GENOMIC ANALYSIS OF TAN SPOT AND STAGONOSPORA NODORUM BLOTCH RESISTANCE IN WHEAT

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

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ABSTRACT

Abeysekara, Nilwala Shyamen, Ph.D., Program of Genomics and Bioinformatics, College of Graduate and Interdisciplinary Studies, North Dakota State University, January 2011. Genomic Analysis of Tan Spot and Stagonospora Nodorum Blotch Resistance in Wheat. Major Professors: Dr. Justin D. Faris and Dr. Timothy L. Friesen.

Host-selective toxins, or necrotrophic effectors, are important determinants of disease in both wheat-*Stagonospora nodorum* and wheat- *Pyrenophora tritici-repentis* pathosystems. This study describes the identification, validation, and genomic analysis of compatible host gene-effector interactions in these systems. In the wheat-*S. nodorum* system, the *Snn4*-SnTox4 interaction was identified and validated using the hexaploid wheat (*Triticum aestivum* L.) recombinant inbred populations 'Arina' x 'Forno' (AF) and Salamouni x 'Katepwa' (SK), respectively. The single dominant gene, *Snn4*, which mapped to the short arm of chromosome 1A in both populations, governs sensitivity to the proteinaceous effector, SnTox4, which is estimated to be 10-30 kDa in size. The compatible *Snn4*-SnTox4 interaction played a significant role in disease development in both the AF and SK populations accounting for 41% and 23.5% of the phenotypic variation, respectively. Effects of the additional minor QTL were largely additive in both genetic backgrounds. Molecular mapping in the SK population using microsatellites and markers developed using bin-mapped expressed sequence tags (ESTs), and from ESTs identified based on colinear studies with rice (*Oryza sativa* L.) and *Brachypodium*, delineated *Tsc2* to a 3.3 cM interval and confirmed its location on 2BS of hexaploid wheat. The compatible *Tsc2*-Ptr ToxB interaction accounted for 54% of the disease variation in the SK population. The marker *XBE444541*, which co-segregated with *Tsc2* is diagnostic of the gene and will be useful in marker –assisted selection (MAS).

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CHAPTER 1.

GENERAL INTRODUCTION

Bread wheat (*Triticum aestivum* L.), which belongs to the family Poaceae, is one of the earliest cereal crops to be cultivated. It was introduced into Europe and Asia about 7000-10000 years ago from the center of origin in the Fertile Crescent, during the spread of agriculture (Salamini et al., 2002). The European colonists introduced wheat into the New World (Americas and Australia) during the last 500 years. Today it is the leading source of vegetable protein in human food, the third most-produced cereal after corn and rice, and the second main human food crop after rice. It is also the third field crop and the leading export crop in the United States. China, India, and United States are the three main producers of wheat in the world (http://faostat.fao.org.2007.retrieved 8-24-2010).

Wheat has two distinct growing seasons. About 70% of the wheat grown in the US is winter wheat, which is sown in the fall and harvested in the spring or summer. About 24% of the planted acreage is spring wheat and 6% is durum wheat (*T. turgidum* L.). Both durum and spring wheat are planted in the spring and harvested in late summer or early fall http://www.ers.usda.gov/briefing/wheat/background.htm.retrieved 8-23-2010).

The six major classes of wheat grown in the US are determined based on the time of the year they are planted and harvested, hardness, color, and the shape of the kernels. Each class of wheat is grown based on rainfall, temperature, soil condition, and tradition. Hard red winter wheat (HRWW), which varies in protein content, is used to produce bread, rolls, and all-purpose flour. HRWW is the predominant export class in the US and the majority is grown in the Great Plains States including Kansas, Oklahoma, Texas, Colorado, and Nebraska. Hard red spring wheat, predominantly grown in North Dakota, Montana,

South Dakota, and Minnesota, contains the highest percentage of protein and is used mainly for bread and hard baked goods. Durum wheat, which is the hardest of all wheats, is predominantly grown in North Dakota and used to make Semolina flour for pasta, macaroni, spaghetti, etc. Soft red winter wheat, which generally has low protein content, is used for cakes, pastries, crackers, and flat bread. Hard white and Soft white are the other two classes of wheat grown in the US (http://www.smallgrains.org/WHFACTS.HTM. retrieved 8-23-2010; http://www.ers.usda.gov/briefing/wheat/background.htm. retrieved 8-23-2010).

Wheat is prone to diseases caused by fungi, bacteria, viruses, insects, and nematodes. The majority of these diseases are caused by fungal pathogens. In recent years, reports of tan spot and Stagonospora nodorum blotch (SNB), two economically important foliar diseases of bread and durum wheat, have become more common in North Dakota and nearby states. Tan spot and SNB are caused by the necrotrophic fungi *Pyrenophora tritici-repentis* and *Stagonospora nodorum*, respectively. The majority of current bread and durum wheat cultivars grown in the region are susceptible to both these diseases (Xu et al., 2004). Both the diseases have the ability to decrease yields and grain quality. Tan spot can cause yield losses ranging from 3 to 50% (Rees and Platz, 1983; Reide et al., 1996) while yield losses due to SNB can also be as high as 50% (Fried and Meister, 1987; King et al., 1983).

Both fungi are known to produce multiple host-selective toxins, or necrotrophic effectors. Host gene-effector interactions reported in these two pathosystems were shown to be important factors in the development of disease. Hence, knowledge regarding the genetics of resistance to tan spot and SNB in common and durum wheat will provide vital

information to breeders for the development of resistant varieties.

Study of the syntenic relationships between genomes of different species, or comparative genomics, will aid in genetic mapping, QTL verification, candidate gene identification, and understanding of genome evolution (Kliebenstein et al., 2001; Murphy et al., 2001; Zhang et al., 2001; Schmidt, 2002). Rice (*Oryza sativa* L.) has long been the model species for the study of grasses including wheat. The availability of a vast array of tools which includes the sequenced genome and the genetic maps, made rice an ideal candidate for the study of genes of agronomic importance in grass species. Recent advances in genome sequencing have made whole genome sequencing a much less difficult task, giving much room for comparative genomics. Recently, with the completion of the genome sequence, *Brachypodium distachyon* (L.), commonly known as the purple false brome, immerged as a promising new model species to study grasses as well. In the course of evolution of the *Pooideae*, *Brachypodium* diverged just prior to the forage grasses and temperate cereals but later than rice (Foote et al., 2004) making it a more close relative to wheat than rice.

As mentioned before, *P. tritici-repentis* and *S. nodorum* produce multiple necrotrophic effectors. Ptr ToxB, is one such effector produced by race 5 isolates of the tan spot fungus *P. tritici-repentis.* Sensitivity to the toxin is governed by a dominant host gene designated *Tsc2*, mapped to the short arm of wheat chromosome 2B in the International Triticeae Mapping Initiative (ITMI) population, which was developed by crossing a synthetic hexaploid wheat (W-7984; synthesized from *Aegilops tauschii* accession CI 18=WPI 219 and the durum wheat variety 'Altar 84') with the hexaploid cultivar 'Opata 85' (CIMMYT-bred spring wheat; Nelson et al. 1995). Ptr ToxB was shown to be

important in the development of disease in the ITMI population and the *Tsc2* locus accounted for 69% of the phenotypic variation (Friesen and Faris, 2004). The B genome donor in the ITMI population, Altar 84, which is a tetraploid, was sensitive to Ptr ToxB while Opata 85 was insensitive.

As mentioned before, both bread and durum wheat are susceptible to tan spot as well as SNB and the complex inheritance of resistance to these diseases makes breeding for resistant varieties a challange. Recent studies have shown that necrotrophic pathogens such as *P. tritici-repentis* and *S. nodorum* exploit biological pathways in plants to create an environment favorable for the spread of the diseases which would be fatal to the survival of biotrophic pathogens. This could create negative impacts when trying to pyramid disease resistance genes in crop species. Therefore, meticulous investigations of such pathosystems in different mapping populations using techniques such as QTL analysis are needed to understand its complex nature and mechanisms underlying specific host-effector interactions. Identification of user-friendly PCR-based markers suitable for marker-assisted selection (MAS) will further facilitate breeding for disease resistance. Whole genome maps developed in mapping populations will not only be useful for QTL analyses and selection of markers for MAS, but also for map-based cloning. Hence the studies reported in this dissertation were carried out to identify host-effector interactions in the wheat-*P. tritici-repentis* and wheat-*S. nodorum* pathosystems and their role in disease development in hexaploid wheat. Other objectives were to validate the findings in different genetic backgrounds and to identify novel sources of disease resistance to tan spot and SNB for future cultivar/variety development. A whole genome map was also developed in one of the hexaploid wheat mapping populations used in these studies. Further, markers suitable

for MAS were also developed or identified. The findings of these studies further add to our understanding of the two complex pathosystems and will be useful in isolating the components involved in host-effector interactions in the two systems in the future.

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CHAPTER 2.

LITERATURE REVIEW

The origin, domestication and evolution of wheat

About 10,000-12,000 years ago, different crop species, including wheat, were domesticated in different geographical regions. These included southern China, Central America, and the Fertile Cresent of the Near East. The Fertile Cresent reffers to a region which spans modern-day Isreal, Lebanon, Jordan, and Western Syria, into southern Turkey and, near the upper reaches of the Tigris and Euphrates rivers, into Iraq and the Western flanks of Iran (Salamini et al., 2002). Wheat belongs to the family Poaceae, tribe Triticeae. The tribe Triticeae also includes other cereal crops such as rye and barley. All cultivated species of wheat belong to the genus *Triticum*. Approximately 35 million years ago (MYA), the Triticeae tribe began separating from its progenitor. Then around 11 MYA, the *Triticum* group separated out. About 500,000 years ago, various polyploid wheat species within the *Triticum* genus arose. The cultivated wheat species we know of today, exhibit three ploidy levels; diploid (einkorn), tetraploid (emmer, durum, rivet, Polish, Persian), and hexaploid (spelt, bread, club and Indian shot) (Gustafson et al., 2009).

The earliest cultivated wheat was the diploid einkorn wheat (*T. monococcum* ssp. *monococcum* L. $2n=2x=14$), which carries the A^mA^m genome and was semi-domesticated from its wild progenitor, *T. monococcum* ssp. *aegilopoides* (Heun et al., 1997). Archeological, botanical and genetic evidence suggest that wild einkon was first grown in the Karacadag mountains in Southeastern Turkey (Heun et al., 1997). There are reports of wild and cultivated einkorn seed remains in the nearby archaeological sites dating from

7500 to 6200 BC. Today, einkorn wheat is a relic crop species, which is rarely planted and found only in some mountainous Mediterranean regions (Salamini et al., 2002; Gustafson et al., 2009).

Domesticated emmer wheat (*T. turgidum* ssp. *dicoccum*, 2n=4x=28, AABB genomes) was domesticated from wild emmer wheat (*T. turgidum* ssp. *dicoccoides*). Until the early Bronze age, emmer was the most important crop in the Fertile Crescent (Bar-Yosef, 1998). The remains of cultivated emmer (*T. turgidum* ssp. *dicoccum*) have also been discovered at several archaeological sites in Syria dating to 7500 BC (Zohary and Hopf, 1993). Domesticated emmer wheats have a non-brittle rachis. It is believed, that these non-shattering forms arose by mutation from primitive wild emmer wheats, which have a brittle rachis. Emmer is still cultivated in Ethiopia, however, the free-threshing *Triticum turgidum* L. var *durum* is the only tetraploid wheat widely cultivated today.

Domesticated einkorn and domesticated emmer cultivation spread across Asia, Europe, and Africa from the Fertile Crescent. Southwestern expansion of domesticated emmer cultivation resulted in overlap with the southern subpopulation of wild emmer (*T. turgidum* ssp. *dicoccoides*). Gene exchanges between the northern domesticated emmer and the southern wild emmer populations or emmer domesticated in the southern region resulted in the creation of a center of domesticated emmer diversity in southern Levant (Luo et al., 2007). This subdivided the domesticated emmer into northern and southern subpopulations with greater gene diversity in the southern subpopulations (Luo et al., 2007). Northeast expansion of domesticated emmer cultivation resulted in overlap with *Aegilops tauschii* populations which resulted in the emergence of hexaploid common wheat (Kihara ,1944).

Modern wheat cultivars primarily consist of the hexaploid bread wheat (*T. aestivum* $2n=6x=42$) and tetraploid durum wheat (*T. turgidum,* $2n=4x=28$). About 10,000-8,000 years ago, after the domestication of tetraploid wheat, a spontaneous hybridization between *T. turgidum* and the diploid goatgrass *Ae. tauschii* (Coss) (2n=2x=14, DD genome), gave rise to *T. aestivum.* Bread wheat is a disomic allohexaploid consisting of three sub genomes A, B and D. It was previously believed that the A genome donor for the tetraploid species, *T. timopheevi* (Zhuk.) Zhuk. (AAGG) and *T. turgidum* was *T. monococcum* (Sax, 1922; Kihara, 1924). However, more recent studies have shown that *T. uratu* contributed the A genomes in both diploid species (Chapman et al., 1976; Nishikawa et al., 1994; Dvorák et al., 1993, 1998).

While the D genome donor of bread wheat is known to be *Ae. tauschii* (McFadden and Sears, 1944, 1946; Kihara 1944), the B genome donor for the polyploids is yet unknown. The B genome likely diverged before the A and D genomes radiated (Gu et al., 2004). It has undergone significant intergenomic changes since the formation of the tetraploid wheats and constitutes the largest component of the polyploid wheat genomes. The large degree of change at the DNA level made the identification of the true B genome donor for both the durum and bread wheat much more challenging. Both the B and G genomes are considered to be modified S genomes having evolved from the same ancestor, which is a close relative of *Ae. speltoides* (2n=2x=14, SS genomes).

The most primitive hexaploid wheat probably would have been a hulled-type (like *T. aestivum* spp. *spelta, macha* or *vavilovii*) in comparison to the current free-threshing types (like *T. aestivum* spp. *aestivum, compactum* or *sphaerococcum*), which were the result of a mutation at the *Q* locus (Muramatsu, 1986). Based on the distribution of *Ae.*

free-threshing durum, first identified in arechealogical sites in Egypt during the Greco-Roman times (Nesbitt and Samuel, 1995), are the tetraploid forms of these Neolithic free-threshing wheats as they show more close genetic relationship to the Mediterranean and Ethiopian subpopulations of domesticated emmer (Luo et al., 2007). All domesticated wheats have increased seed size, reduced number of tillers, more erect growth, and reduced seed dormancy (Dubcovsky and Dvorák, 2007). The *Gpc-B1* gene affects the grain protein concentration (Uauy et al., 2006a). In some genotypes and environments, the functional *Gpc-B1* allele is associated with smaller seeds (Uauy et al., 2006b). This indirect selection for large seeds may have led to the fixation of the nonfunctional *Gpc-B1* allele in both durum and *T. aestivum* (Uauy et al., 2006a). Except for *Q* and *Gpc-B1*, no other genes relevant to the wheat domestication syndrome have been isolated so far.

Polyploid wheat has retained a relatively large proportion of the variability of its tetraploid wild progenitor. This is helpful to compensate for diversity bottlenecks caused by domestication and polyploidy. Insertions and deletions of repetitive elements into coding and regulatory regions have generated new variations in the dynamic wheat genomes which can be expressed as quantitative gene dosage differences. The diversity generated by these dynamic genomes is exploited by polyploid wheat as there is synergy between the high mutation rates and the buffering effects of polyploidy (Dubcovsky and Dvorák, 2007).

The wheat genome is one of the largest plant genomes. The haploid size of the wheat genome is about 16 billion basepairs and it contains more than 80% repetitive DNA. It is about 6 times larger than the maize genome (2500 Mbp), about 40 times larger than the rice genome (390 Mbp), about 140 times larger than the *Arabidopsis* genome (110 Mb), about 60 times larger than the *Brachypodium* genome (272 Mbp), and even about 5 times larger

than the human genome (3300 Mb). Wheat has the ability to tolerate structural and numerical variations in chromosome number for many generations. This has led to the development of several widely used sophisticated cytogenetic stocks such as the nullisomic-tetrasomics, ditelosomics and deletion lines that were created in the wheat genotype "Chinese Spring" background (Endo and Gill, 1996). These cytogenetic stocks are used to assign loci to specific chromosome arms and for the physical mapping of loci.

Recently a group of British scientists released a rough draft of the wheat genome sequenced to 5x coverage on Roche's 454 GS FLX, which is said to cover around 95% of known wheat genes, using the wheat cultivar 'Chinese spring'. This is actually a collection of raw sequence reads which are not assembled. Assembling the whole wheat genome requires further read-throughs, significant work on annotation and assembling the data into individual chromosomes (http://www.sciencedaily.com/releases/2010/08/100827082155.htm, verified on 08-30-2010). Hence cracking the genetic code of wheat completetely still remains a challenge.

Necrotrophic effectors and the inverse gene-for-gene model

Host-selective toxins (HSTs), or necrotrophic effectors, are a special class of pathogen effectors that induce necrosis and cause disease only in specific genotypes of the host that carry a corresponding dominant gene that mediates recognition of the effector (Friesen et al., 2008). These molecules range from low molecular weight metabolites to proteins. Sensitivity to the effector is usually a dominant trait and the effector can reproduce the symptoms of the disease in part or in whole when introduced into plants at relevant concentrations (Walton, 1996; Oliver and Solomon, 2010). Both the production of

the effector by the pathogen and the sensitivity of the host are required for the development of disease. Therefore, necrotrophic effectors are referred to as " agents of compatibility" in contrast to the avirulence determinants which are termed " agents of incompatibility".

Necrotrophic effectors have been documented for over 20 fungal pathogens which include those from the genera *Cochliobolus*, *Alternaria*, *Pyrenophora* and *Stagonospora nodorum*. Victorin (produced by *Cochliobolus victoriae*), AAL-toxin (produced by *Alternaria alternata* f. sp. *Lycopersici)*, T-toxin (produced by *Cochliobolus heterostrophus* race T), PM-toxin (produced by *Mycosphaerella zea-maydis*), HC-toxin (produced by *Cochliobolus carbonum*), and PC-toxin (produced by *Periconia circinata*) are some of the effectors produced by such fungal pathogens (Wolpert et al., 2002). *P. tritici-repentis* and *S. nodorum* have been shown to produce multiple-effectors which will be discussed in detail later.

In necrotrophic systems, the interaction of the necrotrophic effector, produced by the pathogen, with the gene product of the dominant host sensitivity gene is often required for the development of disease. These effectors have a primary virulence function, and are in at least three cases specifically recognized by nucleotide binding site-leucine rich repeat (NBS-LRR) host resistance-like genes which are structurally diverse (Faris et al. 2010; Lorang et al., 2007; Nagy and Bennetzen 2008), and undergo rapid evolution (Oliver and Solomon, 2010). Interestingly, the response mechanisms associated with plant resistance to biotrophs and susceptibility to necrotrophs are quite similar. Typically in classical gene-for-gene type interactions, avirulence gene products confer incompatibility to the pathogen in the presence of the corresponding host resistance gene. This recognition triggers a cascade of signaling events that leads to the induction of host defense responses resulting in

localized programmed cell death, often referred to as the hypersensitive response (HR). The production of reactive oxygen species (ROS), accumulation of phytoalexins, expression of pathogenesis-related (PR) proteins and alterations in the host cell wall are also commonly associated with this active process of induced defense. The absence of either the avirulence gene or the corresponding resistance gene will result in disease (Wolpert et al., 2002).

While the matching products of dominant host and pathogen gene products will result in resistance in some systems, the effectors produced by the pathogen will cause disease in the presence of host produced receptors required for susceptibility in necrotrophic systems (Wolpert et al., 2002). Hence, such necrotrophic effector-host gene interactions are the inverse of the classical gene-for-gene systems. Biotrophs obtain organic nutrients from living cells (Stone 2001) of the host while the necrotrophs survive on nutrients of dead host cells. Hemibiotrophs exhibit both biotrophic and necrotrophic phases during the development of disease (Johal et al., 1995). Gene-for-gene interactions are demonstrated mainly by biotrophic pathogens while the inverse gene-for-gene interactions are seen associated with necrotrophic systems. However, microorganisms that produce avirulence determinants can be biotrophic, necrotrophic or hemibiotrophic, and it is not clear if all the necrotrophic effector-producing fungi are strictly necrotrophic (Wolpert et al., 2002).

Though much is known about the molecular nature of plant disease resistance genes, the exact mechanisms of susceptibility to necrotrophic effectors are largely unknown. The findings of the few plant disease susceptibility genes that have been cloned give us clues of the mysteries yet to unravel (Faris et al. 2010; Lorang et al., 2007; Nagy

and Bennetzen 2008).

The majority of plant disease resistance proteins are intracellular NBS-LRR proteins of which the domain at the N-terminus is either a coiled coil (CC) sequence or a Toll and Interleukin 1 receptor (TIR) (Jones and Dangle, 2006). Surprisingly, the recently cloned susceptibility genes, *LOV1*, *Pc* and *Tsn1*, were all shown to be in the NBS-LRR class of genes (Lorang et al., 2007; Nagy and Bennetzen, 2008; Faris et al., 2010). The *LOV1* gene governs sensitivity to the effector, victorin, produced by *Cochliobolus victoriae*, in *Arabidopsis thaliana*. Sensitivity to the effector Pc toxin, produced by *Periconia circinata,* is governed by the *Pc* gene of Sorghum. *LOV1* encodes a CC-NBS-LRR protein (Lorang et al., 2007) and *Pc* encodes an NBS-LRR protein (Nagy and Bennetzen, 2008). *Tsn1*, which confers sensitivity to the necrotrophic effector ToxA produced by both *S. nodorum* and *P. tritici-repentis* was recently shown to possess a unique structure by having an N-terminal serine/theronine preotein kinase (S/TPK) domain and C-terminal NBS and LRR domains. Faris et al (2010) further demonstrated that the Tsn1 protein does not interact directly with ToxA and that the *Tsn1*-ToxA pathway is associated with the photosynthesis pathway.

It seems that necrotrophic pathogens exploit the same biological pathways used by resistant genes in plants to trigger responses that create an environment favorable for the survival and sporulation of the necrotrophic pathogen, which would otherwise be detrimental to the survival of a biotrophic pathogen (Faris et al., 2010). Hence, the role of resistance genes in conditioning not only resistance but also susceptibility could create repercussions when trying to pyramid resistance genes in crops.

Pyrenophora tritici-repentis

Tan spot

Tan spot (yellow spot/yellow leaf blotch) is one of the major and economically important foliar diseases of wheat, caused by the necrotrophic, homothallic ascomycete *Pyrenophora tritici-repentis* (Died.) Drechs., anamorph *Dreschslera triciti-repentis* (Died.) Shoem. (Syn. *Helminthosporium tritici-repentis* Died.). *P. tritici-repentis* produces multiple-effectors, which include Ptr ToxA, Ptr ToxB, Ptr ToxC and several other partially characterized effectors including two named as Ptr ToxD (Ciuffetti et al., 1998; Ali et al., 2002). The pathogen can attack both durum (*Triticum turgidum* L. ssp. *durum*) and common wheat (*T. aestivum* L.), as well as numerous other grass species (Lamari et al., 2003). The typical reaction on susceptible wheat is tan colored, oval-shaped lesions, which has either a dark brown or black center, surrounded by a chlorotic or yellow border. Sometimes the individual lesions coalesce over a part of or entire leaf surface (Marshall 2009).

Reduction in the photosynthetic area due to tan spot can cause yield losses which range from 3-50% and can also result in kernel weight reductions up to 13%, there by severely affecting wheat production across the globe (da Luz & Hosford, 1980; Shabber & Bockus, 1988; Evans et al., 1999; Bhathal et al., 2003). Reduced tillage practices, which help to retain crop residues on the soil surface, are partially to be blamed for the increase in tan spot incidence because the fungus can survive on infected stubble. Continuous cultivation of wheat, use of susceptible cultivars and shorter crop rotations also contribute to the increasing incidences in tan spot (De Wolf et al., 1998; Ciuffetti and Tuori, 1999).

Life history

Male and female gametes of the same mycelium are formed in pseudothecia which require a cold period to mature. Depending on the geographic location, environmental conditions and crop rotation, the ascospores will act as the primary inoculum to infect wheat plants during spring, fall or winter (Wright and Sutton, 1990). Conidia produced by the asexual stage are disseminated by wind and help to repeat the pathogen life cycle. This will ensure the continuous occurrence of disease throughout the growing season (Schilder and Bergstrom, 1992). Furthermore, factors such as host genotype, environmental conditions and the virulence of the pathogens, influence disease onset, severity, and spread (Ciuffetti and Tuori, 1999).

Race classification

The two genetically distinct symptoms of tan spot, tan necrosis and extensive chlorosis, are observed as a result of the specific host-pathogen interactions (Lamari and Bernier, 1989a). Initially the *P. tritici-repentis* isolates were classified into pathotypes based on their ability to induce necrosis and/or chlorosis on selected wheat genotypes (Lamari and Bernier, 1989a). A race-based classification was later developed, with the identification of *P. tritici-repentis* isolates that could induce the same symptom(s) on different host genotypes. So far, eleven races that produce both necrosis and/or chlorosis on differential wheat cultivars/lines have been identified, of which only the first eight are well-characterized. (Ali and Francl, 2002; Lamari et al., 2003).

Races 1 to 4 were reported to occur in North America and also in the Fertile Crescent (Lamari et al., 1995, Lamari et al., 2005). Race 5 isolates, which were initially reported in Algeria (Lamari et al., 2005), were later found in the United States, Canada, Syria, and Azerbaijan (reviewed in Lamari and Strelkov, 2010). Race 6 isolates are only

found in North Africa (Strelkov et al., 2002). Race 7 and 8 have been reported in Caucasus and the Middle East (Strelkov et al., 2002; Lamari at al., 2003, 2005), and races 10 and 11 have been identified in South America (Singh et al., 2006). Races 1, 7, and 8 produce both necrosis and chlorosis, race 2 produces only necrotic symptoms, races 3, 5 and 6 cause chlorosis (but on different cultivars), and race 4 isolates are avirulent and produce neither symptom (Strelkov and Lamari, 2003).

Even though the differential set consists of six wheat cultivars/lines (Glenlea, 6B662, 6B365, Salamouni, Coulter, and 4B1149) only Glenlea, 6B662, and 6B365 are useful in differentiating the eight well-characterized races of *P. tritici-repentis* (Lamari and Strelkov, 2010). Races that produce only one toxin each (race 2, 3, and 5) are termed 'basic' races and those producing more than one toxin (1, 6, 7, and 8) are known as 'composite' races (Lamari and Strelkov, 2010).

***P. tritici-repentis* effectors**

Ptr ToxA. A 13.2 kDa effector responsible for the necrotic symptoms (Ptr ToxA) was the first Ptr effector to be isolated and shown to be proteinaceous in nature (Lamari et al., 1995). It is produced by the *P. tritici-repentis* races 1, 2 (produce only Ptr ToxA), 7 and 8 (Zhang et al., 1997, Lamari et al., 2003). Ptr ToxA is internalized only in cells of Ptr ToxA-sensitive wheat cultivars (which is mediated by an Arg-Gly-Asp (RGD) vitronectin-like motif found in the mature peptide) and then localize to the chloroplast and cytoplasmic compartments (Manning and Ciuffetti, 2005). Hence sensitivity to Ptr ToxA could be related to protein import (Manning and Ciuffetti, 2005). The single copy *ToxA* gene, which has been cloned independently by two research groups, encodes Ptr ToxA (Balance et al., 1996; Ciuffetti et al., 1997). Several studies have shown that Ptr ToxA sensitivity is highly

correlated with disease susceptibility. Transformation of a nonpathogenic Ptr ToxA deficient *P.tritici-repentis* strain with the *ToxA* gene resulted in a pathogenic Ptr ToxA-producing strain (Ciuffetti et al., 1997). This suggests that Ptr ToxA is a pathogenicity factor. However, other studies suggest that Ptr ToxA is not necessary for pathogenicity and should be considered a virulence factor (Friesen et al., 2003).

The single-copy *ToxA* gene encoding Ptr ToxA was involved in a horizontal transfer from *Stagonospora nodorum*, the causal agent of Stagonospora nodorum blotch in wheat, to *P. tritici-repentis* (Friesen et al., 2006). The SNU16571.1 gene found in *S. nodorum* is 97% identical to the *P. tritici-repentis ToxA* gene. Analysis of Ptr isolates from different geographic regions suggests that the *ToxA* gene is highly conserved (Ballance et al., 1996; Ciuffetti et al., 1997; Friesen et al., 2006). However, there is much sequence variation among *S. nodorum ToxA* sequences compared to the *P. tritici-repentis ToxA* gene sequences suggesting that *ToxA* first occurred in *S. nodorum* and was later transferred to *P. tritici-repentis* (Friesen et al., 2006).

Sensitivity to Ptr ToxA is conditioned by a single dominant gene, *Tsn1*, which has been mapped to the long arm of wheat chromosome 5B in common wheat (Faris et al., 1996) and in durum wheat (Anderson et al., 1999). *Tsn1* also confers sensitivity to ToxA produced by *S. nodorum*, referred to as SnToxA (Liu et al., 2006). The *Tsn1*-Ptr ToxA interaction is light and temperature dependant (Manning and Ciuffetti, 2005) and requires active host processes including transcription, translation and functional H^+- ATPases (Kwon et al., 1998). It has been hypothesized that *Tsn1* is likely to control the effector uptake as only the sensitive genotypes internalize the effector (Manning and Ciuffetti, 2005).

Tsn1 was initially mapped to the long arm of wheat chromosome 5B by genetic linkage mapping (Faris et al., 1996) and located within the deletion bin 5BL 0.75-0.76 on the physical map of 5B (Faris et al. 2000). Haen at al. (2004) delineated the *Tsn1* gene to a 0.8 cM interval based on saturation and high-resolution mapping of the *Tsn1* locus. Lu et al. (2006) conducted genetic linkage mapping with 23 markers derived from bin-mapped ESTs in an attempt to further saturate the *Tsn1* locus. Macro- and microcolinearity studies between the 5B chromosomal region harboring the *Tsn1* gene with rice indicated that the 5B region is highly rearranged relative to rice (Lu and Faris, 2006). Recently, BAC-based physical mapping has led to the cloning of *Tsn1* (Faris et al., 2010). As mentioned before, *Tsn1* includes N-terminal S/TPK and C-terminal NBS-LRR domains, which are disease resistance gene-like features. All three major domains are required for ToxA sensitivity and *Tsn1* transcription is under tight regulation by the circardian clock and light.

Ptr ToxB. A second effector produced by *P. tritici-repentis*, designated Ptr ToxB, is a heat-stable, 6.61 kDa protein (Strelkov et al., 1999). Ptr ToxB was shown to be produced by races 5 (only produce Ptr ToxB), 6, 7, and 8 (Strelkov and Lamari, 2003). It causes chlorosis by a mechanism involving chlorophyll photooxidation, possibly as a result of an inhibition of photosynthesis (Strelkov et al., 1998). Ptr ToxB was initially identified from race 5 isolates of *P. tritici-repentis* (Orolaza et al., 1995). Sensitivity to Ptr ToxB is characterized by extensive chlorosis and it is governed by the *Tsc2* locus at the distal end of the short arm of chromosome 2B (Friesen and Faris, 2004). *Tsc2* was shown to be responsible for the effects of a major QTL associated with resistance to race 5 isolates and accounted for as much as 69% of the phenotypic variation in disease development in the International Triticeae Mapping Initiative (ITMI) population, indicating that Ptr ToxB is a

major virulence factor (Friesen and Faris, 2004). Ptr ToxB is the only chlorosis inducing proteinaceous necrotrophic effector identified so far and hence the characterization of the *Tsc2* gene will be of interest to eventually identify the mechanisms underlying the interactions.

The *ToxB* gene was cloned and characterized by Martinez et al. (2001) using a race 5 isolate of *P. tritici-repentis* from North Dakota and found to be a multi-copy gene unlike the *ToxA* gene. The 261-bp open reading frame of the *ToxB* gene codes for a putative signal peptide of 23 amino acids and the 64 amino acid effector Ptr ToxB (Martinez et al., 2001). Homologs of the *ToxB* gene that differ in their translational regulation have been identified in nonpathogenic isolates that does not produce necrotrophic effectors. *ToxB* homologs were found in the causal agent of brown leaf spot of Bromegrass, *P. bromi* (Died.) Dreschs., and a broad range of other members of the Pleosporaceae which includes *Alternaria* and *Cochliolobolus* (Andrie et al., 2008). The presence of distant putative *ToxB* homologs in *Magnaporthe grisea*, the causal agent of rice blast, indicates that the *ToxB* gene must have arisen in a common ancestor of the Dothideomycetes and Sordariomycetes. Hence, in contrast to *ToxA,* which is considered to have been involved in a horizontal gene transfer, the *ToxB* gene appears to have been vertically transferred from a common ancestor (Andrie at al., 2008).

Ptr ToxC. The *P. tritici-repentis* effector Ptr ToxC also causes chlorosis, but on different host lines and cultivars than does Ptr ToxB (Strelkove and Lamari, 2003). Ptr ToxC is a nonionic, polar, low-molecular mass molecule, not proteinaceous in nature (Effertz et al., 2002) and produced by *P. tritici-repentis* races 1, 3 (only produces Ptr ToxC), 6 and 8. Ptr ToxC has not been purified or fully characterized, but studies have

complex in some genetic backgrounds (Andrie et al., 2007; Friesen et al., 2002; Riede et al., 1996; Tadesse et al., 2007). Genotypes insensitive to known tan spot effectors are not always resistant to the disease (Friesen et al., 2003; Faris and Friesen, 2005; Chu et al., 2008). Faris and Friesen (2005) evaluated a RIL population derived from a cross between the common wheat varieties Grandin and BR34 for reaction to tan spot caused by Ptr races 1, 2, 3, and 5. This study revealed QTLs on the short arm of chromosome 1B and the long arm of chromosome 3B significantly associated with resistance to all four races. The 1B QTL explained 13-29% of the phenotypic variation, and the 3B QTL explained from 13 – 41% of the variation. Neither the 1B nor the 3B QTL were associated with known effector sensitivity genes. Ptr ToxA, which is produced by races 1 and 2, was not a significant factor in the development of disease in this population (Faris and Friesen, 2005).

Using a double haploid wheat mapping population derived from a cross between TA4152-60 and ND495, Chu et al. (2008) identified five genomic regions harboring QTL highly associated with tan spot resistance. None of the QTL, except for one, corresponded to known necrotrophic effector insensitivity loci. Hence markers associated with such QTL are much more useful in breeding for tan spot resistance than only selecting for effector insensitivities.

Stagonospora nodorum

Stagonopora nodorum blotch

Stagonospora (syn. *Septoria*) *nodorum* (Berk.) Castell. and Germano [teleomorph: *Phaeosphaeria* (syn. *Leptosphaeria*) *nodorum* (Müll.) Hedjar.], is a heterothallic, necrotrophic, filamentous, ascomycete fungus pathogenic on wheat, barley and a wide

range of wild grasses (Solomon et al., 2006). In wheat, it affects both the glume and the leaf leading to devastating yield losses up to 50% and reductions in grain quality and grain weight (Eyal 1981; King et al., 1983; Eyal et al., 1987). *S. nodorum* is a member of the Pleosporales, which includes destructive pathogens such as *Leptosphaeria*, *Cochliobolus*, *Alternaria*, and *Pyrenophora* (Solomon et al., 2006).

Life cycle and global migration pattern

The life cycle of *S. nodorum* includes both asexual and sexual stages, which have different mechanisms of dispersal. Ascospores are produced sexually and dispersed by wind. Ascospores and infected seeds act as the major primary inoculum for SNB (Shah et al., 1995, 2000). These ascospores are produced in pseudothecia which mature in wheat residue. Low air temperatures (20 -27 °C), high relative humidity and rainfall can initiate the release of ascospores from stubble of the previous year's crop. During winter, the sexual ascospores mediate long-range air dispersal (Bathgate and Loughman, 2001). Asexual pycnidia and pycnidiospores are formed following the primary infection of the leaves and dispersed over short distances by rain-splash. The splash-dispersed pycnidiospores will then cause secondary spread of the pathogen (Shah et al., 2001). Two to four cycles of asexual infection are usually necessary for the fungus to produce significant disease (Shah and Bergstrom, 2002). Even though the fungus can be readily propagated *in vitro*, it is difficult to reproduce the sexual phase in the laboratory. Hence efforts to conduct genetic analysis by sexual crossing have been unsuccessful to date.

It is suggested that primary infection via infected seeds and the dispersal of infected seeds over long distances due to human activities could contribute significantly to the pattern of genetic variability observed (Bennett et al., 2005). Studies using genetic markers

have been conducted to test the global migration pattern of *S. nodorum* populations. Keller et al. (1997) used restriction fragment length polymorphism (RFLP) markers to compare *S. nodorum* populations collected from Oregon, Texas and Switzerland. However, the population structure of these regions may not be representative of other major wheat growing areas in Asia, Africa, and Australia. Stukenbrock et al. (2006) conducted a study using a set of 12 polymorphic microsatellite loci to characterize the population genetic structure of *S. nodorum* on a global scale. This included nine *S. nodorum* populations from nine different regions in five different continents and consisted of a total of 693 strains. The results of this study indicated that the continental *S. nodorum* populations diverged recently but the population differentiation was reduced due to the occurrence of sufficient migration. While there was moderate population differentiation in continental populations, gene flow among continents was high. The immigrants seem to have originated mainly from populations in Europe, China, and North America based on the migration patterns (Stukenbrock et al., 2006).

Host-effector interactions in the wheat- *S. nodorum* pathosystem

Like *P. tritici-repentis*, *S. nodorum* also produces multiple proteinaceous effectors. These necrotrophic effectors include SnTox1 (10-30 kDa), SnTox2 (6.5 kDa), SnTox3 (10-30 KDa), SnTox4 (10-30 kDa), SnToxA, and other uncharacterized toxins. In recent studies it was shown that SnToxA, as well as SnTox1, SnTox2, SnTox3, and SnTox4 are significant factors in SNB disease. Each effector has been shown to interact either directly or indirectly with single dominant host sensitivity genes designated as *Tsn1* (SnToxA), *Snn1* (SnTox1), *Snn2* (SnTox2), *Snn3* (SnTox3), and *Snn4* (SnTox4). Using four mapping populations segregating for multiple effector sensitivities, disease significance for most of

the toxin sensitivity genes (except for *Snn3*) has been shown to account for a significant percentage (at least 50%) of the disease caused by *S. nodorum* isolates producing each necrotrophic effector (Liu et al., 2004a, 2006; Friesen et al., 2006, 2007, 2008a; Abeysekara et al., 2009; reviewed in Friesen and Faris, 2010). Other than SnToxA and SnTox1, 2, 3, and 4 at least ten additional necrotrophic effectors and their host sensitivity genes have been identified and disease significance data is being collected (Friesen et al. unpublished). These host gene-effector interactions provide strong evidence that the wheat –*S. nodorum* pathosystem is a model inverse gene-for-gene system.

***Snn1*- SnTox1.** Identification and partial purification of SnTox1 was done by Liu et al. (2004a). SnTox1 is a proteinaceous effector 10-30 kDa in size. Sensitivity to SnTox1 is governed by the *Snn1* locus which was genetically and physically mapped to the distal end of the short arm of chromosome 1B (Liu et al., 2004a) using the ITMI population and wheat chromosome deletion stocks (Endo and Gill, 1996), respectively. This is the first study that indicated the presence of a necrotrophic effector-host gene interaction in the SNB system. Using the same mapping population, Liu et al (2004b) identified QTL associated with resistance to SNB using the *S. nodorum* isolate Sn2000. One major QTL on the short arm of chromosome 1B coincided with the effector insensitivity gene *snn1*, and several other QTL with relatively minor effects were idenitifed. This study further demonstrated that SnTox1 produced by Sn2000 was a major virulence factor.

Research has been conducted targeting the cloning of *Snn1*. Colinearity studies have shown that poor conservation of the *Snn1* locus and rice would limit the use of rice genomic information for the cloning of *Snn1*. High-resolution mapping studies have delineated the *Snn1* locus to a 0.46 interval (Reddy et al., 2008). Reddy et al. (2008)

identified two EST markers that co-segregated with the *Snn1* locus and have high homology to known NBS-LRR disease resistance-like genes. Therefore both these ESTs are considered as strong candidates for *Snn1*. Two microsatellite (*Xfcp618* and *Xpsp3000*) markers diagnostic of *Snn1* have also been identified through fine-mapping studies (Reddy et al., 2008; reviewed in Friesen and Faris, 2010). The Chinese Spring 1BS18 chromosome deletion line is used as the differential line for SnTox1.

***Tsn1*- SnToxA.** SnToxA was the second *S. nodorum* effector to be identified (Friesen et al., 2006). This necrotrophic effector first isolated from *P. tritici-repentis* and termed Ptr ToxA, was shown to be involved in a lateral gene transfer event from *S. nodorum* to *P. tritici-repentis* prior to 1941 (Friesen et al., 2006). SnToxA, coded by a gene 99.7% identical to the *Ptr ToxA* gene, was a major factor in SNB disease development in wheat genotypes carrying the *Tsn1* gene (Liu et al., 2006). The wheat *Tsn1* gene, which maps to the long arm of wheat chromosome 5B, confers sensitivity to both Ptr ToxA (Faris et al., 1996) and SnToxA (Liu et al., 2006). Evaluation of the gene sequence of *SnToxA* in a *S. nodorum* collection revealved 11 haplotypes in the *S. nodorum* population in comparison to the single haplotype observed when a *P. tritici-repentis* collection was analysed with the gene sequence of *PtrToxA*. This evidence suggests that the *ToxA* gene was present in *S. nodorum* significantly longer and may have horizontally transferred to *P. tritici-repentis* (Friesen et al., 2006). Faris and Friesen (2009) reported that the *Tsn1*-ToxA interaction is an important factor for the development of SNB disease in common wheat as well as in durum wheat. The progeny line BG261 from the BG population is used as the differential line for SnToxA as it is only sensitive to ToxA.

***Snn2*-SnTox2.** Friesen et al. (2007) identified SnTox2, another necrotrophic

effector produced by *S. nodorum*, and QTL associated with seedling resistance to the *S. nodorum* isolate Sn6, which produces SnToxA and SnTox2, using the BG population. The progeny line BG223 is only sensitive to SnTox2 and therefore selected as the differential line for Tox2. In a previous study, Liu et al. (2006) identified and described the first effector, which they designated as SnToxA, produced by Sn6. SnTox2 is also a proteinaceous effector which is 7-10 kDa in size. Regression analysis revealed four QTL significantly associated with SNB resistance. A single dominant gene, designated *Snn2*, confered susceptibility to SnTox2 and mapped to the short arm of wheat chromosome 2D and underlay the QTL *QSnb.fcu-2DS*. SnToxA causes necrosis in susceptible wheat genotypes carrying *Snn2*. The compatible SnTox2-*Snn2* interaction is light dependant. It was shown that the effects of *Snn2*-SnTox2 and *Tsn1*-SnToxA interactions were almost completely additive and together accounted for the majority of the phenotypic variation (66%) in the BG population (Friesen et al., 2007). A second QTL, *QSnb.fcu-5BL,* was significantly associated with the *Tsn1* locus. The third QTL, *QSnb.fcu-5AL,* which explained 10% of the total phenotypic variation was significantly associated with the marker *Xfcp13* while the fourth QTL detected on the short arm of chromosome 1B (*QSnb.fcu-1BS*) associated with the marker *Xgdm125* explained 5% of the total phenotypic variation (Friesen et al., 2007). This study further demonstrated that the presence of two compatible host-effector interactions led to increased levels of disease and explained the complex quantitative nature of SNB resistance. A recent mapping study led to the identification of two markers, EST-based marker *XTC253803* and the microsatellite marker *Xcfd51*, which delineated the *Snn2* locus to an interval of 4.0 cM and were determined to be suitable for MAS of the gentypes carrying the *Snn2* locus (Zhang et al., 2009).

***Snn3*- SnTox3.** SnTox3 was the fourth *S. nodorum* effector identified and characterized (Friesen et al., 2008a). Sensitivity to this 10-30 kDa effector is governed by the *Snn3* locus in wheat. Like the previously identified *S. nodrum* effectors, SnTox3 is also proteinaceous in nature and induces necrosis in susceptible host genotypes carrying *Snn3*. However, unlike the previously identified intereactions, the *Snn3*-SnTox3 interaction is not light dependant and less important in the development in disease, accounting for a mere 18% of the total phenotypic variation in disease in the BG population (Friesen et al., 2008a). Interestingly, the SnTox3-*Snn3* interaction was only significant in the absence of the compatible *Snn2*-SnTox2 interaction. Hence the *Snn2*-SnTox2 interaction was epistatic to the *Snn3*-SnTox3 interaction. Liu et al (2009) cloned and characterized the SnTox3 encoding gene-*SnTox3*. *SnTox3* is a 693 bp intron-free gene with no strong homology to any known genes and coding for an immature protein of 330 amino acids. *SnTox3* was shown to be present in approximately 60% of a world wide collection of 923 *S. nodorum* isolates (Liu et al., 2009). BG220 is used as the differential line for SnTox3.

Resistance to Stagonospora nodorum blotch

Disease severiety of SNB can be reduced by the use of fungicides and cultural control practices. However, the use of fungicides may not be very cost effective when grain prices are too low. However, reduced tillage practices are preferred by most farmers due to the economic gains of organic farming and reduced environmetal damage. Therefore, host resistance is the most preferred and economically efficient method of controlling SNB. The existing wheat germplasm does not have complete resistance or immunity to SNB (De Wolf et al., 1998, Liu et al., 2004b). However, various levels of partial resistance were reported in wheat and related species (Eyal, 1999). Inheritance of SNB resistance can be

governed by single genes (Kleijer et al., 1977; Ma and Hughes, 1995; Murphy et al., 2000) but is most often governed by multiple genes (Bostwick et al., 1993; Du et al., 1999; Fried and Meister; 1987; Nelson and Gates, 1982; Liu et al., 2004b; Xu et al 2004). Stagonospora nodorum blotch resistance QTL have been identified in almost all of the wheat chromosomes (reviewed in Friesen et al., 2008a). The complex nature of SNB resistance has made the pyramiding of SNB resistance in wheat lines a challenge. Hence indepth knowledge of this complex system is needed in order to understand this system better.

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together they explained 50% of the total phenotypic variation. These results provide further evidence that the wheat – *S. nodorum* pathosystem is a toxin-based inverse gene-for-gene system.

Introduction

Stagonospora nodorum (Berk.) E. Castell. & Germano (telomorph *Phaeosphaeria nodorum* (E. Mull.) Hedjar.), is a necrotrophic filamentous ascomycete fungus that belongs to the Dothideomycete class of the ascomycota. It has high genetic diversity and a heterothallic mating system (McDonald and Linde 2002). *S. nodorum* causes one of the most economically important and destructive foliar diseases of wheat (*Triticum aestivum* L., 2n=6x=42, AABBDD genomes) and related cereals, affecting both the leaves and the glumes. One of the most effective methods of controlling Stagonospora nodorum blotch (SNB) is the use of host resistance. Inheritance of SNB resistance is complex and most often governed by multiple genes (Fried et al. 1987; Bostwick et al. 1993; Du et al. 1999), but monogenic inheritance has also been found in some wheat materials (reviewed in Xu et al. 2004 and Friesen et al. 2008).

Host selective toxins (HSTs) are essential determinants of pathogenicity or virulence and determine host specificity (Wolpert et al. 2002). Most HSTs are characterized as small secondary metabolites and their production in fungi are under the control of complex enzymatic pathways (Panaccione et al. 2002). However, several proteinaceous HSTs have also been reported (Strelkov et al. 1999; Tomas et al. 1990; Barthe et al. 2007; Parada et al. 2008; Sarpeleh et al. 2007).

In *S. nodorum,* four proteinaceous HSTs have been identified and in each case

sensitivity to the HST was conferred by a single dominant host gene. Using segregating wheat populations, the *Tsn1*-ToxA, *Snn1*-SnTox1, *Snn2*-SnTox2, and the *Snn3*-SnTox3 interactions were shown to account for as much as 62, 58, 47, and 17% of the variation in SNB development on leaves, respectively (Liu et al. 2004b, 2006; Friesen et al. 2006, 2007, 2008). Therefore, each of the four host-toxin interactions characterized in the wheat-*S. nodorum* pathosystem plays a significant role in the development of SNB.

Of the four host-toxin interactions identified in the wheat-*S. nodorum* system, *Tsn1*-SnToxA is best characterized. ToxA is a 13.2 kDa proteinaceous HST (Ballance et al. 1989; Tomas et al. 1990; Tuori et al. 1995) that has the ability to cause necrosis in wheat lines carrying the *Tsn1* gene. Manning and Ciuffetti (2005) showed that ToxA is internalized within cells of sensitive wheat cultivars and localized to cytoplasmic compartments and to chloroplasts. Work on the host side has included high resolution mapping of the *Tsn1* locus (Haen et al. 2004; Lu and Faris 2006; Lu et al. 2006) followed by BAC-based physical mapping, and has led to the isolation of *Tsn1* (Faris et al. 2010), which will allow more detailed characterization of the *Tsn1*-ToxA interaction at the molecular level. *SnTox3*, the gene encoding SnTox3 was also cloned and characterized recently (Liu et al., 2009). Host and pathogen components of the two other wheat-*S. nodorum* host-toxin interactions have yet to be isolated.

In previous studies, the Swiss winter wheat cv. 'Arina' was shown to carry major QTL for SNB resistance associated with the glume on chromosome arms 3BS (*QSng.sfr-3BS*) and 4BL (*QSng.sfr-4BL*), whereas the cultivar 'Forno' was shown to be highly susceptible to glume blotch (Schnurbusch et al. 2003). Here, evaluation of the Arina x Forno (AF) population for reaction to SNB on leaves of seedlings led to the identification

and characterization of a novel host-toxin interaction in the wheat-*S. nodorum* pathosystem. This work provides further evidence that the wheat-*S. nodorum* pathosystem is an inverse gene-for-gene system where multiple effector proteins (HSTs) interact with dominant host sensitivity genes to cause disease.

Materials and methods

Plant materials

A recombinant inbred (RI) population consisting of 200 single seed descent F_5 lines developed by an intraspecific cross between the Swiss winter wheat cultivars Arina and Forno was evaluated in this study (Paillard et al. 2003). An F_2 population derived from the cross between Arina and Forno consisting of 50 individuals was infiltrated with the partially purified novel toxin to determine if the host gene/s conferring sensitivity in this population were dominant or recessive in nature. The wheat lines W-7984, BG223, BG220, and BG261, which serve as differentials for SnTox1, SnTox2, SnTox3, and SnToxA, respectively, were infiltrated with culture filtrates of several Swiss *S. nodorum* isolates to determine if the toxin-containing cultures contained novel toxins.

Disease evaluation and toxin bioassays

Swiss *S. nodorum* isolate Sn99CH 1A7a (hereafter referred to as 1A7a) was found to contain at least one novel toxin based on the reactions observed when infiltrated onto the wheat differentials. Inoculum for disease evaluations were prepared from the isolate 1A7a grown in V8-potato dextrose agar (PDA) for 5-7 days as described by Liu et al. (2004b). Three replicates consisting of the 200 AF lines and parents were planted in a completely randomized design (CRD) and used for spore inoculations. Each replicate consisted of 3

cones (Stuewe and Sons, Inc., Corvallis, OR, USA) per line with 3 plants per cone placed in racks of 98 (Stuewe and Sons). Hence each experimental unit consisted of 9 plants per line. The susceptible hard red spring wheat cultivar 'Grandin' was planted around the borders of each rack to eliminate any edge effect. Plants were inoculated at the two to three leaf stage with the conidial suspensions, with $1x10^6$ spores/ml, until runoff as described in Liu et al. (2004b). Inoculated plants were then subjected to 100% relative humidity at 21 °C for 24 h in a mist chamber followed by 6 days of incubation in the growth chamber at 21 °C under a 12 h photoperiod. The second leaf of the inoculated plants was scored on a 0 to 5 lesion type scale (Liu et al. 2004b) 7 days post-inoculation.

Culture filtrates of isolate 1A7a were prepared as described by Liu et al. (2004a) and used to screen the AF population for toxin sensitivity as an initial screen. The fully expanded secondary leaf of each plant was infiltrated with approximately 25 µl of the active culture filtrate using a 1-ml syringe with the needle removed and the infiltrated region was marked with a non-toxic felt pen. Infiltrated leaves were evaluated 3 days after infiltration and were scored based on the presence or absence of necrosis. Experiments were repeated at least twice. After analyzing the results obtained from both the preliminary screens with the culture filtrate and the conidial inoculations (inoculation data), the RI line AF89 was selected as the differential line for the novel toxin (see results-partial characterization of SnTox4).

Toxin partial purification

Acetone precipitated 1A7a culture filtrates (concentrated 6 fold) were subjected to overnight dialysis against water in 3.5 kDa molecular weight cutoff tubing, filtered with a 0.45 µm filter and loaded on to a 1 ml HiTrap SPXL cation exchange column (GE

Healthcare) equilibrated with 20 mM sodium acetate starting buffer (pH 4.3) on a AKTAPrime^{TM}plus system (GE Healthcare). The toxin was eluted using 20 mM sodium acetate + 300 mM NaCl elution buffer, pH 4.3, with a flow rate of 1 ml/min with an elution gradient of 0-300 mM NaCl (pH 4.3) over 20 ml. Fractions were infiltrated onto the differential line AF89 (which shows a sensitive response to the toxin by the development of mottled necrosis), after adjusting the pH by adding 5.0 µl of 1.5 M sodium hydroxide to each 1 ml fraction, to select the active fractions. Fractions with the highest activities were pooled and used to screen the AF population.

Characterization of SnTox4

Pronase (EMD Biosciences, Inc., San Diego, CA, USA), which consists of endo- and exo- proteinases, was used to determine whether the toxin is a protein. A 1 ml fraction of the acetone precipitated 1A7a culture filtrate (6 fold concentration) was treated with pronase (final concentration 1 mg/ml in water). Pronase untreated samples along with pronase alone were kept as controls. All the samples were incubated at 37 °C for 3 h and infiltrated onto the differential line AF89. Pronase treated and untreated samples were also tested on the set of selected 80 AF lines to further confirm the findings.

Active culture filtrates were subjected to ultrafiltration using 30 kDa and 10 kDa Amicon molecular weight cutoff filters (Millipore) in order to determine the size of the toxin. The same subset of 80 AF lines including the differential line AF89 was used to test the partially purified toxin. This set was screened with the concentrates and filtrates of both the 30 kDa and 10 kDa subjected active culture filtrates to verify the presence of SnTox4. Presence of the toxin in the filtrate of each filtration indicated that the active molecule was smaller in size than the filter cutoff (e.g. activity in the filtrate of the 30 kDa filtration

indicated a molecule less than 30 kDa).

Investigation of light dependency for the *Snn4*-SnTox4 interaction was accomplished by testing the SnTox4 differential line AF89, infiltrated with 1A7a culture filtrates containing SnTox4. Two treatments were evaluated including a 48 h dark period after infiltration (treatment 1) and a 16 h photoperiod after infiltration (treatment 2). Three plants were evaluated per replicate per treatment and all plants were subjected to a 16 h photoperiod in the growth chamber at 24 °C with a light intensity of 900 μmol $m^{-2}s^{-1}$ prior to infiltration. All infiltrations were performed immediately after plants had been subjected to an 8 h dark period. At the end of each treatment plants were evaluated for sensitivity to the toxin. The entire experiment was replicated once.

EST marker development and linkage analysis

I assessed the linkage of the genotypic scores of *Snn4* with markers previously mapped in the AF population (Paillard et al. 2003; Schnurbusch et al. 2003, 2004; Tommasini et al. 2007). After it was determined that *Snn4* resided at the distal end of chromosome arm 1AS, NSF-wheat bin mapped EST sequences were downloaded from bin 1AS3-0.86-1.00 (http://wheat.pw.usda.gov/west/binmaps). EST primers were designed using the computer software Primer3 (Rozen and Skaletsky 2000). Each primer was amplified on the parental DNA, which was isolated from plant tissue as described by Faris et al. (2000). PCR conditions were as described in Lu et al. (2006). Amplified products were analyzed on 6% polyacryalmide gels and the polymorphic markers were selected and tested on the 200 individuals of the AF population. Linkage analysis was performed using the computer program MAPMAKER V2.0 (Lander et al. 1987) for Macintosh with the Kosambi mapping function (Kosambi 1944). A maximum θ value of 0.40 and a minimum

LOD threshold of 3.0 were used to identify the linkage groups initially using the "two-point/group" command. The marker order was verified using the "ripple" command with a LOD value of 3.0.

QTL analysis

Previously published linkage maps of the AF population (Paillard et al. 2003; Schnurbusch et al. 2003, 2004; Tommasini et al. 2007) were used to select a subset of 459 markers that gave the best genome coverage to be used for the detection of QTL. Markers significantly associated ($P < 0.001$) with resistance to SNB were identified by simple linear regression using the computer program MapManager QTXb20 (Manly et al., 2001). Composite interval-regression mapping was used to identify chromosomal regions putatively associated with the disease phenotype. A permutation test with 1000 permutations was performed to determine the critical LOD threshold of the AF population which was found to be 3.2 at an experimental-wise error (α) level of 0.05. Additive effects of the QTL were obtained using MapManager QTXb20 (Manly et al. 2001) and markers with significant main effects were further tested for possible significant interactions ($P < 0.0001$) with each other.

Statistical analysis

Statistical analysis was conducted using the computer software packages Data desk (Data description, Inc. Version 4.1), Graphpad (www.graphpad.com/quickcalcs) and SAS (SAS Institute, Inc. Version 9.1). The average disease reaction types calculated from the three replicates were used along with the marker data of the significant markers to conduct the multiple regression analysis using Data desk. Chi squared tests were conducted using the program Graphpad. Bartlett's χ^2 test for homogeneity of variances among replicates

was conducted using SAS, analysis of variance was conducted using the PROC GLM procedure of SAS, and Fischer's protected least significant difference (LSD) was used at α =0.05 to determine the mean separation for the genotypic means.

Results

Identification of an HST produced by the *S. nodorum* isolate 1A7a

Stagonospora nodorum leaf blotch resistance in the AF population has not been studied under field conditions so far. However Stagonospora nodorum glume blotch (SNGB) resistance under natural infestation has been well documented on the same population. Based on the results obtained for SNGB, AF population was chosen for this study in order to see how it responds to Stagonospora nodorum leaf blotch.

Parental lines Arina and Forno of the RI population were evaluated with culture filtrate of the Swiss isolate 1A7a. The results indicated that Arina exhibited a mottled necrotic reaction 3 days after infiltration and was therefore sensitive to a toxic component of the culture filtrate (Figure 3.1D). Forno showed no reaction to the culture filtrate and was therefore considered insensitive (Figure 3.1E). The differential lines for SnToxA (BG261), SnTox1 (W-7984), SnTox2 (BG223) and SnTox3 (BG220) infiltrated with 1A7a culture filtrate resulted in no visible reaction (reaction not shown in Figure 3.1). This indicated that the 1A7a culture filtrate contained at least one new HST, which was designated as SnTox4.

Identification of the host gene conferring sensitivity to SnTox4

The entire AF population was infiltrated with partially purified SnTox4 (see below). The population segregated in a ratio of 95 insensitive: 105 sensitive which fit the expected

1:1 ratio for a single host gene conferring sensitivity (χ^2_{df-1} 0.500, P= 0.4795) in this population. Fifty F_2 plants derived from the cross between Arina and Forno were infiltrated with the partially purified SnTox4 to determine gene action of sensitivity conferred by the host. The F_2 individuals segregated in a ratio of 38 sensitive: 12 insensitive. This fits the expected ratio of 3:1 (sensitive: insensitive) for a single dominant gene conferring sensitivity ($\chi^2_{df=1}$= 0.027, P= 0.8703). I propose to designate the gene conferring sensitivity to SnTox4 as *Snn4*.

Chromosomal location of *Snn4*

I assessed the linkage of the genotypic scores of *Snn4* with markers previously mapped in the AF population (Paillard et al. 2003; Schurbusch et al. 2003, 2004; Tommasini et al. 2007). *Snn4* mapped 1.6 cM distal to the SSR marker *Xcfd58.1* on chromosome arm 1AS (Figure 3.2).

In an attempt to identify more markers linked to the *Snn4* locus, I developed PCR primers (Table 3.1) from ESTs mapped to the wheat 1AS3-0.86-1.00 deletion bin by the NSF-wheat EST project (http://wheat.pw.usda.gov/wEST/binmaps/). Primer sets were designed for 48 ESTs and eleven of these revealed polymorphisms between Arina and Forno, but only four pairs amplified fragments that mapped to chromosome 1A (Table 3.1, Figure 3.2). The EST marker *XBE9590632* mapped 13.9 cM distal to the *Snn4* locus, and *XBG262267* and *XBG262975* co-segregated with each other at 0.9 cM distal to *Snn4*. Therefore, the *Snn4* locus is delineated to a 2.5 cM interval flanked by the EST markers *XBG262267/XBG262975* on the distal side and the SSR marker *Xcfd58.1* on the proximal side.

Figure 3.1. Leaves inoculated with conidia produced by *Stagonospora nodorum* isolate 1A7a or infiltrated with culture filtrate, partially purified SnTox4, or pronase treated cultures. A, B, and C: Arina (A), Forno (B), and AF89 (C) inoculated with conidia produced by *S nodorum* isolate 1A7a. Both Arina and Forno were moderately susceptible to SNB with Arina being more susceptible (average disease reaction type 3.2) to the disease than Forno (average disease reaction type 2.8). D and E: Reaction of Arina (D) and Forno (E) to 1A7a culture filtrates. F, G, H, and I: When the differential line AF89 was infiltrated with the concentrate (F) and filtrate (G) from a 30 kDa filter, and the concentrate of a 10 kDa filter (H), all resulted in faint mottled necrosis. No reaction occurred as the result of infiltration of AF89 with filtrate derived from the 10 kDa filter (I). J, K, and L: Partially purified SnTox4 cultures treated with water alone showed faint mottled necrosis on AF89 (J), and when infiltrated with pronase-treated partially purified SnTox4 cultures (K) or pronase alone (L), AF89 exhibited no reaction. M and N: AF89 developed faint mottled necrosis after 48 hours when infiltrated with partially purified SnTox4 and subjected to a normal light/dark regiment (M), but no reaction occurred on AF89 when plants were kept in complete darkness after infiltration (N).

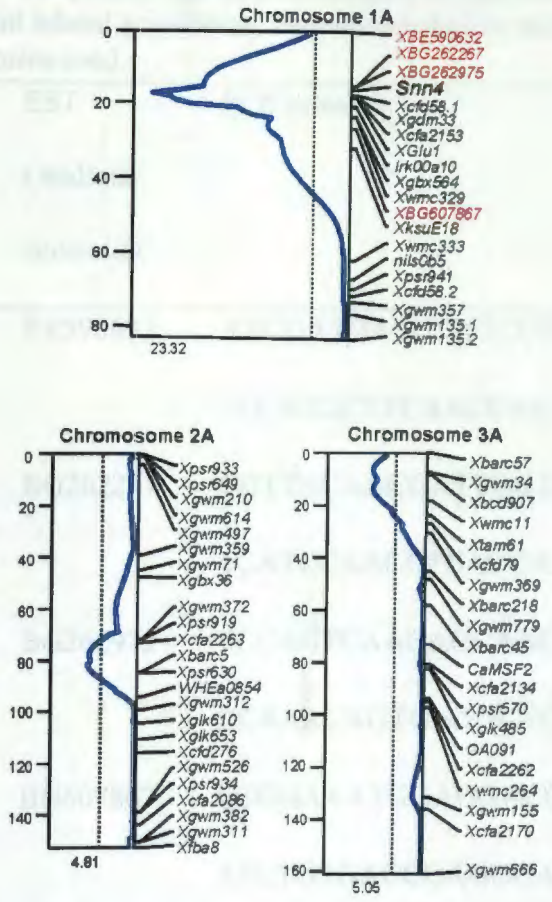

Figure 3.2. Composite interval regression maps of chromosomes 1A, 2A, and 3A generated in the Arina x Forno recombinant inbred population after inoculation of the population with conidia of the *Stagonospora nodorum* isolate 1A7a. A centiMorgan (cM) scale is shown to the left of the maps and the markers are shown to the right. A LOD scale is indicated along the X-axis, and the significant LOD threshold of 3.2 is indicated by the dotted line. Markers defining the QTL or QTL intervals are shown in bold. EST-derived markers developed in this research and mapping to chromosome 1A are shown in red.

The role of a compatible *Snn4*-SnTox4 interaction in causing disease

Three replicates of the AF population along with the parents were inoculated with conidia of 1A7a and rated on a scale of 0-5 based on the severity of the disease (0=highly resistant; 5=highly susceptible) (Liu et al. 2004b). Average disease reaction types for Arina and Forno were 3.2 and 2.8, respectively (Table 3.2, Figure 3.3).

Table 3.2. Average and range of disease reaction types of parents and recombinant inbred lines of the Arina x Forno population for the two allelic state combinations for *Snn4* after inoculation with conidia of *Stagonospora nodorum* isolate 1A7a.

Genotype	Average disease reaction type	Reaction type range
Arina	3.2	2.5-3.5
Forno	2.8	2.0-3.5
Snn4/Snn4	3.0†	2.0 – 4.0
snn4/snn4	2.2†	1.0 -3.5

†Average disease reaction type of *Snn4/Snn4* is significantly different from that of the *snn4/snn4* at the 0.05 level of probability.

The effects of the *Snn4*-SnTox4 interaction in disease caused by 1A7a were investigated by conducting QTL analysis. Simple linear regression and composite interval mapping (CIM) were used to identify molecular markers and genomic regions associated with SNB resistance. One major and two minor QTL were detected on three chromosomal regions. The major QTL designated *QSnb.fcu-1AS* and a minor QTL designated *QSnb.fcu-3AS* located on the chromosome arms 1AS and 3AS, respectively, were significantly associated with the SNB disease resistance contributed by Forno (Table 3.3, Figure 3.2). A third QTL detected on the short arm of chromosome 2A, designated *QSnb.fcu-2AS*, was significantly associated with resistance contributed by Arina. *QSnb.fcu-1AS* peaked at the *Snn4* locus and explained 41% of the phenotypic variation.

QSnb.fcu-2AS peaked between the markers *Xcfa2263* and *Xbarc5*, and *QSnb.fcu-3AS* peaked between the markers *Xbarc57* and *Xbcd907*. *QSnb.fcu-2AS* and *QSnb.fcu-3AS* explained 5.4 and 6.0% of the phenotypic variation, respectively. Together these three QTL explained 50% of the total phenotypic variation in the AF population. No significant QTL× marker interactions were observed at an α level of 0.0001. All the QTL associated with

SNB disease resistance showed significant additive effects with *QSnb.fcu-1AS* providing the largest effect (Table 3.3).

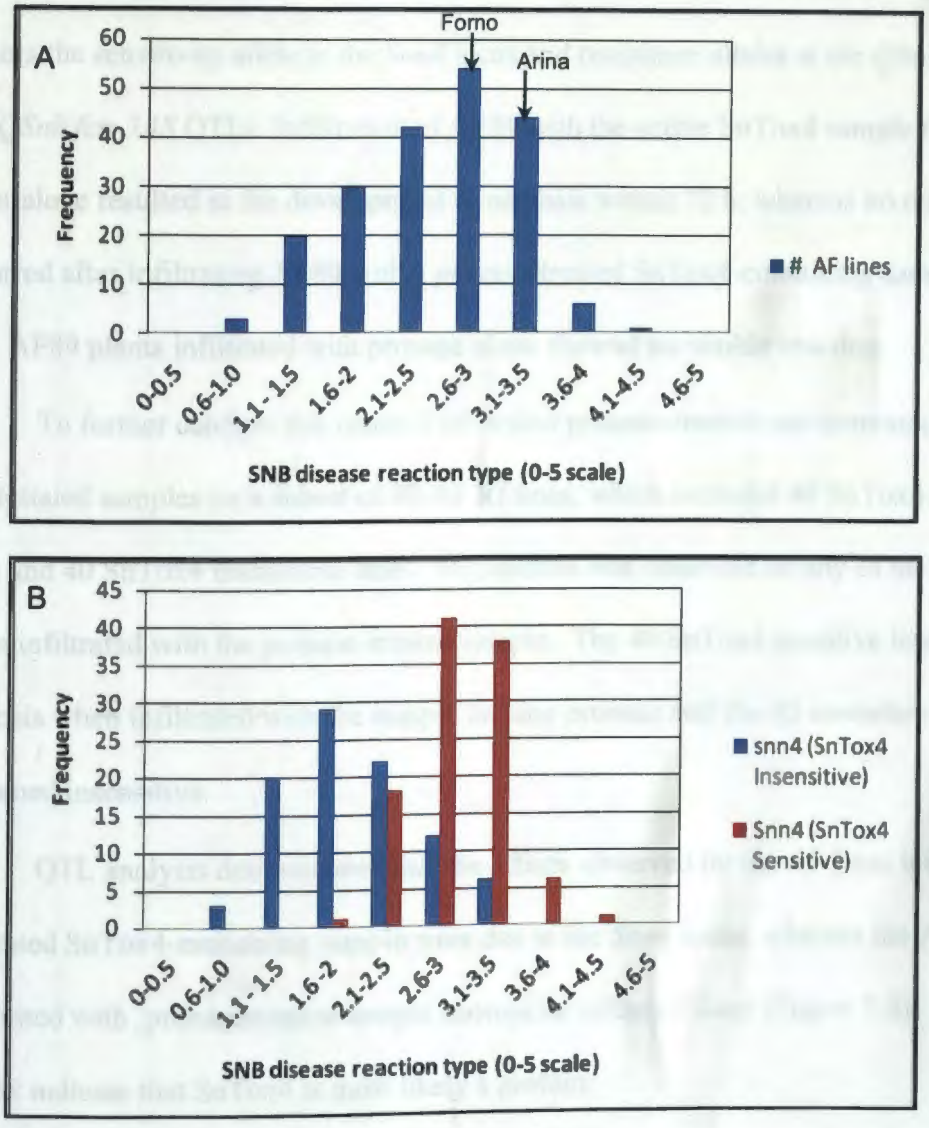

Figure 3.3. Histograms demonstrating the average SNB disease reaction type (obtained from the three replicates of 1A7a conidial inoculations) vs frequency. A: Average disease reaction types of the AF population. B: Average disease reaction types of the SnTox4 sensitive and insensitive AF lines.

Partial characterization of SnTox4

Acetone precipitated active 1A7a culture filtrates were dialyzed and subjected to cation exchange chromatography. The eluted fractions were assayed on the differential line AF89. The toxin eluted between 140 and 180 mM NaCl with the center of the activity peak at 155 mM. The RI line AF89 was chosen as the differential line for SnTox4 because it harbors the sensitivity allele at the *Snn4* locus and resistance alleles at the *QSnb.fcu-2AS* and *QSnb.fcu-3AS* QTLs. Infiltration of AF89 with the active SnTox4 sample treated with water alone resulted in the development of necrosis within 72 h, whereas no reaction occurred after infiltrating AF89 with a pronase-treated SnTox4-containing sample (Figure 3.1). AF89 plants infiltrated with pronase alone showed no visible reaction.

To further confirm this result, I infiltrated pronase-treated and untreated acetone precipitated samples on a subset of 80 AF RI lines, which included 40 SnTox4 sensitive lines and 40 SnTox4 insensitive lines. No reaction was observed on any of the 80 lines when infiltrated with the pronase-treated sample. The 40 SnTox4 sensitive lines showed necrosis when infiltrated with the sample lacking pronase and the 40 insensitive lines remained insensitive.

QTL analysis demonstrated that the effects observed by the AF lines infiltrated with untreated SnTox4-containing sample were due to the *Snn4* locus, whereas the AF lines infiltrated with pronase treated sample showed no effect of *Snn4* (Figure 3.4). These results indicate that SnTox4 is most likely a protein.

To determine the approximate size of SnTox4, the active culture filtrates were subjected to ultrafiltration using 30 kDa and 10 kDa Amicon molecular weight cutoff filters. Both the flow-through and the concentrates were infiltrated on AF89. Using active culture filtrates, both the 30 kDa flow-through and the concentrate caused necrosis (Figure

Table 3.3. QTLs for seedling resistance to Stagonospora nodorum blotch caused by the isolate 1A7a in the Arina xForno recombinant inbred population detected by composite interval mapping using the combined means of three replicates. The chromosomal locations, associated markers, peak positions, R^2, LOD, and additive values are given.

Chromosome arm	QTL designation	Marker or marker interval	Chromosome peak position (cM)	Source of resistance	R^2	LOD†	Additive effect
1AS	*QSnbfcu-1AS*	*Snn4*	170	Forno	0.41	23.32	0.39
2AS	*QSnbfcu-2AS*	*Xcfa2263-Xbarc5*	780	Arina	0.05	4.81	0.17
3AS	*QSnbfcu-3AS*	*Xbarc57-Xbcd907*	60	Forno	0.06	5.05	0.17

†LOD: log of the odds

3.1). However, only the concentrate and not the flow-through of the 10 kDa filter caused necrosis (Figure 3.1). This indicates that the size range of SnTox4 is between 10 and 30 kDa.

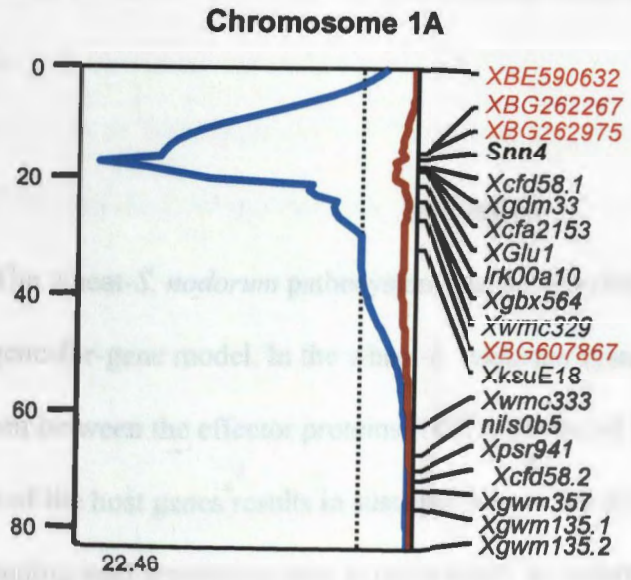

Figure 3.4. Interval regression analysis of chromosome 1A of the Arina x Forno population after infiltration of 80 recombinant inbred lines with partially purified SnTox4 cultures treated with pronase (red line) and without pronase (blue line). Markers are shown to the right of the map and a centiMorgan (cM) scale is indicated to the left. The critical LOD threshold is indicated by the dotted line.

Concentrates and filtrates of both the 10 kD and 30 kD subjected active culture filtrates were used to screen the same subset of 80 AF lines to verify the presence of SnTox4. QTL analysis indicated the presence of SnTox4 in both the concentrate and the filtrate of the 30 kDa filter and only in the concentrate of the 10 kDa filter (data not shown). This result provides further evidence that SnTox4 is in the range of 10-30 kDa.

Light has been shown to be essential for compatibility of other wheat-*S. nodorum* toxin interactions. Therefore to determine if a compatible *Snn4*-SnTox4 interaction is also light dependent, I infiltrated AF89 with partially purified SnTox4 under dark conditions.

As a control, AF89 plants were infiltrated under normal lighting conditions using the same partially purified SnTox4 sample. Infiltrated AF89 plants kept in the dark developed no symptoms, whereas the plants subjected to normal lighting showed necrosis after 48 h (Figure 3.1). This result indicates that a compatible *Snn4*-SnTox4 interaction is dependent on light.

Discussion

The wheat-*S. nodorum* pathosystem can be described as the inverse of Flor's (1956) classic gene-for-gene model. In the wheat-*S. nodorum* system, the direct or indirect interaction between the effector proteins (HSTs) produced by the pathogen and the products of the host genes results in susceptibility to the disease. If either the toxin or the corresponding host sensitivity gene is not present, an incompatible interaction occurs resulting in a resistant response. In a classical gene-for-gene system, the interaction between an effector (Avr gene product) and a corresponding host resistance (R) gene leads to localized host cell death, which is characterized by a hypersensitive response that occurs via programmed cell death. If either the Avr gene or the corresponding host R gene is not present, a compatible reaction occurs, which results in a susceptible reaction. Therefore, the wheat-*S. nodorum* system is a mirror image of the classic gene-for-gene model.

Previous studies on host-toxin interactions in the wheat-*S. nodorum* pathosystem, namely *Snn1*-SnTox1 (Liu et al. 2004b), *Tsn1*-SnToxA (Friesen et al. 2006; Liu et al. 2006), *Snn2*-SnTox2 (Friesen et al. 2007) and *Snn3*-SnTox3 (Friesen et al. 2008), have demonstrated that compatible host-toxin interactions play key roles in the development of SNB. The current work describes the identification and characterization of a fifth HST

produced by *S. nodorum* and demonstrates that a compatible *Snn4*-SnTox4 interaction is a significant factor in the development of disease. This work further broadens the understanding and characterization of the wheat-*S. nodorum* pathosystem. Like the previous four *S. nodorum* toxins that were partially characterized, SnTox4 is proteinaceous in nature. The estimated size of SnTox4 is between 10 and 30 kDa, which is similar to SnTox1 (Liu et al. 2004a) and SnTox3 (Friesen et al. 2008). Our results indicate that a compatible *Snn4*-SnTox4 interaction is light dependent as are compatible *Tsn1*-ToxA, *Snn1*-SnTox1, and *Snn2*-SnTox2 interactions (Manning and Ciuffetti 2005; Friesen et al 2007; Friesen et al. unpublished).

In contrast to the other *S. nodorum* toxins, SnTox4 is unique in that it causes a mottled necrotic reaction as compared to the severe and extensive necrosis caused by the other four toxins. It is possible that the different toxins produced by *S. nodorum* may have different levels of affinity for their host receptors or recognition factors. In this case, it might be possible that toxins with high affinity for host recognition could result in a relatively severe reaction, i.e. severe necrosis, whereas toxins with moderate or low affinity for host recognition may lead to less severe symptoms such as light or mottled necrosis. The isolation of the *Snn4* gene and the gene encoding SnTox4 will allow studies to characterize the interaction and associated pathways at the molecular level, which would provide knowledge regarding the fundamental basis of different host-toxin interactions.

Arina was susceptible to SNB caused by isolate 1A7a with an average disease reaction type of 3.2 and Forno was moderately resistant with an average reaction type of 2.8. Although the difference in parental reaction types was only 0.4, extreme transgressive segregation was observed in the population, which had reaction types ranging from 1.0 to

4.0 indicating that different resistance/susceptibility genes were contributed by both parents. A significant portion of the population was more resistant to 1A7a than was Forno, which indicates that Arina contributed a significant degree of resistance (or Forno contributed susceptibility). However, only one resistance QTL (*QSnb.fcu-2AS*) contributed by Arina was detected, and it explained only 5.4% of the phenotypic variation. It is possible that additional resistance QTLs contributed by Arina went undetected either due to their effects being too minor to detect, or the possibility that they lie within genomic regions not adequately covered by markers in this population. On the contrary, the resistance effects contributed by Forno are significantly accounted for by *QSnb.fcu-1AS* and *QSnb.fcu-3AS*, which explain 41.0 and 6.0% of the variation, respectively.

The *Snn4*-SnTox4 interaction, which was responsible for the effects of the QTL *QSnb.fcu-1AS*, explained most of the variation between Arina and Forno. Because Arina is sensitive to SnTox4 and carries the dominant *Snn4* allele for sensitivity, I consider *QSnb.fcu-1AS* to be a "susceptibility" QTL contributed by Arina. It is possible that the effects of the QTLs on chromosome arms 2AS and 3AS could also be the result of host-toxin interactions not yet identified. Further work examining fractions of 1A7a culture filtrates that lack SnTox4 is needed to determine if additional host-toxin interactions are involved in conferring disease in this population. It is also possible that HST are not the sole casual agents of pathogenesis in the *S. nodorum* system. There can be other resistance mechanisms involving various other non-HST determinants operating at the natural field conditions that contribute to disease development. Preliminary studies on this population with other toxin producing *S. nodorum* isolates have indicated that this population also segregates for the sensitivity to SnToxA and that Forno contributes to the disease

susceptibility (unpublished data). Hence it is possible that the cultivars Arina and/or Forno contain other HST sensitivity genes besides *Snn4*.

The *Snn4* locus is located near the distal end of the short arm of wheat chromosome 1A. The placement of four EST-based markers onto the AF chromosome 1A linkage map demonstrates that *Snn4* is located within the wheat 1AS3-0.86-1.00 deletion bin. This bin is known to be one of the most gene-rich regions of the wheat genome (Sandhu et al. 2001; Peng et al. 2004; Qi et al. 2004) and it contains many important disease resistance genes (see Erayman et al. 2004 for review) including the tan spot chlorosis resistance QTL *QTsc.ndsu-1AS* (Faris et al. 1997; 1999) and the *Tsc1* gene, which confers sensitivity to the tan spot chlorosis-inducing toxin Ptr ToxC (Effertz et al. 2002). Although, *Tsc1* and *Snn4* both confer sensitivity to toxins, they are not likely to be the same gene. I tested the wheat cultivar Opata 85, which carries the *Tsc1* gene and is sensitive to Ptr ToxC, with partially purified SnTox4 cultures and found it to be insensitive. Also, SnTox4 is likely a protein whereas Ptr ToxC is not. Therefore the two toxins likely have different targets and disease is induced by separate mechanisms.

Previously identified toxin sensitivity genes *Tsn1, Snn1, Snn2* and *Snn3* have been mapped to wheat chromosomes 5BL, 1BS, 2DS and 5BS respectively. *Snn1*, which confers sensitivity to SnTox1, is located near the distal end of the short arm of chromosome 1B (Liu et al. 2004a; Reddy et al. 2008). It is possible that *Snn1* and *Snn4* are homoeoallelic. However, there are no common markers between the maps generated by Liu et al. (2004a) or Reddy et al. (2008) and the map of chromosome 1A developed in the AF population. The addition of more EST-based markers to the AF map will allow for better comparisons to be made between the AF chromosome 1A map and the 1B map developed by Reddy et

al. (2008), which will help determine if *Snn1* and *Snn4* are homoeoalleles and possibly derived from a common origin.

The wheat-*S. nodorum* pathosystem now consists of five genetically well characterized host-toxin interactions. All five interactions are similar in that single dominant genes in the host confer sensitivity to the toxins, and all five play prominent roles in disease development. However, differences among the interactions exist regarding light dependence, toxin size, and now with the characterization of the *Snn4*-SnTox4 interaction, the type of symptoms that develop. Therefore, there is likely diversity in the mechanisms exploited by *S. nodorum* to cause disease. It is not yet known how many different host-toxin interactions may be involved in the wheat-*S. nodorum* pathosystem, but it is the system with the most HST-host gene interactions characterized to date and may serve well as a model for other pathosystems involving necrotrophic fungal pathogens.

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CHAPTER 4.

MARKER DEVELOPMENT AND SATURATION MAPPING OF THE TAN SPOT Ptr TOXB SENSITIVITY LOCUS *Tsc2* IN HEXAPLOID WHEAT

Abstract

Ptr ToxB is a host-selective toxin produced by the tan spot fungus, *Pyrenophora tritici-repentis,* that induces chlorosis in wheat lines harboring the *Tsc2* gene, which was previously mapped to chromosome arm 2BS in tetraploid wheat (*Triticum turgidum* L.). The objectives of this study were to determine the chromosomal location of *Tsc2* in hexaploid wheat, evaluate the effects of the *Tsc2*-Ptr ToxB interaction on disease development, develop markers suitable for marker-assisted selection against *Tsc2*, and to determine the utility of *Brachypodium* and rice (*Oryza sativa* L.) genomic information for genomic analysis of the *Tsc2* locus. Molecular mapping in a recombinant inbred population derived from the Ptr ToxB-sensitive cultivar 'Katepwa' and the Ptr ToxB-insensitive landrace Salamouni (SK population) confirmed the location of *Tsc2* on 2BS in hexaploid wheat (*T. aestivum* L.). Analysis of an F_2 population derived from the same parents indicated that a single dominant gene governed Ptr ToxB sensitivity. Thirteen microsatellite markers were used to construct a basic linkage map of the *Tsc2* region in the SK population, and 14 additional markers developed from bin-mapped expressed sequence tags (ESTs) and from ESTs identified based on colinearity with rice and *Brachypodium* were mapped to the *Tsc2* region. *Tsc2* was delineated to a 3.3 cM interval and co-segregated with marker *XBE444541*. Spore inoculations and composite interval-regression mapping demonstrated that a compatible *Tsc2*-Ptr ToxB interaction accounted for 54% of

the variation in disease expression. Analysis of Ptr ToxB-sensitive and -insensitive genotypes suggested that *XBE444541* is diagnostic for *Tsc2* and should be useful for marker-assisted selection.

Introduction

Many foliar diseases, such as tan spot (also known as yellow spot) of wheat (*Triticum aestivum* L.) caused by *Pyrenophora tritici-repentis*, result in major yield losses throughout the world, and some are caused by fungi that produce host-selective toxins (HSTs). Host selective toxins produced by necrotrophic fungal pathogens behave as effectors to induce disease susceptibility in hosts harboring genes that mediate effector recognition. The ascomycete fungus *Pyrenophora tritici-repentis* (Died.) Drechs., anamorph *Dreschslera tritici-repentis* (Died.) Shoem., is a necrotroph that causes tan spot in wheat, and it produces several HSTs (Lamari et al., 2003) including Ptr ToxA, Ptr ToxB, Ptr ToxC and several other partially characterized toxins including two named as Ptr ToxD (Tomás and Bockus, 1987; Tuori et al., 1995; Orolaza et al., 2005; Effertz et al., 2002; Ciuffetti et al., 1998; Ciuffetti et al., 2003; Meinhardt et al., 2003; Ali and ., 2002).

Ptr ToxA is a 13.2 kDa proteinaceous HST that causes necrosis in sensitive wheat genotypes and is produced by *P. tritici-repentis* races 1, 2, 7, and 8 (Zhang et al., 1997; Lamari et al., 2003). The wheat *Tsn1* gene, which maps to the long arm of wheat chromosome 5B, confers sensitivity to Ptr ToxA (Faris et al., 1996) Fine mapping of the *Tsn1* locus (Haen et al., 2004; Lu and Faris 2006; Lu et al., 2006) and bacterial artificial chromosome (BAC)-based physical mapping led to the cloning of *Tsn1* (Faris et al., 2010). *Tsn1* encodes a protein harboring resistance gene-like features including serine/threonine

protein kinase, nucleotide binding (NB), and leucine-rich repeat (LRR) domains.

Ptr ToxC is a nonionic, polar, low molecular mass molecule that, like Ptr ToxB, causes chlorosis but on host genotypes harboring the *Tsc1* gene residing on the short arm of wheat chromosome 1A (Effertz et al., 2002; Strelkov and Lamari, 2003). Unlike Ptr ToxA and Ptr ToxB, Ptr ToxC is non-proteinaceous and is produced by races 3, 6, and 8 (Strelkov and Lamari, 2003).

Ptr ToxB is produced by *P. tritici-repentis* races 5, 6, 7, and 8 and is a 6.61 KDa proteinaceous HST (Lamari et al., 2003; Strelkov et al., 1999) that induces chlorosis in sensitive wheat genotypes. *ToxB* is a complex locus comprised of a 261 bp open reading frame (ORF). It has been cloned and found to be a multi-copy gene, in comparison to the single-copy *ToxA* gene (Martinez et al., 2001). There is sequence variability of the *ToxB* gene among *P. tritici-repentis* races and homologues of the *ToxB* gene have been identified from non-pathogenic isolates that do not produce Ptr ToxB (Martinez et al., 2004; Strelkov et al., 2006). *ToxB* homologues have also been found in *P. bromi* (Died.) Drechs., and other members of the Ascomycota, suggesting an origin in early ancestors of the Ascomycota (Andrie et al., 2008). However the role(s) of these homologues are yet to be clearly identified.

Friesen and Faris (2004) mapped the gene conferring Ptr ToxB sensitivity to the distal end of the short arm of chromosome 2B using the International Triticeae Mapping Initiative (ITMI) mapping population, which was derived from the synthetic hexaploid wheat W-7984 and the hexaploid variety 'Opata 85', and designated the gene *Tsc2*. The *Tsc2* locus defined a major QTL associated with resistance to the race 5 isolate DW5 and accounted for 69% of the phenotypic variation in disease development. Therefore, a

compatible *Tsc2*-Ptr ToxB interaction played a major role in the development of tan spot. However, the *Tsc2*-Ptr ToxB interaction was not the only factor responsible for disease because QTLs with minor effects were also identified on chromosome arms 2AS and 4AL.

Studies on the inheritance and mapping of resistance to *P. tritici-repentis* race 5 isolates have also been conducted by Singh et al. (2008) who analyzed the inheritance of resistance to the race 5 isolate DW13 in multiple populations and reported that a single dominant gene governed resistance in each population. However, using conidia and culture filtrates derived from the same isolate, Singh et al. (2010) evaluated a mapping population developed from the hexaploid wheat lines 'Steele-ND' and ND375 and reported that a single recessive gene on the short arm of chromosome 2B governed resistance. The discrepancies in gene action between the two studies were attributed to variation in the expression of chlorotic symptoms caused by environmental influences.

The objectives of this study were to 1) validate the gene action and chromosomal location of *Tsc2* in an intervarietal hexaploid wheat population, 2) determine the effects of a compatible *Tsc2*-Ptr ToxB interaction on the development of disease caused by race 5 in the population, 3) develop user-friendly PCR-based markers suitable for marker-assisted selection (MAS) against Ptr ToxB sensitivity conferred by the *Tsc2* locus, and 4) evaluate the utility of rice (*Oryza sativa* L.) and *Brachypodium* genomic sequences for fine-mapping of the *Tsc2* region. Ptr ToxB is the only chlorosis-inducing proteinaceous HST identified so far and hence the characterization of the *Tsc2* gene will provide knowledge regarding mechanisms underlying the *Tsc2*-Ptr ToxB interaction at the molecular level. Knowledge of the differences and similarities of the mechanisms leading to chlorosis and necrosis will advance our knowledge of the wheat-*P. tritici-repentis* pathosystem.

Materials and methods

Plant materials

A segregating population of 150 F_2 plants derived from a cross between the Ptr ToxB-sensitive hexaploid wheat cultivar 'Katepwa' and the Ptr ToxB-insensitive hexaploid landrace Salamouni was developed initially. A total of 121 plants were advanced to the F_7 generation by single seed descent (SSD) to develop recombinant inbred lines (RILs). Plants were grown in cones containing SB100 (SunGr- Sunshine) soil mix with 10-20 granules of Osmocote (Scotts Company LLC, Marysville, OH, USA) added to each cone. Plants were advanced in the greenhouse at an average temperature of 21 °C with a 16 h photoperiod. Three lines were later found to be largely heterozygous for the *Tsc2* genomic region and were thus eliminated from the analysis. Therefore, the resulting Salamouni × Katepwa (SK) recombinant inbred population used for mapping and phenotypic analysis in this research consisted of 118 lines.

Disease evaluations and statistical analysis

The SK population was screened with DW5, a race 5 isolate of *P. tritici-repentis* known to produce Ptr ToxB (Friesen and Faris 2004). DW5 was grown on V8- potato dextrose agar (Difco ™ PDA, Becton, Dickinson and Company, Sparks, MD, USA) plates for 5-7 days in the dark, and inoculum was prepared for disease evaluations as described by Lamari and Bernier (1989) and Ali et al. (2010). Parents and the 118 SK RILs were planted in a completely randomized design (CRD) in three replicates for DW5 conidial inoculations. Each replicate consisted of a single cone (Stuewe and Sons, Inc., Corvallis, OR, USA) per line with 3 plants per cone placed in racks of 98 (Stuewe and Sons, Inc., Corvallis, OR, USA). Thus, an experimental unit consisted of 3 plants per line. The tan

spot-susceptible hard red spring wheat cultivar 'Grandin' was planted in the borders of each rack in order to reduce any edge effect. Plants were inoculated until runoff at the two- to three-leaf stage with 3000 spores mL^{-1} and 2 drops of Tween20 (polyoxyethylene sorbitan monolaurate, J.T. Baker Chemical Co., Phillipsburg, NJ, USA) per 100 mL of inoculum. Inoculated plants were placed in a mist chamber with 100% relative humidity at 21 °C for 24 h and then subjected to 6 days of incubation in the growth chamber at 21 °C under a 12 h photoperiod. Inoculated plants were rated using a 1 to 5 lesion type scale (Lamari and Bernier, 1989) at 7 days post-inoculation. Chi-square tests were conducted using the program Graphpad (http://www.graphpad.com/quickcalcs) and homogeneity of variances among the three replicates were determined by Bartlett's χ^2 test using SAS (SAS Institute Inc., 2003). Mean separation of the genotypic means were determined by Fisher's protected least significant difference (LSD) at an α level of 0.05.

Ptr ToxB production and screening

Cultures containing Ptr ToxB were obtained by expressing the *ToxB* gene using the same procedure as for *SnTox3* described by Liu et al. (2009). The commercial kit developed for the constitutive expression and purification of recombinant proteins (Invitrogen, Carlsbad, CA, USA), which includes the yeast strain *Pichia pastoris* X33 and the vector pGAPZA, was used for this purpose. The *ToxB* coding region was amplified from isolate DW5 cDNA using primer pair ToxB_LEcoRI (GAATTCATGCTACTTGCTGTGGCTATCCT) and ToxB_RXbaI (TCTAGACTAACAACGTCCTCCACTTGCCA) and cloned into the pCR4-TOPO vector using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA). After confirmation for sequence identity, the *ToxB* gene was released from the pCR4-TOPO vector by restriction

digestion with *Eco*RI and *Xba*I, cloned into the pGAPZA vector, and transformed into the wild type yeast strain *P. pastoris* X33. Competent *P. pastoris* cells were prepared and transformed using the *Pichia* EasyComp kit (Invitrogen, Carlsbad, CA, USA) as described in the user manual. Fully expanded secondary leaves were infiltrated with Ptr ToxB cultures, and the infiltrated plants were kept in the growth chamber at 21 °C under a 12 h photoperiod. Plants were evaluated 4 days after infiltration and scored as sensitive or insensitive based on the presence or absence of chlorosis. One hundred fourteen F_2 plants were infiltrated with the Ptr ToxB cultures two times (separated by six days), and the 118 lines of the SK recombinant inbred population along with parents were screened for reaction to Ptr ToxB cultures at five different times.

SSR marker identification and bin-mapped EST marker development

Deoxyribonucleic acid (DNA) was isolated from the plant tissues of the entire SK population and of the parental lines as described by Faris et al. (2000). Linkage maps consisting of over 400 simple sequence repeat (SSR) markers spanning all 21 chromosomes were developed for the SK population, and details of the whole-genome maps are provided in Chapter 5. Here, once a skeletal map of chromosome 2B was assembled, previously published genetic and physical maps of wheat were surveyed to identify additional SSR markers mapped to the short arm of chromosome of 2B, and they were selected from the following primer sets: MAG (Xue et al. 2008), GWM (Röder et al. 1998), WMC (Somers et al. 2004), HBG (Torada et al. 2006), CFD (Sourdille et al. 2004), and BARC (Song et al. 2005).

National Science Foundation (NSF)-wheat bin mapped expressed sequence tagged (EST) sequences from bin 2BS3 0.84-1.00 were downloaded from the Graingenes database

(http://compbio.dfci.harvard.edu/cgi-bin/tgi/Blast/index.cgi). These TC sequences were then subjected to BLASTn and tBLASTx searches against the rice and *Brachypodium* genomic sequences using Gramene release 31.0 (http://www.gramene.org/Multi/blastview) and Brachyblast (http://blast.brachybase.org/), respectively. The predicted proteins for the ESTs were identified by subjecting the TC sequences to BLASTx searches against the National Center for Biotechnology Information (NCBI) nonredundant database. An *e* value of *e*-20 was set as the threshold for significant matches for the searches conducted.

Additional markers were developed based on the sequences of genes residing in the corresponding regions of rice and *Brachypodium* chromosomes that were colinear with the *Tsc2* region of wheat chromosome arm 2BS. Deduced cDNA sequences of 67 genes residing within a 1.37-Mb region of *Brachypodium* chromosome 5 that corresponded to the *Tsc2* marker interval of wheat were downloaded from the *Brachypodium* web site (http://www.brachybase.org/). Similarly, deduced cDNA sequences of 28 rice genes that reside within a 1.81-Mb region of rice chromosome 4 corresponding to the *Tsc2* marker interval that had significant similarity to the downloaded *Brachypodium* sequences were downloaded from Gramene release 31.0 (http://www.gramene.org). Downloaded *Brachypodium* and rice cDNA sequences were used as queries in BLASTn searches of the DFCI wheat gene index database release 12.0 (http://compbio.dfci.harvard.edu/cgi-bin/tgi/Blast/index.cgi) to identify the corresponding wheat TC sequences. A total of 48 primer sets were developed from the identified wheat TC sequences using PRIMER3 and evaluated by PCR using the methods described above.

Linkage and regression analysis

Linkage analysis was performed using the computer program MAPMAKER V2.0

(Lander et al., 1987) for Macintosh, and the Kosambi mapping function (Kosambi, 1944) was used to calculate linkage distances. The marker order was verified using the "ripple" command with a LOD value of 3.0. Markers that could not be assigned to the map at a LOD value of 3 were placed in the most likely positions along the map.

Composite interval-regression mapping was conducted with the computer programs QGene (Joehanes and Nelson, 2008) and MapManager QTX (Manly et al., 2001) using the entire marker data set (>400 markers spanning all chromosomes) to determine the amount of variation in disease expression explained by a compatible *Tsc2*-Ptr ToxB interaction as described in Faris and Friesen (2009). A critical LOD threshold of 3.3 was determined by performing a permutation test with 1000 iterations.

Results

Genetic analysis of Ptr ToxB sensitivity

Salamouni and Katepwa exhibited insensitive and sensitive reactions to Ptr ToxB, respectively (Figure 4.1). The SK population segregated in a ratio of 52 insensitive: 66 sensitive when infiltrated with Ptr ToxB cultures, which fit the expected 1:1 ratio for a single host gene conferring Ptr ToxB sensitivity ($\chi^2_{df=1}$ 1.66, $P = 0.1975$). A total of 114 F_2 plants derived from a Salamouni by Katepwa cross were infiltrated with Ptr ToxB cultures and showed a segregation ratio of 91 sensitive: 23 insensitive, which fit the expected 3:1 ratio for a single gene ($\chi^2_{df=2}$ 1.41, $P = 0.50$) and indicated that Ptr ToxB sensitivity is conferred by the dominant allele.

Mapping the *Tsc2* locus using SSRs and bin-mapped EST-derived markers

Thirteen SSR markers were mapped to the chromosome 2B region in the SK

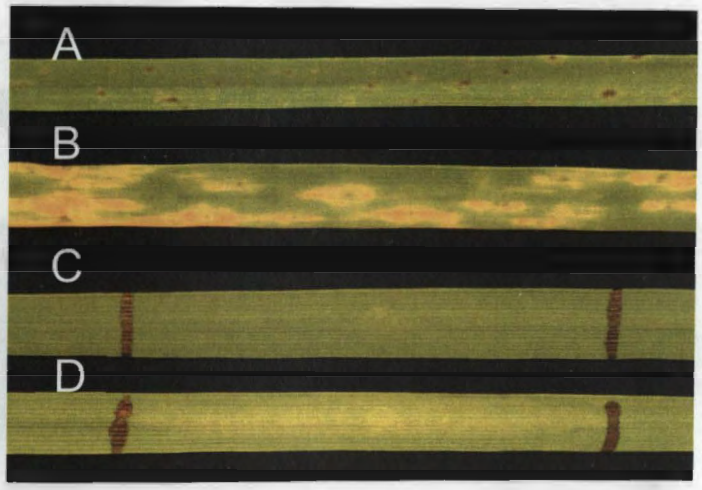

Figure 4.1. Secondary leaves inoculated with conidia of DW5, a race 5 isolate produced by the tan spot fungus *Pyrenophora tritici-repentis*, or infiltrated with Ptr ToxB. A and B: Reaction of Salamouni (A) (average disease reaction type 1.8) and Katepwa (B) (average disease reaction type 3.7) to conidial inoculations with the *P. tritici-repentis* race 5 isolate DW5. C and D: Reaction of Salamouni (C) and Katepwa (D) infiltrated with Ptr ToxB. The boundaries of the infiltrated regions are marked in black.

population that corresponded to the wheat deletion bin 2BS3 0.84-1.00, which was the region expected to harbor *Tsc2* (Figure 4.2). Of the primer sets developed from 50 bin-mapped ESTs, 24 revealed polymorphisms between Salamouni and Katepwa. Six of these amplified fragments mapped to chromosome 2B (Table 4.1, Figure 4.2).

Ten ESTs that were monomorphic as STS markers revealed RFLPs when used as probes in Southern hybridization experiments, and three of these mapped to the chromosome 2B region corresponding to deletion bin 2BS3 0.84-1.00 (Table 4.1, Figure 4.2). *Tsc2* mapped 2.7 cM proximal to the SSR marker *Xmag681* and 0.6 cM distal to the EST-STS marker *XBE517745*. The EST-RFLP marker *XBE444541*, which was later converted to an STS marker (see below), co-segregated with *Tsc2*. These results confirmed that *Tsc2* is located within the 2BS3 0.84-1.00 deletion bin. The genetic map of

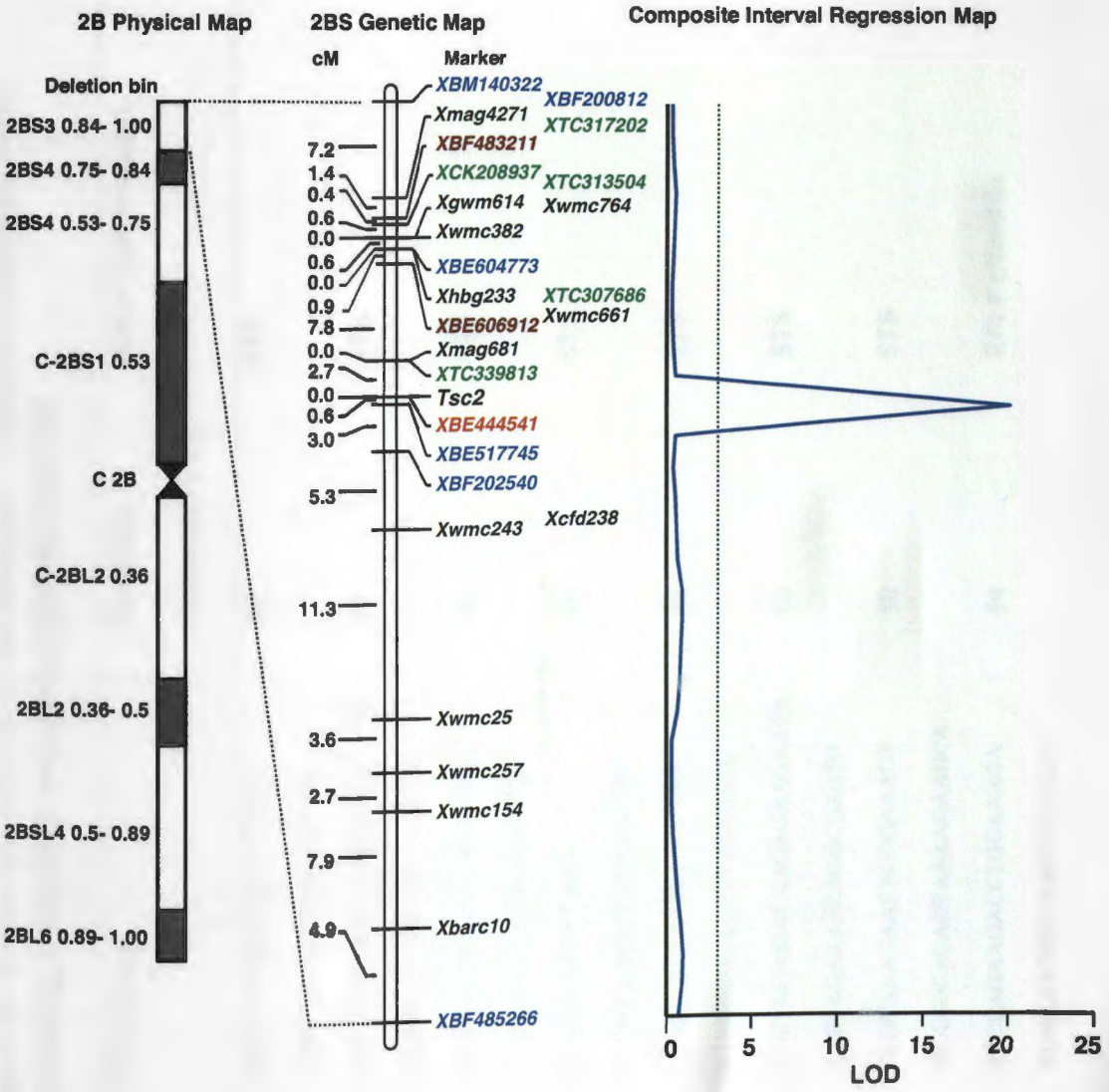

Figure 4.2. The genetic linkage map of the *Tsc2* region (middle) corresponds to the deletion bin 2BS3 0.84-1.00 on the physical map (left). Markers are indicated to the right of the genetic map. Microsatellite (SSR) markers are shown in black; STS and RFLP markers derived from bin-mapped ESTs are shown in blue and burgundy, respectively; the marker *XBE444541*, which was derived from a bin-mapped EST and initially mapped as an RFLP then converted to an STS marker, is shown in bright red; and markers developed from ESTs identified based on colinearity with rice and *Brachypodium* are shown in green. The composite interval regression map of the *Tsc2* region after inoculation with the *Pyrenophora tritici-repentis* race 5 isolate DW5 is on the right. The critical LOD threshold of 3.30 is indicated by the dotted line, and the LOD scale is indicated at the bottom.

Table 4.1. Expressed sequence markers mapped on chromosome arm 2BS in the Salamouni × Katepwa recombinant inbred population. The source, primer sequences, annealing temperatures, and marker types are indicated.

Source	Marker	PCR primers	Annealing temperature (°C)	Marker type (enzyme)
2BS-3	*XBM140322*	AAGGCTCACCAACAACTCC CTCGGAACAGCAACATTACC	50	STS
2BS-3	*XBF200812*	GCATTCCCTCTAACGACTGA TTCTTTGATAACCATCCGTAGG	50	STS
2BS-3	*XBE604773*	GAGAGGCGCAAACAAAATAA AACATCCTCATAGCCATCCA	50	STS
2BS-3	*XBE444541*†	TGGACCAGTATGAGA TTCTGGAGGATGTTGAGCAC	55	STS
2BS-3	*XBE517745*	CTCACCTCCATTGCCTCTAA AGAACCGAAGATTGCTGATG	50	STS
2BS-3	*XBF202540*	TGGTTATGGACAAGAGAAGAAAA AGGTAGAAATGAGGCGAGTG	50	STS
2BS-3	*XBF485266*	CAGCAAAGAACACGGAAAGT GCAAGCACAGTAAAAGAAGCA	50	STS
2BS-3	*XBE606912*	GTCCAGAAGACCTGCAAAGA AGACAAGGTGAACTCCCACA	54	RFLP (*Hin*dIII)

Table 4.1. Continued

Source	Marker	PCR primers	Annealing temperature (°C)	Marker type (enzyme)
2BS-3	*XBF483211*	GATACACCAATGACGGAACAA GCCAAGAAGGCTCCAATAAG	50	RFLP (*Eco*RI)
Rice and *Brachypodium* Colinearity	*XTC317202*	AGGCATTTGGTCATTTTGG GAAGGCATACTCAACAGAAATCA	50	STS
Rice and *Brachypodium* Colinearity	*XTCK208937*	TCTCATTAATGGCGCTCTCC GAGGTTTCTTCTTGCGCTTG	52	STS
Rice and *Brachypodium* Colinearity	*XTC313504*	CTCGTCATGGGGTGACTTTT TGGATCTGCTCGTCAAAGTG	50	STS
Rice and *Brachypodium* Colinearity	*XTC307686*	AAGTCGCGTTGATGCAATTA TTGACGTTTGCAACAGTTGAT	54	STS
Brachypodium Colinearity	*XTC339813*	CGTTCTTTGCACATCACTAA TGGAAATGCCACATCACTGT	50	STS

[†]Marker *XBE444541* was initially mapped as an RFLP marker and later converted to a PCR-based STS marker.

the region developed using SSRs and bin-mapped EST-based markers consisted of 22 DNA markers in addition to the *Tsc2* locus and spanned a genetic distance of 60.9 cM.

Comparative analysis and additional marker development based on colinearity with rice and *Brachypodium*

The sequences of the ESTs that mapped to chromosome arm 2BS in the SK population were used as query sequences to search the rice and *Brachypodium* genome sequences to identify putative orthologs. Six of the nine mapped wheat EST sequences had similarity to sequences on *Brachypodium* chromosome 5 (Table 4.2), and five of these six had similarity to sequences on rice chromosome 4 (Table 4.3). One EST (BF483211), which had similarity to a sequence on *Brachypodium* chromosome 5, had similarity to a sequence on rice chromosome 10 (Table 4.3). The remaining three mapped EST sequences, BM140322, BF200812, and BE606912, had no significant similarity to any rice or *Brachypodium* sequences (Tables 4.2 and 4.3). Furthermore, BM140322 and BE606912 had no similarity to any protein sequences in the NCBI database, whereas putative homologs for the other seven mapped ESTs were identified (Table 4.4).

The SK 2BS genetic linkage map and *Brachypodium* chromosome 5 were colinear with only one exception (Figure 4.3). The sequence represented by marker *XBE604773* was inverted relative to that of marker *XBF483211* in *Brachypodium* compared to wheat. Colinearity was also well conserved among the five EST markers mapped to wheat 2BS and their putative orthologs on rice chromosome 4 except that the sequence represented by marker *XBF485266* was inverted relative to the positions of the other four markers.

The comparative analysis indicated that good levels of colinearity existed within a 1.81-Mb region of rice chromosome 4, a 1.37-Mb region of *Brachypodium* chromosome 5,

Table 4.2. Putative *Brachypodium* orthologs of the EST/TC sequences mapped on chromosome 2B in the Salamouni×Katepwa recombinant inbred population based on significant BLASTn and tBLASTx hits to *Brachypodium* genomic sequences.

		BLASTn				tBLASTx			
Marker	TC[†]	Gene	e value	Position (bp)	Chr.[‡]	Gene	e value	Position (bp)	Chr.
XBM140322	TC312601		ns[§]				ns		
XBF200812	TC339576		ns			Bradi5g214001	2e-81	24,030,676	5
XTC317202	TC317202	Bradi5g01430	8e-94	1,399,986	5	Bradi5g014301, 02	2e-95	1,400,019	5
XBF483211	TC319144	Bradi5g017401	3e-65	1,760,470	5	Bradi5g017401	1e-119	1,760,449	5
XCK208937	N/A[¶]	Bradi5g018701	1e-21	1,898,167	5	Bradi5g018701	7e-27	1,897,704	5
XTC313504	TC313504	No hits	2e-43	1,791,258	5	No hits	0	1,791,751	5
XBE604773	TC288658	Bradi5g01230	2e-83	1,144,639	5	Bradi5g012301	0	1,144,638	5
XTC307686	TC307686	No hits	2e-66	4,218	5	Bradi5g013401	1e-106	1,263,938	5
XBE606912	TC320680		ns				ns		
XTC339813	TC339813	Bradi5g01940	0	2,011,335	5	Bradi5g019401	0	2,011,053	5
XBE444541	TC289693	Bradi5g02160	1e-77	2,253,564	5	Bradi5g021601	1e-159	2,252,432	5
XBE517745	TC323680	Bradi5g023001	4e-62	2,408,534	5	Bradi5g023001	1e-142	2,408,533	5
XBF202540	TC358868	Bradi5g02400	1e-33	2,518,619	5	Bradi5g024001 -04	2e-78	2,518,619	5
XBF485266	TC296434	Bradi5g03810	1e-104	4,774,323	5	Bradi5g038101	1e-148	4,772,425	5

[†]TC: tentative consensus; [‡]Chr: chromosome; [§]ns: not significant; [¶]N/A: not available

Table 4.3. Putative rice orthologs of the EST/TC sequences mapped on chromosome 2B in the Salamouni × Katepwa recombinant inbred population based on significant BLASTn and tBLASTx hits to rice genomic sequences.

		BLASTn				tBLASTx			
Marker	TC[†]	Gene	e value	Position (bp)	Chr.[‡]	Gene	e value	Position (bp)	Chr.
XBM140322	TC312601		ns[§]				ns		
XBF200812	TC339576		ns			LOC_Os02g32770	2.70e-29	19,453,542	2
XTC317202	TC317202	LOC_OS04g02870	4.90e-159	1,120,206	4	LOC_OS04g02870	130e-66	1,118,608	4
XBF483211	TC319144	LOC_Os10g22560	580e-19	11,621,740	10	LOC_Os07g01070	430e-55	41,699	7
XCK208937	N/A[¶]	LOC_Os04g02510	82e-94	913,265	4	LOC_Os04g02510	15e-34	913,703	4
XTC313504	TC313504	LOC_Os04g27340	600e-21	15,987,294	4	LOC_Os04g27670	14e-119	16,180,747	4
XBE604773	TC288658	LOC_Os04g04254	190e-292	1,987,260	4	LOC_Os04g04254	540e-189	1,987,261	4
XTC307686	TC307686	No hits	38e-59	30,386	9	LOC_Os04g03990	11e-68	1,833,746	4
XBE606912	TC320680		ns				ns		
XTC339813	TC339813		ns			LOC_Os04g02110	340e-124	684,478	4
XBE444541	TC289693	LOC_Os04g01590	320e-282	399,306	4	LOC_Os04g01590	130e-137	399,307	4
XBE517745	TC323680	LOC_Os04g01480	290e-211	325,319	4	LOC_Os04g01480	160e-120	327,479	4
XBF202540	TC358868	LOC_Os04g01230	170e-53	179,571	4	LOC_Os04g01230	450e-50	178,955	4
XBF485266	TC296434	LOC_Os04g14790	200e-203	8,304,461	4	LOC_Os04g14790	690e-118	8,302,979	4

[†]TC: tentative concensus; [‡]Chr: chromosome; [§]ns: not significant; [¶]N/A: not available.

Table 4.4. Putative proteins of the EST markers mapped to wheat chromosome arm 2BS based on BLASTx searches.

GenBank accession	Marker	TC[†]	Predicted protein based on NCBI BLASTx	e value
BM140322	***XBM140322***	**TC312601**	**N/A[‡]**	**ns[§]**
BF200812	*XBF200812*	TC339576	cytochrome P450 [*Triticum aestivum*]	6e-55
N/A	*XTC317202*	TC317202	Os04g0118900 [Oryza sativa (japonica cultivar-group)]	2e-101
BF483211	*XBF483211*	TC319144	hypothetical protein SORBIDRAFT_01g027250 [*Sorghum bicolor*]	1e-91
N/A	*XCK208937*	CK208937	hypothetical protein OsI_14623 [Oryza sativa Indica Group]	4e-33
N/A	*XTC313504*	TC313504	OSIGBa0106G08.3 [Oryza sativa (indica cultivar-group)]	3e-164
BE604773	*XBE604773*	TC288658	UDP-glucose:sterol glucosyltransferase [*Avena sativa*]	0
N/A	*XTC307686*	TC307686	Os04g0129200 [Oryza sativa (japonica cultivar-group)]	7e-96
BE606912	*XBE606912*	TC320680	N/A	ns
N/A	*XTC339813*	TC339813	Vrga1 [*Aegilops ventricosa*]	3e-153
BE444541	*XBE444541*	TC289693	hypothetical protein SORBIDRAFT_06g000580 [*Sorghum bicolor*]	9e-173
BE517745	*XBE517745*	TC323680	hypothetical protein OsI_14559 [*Oryza sativa* Indica Group]	1e-164
BF202540	*XBF202540*	TC358868	OSIGBa0123D13.3 [*Oryza sativa* (indica cultivar-group)]	8e-112
BF485266	*XBF485266*	TC296434	hypothetical protein OsJ_13895 [*Oryza sativa* Japonica Group]	3e-143

[†]TC: tentative consensus; [‡]N/A: not applicable; [§]ns: not significant

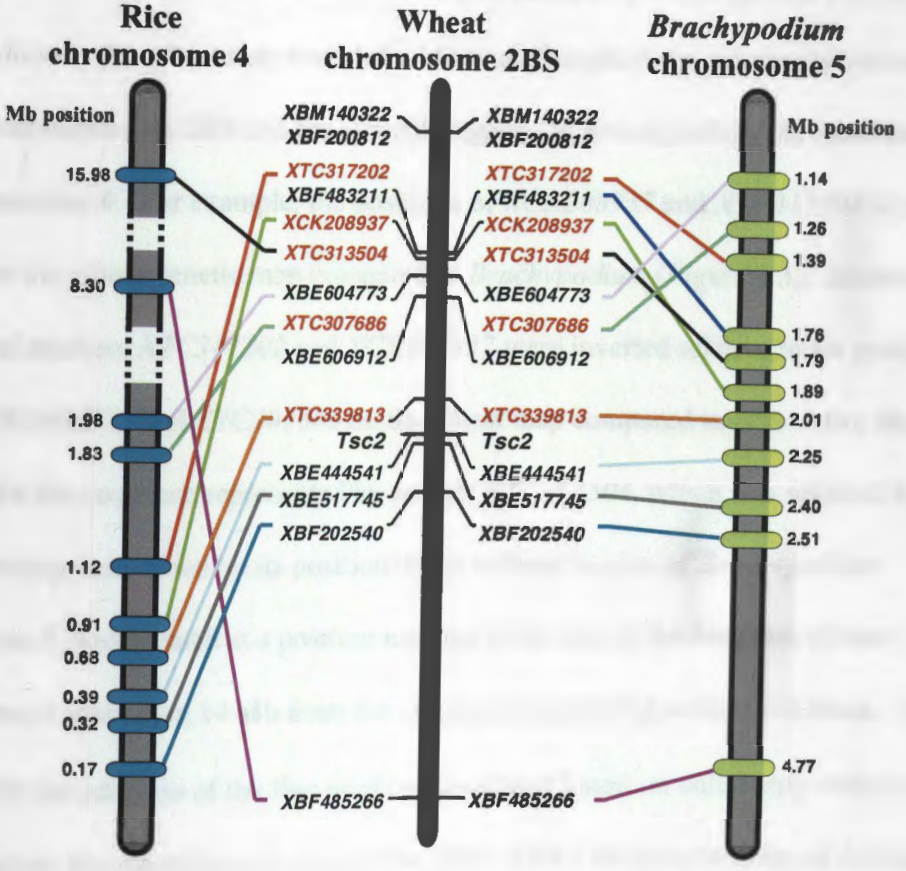

Figure 4.3. Comparison of the *Tsc2* region in wheat chromosome 2BS (middle) with rice chromosome 4 (left) and *Brachypodium* chromosome 5 (right). Markers developed based on bin-mapped ESTs are indicated in black, and markers developed based on colinearity with rice and *Brachypodium* are indicated in red. The Mb positions of colinear genes are indicated to the left of rice chromosome 4 and to the right of *Brachypodium* chromosome 5. Hatched regions of rice chromosome 4 indicate they are not to scale.

and the region between the markers *XBE604773* and *XBF202540* on wheat chromosome 2B (Figure 4.3).Forty-eight primer sets were developed from gene sequences within the 1.37-Mb segment of *Brachypodium*, and 17 of these revealed polymorphisms between Salamouni and Katepwa. Of these 17, five (*XTC317202, XCK208937, XTC313504, XTC307686* and *XTC339813*) detected loci on chromosome 2B and were placed on the linkage map (Figures 2, 3). *XTC339813* co-segregated with the SSR marker *Xmag681*, and the remaining four loci were more distal to *Tsc2*.

(TC289693) and the sequence of the putative rice ortholog (LOC_Os04g01590) were aligned using ClustalW (http://www.ebi.ac.uk/Tools/clustalw2/index.html). Eight primer sets were developed from the TC sequence by targeting the boundaries of introns identified based on annotation of the rice gene sequence. Several of the primer pairs revealed indel polymorphisms between Salamouni and Katepwa, and the one giving the clearest profile was selected for genotyping the SK population (Table 4.1). The *XBE444541* EST-STS marker co-segregated with the corresponding RFLP marker confirming that both represent the same locus and co-segregate with *Tsc2*.

To evaluate the utility of the *XBE444541* EST-STS marker for use in MAS programs, seven Ptr ToxB-insensitive and seven Ptr ToxB-sensitive wheat cultivars were genotyped. A 340-bp fragment amplified in Katepwa was specific to Ptr ToxB-sensitive wheat genotypes, whereas Salamouni and the other Ptr ToxB-insensitive genotypes yielded a 505-bp allele (Figure 4.4).

Effects of a compatible *Tsc2*-Ptr ToxB interaction on tan spot development in the SK population

The average disease reaction types for Salamouni and Katepwa after inoculation with the race 5 isolate DW5 were 1.8 and 3.7 respectively (Table 4.5, Figure 4.1). The three replicates of the SK population inoculations were homogeneous based on Bartlett's χ^2 test for homogeneity ($\chi^2_{df=2}$ 3.35, $P = 0.1868$), and therefore average reaction types were calculated for each RIL and used in the analysis. The average disease reaction types of the SK population ranged from 1.5 to 4.0 with an overall mean of 2.8. The mean disease reaction type of Ptr ToxB-sensitive RILs was 3.2 and ranged from 2.0 to 3.8, whereas the mean disease reaction type of Ptr ToxB-insensitive RILs was 2.3 and ranged from 1.3 to

3.8 (Table 4.5, Figure 4.5).

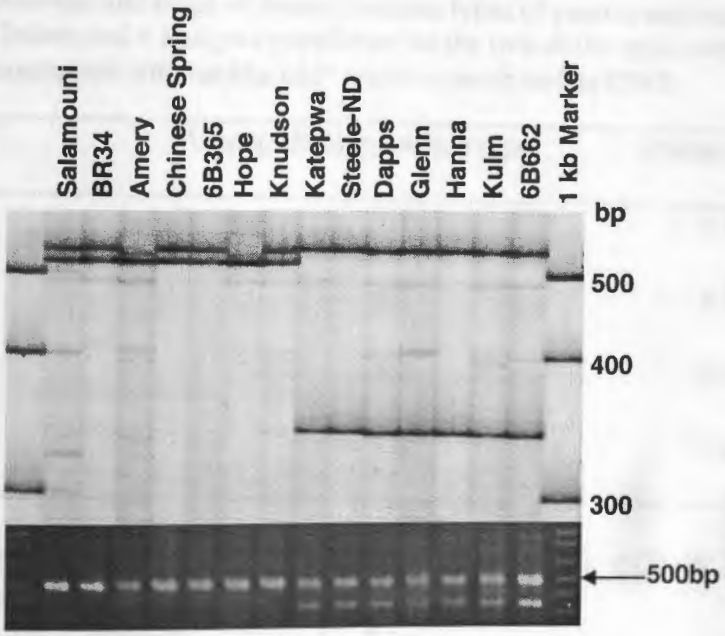

Figure 4.4. Polyacrylamide (top) and agarose (bottom) gel electrophoresis of PCR products amplified from seven Ptr ToxB-insensitive genotypes (lanes 2-8; cultivars Salamouni through 'Knudson') and seven Ptr ToxB-sensitive genotypes (lanes 9-15; cultivars Katepwa through 6B662) with the primer set for marker *XBE444541*. Lanes are annotated across the top. A 1 kb ladder is shown in lanes 1 and 16.

Composite interval regression mapping indicated that the *Tsc2* locus defined the peak of a major QTL with an LOD of 20.02 on chromosome 2B (Figure 4.2). The *Tsc2* locus explained 54% of the variation in disease expression caused by the DW5 isolate, and resistance was contributed by Salamouni, the Ptr ToxB-insensitive parent.

Discussion

Wheat-*P. tritici-repentis* interactions have gained much attention over the past few decades due to the devastating impacts of tan spot on wheat production throughout the world. To date, three of the HSTs produced by the tan spot fungus, Ptr ToxA, Ptr ToxB and Ptr ToxC, have been characterized and the chromosomal locations of the corresponding

Table 4.5. Average and range of disease reaction types of parents and recombinant inbred lines of the Salamouni × Katepwa population for the two allelic state combinations for *Tsc2* after inoculation with conidia of *P. tritici-repentis* isolate DW5.

Genotype	Average disease reaction type	Reaction type range
Salamouni	1.8	1.5 – 2.0
Katepwa	3.7	3.5 – 4.0
Tsc2Tsc2	3.2†	2.0 – 3.8
tsc2tsc2	2.3†	1.3 – 3.8

†Average disease reaction type of *Tsc2Tsc2* is significantly different from that of the *tsc2tsc2* at the 0.05 level of probability.

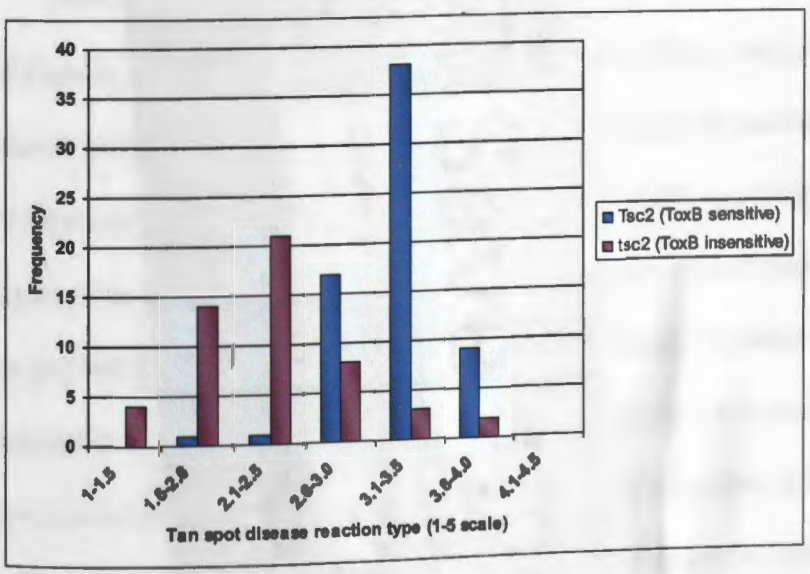

Figure 4.5. Histogram of the average tan spot disease reaction types of the Ptr ToxB-insensitive and -sensitive recombinant inbred lines versus frequency in the Salamouni × Katepwa population.

host sensitivity genes, *Tsn1* (Faris et al., 1996), *Tsc1* (Effertz et al., 2002), and *Tsc2* (Friesen and Faris, 2004) have been reported. Of the three wheat-*P. tritici-repentis*

interactions, only *Tsn1*-ToxA has been studied in detail.

Tsc2 was initially mapped to chromosome arm 2BS in the ITMI population, which was developed by crossing a synthetic hexaploid wheat W-7984 with the hard red spring wheat variety 'Opata 85'. W-7984 was synthesized from the durum wheat variety 'Altar 84' and *Aegilops tauschii* accession CI 18-WPI 219. The B genome donor in the ITMI population, Altar 84, is a tetraploid and was shown to be sensitive to Ptr ToxB whereas Opata 85 was insensitive. Therefore, the *Tsc2* gene was derived from the durum variety Altar 84. In this study, the genomic position of *Tsc2* harbored by the hexaploid variety Katepwa was also located on 2BS, which indicates that Ptr ToxB sensitivity is controlled by the *Tsc2* locus on 2BS in both tetraploid and hexaploid wheat.

Previous reports regarding the inheritance of resistance to tan spot race 5 isolates have been in disagreement. Singh et al. (2008) reported that a single dominant gene governed resistance to race 5, whereas Singh et al. (2010) indicated that a single recessive gene was responsible for conferring resistance. Our results agree with the latter in that analysis of an F_2 population with Ptr ToxB clearly demonstrated that a single dominant gene governs sensitivity, and because sensitivity is highly correlated with susceptibility, resistance to isolate DW5 would primarily be governed by the recessive *tsc2* allele. Investigating gene action and inheritance by conidial inoculations is difficult due to possible environmental influences, the effects of minor genes on conferring resistance to race 5 (Friesen and Faris, 2004), and the fact that experiments involving inoculation of F_1 and F_2 plants cannot be replicated. However, Ptr ToxB infiltrations, especially with *Pichia*-produced cultures, circumvent the effects of other minor genes, are less affected by

environmental variables, and can be repeated multiple times on the same plant to obtain accurate results.

The bin-mapped ESTs provide a useful source of sequences for marker development, and they have been used extensively to saturate genomic regions of wheat that harbor targeted loci (Lu et al., 2006; Reddy et al., 2008; Zhang et al., 2009). Here, I mapped nine markers derived from bin-mapped ESTs to the *Tsc2* region on 2BS. While this is only 18% of the 50 ESTs selected for marker development, additional efforts to identify single nucleotide polymorphisms (SNPs) through allele sequencing would likely prove to be effective for mapping more of the ESTs to the *Tsc2* region in the SK population.

The usefulness of rice and *Brachypodium* genomic information in the development of markers and genomic analysis of the *Tsc2* region in wheat was also investigated and led to the development of five additional markers near the *Tsc2* locus. Both *Brachypodium* chromosome 5 and rice chromosome 4 were perfectly colinear with the wheat *Tsc2* region between markers *XTC339813* and *XBF202540.* However, some disruptions in colinearity were observed between wheat and *Brachypodium* in the region distal to *Tsc2*, and between wheat and rice in regions both proximal and distal to *Tsc2*. Regardless, it is evident that the degree of colinearity between wheat chromosome arm 2BS and *Brachypodium* chromosome 5 was better than that of 2BS and rice chromosome 4 at the macro level of resolution, which agrees with other studies demonstrating that *Brachypodium* is more closely related to wheat than rice is (Vogel et al., 2006; Bossolini et al., 2007; Faris et al., 2008; Huo et al., 2009). Therefore *Brachypodium* genomic information may be more useful for conducting further genomic analysis and additional marker development in wheat

compared to rice. The *Brachypodium* orthologs of markers *XTC339813* and *XBE517745*, which flank *Tsc2*, lie approximately 390 kb apart. Forty-three genes lie within this interval, of which the majority have either no significant hits to any proteins of known function or code for yet unidentified hypothetical proteins. Therefore, none of these genes seem to be strong candidates for *Tsc2*. However, this gene information could be useful for the development of additional markers for use in targeting the *Tsc2* locus.

Pyrenophora tritici-repentis race 5 isolates were first identified in Algeria (Orolaza et al., 1995) and later found in North Dakota, USA (Ali et al., 1999). A primary objective of this research was to develop markers tightly linked to the *Tsc2* locus that would be suitable for use in MAS schemes. Marker-assisted selection using co-dominant markers is less expensive and less time consuming compared to conventional disease screening or toxin infiltrations, especially when backcrossing to pyramid recessive resistance genes because genotypes that are homozygous susceptible cannot be distinguished from heterozygotes when screening by toxin infiltrations or spore inoculations. The PCR-based marker *XBE444541*, which co-segregates with *Tsc2,* will be useful for high-resolution mapping and eventual cloning of *Tsc2*. In addition, the evaluation of *XBE444541* in 14 wheat cultivars indicates the potential diagnostic capabilities of this marker suggesting that it should be useful for association mapping studies and MAS schemes. The SSR marker *Xmag681* and the EST-STS markers *XTC317202* and *XBE517745*, which together delineate *Tsc2* to a 3.3-cM interval, are all viable alternatives for use in MAS against Ptr ToxB sensitivity.

Analysis of a compatible *Tsc2*-Ptr ToxB interaction in the SK population confirmed that it plays a major role in conferring susceptibility to race 5 isolates of *P. tritici-repentis*

by explaining 54% of the variation in disease expression. Although genome-wide QTL scans revealed no additional loci significantly associated with disease caused by DW5 (data not shown), some Ptr ToxB-insensitive lines were susceptible to the disease (Figure 4.5) suggesting that other factors with minor effects could be involved as well. The reaction type ranges for *Tsc2Tsc2* (2.0-3.8) and *tsc2tsc2* (1.3-3.8) allelic combinations also suggest that there are other factors contributing to disease development in the SK population other than the *Tsc2*-Ptr ToxB interaction. Friesen and Faris (2004) reported three QTL with minor effects for resistance to *P. tritici-repentis* race 5 residing on chromosome arms 2AS, 4AL and 2BL. Others have reported the action of minor QTL and race nonspecific QTL as well. For example, Chu et al. (2008) reported novel QTL located on 2AS and 5BL that conferred resistance to *P. tritici-repentis* races 1, 2 and 5, and Faris and Friesen (2005) reported QTL on chromosome arms 1BS and 3BL that conferred resistance to races 1, 2, 3, and 5. These results expose the complexity of the wheat-*P. tritici-repentis* pathosystem and emphasize the importance of conducting replicated experiments and using QTL analysis for characterizing wheat-*P. tritici-repentis* interactions.

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CHAPTER 5.

WHOLE GENOME QTL ANALYSIS OF STAGONOSPORA NODORUM BLOTCH RESISTANCE AND VALIDATION OF THE SnTOX4-*Snn4* INTERACTION IN HEXAPLOID WHEAT

Abstract

Necrotrophic effectors (also known as host-selective toxins) are important determinants of disease in the wheat-*Stagonospora nodorum* pathosystem. To date, five necrotrophic effector-host gene interactions have been identified in this system. Most of these interactions have additive effects while some are epistatic. The *Snn4*-SnTox4 interaction was originally identified in a recombinant inbred population derived from a cross between the Swiss winter wheat varieties 'Arina' and 'Forno' using the *S. nodorum* isolate Sn99CH 1A7a. Here, I used a recombinant inbred population consisting of 121 lines developed from a cross between the hexaploid land race Salamouni and the hexaploid wheat cultivar 'Katepwa' (SK population). The SK population was used for the construction of linkage maps and QTL detection using the Swiss *S. nodorum* isolate Sn99CH 1A7a. The linkage maps developed in the SK population spanned 3,228.0 cM and consisted of 441 SSRs, 9 RFLPs, 29 EST-STS markers and 5 phenotypic markers. The average marker density was 6.7 cM/marker. Two QTL, designated *QSnb.fcu-1A* and *QSnb.fcu-7A* on chromosome arms 1AS and 7AS, respectively, were associated with disease caused by the *S. nodorum* isolate Sn99CH 1A7a. The effects of *QSnb.fcu-1A* were determined by the *Snn4*-SnTox4 interaction and accounted for 23.5% of the phenotypic variation in this population whereas *QSnb.fcu-7A* accounted for 16.4% of the phenotypic

variation for disease but was not associated with any known effector sensitivity locus. The effects of both QTL were largely additive and collectively accounted for 35.7% of the total phenotypic variation. The results of this research validate the effects of a compatible *Snn4*-SnTox4 interaction in a different genetic background, and it provides knowledge regarding genomic regions and molecular markers that can be used to improve SNB resistance in wheat germplasm.

Introduction

Wheat (*Triticum aestivum* L.) is the third most-produced cereal and ranks second among food crop species in the world [FAOSTAT (http://faostat.fao.org).2007.retrieved 8-24-2010]. Like any other crop species, wheat is also prone to diseases caused by a number of pathogens, the majority of which are caused by fungi. *Stagonospora nodorum* (Berk.) Castell. & Germano (teleomorph: *Phaeosphaeria nodorum* (Müll.) Hedjar.) is a necrotrophic fungal pathogen that causes Stagonospora nodorum blotch (SNB), a major disease in wheat. SNB causes yield losses and reduction in grain quality across the globe (Eyal 1981; King et al., 1983; Eyal et al., 1987). This filamentous ascomycete produces multiple necrotrophic effectors, also known as host-selective toxins (HSTs), that are major disease determinants. *S. nodorum* is pathogenic on wheat, barley, and a wide range of wild grasses (Solomon et al., 2006), and has the ability to infect both the glume and the leaf, causing glume blotch and leaf blotch, respectively.

Necrotrophic effectors are a special class of pathogen effectors which range from low molecular weight metabolites to proteins, and induce cell death resulting in disease in specific genotypes of the host (Friesen et al., 2008a; Wolpert et al., 2002). Sensitivity to

these effectors is more often a dominant trait and the effectors can reproduce the symptoms of the disease in part or in whole when introduced into plants at appropriate concentrations (Walton, 1996; Oliver and Solomon, 2010). In necrotrophic systems such as *S. nodorum* or *Pyrenophora tritici-repentis* (the fungal pathogen that causes tan spot of wheat), the effectors produced by the pathogen are known to cause disease in the presence of corresponding host gene products required for susceptibility (Wolpert et al., 2002).

In recent years, resistance to wheat diseases such as SNB and tan spot, which are caused by fungal pathogens that produce necrotrophic effectors, has been characterized in host mapping populations derived from diverse wheat genotypes. Whole genome maps developed in such mapping populations have become an important tool, not only for the identification of quantitative trait loci (QTL) associated with agronomically important quantitative traits, but also for map-based cloning and marker-assisted selection. Genetic map construction requires a good mapping population and high-throughput, user-friendly molecular markers such as simple sequence repeats (SSR) or microsatellites. However, detecting polymorphism in wheat populations derived from intra-specific or inter-varietal crosses can be difficult as wheat has a narrow genetic base (Messmer et al., 1999).

Wheat mapping populations that segregate for multiple effector sensitivities have been used in comprehensive QTL studies of the wheat-*S. nodorum* pathosystem. These studies have used genetic maps comprised of a combination of molecular marker types to add to our understanding of the multigenic inheritance of resistance to *S. nodorum*. QTL associated with SNB expression have been detected in almost all of the hexaploid wheat chromosomes. However, no necrotrophic effector-host gene interactions were reported for the majority of these studies (reviewed in Friesen et al., 2008a). To date, five necrotrophic

effectors produced by *S. nodorum* (SnToxA, SnTox1, SnTox2, SnTox3, and SnTox4), and the corresponding host sensitivity genes (*Tsn1*, *Snn1*, *Snn2*, *Snn3*, *Snn4*), which are all dominant in nature, have been documented (Liu et al., 2004, 2006, Friesen et al., 2006, 2007, 2008b; Abeysekara et al., 2009; reviewed in Friesen and Faris, 2010). The results of these studies indicate that each of the effectors play significant roles in the development of SNB. Several other uncharacterized necrotrophic effectors are also known to be produced by *S. nodorum* (Friesen et al., unpublished).

The ToxA-*Tsn1* interaction is the most thoroughly characterized of the five necrotrophic effector-host gene interactions in the wheat-*S. nodorum* pathosystem. Since ToxA was first discovered in *P. tritici-repentis*, the majority of the early work on the ToxA-*Tsn1* interaction was done in relation to the *P. tritici-repentis*-wheat interaction. Approximately two decades after its discovery in *P. tritici-repentis*, *ToxA* was identified in *S. nodorum* and was shown to have been involved in a recent lateral gene transfer event from *S. nodorum* to *P. tritici-repentis* (Friesen et al., 2006).

The compatible wheat-*S. nodorum* effector interactions can have additive (Friesen et al., 2007, 2008b, 2009) or epistatic effects (Friesen et al., 2008b) making this system complex. Evaluation of the BG recombinant inbred line (RIL) population with the *S. nodorum* isolate Sn6 revealed that the presence of both the SnToxA-*Tsn1* and SnTox2-*Snn2* interactions can be additive and result in higher susceptibility to disease than when just one interaction is present (Friesen et al., 2007). However, analysis of the same population with different *S. nodorum* isolates showed that both SnToxA-*Tsn1* and SnTox2-*Snn2* interactions are epistatic to the SnTox3-*Snn3* interaction (Friesen et al., 2008b).

The latest addition to the wheat–*S. nodorum* pathosystem, the SnTox4-*Snn4*

interaction, was studied by evaluating an RIL population derived from a cross between the Swiss winter wheat varieties 'Arina' and 'Forno' (AF population) with the Swiss *S. nodorum* isolate Sn99CH 1A7a (Abeysekara et al., 2009). SnTox4 was estimated to be 10-30 kDa in size and proteinaceous in nature. Arina was sensitive to SnTox4 while Forno was insensitive. Infiltration of culture filtrates containing SnTox4 resulted in mottled necrosis in the susceptible wheat variety Arina as opposed to the severe necrosis induced by the other necrotrophic effector-host interactions. *Snn4*, a single dominant gene which governs sensitivity to SnTox4, was mapped to the short arm of wheat chromosome 1A and accounted for 41% of the phenotypic variation. The effects of this major QTL and two additional minor QTL detected on the short arms of chromosomes 2A and 3A were largely additive explaining 50% of the total phenotypic variation.

Our preliminary research indicated that the wheat hexaploid landrace Salamouni was resistant to numerous isolates of *S. nodorum*, whereas the cultivar Katepwa was susceptible. Therefore, the objectives of this research were to develop an RIL population derived from Salamouni and Katepwa, construct whole-genome genetic linkage maps in the RIL population, and identify QTL and/or host-effector interactions associated with SNB caused by the *S. nodorum* isolate Sn99CH 1A7a.

Materials and methods

Plant materials

A RIL population, hereafter referred to as the SK population, consisting of 121 $F_{2:7}$ plants developed from a cross between the hexaploid wheat cultivar Katepwa and the hexaploid landrace Salamouni was used for whole genome linkage map construction and

phenotypic analysis. Details regarding the development of the SK population are described in Chapter 4.

Molecular markers

Genomic DNA was isolated from the plant tissues as described by Faris et al. (2000). Initially, 1,500 simple sequence repeat (SSR) markers scattered throughout the entire wheat genome were selected and used to screen the parental lines (Salamouni and Katepwa) for polymorphism. Selected SSR markers were amplified with the primer sets Ac (http://wheat.pw.usda.gov/GG2/index.shtml), BARC (Song et al. 2005), CFD (Sourdille et al. 2004), DuPw (http://wheat.pw.usda.gov/GG2/index.shtml), GDM (Pestsova et al. 2000), GWM (Röder et al. 1998), KSUM (Yu et al., 2004) and WMC (Somers et al. 2004; http://wheat.pw.usda.gov/ggpages/SSR/WMC). A total of 405 polymorphic SSR markers were selected to genotype the entire SK population. Genotyping of the parental lines and the 121 RILs of the SK population was carried out at the USDA-ARS Small Grains Genotyping Lab, Fargo, ND. PCR conditions and multiplexing of the PCR products, amplified with four different dyes, to a final volume of 10 µl were as described by Tsilo et al. (2010). An ABI 3130XL Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) was used to separate the amplified PCR products by capillary electrophoresis after denaturing the multiplexed PCR products at 95 °C for 5 min and cooling on ice. Separated fragments were analyzed using the software GeneMapper v3.7 (Applied Biosystems, Foster City, CA, USA) according to the instructions in the user manual. Each data point was also manually checked for errors in allele calling (identification of alleles).

Simple sequence repeat and expressed sequence tagged – sequence tagged site (EST–STS) markers were developed for chromosome arm 2BS using previously published

genetic and physical maps of wheat, NSF-wheat bin mapped EST sequences from bin 2BS3 0.84-1.00, and based on colinearity with rice and *Brachypodium* (Abeysekara et al. accepted). The EST-STS markers developed using the 2DS5-0.47-1.00 deletion bin-mapped ESTs in a previous study (Zhang et al. 2009) were also used to survey Salamouni and Katepwa for polymorphism. The 121 RILs were genotyped with the polymorphic 2BS SSR and 2BS/2DS EST-STS markers using PCR conditions described by Lu et al. (2006). Amplified PCR products were separated on 6% polyacrylamide gels and visualized using a Typoon 6410 variable mode imager (GE Healthcare, Waukesha, WI, USA) after staining with SYBR Green II (Sigma, St. Louis, MO, USA). Corresponding EST clones of the monomorphic 2BS EST-STS markers were used as probes for RFLP analysis using procedures described by Faris et al. (2000).

Disease evaluations and statistical analysis

The Swiss *S. nodorum* isolate Sn99CH 1A7a (here after referred to as 1A7a) which produces SnTox4 was obtained from Bruce McDonald (ETH Zurich, Switzerland) and was used to screen the SK population. Inoculum for disease evaluations were prepared as described by Liu et al. (2004) using mycelial plugs of 1A7a grown in V8- potato dextrose agar (Difco ^{TM} PDA, Becton, Dickinson and Company, Sparks, MD, USA) plates for 5-7 days under light. Parents and the SK population planted in a completely randomized design (CRD) in three replicates were used for 1A7a conidial inoculations. Three seeds of each SK progeny line were planted in single conetainers (Stuewe and Sons, Inc., Corvallis, OR, USA). Cones were placed in racks capable of holding 98 conetainers with the outside cones being planted to the susceptible cultivar Grandin to reduce any edge effect. Two racks of 98 cones made up one replicate. Therefore the experimental unit consisted of 3 plants per

line. Plants were inoculated until runoff with the conidial suspensions containing 1×10^6 spores mL^{-1} at the two- to three-leaf stage. Two drops of Tween20 (polyoxyethylene sorbitan monolaurate, J.T. Baker Chemical Co., Phillipsburg, NJ, USA) per 100 mL of inoculum were added as a surfactant prior to inoculation. Inoculated plants were subjected to 6 days of incubation in the growth chamber at 21 °C under a 12 h photoperiod after 24 h at 21 °C in a mist chamber with 100% relative humidity. Secondary leaves of the inoculated plants were scored on a 0 to 5 lesion type scale (Liu et al. 2004) seven days post-inoculation.

Bartlett's χ^2 test using SAS (SAS Institute Inc. Version 9.1) was carried out to determine the homogeneity of variances among the three replicates. The computer program Graphpad (http://www.graphpad.com/quickcalcs) and Fischer's protected least significant difference (LSD) at an α level of 0.05 was used to conduct the Chi square tests and to determine the separation of the genotypic means, respectively. Analysis of variance and multiple regression analysis were conducted using Minitab (Minitab Inc. Version 16.0).

Necrotrophic effector evaluations

Culture filtrates (CF) containing SnTox4 were prepared using the isolate 1A7a as described by Liu et al. (2004). All the culture filtrates were tested for toxin activity prior to infiltration using the SnTox4 differential line AF89. Plants were kept in the growth chamber at 21 °C under a 12 h photoperiod after infiltrating the fully expanded secondary leaf of each plant with 1A7a CF. Infiltrated plants were evaluated 4 days post-infiltration and scored as sensitive or insensitive based on the presence or absence of necrosis.

Additionally, the SK population and the parents were infiltrated with Ptr ToxA, SnTox1, and SnTox3. Necrotrophic effectors were produced following the same procedure

described for SnTox3 production (Liu et al. 2009) using the commercial kit developed for the constitutive expression and purification of recombinant proteins (Invitrogen, Carlsbad, CA, USA). Briefly, the necrotrophic effector genes were cloned into the pGAPZA vector and transformed into the wild type yeast strain *Pichia pastoris* X33. Preparation and transformation of the competent *P. pastoris* cells using the *Pichia* EasyComp kit (Invitrogen, Carlsbad, CA, USA) are as described in the user manual. The parents and the 121 individuals of the SK population were screened for reaction to necrotrophic effector sensitivities three times.

Whole genome map construction and QTL analysis

Genetic linkage maps were constructed using the computer program MAPMAKER v2.0 for Macintosh (Lander et al. 1987) using a logarithm of the odds (LOD) value of 3.0 as described in Liu et al. (2005). The Kosambi mapping function (Kosambi, 1944) was used to calculate centiMorgan (cM) distances. Previously published consensus and physical maps of wheat (Sourdille et al. 2004) were surveyed to compare the markers on each chromosome and to estimate the centromere positions on the maps, respectively.

QTL analysis was performed using composite interval-regression mapping (CIM) with the computer programs QGene (Joehanes and Nelson, 2008) and MapManager QTX (Manly et al., 2001). A permutation test with 1000 iterations was executed to determine the critical LOD threshold, which was found to be 3.23.

Results

Marker analysis and linkage map construction

Parental lines of the SK population, Salamouni and Katepwa, were screened with

Segregation of the SK population for reaction to the SnTox4 cultures indicated that a single gene governed sensitivity (Table 5.3), and molecular mapping of response to SnTox4 as a phenotypic marker placed the locus on the short arm of chromosome 1A (Figure 5.1) in a position that closely agreed with that in the Arina x Forno population (Abeysekara et al. 2009). These results strongly indicated that the *Snn4*-SnTox4 interaction was the interaction observed as opposed to a yet unidentified interaction.

Salamouni was also sensitive to SnTox1 and insensitive to SnToxA and SnTox3, whereas Katepwa was insensitive to SnTox1 and sensitive to SnToxA and SnTox3 (data not shown). Therefore, Salamouni carried the dominant alleles, *Snn4* and *Snn1*, and the recessive alleles, *tsn1,* and *snn3*, whereas Katepwa carried the *snn4*, *snn1*, *Tsn1*, and *Snn3* alleles. Segregation analysis confirmed that each of these effectors was governed by a single gene (Table 5.3), and genomic mapping of the effector sensitivity loci in the SK population (Figure 5.1) agreed closely with their known chromosomal locations determined by previous research (Friesen and Faris 2010 for review). There were no visible reactions in either parental line when infiltrated with culture filtrates containing SnTox2 (data not shown), suggesting that the SK population does not segregate for SnTox2 sensitivity.

Identification of QTL associated with SNB caused by isolate 1A7a in the SK population

Average disease reaction types for each RIL was used for the QTL analysis as all three replicates of the SK population inoculated with the *S. nodorum* isolate 1A7a were found to be homogeneous (Bartlett's χ^2 for homogeneity: $\chi^2_{df=2}$ 4.436 P= 0.1088). Salamouni was moderately resistant to 1A7a, whereas Katepwa was moderately susceptible to 1A7a (Figure 5.3). The average disease reaction types of Salamouni and Katepwa, were

Table 5.1. Number of simple sequence repeats (SSR), restriction fragment length polymorphism (RFLP), expressed sequence tag-sequenced tagged site (EST-STS) and other markers mapped in each wheat chromosome/genome in the Salamouni x Katepwa recombinant inbred population, the total length of each linkage group and the marker density.

Chromosome	Marker						
	SSR	RFLP	EST-STS	Morphological	Total	Length (cM)	Marker density (cM/marker)
1A	22	1	3	1	27	138.6	5.1
1B	32		1	1	34	202.5	6.0
1D	10				10	98.5	9.9
2A	26	2	1		29	190.7	6.6
2B	34	2	15	1	52	200.9	3.9
2D	32	2	2		36	212.7	5.9
3A	23				23	170.1	7.4
3B	30	1	1		32	223.8	7.0
3D	16		2		18	152.6	8.5
4A	19				19	167.0	8.8
4B	12		1		13	62.0	4.8
4D	13				13	52.7	4.1
5A	17		1		18	94.6	5.3
5B	27			2	29	141.2	4.9
5D	28				28	228.8	8.2
6A	9	1			10	166.1	16.6
6B	12				12	43.8	3.7
6D	13				13	115.0	8.8
7A	19		1		20	122.1	6.1
7B	20		1		21	153.3	7.3
7D	27				27	291.0	10.8
A genome	135	4	6	1	146	1049.2	7.2
B genome	167	3	19	4	193	1027.5	5.3
D genome	139	2	4	0	145	1151.3	7.9
Total	441	9	29	5	484	3228.0	6.7

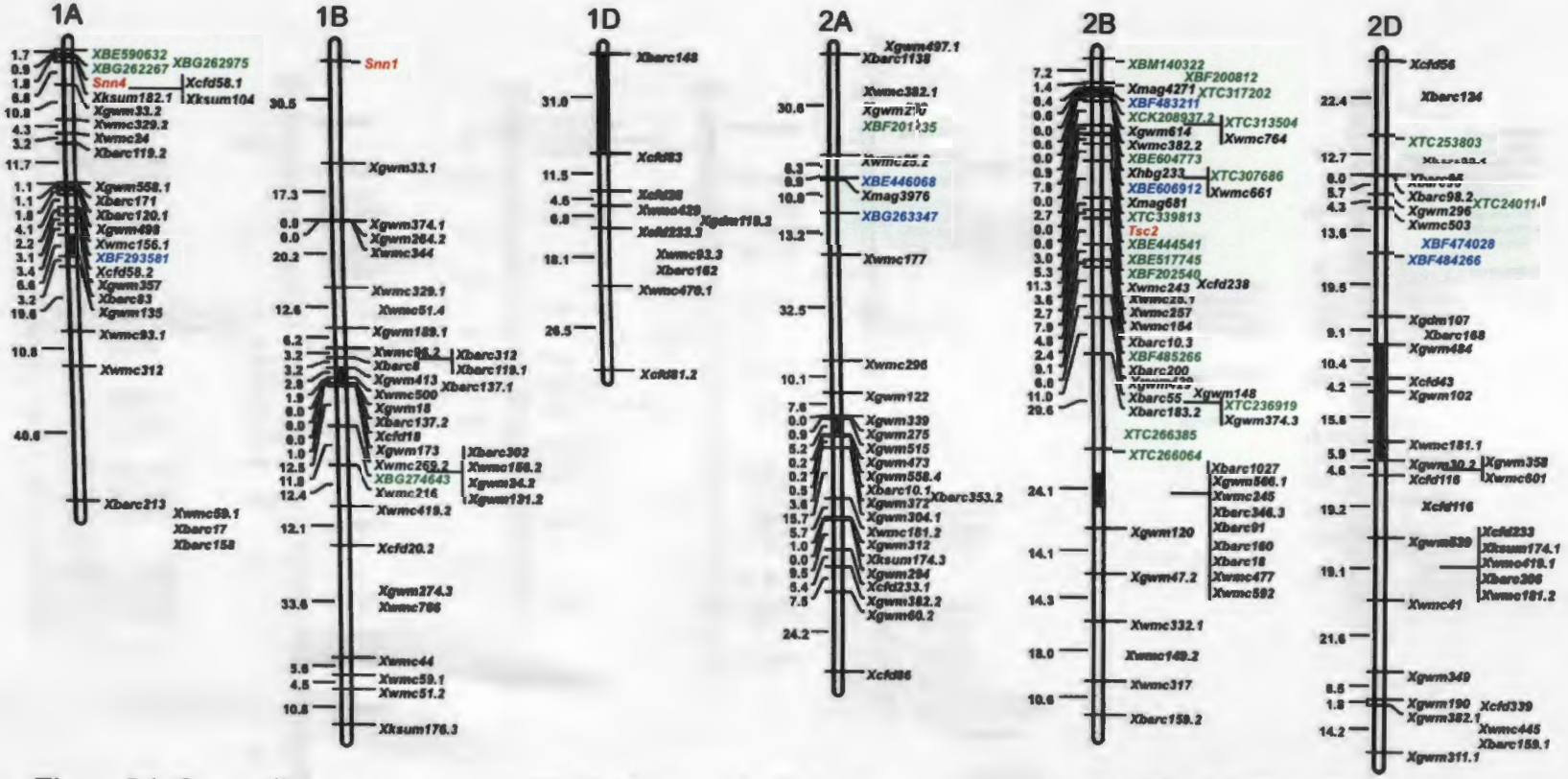

Figure 5.1. Genetic linkage maps generated in the Salamouni x Katepwa population with 441 SSRs, 9 RFLPs, 29 EST-STS markers and 5 phenotypic markers. SSR markers are indicated in black, RFLP markers are indicated in blue, EST-STS markers are shown in green and phenotypic markers are shown in red. Markers that mapped at LOD < 3.0 are shown in their most likely linkage positions in the maps and without lines drawn across the chromosomes. Black regions in the chromosomes indicate the approximate positions of centromeres.

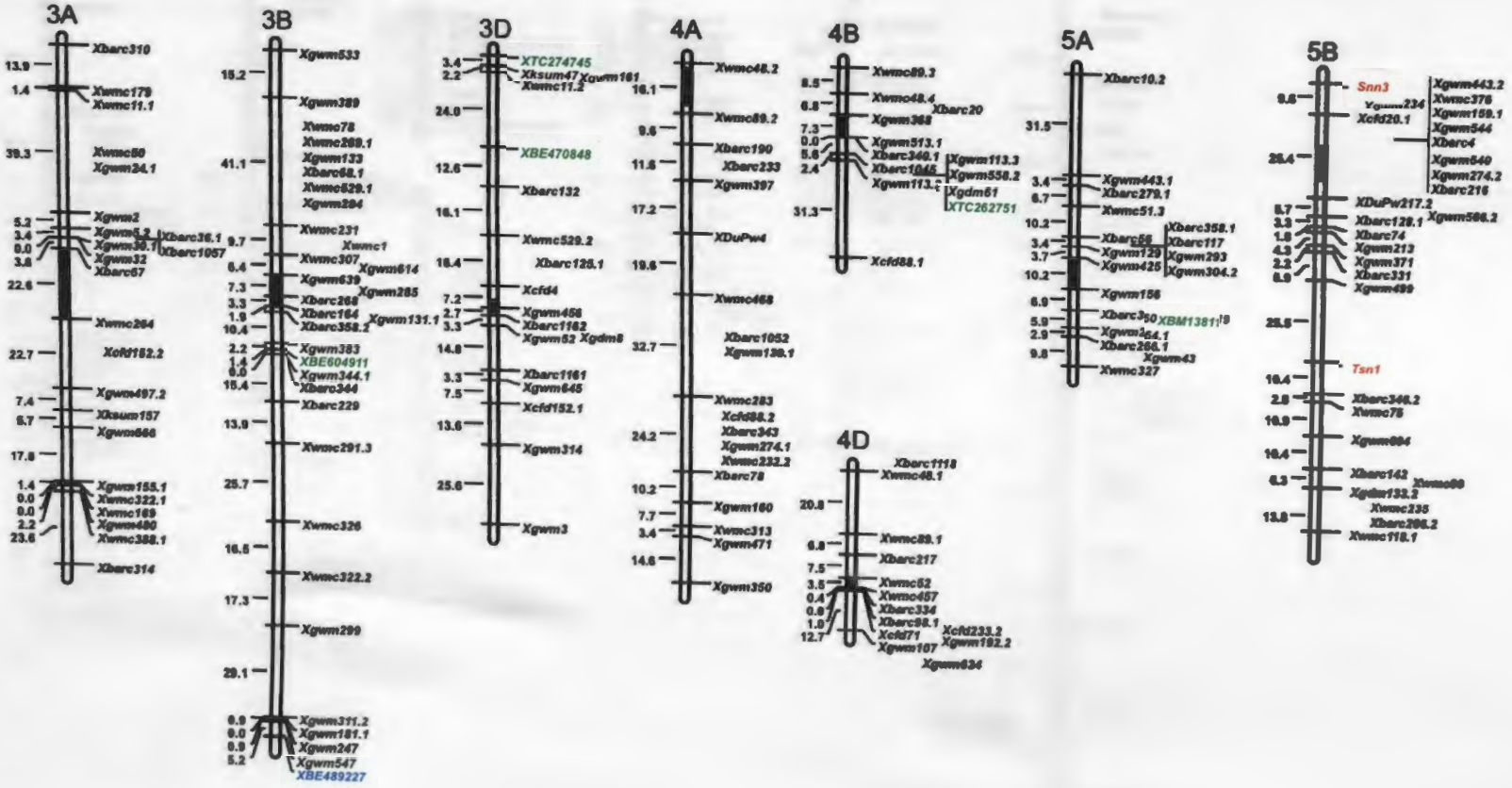

Figure 5.1. Continued

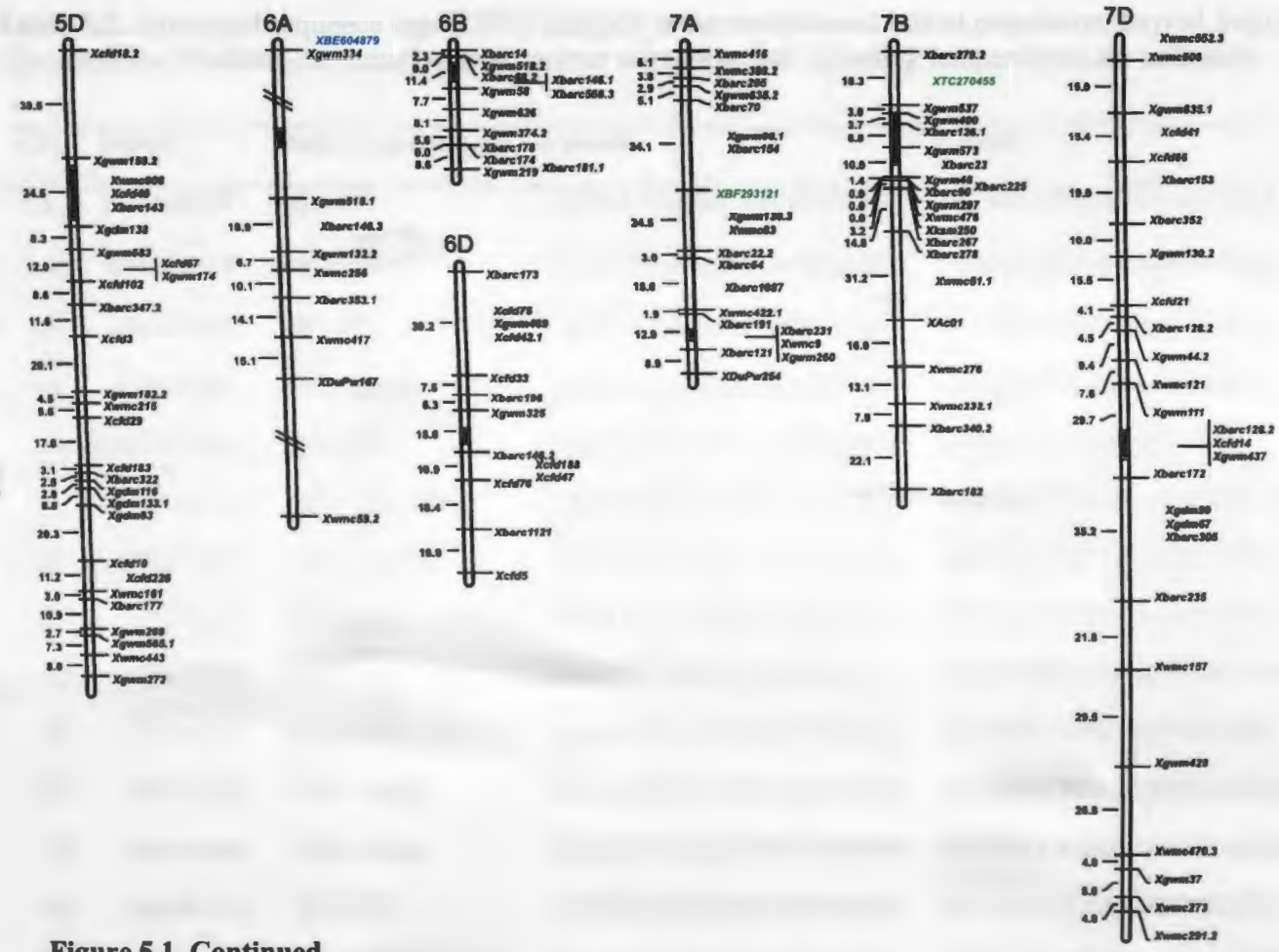

Figure 5.1. Continued

Table 5.2. Expressed sequence tags (ESTs) mapped in the recombinant inbred population derived from Salamouni and Katepwa. Chromosome location (chr.), marker type, primer sequences and annealing temperatures are indicated.

Chr.	Marker	Marker type (Enzyme)	F primer	R primer	Anealing temp. (°C)
1A	*XBE590632*‡	EST-STS	AACGATGATCCATCCGTCTT	TCCATCCTTCAACCACAACA	53
1A	*XBG262975*‡	EST-STS	TCCAGTCAACAGCAACCATC	CCAAACAGTGAAGCTGCAAA	54
1A	*XBG262267*‡	EST-STS	CGTTACAACGATTGGTGCAT	TCATCCAACCTCACCAACCA	53
1A	*XBF293581*	RFLP (*Dra*I)	AAACGAAAAGTTGGATCGTG	GATTGGCAGAGAATGTGCTT	51
1B	*XBG274643*	EST-STS	TGAAGACCTGCCTGAGTTGT	CTCCGTCCGTTTTGCTGT	55
2A	*XBE446068*	RFLP (*Hin*dIII)	ATGGCTTGGTTTCCCTTTTT	TCTTTGTAGGTTATTTGTCAGTCGTC	53
2A	*XBG263347*	RFLP (*Dra*I)	TGAGCATTGGAGGGATTTAG	AGCCTGGTTTCTTGTCTTCTC	50
2A	*XBF201235*	EST-STS	ATGCCCGCTGTTTATTGTAG	TGTGATTTTCCTTGCTGTCC	53
2D	*XTC253803*	EST-STS	TGCTTTTGTGCCAGATGATG	CCACCGGGACAAGTCAGATA	53
2D	*XTC240114*	EST-STS	GGAATTAACCGAGCTGCGTA	GCGGCCTCTGTATCTTGATT	54
2D	*XBF474028*	RFLP (*Bgl*II)	GAAAATTACCGCAAATCGTG	ACTTGCCTGAATATCGCTTG	50
2D	*XBF484266*	RFLP (*Dra*I)	GCAGGTTAGCTGTCCAAAAA	TTCTTCCAGAGCAAGGACAC	53
3B	*XBE604911*	EST-STS	ACCACGGAAGGTATCAGCAT	CGCAAGTCCATTCTTTTCTC	50
3B	*XBE489227*	RFLP (*Bgl*II)	TGAAACCTGGCTTGTCATTT	TGGAATAGGCCAAAGAGATG	52
3D	*XTC274745*	EST-STS	TACCTCCCAGATGGGTTCCA	CGACAGTGACCACAGTCAGC	54

Table 5.2. Continued

Chr.	Marker	Marker type (Enzyme)	F primer	R primer	Anealing temp. (°C)
3D	*XBE470848*	EST-STS	GGAAATGCAATGGAACTGTC	AGCAGCAACTTGGATCTCTG	50
4B	*XTC262751*	EST-STS	AACAGACCATTGCCCCAATC	AGATTTGCATCCCTCCGTTG	55
5A	*XBM138119*	EST-STS	ACTCACCCTAGGCGGTCTCT	TATTCTCATCCGTGCAGTCG	54
6A	*XBE604879*	RFLP (*Eco*RI)	CATGAACAAATGCAACAAGC	TGACAAGAACAACCAACTGC	50
7A	*XBF293121*	EST-STS	TATCTTAGCCCACAGGCATC	TGAAGCCGAAGTCATTTGAT	50
7B	*XTC270455*	EST-STS	CGACATCGAGCTGATGAAGC	AGTAAGGTCGCACCCGATGT	55
2B	*XBM140322*§	EST-STS	AAGGCTCACCAACAACTCC	CTCGGAACAGCAACATTACC	50
2B	*XBF200812*§	EST-STS	GCATTCCCTCTAACGACTGA	TTCTTTGATAACCATCCGTAGG	50
2B	*XBE604773*§	EST-STS	GAGAGGCGCAAACAAAATAA	AACATCCTCATAGCCATCCA	50
2B	*XBE444541*§	EST-STS	TGGACCAGTATGAGA	TTCTGGAGGATGTTGAGCAC	55
2B	*XBE517745*§	EST-STS	CTCACCTCCATTGCCTCTAA	AGAACCGAAGATTGCTGATG	50
2B	*XBF202540*§	EST-STS	TGGTTATGGACAAGAGAAGA AAA	AGGTAGAAATGAGGCGAGTG	50
2B	*XBF485266*§	EST-STS	CAGCAAAGAACACGGAAAGT	GCAAGCACAGTAAAAGAAGCA	50
2B	*XBE606912*§	RFLP (*Hin*dIII)	GTCCAGAAGACCTGCAAAGA	AGACAAGGTGAACTCCCACA	54
2B	*XBF483211*§	RFLP (*Eco*RI)	GATACACCAATGACGGAACA A	GCCAAGAAGGCTCCAATAAG	50

Table 5.2. Continued

Chr.	Marker	Marker type (Enzyme)	F primer	R primer	Anealing temp. (°C)
2B	*XTC313504*[§]	EST-STS	CTCGTCATGGGGTGACTTTT	TGGATCTGCTCGTCAAAGTG	50
2B	*XTC317202*[§]	EST-STS	AGGCATTTGGTCATTTTGG	GAAGGCATACTCAACAGAAATCA	50
2B	*XCK208937.2*[‡]	EST-STS	TCTCATTAATGGCGCTCTCC	GAGGTTTCTTCTTGCGCTTG	52
2B	*XTC307686*[§]	EST-STS	AAGTCGCGTTGATGCAATTA	TTGACGTTTGCAACAGTTGAT	54
2B	*XTC339813*[§]	EST-STS	CGTTCTTTGCACATCACTAA	TGGAAATGCCACATCACTGT	50
2B	*XTC236919*	EST-STS	GCTCATATCGGAGGCTAGTTC	AAGTGCCCAACACTGGAAAG	50
2B	*XTC266385*	EST-STS	GCCGATTCATACCCACTCAA	ACTGCCTCTCCGCAGTTACA	50
2B	*XTC266064*	EST-STS	GATCAACACCAGGGAGCACA	TTCCTGGAAACGGTGAGTGA	50

[‡]mapped to the wheat chromosome arm 1AS in the Arina x Forno population, initially (Chapter 3).
[§] mapped to the wheat chromosome 2B in the Salamouni x Katepwa population in a different study (Chapter 4)

Figure 5.2. Secondary leaves inoculated with conidia of the Swiss *S. nodorum* isolate Sn99CH 1A7a or infiltrated with the Sn99CH 1A7a culture filtrate containing SnTox4. A and B: Disease reaction of Salamouni (A) (average disease reaction type 2.20) and Katepwa (B) (average disease reaction type 3.10) to conidial inoculations with Sn99CH 1A7a. C and D: Reaction of Salamouni (C) and Katepwa (D) infiltrated with Sn99CH 1A7a culture filtrate containing SnTox4. The boundaries of the infiltrated regions are marked in black.

Table 5.3. Analysis of the necrotrophic effectors segregating in the Salamouni x Katepwa recombinant inbred population. Chromosomal locations and closely related markers of the corresponding host genes are also indicated.

Effector	Host gene	Chromosomal location	Markers	Sensitive RILs†	Insensitive RILs	$\chi^2_{df=1}$	*P*
Ptr ToxA	*Tsn1*	5BL	*Xgwm499, Xbarc346.2*	64	57	0.405	0.5245
SnTox1	*Snn1*	1BS	*Xgwm33.1*	63	58	0.207	0.6494
SnTox3	*Snn3*	5BS	*Xcfd20.1*	69	52	2.388	0.1222
SnTox4	*Snn4*	1AS	*XBG262267, Xksum182.1*	54	67	1.397	0.2373

† RILs: recombinant inbred lines

2.2 and 3.1, respectively (Table 5.4, Figure 5.4), and those of the SK population ranged from 1.0 to 4.2 with an overall mean of 2.6.

Composite interval regression mapping indicated the presence of significant QTL on the short arms of chromosomes 1AS and 7AS designated *QSnb.fcu-1A* and *QSnb.fcu-7A*, respectively (Figure 5.3). *QSnb.fcu-1A* was defined by the *Snn4* locus with a LOD of 7.03 (Figure 5.3, Table 5.5) and *QSnb.fcu-7A* peaked at the EST-STS marker *XBF293121* with a LOD of 4.64 (Figure 5.3, Table 5.5). Resistance effects at both QTL were contributed by Katepwa. *QSnb.fcu-1A* and *QSnb.fcu.7AS* accounted for 23.5% and 16.4% of the phenotypic variation in disease in this population, respectively (Table 5.5). Whereas the effects of *QSnb.fcu-1A* were due to a compatible *Snn4*-SnTox4 interaction, *QSnb.fcu.7AS* was not associated with any of the known effector sensitivity loci. There were no significant QTL x QTL interactions observed for reaction to isolate 1A7a.

The average disease reaction types of the SnTox4-sensitive RILs ranged from 2.0 to 4.2 with a mean of 3.0, and those of the SnTox4-insensitive RILs ranged from 1.0 to 4.2 with a mean disease reaction type of 2.3 (Table 5.4, Figure 5.4). Recombinant inbred lines harboring the *Snn4* and *XBF293121* alleles from Salamouni ($Snn4^S/XBF293121^S$) showed relatively higher susceptibility (average disease reaction type of 3.2) to SNB than the RILs harboring $snn4^K/XBF293121^K$ which had an average disease reaction type of 2.0 (Table 5.4, Figure 5.5). The average disease reaction type in the presence of either $Snn4^S$ or $XBF293121^S$ were similar (2.7 and 2.5 respectively) but were less than the values observed for $Snn4^S/XBF293121^S$ and higher than for those of $snn4^K/XBF293121^K$ (Table 5.4, Figure 5.5). Hence the RILs harboring both $Snn4^S$ and $XBF293121^S$ were more susceptible to SNB than RILs harboring just one allele. This suggests that the *Snn4*-SnTox4 interaction as well as the interaction at the locus defined by the marker *XBF293121,* contribute individually to disease caused by 1A7a in this population and the two loci appear to be largely additive.

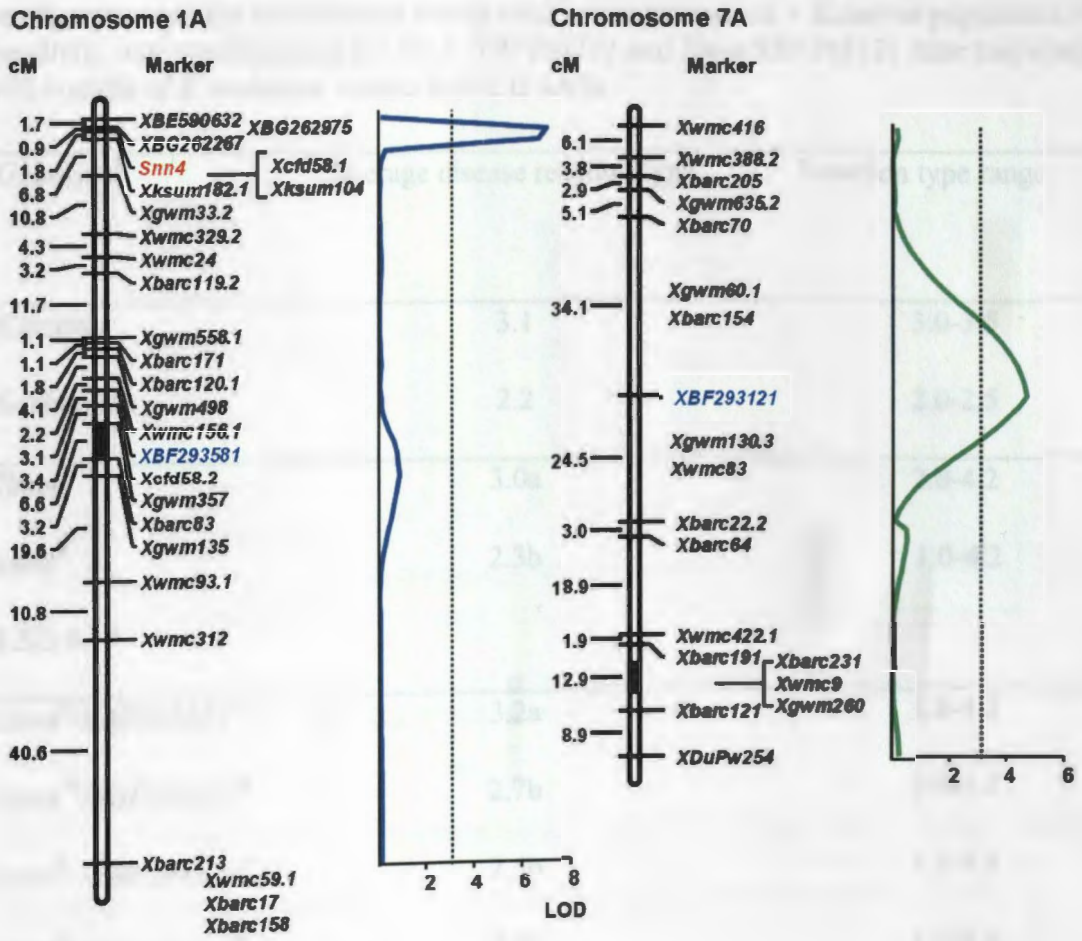

Figure 5.3. Composite interval mapping of QTL associated with Stagonospora nodorum blotch on chromosomes 1A and 7A in the Salamouni x Katepwa recombinant inbred population. Genetic maps of the chromosomes with the positions of the marker loci and the centiMorgan (cM) distances between loci are shown on the left. The LOD threshold of 3.23 is indicated by the dotted line.

Multiple regression analysis using the markers *Snn4* and *XBF293121* showed that combined, the interactions accounted for 35.7 % of the total variation in SNB disease in the SK population. The correlation between SNB disease and *Snn4* is slightly higher than that between SNB disease and *XBF293121* (Pearson's product moment correlation – 0.485 and 0.405 respectively).

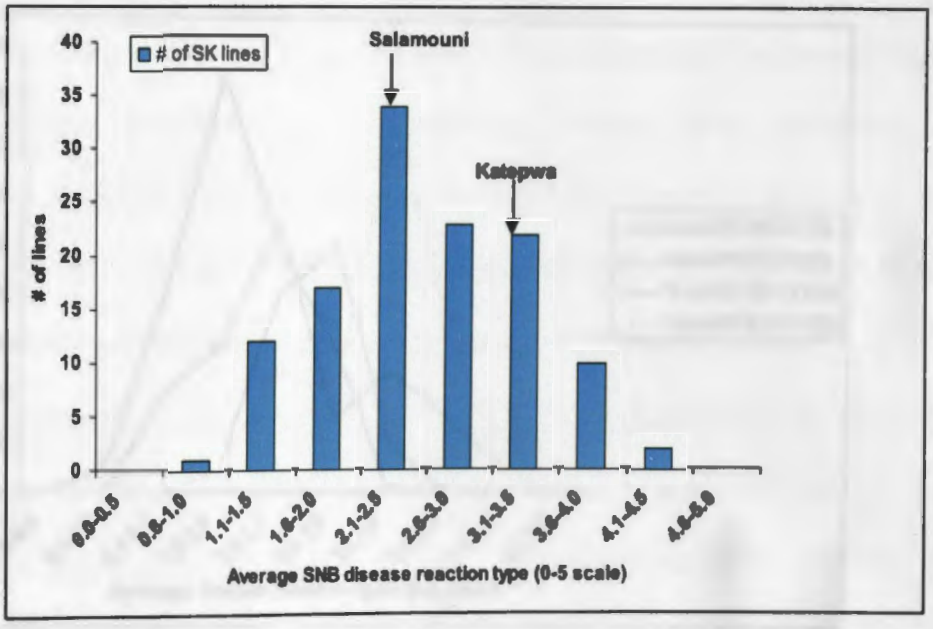

Figure 5.4. Frequency of the average Stagonospora nodorum blotch disease reaction types of the Salamouni x Katepwa recombinant inbred population

Table 5.5. Composite interval regression mapping analysis of significant QTLs associated with resistance to the Swiss *S. nodorum* isolate Sn99CH 1A7a, their marker interval and phenotypic variance explained (R^2) in the Salamouni × Katepwa recombinant inbred population.

Chr. Arm[†]	QTL[‡]	Marker /Marker interval	R^2	LOD[§]	Additive effects[¶]
1AS	*QSnb.fcu-1AS*	*Snn4*	0.23	7.03	-0.36
7AS	*QSnb.fcu-7AS*	*XBF293121*	0.16	4.64	-0.30

[†]Chr. arm- Chromosome arm
[‡]QTL- Quantitative trait loci
[§]LOD- Logarithm of the Odds
[¶]Negative additive effects indicate that Katepwa contributed the alleles for resistance

Discussion

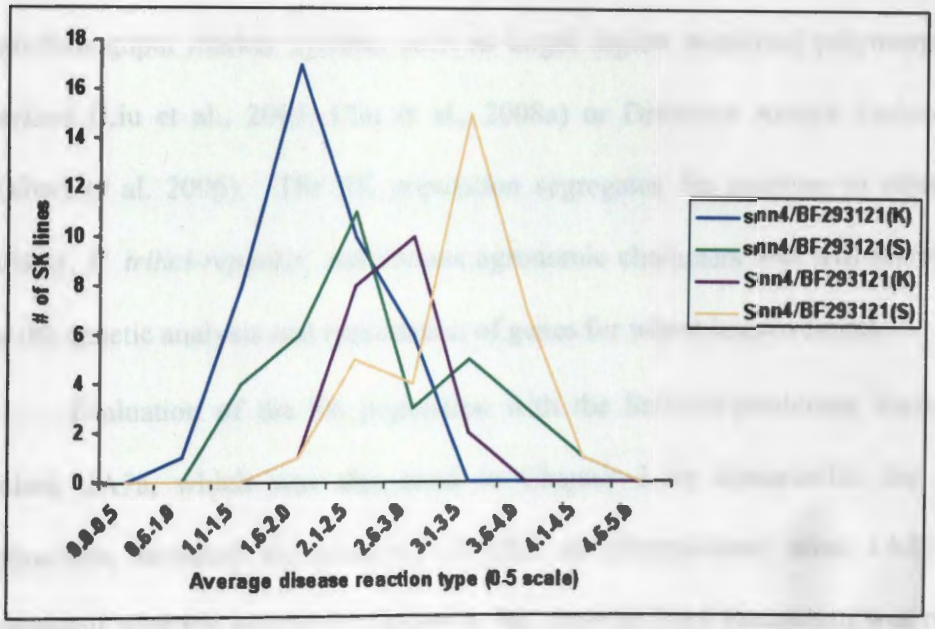

Figure 5.5. Frequency polygon of the average Stagonospora nodorum blotch disease reaction types of the recombinant inbred lines of the Salamouni x Katepwa population for the allelic state combinations of *Snn4/XBF293121* after inoculation with conidia of the *Stagonospora nodorum* isolate Sn99CH 1A7a.

Previously published hexaploid wheat maps have varying genetic lengths (Chalmers et al., 2001: Groos et al., 2002; Paillard et al., 2003; Somers et al., 2004; Song et al., 2005; Liu et al., 2005; Torada et al., 2006; Chu et al., 2008a). Assuming the genetic length of the entire hexaploid wheat genome to be about 4,000 cM, the genetic linkage map constructed in the SK population, which consists of a total of 484 markers, has a genome coverage of about 80%. The markers were not evenly distributed on chromosomes and I observed 17 gaps that ranged from 30-50 cM in size. These gaps were found in the distal ends of the short and long arms of the chromosomes. In order to obtain more complete genome coverage these gaps will need to be filled and the linkage groups should be extended into telomeric regions where the frequency of recombination is relatively high.

This objective can be achieved by targeting the gaps with additional SSRs or employing high-throughput marker systems such as target region amplified polymorphism (TRAP) markers (Liu et al., 2005; Chu et al., 2008a) or Diversity Arrays Technology (DArT) (Akbari et al. 2006). The SK population segregates for reaction to other *S. nodorum* isolates, *P. tritici-repentis*, and various agronomic characters and will therefore be useful for the genetic analysis and exploitation of genes for wheat improvement.

Evaluation of the SK population with the SnTox4-producing Swiss *S. nodorum* isolate, 1A7a, which was also used in Chapter 3 to characterize the *Snn4*-SnTox4 interaction, revealed the presence of QTL on chromosome arms 1AS and 7AS. In agreement with the results of Chapter 3, the *Snn4*-SnTox4 interaction was responsible for the effects observed for the QTL *QSnb.fcu-1A*. The compatible *Snn4*-SnTox4 interaction caused mottled necrosis in the SnTox4 sensitive parents in both the AF and SK populations. There was moderately high correlation between the average SNB disease and SnTox4 reaction in the AF population while that of the SK population was relatively low. Therefore, the *Snn4*-SnTox4 interaction seems to play a more significant role in causing SNB in the AF population compared to the SK population. Nevertheless, the QTL defined by the *Snn4*-SnTox4 interaction had the largest effect in both of these populations.

The QTL *QSnb.fcu-7A* detected on the short arm of chromosome 7A was not associated with any of the currently known effector–sensitivity loci. Even though infiltration of the SK population with culture filtrate of 1A7a only identifies the presence of SnTox4, it is possible that the 7AS locus has a corresponding virulence factor, which could be a yet unidentified or uncharacterized necrotrophic effector. Either the effects at the 7AS locus are not due to a necrotrophic effector, or the concentration of the effector produced in

culture was insufficient to induce a visible phenotype. No SNB resistant QTL have been previously identified on chromosome 7A suggesting that this novel resistance QTL may be useful for the development of wheat varieties with improved SNB resistance.

Results of the infiltration and inoculation experiments of the parental lines of the SK population revealed, what appeared to be, contradictory results. Salamouni was sensitive to the culture filtrate of 1A7a but moderately resistant to SNB caused by 1A7a, whereas Katepwa was insensitive to the culture filtrate but moderately susceptible to SNB caused by 1A7a with the difference between the reaction types of the two parental lines being 0.90. The range of the average disease reaction types of the SK population inoculated with 1A7a showed transgressive segregation. Susceptibility alleles for both the *QSnb.fcu-1A* and *QSnb.fcu-7A* QTLs were contributed by the SnTox4-sensitive parent Salamouni. Therefore, one would expect Salamouni to be more susceptible to SNB than Katepwa, but the opposite was observed and therefore additional disease determinants must exist. Multiple susceptibility factors in Katepwa or resistance factors in Salamouni with minor effects may have gone undetected due to the relatively small population size, which could explain the apparent contradiction. Good evidence for this is provided by the fact that Salamouni and Katepwa had average disease reaction types of 2.2 and 3.1, respectively, whereas RILs having Salamouni alleles at both QTL had an average reaction type of 3.2 and RILs having Katepwa alleles at both QTL had an average reaction type of 2.0.

Additionally, there could be epistatic interactions, which are also difficult to detect in small populations. As mentioned before, it has been shown that host-effector interactions in the wheat-*S. nodorum* system can have additive or epistatic effects (Friesen et al., 2008a). Friesen et al. (2008b) showed that the presence of a compatible SnToxA-*Tsn1*

interaction could mask the effects of a compatible SnTox3-*Snn3* interaction. Previous studies conducted on the wheat-tan spot system have also indicated that certain genotypes insensitive to known tan spot effectors can demonstrate susceptibility to disease and vise versa (Faris and Friesen, 2005; Chu et al., 2008b). Similar results have been reported in the wheat-SNB system as well. Liu et al. (2004) showed that the parental lines W-7984 and 'Opata 85' differ significantly in their responses to SnTox1 and disease development, where W-7984 is sensitive to SnTox1 but is the more resistant parent. Our results indicate that the same could be true in the SK population.

The two EST markers, *XBG262267* and *XBE590632*, closely linked to *Snn4* were found to detect the same marker alleles in both Salamouni and Arina. Therefore, these two EST markers can potentially be useful as markers for marker-assisted selection and genotyping of lines carrying *Snn4*. Once SnTox4 has been purified, more robust screening of diverse wheat genotypes can be conducted to determine the efficacy of these markers for detecting *Snn4* alleles.

There is mounting evidence that necrotrophic pathogens exploit biological pathways in plants to trigger responses that create an environment favorable for the survival and sporulation of the necrotrophic pathogen. Interestingly, these appear to involve classical resistance-like genes and therefore likely are the same pathways, which are detrimental to the survival of a biotrophic pathogen (Faris et al. 2010). Hence, the role of resistance genes in conditioning not only resistance, but also susceptibility, could create repercussions when trying to pyramid resistance genes in crops.

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