro transcribed virus RNA. In the tube with the resuspended plasmid DNA, 2ul of 10mg/mL Invitrogen Proteinase K (cat. no. 25530-049) was added with 5ul of 10% SDS. The solution was vortexed for ~1 second and incubate at 50° C for 1 hour. Following incubation phenol:chloroform (24:1) was added 1:1 (vol:vol) to each tube and vortexed for 10 seconds followed by centrifugation at maximum speed for 5 minutes. The supernatant was transferred to a clean 1.5 ml tube and chloroform:isoamyl (24:1) was added at 1:1 (vol:vol) and vortexed for 10 seconds. The solution was then centrifuge at max speed for 5 minutes and supernatant placed in a new tube followed by a second chloroform:isoamyl extraction. The DNA was precipitated by adding 1/10 volumes of 3M NaOAc, 1ul of glycogen, and 2.5 volumes of 95% ethanol, followed by mixing by inversion. The solution was placed in -20° C for 15 minutes followed by centrifugation at max speed for 20 minutes, supernatant was poured off and DNA pellets washed with 75% ethanol. After pouring off and removing residual ethanol the DNA was allowed to dry at room temperature and resuspend in 20ul of sterile H₂O. Following extraction the construct were linearized prior to in-vitro transcription. The Alpha construct was linearized with MluI, Beta construct SpeI, and all gamma constructs cloned into pSL38.1 were linearized with MluI following the manufactures standard protocol (NEB). A second Proteinase K treatment was performed to remove all remaining enzymes. All of the constructs were utilized as template for in vitro-transcription using the T7 mMessage mMachine transcription kit from Life Technologies (AM1344) enzyme and buffer kit. One reaction set up constitutes: DNA-0.625ul, H₂O-0.125ul,

Buffer-0.250ul, Enzyme-0.250ul and 2xNTPs-1.25ul. This reaction was run as per the manufacturer's protocol.

Plant Infection

After the tripartite gRNAs were transcribed in-vitro the barley seedlings were mechanically inoculated. For this experiment the cvs Steptoe (S), Harrington (S) and Morex (R) were used. A rack of containers (98) of each variety was planted and grown in a growth chamber with parameters: 14 hours light at 22.5C and 10 hours dark at 19.1C for approximately ten days, until the primary leaf was fully expanded and the secondary leaf was approximately 4-7cm long. The target number of infected plants was at least 20 plants of each genotype for each silencing experiment (BSMV-*asHvWAK2* and BSMV-*asHvWAK345*, and BSMV-MCS) and 10 each for non-inoculated controls. Following virus infection the plants were grown until symptoms appeared and then the plants were separated and labeled in a double blind manner according to the silencing construct being tested. The silencing experiments were set up as follows: 1) Alpha, Beta, MCS; 2) Alpha, Beta, BSMV-*asWAK2*; 3) Alpha, Beta BSMV-*asWAK345*; 4) Alpha, Beta, BSMV-*asWAK2* + BSMV-*asWAK345*; and 5) Water + FES. FES was prepared by making GP buffer – (10x) 18.77g Glycine, 26.13 g K₂HPO₄ in 500mL ddH₂O, Autoclave 20 minutes. In 50mL of 10X GP buffer 2.5g Sodium Pyrophosphate, 2.5g Bentonite (Fluka cat. no. 11959), and 2.5g Celite 545 AW Course (Fluka cat. no. 22141) was added to make the material used for viral infection. 250mL of ddH₂O was then added to the 10X GP buffer/compound mixture and autoclaved for 20 minutes. FES is used as an abrasive wound producer so the RNA virus is able to enter the strong cell walls of the plant. To prepare the inoculum 1.25ul of each in-vitro transcription reaction was used to inoculate each seedling at the second leaf stage. The volume then is 75ul for each construct per treatment. FES is used in a 6:1

ratio to the RNA. For the treatments with one knockout construct (*WAK2*, *WAK345*, *MCS*) there was $1.25\text{ul}/\text{construct} \times 3 = 3.75\text{ul}$ of construct times the ratio $6 = 22.5\text{ul}$ of FES/sample. That is 3.75ul of RNA + 22.5ul of FES = 26.25ul per sample. Since 20 plants per variety (3) are being tested there needs to be enough for 60 plants. $22.5 \times 60 = 1350\text{ul}$ of FES with 225ul of RNA. In the case of the treatment with two knockout constructs (*HvWAK2* and *HvWAK345*) 300ul of RNA is used, or 5ul per sample. To maintain the same inoculation volume between treatments with both one and two knockout constructs the volume of 5ul of RNA was subtracted from the total amount per sample for one knockout construct, $26.25 - 5 = 21.25\text{ul}$ of FES/plant. So $21.25\text{ul} \times 60\text{plants} = 1275\text{ul}$ of FES total.

Once the leaves have reached the stage where the second leaf is extending approximately 6 cm, which is approximately 10 days after emergence, the secondary leaf of each seedling was rub inoculated using $25.0\text{ul}/\text{plant}$ of FES/RNA solution for all treatments. The solution was pipetted onto a gloved forefinger and pulling up from the base of the leaf to the top gently as not to tear the leaf. It is important to hear the glove squeaking while performing the procedure. This ensures the exterior walls are broken allowing the virus to enter. Following inoculation the seedlings were placed in a dark mist chamber for 16 hours. The seedlings were removed the following day and placed back into the growth chamber.

VIGS seedling inoculation with *Cochliobolus sativus* ND85F

Following inoculation of seedlings with the BSMV-VIGS constructs the symptoms take approximately one week to develop as the third leaf begins to expand chlorotic stripes and mottling become visible. Full virus symptoms are expressed on the fully expanded fourth leaf and this new growth, with high virus titer, is ideal for assessing disease as the highest level of gene silencing occurs at this stage (Bruun-Rasmussen 2007). The fourth leaves with virus

symptoms are then inoculated with *C. sativus* ND85F at 5,000 spores/mL and evaluated for disease resistance 7 days after infection using the Fetch scale (Fetch 1999). The plants were rated in a double blind manner. The treatment was coded with a number and the variety was coded with a number.

Results

VIGS knockout vectors

The BSMV-VIGS constructs described in the materials and methods were produced to post-transcriptionally silence the four *Rcs5* WAK candidate genes identified on barley chromosome 7H (Drader 2010). The WAKs are currently designated *HvWAK2*, *HvWAK3*, *HvWAK4* and *HvWAK5*. The genes were previously given the nomenclature ctg19, ctg37, ctg16 and ctg35, however, we changed the nomenclature to ctg19=*HvWAK2*, ctg37=*HvWAK3*, ctg16=*HvWAK4* and ctg35=*HvWAK5*. We chose to rename the putative *rsc5* genes because they blastx as WAKs across multiple databases (IPK, GrainGenes, NCBI) and need proper names that are understandable rather than a contig number. The genes *HvWAK2* and *HvWAK3* reside within the previously described *Rcs5* interval and were shown to be constitutively expressed (Drader 2010). The continuous expression level of both genes under infection and non-infection conditions was the basis for choosing these two WAK genes in the delimited interval as the candidate *Rcs5* genes. However, these analyses determined that two other candidate genes within the region needed to be considered based on the results gathered here: *HvWAK4*, *HvWAK5*. Interestingly the WAKs in the region fell into two groups due to an extremely high level of sequence similarity between *HvWAKs3-5* and a lower similarity with *HvWAK2* (See supplement 1). The two primary candidate genes *HvWAK2* and *HvWAK3* are both

approximately the same size with 2.5kb and 2.7kb in length respectively with 4 exons and 3 introns (Figure 2.3). The exons code for: S/TP kinase domain, elongation growth factor and a transmembrane domain. These domains are typical of all WAKs identified to date (Kanneganti 2008) and are present in the other candidate WAKs in the region.

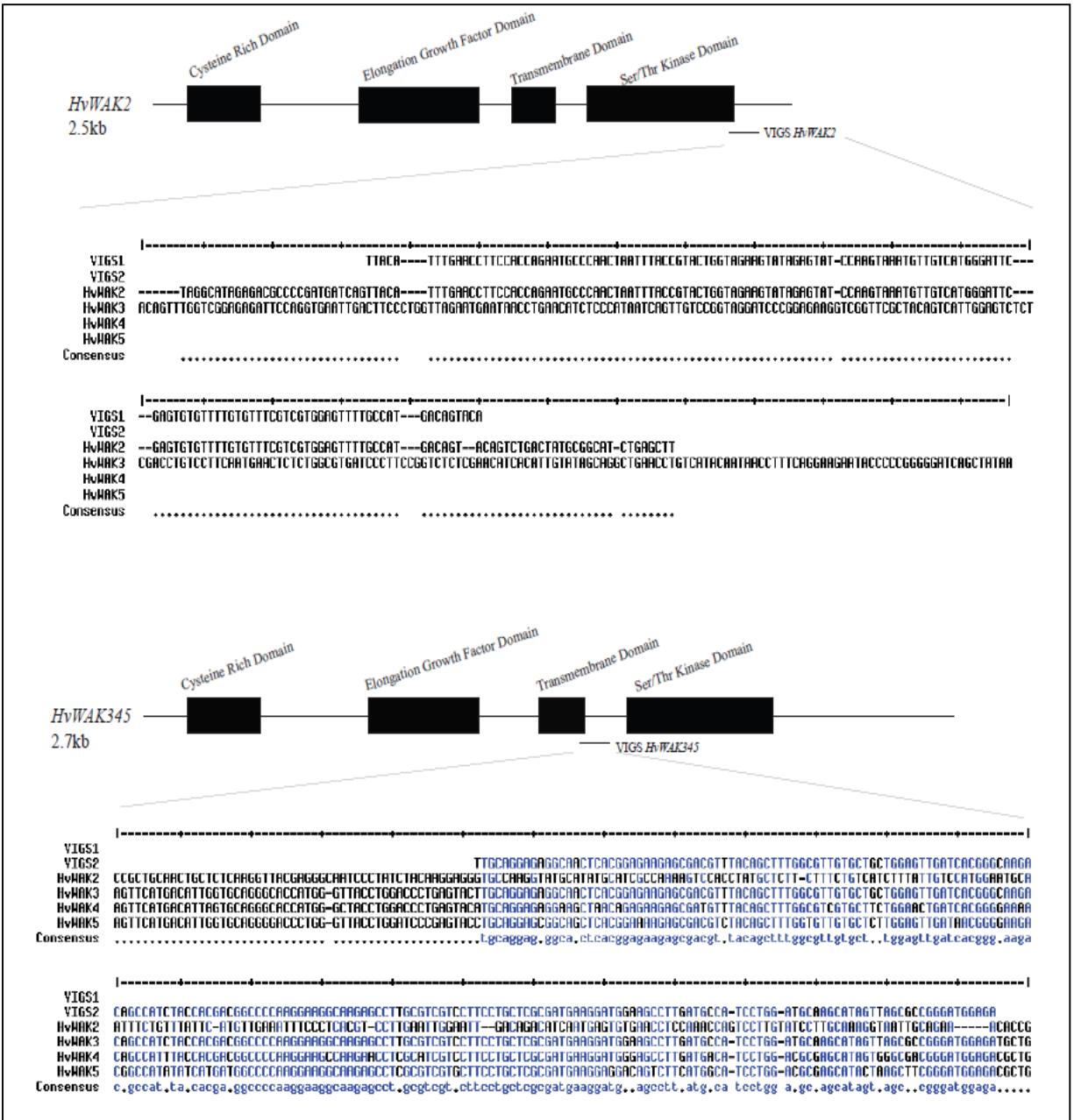


Figure 2.3. Gene structure of *HvWAK2* and *HvWAK345* with the knockout construct and alignments. The four domains- Cysteine rich domain, EGF, TM domain and S/TPK domain are the hallmarks of WAKs. The *HvWAK2* VIGS silencing vector was specific to the 3' UTR of the gene. The *HvWAK345* VIGS silencing vector was specific to the third exon and third intron of *HvWAKs3-5*, each designated with a small line just below the annotation of the WAK genes. The sequence information in blue is the consensus sequence.

Virus Induced Gene Silencing (VIGS)

VIGS provides a method of testing the effect of reduced specific gene expression on phenotypes in barley by employing post-transcriptional gene silencing. Under normal *C. sativus* infection conditions, cvs. Steptoe and Harrington exhibit numerous elongated necrotic lesions indicating susceptibility, while cv. Morex exhibits fewer and smaller necrotic spots indicating resistance (Figure 2.6). Spot-blotch lesions in both Steptoe and Morex were increased slightly in size and frequency when infected with virus carrying a control MCS gene fragment, the multiple cloning site of pBluescript, which has no homology with any known barley genes. Therefore, the consistently observed decrease in lesion size in cultivars Steptoe and Harrington compared to the BSMV- MCS control indicated an increase in resistance suggesting that the corresponding gene *Rcs5* gene may represent dominant susceptibility or recessive source of resistance. The increased lesion size in the BSMV-MCS treated cultivars may be due to an increased susceptibility of barley to the pathogen due to virus infection.

Morex was consistently resistance in the VIGS experiment (Table 2.1 and Figure 2.4). This initially suggested that the genes tested were not the *rsc5* gene. The silencing of the *HvWAK2* had no apparent phenotypic shift on any of the cultivars, effectively eliminating it as a possible candidate gene. The combined silencing of *HvWAK2* and *HvWAK345* displayed wild type scores (Table 2.1), that is Morex was resistant and Steptoe and Harrington remained susceptible. The most notable observation was the silencing of the *HvWAK345* caused an increase in resistance in both Harrington and Steptoe while having no effect on the resistant cv Morex. Previous sequencing and amplification attempts of Harrington indicated the lack of *HvWAK3* (Drader 2010). Further sequence analysis and gene comparisons indicated that the knockout vector was not specific to just *HvWAK3*, but putatively silenced *HvWAKs3-5* present in

the region (Figures 2.3-2.6, 2.8). A genetic and physical map was also constructed by Dr. Drader that shows the fine mapping of the *rcs5* region (Figure 2.7).

Table 2.1. Average of disease rating scores 14 days after VIGS inoculation and 7 days after infection with *C. sativus*.

	Stepoe (S) scores*	Harrington (S) scores*	Morex (R) scores*
MCS	5.3	6.5	3.25
<i>HvWAK2</i>	5	6	3.1
<i>HvWAK345</i>	4.15**	4.7**	3.1
<i>HvWAK2</i> + <i>HvWAK345</i>	4.9	6.5	3.2
FES + H₂O	5.75	5.5	3.25
No BSMV inoculation No FES inoculation	5.3	5.3	3.4

*The rating scale used was Fetch 1999 and has a range of scores from 1-9 where 1-3 low host-parasite compatibility, 4-5 intermediate compatibility and 6-9 high compatibility.

**The bolded scores indicate the treatments that had significant differences between treatments (p=>0.0001)



Figure 2.4. Susceptible variety Steptoe across all VIGS treatments, FES+H₂O, *HvWAK2*, *HvWAK345*, *HvWAK2 + HvWAK345*, and MCS. This image shows the increase in resistance of Steptoe following VIGS inoculation with construct *HvWAK345* but little difference is observed following infection with VIGS constructs *HvWAK2* and *HvWAK2 + HvWAK345*.

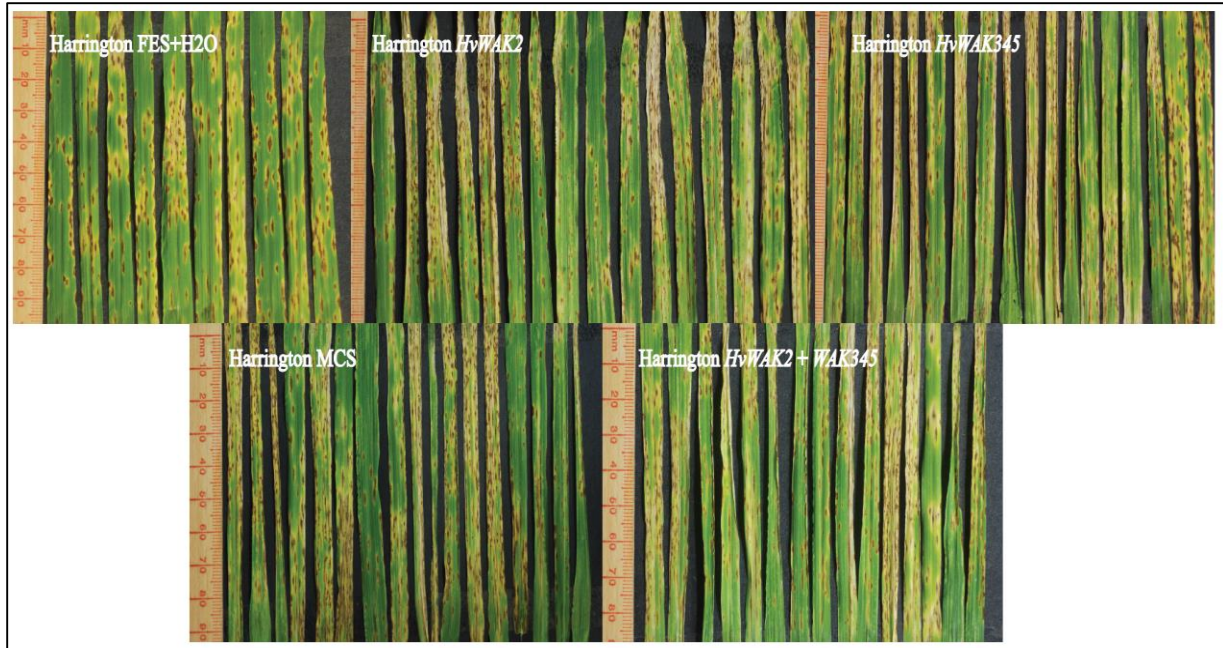


Figure 2.5. Susceptible variety Harrington across all VIGS treatments FES+H₂O, *HvWAK2*, *HvWAK345*, *HvWAK2* + *HvWAK345*, and MCS. Just as observed with Steptoe we observe an increase in resistance with the silencing of *HvWAK345* but no difference is observed when both constructs are silenced or when *HvWAK2* is being silenced.

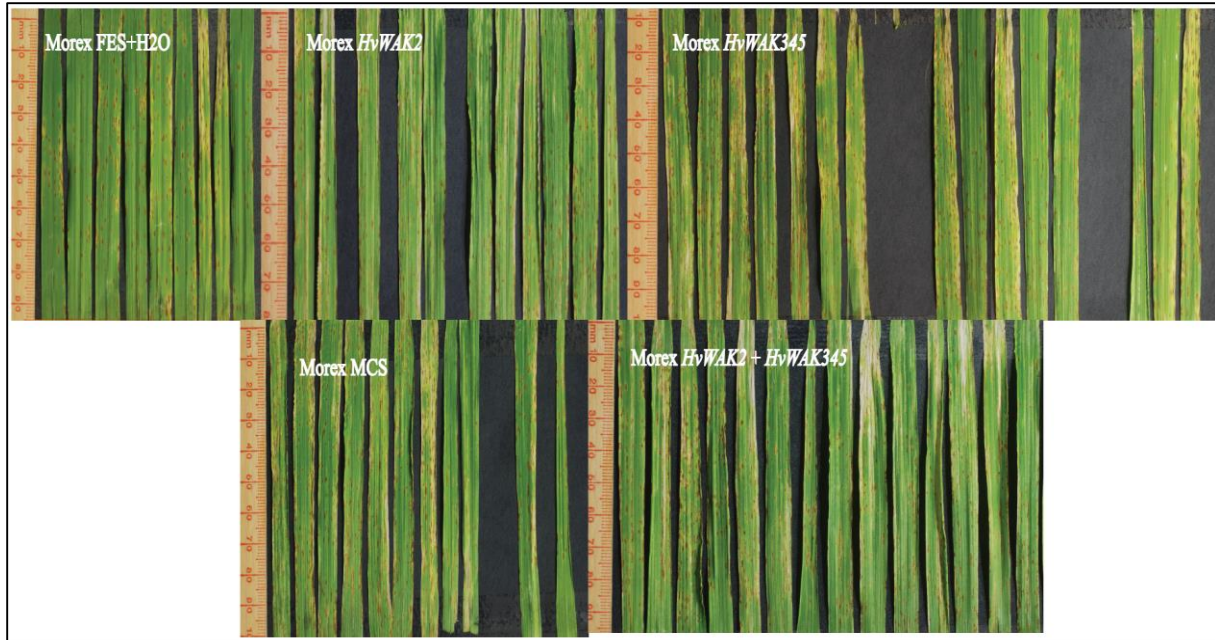


Figure 2.6. Resistant variety Morex across all VIGS treatments FES+H₂O, *HvWAK2*, *HvWAK345*, *HvWAK2 + HvWAK345*, and MCS. Following the treatments it was observed and validated statistically that there are no differences between any of the treatments indicating the *rcs5* gene is not a dominant resistant gene but rather a dominant susceptibility gene.

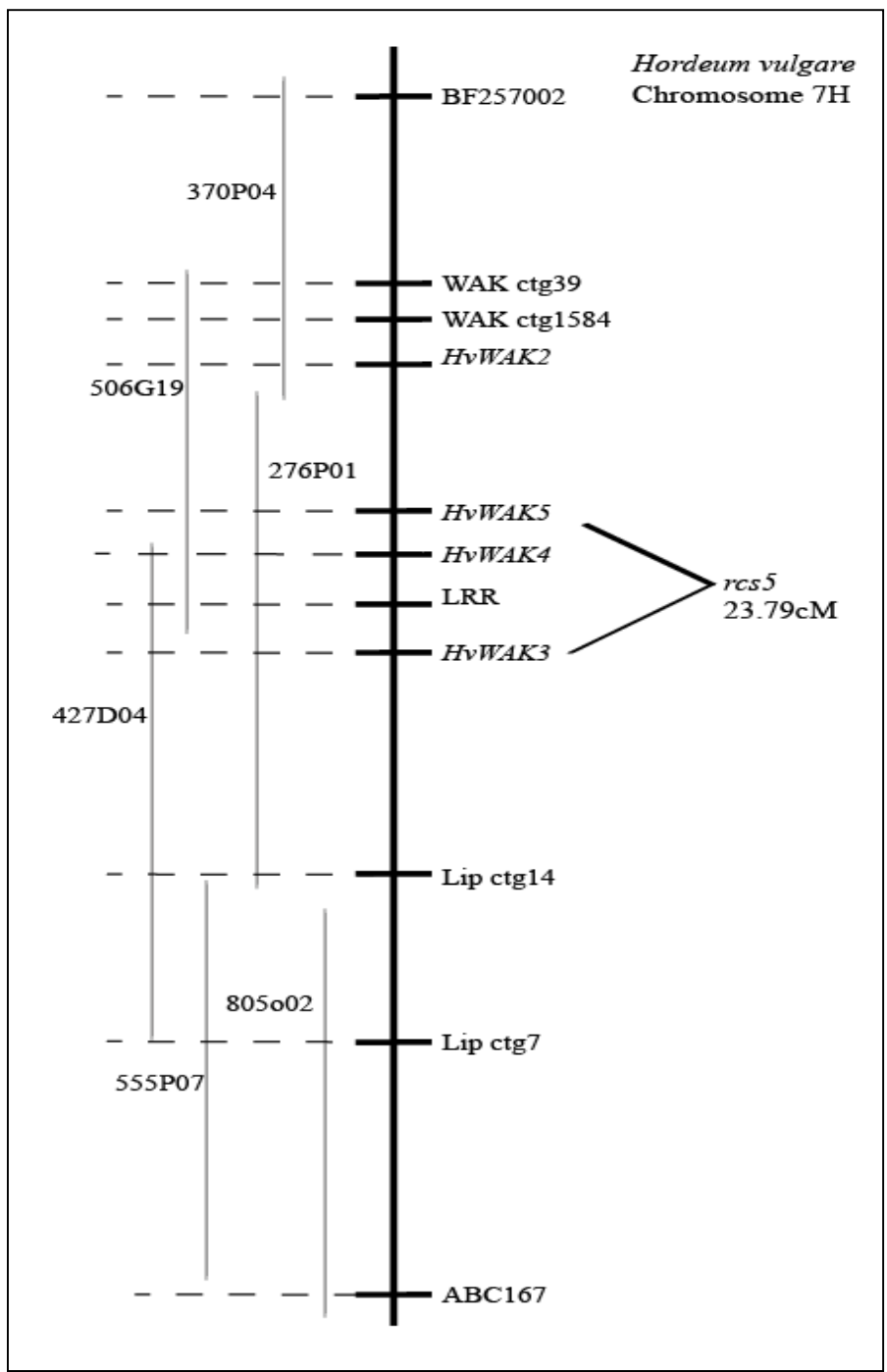


Figure 2.7. Simplified Genetic map of the region Modified from Drader 2012 unpublished. The *rcs5* interval is indicated with the two sided arrow encompassing *HvWAK3-5*. The BAC contigs used to sequence the region are on the left. The dotted lines to the left of each marker indicate where the marker is on each of the BACs.

VIGS analysis

All of the ratings provided in Table 2.1 were analyzed using JMP 10.0 statistical software based off of ordinal data in a quantitative (disease scores based on lesion size, Fetch 1999) manner using parametric statistical analysis tests Oneway ANOVA (all treatments) and Tukey-Kramer (treatment means) HSD. Each cultivar was analyzed for each treatment. These data demonstrate a significant difference between treatments for both of the susceptible varieties ($p < 0.0001$) with no change observed in the resistant variety ($p = 0.1216$). This is why a Tukey-Kramer test was not conducted on this variety. This indicates, based on statistical data, that the *rcs5* gene is a susceptibility factor rather than a resistance factor as previously hypothesized (Drader 2010). This is very important as it is another system that can be used to study NETS and dominant susceptibility pathways.

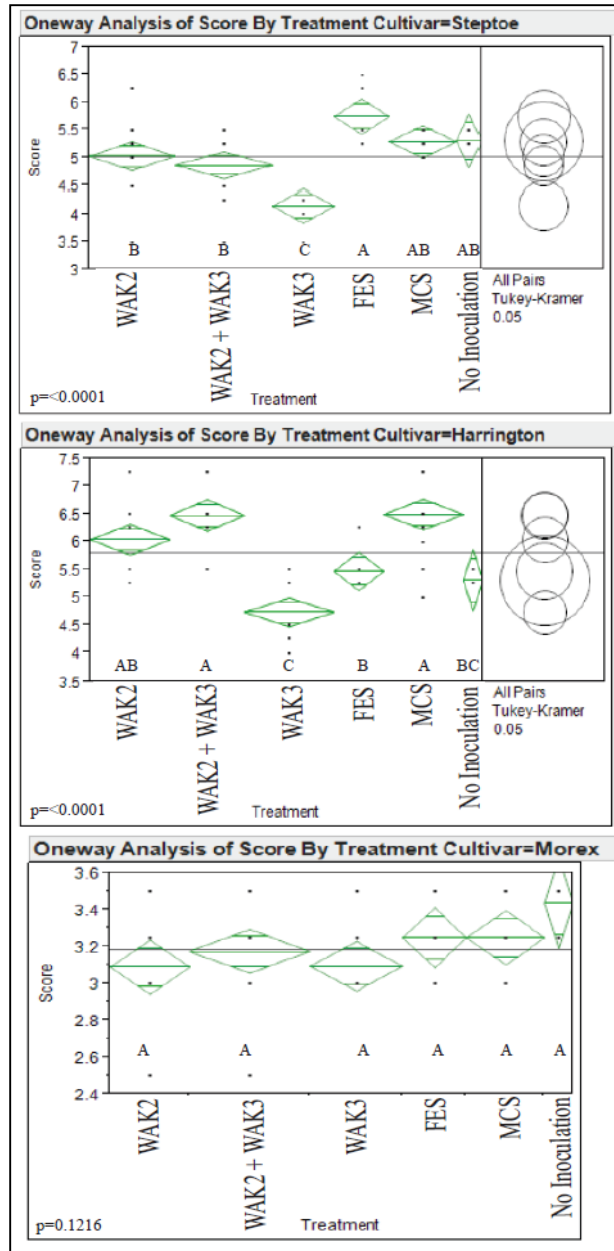


Figure 2.8. Statistical Analysis of Steptoe, Harrington and Morex following all 6 treatments. These data indicate there was no significant difference between any of the Morex (R) treatments ($p=0.1216$) however there were differences with the susceptible varieties Steptoe ($p<0.0001$) and Harrington ($p<0.0001$). Because of these differences a Tukey-Kramer analysis was performed in order to compare the mean scores of each treatment that where the scores were significantly different across treatments.

Discussion

The use of NDB112 as the major source of Spot Blotch resistance, derived mainly from the putative dominant gene *Rcs5*, has been used for decades to combat the foliar pathogen *Cochliobolus sativus* (Wilcoxson 1990). However, the identity of the *Rcs5* gene is unknown. Physical (Bilgic 2005, Bilgic 2006, Drader 2009) and high-resolution genetic mapping of the *Rcs5* locus has identified 4 candidate WAK genes and one LRR (Drader 2012). Our VIGS experiment eliminated *HvWAK2* as the source for susceptibility as none of the lines tested including the putative *Rcs5* containing cv Morex and two susceptible cvs Harrington and Steptoe showed a differential reaction after inoculation with the two BSMV-VIGS constructs BSMV-*asHvWAK2* and BSMV-*asHvWAK345* compared to the positive BSMV-MCS control. Although *HvWAK2* was eliminated as a candidate *Rcs5* gene this analysis is still preliminary and must be repeated as the gene could play a minor role in susceptibility or resistance against the pathogen. The BSMV-VIGS silencing construct BSMV-*asHvWAK345* was of the most interest as post transcriptional gene silencing with this construct did give an unexpected differential reaction. After the putative silencing of the *HvWAK345* genes, a shift from compatibility/susceptibility in cvs Harrington and Steptoe towards incompatibility/resistance was observed (Figures 2.4-2.5 and Table 2.1). The susceptible cv Harrington, does not apparently contain *HvWAK3* based on lack of amplification and lack of sequencing data, thus the shift in susceptibility towards resistance in this cv suggests that *HvWAKs4* or *5* may play a role as dominant susceptibility targets or there is redundant functions as dominant susceptibility targets in the *HvWAK345* gene family. This result led us to question the specificity of the BSMV-*asHvWAK345* silencing construct. BLAST analyses with the NCBI (National Center for Biotechnology Information) database and the IPK genome sequence server (Institut für Pflanzengenetik und

Kulturpflanzenforschung), resulted in *HvWAK3*, *HvWAK4* and *HvWAK5* nucleotide alignments indicating the possibility that the DNA fragment utilized to design the silencing construct was silencing the *HvWAK345* gene family. All the genes within the region have identical protein domain structures containing a predicted extracellular domain with an elongation growth factor domain, a transmembrane domain and a C-terminal intracellular S/TPK. The genes also share over 80% similarity to each other at each of the four exons (for the four domains) with a majority of the exons sharing long stretches of similar sequence reducing the ability to design specific primers that would produce amplicons with sufficient divergency to develop specific silencing constructs. The silencing of the WAK gene family did not allow us to determine which gene is giving the observed phenotype shift towards resistance in the two susceptible cultivars tested, but it is evidence that a WAK (most probably *HvWAK4* or *HvWAK5*) is a susceptibility targeted by the pathogen. This hypothesis is substantiated by the observations that the resistant cv Morex had no apparent shift in phenotype following BSMV-VIGS silencing. How *HvWAK4* or *HvWAK5*, and possibly *HvWAK3* are involved in susceptibility, if at all, is still unknown and requires further testing and qPCR to quantitate the levels of silencing of each gene with each construct. Highly specific knockout vectors need to be developed in order to further assess and validate each gene in this WAK gene family. However, based on these data it is most likely that *HvWAK4* or *HvWAK5* are dominant susceptibility factors targeted by *C. sativus* in a necrotrophic inverse gene-for-gene manner (Liu 2014).

Previous analyses on data gathered by Drader et al. 2010, suggested that both *HvWAK2* and *HvWAK3* were constitutively expressed under both disease and non-diseased conditions. This was the basis for considering these two WAK genes as the most likely *Rcs5* candidates and used in their subsequent VIGS experiments. The constitutively expressed WAK receptor was

believed to behave as a dominant resistance gene in a gene-for-gene manner with a direct or indirect interaction between the R-gene and pathogen effector leading to a resistance response (Dader 2012). However, the WAK may recognize cell wall damage, such as pectin degradation or other cell wall component, or directly interact with a necrotrophic effector leading to a PCD response that the pathogen in its necrotrophic phase utilizes to complete its life cycle resulting in more necrosis and disease. The data presented here follows the necrotrophic effector triggered susceptibility (NETS) or inverse gene-for-gene model as silencing the gene in the susceptible lines resulted in more resistance suggesting a functional dominant susceptibility gene present in the susceptible lines rather than dominant resistance gene in the resistant lines.

The mechanism for the specificity of *rcs5* to *C. sativus* pathotype 1, including ND85F, is still unknown as the other pathotypes (0 and 2) of the fungus confer differing responses indicating the presence or absence of other NEs or AVR genes in these pathotypes (Biligić 2006). Additionally the hypothesis that *rcs5* is constitutively expressed (Drader 2012) may be inaccurate as studies have shown certain WAK genes are only expressed under certain conditions such as drought or pathogenic responses (He 1998, Wagner 2001, Anderson 2001, Sivaguru 2003, Kohorn 2012). Other WAK genes found to interact with pathogens, through unknown mechanisms, are *WAK1,2* and *3* in *Arabidopsis* which are induced by infection from *Phytophthora parasitica* and other stress signals such as ozone and benzothiadazole, an activator of systemic acquired immunity (Denoux 2008). *WAK1* overexpression was found to confer increased resistance to *Botrytis cinerea* a necrotrophic fungus (Brutus 2010). Rice also produces a WAK that has been found to be involved in resistance to *Magnaporthe oryzae*. This particular gene, *OsWAK1* is induced from infection as well as SA and methylated-JA treatment, but not ABA, which has, in other systems, been shown to resist biotrophic pathogens (Li 2009).

WAKs have been shown in multiple studies to be receptors of pectin fragments with subsequent downstream induction of the MAPK pathway, which has been shown to be involved in responses to pathogen infection (Kohorn 2006, 2009, 2011; Brutus 2010). These observations make the WAKs in the *rsc5* region good candidates as the action of the fungus induces severe damage to the host cell, no doubt disrupting pectin in susceptible cultivars, causing cell wall molecules to fragment and subsequent release of the cells contents (Figure 2.9). Pectin fragments have also been shown to compete for WAK binding (Kohorn 2011). Interestingly WAKs also appear to bind covalently to pectin as pectinase treatment was the only treatment, among other cell wall degrading enzymes, that released WAKs from the cell wall (Wagner 2001). An alternative hypothesis is the *rsc5* WAK interacts with a specific effector produced by the pathogen (Figure 2.9). Additionally, the *rsc5* WAK gene could perform a unique function not yet observed demonstrating even further the need to clone and perform functional analysis on the gene.

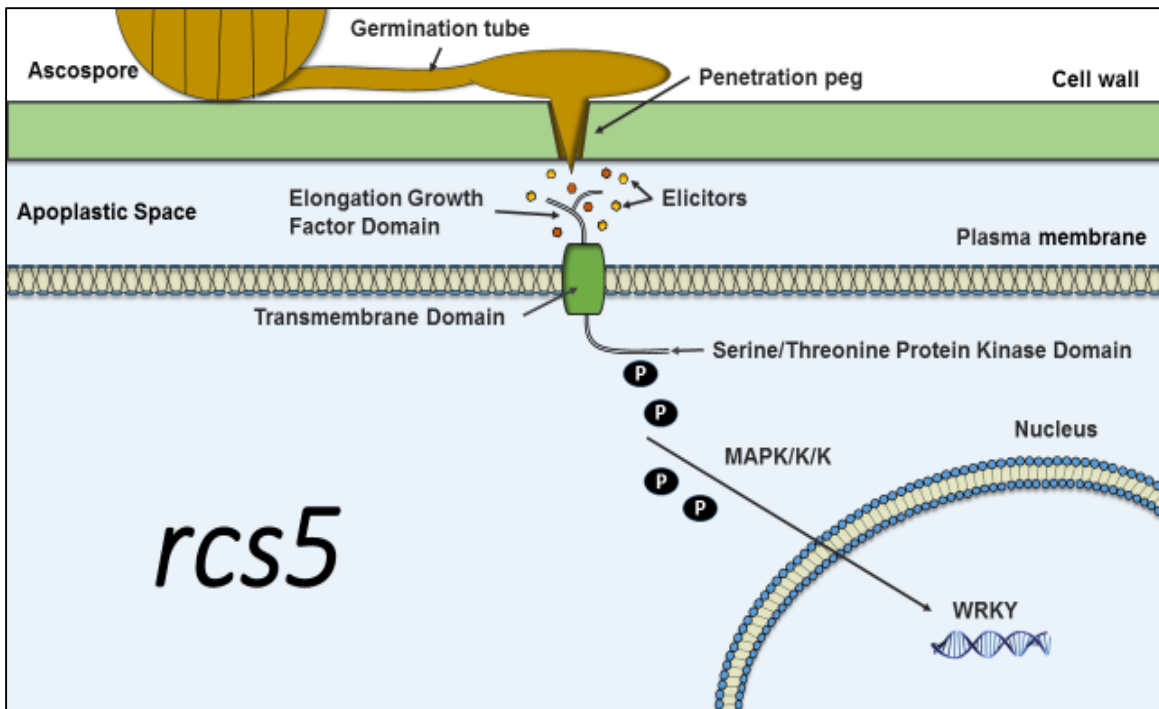


Figure 2.9. Putative Dominant Susceptibility model for *rcs5*. The WAK responds to an unknown elicitor and triggers the MAPK pathway and eventually WRKY transcription factors that have been shown to be involved in disease resistance and susceptibility pathways.

Final identification of the *rcs5* gene will be dictated by the results of VIGS knock down experiments, which will identify the gene from the remaining WAK genes HvWAK3, HvWAK4 and HvWAK5. It is a possibility that the closely related WAK genes are all susceptibility targets and contribute to susceptibility in a quantitative manner similar to what is seen in the *Stagonospora nadorum* pathosystem (Friesen 2007) (Figure 2.9). In this pathosystem it has recently been determined that the *Snn1* gene which confers dominant susceptibility to *S. nadorum* is in fact a WAK gene from wheat (Dr. Faris, personal communication). Although further analyses are required including more silencing experiments and qPCR analyses, we have narrowed the *rcs5* candidate list down to two candidate susceptibility genes that putatively act as necrotrophic effector targets that induce PCD pathways utilized by the pathogen in its necrotrophic phase to cause disease.

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CONCLUSION

The research described in this thesis had two main goals: 1) Identify the *nec3* gene on chromosome 6H of barley, and 2) Identify and validate the spot blotch resistance gene, *Rcs5*, based on the delimitation of four WAK candidate genes on Chromosome 7H in barley. To this end, progress was made towards accomplishing both of these goals and from the data presented we further characterized both genes and were able to develop novel models of their functions in pathogen elicited programmed cell death.

In the *nec3* project we were able to reduce the resolution of the *nec3* region from 13cM between markers CMWG652a and HARV32_5771 (Keisa 2012) to 5.4cM between markers GBM1053 and GBM1423 with co-segregating marker GBM1212, which reside at approximately 49.07-49.22cM on Bowman Chromosome 6H using a newly identified *nec3* mutant with a Bowman background designated *gamma1*. Previously the *nec3* mutants (*nec3.d*, *nec3.e*, FN362 and FN363) were thought to be Lesion Mimic Mutants (LMMs), which are physiological mutants that produce spontaneous necrosis phenotypes without elicitation from abiotic or biotic stresses or challenge. However, we were able to demonstrate that the *nec3* phenotype is expressed following infection with various pathogens including the necrotrophic ascomycetes *C. sativus* and *P. teres* f. *maculata* and *teres*, the biotrophic ascomycete *E. graminis* and the bacterial pathogen *X. translucens* pv. *undulosa*. RNAseq was performed on Bowman wild type and the *gamma1* mutant to identify candidate genes within the 5.4cM region following one week growth with no inoculation and one week growth with inoculation with *C. sativus*. Currently the candidate genes are being validated or eliminated via allele analyses. Future work includes creating a higher resolution map and identification of the *nec3* gene.

My research on the *rcs5* validation project resulted in the identification of 2 strong candidate *rcs5* genes previously delimited by Dr. Tom Drader (Drader 2012). Originally *HvWAK2* and *HvWAK3* were considered the only candidate genes since they are expressed under both disease and non-disease conditions. However, this may not be the requirement for delimiting the WAK *rcs5* candidate gene list as many WAKS are expressed only under certain conditions (Li 2009). We were able to eliminate *HvWAK2* from the candidate gene list as it showed no change in phenotype after the VIGS experiment. However, the silencing construct produced for *HvWAK3*, 4 and 5 resulted in increased resistance in the susceptible cvs Steptoe and Harrington with no shift in phenotype observed in the resistant cultivar Morex. These data suggested that the *rcs5* gene is probably not a dominant resistance gene but is rather a dominant susceptibility gene. Future work includes identifying the *rcs5* gene from the two possible candidates, *HvWAK4*, and *HvWAK5*.

APPENDIX

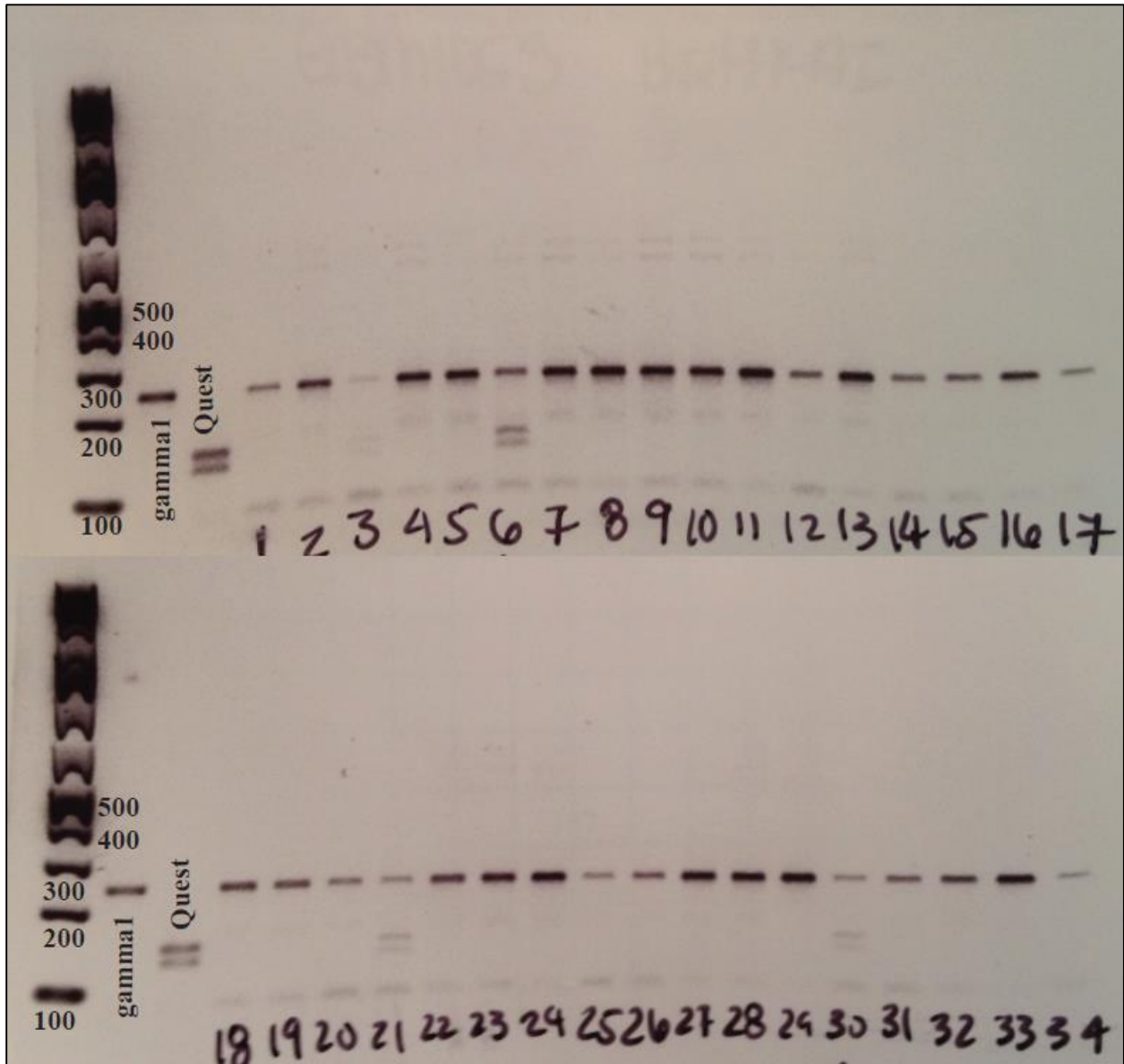


Figure A1. GBM1053 CAPS marker. Amplified PCR product was digested with BSIHKAI and analyzed on a 1.5% gel. A 1kb plus ladder from Life Technologies is on the far left with size designations. Gamma1 has a single amplicon at 250bp however Quest has two amplicons at 150 and 170 providing easy analysis.

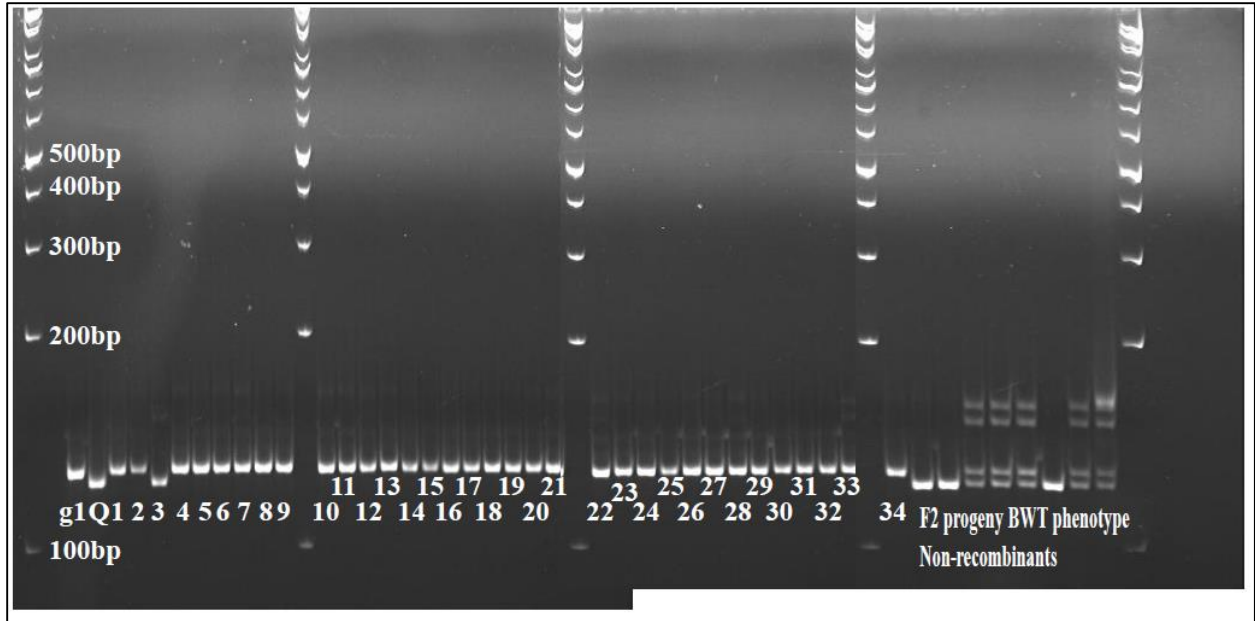


Figure A2. GBM1212 *nec3* co-segregating marker. Amplified PCR product was separated and visualized on a 12.5% Polyacrylamide gel. All lines amplified the expected 150bp product for the gamma1 genotype making this a co-segregating marker. The third recombinant gamma1.3 is not Quest like (A3).

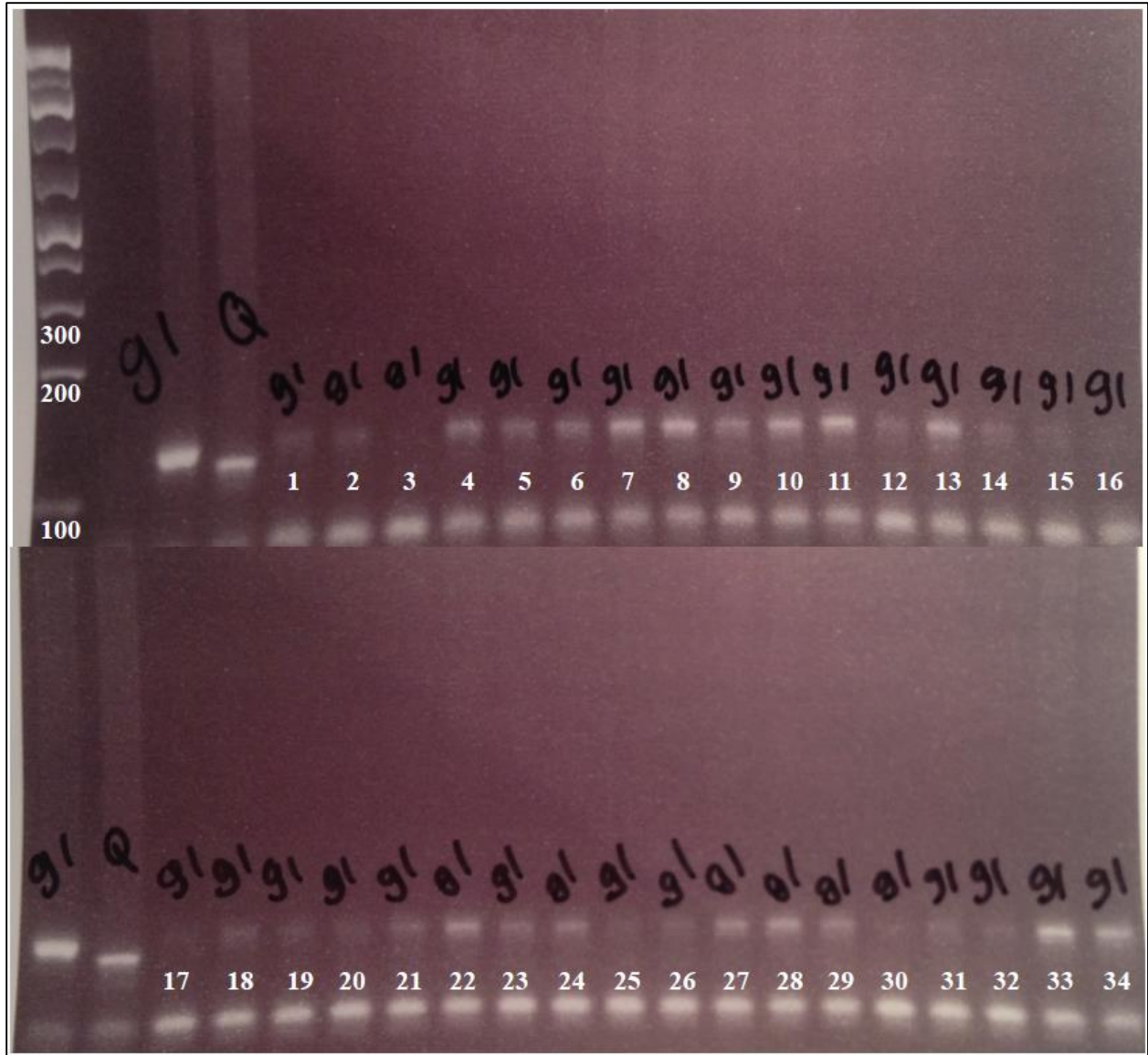


Figure A3. Bmag0807 *nec3* co-segregating marker. All of the 34 recombinants amplified for the gamma1 genotype making this a second co-segregating marker with an amplicon size of 120bp.

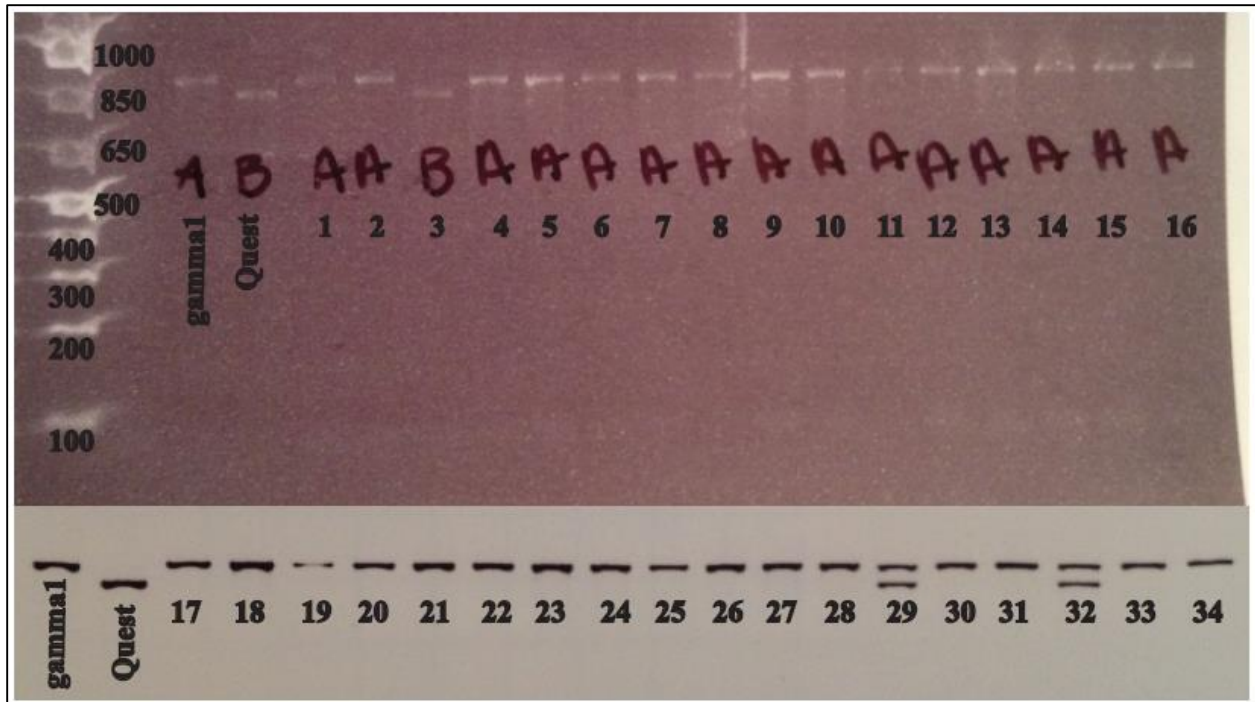


Figure A4. GBM1423 CAPS marker. Amplified PCR product was digested with StyI and analyzed on a 1.5% gel. A 1kb plus ladder from Life Technologies is on the far left with size designations. Gamma1 has a single amplicon at 900bp and Quest has a single amplicon at 850bp.

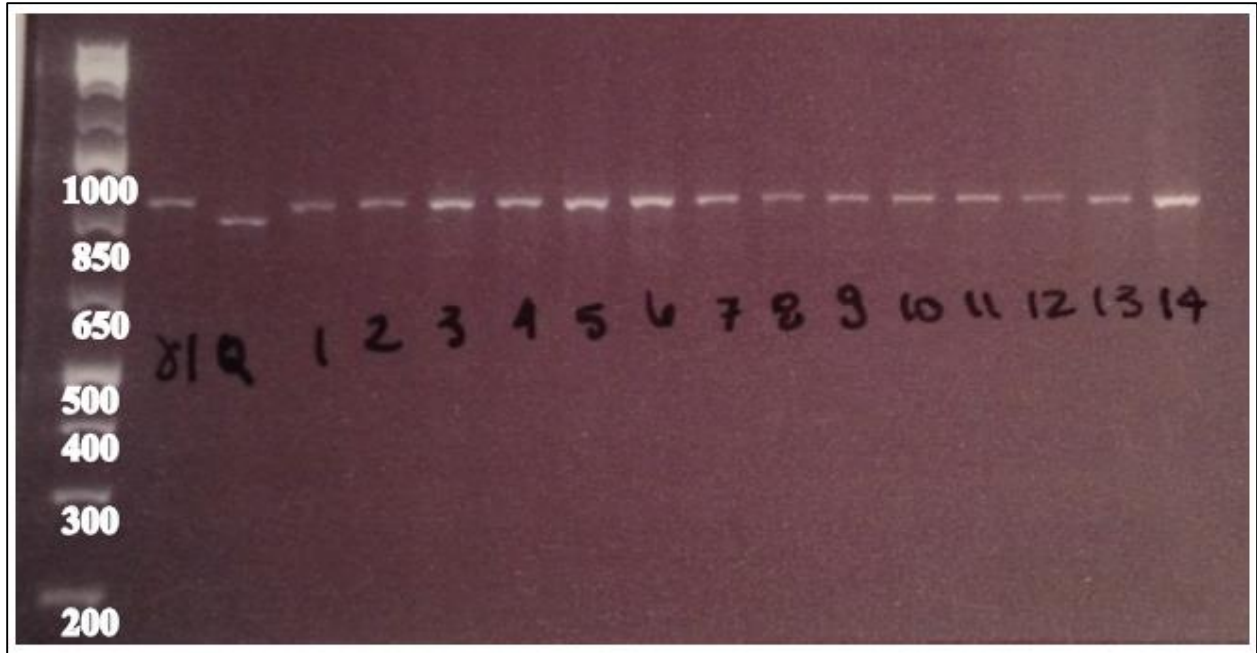


Figure A5. Re-analysis of putative double recombinant gamma1.3. GBM1423 was used to screen gamma1.3, a putative double recombinant. 15 plants were sown and 14 emerged. All of the plants were shown to be gamma1 removing gamma1.3 from the recombination list.

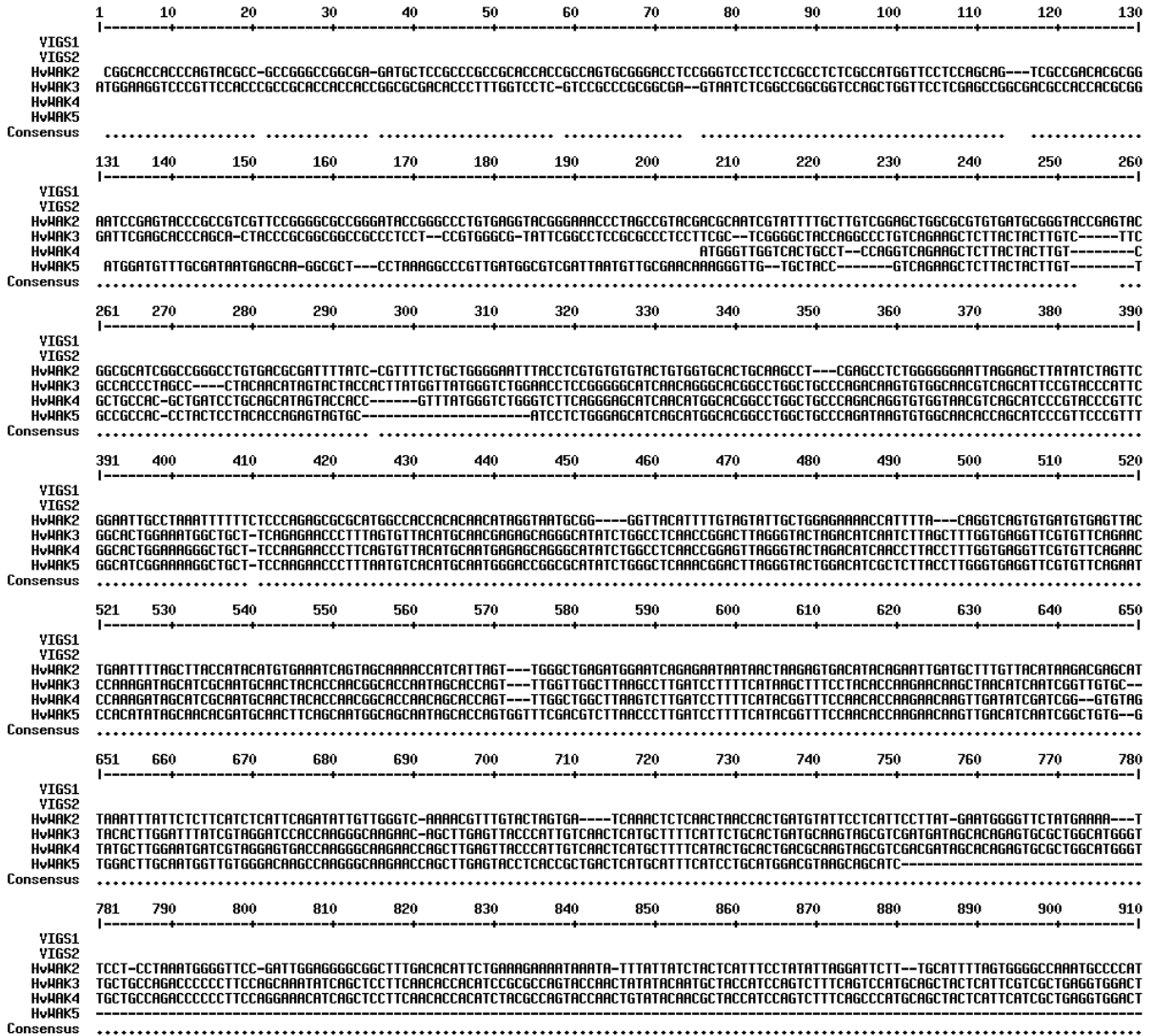


Figure A6. Alignment of *rcs5* candidate genes with each other and the knockout vectors.
 Pane 1: It should be noted that VIGS1=*HvWAK2* silencing vector and VIGS2=*HvWAK3-5* silencing vector.

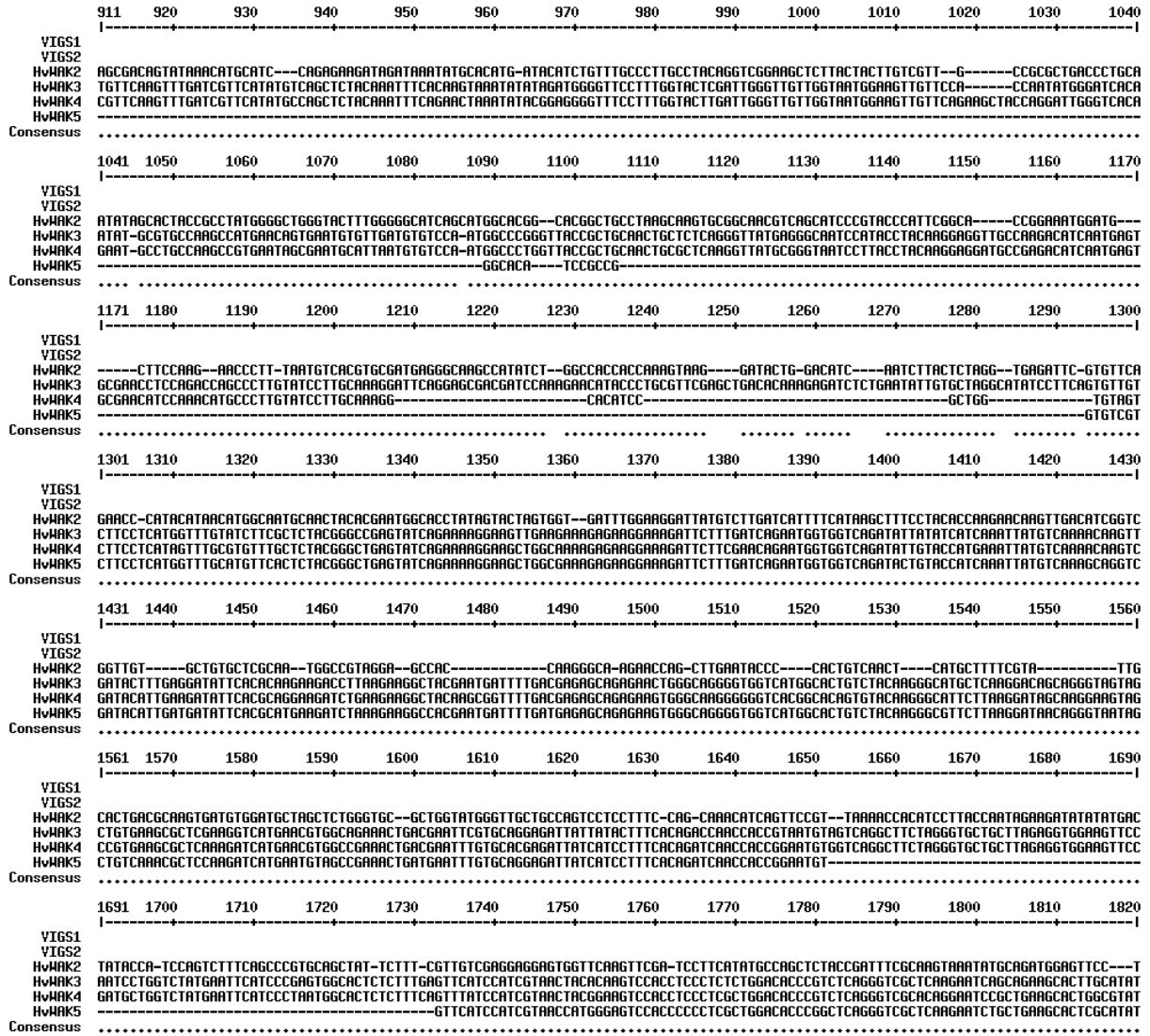


Figure A6. Alignment of *rcs5* candidate genes with each other and the knockout vectors (continued)
 Pane 2

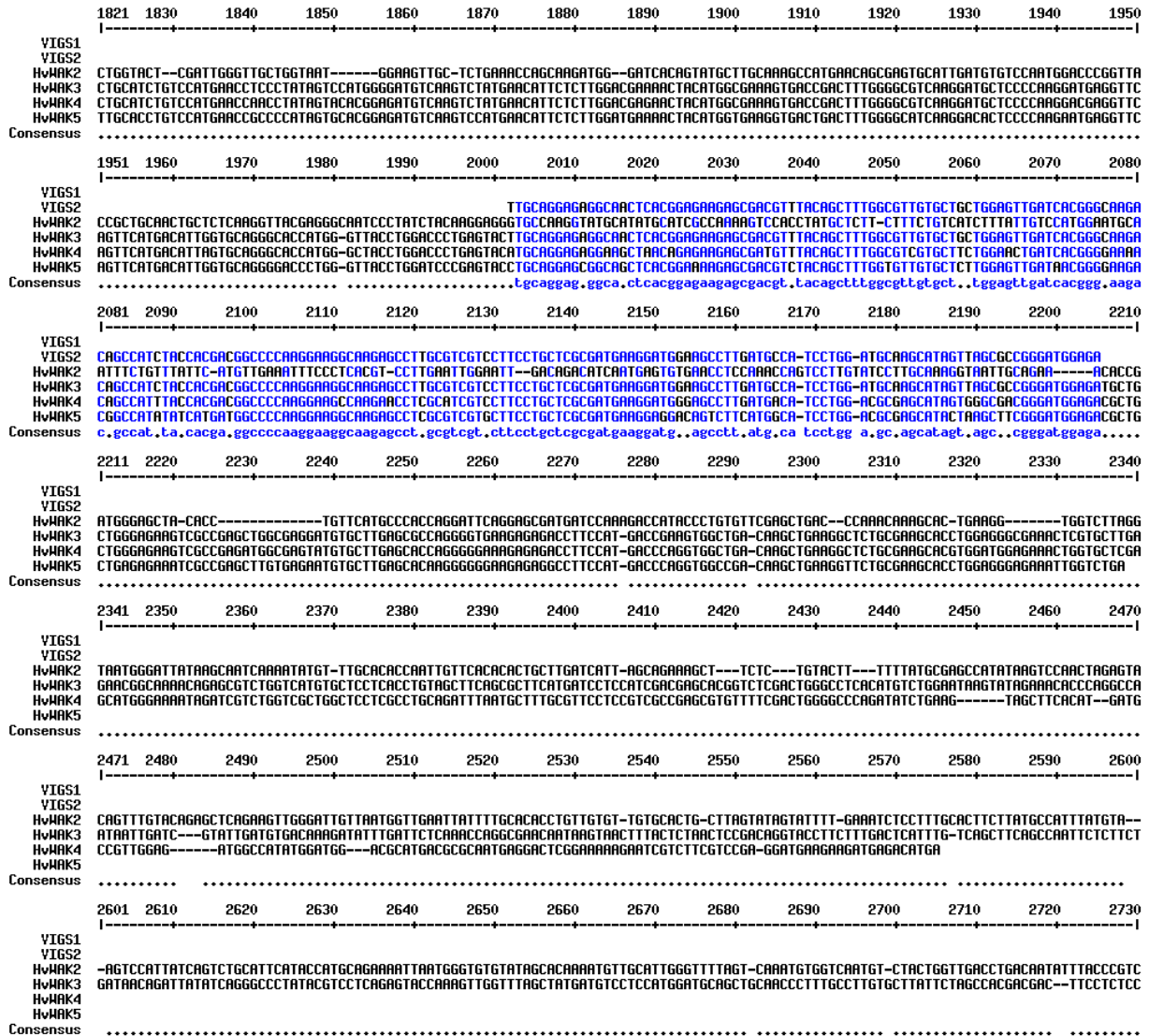


Figure A6. Alignment of *rcs5* candidate genes with each other and the knockout vectors (continued).

Pane 3: In this pane we are able to see the VIGS2 BSMV knockout vector which was specific to *HvWAKs3-5*. The alignment shows the likely chance that this vector silenced all three genes since the stretches of similar (blue) are equal to and greater than 15 base pairs which is the size the DICER/RISC system utilizes. This silencing vector targets a region between exons 3 and 4.

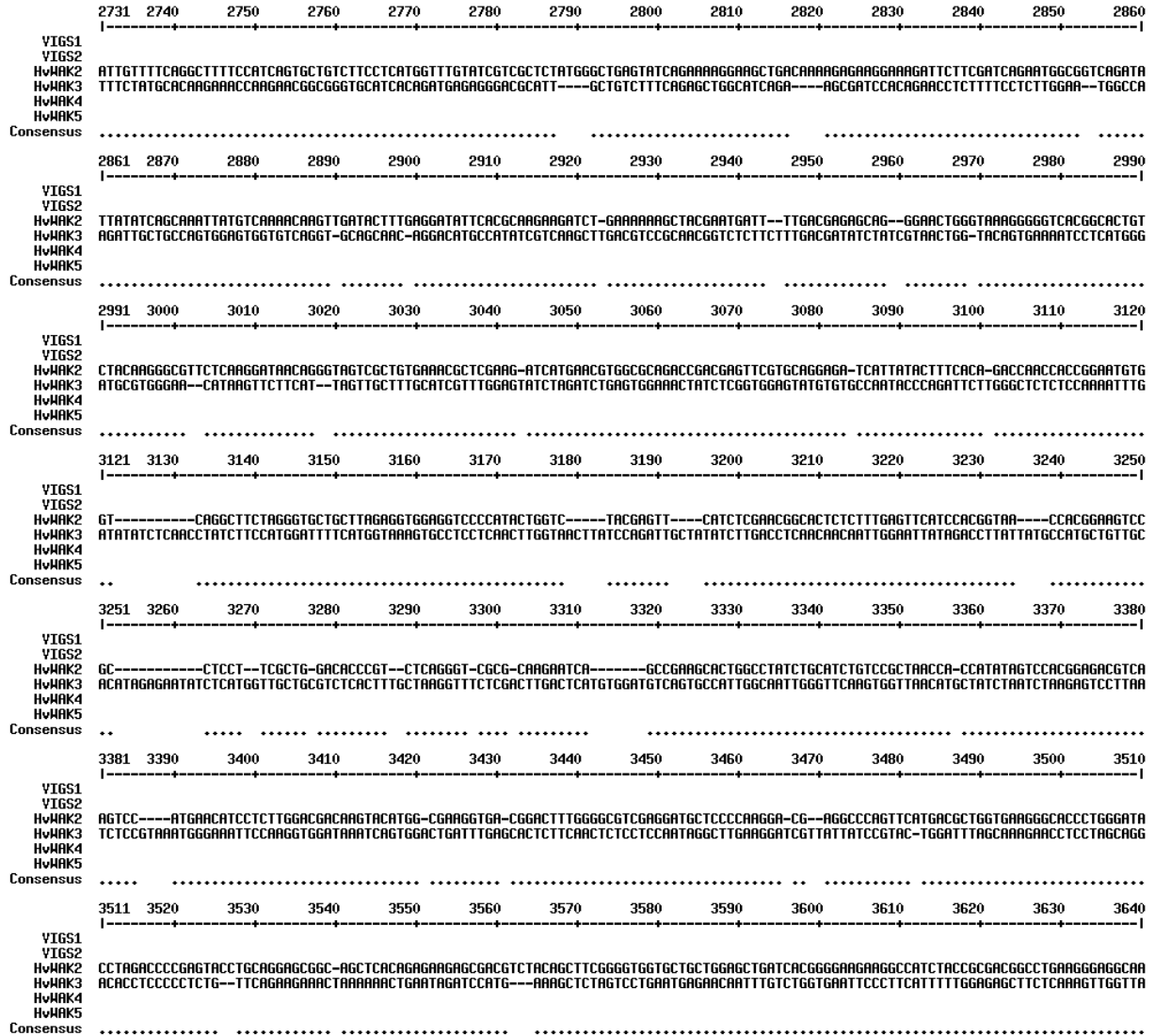


Figure A6. Alignment of *rcs5* candidate genes with each other and the knockout vectors (continued).
 Pane 4

