IDENTIFICATION OF MULTIPLE VIRULENCE LOCI IN *PYRENOPHORA TERES* F. *TERES ASSOCIATED WITH NET FORM NET BLOTCH IN BARLEY*

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Identification of multiple virulence loci in *Pyrenophora teres* f. *teres* associated with

net form net blotch in barley

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MASTER OF SCIENCE

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ABSTRACT

The necrotrophic fungal pathogen *Pyrenophora teres* f. *teres* causes the foliar disease net form net blotch (NFNB) on barley (*Hordeum vulgare)*. To investigate the genetics of virulence in the barley- *P. teres* f. *teres* pathosystem, we used 118 progeny derived from a cross between the isolates 15A and 6A. The barley lines, chosen based on their different reactions to 15A and 6A, were evaluated for NFNB caused by the 15A × 6A progeny. Genetic maps generated with SSR and AFLP markers in the fungal population were scanned for quantitative trait loci (QTL) associated with virulence in *P. teres* f. *teres.* Two QTL were identified in Rika, two in Kombar and PI356715 had a single unique QTL. Therefore, a total of five virulence loci were identified in this pathogen population based on inoculation on three different barley lines.

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LITERATURE REVIEW

Host

Barley, *Hordeum vulgare*, which originated in the Fertile Crescent region of Asia, was likely domesticated prior to 8,000 B.C. (Harlan 1979; Mathre 1997). Barley is a profitable crop throughout the world because of its ability to adapt to different altitudes as well as a wide range of rainfall conditions (Lemaux et al. 1999). The United States is one of the top ten leading countries for barley production and one of the top four for production value, trailing behind France, Australia, and Ukraine (FAOSTAT 2012). In the United States, over 2.8 million acres of barley was sown annually from 2009- 2011; of this at least 500,000 acres was planted annually in North Dakota alone, reaching as high as 1.2 million acres in 2009 (American Malting Barley Association, Inc. 2011). In 2010, North Dakota was the leading state in the U.S. for barley production, harvesting over 43 million bushels, nearly a quarter of the total 180 million bushels produced in the United States as a whole that year (USDA NASS 2011). Commonly used barley cultivars such as Tradition, Lacey, Celebration, Robust, Stellar-ND, and Pinnacle, which currently account for 80% of the total barley planted in North Dakota (USDA NASS 2011), are all susceptible to net form net blotch's causal agent *P. teres* f. *teres*. Because of this prevalent susceptibility, the genetic characterization of the mechanism of host resistance and pathogen virulence is necessary for effective and consistent control of the disease.

Pathogen

Pyrenophora teres f. *teres* is an ascomycete that causes the foliar disease net form net blotch (NFNB) on barley. Although *P. teres* is frequently only seen on the leaves of barley species, greenhouse inoculations have shown several other cereal host species to be susceptible

to the pathogen, including both wheat and rye (Shipton et al. 1973). NFNB, which is prevalent on barley worldwide, received its name because of the net-like lesions that develop after the pathogen has infected the leaves of susceptible barley lines (Atanasoff and Johnson 1920; Shipton et al. 1973). Although the disease is most severe in regions of the world with temperate climate paired with high rainfall and high humidity, it can also occur in regions where little rainfall is present (Steffenson and Webster 1992). Outbreaks of NFNB often produce yield losses of 10-40% by means of reduced kernel size, although the entire crop can be destroyed in some extreme cases (Mathre 1997). The pathogen itself is a heterothallic fungus that produces the primary inoculum (ascospores) for the disease in pseudothecia in crop stubble; the secondary inoculum however is most often in the form of asexual spores called conidia (Liu et al. 2010). Smedegård-Petersen (1971) found that there were two different forms of *P. teres, P. teres* f. *teres* and *P. teres* f. *maculata*, which caused NFNB and spot form net blotch (SFNB), respectively. Although both pathogens are a form of *P. teres*, multiple studies have shown evidence for the two forms being separate species (reviewed in McLean et al. 2009). More recently, the two forms have been distinguished with the use of mating type locus markers (Lu et al. 2012).

Both qualitative and quantitative resistance to *P. teres* f. *teres* has been characterized in barley. The qualitative relationship between the pathogen and host has been proposed to follow a gene-for-gene model similar to what was previously observed with flax rust by Flor (1956), which involves the presence of both dominant resistance (*R*) genes in the host and avirulence (*Avr*) genes in the pathogen. Several studies have been performed on the NFNB system, which claim that both *Avr* genes and *R* genes are present within this host-pathogen system (Weiland et al. 1999; Lai et al. 2007; Beattie et al. 2007; Mode and Schaller 1985; Manninen et al. 2006; Cakir et al. 2003; Ma et al. 2004; Emebiri et al. 2005; Friesen et al. 2006; Grewel et al. 2008).

However, more recent information from our lab has shown that both gene-for-gene (Friesen et al. 2006) and inverse gene-for-gene interactions (Abu Qamar et al. 2008; Liu et al. 2010) are likely involved. An inverse gene-for-gene interaction likely involves necrotrophic effectors (Synonym: host-selective toxins) that interact with susceptibility targets in the host (Friesen and Faris 2010; Oliver et al. 2012). Quantitative resistance involving necrotrophic pathogens that are host specialists can often be best explained by an inverse gene-for-gene model with the individual quantitative interactions often being additive in nature (Reviewed in Oliver et al. 2012; Friesen and Faris 2010; Friesen et al. 2008).

Life Cycle

The fungus, *P. teres* f. *teres*, is known to produce pseudothecia, which appear as dark spots on the host debris left in the field, these sexual fruiting bodies act as the over-wintering structures and the source of the primary inoculum for the disease (Mathre 1997). Because *P. teres* is heterothallic, both mating types must be present in order for the ascospores within the pseudothecia to be formed (Rau et al. 2005). Within the ascocarp are bitunicate asci with eight ascospores each, the result of meiosis and one mitotic division. These ascospores have been observed as being light brown in color and as having both transverse and longitudinal septa (Webster 1951). The ascospores act as primary inoculum and are spread easily by wind after maturing and being discharged from the asci; however, it has also been noted that diseased seeds can be a source of primary inoculum if they are sown without seed treatment (Jordan 1981; McLean et al. 2009). The secondary inoculum is commonly observed as conidia, which first appear after the fungus has colonized and begins to reproduce asexually. Conidia are produced on stalk-like structures called conidiophores and are described as being slightly rod shaped and yellow/brown in color; much like the primary inoculum, ascospores, the conidia are also spread

to new uninfected plants by wind (Mathre 1997; McLean et al. 2009). Once mature and dispersed, the fungal spores need the proper environment before spore germination and infection of the host can take place; including proper humidity, temperature, and leaf wetness (Jordan 1981). Spore germination can take place within a matter of hours in the presence of moisture and favorable temperatures (Shipton et al. 1973; Kenneth 1962), allowing the pathogen to infect a susceptible host quickly. NFNB is a destructive disease of barley worldwide due to *P. teres* f. *teres* being able to successfully penetrate and survive on the host in temperate environments. Although NFNB is most prevalent in temperate climates with high rainfall and high humidity, as noted previously, it can also persist in areas with little rainfall. (Steffenson and Webster 1992)

Disease Development

The physical interaction between the pathogen and the host begins as soon as a fungal spore attaches to the host surface and begins to germinate. A germ tube is produced from one end of the spore and grows along the surface of the host as hyphae before forming an appressorium. Beneath the appressorium, a penetration peg is formed. By using a great deal of pressure, cuticle/ cell wall degrading enzymes, or both, the penetration peg formed from the appressorium, breaks through the hosts cell wall and into the epidermal cells (Van Caeseele and Grumbles 1979; Keon and Hargreaves 1983). After the fungus has penetrated the host cell wall, the fungal hyphae moves within the mesophyll of the host and begins to gain nutrient from the host (Keon and Hargreaves 1983). According to Singh et al. (1963), temperature plays a vital role in disease development with 25°C being the optimal temperature for disease development; however, NFNB can be observed in a range of temperatures.

Although the disease observed on the leaves of the host is often considered the most important economically, the pathogen can also infect other areas of the plant including both the stem and the kernels. Along with the pathogen remaining on the stems and plant debris from the previous year, sowing of infected seed can contribute to the primary inoculum. Due to reduced tillage and no tillage practices, which have become more popular in an attempt to increase soil conservation, more plant debris is available from previous years allowing the pathogen to overwinter more easily and more successfully (Liu et al. 2010). Once the primary inoculum has penetrated the leaves, disease symptoms (lesions) can be observed as soon as 24 hours after penetration. The visible symptoms of infection begin with small pin-point lesions at the sight of fungal penetration. As the pathogen progresses through the susceptible host, these lesions become less circular and begin to take on the familiar net-like form. The disease symptoms are stopped as small circular lesions in resistant barley lines but are known to form chlorotic regions and large brown net-like lesions on the more susceptible lines (Mathre 1997).

Toxin Production

Smedegard-Petersen (1976) showed that symptoms of toxins produced by *P. teres* include water soaking, chlorosis, and either spot or net-like necrosis depending on the form of *P. teres* being observed. Smedegard-Petersen (1976) also noted that it was the non-necrotic symptoms that were most important because they affected the plant to a higher degree than the necrotic lesions, explaining that these lesions "were harmLess to the host when occurring alone as in resistant plants" (Smedegard-Petersen 1976). At this point in time only two toxins had been characterized in *P. teres*. Smedegard-Peterson (1976) showed that these toxins "contribute to virulence but do not determine pathogenicity". However, by 1979, three distinct non-proteinaceous toxins had been identified as products of *P. teres*, all of which were able to cause

necrotic/ cholorotic lesions on the host, termed as toxins A, B, and C (Bach et al. 1979). Both toxin A and B could cause chlorosis and necrosis with concentrations of 400 µg per gram of fresh weight; however, concentrations of these two toxins needed to be much higher in order to cause the symptom of water soaking (Smedegard-Peterson 1976). Of the three toxins produced, toxin C was the most commonly found and the most effective at causing chlorotic symptoms; however, a strong correlation with susceptibility of the host to the pathogen was never made (Weiergang et al. 2002).

Both *P. teres* f. *teres* and *P. teres* f. *maculata* are capable of producing multiple low molecular weight compounds (LMWCs) and metabolites, although, Sarpeleh et al. (2009) stated that when grown in identical conditions, *P. teres* f. *maculata* frequently produced more toxins than *P. teres* f. *teres*. Both forms of the fungal pathogen are able to produce toxins found in multiple classes, including marasmines, pyrenolides and pyrenolines (Sarpeleh et al. 2009). The chlorotic regions observed on the diseased plants are likely due to effectors or toxins that are produced by the fungus that result in the induction of programed cell death (PCD) (Sarpeleh et al. 2009). In some cases, no fungal hyphae were present in the cells surrounding the chlorosis, implying the presence of a toxin (reviewed in Liu et al. 2010).

Sarpeleh et al. (2008) stated that the proteins produced by the pathogen are dependent on factors such as temperature to remain functional. Active toxic proteins need to remain below 60 °C to cause lesions on the host; the loss of function of these toxic proteins could be due to "low plant cell metabolism, protein inactivation, or a loss of enzyme activity". These proteins are also known to be most effective on young leaves and also rely heavily on the presence of light. The disease symptoms observed on the plants has been found to be from an interaction from both LMWCs and proteinaceous metabolites. It is speculated that the LMWCs are responsible for

causing "yellowing and water soaking" type lesions on the leaves while the proteinaceous metabolites are responsible for causing only the brown necrotic lesions. Neither LMWCs or proteinaceous metabolites cause both types of lesions, only together do they cause the chlorotic and necrotic symptoms recognized with both SFNB and NFNB (Sarpeleh et al. 2007). Water soaking, which is often the first symptom observed, "appears to occur due to a perturbation of the membranes of the host plant and leads to electrolyte leakage because of damage to the plasma membrane by the phytotoxins" (Strange 2007). Sarpeleh et al. (2007) also claimed that the proteinaceous metabolites found to cause necrotic lesions on barley were highly specific, even though they were shown to be produced by both *P. teres* forms, indicating the opposite. The reaction of different metabolites was different on different barley lines. The LMWCs, however, differ from the metabolites slightly; although they are also light and temperature dependent, they are non-host selective (Sarpeleh et al. 2009).

Host Resistance

In 1955, incompletely dominant resistance genes were identified and found to be effective against *P. teres* (Schaller 1955; Mode and Schaller 1958). By 1958, single dominant resistant genes had also been discovered (Frecha 1958). Shortly after, it was established that different strains of *P. teres* had different virulences (Khan and Boyd 1969), which led researchers to focus on host and pathogen variability within the system. In 1977, the first set of recessive resistance genes were found (Bockelman et al. 1977), implying that there potentially were necrotrophic effectors produced by the pathogen corresponding to different dominant susceptibility genes in the host. Weiland et al. (1999) found that five random amplified polymorphic DNA (RAPD) markers were associated with avirulence within a segregating *P. teres* f. *teres* population; this locus, named *Avr*Har, implied the presence of a corresponding

resistance gene within the barley line Harbin according to the gene-for-gene theory. Single dominant resistance genes had been identified in barley lines Harbin and Ming (Mode and Schaller 1958) before the Weiland et al. (1999) discovery of *Avr*Har.

It is believed that resistance genes often cluster together in the same region or there are multiple alleles of a single resistance locus on the chromosome (Liu et al. 2010). Several genes, which may be clustered, have been identified within the barley 6H chromosome (Abu Qamar et al. 2008). These resistance and susceptibility genes have been identified in multiple studies, with the use of lines originating from several locations throughout the world including Finland, Canada, the United States, and Australia (Friesen et al. 2006; Cakir et al. 2003; Emebiri et al. 2005; Manninen et al. 2006). Based on the fact that these lines did not originate from a single location, it can be hypothesized that the clustering of genes observed on the 6H chromosome is likely not limited to barley lines that originated from a specific region but rather are derived from diverse backgrounds. By looking at barley populations such as Rika × Kombar, we have identified multiple dominant susceptibility genes (Abu Qamar et al. 2008), including *rpt.r* and *rpt.k*, which are present in Rika and Kombar, respectively. Both *rpt.r* and *rpt.k* are located close to each other on chromosome 6H even though the parental barley lines originated in Sweden and the United States, two locations which likely have very different host genetics and pathogen selection pressures.

Pathogen Virulence

According to the disease triangle, the ability of the pathogen to successfully cause disease is due to three aspects: a susceptible host, disease favorable environment, and a virulent pathogen; if any of these factors are missing, disease will not occur (Francl 2001). There are

currently two types of interactions commonly associated with the *P. teres* f. *teres*-barley pathosystem, the gene-for-gene model and the inverse gene-for-gene model. Because both resistance and susceptibility genes have already been identified within this system, it is likely that either model or a mixture of the two models is present. In the classical gene-for-gene model, both avirulence genes within the pathogen and resistance genes within the host are required for an incompatible (resistant) interaction; however, in an inverse gene-for-gene model, both virulence genes found in the pathogen and susceptibility genes found in the host are required in order for disease symptoms to occur (Friesen et al. 2008; Flor 1956).

Weiland et al. (1999) identified one major gene associated with avirulence/virulence in a pathogen population created from the cross of an isolate conferring high virulence and an isolate conferring low virulence on the barley cultivar Harbin. In addition, two genes were identified in the same population to have an effect on virulence in *P. teres* Lai et al. (2007). However, Lai et al. (2007) could not confirm if the genes identified encoded virulence factors or avirulence factors. Lai et al. (2007) also found that *AvrHar* and *AvrPra2*, two avirulence genes on barley cultivars Harbin and Prato, co-segregated but in repulsion; implying that they were closely linked or the same gene (Lai et al. 2007). Afanasenko et al. (2007) preformed a study looking at the gene-for-gene relationship between barley and *P. teres* f. *teres*, proposing an interaction much like what Flor (1956) described in the flax rust system. Afanasenko et al. (2007) concluded not only that barley had multiple resistance genes but also that there were multiple genes within *P. teres* f. *teres* accounting for differences in pathogen virulence, similarly they found a correlation between the number of resistance genes identified in the barley lines used in their study and the number of avirulence genes within the different isolates used. Afanasenko et al. (2007) also discussed the possibility of suppressor genes within *P. teres* f. *teres*, suggesting that these genes

may account for what appeared to be active virulence genes in the pathogen. The use of suppressor genes was observed by Jones et al. (1988) in the flax rust system; in this system mutated suppressor genes found within the pathogen affected the virulence by activating or inactivating both the virulence and avirulence genes. However, these differences can also be explained without the presence of suppressor genes if the host is assumed to have dominant susceptibility genes rather than dominant resistance genes.

Molecular Markers

There have been several different types of molecular markers used to map virulence associated with *P. teres* f. *teres*. Markers are used to identify areas of interest within a genome, markers segregate with genes of interest allowing for the location of a gene to be identified without knowing the actual gene sequence (reviewed by Loxdale and Lushai 1998). Although there are several types of markers that can be used to produce a genetic map, AFLP and SSR markers have been used often in the identification of virulence loci within a pathogen like *P. teres* f. *teres* because they are PCR based, easy to use, and are highly repeatable from lab to lab. Amplified fragment length polymorphism (AFLP) markers are generated using two different selective restriction enzymes, one rare cutter and one more frequent cutter, to digest and amplify the fungal genomic DNA into smaller fragments typically ranging from 45-500 nucleotides in size (Vos et al. 1995). Depending on where these restriction enzymes cut the DNA, different size fragments are amplified and will separate when the samples are run on an agarose or polyacrylamide gel. If the marker is polymorphic, the DNA amplicon of the parental isolates will differ, allowing for the progeny to be scored according to their likeness to one parent isolate or the other.

Another type of marker often used with the creation of genetic maps is the simple sequence repeat (SSR) marker, often referred to as a microsatellite marker. SSR primers are created by taking advantage of the unique DNA sequences flanking simple sequence repeats in the fungal genome. The fungal isolates should only differ in the repeated regions allowing primers to be made based on the DNA sequence found immediately before and after the repeats. The differing number of repeats affects the size of the amplicon seen when separating the DNA on a gel (Röder et al. 1998).

By using multiple forms of molecular markers, it is possible to increase the saturation of markers along the organism's genome. High saturation and marker coverage make it easier for the identification and fine mapping of gene regions associated with a phenotype because there is less distance between each marker and a smaller area of focus especially when looking at quantitative trait loci. Ellwood et al. (2010) and Lai et al (2007) used both AFLP and SSR markers in the generation of the *P. teres* f. *teres* genetic linkage maps and ultimately in the identification of virulence/avirulence factors.

QTL Analysis

Once a genetic map has been created, phenotypic data can be used in conjunction with the linkage map to locate and characterize quantitative trait loci (QTL) within the genome of interest. QTL analysis is used to identify areas within the genome that harbor loci contributing quantitatively to a trait (Kearsey 1998). By identifying these loci of interest, phenotypic traits can be limited to specific regions of a chromosome, eventually allowing for the identification of genes (Miles et al. 2008). These genes can be dominant, incompletely dominant, or work additively (Grewal et al. 2008). Single genes associated with disease resistance/susceptibility are sometimes difficult to identify because these genes are often part of a group of multiple genes

contributing quantitatively, rather than a single qualitative gene. Quantitative genes often contribute to a trait in an additive manner where additional genes contribute to a stronger phenotype. In the case of additive genes involved in disease, additional genes would increase susceptibility or resistance depending on the action of the genes. (Grewal et al. 2008). QTL analysis allows for the identification and characterization of multiple genes from the same or different chromosomes in a mapped genome. Genes that contribute quantitatively to a trait work together, each adding different degrees of significance to the quantitative trait outcome (Weiland 1999).

QTL analysis has been used to identify genes within several organisms including plants, animals, and fungi (Kearsley 1998; Miles et al. 2008). As noted previously, several genes have already been identified in the *P. teres* f. *teres*-barley pathosystem; with several host susceptibility and resistance genes being identified on barley chromosome 6H (Friesen et al. 2006; Cakir et al. 2003; Emebiri et al. 2005; Manninen et al. 2006) and several avirulence and virulence genes being identified within the pathogen as well (Weiland et al. 1999; Lai et al. 2007; Afanasenko et al. 2007). In the case of the host, markers associated with disease QTL can be used in a marker assisted selection scheme to pyramid multiple genes associated with a resistant phenotype (ZhengQiang et al. 2004).

With the improvement of statistical programs created to identify QTL with the input of genetic maps and molecular marker data, smaller effect loci can be identified within both the host and pathogen, ultimately resulting in the cloning of the genes underlying these QTL. Here we hope to succeed in identifying and characterizing additional virulence genes within *P. teres* f. *teres* with the ultimate goal of characterizing the genes underlying the QTL. By identifying these genes within the pathogen, we can more effectively identify and understand the

corresponding genes in the host that cause susceptibility. This information can be provided to breeders to more effectively breed for the control of disease.

Fungal Genome

Ellwood et al. (2010) reported a fungal genetic map for *P. teres* f. *teres*. *P. teres* f. *teres* was the third plant pathogenic ascomycete to be assembled and published, with the previously sequenced and assembled fungi being *Magnaporthe grisea* and *Stagonospora nodorum*. Much like these two fungal genomes, the predicted number of genes within *P. teres* f. *teres* was estimated at 11,799 genes, many of which are likely important for pathogenicity and virulence (Ellwood et al. 2010). The published map of *P. teres* f. *teres* consisted of 25 linkage groups, which resulted in a map of 2,477.7 cM in length. These linkage groups were created by using 243 markers, including AFLP, SSR, random amplified polymorphic DNA (RAPD), and the mating type locus. *P. teres* f. *teres* is thought to have 9-11 chromosomes, and the genome size has been reported to range from 35.5-42.3 Mbp. A large portion of the size could be due to the presence of transposable elements, recombinogenic, repetitive elements and possible repetitive DNA within the genome; although the amount of these within the genome of *P. teres* is still unknown (Ellwood et al. 2010).

Mehrabi et al. (2007) found with *Mycosphaerella graminicola*, a wheat foliar pathogen, that both effectors and other host-specific genes are often found near areas known to have one or more transposons present. The identification of secreted proteins, avirulent proteins, and necrotrophic effects are of great interest. Completed genomes such as this can be used to identify candidate genes that code for such proteins. Ellwood et al. (2010) found that there were 1,031 genes within the *P. teres* f. *teres* genome that are predicted to encode for secreted proteins.

The products of necrotrophic effector genes are often small, secreted, cysteine-rich proteins. These proteins are unique with little conservation, allowing for greater host specificity (Ellwood et al. 2010; Stergiopoulos and de Wit 2009).

Large strides have been made in the understanding of the barley-*P. teres* f. *teres* pathosystem since it was first discovered. By using knowledge obtained from the previous studies on disease development, favorable environmental conditions, molecular markers, and QTL analysis, we will continue by identifying virulence loci within the pathogen. Virulence loci are identified by QTL analysis with the use of molecular markers such as AFLP and SSR markers to create genetic linkage maps of the *P. teres* f. *teres* 15A × 6A population. The genetic maps will be combined with phenotypic data collected from inoculations of progeny from this same population on a set of barley lines that differ for resistance/susceptibility to the parental isolates used in the population. Once these loci are identified, sequenced and assembled, the published *P. teres* f. *teres* genome material can be used to help identify the specific virulence genes within the pathogen and eventually lead to the identification of specific susceptibility genes within the host.

IDENTIFICATION OF MULTIPLE VIRULENCE LOCI IN *PYRENOPHORA TERES* F. *TERES* ASSOCIATED WITH NET FORM NET BLOTCH IN BARLEY

Introduction

Pyrenophora teres f. *teres* is an ascomycete that causes the foliar disease net form net blotch (NFNB) on barley (*Hordeum vulgare)*. NFNB, which is prevalent on barley worldwide, received its name because of the net-like lesions that developed after the pathogen had penetrated the leaves of susceptible barley lines (Atanasoff and Johnson 1920; Shipton et al. 1973). Although the disease is most severe in regions of the world with temperate climate paired with high rainfall and high humidity, it can also occur in regions with a variety of temperatures as well as where little rainfall is present (Steffenson and Webster 1992). Outbreaks of NFNB often produce yield losses of 10-40%, by means of reduced kernel size, although the entire crop can be destroyed in extreme situations where the environment is ideal for the pathogen to infect a susceptible host (Mathre 1997).

There are both qualitative and quantitative resistance sources to *P. teres* f. *teres* The qualitative relationship between the pathogen and host has been proposed to follow a gene-for-gene model, in which disease resistance requires the presence of both a resistance (*R*) gene in the host and an avirulence (*Avr*) gene (Afanasenko et al. 2007; Flor 1956) in the pathogen. *P. teres* f. *teres* is an ideal pathogen for investigating a host pathogen interaction due to several avirulence/virulence genes being present in the pathogen (Weiland et al. 1999; Lai et al. 2007; Beattie et al. 2007; Mode and Schaller 1985) and several resistance/susceptibility genes being present in the host (Friesen et al. 2006; Cakir et al. 2003; Emebiri et al. 2005; Manninen et al. 2006; Abu Qamar et al. 2008). Recent information from our lab has shown that both gene-for-

gene (Friesen et al. 2006) and inverse gene-for-gene interactions (Abu Qamar et al. 2008; Liu et al. 2010) are involved in disease resistance and susceptibility. An inverse gene-for-gene interaction would likely involve one or more necrotrophic effectors (synonym: host-selective toxins) that interact with susceptibility targets in the host (Friesen et al. 2008). In this case, quantitative resistance would be best explained by an inverse gene-for-gene model.

In this study, we aimed to identify virulence loci within the NFNB pathogen *P. teres* f. *teres* that were associated with various barley lines that differed for their reaction to parental isolates 15A and 6A. *P. teres* f. *teres* isolates 15A and 6A were used to identify several barley lines that showed a differential reaction (i.e. they were susceptible to one parent and resistant to the other). 15A×6A progeny were inoculated on these barley lines and the phenotypic data were used along with marker data to identify QTL within the fungal genome that were associated with virulence.

Materials and Methods

Development of a *P. Teres* F. *Teres* Population

Isolates 15A (Steffenson and Webster 1992) and 6A (Wu et al. 2003) were both collected from California but have different virulence patterns on the lines used in this study. A population of 118 progeny from a cross between *P. teres* f. *teres* isolates 15A and 6A was created as described by Lai et al. (2007). Briefly, 100µl of 15A and 6A inoculum (4000 spores/mL diluted in water) was pipetted onto opposite ends of a sterilized wheat stem. Five wheat stems were placed on one plate of Sach's media (1g $CaNO_3$, 0.25g $MgSO_4 7H_2O$, trace $FeCl_3$, 0.25g K_2HPO, 4g $CaCO_3$, 20g Agar, H_2O to 1L) and stored in the dark at 13°C until fruiting bodies began to develop on the wheat stems (approximately 3 months); after this point

each wheat stem was transferred to a lid of a water agar plate and placed in the same incubator, with the water agar above the wheat stem. The samples were placed in a 12 hr light/dark cycle; the ascospores from the cross were randomly collected from a water agar plate that was placed over the pseudothecia. Individually collected ascospores were then allowed to germinate and a small portion of this fungal sample was then spread across a section of water agar so that single spores could be collected. Each progeny was transferred to a V8PDA (150mL V8 juice, 10g difco PDA, 3g CaCO3, 10g agar, and 850mL distilled water) plate and progeny were single-spored twice by isolating individual conidia to ensure genetic purity of the samples. Pure isolates were stored at -20°C as 8mm diameter dried plugs after being grown and collected according to Lai et al. (2007).

Inoculation of Barley Lines

Conidia from 15A, 6A, and the 118 progeny were collected for inoculum to be used on a collection of barley genotypes consisting of Rika, Kombar, Hector, NDB112, and PI 356715, a barley core collection line obtained from the USDA-ARS National Small Grains Collection. The genotypes Hector and NDB112 served as susceptible and resistant checks, respectively, to ensure even inoculations between progeny isolate applications. The inoculum was generated by placing a lyophilized plug on a V8-PDA plate for 5-7 d at 20°C in a dark cabinet, 24 hrs at room temperature in the light, followed by a 24 hr dark period at 13°C. After the isolates had grown out and gone through the above light cycles, the plates were flooded with 100 mL of sterile distilled water and gently scraped with an inoculating loop. The collected inoculum was then diluted to a concentration of 2000 conidia per mL and 3 drops from an eyedropper (approximately 30µl) of Tween 20 was added to the inoculum per every 50mL of spore suspension. Plants were grown to the 2.5 leaf stage, (approximately 14 d) according to Lai et al.

(2007). The plants were grown in racks of 49 cone-tainers with a border of Robust barley surrounding the lines of interest to reduce any edge effect. Each rack of plants was inoculated according to Friesen et al. (2006), by using a paint sprayer (Huskey, model # HDS790) for application until each leaf was almost to the point of inoculum runoff. After the plants were inoculated they were placed in mist chambers for 24 hrs at 100% humidity, a temperature of 21°C and a 24 h light cycle. Plants were then moved to a growth chamber under a 12hr photoperiod at 21°C until evaluation. Seven days after inoculation, the plants were evaluated for disease symptoms and rated based on the Tekauz (1985) scale. A total of three replicates across the barley line set were completed for each parental and progeny isolate.

Fungal DNA Extraction

Fungal DNA was extracted from all collected isolates by placing a dried plug on a V8-PDA plate and allowing it to grow in the dark for 7-10 d. After the fungal isolate had covered the plate, the arial mycelial tissue was removed and placed in a 2mL microcenterfuge tube before being placed in a lyophilizer overnight. After all the mycelial tissue samples had been collected and dried, each sample was ground using a small pestle and 500µl of lysing solution (Qiagen BioSprint 15 Plant extraction kit (360)) was added to the ground tissue; the solution was vortexed and centrifuged for 5 min at 6,000g. DNA was extracted using the Qiagen BioSprint 15 Plant extraction kit (360) (200µl alcohol + 20µl MagAttract Suspension + 200µl DNA supernatant, 500µl RPW buffer, 1000µl of 100% ethanol split between two wells, and 200µl sterile water or TE buffer).

AFLP and SSR Marker Selection

The fungal DNA was then used for SSR (Röder et al. 1998) and AFLP analysis (Vos et al. 1995) on polyacrylamide gels with the LI-COR IR^2 DNA sequencer model 4200 global edition. For AFLP analysis, forty-nine AFLP marker combinations were used to generate markers for the genetic mapping. PCR was done by using the *Eco*RI (EcoRI adapter1: 5'-CTCGTAGACTGCGTACC; EcoRI adapter2: CATCTGACGCATGGTTAA-5)' and *Mse*I (MseI adapter1: 5'-GACGATGAGTCCTGAG; MseI adapter2: TACTCAGGACTCAT-5') adaptor sequences with two bases added at the 3' ends (Lai et al. 2007) (Table 1). Because two bases were added, the AFLP primers and fungal DNA were fixed using the following PCR conditions adapted from Vos et al. (1995). The procedure was started with the *EcoRI* adapter at 5µM (25µl *Eco*RI forward adapter + 25µl *EcoRI* reverse adapter + 450µl TE buffer) and the *Mse*I adaptor at 50µM (250 µl forward *Mse*I forward adapter + 250µl reverse *Mse*I adapter) were denatured at 95°C for 5 min. Next, 5.5µl of template DNA diluted to 10-20ng/µl and 5.5µl of the ligation master mix [1µl T4 buffer 10X + 1µl NaCl 0.5M + 0.5µl BSA @ 1mg/mL + 1µl enzyme master mix (0.1µl T4 Buffer 10X+ 0.1µl NaCl+ 0.05 BSA @ l mg/mL+ 0.1µl *Mse*I: 1 unit + 0.12µl *EcoRI*: 5 units+ 0.2µl T4 ligase: 1 unit+ 0.33µl distilled H_2O) + 1µl *Mse*I adapter + 1µl *EcoRI* adapter] were mixed together and run for 2 hrs at 37 °C, then diluted 1:10 with TE buffer. 2.5µl of the diluted adaptor ligated DNA was then mixed with 27.5µl pre-amplification master mix (3µl buffer without $MgCl_2$ + 0.9µl $MgCl_2$ + 1µl *EcoRI* primer 50ng/µl + 1µl *Mse*I primer 50ng/mL + 6µl dNTPs 1 mM each + 0.5µl *Taq* polymerase + 15.1µl ddH_20) and run for 20 cycles at 94°C for 30s, 56 °C for 1min and 72 for 1 min. To ensure all the previous steps, denaturing, ligation and pre-amplification had succeeded; 2µl of the pre-amplification DNA and

Table 1. AFLP Primer Data.

MSE I primer	Eco RI primer	# of markers mapped	# of markers
M-AA	E-CC	3	3
M-AA	E-CG	3	3
M-AA	E-GC	2	7
M-AC	E-AT	3	3
M-AC	E-CT	3	3
M-AG	E-AT	4	4
M-AG	E-CC	1	3
M-AG	E-CT	4	6
M-AG	E-GC	1	1
M-AT	E-CA	5	8
M-AT	E-CC	2	3
M-AT	E-CG	2	2
M-AT	E-CT	2	3
M-AT	E-GC	6	8
M-AT	E-GG	5	4
M-AT	E-GT	4	5
M-CA	E-AA	3	4
M-CA	E-AC	5	5
M-CA	E-AG	7	8
M-CA	E-AT	4	7
M-CA	E-CA	3	3
M-CA	E-CC	2	2
M-CA	E-CG	2	3
M-CA	E-CT	3	3
M-CC	E-CA	1	4
M-CC	E-CT	4	3
M-CC	E-GT	5	4
M-CG	E-AA	4	5
M-CG	E-AT	1	2
M-CG	E-CC	5	5
M-CG	E-CT	1	2
M-CT	E-AA	3	4
M-AT	E-CT	5	7
M-CT	E-AT	6	8
M-CT	E-CC	2	2
M-CT	E-GC	2	3
M-GA	E-AT	3	3
M-GA	E-CA	1	5
M-GA	E-GC	3	6
M-GC	E-CT	3	4
M-GG	E-AG	1	7
M-GG	E-AT	5	7
M-GG	E-CA	4	5
M-GT	E-AT	4	6
M-GT	E-CC	8	7
M-GT	E-CT	3	5
M-GT	E-AA	0	4
M-GT	E-AC	0	3

49 AFLP marker combinations were used. The first column lists the *Mse*I primer plus the two-nucleotide extension. The second column lists the *Eco*RI primer plus the two-nucleotide extension. All of the markers created from the combination of the *Mse*I and *Eco*RI primers are listed in the last column, however not all markers identified were included in the final genetic map. The markers mapped from each of the combinations are listed in the third column.

1µl blue juice (35mL glycerol + 2.5mL 10× NEB buffer + 2mL 0.5M EDTA + 1mL 10% SDS + 50mg bromphenol blue + H_2O to 50mL) was run on a 1% agarose gel at 100v for 40 min. After the DNA was ran on the gel to ensure a successful digestion, pre-amplifaction DNA was diluted 1:40 with TE buffer. A 2µl aliquot of the diluted pre-amplification DNA was then mixed with 16µl of the selective amplification master mix (2µl 10×Taq Buffer without $MgCl_2$ + 0.2µl *Taq* polymerase + 0.8µl $MgCl_2$ + 2.4µl dNTPs 1mM each + 0.08µl *EcoRI*-NNN primer 10µM + 0.4µl *Mse*I-NNN primer 10µM + 10.12µl H_2O and ran at 94°C for 30 sec, 65°C for 30 sec, 72°C for 1 min, 94°C for 30 sec, 65°C for 30 sec and 72°C for 1 min. The cycle of 94°C for 30 sec, 65°C for 30 sec and 72°C for 1 min was repeated eleven times with the 65°C temperature decreasing by 0.7°C each cycle. After the eleventh cycle, the procedure continued with 94°C for 30 sec, 56°C for 30 sec and 70°C for 1 min. These last three steps were repeated for 22 cycles before the process was completed and ready to be separated on a polyacrylamide gel.

Fifty-five SSR primer sets were tested on the parental isolates 15A and 6A to identify polymorphism (Table 2); of the primer sets tested 41 were found to be polymorphic and were used on the entire 15A×6A mapping population. To do this, 2µl of fungal template DNA at 10-20ng/µl was added to 8µl SSR master mix (1µl 10× Biolase buffer + 2µl dNTPs 1mM each + 0.3µl forward primer 10pmol/µl + 0.3µL reverse primer 10pmol/µL + 0.3µL M13 1pmol/µL + 0.3µL Taq polymerase + 3.8µL H_2O); the samples were then placed in the thermo cycler and programmed to run at 95°C for 5min, 95°C for 20 sec, 50°C for 20 sec, 72°C for 30 sec, then repeated the 2^{nd} -4^{th} step for three cycles, 95°C for 20sec, 52°C for 20 sec, 72°C for 30 sec, then repeated the 6^{th} – 8^{th} step for 32 cycles and then brought down to 4°C for storage.

After the AFLP and SSR procedures had been completed, 2µL of Li-cor loading dye was added to each of the sample wells and approximately 2µL of the sample was loaded onto a

Table 2. SSR Primer Data.

Marker name	Repeat motif	Forward primer sequence	Reverse primer sequence
CTG15	CTG	GATGCAGCTCCTCATTTGTG	GTATCCATTTGTCTCGTGATGG
AGC15	AGC	TCAATCTCGGCATTCTTCCT	GTAACCCGTAGCCCGTAGC
GTG17	GTG	GATGCCAGAAGAATGGTAGAAGA	AGTAGCACAAGCGAACAAGATG
GGA57	GGA	TTTACGAATGATGAGCTTGTTG	TGGTTTCTCTAGCACCTTATCC
CTA14	CTA	TTAAATAGAGGTGGGTTGCTG	CGATGGCGATATGAGTAAGATT
GAT13	GAT	CCGGCCACCTACTTACTTAGATT	GCGACTTACATCTGCATACCC
CAG31	CAG	ACACAGAGCTGAAGCAGAAATG	AAGTTGTGGCTGTTGCTGATA
ATC16	ATC	ACGTTTCAAGCATTTACATTCG	TTCCTGCTCAACCAGTCTACAA
TGT12	TGT	GTTGGAAGGCCATAAGATGC	AATTGGAAGTCATAGCGACACA
GTC15	GTC	CCCGAGAACTCACTCTGCT	GAAGCCAGAAGCTAGACTCACC
CTG19	CTG	CCGGATTCATACCCACTATGTT	CGCCTTCATATAACGAGACTACA
TCA29	TCA	GCTCTTCCTGCTTCTGTAGGAC	GCATCCAATATCTTTGCGTGTA
CTA18	CTA	TGGCTATATCAAACGTAATGTCG	ATTTGGGTAAATGCTGACGAGT
CGA24	CGA	CAGAGGATAGCGTGTTTGAGG	GCCATTGCCCTCGACTAA
CTG35	CTG	TTGTAGGCAAGGCATCATCTAA	CAGATACATCAGACAAGCACTGG
GCA85	GCA	CGGTCACAGTCTCTCCGTAT	TTGTTGGTATTGTGGCTGGA
TGA16	TGA	GGATAGTTTCTCCAGCTCGTTC	AACCAGGCATCGACTTCTTC
ACA18	ACA	CATGTCGCTGCTCCTACACT	GGCCAGACTATTGGAATGTGA
TGC66	TGC	CATAATAGTACACACGCCATGC	GTAGGGAGCAGCGACGAC
CAT15	CAT	CGTCGTATCTCGATCTGTTCAT	CCCATGTGCTAGATTTGAGTGT
TGT32	TGT	ACTGTTGAGGTGCTGAAGATGA	CAGATTGGACGACAAGACTCAC
ACA14	ACA	CGCTGGTACATCTTCATTTCAG	CGCATAGTTGGAGTGTGGATAG
AGC14	AGC	CATGCAGAGTGGTCAGAAAGAC	TGCTCCACACCATGTTTACTTT
GCT51	GCT	CGCTGATACTGAGCAAACTCAT	CTCTTATCTGGCAAAGGTTCAG
CAG31	CAG	ACACAGAGCTGAAGCAGAAATG	AAGTTGTGGCTGTTGCTGATA
GAC27	GAC	GTATGGCCTCTCTTGCAGTTC	GTCCACCTGGCGAAGTATCTA

SSR primers used in the generations of the *P. teres* f. *teres* genetic maps. In the first column, the repeated motif is identified followed by the forward and reverse sequence of each primer.

polyacrylamide gel (20mL rapid gel polyacrylamide + 20µL Temid + 120µL APS) and run for 2 hrs or until the polymorphic bands were observed, shorter gel time was only used for SSR marker runs. Fragments that were polymorphic between the two parental isolates and progeny were scored. 15A and 6A marker types were assigned 0s and 1s respectively, for subsequent mapping. Marker nomenclature was based on the primer combination and base pair size according to the marker standard.

Genetic Map Construction

Linkage groups were created using Mapmaker v2.0 for Macintosh (Landers et al. 1987) using the Kosambi mapping function according to Liu et al. (2005). The "group", "ripple", and "first order" functions were used starting with large groups with a minimum LOD of 3.0 and working into smaller groups with a LOD threshold of 12.0. Once linkage groups were assembled with a minimum LOD of 3.0, maps were generated. Once the order of the group had been established, any markers that had been removed from the original group because of distance extremes were put back into the program to ensure they were accurately removed from the current map. A total of 24 linkage groups were created.

QTL Analysis

The average value of the three replicates of phenotypic data and linkage map data were put into the *Q Gene* program (Joehanes and Nelson 2008) as described in Liu et al. (2008), using the 'resampling' function to calculate the significance value for each of the barley genotypes inoculated, at $\alpha = 0.05$ (Table 3). QTL were observed using the 'single trait multiple interval mapping' and 'composite' functions. The QTL found on unlinked markers were checked to ensure that the markers were not originally associated with markers in linkage groups that also

Table 3. Barley Lines Used In Study with Reaction to Parental Isolates and Virulence QTL.

Barley Line	15A	Avg Score	6A	Avg Score	qtl.VirK1	qtl.VirR1	qtl.VirP1	qtl.VirR2	qtl.VirK2
Rika	Avr	2	Vir	6.3	X	**0.31**	X	**0.14**	X
Kombar	Vir	7.2	Avr	1.6	**0.26**	X	X	X	**0.21**
PI 356715	Vir	6.3	Avr	1.8	X	X	**0.52**	X	X

All of the barley lines inoculated with the 15A × 6A *P. teres* f. *teres* population are listed in the column furthest to the left, followed by the virulence reaction of the parental isolates on each line and the disease score average from all three replicates. 6A is virulent on barley line Rika while 15A is virulent on barley lines Kombar and PI 356715. The R^2 values (α= 0.05) for each of the QTL identified are listed in the same row as the associated barley line. Virulence qtl.VirR1 and qtl.VirR2 were significant when 15A × 6A progeny are inoculated on Rika, virulence qtl.VirK1 and qtl.VirK2 were significant when 15A × 6A progeny are inoculated on Kombar, and virulent qtl.VirP1 was only significant when *P. teres* f. *teres* progeny were inoculated on PI 356715. In total, five different virulence QTL where identified, three coming from parental isolate 15A and two coming from parental isolate 6A.

showed QTL or had incomplete QTL arches observed. By doing this, each QTL was verified as an individual and significant QTL. Five linkage groups were found to have QTL present on them. To look at the influence of multiple QTL on the virulence trait, multiple regression tests were performed with DataDesk 4.1 by using the "regression" function.

Statistical Analysis

To test for homogeneity between the three replicates for each barley genotype individually, a Barlett's χ^2 test was used. In this analysis, the value obtained was compared to the χ^2 table value at P=0.05 with 2 degrees of freedom. Replicates that were not significantly different at the P=0.05 level were combined for QTL analysis.

The least significant difference (LSD) test was used to look for significant differences between average disease scores when the 15A×6A *P. teres* f. *teres* population was separated into groups that contained different QTL pairings. SAS 9.3 (SAS Institute Inc. 2011) was used to perform this test at α=0.05. If the difference between two groups was greater than the LSD value calculated, then there was a significant difference between the two groups.

Results

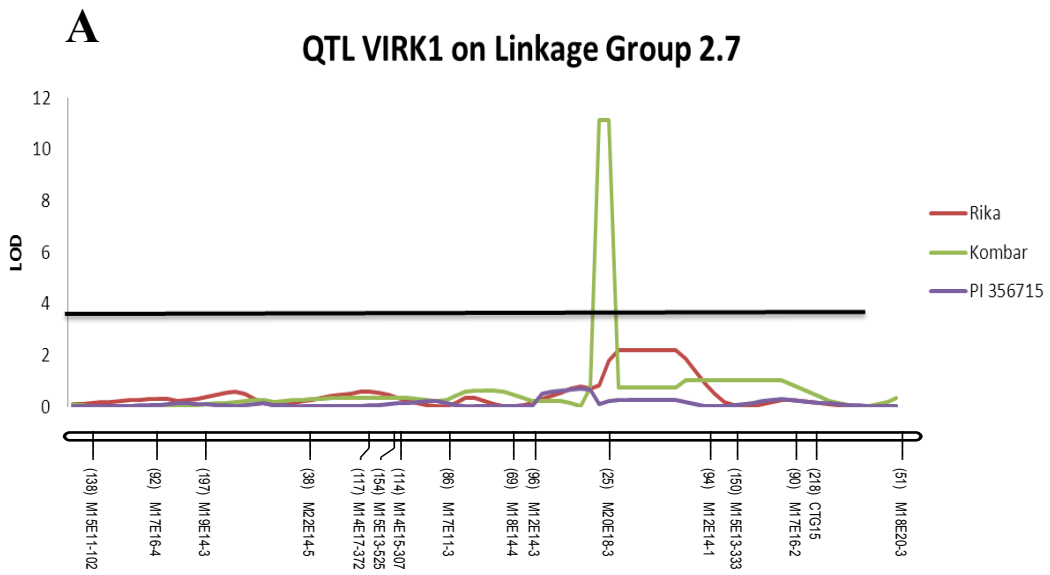
In all, five different virulence QTL were identified in the *P. teres* f. *teres* 6A × 15A population. Two virulence QTL were found to be associated with Rika and two different virulence QTL were identified in association with the barley line Kombar. A single unique virulence QTL was associated with PI 356715. (Table 3, Figure 1)

Virulence QTL were analyzed with the use of the average of the three replicates performed as inoculations on the barley genotypes Rika, Kombar and PI 356715. Each of the three replicates were tested for homogeneity by the use of Barlett's χ^2 test (Chu et al. 2010), when P= 0.05 all reactions were found to be homogenous between replicates (Table 4).

QTL Associated with Barley Line Kombar

Kombar, on which 15A was virulent and 6A was avirulent (Figure 2), was used to identify two virulence QTL. The first virulence QTL, qtl.VirK1 (Figure 1A), was found on linkage group 2.7 (Figure 3) and had an R^2 value of 0.26 (Table 3). The second QTL, qtl.VirK2 (Figure 1E), was found on an unlinked marker (M13E18-3) and had an R^2 value of 0.21 (Table 3). The R^2 value of 0.394 was found when a multiple regression analysis was performed using the 15A (virulent parent) markers (M17E11-3 and M13E18-3) most closely associated with each of the QTL (Table 5). A least significant difference (LSD) test was also performed on the

Figure 1. Five Virulence QTL Identified.



Above is the graphical depiction of the five virulence QTL identified in the 15A × 6A population. The X-axis is the linkage group depicted below the graph symbolizing where the QTL lie in relation to the markers within the linkage groups. The Y-axis shows the LOD values. The black line on the graphs represents the LOD threshold at 3.8. **A.** Virulence QTL, qtl.VirK1 on linkage group 2.7 has a LOD value of 11 (R^2=0.26), is contributed by the parental isolate 15A and is associated with barley line Kombar. **B.** Virulence QTL, qtl.VirR1 on linkage group 9.1 has a LOD value of 14.0 (R^2=0.31), is contributed by the parental isolate 6A and is associated with barley line Rika. **C.** Virulence QTL, qtl.VirP1 on linkage group 8.1 has a LOD value of 19 (R^2=0.52), is contributed by the parental isolate 15A and is associated with barley line PI 356715. **D.** Virulence QTL, qtl.VirR2 on linkage group 17.1 has a LOD value of 7.8 (R^2=0.14), is contributed by the parental isolate 6A and is associated with barley line Rika. **E.** Virulence QTL, qtl.VirK2 found associated with the unlinked marker M13E18-3 has a LOD value of 8.9 (R^2=0.21), is contributed by the parental isolate 15A and is associated with barley line Kombar.

Figure 1. Five Virulence QTL Identified (continued).

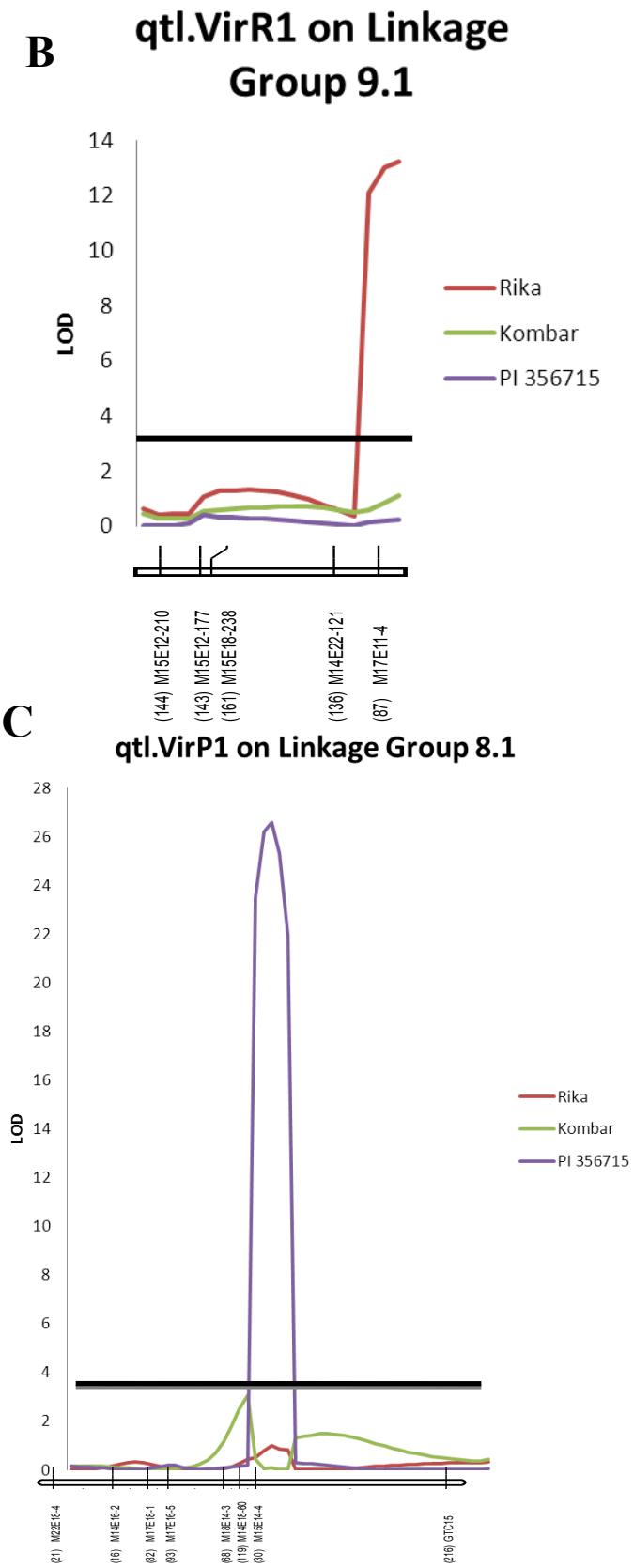


Figure 1. Five Virulence QTL Identified (continued).

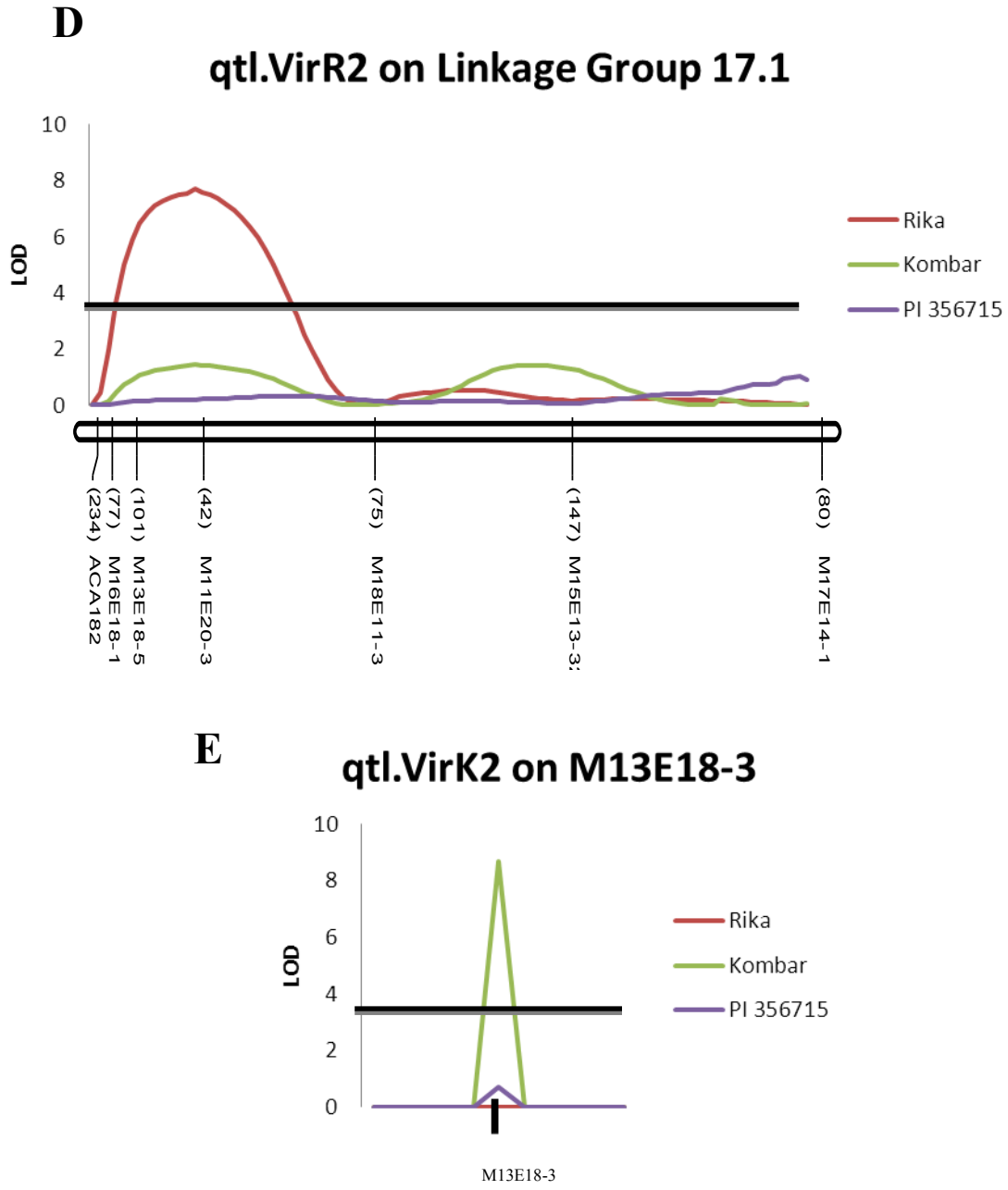


Table 4. Bartlett's χ^2 Test Analysis.

Barley Line	χ^2 (df=2)	P value
Rika	1.6	>0.30
Kombar	3.41	>0.10
PI 356715	2.89	>0.20

To check for homogeneity between the three replicates, a Bartlett's χ^2 test was performed on each of the barley lines used in this study. Replicates were not significantly different at the *P*=0.05 level.

phenotypic data from four genotypic classes of the 15A × 6A progeny including qtl.VirK1/qtl.VirK2, qtl.VirK1/qtl.virk2, qtl.virk1/qtl.VirK2, and qtl.virk1/qtl.virk2 (Table 6). The LSD analysis showed that there was a significance difference between the genotypic classes that had both QTL (i.e. qtl.VirK1/qtl.VirK2) and genotypes that had either qtl.VirK1 or qtl.VirK2 (i.e. qtl.VirK1/qtl.virk2 or qtl.virk1/qtl.VirK2). There was also a significant difference between genotypes harboring neither QTL (qtl.virk1/qtl.virk2) and those genotypic classes harboring one or the other QTL (i.e. qtl.VirK1/qtl.virk2 or qtl.virk1/qtl.VirK2), however there was no significant difference between genotypic classes harboring only one of the virulence QTL (i.e. qtl.VirK1/qtl.virk2 or qtl.virk1/qtl.VirK2).

QTL Associated with Barley Line Rika

Rika, on which 6A was virulent and 15A was avirulent (Figure 2), was used to identify two virulence QTL, both QTL were different from those identified on Kombar. qtl.VirR1 (Figure 1B) was found on linkage group 9.1 (Figure 3) having an R^2 value of 0.31 (Table 3). qtl.VirR2 (Figure 1D) was found on linkage group 17.1 (Figure 3) and had an R^2 value of 0.14 (Table 3). The R^2 value of 0.394 was found when a multiple regression analysis was performed using the 6A (virulent parent) markers (M17E11-4 and M17E14-1) most closely associated with each of

the QTL (qtl.VirR1 and qtl.VirR2) (Table 5). A least significant difference (LSD) test was also performed on the phenotypic data from four genotypic classes of the 15A × 6A progeny including qtl.VirR1/qtl.VirR2, qtl.VirR1/qtl.virr2, qtl.virr1/qtl.VirR2, and qtl.virr1/qtl.virr2 (Table 6). The LSD analysis showed that there was no significant difference between the genotypic classes that had both QTL (i.e. qtl.VirR1 and qtl.VirR2) and the genotypic that had only qtl.VirR1, the more significant of the two QTL identified in inoculations on Rika. There was a significant difference however between the genotypic classes harboring only one of the QTL (i.e. qtl.VirR1/qtl.virr2 vs qtl.virr1/qtl.VirR2) (Table 6). There was also a significant difference between genotypes harboring neither QTL (qtl.virr1/qtl.virr2) and those genotypic classes harboring one or both of the other QTL (i.e. qtl.VirR1/qtl.VirR2, qtl.VirR1/qtl.virr2, or qtl.virr1/qtl.VirR2).

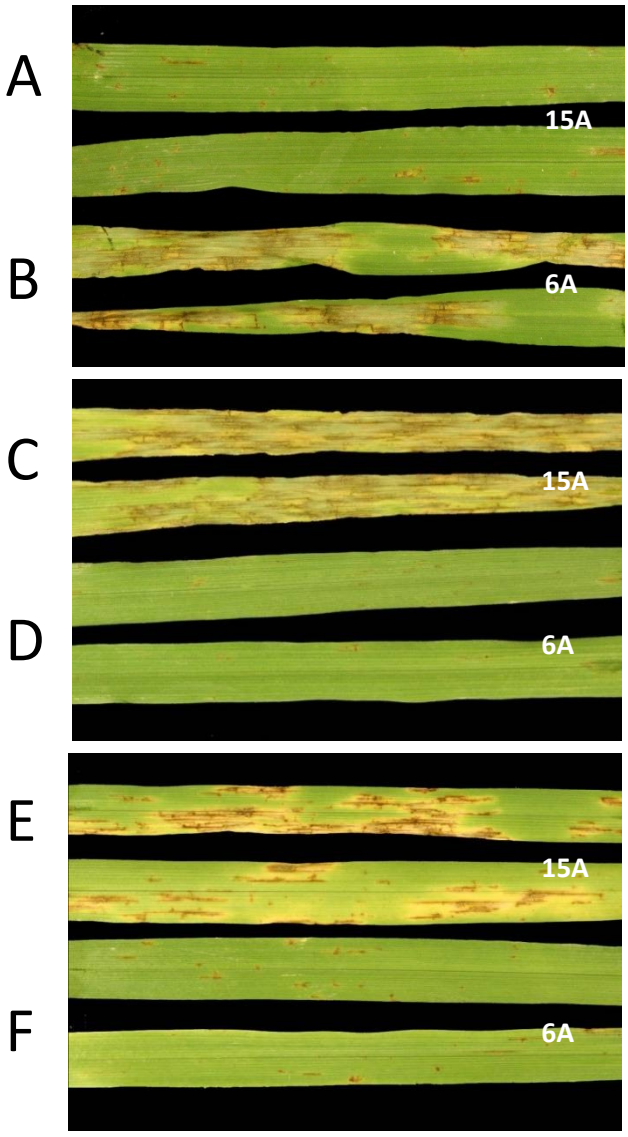
QTL Associated with Barley Line PI 356715

PI 356715 had a similar disease reaction for the parental isolates as that observed on Kombar (Figure 2), however the virulence pattern of the progeny was different and only one major virulence QTL was observed and this QTL, qtl.VirP1 (Figure 1C), was unique to only this genotype. Virulence QTL, qtl.VirP1 was observed on linkage group 8.1 (Figure 3) and had an R^2 value of 0.52 (Table 3). No other significant QTL were observed in association with this barley line.

Discussion

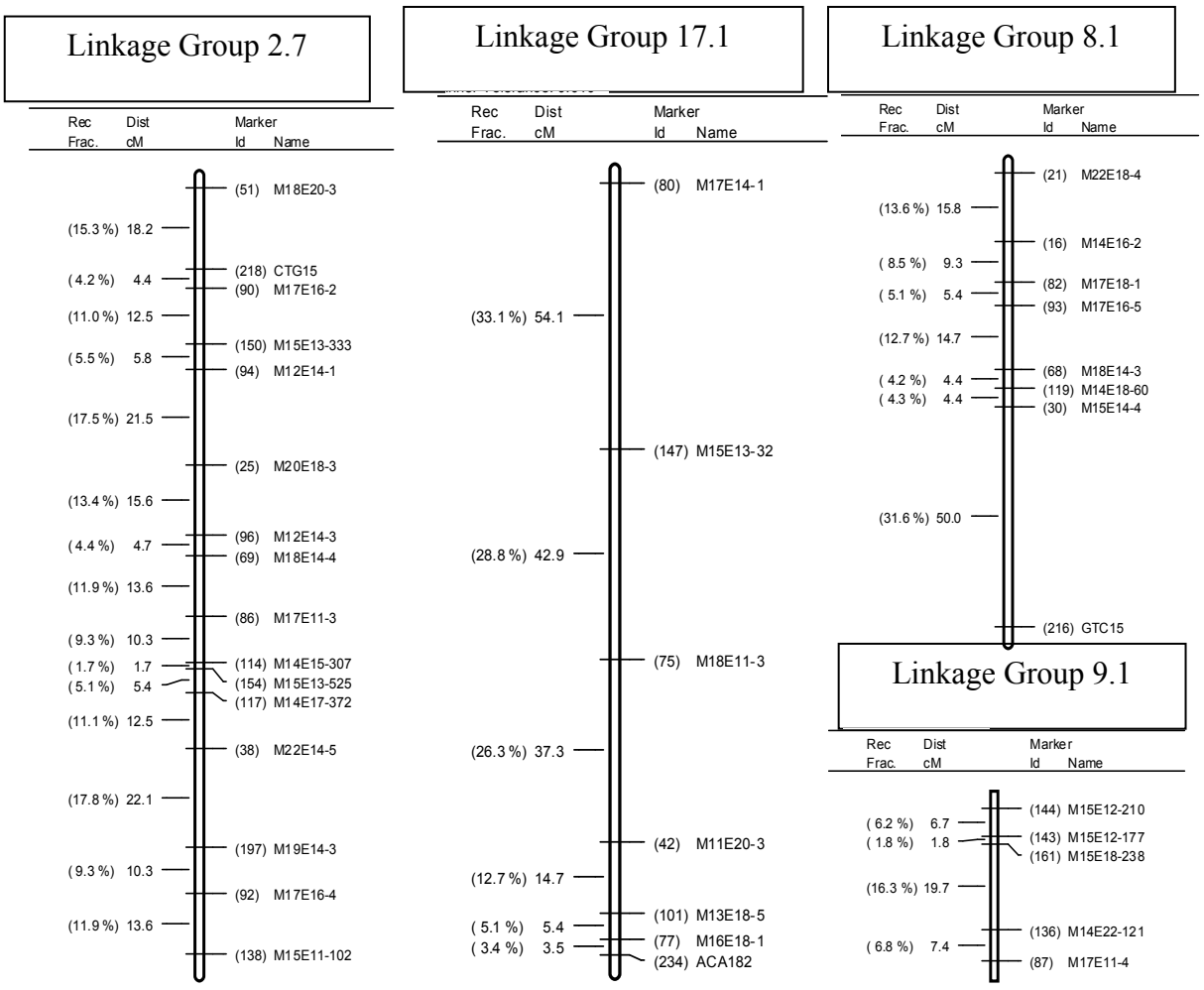
According to both the gene-for-gene and inverse gene-for-gene models, if each of the virulence QTL identified in this *P. teres* f. *teres* population represents unique effectors that

Figure 2. Phenotypic Reactions of Parental Isolates on Barley Line Rika, Kombar and PI356715.



Reactions observed seven days after parental isolates 15A and 6A were inoculated on barley lines Rika (A-B), Kombar (C-D), and PI 356715(E-F). All lines were chosen for this study based on their differential reaction types when inoculated with the parental isolates. 6A is virulent on barley line Rika (B). 15A is virulent on barley lines Kombar(C) and PI 356715 (E).

Figure 3. Linkage Groups of *P. teres* f. *teres* with QTL Identified on.



Linkage groups harboring QTL are listed in order by map size. Linkage groups 2.7, 9.3 and 8.1 each harbor virulence loci conferred from parental isolate 15A. Linkage groups 17.1 and 9.1 each harbor virulence loci conferred from parental isolate 6A.

Table 5. Multiple Regression Analysis.

Marker	QTL	Rika R^2	Kombar R^2	PI356715 R^2
M17E11-3	VirK1	NS	**0.26**	NS
M17E11-4	VirR1	**0.31**	NS	NS
M14E18-60	VirP1	NS	NS	**0.52**
M17E14-1	VirR2	**0.14**	NS	NS
M13E18-3	VirK2	NS	**0.21**	NS
Multiple Regression		**0.394**	**0.394**	NS

R^2 values were calculated with the single trait- multiple interval mapping function of Qgene for each of the five QTL identified. The multiple regression analysis was performed using DataDesk 4.1 (Data Description, Inc., Ithaca, NY) at a significance level of $P< 0.005$. The markers most closely related to qtl.VirK1 and qtl.VirK2 were compared with phenotypic data from Kombar, while the markers most closely related to qtl.VirR1 and qtl.VirR2 were compared with phenotypic data from Rika.

Table 6. Least Significant Difference Analysis.

A. Rika

	# of isolates	Avg Score
15A	X	2
6A	X	7.2
qtl.VirR1/qtl.VirR2	21	7.63 A
qtl.VirR1/qtl.virr2	41	7.27 A
qtl.virr1/qtl.VirR2	19	6.33 B
qtl.virr1/qtl.virr2	37	4.34 C
LSD= 0.7362 when P<0.0001		

B. Kombar

	# of isolates	Avg Score
15A	X	7.2
6A	X	1.7
qtl.VirK1/qtl.VirK2	38	7.25 A
qtl.VirK1/qtl.virk2	33	5.99 B
qtl.virk1/qtl.VirK2	22	5.95 B
qtl.virk1/qtl.virk2	25	3.44 C
LSD= 0.8323 when P<0.0001		

C. PI356715

	# of isolates	Avg Score
15A	X	6.3
6A	X	1.8
qtl.VirP1	66	5.62 A
qtl.virp1	52	2.39 B
LSD= 0.5819 when P<0.0001		

Average disease reaction types for all possible genotypic classes evaluated for significant differences using a least significant difference (LSD) test. The genotypic class is shown in the first column of each table followed by the number of progeny identified in each genotypic class and average disease reaction type score for progeny included in that genotypic class. The presence of the virulence locus underlying each QTL is shown by capital letters (e.g. qtl.VirR1) and those lacking the virulence locus underlying the virulence QTL are designated by lowercase letters (e.g. qtl.virr1). Average disease reaction scores that are followed by different letters are significantly different at the *P*= 0.0001 level. **A.** The least significant difference value is 0.7362. qtl.VirR1/qtl.VirR2 is not significantly different from qtl.VirR1/qtl.virr2, however each of the other groups is significantly different from these two groups as well as from each other. **B.** The least significant difference value is 0.8323. qtl.VirK1/qtl.virk2 and qtl.virk1/qtl.VirK2 are not significantly different from each other; however, each of the remaining groups is significantly different from each other and each of these two groups. **C.** The least significant difference value is 0.5819. Both groups of isolates inoculated on this barley line are significantly different.

interact with a unique barley gene, there should be an equal number of susceptibility/resistance genes in the host as there are virulence / avirulence loci in the pathogen. There were five virulence QTL identified in this study, indicating the presence of five different effector genes within the pathogen. Four different QTL, qtl.VirR1, qtl.VirR2, qtl.VirK1 and qtl.VirK2, were associated with barley lines Rika and Kombar with qtl.VirR1 and qtl.VirR2 being associated with Rika and qtl.VirK1 and qtl.VirK2 being associated with Kombar. From previous research, we know that barley chromosome 6H harbors a region with multiple NFNB resistance/susceptibility genes; both Rika and Kombar are among the lines to harbor dominant susceptibility genes within this region and more specifically, susceptibility associated with both 15A and 6A map to the 6H region, but in repulsion (Abu Qamar et al. 2008). Originally, based on segregation ratios, it was thought that there were only two single dominant susceptibility genes on the 6H chromosome region containing *rpt.r* and *rpt.k*, one in Rika and one in Kombar. However, based on the results presented in this study, it is likely that there are at least four *P. teres* f. *teres* effector gene products that interact, directly or indirectly with four closely linked genes found in the *rpt.r*/*rpt.k* region on barley chromosome 6H. Because we do not currently know a great deal about this region of chromosome 6H or gene clustering in this region, it is possible that the virulence QTL associated with PI 356715 may also interact with genes found within this same region. Populations involving PI 356715 are being developed to characterize the genetics of resistance/susceptibility in this line.

To characterize the impact on virulence of isolates harboring multiple virulence QTL associated with Rika and Kombar, a multiple regression analysis was performed. If the virulence QTL associated with Rika and Kombar were completely additive, then qtl.VirR1 and qtl.VirR2 would account for 45% of the disease observed and qtl.VirK1 and qtl.Virk2 would account for

47% of the disease observed on Rika and Kombar, respectively. Qtl.VirR1 and qtl.VirR2 together accounted for 39.4% (Table 5) of the disease observed on Rika and qtl.VirK1 and qtl.VirK2 accounted for 39.4% (Table 5) of the disease observed on Kombar. These results imply that the virulence QTL are not completely additive but that the pathogen does benefit from harboring both virulence loci in the presence of the corresponding susceptibility genes in the host, as opposed to just harboring one or the other. This is also supported by the data in Table 6 where the different progeny genotypes were compared. The genotypes were derived based on the closest markers to the different QTL. For virulence on Kombar, the four genotypic classes included qtl.VirK1/qtl.VirK2, qtl.VirK1/qtl.virk2, qtl.virk1/qtl.VirK2, and qtl.virk1/qtl.virk2. Genotypes harboring markers coming from the virulent parent 15A for both QTL were significantly different than the other three genotypic classes. Genotypic classes harboring 15A markers for only qtl.VirK1 or only qtl.VirK2 were not significantly different from one another; however, each of these two genotypic classes was significantly greater than the genotypic class that harbored neither 15A (virulence) QTL. This is yet another strong indication that isolates harboring multiple virulence genes have the ability to cause more severe disease in the presence of the corresponding susceptibility genes in the host.

Somewhat different results were found for the inoculation data collected on the barley line Rika where no significant differences were observed between lines harboring both qtl.VirR1 and qtl.VirR2 compared to those harboring markers for qtl.VirR1 only; however, there was a difference between genotypes harboring markers for qtl.VirR1 alone and qtl.VirR2 alone. This can be explained by the significance levels of qtl.VirR1 (R^2=0.31) and qtl.VirR2 (R^2=0.14). Based on the lower level of significance of qtl.VirR2, we would expect a significant difference between genotypic classes harboring only qtl.VirR1 or only qtl.VirR2 and due to the population

size we do not see a significant difference between genotypes harboring both qtl.VirR1 and qtl.VirR2 and the genotypic class harboring only qtl.VirR1.

In this study, we account for roughly half of the virulence variation observed on these two lines, it is possible, even likely, due to the small size of this population, that there are other QTL with smaller effects associated with these lines, which were not detected or that the level of marker saturation was not adequate to identify all QTL. This is evidenced by the fact that when looking at the different genotypic classes of the 15A×6A progeny, some progeny that contained none of the markers for virulence on their corresponding host line, still conferred virulent reactions. This is a good indication that one or more virulence loci/QTL remain unaccounted for. It is also possible that due to recombination between the markers and the actual genes conferring virulence, the genotypic classes do not reflect the actual virulence loci harbored by each progeny isolate. Additionally, it is likely that some of the remaining variability that was not accounted for by the QTL analysis was due to phenotyping error. Even though the phenotyping was not significantly different between replicates there was still variability between inoculations of individual progeny isolates indicating that error was present.

According to the zigzag model presented by Jones and Dangl (2006), pathogens and their hosts are in constant battle for survival. The host has a network of resistance genes that are effective at recognizing pathogen effectors resulting in a resistance response involving defense response pathways. The pathogen eludes this defense mechanism through elimination or mutation of these recognized effectors. This model was developed based on research mostly from biotrophic and bacterial systems and does not necessarily apply to pathogens with a necrotrophic lifestyle. In the case of necrotrophic pathogens such as *P. teres* f. *teres*, the

pathogen may be using this host gene/effector recognition to its advantage to cause effector triggered susceptibility (Liu et al. 2012) rather than immunity. Rather than the host adapting to the presence of the pathogen by evolving resistance genes (R genes) involved in recognition of pathogen produced effectors, in the necrotrophic model, it is the pathogen that makes the first move by secreting effectors that are "recognized" by host pathways that in several cases have been shown to have hallmarks of defense response (Liu et al. 2012, Faris et al. 2010, Lorange et al. 2012). These effectors trigger defense response pathways often resulting in programmed cell death (PCD) where the necrotrophic pathogen is able to take advantage of the nutrient from the dying tissue (Liu et al. 2012).

By looking at the differences in QTL associated with the different barley lines, we can begin to estimate the number of major susceptibility genes present in each of the lines used in this study. For instance, Rika likely has at least two susceptibility genes because two virulence QTL were found to be associated with this line. Kombar appears to have at least two different susceptibility genes as well. A single unique virulence QTL is associated with barley line PI 356715, which indicates the probability of another unique susceptibility gene in PI 356715 on top of the four potential genes identified in Rika and Kombar. In total, it is likely that at least five unique susceptibility genes are present in these three barley genotypes; two in Rika, two in Kombar, and one in PI 356715.

As noted previously, only one virulence QTL was associated PI 356715. However, this single virulence QTL accounted for 52% of the disease observed on PI 356715. It differs from the other lines used in this study because all of the pathogen virulence identified is represented in one major QTL, qtl.VirP1, and possibly other smaller unidentified QTL rather than multiple virulence QTL as seen on the other lines. In Figure 2, one can see that the disease occurrence on

PI 356715 with the virulent isolate 15A, is less than that of 15A on Kombar or 6A on Rika, having an average disease reaction type of 6.3 rather than 7.2 as seen on Rika and Kombar when inoculated with their virulent parental isolates 6A and 15A, respectively. A relationship between QTL number and disease severity could be formed to explain that when a single QTL is present, such as in PI356715, the disease severity is lower than when two QTL are present, such as in Rika and Kombar (Table 6). In general, this shows that the presence of multiple effector-host gene interactions is more effective in inducing high levels of disease than a single interaction on its own. Therefore, the lower levels of disease (i.e. less severe lesions) observed on PI356715 could be explained by the presence of only one major effector interaction in contrast to the multiple effector interactions that are present in the other lines.

This research is the first step in the identification of virulence effectors within *P. teres* f. *teres*. Once candidate genes within the QTL are identified and validated, the sequence of the effectors can be compared, looking for conserved regions that can be used later to help identify other effectors with similar characteristics within the pathogen genome. Isolates identified from the 15A × 6A population known to harbor only one of the five virulence QTL identified can now be used to inoculate barley populations such as the Rika × Kombar population or a population made from a cross with PI 356715 to aid in the identification of the susceptibility genes that interact with these five virulence genes identified in *P. teres* f. *teres*.

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