Purification and Characterization of *Stagonospora nodorum* Toxins and Mapping of Toxin Insensitivity

---

By

Pratisara Bajracharya
2015

A Thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

in partial fulfillment of the requirements of the degree of

MASTER OF SCIENCE

Department of Plant Science

University of Manitoba

Winnipeg

ABSTRACT

Stagonospora nodorum is a pathogenic fungus of wheat causing Stagonospora nodorum blotch disease, an important disease in western Canada. *S. nodorum* produces a multitude of host selective toxins (HSTs), which when recognized by corresponding sensitivity gene in wheat results in a compatible interaction. In this study, novel HST-host sensitivity gene interactions were investigated. Two different putative HSTs were identified. SnTox3 was likely one of the HSTs present in *S. nodorum* isolate Swift Current culture filtrate as the chromosomal location of the compatible sensitivity gene corresponded to that of *Snn3* locus. Another putative HST interacting with *Tsn1* or a tightly linked sensitivity gene was identified from *S. nodorum* isolate Langham. SNOG_15679, a candidate gene for production of this putative HST was heterologously expressed in *Pichia pastoris* which caused chlorosis on a sensitive host. Additional tests will be required to confirm the bioactivity of putative novel HST(s) produced by isolate Langham.

ACKNOWLEDGEMENTS

I would like to thank my advisor, Dr. Curt McCartney for his guidance and support. I would like to thank my co-advisor, Dr. Fouad Daayf and my committee members, Dr. Christof Rampitsch, Dr. Anita Brûlé-Babel and Dr. Georg Hausner for their suggestions. I would like to thank Drs. Jeanie Gilbert and Sylvie Cloutier for allowing me to use their equipment.

I would also like to thank Monika Eng, Leslie Bezte, and Yanfen Zheng for the technical assistance in molecular genetics lab. I am grateful for receiving the technical assistance in Dr. Rampitsch's and Dr. Bykova's proteomics lab. A special thanks to Jacqueline Ching, Brenda Hoehn, Eva Beimcik, Slavica Djuric-Ciganovic, and Natalie Middlestead for their help for protein work and also for always encouraging me. Thanks to Natasa Radovanovic for the technical assistance in molecular work. Also, thanks to Lorne Adams for his technical assistance. Thanks to Tao Fan for his help with mass spectrometry.

I am grateful to NSERC for funding this research. Thank you to Willy Wiebe Graduate Fellowship in Agriculture and Food Science.

Thanks to my Mom and Dad for unconditional love and support. Thanks to my brother, Anik, my extended family and my friends. Thanks to Vinod Katuwal Chhetri for standing by me through thick and thin. Thanks for being my rock, I couldn't have done it without you.

DEDICATION

This thesis is dedicated to the loving memories of my grandfather, Mr. Narendra Bilas Bajracharya. Special dedications to my mom (Mrs. Ramita Bajracharya) and dad (Mr. Ashok Bilas Bajracharya) who gave up their dreams to fulfill mine.

Table of Contents

LIST OF TABLES

LIST OF FIGURES

FOREWORD

This thesis is written in manuscript style. Each manuscript is composed of an abstract, introduction, materials and methods, results and discussion. This thesis includes a general introduction, literature review, two manuscripts, a general discussion and conclusions and literature cited. The manuscripts will be submitted to Canadian Journal of Plant Pathology.

1. INTRODUCTION

Wheat is cultivated in most parts of the world including Canada, the United States of America, Australia, Russia, China, India, and many others (Gustafson et al. 2009, p. 6). Wheat is a preferred food staple in many countries and therefore with the exponentially increasing world population, the demand for wheat and wheat based products is also on the rise (Gustafson et al. 2009, p. 5-8). There are two different commercially important wheat species: 1) *Triticum aestivum* (hexaploid) 2) *Triticum turgidum* ssp. *durum* (tetraploid). The cultivars belonging to both species are used in making different wheat based products.

In the midst of growing demands for wheat, diseases affecting wheat pose a huge challenge to the wheat industry (Carver 2009). Wheat is affected by a variety of plant diseases which include, but are not limited to, leaf rust, stem rust, stripe rust, various root rots, powdery mildew, Stagonospora nodorum blotch, Septoria tritici blotch, tan spot and Fusarium head blight (Carver 2009). The epidemics caused by these diseases are capable of jeopardizing the world supply of wheat. Therefore, the challenge of meeting the world's food demand also fuels the need for improving wheat varieties mainly in terms of disease resistance.

Stagonospora nodorum blotch (SNB) is one of the diseases of wheat that affect the leaves and glumes (Solomon et al. 2006a). The disease is referred to as glume blotch when the glumes are affected (Solomon et al. 2006a). Although SNB epidemics can be managed with the use of fungicides, sustainable SNB management through genetic resistance is preferable. However, breeding for SNB resistance has proven to be difficult due to lack of complete resistance in the currently available resistance sources (King et al. 1983; Solomon et al. 2006a; Oliver et al.

2012). The etiological agent of SNB and glume blotch is the necrotrophic ascomycete *Stagonospora nodorum* (Solomon et al. 2006a). The SNB symptoms on leaves begin as a small dark brown lesion which gradually progresses into larger necrotic lesions and eventually coalesce to form a large mass of necrotized tissue (Solomon et al. 2006a). The necrotic activity observed during *S. nodorum* infection has been attributed to a myriad of HSTs produced by the pathogen (Liu, Faris, et al. 2004; Friesen et al. 2006; Friesen et al. 2007; Friesen, Faris, et al. 2008; Liu et al. 2009; Abeysekara et al. 2009; Friesen et al. 2012).

A total of six HSTs have been recognized and purified from *S. nodorum*. SnTox1 (Liu, Faris, et al. 2004) was the first *S. nodorum* HST to be identified. Subsequently, the identification of other HSTs followed, which included SnToxA (Friesen et al. 2006), SnTox2 (Friesen et al. 2007), SnTox3 (Liu et al. 2009), SnTox4 (Abeysekara et al. 2009) and SnTox5 (Friesen et al. 2012). All *S. nodorum* HSTs identified to date have been found to produce a necrotizing effect on wheat leaves when a compatible sensitivity gene was present in the host (Liu, Faris, et al. 2004; Friesen et al. 2006; Friesen et al. 2007; Liu et al. 2009; Abeysekara et al. 2009; Friesen et al. 2012).

Among all the identified HSTs of *S. nodorum*, SnToxA has been the most thoroughly studied HST. The 99.7% similarity between the SnToxA and the PtrToxA genes was one of the reasons for heightened interest in SnToxA compared to other *S. nodorum* HSTs (Friesen et al. 2006). Eleven different haplotypes of ToxA were found in *S. nodorum* whereas only one haplotype of ToxA was present in *Pyrenophora tritici-repentis,* which along with other circumstantial evidence, indicated that ToxA was laterally transferred from *S. nodorum* to *P. tritici-repentis* (Friesen et al. 2006; Friesen, Faris, et al. 2008). Friesen et al. (2006) with the help

of ToxA-disrupted mutants suggested that *Tsn1* gene was responsible for SnToxA activity in ToxA sensitive (*Tsn1* positive) wheat lines.

Each of the identified *S. nodorum* HSTs have been shown to adequately reproduce disease symptoms (Liu, Faris, et al. 2004; Friesen et al. 2006; Friesen et al. 2007; Liu et al. 2009; Abeysekara et al. 2009; Friesen et al. 2012). The variety of HSTs produced by *S. nodorum* and the availability of corresponding HST sensitivity loci in the wheat cultivars tend to determine the severity of disease symptoms (Oliver et al. 2012). However, how these HSTs are deployed to produce disease in a natural setting is unknown. Additionally, evidence from different studies suggests that *S. nodorum* is armed with a multitude of HSTs (Friesen, Faris, et al. 2008). A previous study done by Matlock et al. (2012) grouped western Canadian *S. nodorum* isolates into nine Unweighted Pair Group Method with Arithmetic Mean (UPGMA) clusters differing in their virulence. The *S. nodorum* isolates from Manitoba and Saskatchewan were also found to have host specificity (Matlock et al. 2012). This suggested that additional HSTs interacting with novel loci on wheat chromosomes were being produced by Canadian isolates of *S. nodorum*. Therefore, this study is focused on 1) Identification, purification and characterization of novel HSTs and 2) Mapping of HST sensitivity genes in the host.

2. LITERATURE REVIEW

2.1. Stagonospora nodorum blotch (SNB) disease

Stagonospora nodorum blotch disease is a common disease of wheat that causes necrotic lesions on leaves. The worldwide occurrence of SNB and the associated losses makes it a nuisance for wheat growers across the world (King et al. 1983; Eyal 1999; Solomon et al. 2006a). Recently, the study of host specific toxins and the wheat-*S. nodorum* pathosystem has provided an improved understanding of the disease mechanism of *S. nodorum* (Friesen et al. 2007; Friesen, Faris, et al. 2008).

2.1.1. Global distribution and losses

S. nodorum is a common pathogen in wheat growing areas worldwide (King et al. 1983; Solomon et al. 2006a). Isolates of *S. nodorum* have been collected from different regions in North America, South America, Asia, Australia, Europe and Middle East (Friesen et al. 2006; Stukenbrock & Mcdonald 2007). *S. nodorum* causes one of the major losses in the wheat industry in western Australia with losses adding up to $108 million per year (Murray & Brennan 2009). The yield reduction due to tan spot and SNB was found to be comparable and together it accounted for 18-31% yield reduction (Bhathal et al. 2003). In Saskatchewan, Canada, *S. nodorum* was reported as a major species in comparison to other species constituting the Septoria complex (Ma & Hughes 1993). SNB used to be a significant disease in Europe which has been gradually replaced by another leaf spotting disease caused by *Septoria tritici* (Oliver et al. 2012).

In the United States, SNB mainly affects winter wheat and has devastating effects especially in the southern United States due to favorable environmental conditions for *S. nodorum* (Crook et al. 2012).

2.1.2. Leaf spot complex in western Canada

The leaf spotting diseases are among the most prevalent diseases of wheat in western Canada (Gilbert et al. 1998; Gilbert & Woods 2001; Fernandez et al 2002, 2008). The common leaf spotting pathogens in western Canada are *Stagonospora nodorum* (Berk.) Castellani and Germano, *Septoria tritici* Roberge in Desmaz, *Pyrenophora tritici-repentis* (Died.) Drechs. and *Cochliobolus sativus* (Ito and Kuribayashi) Drechs. ex Dastur (Gilbert et al. 1998). *S. nodorum*, *S. tritici*, *P. tritici-repentis* and *C. sativus* cause Stagonospora nodorum blotch (SNB), Septoria tritici blotch (STB), tan spot and spot blotch, respectively.

2.2. Pathogen

2.2.1. General biology

S. nodorum is a heterothallic ascomycete belonging to the order pleosporales (Solomon et al. 2006a). *Phaeosphaeria nodorum* (Müller) Hedjaroude is the teleomorphic form of *Stagonospora nodorum* (Solomon et al. 2006a). The synonymous names *Septoria nodorum* (Berk.) Berk. in Berk. and Broome and *Leptosphaeria nodorum* E. Müller are often used for describing *Stagonospora nodorum* and *Phaeosphaeria nodorum* respectively (Menzies and Gilbert, 2003; Solomon et al. 2006a). *Septoria* is a large genus that consists of many plant

pathogens (Cunfer & Ueng 1999). *Septoria* was previously thought to accommodate the Septoria nodorum blotch pathogen which we now know as *Stagonospora nodorum* (Cunfer & Ueng 1999). A recent study by Quaedvlieg et al. (2013) found Septoria nodorum blotch pathogen (*Stagonospora nodorum*) to be different from genus *Stagonospora* and suggested its placement under a separate genus *Parastagonospora*.

2.2.2. Disease cycle and epidemiology

The spores of *S nodorum* germinate to produce hyphae that invade and colonize the host tissue (Oliver et al. 2012). Sexual reproduction is characterized by production of pseudothecia, which are responsible for production of ascospores (Oliver et al. 2012). The initial source of inoculum for SNB disease is mostly supplied by the ascospores that overwinter on the stubble (Solomon et al. 2006a). The dissemination of ascospores over long distances is facilitated by wind (Bathgate & Loughman 2001; Solomon et al. 2006a; Sommerhalder et al. 2010). Alternatively, SNB infected seeds can also serve as a source of inoculum (Solomon et al. 2006a).

S. nodorum produces secondary mitotic inoculum called pycnidiospores (Solomon et al. 2006a). The pycnidia containing pycnidiospores are developed in the colonized host tissue (Oliver et al. 2012). The asexual reproduction of *S nodorum* is completed when the pycnidiospores infect healthy tissue (Oliver et al. 2012). Pycnidiospores produce repeated cycles of infections during the same growing season (Shah et al. 2001; Solomon et al. 2006a). The ability of *S. nodorum* to undergo repeated asexual cycles resulting in production of pycnidiospores causing multiple infections may result in larger yield losses (Oliver et al. 2012).

The early symptoms of SNB infection start as a very tiny dark brown lesion on the leaves. As the disease progresses, the lesions become larger, elongated and necrotic (Solomon et al. 2006a; Oliver et al. 2012). These necrotic lesions also develop a chlorotic halo (Solomon et al. 2006a; Oliver et al. 2012). In case of severe infection, the lesions coalesce to create a large infected area (Solomon et al. 2006a; Oliver et al. 2012). When conditions are favorable, the infected area develops pycnidia which will eventually produce pycnidiospores (Solomon et al. 2006a; Oliver et al. 2012). Pycnidiospores ooze out of pycnidia in a light pink colored secretion.

2.2.3. Population genetics

The new genotypes generated from sexual reproduction and the ascospores disseminated from long distances are the two major contributors in epidemics caused by the heterothallic fungus, *S. nodorum* (Sommerhalder et al. 2010). The study of mating types in Central Asia (Vergnes et al. 2006), Western Australia (Solomon et al. 2004), Sweden (Blixt et al. 2008), and North-Central and Midwestern United States (Adhikari et al. 2007) reported the presence of both mating types, MAT1-1 and MAT1-2 in the respective *S. nodorum* populations. A 1:1 ratio of MAT1-1 and MAT1-2 was reported by multiple mating type distribution studies (Sommerhalder et al. 2006; Adhikari et al. 2007; Blixt et al. 2008) which suggested frequent sexual reproduction.

A high genetic diversity has been observed within the populations of *S. nodorum* studied by different researchers (McDonald 1994; Keller et al. 1997; Murphy et al. 2000; Sommerhalder et al. 2006; Blixt et al. 2008). For example, Blixt et al. (2008) reported 93 genotypes in five populations among which 15 genotypes were reported in at least three fields, nine genotypes were reported in two fields and the remaining 69 genotypes were reported only once. A recent

study of allelic richness in the Iranian *S. nodorum* population McDonald et al. (2012) showed allelic richness results that were comparable to the results for Chinese, Swiss and New York *S. nodorum* populations previously reported by Stukenbrock et al. (2006). However, the Iranian *S. nodorum* population was reported to have high number of unique alleles and multilocus haplotypes [i.e. highest diversity seen in "fertile crescent"] suggesting the Fertile Crescent as the point of origin for *S. nodorum* (McDonald et al. 2012).

2.2.4. Infection process

The infection process by necrotrophic pathogen *S. nodorum* involves spore landing, germination and adherence on wheat leaves, penetration of the leaf surface and intercellular growth (Karjalainen & Lounatmaa 1986; Solomon et al. 2006b; Newey et al. 2007). The dissemination of pycnidiospores is dependent on rain splash (Solomon et al. 2006a). The disseminated pycnidiospore germinates once it lands on the leaf surface and establishes itself with the help of an amorphous substance or mucilagineous sheet (Karjalainen & Lounatmaa 1986). Solomon et al. (2006b) found that the spores usually germinated in a unipolar fashion but bipolar germination was also observed sporadically.

Following germination the hyphae continue growing on the leaf surface and eventually penetrate directly through the cell wall or through stomata (Karjalainen & Lounatmaa 1986; Solomon et al. 2006b). The use of an appresorium for penetration of the cuticle and epidermal cells of the cell wall has been observed (Bird & Ride 1981; Karjalainen & Lounatmaa 1986). Solomon et al. (2006b) successfully demonstrated that *S. nodorum* is able to invade the leaf tissue through stomatal openings. Stomatal penetration is believed to be an energy efficient

mechanism of penetration as the pathogen benefits from the natural openings on the leaf surface without the need to invade any tissue (Solomon et al. 2006b).

Once *S. nodorum* successfully penetrates the leaf surface, it continues intercellular growth as far as the mesophyll tissue (Bird & Ride 1981; Solomon et al. 2006b). However, the vascular infection is prevented by lignified sclerenchymatous tissue (Bird & Ride 1981; Solomon et al. 2006b). As the pathogen continues the intercellular growth, the disintegration of most epidermal and mesophyll cells is observed by the sixth day post inoculation (Bird & Ride 1981; Solomon et al. 2006b). Although the exact reason for this tissue disintegration is not known, different host selective toxins (HSTs) in *S. nodorum* that induce host necrosis and/or chlorosis are likely involved in the death of the host tissue (Bird & Ride 1981; Solomon et al. 2006b; Friesen, Faris, et al. 2008). Eventually, the leaf tissue completely collapses and pycnidia containing pycnidiospores start forming on the leaf surface which serves to infect other healthy host tissue (Solomon et al. 2006b).

2.2.5. Physiological specialization

Physiological specialization of *S. nodorum* is suspected but a conventional race classification system has not been used to illustrate the physiological specialization (Eyal 1999; Ali & Adhikari 2008; Matlock et al. 2012). The western Canadian *S. nodorum* isolates were grouped into nine different clusters based on the interactions between the host and the *S. nodorum* isolates (Matlock et al. 2012). A significant *S. nodorum* isolate x host interaction suggested the presence of host specificity (Matlock et al. 2012). The observed qualitative interactions between isolate and wheat lines were found to be significant and was presumed to

account for the host specificity (Matlock et al. 2012). Several other studies have demonstrated the presence of highly aggressive and less aggressive isolates of *S. nodorum* with moderate host specificity (Eyal 1999). Matlock et al. (2012) reported that the observed physiological specialization in *S. nodorum* is consistent with the presence of an inverse gene-for-gene interaction in wheat-*S. nodorum* pathosystem.

2.2.6. Host range

Wheat and barley are both susceptible to infection by *S. nodorum* (Eyal 1999; Solomon et al. 2006a). *S. nodorum* can also infect wild grass species, such as *Bromus inermis* and *Agropyron* species (Krupinsky 1982; Krupinsky 1986; Eyal 1999; Solomon et al. 2006a). The *S. nodorum* isolates from perennial grass species were reported to produce disease symptoms in wheat, which suggests that perennial grass species may be used as alternative hosts by *S. nodorum* (Krupinsky 1997).

2.3. SNB management

The ascospores released by pseudothecia present on the crop stubble and other residues provide a significant source of inoculum for SNB (King et al. 1983). Cultural practices like tillage, burning of crop residues and crop rotation are important strategies in reducing the primary inoculum for SNB (King et al. 1983; Eyal 1999). According to a comparative study of conventional and conservation till fields in southern Manitoba, a higher number of isolations of *S. nodorum* was observed from conventional till fields in the eastern region only (Gilbert &

Woods 2001). A proper crop rotation is expected to reduce wheat diseases irrespective of the tillage system used (Bailey 1996). In a study of leaf spot and root rot diseases of wheat, continuous rotations with wheat caused 28% more yield loss due to leaf spot diseases (Bailey 1996). In another study in southern Manitoba, no significant effect of crop rotation on *S. nodorum* and *P. tritici-repentis* isolation frequency was observed when the rotations included non-cereal crops (Gilbert & Woods 2001).

SNB can be effectively managed with the use of commercially available fungicides (Oliver et al. 2012)**.** Fungicides have been used to treat wheat seeds in the past (King et al. 1983). The organo-mercury compounds were reported as effective seed treatment fungicides (Shipton et al. 1971). The non-mercurial alternative like carboxin has also been used in wheat seed treatment which was eventually replaced by systemic triazole ergosterol biosynthesis inhibiting fungicides (Solomon et al. 2006a). Recently, the use of strobilurins as foliar fungicides has become wide-spread. Although fungicides are readily available and effective against SNB, the resistance against fungicide and cost effectiveness are some of the major concerns (King et al. 1983; Solomon et al. 2006a; Oliver et al. 2012).

Biological control of *S. nodorum* is an environment friendly alternative for SNB management. Microorganisms that are non-pathogenic to the host plant can potentially act as biological control agents (Nolan & Cooke 2000). According to Nolan & Cooke (2000), the treatment of wheat plants (cultivars Hussar and Brigadier) with *Drechslera teres* prior to inoculation with a mixture of *S. nodorum* and *Septoria tritici* significantly reduced the symptoms of both diseases. In the same study, pre-treatment of wheat cultivar Brigadier with *D. teres* followed by inoculation of *S. nodorum* caused an initial increase in disease symptoms (Nolan & Cooke 2000). The mechanism by which *D. teres* impart biological control on *S. nodorum* and *S.*

tritici is not understood. Therefore, due to the limited knowledge of biological control agents, limited efficiency of cultural practices alone, and cost and long term efficacy issues related to fungicides, sustainable SNB management remains an issue.

Genetic host resistance is an important component of integrated pest management. Genetic control of SNB, although desirable, has been difficult to achieve as most existing cultivars of wheat have only partial resistance to SNB (Aguilar et al. 2005; Solomon et al. 2006b). SNB resistance has been reported to be a quantitative trait (Wicki et al. 1999; Aguilar et al. 2005; Solomon et al. 2006a) and many QTL related to *S. nodorum* leaf blotch (Czembor et al. 2003; Liu, Friesen, et al. 2004) and glume blotch (Schnurbusch et al. 2003) resistance have been identified.

2.4. Host-pathogen interactions

Plants are at constant risk of infection by plant pathogens. The susceptibility or resistance of the host to the pathogen is dependent on the effectiveness of the defense responses deployed by the plants. The defense response against plant pathogens has been classified into two different types: 1) Pathogen associated molecular patterns (PAMP) triggered immunity (PTI) 2) Effector triggered immunity (ETI) (Chisholm et al. 2006; Sacco and Moffett 2009).

PTI is the first line of plant defense once the pathogens have evaded the plant cell wall. PAMP refers to a general group of pathogen-associated components like oligosaccharides, polypeptides, glycoproteins and lipids (Nürnberger et al. 2004; Sacco and Moffett 2009). As the name indicates, pathogen associated molecular patterns (PAMP) occur in a pattern common to different pathogens but are non-native to the plant (Nürnberger et al. 2004). The PAMP (e.g.

bacterial flagellin), when recognized by the receptors on the plant cell, trigger a series of Mitogen-activated protein kinases (MAPKs) reactions which in turn activates the WRKY transcription factors thereby triggering a non-specific defense response against the pathogens, known as PAMP-triggered immunity (PTI) (Nürnberger et al. 2004; Chisholm et al. 2006).

Phytopathogens have evolved to overcome PTI in plants (Chisholm et al. 2006). Bacterial phytopathogens are known to produce effectors that are directly fed into the plant cells via Type III secretion systems (Chisholm et al. 2006). The bacterial effectors are mostly proteases, protein phosphatases, protease inhibitors, chitin binding agents or ubiquitin conjugating enzymes (Chisholm et al. 2006). Fungi and oomycetes also produce effectors, but the mechanism of transport of these effectors is not understood (Chisholm et al. 2006). The effectors produced by phytopathogens are suspected to interfere with the normal plant physiology to facilitate pathogen growth (Chisholm et al. 2006). Plant defense mechanisms have evolved alongside these pathogens to recognize these effectors causing a defense response termed as Effector triggered immunity (ETI) (Sacco and Moffett 2009).

Host-pathogen interactions involving necrotrophic pathogens like *S. nodorum* and *P. tritici-repentis* utilize effectors/ host selective toxins (HSTs) to induce susceptibility in the host (Ciuffetti & Tuori 1999; Friesen, Faris, et al. 2008). The effector-induced host-pathogen interaction in wheat-*S. nodorum* pathosystem has been termed as Effector triggered susceptibility (ETS) (Liu et al. 2009). A typical ETS response is observed when an effector from the pathogen is recognized by the compatible sensitivity gene in the host (Liu et al. 2009).

2.4.1. Gene-for-gene hypothesis

The ETI response as a result of effector recognition by the plant forms the basis of the gene-for-gene theory proposed by Flor in 1956 (Chisholm et al. 2006; Sacco and Moffett 2009,p. 95). Flor (1956) proposed the gene-for-gene hypothesis which suggests that "for each gene that conditions a reaction in the host, there is a corresponding gene in the parasite that conditions pathogenicity". The implications of gene-for-gene hypothesis holds true for biotrophic pathogens where recognition of effectors produced by the pathogen once recognized by the corresponding resistance (R) gene on the host makes the host resistant. The effectors of biotrophic fungi are the product of an avirulence (Avr) gene whereas the R proteins are the product of a resistance (R) gene (Vleeshouwers & Oliver 2014).

2.4.2. Host specific toxin (HST) model, known toxins, and host sensitivity genes

HST model

The host-specific toxins (HSTs) are the group of phytotoxins that are only active against specific host genotypes. *S. nodorum* relies on HSTs for virulence (Friesen, Faris, et al. 2008). The HST when recognized by the corresponding sensitivity gene in wheat triggers a susceptible host reaction (Lamari & Bernier 1989; Friesen & Faris 2010). This type of relationship was first identified in *Pyrenophora tritici-repentis* (Ballance et al. 1989) and has been termed as an inverse gene-for-gene relationship (Friesen & Faris 2010). *S. nodorum* is known to produce numerous HSTs that are significant for disease development (Friesen, Faris, et al. 2008).

The involvement of phytotoxins in different pathosystems have been suspected for a long time but only a few of these toxins have been purified to date (Walton & Panaccione 1993; Walton 1996; Markham & Hille 2001; Wolpert et al. 2002; Friesen, Faris, et al. 2008; Ciuffetti et al. 2010). Most of the phytotoxins identified are known to be secondary metabolites belonging to different chemical groups (Walton & Panaccione 1993; Walton 1996; Markham & Hille 2001; Wolpert et al. 2002). However, in the recent decade, many HSTs that have been identified are proteins (Friesen, Faris, et al. 2008; Ciuffetti et al. 2010). In the recent years, HSTs produced by necrotrophic pathogens have been referred to as necrotrophic effectors (NE) (Friesen, Faris, et al. 2008; Oliver & Solomon 2010; Liu et al. 2012). Some of the known HSTs have been elaborated.

Known HSTs and host sensitivity genes

ToxA

The identification of ToxA in *P. tritici-repentis* (Ptr) and recognition of its host-specific toxicity was a milestone in the study of host-pathogen interactions of necrotrophic pathogens (Tomas & Bockus 1987; Lamari & Bernier 1989; Lamari 1991). ToxA was purified from Ptr by many researchers and was subsequently designated as PtrToxA (Ballance et al. 1989; Tomas et al. 1990; Tuori & Ciuffetti 1995; Zhang et al. 1997; Ciuffetti & Tuori 1999). The sensitivity to purified PtrToxA was found to be cultivar-specific (Tomas & Bockus 1987; Tomas et al. 1990). Therefore, the quest for the specific gene(s) in wheat interacting with PtrToxA became imperative. A single dominant gene in wheat cultivars was found to be responsible for PtrToxA sensitivity (Lamari & Bernier 1989; Lamari 1991; Ciuffetti & Tuori 1999). The recessive gene responsible for insensitivity to PtrToxA was designated as *tsn1* and was found to be located on

the long arm of chromosome 5B (Faris et al. 1996; Stock et al. 1996; Ciuffetti & Tuori 1999). The dominant allele of the same gene (*Tsn1*) is known to impart sensitivity to PtrToxA (Gamba et al. 1998; Ciuffetti & Tuori 1999; Haen et al. 2004; Horbach et al. 2011).

In the following years, it was demonstrated that ToxA was also produced by *S. nodorum* and was designated as SnToxA (Friesen et al. 2006). Friesen et al. (2006) developed SnToxA disrupted mutants, which showed that SnToxA also interacts with *Tsn1*. Liu et al. (2006) demonstrated that both SnToxA and PtrToxA induce necrosis by interacting with *Tsn1*, revealing that ToxA is an important HST for both *S. nodorum* and *P. tritici-repentis*. Given the structural and functional similarities of SnToxA and PtrToxA, a common ancestry for these toxins was suspected which eventually led to the conclusion that ToxA was interspecifically transferred from *S. nodorum* to *P. tritici- repentis* (Friesen et al. 2006).

Among all the HSTs identified in *S. nodorum*, ToxA is one of the most extensively studied HST. ToxA has been reported as a proteinaceous necrotic HST and was found to be of varying molecular weight by different researchers (Ballance et al. 1989; Tomas et al. 1990; Tuori & Ciuffetti 1995; Zhang et al. 1997; Ciuffetti & Tuori 1999). Initially, ToxA was believed to be produced as a 19.7kD immature protein by a single gene (Ballance et al 1996; Ciuffetti et al. 1997). The immature ToxA is comprised of a pre-pro-protein harboring a 22 amino acid putative signal peptide on the N-terminal region, N-terminal domain, and a C terminal domain (Ciuffetti et al. 1997). A mature ToxA protein is believed to be a 13.2kD protein corresponding to the C-terminal domain, whereas the function of N-terminal domain in the precursor of ToxA protein was undetermined (Ciuffetti et al. 1997). Tuori et al. (2000) investigated the toxicity and function of the N-terminal domain and demonstrated that when the N-terminal domain remained intact in a mature ToxA, the toxicity of ToxA remained unaffected and in fact enhanced the

toxicity of ToxA. The structural study of ToxA (Sarma et al. 2005) was consistent with previous ToxA mutation studies (Tuori et al. 2000; Meinhardt et al. 2002; Manning et al. 2004), which demonstrated that the RGD (Arginine-Glycine-Aspartic acid) motif is crucial in maintaining ToxA function. ToxA is believed to be recognized by the plant via interaction of the RGD motif and integrin-like receptors in the plant cells (Meinhardt et al. 2002; Sarma et al. 2005).

Tsn1 was sequenced by map-based cloning, which revealed that it is comprised of NBS-LRR (Nucleotide binding site- leucine rich repeat) and S/TPK (serine/ threonine protein kinase) domains (Faris et al. 2010). The presence of the NBS-LRR domain (typical of most resistance genes) indicates that necrotrophs, such as *S. nodorum* are capable of surmounting the plant defense mechanism by producing effectors that directly or indirectly interact with these resistance-like genes and their products in host plants (Lorang et al. 2007; Hammond-Kosack & Rudd 2008; Faris et al. 2010). The presence of functional *Tsn1* gene and internalization of ToxA has been demonstrated to be important for ToxA activity (Manning & Ciuffetti 2005; Faris et al. 2010). However, the lack of a transmembrane domain in the *Tsn1* protein and lack of evidence of direct interaction of *Tsn1* protein with ToxA suggests that *Tsn1* does not have a direct role in receptor-mediated recognition of ToxA (Manning & Ciuffetti 2005; Faris et al. 2010).

Many studies have been directed towards understanding the mechanism of ToxA-host cell interaction, ToxA recognition and localization. One such study suggested that ToxA gains entry only into the cells of ToxA sensitive wheat lines by the process of receptor mediated endocytosis (Manning & Ciuffetti 2005). In the same study, the fluorescence produced by ToxA fused with green fluorescent protein (GFP) was found to be associated with chloroplast and in distinct regions of cytoplasm, which confirmed immunolocalization in these areas (Manning & Ciuffetti 2005). ToxA has been demonstrated to interact with a plant protein that is present on

chloroplast membrane and stroma. Localization in the chloroplast suggests the chloroplast is a potential target for induction of necrosis following ToxA-ToxA binding protein 1 (ToxABP1) interaction (Manning & Ciuffetti 2005; Manning et al. 2007). In a subsequent study, Manning et al. (2009) demonstrated that alterations in photosystem I and II led to accumulation of reactive oxygen species (ROS) in the ToxA sensitive host, which when exposed to light, triggered cell death. Tai et al. (2007) demonstrated direct interaction of plastocyanin with ToxA. A recent study by Lu et al. (2014) suggested that PR-1 type pathogenesis related proteins (PR-1-5) interact with ToxA to induce necrotic symptoms in the host.

SnTox1

In addition to ToxA, many other HSTs have been identified in *S. nodorum*. SnTox1 was the first HST to be identified in *S. nodorum.* SnToxA was reported to be a proteinaceous HST which was estimated to be between 10-30kDa in size (Liu, Faris, et al. 2004). The actual size of mature SnTox1 protein (10.33 kDa) was unknown until the molecular cloning of the SnTox1 gene which also revealed that SnTox1 was initially produced as a longer protein (117 amino acids) with a signal peptide (Liu et al. 2012). The wheat gene conferring sensitivity to SnTox1 was successfully located on chromosome 1BS and was named *Snn1* (Liu, Faris, et al. 2004). The physical location of *Snn1* was predicted based on the study of 1BS deletion lines which also placed several DNA markers in the close vicinity with *Snn1* (Liu, Faris, et al. 2004). A compatible SnTox1-*Snn1* interaction was demonstrated to cause necrosis in wheat (Liu, Faris, et al. 2004) whereas disruption of SnTox1 in a virulent isolate consequently made the isolate avirulent, suggesting SnTox1 was a virulence factor (Liu et al. 2012).

SnTox2

SnTox2 is another HST that has been identified in *S. nodorum* (Friesen et al. 2007). SnTox2 is estimated to be a 7-10 kDa sized protein (Friesen et al. 2007). SnTox2 was identified from the same *S. nodorum* isolate that also produced SnToxA and is known to produce a necrotic reaction like SnToxA (Friesen et al. 2007). The gene in wheat that was responsible for SnTox2 sensitivity was determined to be a single dominant gene that mapped on chromosome 2DS and was designated *Snn2* (Friesen et al. 2007).

SnTox3

Subsequently, another necrotic HST was identified, which was named SnTox3 (Friesen, Zhang, et al. 2008). The mature SnTox3 protein is approximately 18 kDa in size and is initially produced as a precursor protein composed of 230 amino acids (Liu et al. 2009). Addition of a functional SnTox3 gene to an avirulent *S. nodorum* isolate via artificial transformation resulted in a newly virulent isolate producing SnTox3, which indicated self-sufficiency of SnTox3 as a virulence factor (Liu et al. 2009). A study of SnTox3 in the global *S. nodorum* population indicated the presence of four different amino acid sequences all of which represented alterations in SnTox3 protein in four different ways (Liu et al. 2009). An interesting finding of the same study was that all four SnTox3 variants were equally potent at producing a sensitive reaction in the host (Liu et al. 2009). The sensitivity gene corresponding to SnTox3 was designated *Snn3* and was mapped 1.4 centiMorgans (cM) away from the microsatellite marker *Xcfd20* on short arm of chromosome 5B (Friesen, Zhang, et al. 2008). The sensitivity to SnTox3 due to *Snn3* was determined to be a dominant trait (Friesen, Zhang, et al. 2008). More recently, another gene interacting with SnTox3 was identified in *Aegilops tauschii* and was mapped on the short arm of

chromosome 5D (Zhang et al. 2011). It is believed that the genes interacting with SnTox3 are homoeologous and were therefore designated as *Snn3-B1* and *Snn3-D1* (Zhang et al. 2011).

SnTox4

SnTox4 is a proteinaceous HST (Abeysekara et al. 2009). The exact size of SnTox4 is not known, but is estimated to be between 10 and 30 kDa (Abeysekara et al. 2009). A single dominant host gene interacting with SnTox4 has been identified and designated *Snn4* (Abeysekara et al. 2009). A successful SnTox4-*Snn4* interaction leads to a sensitive reaction in host plants which has been described as "mottled necrotic reaction" which differs in the severity of the necrosis observed (Abeysekara et al. 2009). *Snn4* gene has been mapped on the short arm of chromosome 1B (Abeysekara et al. 2009).

SnTox5

SnTox5 is the most recent HST to be identified in *S. nodorum*. The sensitivity gene in wheat that interacts with SnTox5 was mapped on the long arm of chromosome 4B and was named *Snn5* (Friesen et al. 2012). A compatible SnTox5-Snn5 interaction also leads to a sensitive reaction in the host plant which is characterized by a necrotic reaction (Friesen et al. 2012). Although, the exact size of SnTox5 is not known, it is estimated to be in a range of 10-30 kDa (Friesen et al. 2012).

2.4.3. HSTs in *Pyrenophora tritici-repentis*

In addition to Ptr ToxA, two other HSTs have been identified in *P. tritici-repentis*. Among the HSTs identified in *P. tritici-repentis*, Ptr ToxA is a necrotic toxin whereas the other two HSTs are found to cause chlorosis. The chlorosis toxins of *P. tritici-repentis* are described as follows:

Ptr ToxB

ToxB was successfully purified by Strelkov et al. (1999) from a race 5 isolate of *P. tritici-repentis* and was reported to be a small protein of 6.61 kDa causing chlorosis in sensitive wheat cultivars. Ptr ToxB was previously identified as Ptr chlorosis toxin (Strelkov et al. 1998). Following the purification of Ptr ToxB, only the partial amino acid sequence of Ptr ToxB was known which served as a basis for tracing the Ptr ToxB gene and eventually led to heterologous expression of Ptr ToxB (Strelkov et al. 1999; Martinez et al. 2001). Although discrepancies in amino acid sequence were found in comparison to the Ptr ToxB amino acid sequence proposed by Strelkov et al. (1999), the heterologously expressed Ptr ToxB retained bioactivity proving that Ptr ToxB is a HST causing chlorosis (Martinez et al. 2001). Kim & Strelkov (2007) with the help of heterologous expression of the Ptr ToxB gene from virulent and avirulent isolates demonstrated that change in nucleotide and/or amino acid sequence may have significant effect on the severity of toxin activity. A single dominant gene was found to be responsible for sensitivity towards Ptr ToxB (Orolaza 1995). The Ptr ToxB sensitivity gene (*Tsc2*) was mapped on chromosome 2BS (Friesen & Faris 2004).

Ptr ToxC

Ptr ToxC is a chlorosis toxin produced by *P. tritici-repentis* which is a non-protein in nature (Effertz et al. 2002; Lamari & Strelkov 2010). Effertz et al. (2002) partially purified Ptr ToxC using three different purification methods and concluded that Ptr ToxC was a small molecule which was polar and nonionic unlike existing HSTs. The Ptr ToxC insensitivity gene *tsc1* mapped 5.7 cM away from the marker *XGli1* on the short arm of chromosome 1A (Effertz et al. 2002).

2.4.4. Other toxin systems

Besides, *S. nodorum* and *Pyrenophora tritici-repentis*, many other plant pathogenic fungi are known to produce phytotoxins. Some of the characterized toxins have been described.

Victorin

Victorin is a toxin produced by *Cochliobolus victoriae* which causes Victoria blight of oats (Meehan & Murphy 1946; Meehan & Murphy 1947; Wolpert et al. 2002). Victoria blight was introduced in the United States as a result of introgression of *Puccinia coronata* resistance gene (*Pc-2*) in oats (Walton 1996). The *Pc-2* gene is thought to be the same as or closely linked to *Hv-1* or *Vb* gene which imparts sensitivity to victorin toxin (Walton & Panaccione 1993; Walton 1996).

Victorin is one of the longest known and most potent phytotoxins, which is effective at a concentration as low as 10 pM (Walton & Panaccione 1993; Walton 1996; Markham & Hille

2001). The victorin toxin is a halogenated cyclic-pentapeptide. (Walton 1996; Markham & Hille 2001). Victorin B, victorin C, victorin D, victorin E and victorine are different forms of victorin to be identified among which victorin C is the most common victorin (Navarre et al. 1995). Another form of victorin named HV toxin M was also reported by (Kono et al. 1986). The *TOX 3* gene regulates the production of victorin in *C. victoriae*. (Walton & Panaccione 1993). Victorin has been demonstrated to bind to a 100 kDa protein found in oats which was later identified as pyridoxal phosphate containing P-subunit of glycine decarboxylase (GD) (Navarre et al. 1995; Walton 1996).

HC-toxin

HC-toxin is produced by race 1 of *Cochliobolus carbonum* which is a causal agent of leaf spot disease of maize (Markham & Hille 2001). *TOX 2* gene is known to regulate HC-toxin production in *C. carbonum* (Bronson 1991; Walton 1996). However, the sensitivity of specific maize varieties to HC-toxin is a direct result of the presence of a single recessive locus (*hm1/hm1*) (Walton 1996). The HC-toxin inhibits root growth in sensitive maize (Walton 1996).

T-toxin

T-toxin is a phytotoxin typical to T race of *Cochliobolus heterostrophus* (Walton 1996). *C. heterostrophus* is an etiological agent of Southern corn leaf blight which relies on T-toxin for its virulence (Walton 1996). The presence of T-cytoplasmic male stertility (T-CMS) type cytoplasm in maize cultivars massively increases the sensitivity towards T-toxin in comparison

to normal maize cytoplasm (Walton 1996). T-toxin biosynthesis genes are harbored by two different loci *TOX 1A* and *TOX1B* which are the results of translocation of the *TOX 1* locus (Kodama et al. 1999). The genes related to T-toxin biosynthesis have been identified within and outside *TOX 1* loci (Markham & Hille 2001).

Toxins produced by the genus *Alternaria*

Members of the genus *Alternaria* produce phytotoxins belonging to various chemical groups (Wolpert et al. 2002). *A. alternata f. sp. lycopersici* produces AAL toxin which bears resemblance with a mycotoxin produced by *Fusarium moniliforme* (Gilchrist et al. 1995; Markham & Hille 2001). AAL toxin is a aminopentol ester and the AAL toxin sensitivity in tomato is recessive (Markham & Hille 2001; Wolpert et al. 2002). AM toxin is toxin produced by *A. alternata f. sp. mali*, which causes Alternaria blotch in apple (Markham & Hille 2001). Chemically, AM toxin has been recognized as a cyclic tetrapeptide and host sensitivity to the AM toxin is reported to be a dominant trait (Wolpert et al. 2002). *A. alternata f. sp. kikuchiana* is known to produce AK toxin which is responsible for susceptibility of Japanese pears to Black spot disease (Markham & Hille 2001). AK toxin is an epoxy-decatrienoic ester and the sensitivity of Japanese pears to AK toxin is a dominant trait (Markham & Hille 2001; Wolpert et al. 2002). Additionally, genus *Alternaria* also produces AF and ACT toxins which are also reported to be epoxy-decatrienoic esters like AK toxin (Markham & Hille 2001; Wolpert et al. 2002). The sensitivity of the hosts to both AF and ACT toxin is dominant (Wolpert et al. 2002). AF toxin is produced by *A. alternata f. sp. fragariae* and ACT toxin is produced by *A. alternata f. sp. citri* (Otani et al. 1995; Markham & Hille 2001).

CDiT1 toxin

Recently, a cell death-inducing toxin called Cell death in tomato 1 (CDiT1) toxin has been identified in *Pyrenochaeta lycopersici*, an etiological agent of corky root rot (CRR) disease of tomato (Clergeot et al. 2012). CDiT1 consists of two identical subunits collectively weighing approximately 35 kDa (Clergeot et al. 2012). The cell death induction was found to be much more severe in *Solanum lycopersicum* than in comparison to other hosts of *P. lycopersici* when infiltrated with CDiT1 (Clergeot et al. 2012).

Miscellaneous toxins

In addition to all the above-mentioned phytotoxins, there are many other phytotoxins that are produced by other pathogens. Peritoxin (PC Toxin) is produced by *Periconia circinata*, a pathogen in sorghum (Wolpert et al. 2002). PC Toxin is chemically a peptidyl chlorinated polyketide (Wolpert et al. 2002). HS toxin is a glycosylated sesquiterpene produced by *Bipolaris sacchari* which is a pathogen of sugarcane (Wolpert et al. 2002). PM toxin is a linear polyketol produced by *Mycosphaerella zeae-maydis* that infects corn (Wolpert et al. 2002). Therefore, many chemically diverse phytotoxins have been identified in plant pathogens. Possibly, many others are either under investigation or yet to be discovered.

2.4.5. Other types of SNB resistance

The SNB resistance has been reported to be a quantitative trait (Wicki et al. 1999; Solomon et al. 2006a). Many quantitative trait loci (QTL) related to *S. nodorum* leaf and glume

blotch resistance have been identified (Schnurbusch et al. 2003; Liu, Friesen, et al. 2004; Aguilar et al. 2005; Uphaus et al. 2007; Shankar et al. 2008; Gonzalez-Hernandez et al. 2009; Francki et al. 2011). Among the identified QTL, some have been associated with the toxin insensitivity loci (Liu, Friesen, et al. 2004), but others do not correspond to any known toxin insensitivity loci (Gonzalez-Hernandez et al. 2009; Francki et al. 2011).

In a QTL analysis using a recombinant inbred (RI) population based on the cross between wheat cultivars Forno x Oberkulmer (*Triticum aestivum L*. x *Triticum aestivum L*.), 11 different QTL were reported for leaf blotch and ten different QTL were reported for glume blotch (Aguilar et al. 2005). Two different glume blotch resistance QTL were identified on chromosomes 3BS and 4BL using a single seed descent (SSD) population based on Arina x Forno (Schnurbusch et al. 2003). In the same study, a third glume blotch resistance QTL (*QSng.sfr-5BL*) was identified on chromosome 5BL overlapping with QTL for plant height and heading time (Schnurbusch et al. 2003). A study in Australia reported QTL for flag leaf resistance and glume resistance on chromosome 2DL and 4BL, respectively (Shankar et al. 2008). The QTL analysis using the RI population based on two unrelated parents partially resistant to SNB detected two different glume blotch resistance QTL *QSng.pur-2DL.1* and *QSng.pur-2DL.2* (Uphaus et al. 2007). The QTL *QSng.pur-2DL.1* and *QSng.pur-2DL.2* were common for the studies in United States and Australia (Uphaus et al. 2007). Gonzalez-Hernandez et al. (2009) reported a SNB resistance QTL on chromosome 5BL at a distance of 8.8cM distal to *tsn1* locus and the resistance allele was found to be contributed by *T. turgidum var. dicoccoides*. Recently, new QTL for flag leaf resistance to SNB were located on chromosomes 1BS and 2AS which were not found to be associated with the known HST insensitivity loci (Francki et al. 2011).

Additionally, partial resistance QTL for SNB have been reported (Czembor et al. 2003; Arseniuk et al. 2004). Arseniuk et al. (2004) studied the factors affecting partial resistance, such as length of incubation period, disease severity and length of latent period. This allowed the identification of QTL for partial resistance to SNB. A QTL for partial resistance was located on chromosome 6AL which accounted for 36% and 14% phenotypic variation due to disease severity and length of incubation period, respectively (Arseniuk et al. 2004). The same study also reported a putative QTL for partial resistance on chromosome 6D (Arseniuk et al. 2004). The QTL for partial resistance to SNB were reported on chromosomes 2B, 3B, 5B and 5D (Czembor et al. 2003). Several broad spectrum resistance (BSR) QTL to Fusarium head blight (FHB), Septoria tritici blotch (STB) and Stagonospora nodorum glume blotch (SGB) have been identified (Miedaner et al. 2012). Among the identified BSR QTL, none has been associated with resistance to all three diseases (FHB, STB and SGB) but BSR QTL associated with resistance to at least two diseases have been identified (Miedaner et al. 2012). A BSR QTL for resistance to STB and SGB and another for resistance to FHB and SGB have been identified during meta-QTL analysis using Arina x Forno mapping population (Miedaner et al. 2012).

2.5. Biochemistry and proteomics in the study of HSTs

The techniques in biochemistry and proteomics have been crucial in the study of HSTs in *S. nodorum* and other necrotrophs. ToxA was the first HST to be identified in Ptr (Tomas & Bockus 1987; Lamari & Bernier 1989; Lamari 1991). ToxA has been purified by many researchers using a series of filtrations and liquid chromatography techniques (Ballance et al. 1989; Tomas et al. 1990; Tuori & Ciuffetti 1995; Zhang et al. 1997). Some of the earliest studies

for purification of ToxA used multiple filtrations and column chromatography techniques. For example, Ballance et al. (1989) used a ultrafiltration membrane to filter the crude culture filtrate containing ToxA. Subsequently, the filtrate containing ToxA was further purified and concentrated using sephadex G-100 columns and CM-cellulose columns. In addition to the sephadex and cellulose columns, other chromatography techniques like Mono-S fast protein liquid chromatography (FPLC) and High performance liquid chromatography (HPLC) have also been used in the purification of HSTs in Ptr (Tomas et al. 1990; Tuori & Ciuffetti 1995). Likewise, anion and/or cation exchange FPLC and gel filtration systems have also been used in purification of HSTs like SnTox1, SnTox2, SnTox3, SnTox4 and SnTox5 in *S. nodorum* (Liu, Faris, et al. 2004; Friesen et al. 2007; Liu et al. 2009; Abeysekara et al. 2009; Friesen et al. 2012). However, in most cases, only partial purification of HSTs is possible.

Gel electrophoresis techniques separate the proteins according to their molecular weight, which allows the identification of candidate protein(s) for a HST (Liu et al. 2009; Liu et al. 2012). The candidate proteins identified by gel electrophoresis can be analyzed by mass spectrometry and identified using a database search (Rampitsch & Bykova 2009). Ballance et al. (1989) attempted amino acid analysis, spectrophotometry and tryptophan estimation of the purified PtrToxA. The amino acid analysis of PtrToxA was repeated by other researchers (Tomas et al. 1990; Tuori & Ciuffetti 1995) and was found to be consistent with the results obtained by Ballance et al. (1989). The other aspects of the purified HSTs have also been investigated, which includes determination of heat stability and protease treatment to confirm the protein nature of the purified HST (Ballance et al. 1989; Tuori & Ciuffetti 1995; Liu et al. 2009; Liu et al. 2012). Proteomics and metabolomics were used to investigate the effect of ToxA on wheat by Vincent

et al. (2012). The same study reported the abundance of 91 proteins and 101 metabolites as a result of ToxA infiltration in wheat (Vincent et al. 2012).

2.6. Genetic mapping

The relative location of gene(s) controlling qualitative and quantitative traits can be determined using a genetic map. The mapping can be grouped into two different types based on the type of trait under study: 1) Linkage mapping and 2) QTL mapping.

Qualitative traits are mapped using linkage analysis. Linkage mapping groups the available molecular markers into different linkage groups and arranges markers in these linkage groups in the most likely order. The phenotypic data for a qualitative trait is treated as a molecular marker locus during linkage mapping (Liu, Faris, et al. 2004). The sensitivity of wheat cultivars to *S. nodorum* HSTs is a qualitative trait. Therefore, linkage maps can be used to map the location of HST sensitivity gene(s) relative to molecular markers. Liu, Faris, et al. (2004) mapped the *Snn1* gene that confer sensitivity to SnTox1 using synthetic hexaploid wheat W-7984/hard red spring wheat cv. Opata 85 based recombinant inbred population. The SnTox1 sensitivity gene was determined to be located at a distance of 4.7 centimorgans (cM) from the marker *XksuD14* on the distal end of chromosome 1BS (Liu, Faris, et al. 2004). The *Snn2* gene imparting sensitivity to SnTox2 was also mapped using linkage analysis and was found to be located on distal end of chromosome 2D (Friesen et al. 2007). The *Snn2* gene was estimated to be at distances of 7.6 cM and 5.9 cM from microsatellite markers *Xgwm614.1* and *Xbarc95*, respectively (Friesen et al. 2007). The location of other HST sensitivity genes *Snn3* (SnTox3)

(Liu et al. 2009), *Snn4* (SnTox4) (Abeysekara et al. 2009) and *Snn5* (SnTox5) (Friesen et al. 2012) were also mapped using linkage mapping.

Quantitative traits are complex in nature and are controlled by multiple genes, such that discrete classes are not present (Sleper & Poehlman 2006). The location of a gene controlling a quantitative trait on the chromosome is termed as a quantitative trait locus (QTL) (Sleper & Poehlman 2006, p. 53-70). QTL analysis is used to locate the genetic location of the QTL. Plant disease resistance can be a quantitative trait in some cases. For example, disease resistance to SNB has been shown to be a quantitative trait and hence related QTL have been mapped on different chromosomes of wheat (Solomon et al. 2006a). Francki et al. (2011) recently reported new QTL for flag leaf resistance for SNB on chromosomes 2AS and 1BS. A major QTL coinciding with SnTox1 insensitivity (*snn1*) gene was mapped on chromosome 1BS (Liu, Friesen, et al. 2004). The same study also found many other minor QTL with varying effects on chromosomes 3AS, 3DL, 4AL, 4BL, 5DL, 6AL and 7BL (Liu, Friesen, et al. 2004).

2.7. Applications of HSTs in breeding for resistance

The use of HSTs as an aid to conventional breeding is a relatively new idea. Conventional breeding can be labour intensive and time consuming. HST-aided breeding is an important tool for breeding programs, especially in the regions where SNB epidemics are more prevalent. In the recent years, many HSTs have been identified and purified in different pathosystems including *S. nodorum* (Friesen, Faris, et al. 2008; Ciuffetti et al. 2010). Among the five identified HSTs of *S. nodorum*, SnTox1 (Liu, Faris, et al. 2004), SnToxA (Friesen et al. 2006) and SnTox3 (Friesen, Zhang, et al. 2008) have been well studied. One method of applying

purified HSTs directly to the breeding programs is by screening the parental lines used for breeding with the available HSTs (Vleeshouwers & Oliver 2014). This method has aided breeding programs in Australia where breeders were able to eliminate the sensitive alleles corresponding to the available toxin preparations based on infiltration assays (Vleeshouwers & Oliver 2014). Additionally, HST-aided breeding can also make field trials more efficient by allowing elimination of wheat cultivars or lines sensitive to specific HSTs. However, the direct HST infiltration in order to identify sensitive alleles is not an ideal method for achieving complete resistance because only a handful of HSTs have been fully characterized (Tuori et al. 2000; Oliver et al. 2009; Liu et al. 2009; Liu et al. 2012). Therefore, any unidentified or uncharacterized source of susceptibility for which the HST is not available cannot be eliminated with HSTs. Another use of HST is in the genetic analyses of the host. HSTs can be used to accurately map the corresponding sensitivity genes. Therefore, these genes, once located can be used to develop closely linked or gene-based markers ideal for marker-assisted selection. However, developing closely linked, diagnostic markers for marker-assisted selection can be time consuming and difficult process. Overall, identification, purification and characterization of HSTs are important precursor steps in HST-assisted breeding for SNB resistance.

3. IDENTIFICATION OF A NOVEL *STAGONOSPORA NODORUM* TOXIN AND MAPPING OF TOXIN SENSITIVITY IN WHEAT

3.1. Abstract

Stagonospora nodorum is a necrotrophic ascomycete causing foliar and glume diseases in wheat referred to as Stagonospora nodorum blotch and Stagonospora nodorum glume blotch, respectively. The other hosts of *S. nodorum* include barley and wild grass species. The worldwide occurrence of *S. nodorum* is well documented. The wheat-*S. nodorum* pathosystem relies on inverse gene-for-gene theory. *S. nodorum* produces a multitude of proteinaceous host selective toxins (HSTs) which include SnToxA, SnTox1, SnTox2, SnTox3, SnTox4 and SnTox5. The recognition of a HST by a specific sensitivity gene in wheat causes a susceptible reaction in wheat. In this study, novel HST-host sensitivity gene interactions were investigated. The wheat cultivar Laura was found to be sensitive and another wheat line 86ISMN 2137 was found to be insensitive to the HST(s) produced by *S. nodorum* isolate Swift Current in liquid media. The sensitivity gene for this HST was mapped by infiltrating the culture filtrate of isolate Swift Current into the Kenyon/86ISMN 2137 recombinant inbred line (RI) and Laura/86ISMN 2137 double haploid (DH) populations. The chromosomal location of the putative sensitivity gene corresponded to that of *Snn3* locus. Therefore, one of the HSTs present in Swift Current culture filtrate was identified to be SnTox3. Additionally, SnTox3 sensitivity in Kenyon/86ISMN 2137 recombinant inbred (RI) population was confirmed and mapped using recombinant SnTox3. The Kenyon/86ISMN 2137 RI population was previously thought to be insensitive to SnTox3.

3.2. Introduction

Stagonospora nodorum (teleomorph: *Phaeosphaeria nodorum*) is related to other plant pathogens such as *Pyrenophora tritici-repentis*, *Cochliobolus heterostrophus* and *Leptosphaeria maculans* in the phylum Ascomycota. *Stagonospora nodorum* is the causal agent for Stagonospora nodorum blotch (SNB) disease in tetraploid and hexaploid wheat (Eyal, 1981; King et al., 1983; Murray and Brennan, 2009; Oliver, et al., 2012).

SNB can be managed by the application of conventional foliar fungicides. However, a growing concern for fungicide resistance threatens the traditional SNB management strategies with increased fungicide use around the world (Blixt et al. 2009; Murray & Brennan 2009; Oliver et al. 2012). Genetic resistance is also an effective long term SNB management strategy. However, introgression of the SNB resistance into wheat varieties has proven difficult due to the polygenic nature of SNB resistance and also due to the lack of understanding of host pathogen interactions in the wheat-*S. nodorum* pathosystem. It wasn't until the unlocking of host-pathogen interaction mechanism involving Host selective toxins (HSTs) in *S. nodorum* by Liu, Faris, et al. (2004) that the new avenue for achieving SNB resistance was realized. Subsequently, a significant quantitative trait locus (QTL) for toxin insensitivity was identified and was mapped on chromosome 1BS of wheat. The gene was later found to be the *Snn1* toxin insensitivity gene (Liu, Friesen, et al. 2004).

Subsequently, other HST(s) have been purified and their sensitivity loci in the host have been reported. ToxA is one of the first purified and best characterized HST, which was first identified in *Pyrenophora tritici-repentis* by Ballance et al. (1989). ToxA is believed to be transferred from *S. nodorum* to *P. tritici-repentis* as a result of interspecific virulence gene

transfer (Friesen et al. 2006). Liu et al. (2006) demonstrated that SnToxA-*Tsn1* gene interaction is comparable to PtrToxA-*Tsn1* gene interaction. In the following years, SnTox2-Snn2 (Friesen et al. 2007), SnTox3-Snn3 (Friesen, Zhang, et al. 2008; Liu et al. 2009), SnTox4-Snn4 (Abeysekara et al. 2009) and SnTox5-Snn5 (Friesen et al. 2012) interactions were characterized. Although a total of six different HSTs have been identified to date, there are no set rules to predict how many more interactions are yet to be identified. At present, searching for effective resistance against SNB and understanding the underlying interactions in the *Stagonospora nodorum*-wheat pathosystem relies on the discovery of new HST(s) and unlocking their significance in the pathosystem. The objectives of this study were to purify and characterize HST produced by *S. nodorum* and genetically map the host sensitivity to the purified HST.

3.3. Materials and methods

Plant material

The Kenyon/86ISMN 2137 recombinant inbred line (RI) population consisting of 125 lines and the Laura/86ISMN 2137 double haploid (DH) population consisting of 122 lines were developed by single seed descent and the maize pollination methods, respectively. Kenyon and Laura were susceptible parents whereas 86ISMN 2137 was a resistant parent. The Kenyon/86ISMN 2137 RI population and Laura/86ISMN 2137 DH population were used in mapping of HST sensitivity genes.

Various wheat lines were used for bioassays to test known HSTs, purified HST(s) and culture filtrate. As part of initial screening step, various wheat lines (Table 3.1) with different genetic background were tested for sensitivity to known toxins. The differential lines BG261,

BG220, BG231 and M6 were obtained from Dr. Justin Faris's labs at United States Department of Agriculture, Fargo, ND, U.S.A. The differential lines, parental lines and a subset of the Kenyon/86ISMN2137 RIL population were used to test the HST(s) produced in culture filtrate and to test the bioactivity of purified HST.

The Kenyon/86ISMN 2137 RI population and Laura/86ISMN 2137 DH population for mapping and the seedlings for bioassay were planted in a similar arrangement. Kenyon and 86ISMN 2137 were included as sensitive and insensitive checks, respectively. Two seeds of each line were planted in a cone-tainer cell (Ray Leach cone-tainers, Tangent, OR). A total of two cone-tainer cells were used for planting four seeds from each line using the potting mix Sunshine Mix #5 (Sun Gro Horticulture Canada Ltd.).

Bioassay

Bioassays were used for the following: 1) To screen different parental lines 2) To test for the presence of HST in culture filtrate 3) To test the bioactivity of purified HST and 4) To map HST sensitivity. The known HSTs SnToxA (Friesen et al. 2006), SnTox1 (Liu, Faris, et al. 2004) and SnTox3 (Liu et al. 2009) were obtained from Dr. Timothy Friesen's lab at United States Department of Agriculture, Fargo, ND, U.S.A.

The second leaf of each seedling was used to perform bioassays. These leaves were injected with approximately 100 µl of a known HST, culture filtrate or a purified HST. The infiltrated seedlings from all bioassays were incubated for three days in a growth cabinet maintained at 21/19°C (day/night) with 16 hour photoperiod. After three days, the infiltrated plants were rated using a scale of 0 to 3 where 0 indicated insensitive reaction, 1 indicated faint

chlorosis, 2 indicated necrosis and/or chlorosis and 3 indicated severe necrosis. The rating scale of 0 to 3 was developed in Dr. Tim Friesen's lab (personal communication).

Toxin production

S. nodorum isolates were isolated from infected leaf tissue. A single spore culture of each isolate was used to inoculate double strength (2X) V8 agar medium (314 ml V8 juice, 3 g $CaCO_3$, 15 g Agar in 686 ml distilled water). The inoculated 2X V8 agar medium was incubated under fluorescent light for 8-9 days until pink colored pycnidiospores started oozing on the surface. To this culture, 4 ml of sterile distilled water was added in order to suspend the oozing pycnidiospres. 80 µl of the pycinidiospore suspension was used to inoculate 125 ml of Fries medium (5 g $(NH_4)_2C_4H_4O_6$, 1 g NH_4NO_3, 0.5 g $MgSO_4.7H_2O$, 1.3 g KH_2PO_4, 2.6 g K_2HPO_4, 30 g sucrose, 1 g yeast extract and 2 ml trace elements in 1000 ml distilled water). Trace elements for Fries medium were prepared by suspending 167 mg LiCl, 107 mg CuCl, 34 mg H_2MoO_4, 72 mg $MnCl_2$-$4H_2O$, 80 mg $CoCl_2$-$4H_2O$ in a final volume of 1000 ml water. Corning 175 cm^2 angled neck flasks (Corning Incorporated, Corning, NY) were used for all Fries medium cultures. The Fries medium culture was then incubated at room temperature in natural light for three days with shaking at 80 rpm. After three days, the flasks were subjected to stationary incubation in an incubator maintained at 21°C and dark conditions. The Fries medium culture was used for a bioassay 12 days post-inoculation into Fries medium. This 12-day-old Fries medium culture was filtered through two layers of miracloth (Merck KGaA Darmstadt, Germany) before infiltration into seedlings.

Putative toxin isolation and identification

Following positive identification in the bioassay, the *S. nodorum* culture was further filtered using 0.45 µm Millipore Durapore membrane (EMD Millipore Billerica, MA) and dialyzed overnight against approximately 3 liters of cold Mili-Q purified water. Forty ml of dialyzed filtrate was then subjected to Cation Exchange Chromatography using 5 ml SP XL Sepharose column in an *ÄKTA Prime* Fast Protein Liquid Chromatography (FPLC) unit (AktaPrime: GE Healthcare, UK) and 40 fractions were collected. The fractions were eluted using a sodium chloride gradient ranging from 0 to 300 mM. In another bioassay, each of the 40 fractions were separately infiltrated into Kenyon (susceptible/sensitive parent) and 86ISMN2137 (resistant/insensitive parent) to identify an active fraction. The fractions that tested positive in the bioassay along with fractions preceding and following the active fractions were separated by Tris-tricine gel electrophoresis.

The Tris-tricine gels were cast by pouring 12 % separating and 3 % stacking gel into a Mini-PROTEAN Tetra Cell system (Bio-Rad, Hercules, CA). Running buffer for the electrophoresis consisted of 1X Cathode buffer (From 10X stock: 1 M Tris pH 8.25, 1 M Tricine and 1% (w/v) sodium dodecyl sulfate) and 1X Anode buffer (From 10X stock: 2 M Tris pH 8.9). The 15 µl of each sample was mixed with 6 µl of 6X sample buffer (3.5 ml 1M Tris pH 6.8, 1.028 g SDS, 0.93 g dithiothreitol (DTT), 3.6 ml glycerol and 1.2 mg bromophenol blue in a total volume of 10 ml) and were incubated in a boiling water bath for 5 min. The samples were then separated by electrophoresis for three hours using constant voltage of 80 V. Active fractions with a similar profile on the Tris-tricine gel were pooled and electrophoresis of a small aliquot of each pooled sample was repeated on a Tris-tricine gel and also used for differential line infiltration. After electrophoresis, the candidate band was excised, alkylated and digested with

trypsin as described by Rampitsch and Bykova (2009, p. 93-110). The digested protein was then analyzed by LC-MS. The MS/MS spectra were then queried against *Stagonospora nodorum* database [*Stagonospora nodorum* Sequencing Project, Broad Institute of Harvard and MIT (http://www.broadinstitute.org/) (12,379 sequences)] using Mascot (v2.4).

Statistical analysis

Least square means were calculated for the toxin infiltration data (0-3 scale) with JMP Genomics 6.0 (SAS Institute Inc.) using a mixed model where wheat lines were considered fixed effects and replicate was considered a random effect. These least square means were used for mapping the sensitivity gene.

The χ^2 analysis was conducted for the phenotypic data obtained from the Swift Current culture filtrate infiltration in Laura/86ISMN 2137 DH population. The Laura/86ISMN 2137 DH population was phenotyped based on a HST sensitivity scale of 0 to 3, where 0 refers to no visible necrosis/ chlorosis and 3 refers to severe necrosis. RILs with a mean ranging from 0-0.5 were considered insensitive to the HST and RILs with a mean > 0.5 were considered sensitive. A χ^2 test was used to determine if the observed segregation ratio fit Mendelian segregation ratios. The χ^2 value was calculated as follows:

$$\chi 2 = \frac{\Sigma[(\text{observed number} - \text{expected number}) - 1/2]\text{^}2}{\text{expected number}}$$

Where, reduction of 1/2 from the absolute value of difference between observed and expected number is the Yates correction term (Strickberger 1985), which is used when only two phenotypic classes are observed.

The significance of calculated χ^2 value was calculated at 5% confidence limit and 1 degree of freedom (df).

Linkage and QTL mapping

Ninety-seven RILs of the Kenyon/86ISMN 2137 population were previously genotyped with a combination of simple sequence repeat (SSR), diversity array technology (DArT), and single nucleotide polymorphism (SNP) markers. DNA was extracted from freeze-dried seedling leaf tissue with the Qiagen Dneasy 96 Plant Kit (Qiagen, Mississauga, ON). Hoechst 33258 stain was used to quantify DNA by fluorimetry. DArT markers were assayed by Diversity Arrays Technology Pty. Ltd (Yarralumla, Australia). The SNP markers were genotyped using the Illumina Infinium 9K wheat SNP beadchip, as per manufacturer's instructions. Each SSR PCR reaction was 10 µl in volume and was composed of 25 ng of template DNA, 1x PCR buffer (Applied Biosystems,Foster City, CA), 1.5 mM $MgCl_2$, 0.5 U of Taq DNA polymerase (Gibco/BRL, Mississauga, ON), 200 µM each dNTP, 20 µM forward primer, 180 µM 6-FAM/HEX/NED-labelled M13 primer (5' – 3' CACGACGTTGTAAAACGAC; Applied Biosystems, Foster City, CA) and 200 µM reverse primer. SSR PCR reactions were performed in a 384 well PCR plates. A 5', 19 nucleotide M13 tail (5' - 3', CACGACGTTGTAAAACGAC) (Schuelke 2000) modification was added to all forward microsatellite primers. The denaturation of reaction mixture was performed at 94°C for 2 min, followed by 30 cycles of 95°C for 1 min, 49/58°C for 50 s, 73°C for 1 min, with a final extension at 73°C for 5 min. An ABI Prism 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA) was used to resolve Microsatellite PCR amplicons. To this genetic map, the *Snn3* and *Tsn1* loci were added with the toxin reaction data

from SnTox3 and SnToxA, respectively. The linkage map was developed with MapDisto version 1.7.5 (Lorieux, 2012).

One hundred and four DH lines of the Laura/86ISMN 2137 population were genotyped with simple sequence repeat (SSR) markers on chromosomes 2A and 5B (Somers et al. 2004). PCR and electrophoresis conditions were the same as described for the Kenyon/86ISMN 2137 RIL population. The linkage map was developed with MapDisto version 1.7.5 (Lorieux, 2012**).**

QTL analysis was conducted with QGene version 4.3.10 (Joehanes and Nelson 2008) using simple interval mapping. LOD significance thresholds were determined by permutation testing (10,000 permutations; Churchill and Doerge 1994).

3.4. Results

The parental lines were screened for sensitivity to known HSTs SnToxA, SnTox1 and SnTox3. Among 40 wheat lines (including parental lines) screened by infiltration of the HST(s), most wheat lines had sensitivity to either SnToxA or SnTox3 (Table 3.1). Some wheat lines were sensitive to both SnToxA and SnTox3. Glenlea and SYN HEX Elite #44 were sensitive to SnToxA and SnTox1. SYN HEX Elite #85 and SYN HEX Elite #25 were the only lines sensitive to SnTox3 and SnTox1. SC8021-V2 was the only wheat line sensitive to all three toxins, although the sensitivity due to SnTox3 and SnTox1 was evident as a faint chlorosis. Fourteen wheat lines were insensitive to the three known HSTs. *Triticum turgidum* PI352519 was one of the fourteen wheat lines insensitive to all three known HSTs. BG261 and M6 are differential lines for SnToxA and SnTox1 respectively which tested sensitive to their respective HST.

However, upon infiltration with SnTox3, BG220 did not exhibit a sensitive reaction despite of being a differential line for SnTox3.

Table 3.1. Sensitivity of wheat lines to SnToxA, SnTox3 and SnTox1

Wheat Lines	SnToxA	SnTox3	SnTox1
Kenyon	+++	+++	-
Superb	+++	+++	-
Genesis	+++	+++	-
HW04300	+++	++	-
Kane	+++	+	-
Coulter	+++	-	-
RL4452	+++	-	-
AC Domain	+++	-	-
BW278	+++	-	-
98W1147	+++	-	-
AC Karma	-	+++	-
Altar Synthetic	-	+++	-
AC Foremost	-	+++	-
Laura	-	+++	-
Nyubai	-	+++	-
Septoria Synthetics57	-	+++	-
CDC Alsask	-	++	-
IR05157	-	++	-
92MREHTR28B	-	++	-
SYN HEX Elite#9	-	-	+
Glenlea	+++	-	+
SYN HEX Elite#44	+++	-	+
SYN HEX Elite#1	*	-	+
SYN HEX Elite# 85	*	+++	+
SYN HEX Elite#25	-	+++	+
SC8021-V2	+++	+	+
86ISMN2137	-	-	-
RL5407	-	-	-
Wuhan-1	-	-	-
Erik	-	-	-
BW880	-	-	-
CNT2	-	-	-
SK0263	-	-	-
SK0505	-	-	-
SYN HEX Elite#67	-	-	-
SYN HEX Elite#89	-	-	-
T. turgidum (PI352519)	-	-	-
Thatcher	-	-	-
BR34	-	-	-
2000 Spelt#20	-	-	-
BG261(*SnToxA differential*)	+++	-	-
BG220(*SnTox3 differential*)	-	-	-
M6 (*SnTox1 differential*)	-	-	++

Note: +++ Indicates high necrosis rating of 3 *- Indicates necrosis and chlorosis rating of 0*
++ Indicates necrosis and or chlorosis rating of 2 ** Indicates missing data*
+ Indicates faint chlorosis rating of 1

Fig. 3.1. Kenyon/86ISMN 2137 5B map demonstrating the map location of *Tsn1* and *Snn3* on the long and short arm of chromosome 5B respectively.

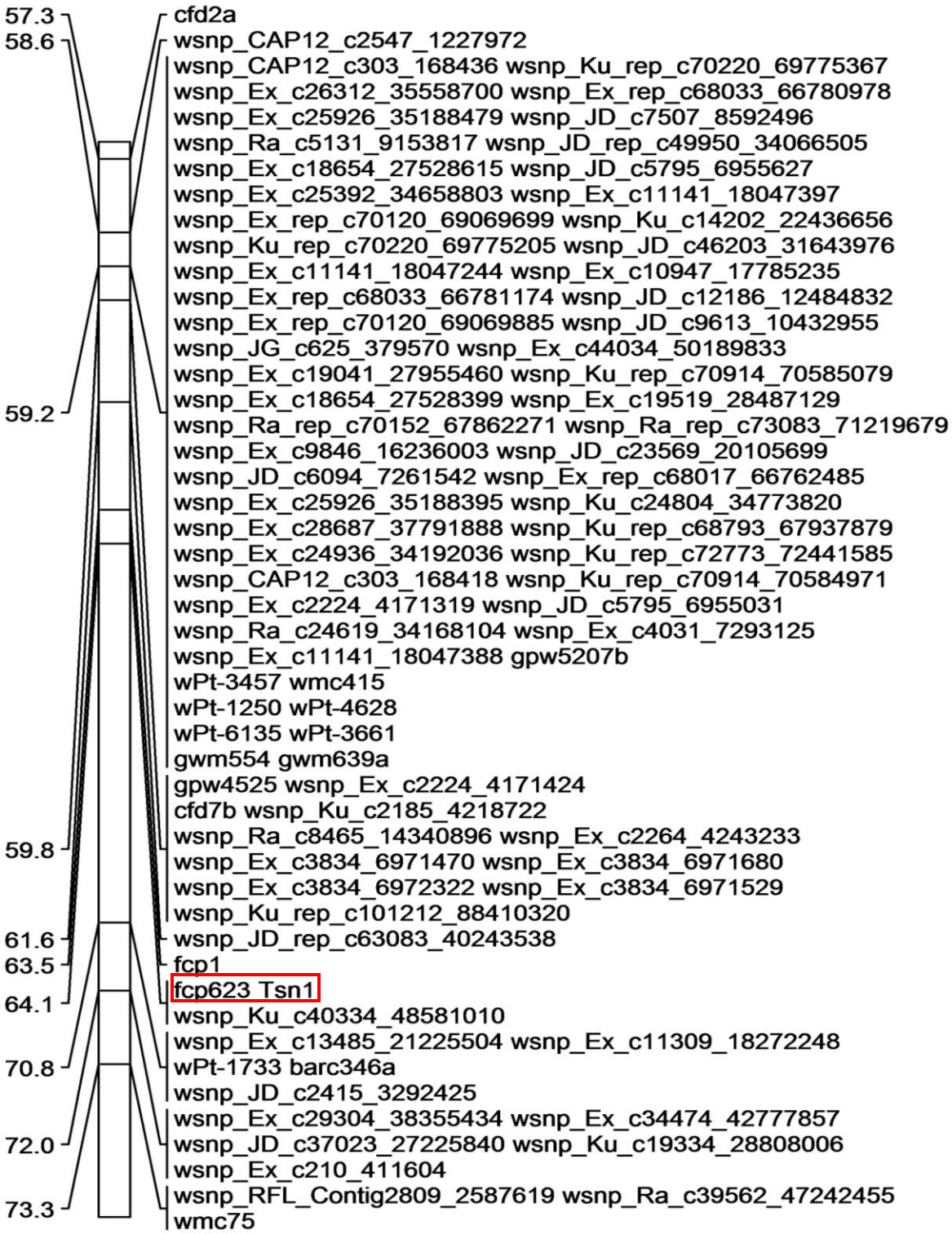

Fig. 3.2A. A segment of genetic map of 5BL chromosome of wheat showing *Tsn1* gene mapped in perfect alignment with gene based marker fcp623

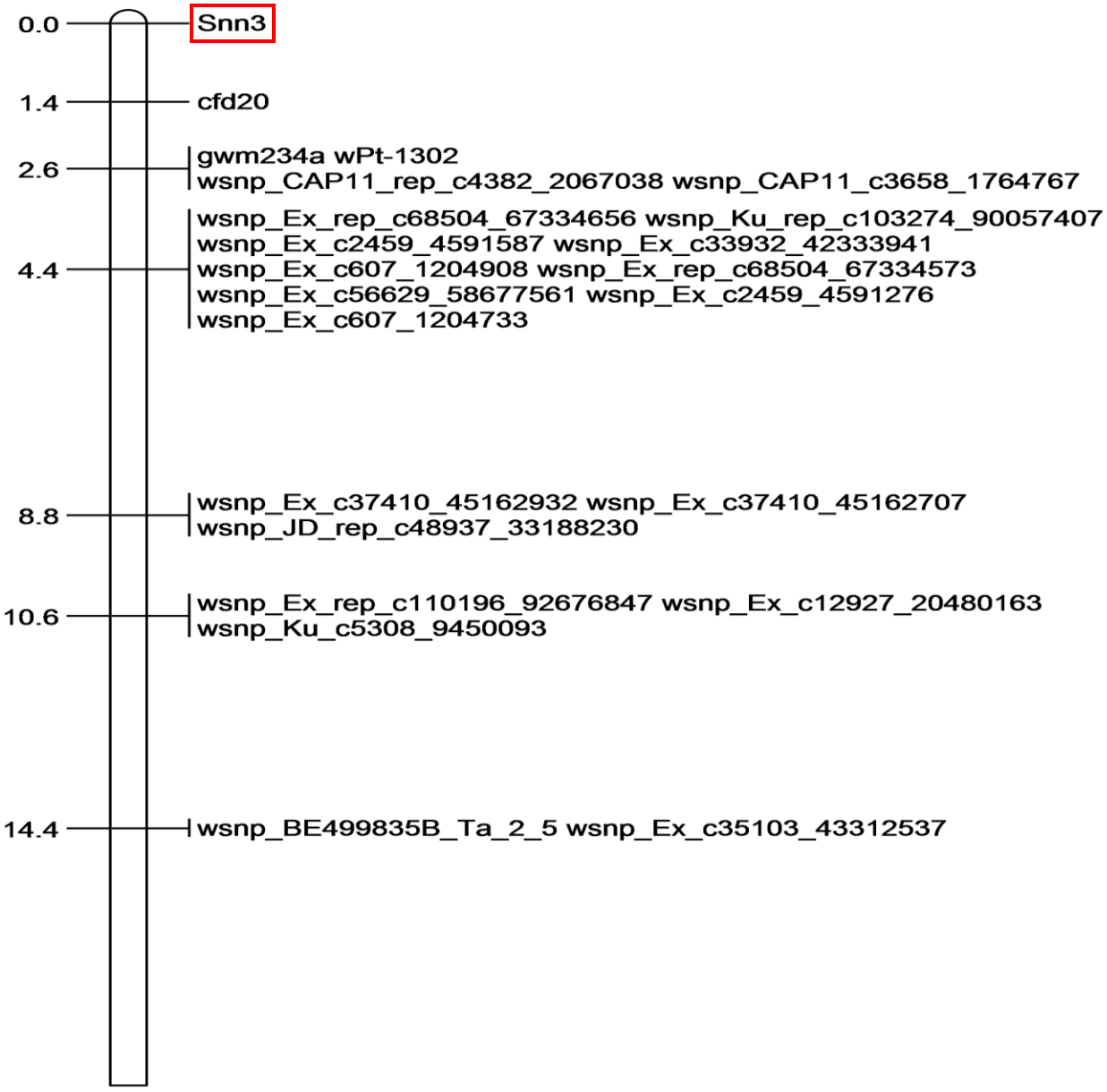

Fig. 3.2B. A segment of genetic map of 5BS chromosome of wheat showing *Snn3* gene mapped 1.4 cM distal to cfd20

The Kenyon/86ISMN 2137 RIL population was injected with the HSTs SnToxA and SnTox3 in order to map *Tsn1* and *Snn3*, respectively. The results of *Tsn1* and *Snn3* mapping confirmed the presence of sensitivity to both SnToxA and SnTox3 in Kenyon/86ISMN 2137 RIL

population (Fig. 3.1). The SnToxA sensitivity gene *Tsn1* mapped on chromosome 5BL in the same linkage bin as marker *fcp623*, which is based upon the gene sequence of *Tsn1* (Fig. 3.2A). Likewise, SnTox3 sensitivity gene *Snn3* mapped on the distal end of chromosome 5BS. *Snn3* was mapped 1.4cM distal of the microsatellite marker *cfd20* (Fig. 3.2B). The chromosomal loci identified for the sensitivity genes *Tsn1* and *Snn3* were consistent with the locations previously published by (Faris et al., 1996; Haen et al., 2004; Liu et al., 2006) and (Zhang et al 2011). The previously published locations of *Tsn1* and *Snn3* were based on culture filtrate infiltration and/or infiltration of purified toxins.

Toxin identification

Among 10 *S. nodorum* isolates tested for toxin production, only three isolates, 06SN002, Langham and Swift Current produced HSTs in Fries medium. According to the differential line bioassays for 06SN002 and Langham culture filtrates, the sensitivity gene for the HST(s) present in the culture filtrate was most likely *Tsn1* or a linked gene on chromosome 5B (Table 3.2). The HST(s) produced by isolates 06SN002 and Langham are further discussed in chapter 4. Bioassay of the differential set with Swift Current culture filtrate suggested the tentative chromosomal location of the sensitivity gene was 2A.2 or on chromosome 5B near *Tsn1* (Table 3.2). The tentative chromosomal location of the sensitivity gene(s) was devised on the basis of bioassay results from the differential lines #1 through #8 with one or more potential sensitivity loci (Table 3.3). A further confirmation of presence/absence of any previously identified HST(s) is done on the basis of differential lines #9 through #23 (Table 3.3). The infiltration of differential lines #1 through #8 indicated the sensitivity gene(s) compatible to the HST(s) being produced in Swift Current culture filtrate was potentially located at 2A.2 and/or *Tsn1*. At least one of the HSTs in

the Swift Current culture filtrate was believed to be interacting with *Tsn1* or a linked gene. However, it is possible that the sensitivity observed at *Tsn1* was due to SnToxA. Additionally, the sensitivity imparted by compatible reaction at the 2A.2 locus was thought to be due to one or more potentially novel HSTs.

Table 3.2. List of isolates used in HST production and tentative chromosomal location of sensitivity gene corresponding to the HST being produced

***S. nodorum* isolates**	**Toxin/HST production**	**Tentative chromosomal location of sensitivity gene**
06SN010	-	-
06SN002	+	5B
Langham	+	5B
Kyle	-	-
Kelvington	-	-
Rosetown#9	-	-
Swift Current	+	5B/ 2A.2
06SN007	-	-
06SN014	-	-
06SN008	-	-

+ indicates presence of HST production
- indicates absence of HST production

Table 3.3. Demonstration of sensitivity of differential lines to known toxins SnToxA and SnTox3, potential sensitivity loci and result of Swift Current culture filtrate infiltration in differential lines

SN	Wheat lines	SnToxA sensitivity	SnTox3 sensitivity	Postulated susceptibility /sensitivity QTL alleles	Swift Current culture filtrate reaction	Pooled fraction (#5- #9) infiltration reaction	Pooled fraction (#10 and #11) infiltration reaction
1	Line 28	Insensitive	Insensitive	2A.2 / 2D	**Sensitive**	Insensitive	Insensitive
2	Line 44	**Sensitive**	**Sensitive**	2D/ *Tsn1*	**Sensitive**	**Sensitive**	Insensitive
3	Line 50	Insensitive	**Sensitive**	6D	Insensitive	Insensitive	Insensitive
4	Line 52	**Sensitive**	Insensitive	2A.2 / *Tsn1*	**Sensitive**	**Sensitive**	Insensitive
5	Line 64	**Sensitive**	Insensitive	2A.1/ *Tsn1*	**Sensitive**	**Sensitive**	Insensitive
6	Line 83	Insensitive	Insensitive	*	**Sensitive**	Insensitive	Insensitive
7	Line 85	Insensitive	Insensitive	2D/ 6D	**Sensitive**	Insensitive	Insensitive
8	Line 88	Insensitive	**Sensitive**	2A.1/ 2D	Insensitive	Insensitive	Insensitive
9	BG261	**Sensitive**	*****	*	**Sensitive**	**Sensitive**	Insensitive
10	BG223	*	*	*	**Sensitive**	Insensitive	Insensitive
11	BG220	*****	**Sensitive**	*	**Sensitive**	**Sensitive**	*****
12	M6	*	*	*	Insensitive	Insensitive	*
13	Kenyon	**Sensitive**	**Sensitive**	*	**Sensitive**	**Sensitive**	Insensitive
14	86ISMN 2137	Insensitive	Insensitive	*	Insensitive	Insensitive	Insensitive
15	Laura	Insensitive	**Sensitive**	*	**Sensitive**	Insensitive	**Sensitive**
16	Superb	**Sensitive**	**Sensitive**	*	**Sensitive**	**Sensitive**	Insensitive
17	BW880	Insensitive	Insensitive	*	**Sensitive**	Insensitive	*****
18	CDC Alsasak	Insensitive	**Sensitive**	*	**Sensitive**	Insensitive	Insensitive
19	Grandin	*	*	*	**Sensitive**	**Sensitive**	*****
20	BR34	Insensitive	Insensitive	*	Insensitive	Insensitive	*
21	Glenlea	**Sensitive**	Insensitive	*	**Sensitive**	**Sensitive**	Insensitive
22	Amazon	Insensitive	*	*	**Sensitive**	Insensitive	Insensitive
23	Erik	Insensitive	Insensitive	*	**Sensitive**	Insensitive	Insensitive

** Indicates unknown sensitivity data*
"Sensitive" Indicates type 1, 2 or 3 ratings
"Insensitive" Indicates type 0 rating
Postulated sensitivity data was derived from McCartney et al. (unpublished data)

The sensitivity observed in the differential lines could not have been due to 2A.1 or 2D loci because Line 88 which harbors susceptibility alleles at both of these loci remained insensitive to the infiltrated culture filtrate. Likewise, locus 6D could not have contributed to the observed sensitivity because Line 50 which harbors locus 6D remained insensitive. Furthermore,

the sensitive reaction produced by wheat lines BG261, Superb and Glenlea suggested the presence of SnToxA in the Swift Current culture filtrate. The observed sensitivity in wheat lines BG220, Laura, Superb and CDC Alsask also suggested the presence of SnTox3. A faint chlorosis produced by wheat line BG223 after infiltration of Swift Current culture filtrate suggested a low concentration of SnTox2. Simultaneously, the sensitive reaction of Amazon and Erik indicated presence of HST other than SnToxA as both the lines are insensitive to SnToxA (Table 3.3).

Putative toxin identification

Cation exchange chromatography was able to partially purify the complex protein mixture in the Swift Current culture filtrate. The purified proteins resolved as two closely spaced peaks on the chromatogram. The first peak was observed at approximately 54 mM sodium chloride and a second peak was obvious at 81 mM sodium chloride (Fig. 3.3). Among all the fractions from the chromatography that were tested on Kenyon (susceptible) and 86ISMN 2137 (resistant) parents during a second bioassay, active fractions #5, #6, #7, #8, #9, #10, #11 and #12 were identified. The aliquots of fractions #5, #6, #7, #8 and #9 analyzed by Tris-tricine gel electrophoresis indicated a distinct band approximately 25 relative molecular weight (M_r) (Fig. 3.4A). Additionally, for fractions #10 and #11 a band near 25 M_r was visible along with other faint bands at a different size range (Fig. 3.4B). Although fraction #12 was identified as an active fraction, no protein band was visible on the Tris-tricine gel. Likewise, the non-active fractions #3, #4 and #13 also failed to produce any visible protein band(s) on Tris-tricine gel (Figs. 3.4A and 3.4B). The active fractions #5, #6, #7, #8 and #9 were pooled and simultaneously fractions #10 and #11 were pooled on the basis of similar protein profiles observed on the Tris-tricine gel electrophoresis. The infiltration of pooled fractions (#5 - #9) into the differential set of wheat

lines indicated similar results as culture filtrate infiltration into differential lines (Table 3.3) which also suggested the interacting HST to be SnToxA. However, the infiltration of pooled fractions containing fraction 10 and fraction 11 into the leaves produced anomalous results. Although one of the bands in these fractions corresponded to the size of ToxA, *Tsn1* positive lines were insensitive to the same fraction. In fact, "Laura" which is known to be insensitive to ToxA showed sensitivity to the HST present in pooled fractions 10 and 11. Therefore, this suggested that the band seen on the Tris-tricine gel that had a comparable size to ToxA was most likely an unrelated protein produced by isolate Swift Current. A second Tris-tricine gel electrophoresis of pooled sample containing fractions #5, #6, #7, #8 and #9 indicated the presence of a single likely candidate protein for HST(s) which was approximately 25 kDa (Fig. 3.5). However, a likely candidate HST could not be identified from another Tris-tricine gel electrophoresis of pooled sample containing fractions #10 and #11 (Fig. 3.5). The Mass spectrometry and database search for this 25 kDa protein identified sequence similarity with SNOG_15679 which is a hypothetical gene in *Phaeosphaeria nodorum*.

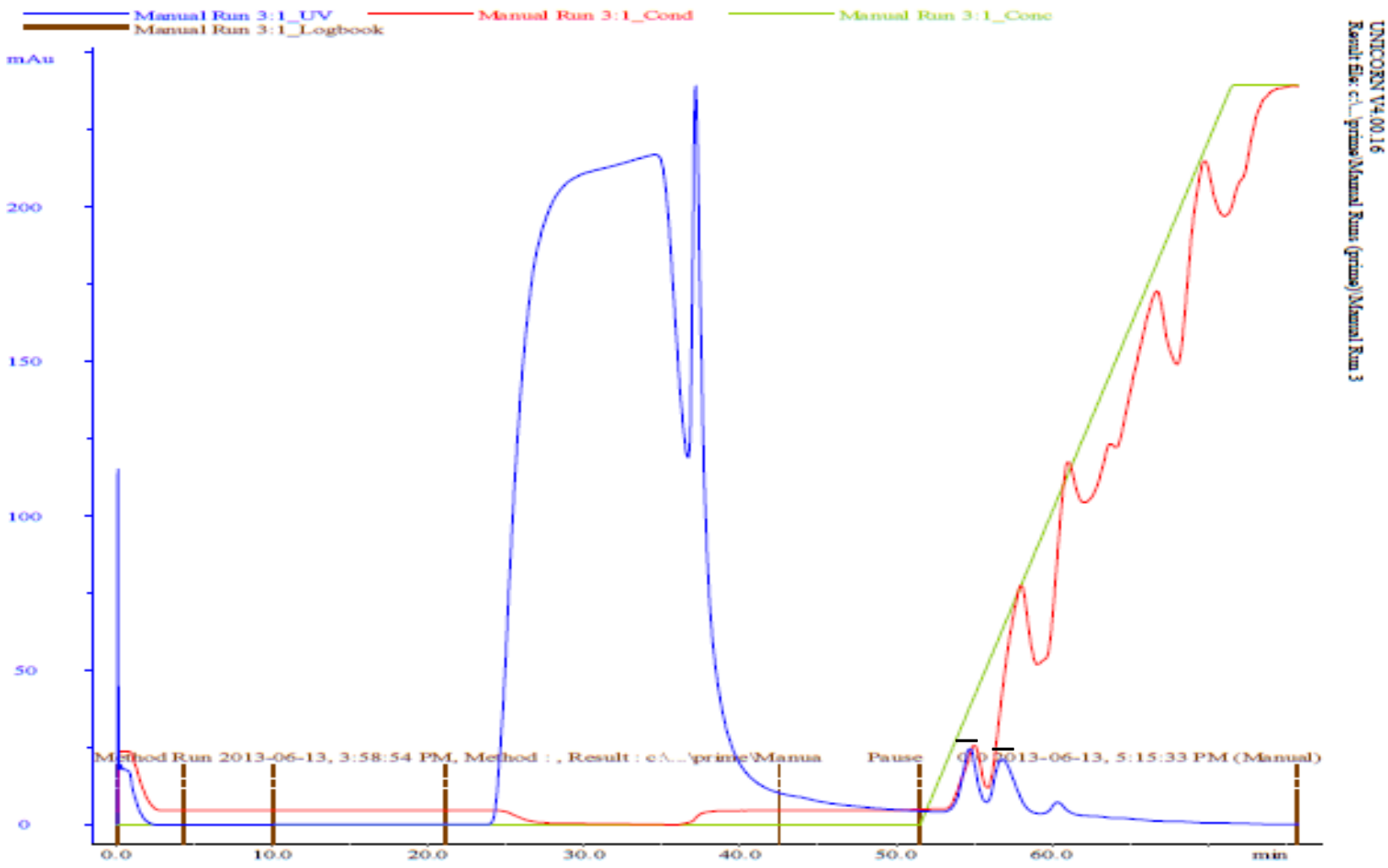

Fig. 3.3. A chromatogram produced after cation exchange chromatography of Swift Current culture filtrate showing the resolution of two distinct peaks corresponding to the active fractions identified in the bioassay. The resolved peaks are indicated in the chromatogram with a bar.

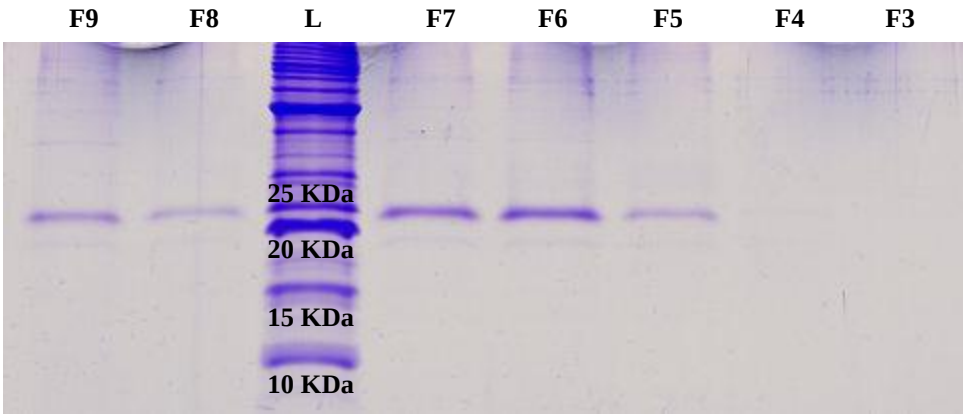

Fig. 3.4A. Tris-tricine gel analysis for fractions obtained from cation exchange chromatography of Swift Current culture filtrate. F3-F9, Fraction 3 to Fraction 9; L, Benchmark protein ladder (Invitrogen).

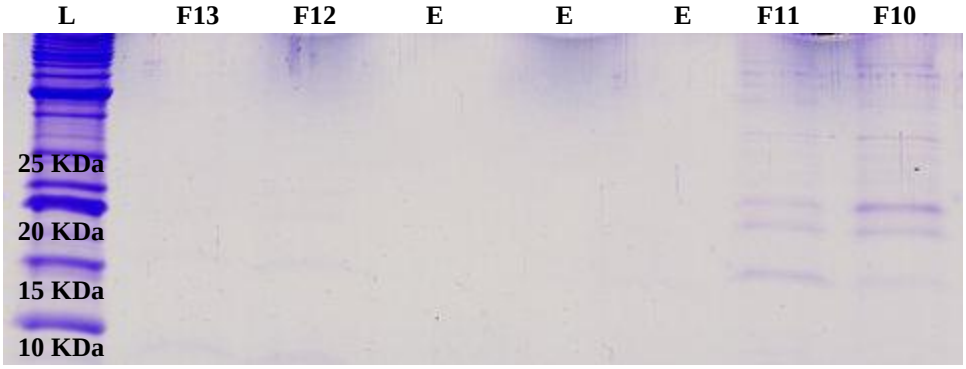

Fig. 3.4B. Tris-tricine gel analysis for fractions obtained from cation exchange chromatography of Swift Current culture filtrate. L, Benchmark protein ladder (Invitrogen); F10-F13, Fraction 10 - Fraction 13; E, Empty.

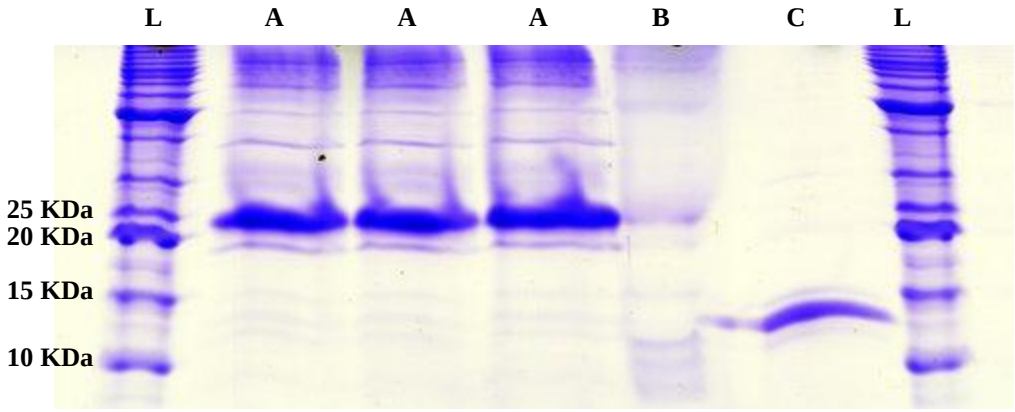

Fig. 3.5. Tris-tricine gel analysis for pooled fractions obtained from cation exchange chromatography of Swift Current culture filtrate. L, Benchmark protein ladder (Invitrogen); A, pooled fractions 5, 6, 7, 8, and 9; B, pooled fractions 10 and 11; C, PtrToxA.

Segregation of mapping population to Swift Current culture filtrate

The Kenyon/86ISMN 2137 RI population segregated in a 68:56 sensitive:insensitive ratio. The Kenyon/86ISMN 2137 RI population did not show any clear pattern of segregation in response to Swift Current culture filtrate infiltration (Fig. 3.6). The Laura/86ISMN 2137 DH population segregated in a 51:40 sensitive:insensitive ratio following infiltration of Swift Current culture filtrate (Fig. 3.7). The observed ratio of 51:40 fit the expected 1:1 single gene segregation ratio, which was supported by a non-significant χ^2 value (χ^2= 1.098, df=1).

Linkage mapping

The Kenyon/86ISMN 2137 linkage map was 2,655 cM in length and consisted of 25 linkage groups and 3,087 loci. Most of the wheat genome was covered by 22 linkage groups, with chromosome 1A consisting of two linkage groups. The three remaining linkage groups were small (7, 13, and 15 cM) and were not assigned to chromosomes.

The Laura/86ISMN 2137 linkage map consisted of three linkage groups, 2A, 5B, and 6D. The 2A map was 28.1 cM in length and consisted of 18 SSR loci. The 2A map encompassed a region affecting seedling SNB resistance in the Kenyon/86ISMN 2137 population (McCartney, unpublished data). The 5B linkage map was 98.8 cM and consisted of 16 SSR loci. The 5B map encompassed *Snn3* on the distal end of the short arm, and past *Tsn1* on the long arm. The 6D map was 70 cM in length and spanned the region of a non-significant putative QTL for reaction to the Swift Current culture filtrate in the Kenyon/86ISMN 2137 RI population.

QTL mapping of sensitivity to Swift Current culture filtrate

Simple interval mapping identified two QTL controlling reaction to the Swift Current culture filtrate in the Kenyon / 86ISMN 2137 RIL population (Table 3.4). Both QTL were located on chromosome 5B at the locations of *Snn3* and *Tsn1* (Fig. 3.8). The QTL mapping to the *Tsn1* locus explained more phenotypic variation than the QTL mapping to the *Snn3* locus, although their additive effects were of similar magnitude. In both cases, Kenyon alleles conferred sensitivity to the Swift Current culture filtrate.

For the Laura/86ISMN 2137 DH population, one QTL was detected for reaction to the Swift Current culture filtrate (Table 3.4). This QTL was located on the short arm of chromosome 5B (Fig. 3.9). The linkage map for chromosome 5B did not extend to the end of the short arm, where *Snn3* is located. Unfortunately, no polymorphic SSR markers could be identified for that region of the chromosome. The detected QTL reached its peak 2.1 cM from the end of the distal end of chromosome 5BS. Laura contributed sensitivity to the Swift Current culture filtrate at this QTL. No other QTL were detected on chromosomes 2A, 5B, and 6D.

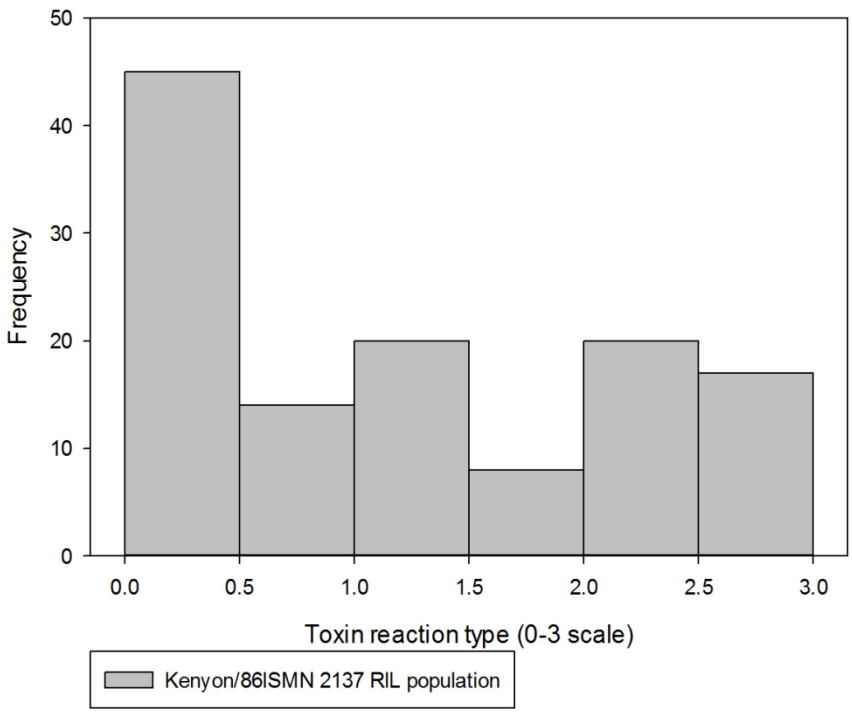

Fig. 3.6. A histogram showing frequency of sensitive and insensitive RI lines in response to the infiltration of Swift Current culture filtrate in Kenyon/86ISMN 2137 RI population. The sensitive parent (Kenyon) and insensitive parent (86ISMN 2137) showed type 3 and type 0 toxin reaction respectively.

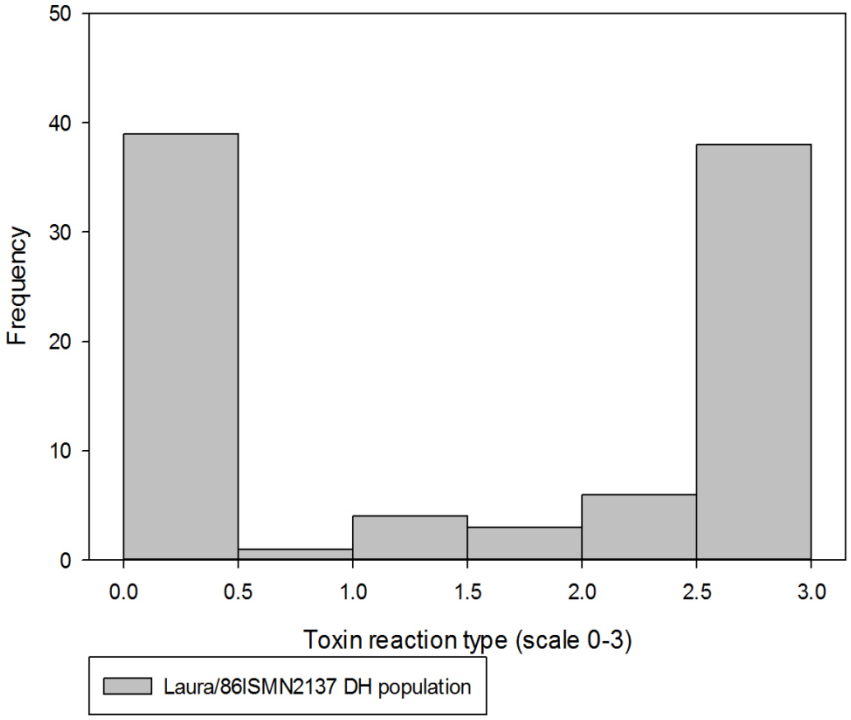

Fig. 3.7. A histogram showing frequency of sensitive and insensitive DH lines in response to the infiltration of Swift Current culture filtrate in Laura/86ISMN 2137 DH population. The sensitive parent (Laura) and insensitive parent (86ISMN 2137) showed type 3 and type 0 toxin reaction respectively.

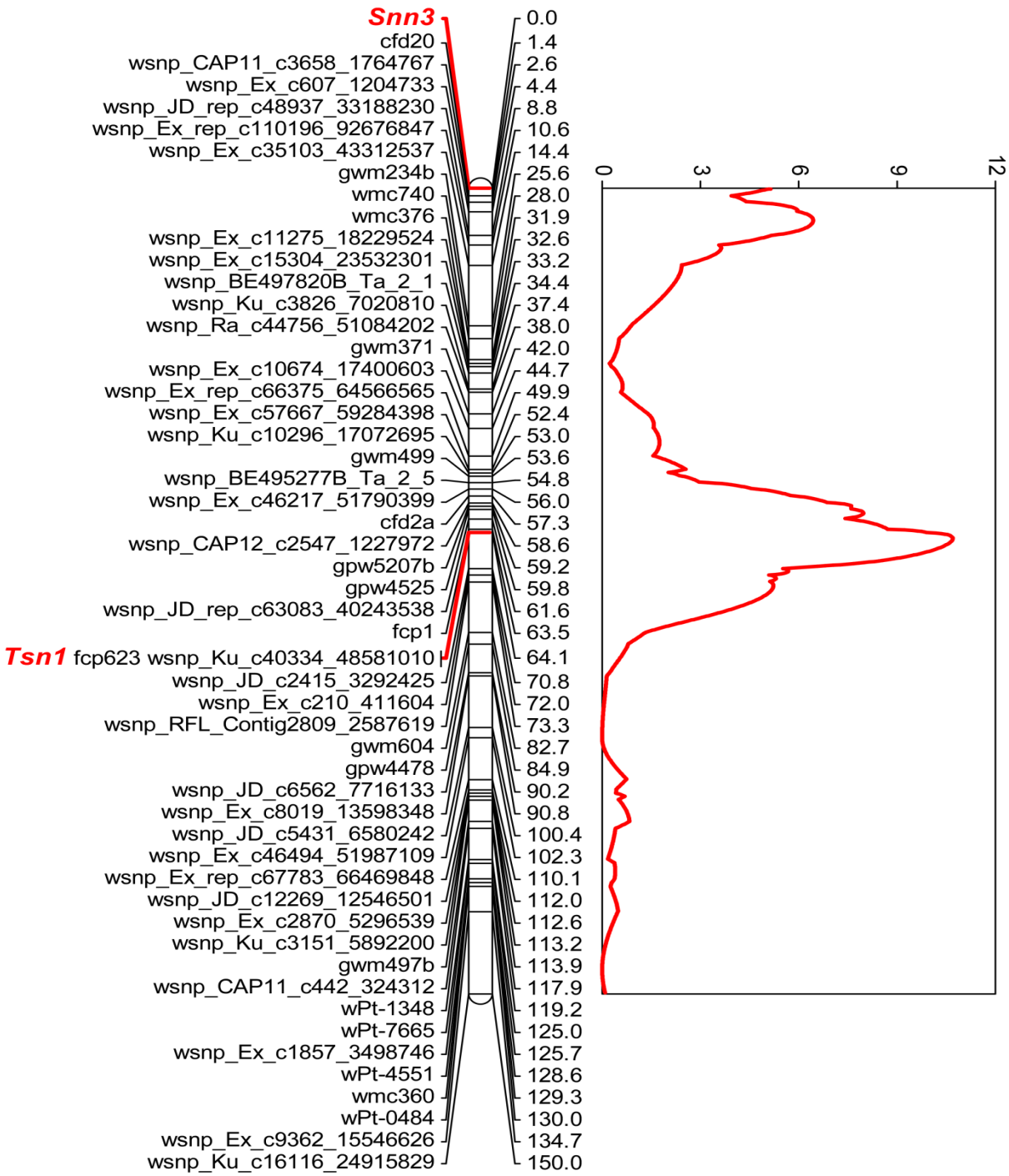

Fig. 3.8. QTL scan showing two QTL corresponding to *Snn3* and *Tsn1* loci resulting from infiltration of Swift Current culture filtrate in Kenyon/86ISMN 2137 RIL population

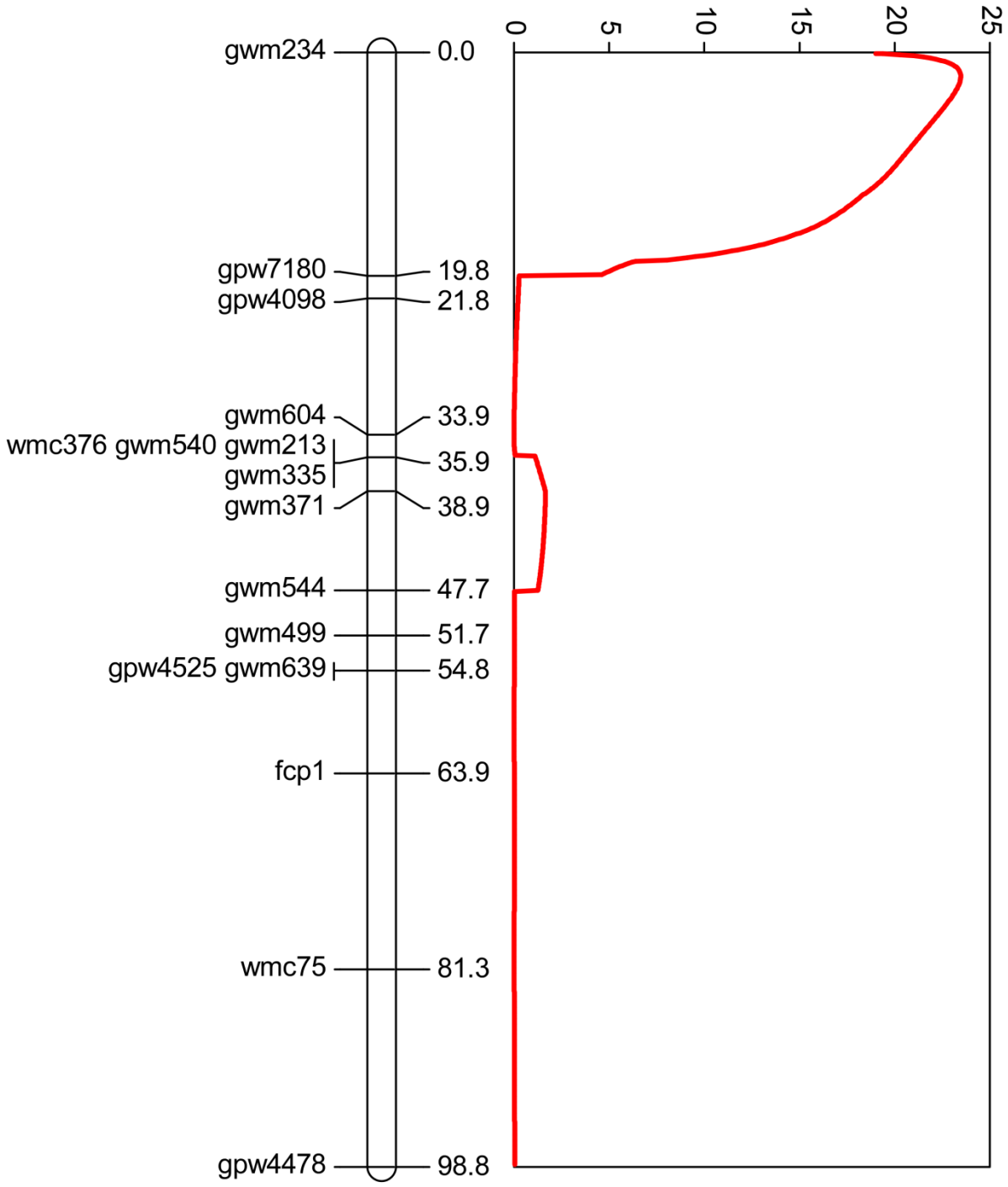

Fig. 3.9. QTL scan showing a partial QTL corresponding to *Snn3* locus resulting from infiltration of Swift Current culture filtrate in Laura/86ISMN 2137 DH population

Table 3.4. Statistics for QTL derived from infiltration of Kenyon/86ISMN 2137 and Laura/86ISMN 2137 mapping populations with Swift Current culture filtrate

Population	Chromosome	Map Position (cM)	LOD	Additive Effect[a]	R^2 (%)	LOD Threshold[b]
Kenyon / 86ISMN 2137	5B	6.0	6.44	0.57	26.3	3.59
Kenyon / 86ISMN 2137	5B	65.2	10.70	0.71	39.8	3.59
Laura / 86ISMN 2137	5B	2.1	23.47	1.06	64.6	1.90

[a]Relative effect of Kenyon or Laura alleles on the trait mean

[b]10,000 permutations

3.5. Discussion

Before the first host specific toxin (HST) in *S. nodorum* was reported in 2004 by Liu et al., *S. nodorum* was thought to rely solely on non-specific toxins like mellein and septorin for disease development in wheat (Keller et al. 1994; Wicki et al. 1999; Liu, Friesen, et al. 2004). The discovery of the first HST in *S. nodorum* opened a new avenue for studying host-pathogen interactions in this pathosystem and breeding for SNB resistance. It also simultaneously triggered the exploration for other HSTs (Friesen, Faris, et al. 2008; Oliver et al. 2012). Although the role of HSTs in disease development is indisputable, the truth remains that the actual mode of action of individual HST and the interaction among different HSTs is unclear. The involvement of HST in disarming or destroying the plant defense mechanism is speculated but supported by little or no evidence. Recently, Du Fall and Solomon (2013) demonstrated that SnToxA escalates production of serotonin and other metabolites of the tryptophan metabolic pathway when infiltrated into wheat cultivars harboring the *Tsn1* locus. The same study also established serotonin as a phytoalexin associated with interference of spore production and maturation in *S.*

nodorum (Du Fall & Solomon 2013). Additionally, Du Fall & Solomon (2013) found that production of serotonin is decreased during inoculation of wheat with *S. nodorum* spores, suggesting that *S. nodorum* has to overcome the host defence mechanism before establishing itself and causing susceptibility in wheat. Although Du Fall & Solomon (2013) successfully established and emphasized the necessity to overcome plant defences by necrotrophs, the mechanisms involved could not be explained. In absence of convincing evidence, it can only be suspected that the wheat-*S. nodorum* pathosystem is possibly a sophisticated pathosystem capable of manufacturing the HSTs that counter act one another or work in conjunction with one another eventually overcoming the host defense mechanism. In order to understand the complicated underlying mechanism of *S. nodorum*-wheat pathosystem, unlocking all or most of HST(s) produced by *S. nodorum* is crucial.

In this study we attempted to identify and purify novel HST(s). The initial screening of parental Canadian wheat lines was intended to generate background information about toxin sensitivity to known HSTs which would potentially allow isolation of the activity of any new toxin(s). This screening also aimed at identification of additional lines which could be used in development of additional RIL or DH populations segregating for sensitivity to one or more known HST(s). One peculiar observation during screening of wheat lines was the insensitivity or low sensitivity of Line BG220 to SnTox3, which is the differential line for SnTox3. This could be explained as a mere concentration issue as this line was previously demonstrated to be sensitive to SnTox3 (Friesen, Zhang, et al. 2008). The Kenyon/86ISMN 2137 mapping population was not thought to be sensitive to SnTox3 since no SNB resistance QTL were identified in the population when it was individually inoculated with conidia of seven *S. nodorum* isolates in seedling tests. Interestingly, the infiltration of SnTox3 in parental lines and

Kenyon/86ISMN 2137 RIL population revealed the presence of SnTox3 sensitivity in Canadian wheat varieties and breeding lines. While the failure of the conidial inoculation study to identify SnTox3 sensitivity is surprising, it could be partly explained as simply the effect of *S. nodorum* isolates that did not produce SnTox3 under the specific conditions of that study. Additionally, the absence of significant *SnTox3-Snn3* interaction could also be due to the epistatic effect of other toxin interactions on the *SnTox3-Snn3* interaction. This is supported by previously published data by Friesen et al., (2008) which showed that SnTox2-*Snn2* interaction was epistatic on SnTox3-*Snn3* interaction and SnTox3-*Snn3* interaction doesn't seem to contribute to a significant interaction in presence of SnToxA-*Tsn1* interaction. Therefore, the study of the effect of an individual toxin is facilitated by its purification in a pure or partially pure state. In contrary, the true contribution of a particular HST for susceptibility to SNB can only be determined when the effect of HST under question is being studied in conjunction with the effect of other known HSTs.

During the early attempts to identify the candidate protein for HST, the differential set and Kenyon/86ISMN 2137 RI lines infiltration with culture filtrate of the *S. nodorum* isolate (Swift Current) indicated presence of sensitivity at *Tsn1*. Therefore, presence of some SnToxA in the culture filtrate was suspected. Even after purification by fast protein liquid chromatography (FPLC), the differential line infiltration of fractions indicated presence of SnToxA. However, the Tris-tricine gel analysis of the same set of fractions after pooling failed to produce any protein band corresponding to SnToxA on the gel. However, the absence of visible band of SnToxA on Tris-tricine gel did not eliminate the possibility of presence of SnToxA in the liquid media cultures of *S. nodorum*. In fact, the absence of a visible SnToxA band on the Tris-tricine gel may be the result of a low concentration of SnToxA that is still biologically active. Friesen et al.

(2006) showed that PtrToxA and SnToxA are the gene products of ToxA gene in *Pyrenophora tritici-repentis* and *S. nodorum*, respectively, which interacts with the same sensitivity gene (*Tsn1*) in wheat. Also, 2µg or higher amount of PtrToxA has been previously demonstrated on the SDS PAGE (Sodium dodecyl sulfate polyacrylamide gel electrophoresis) gel and native gels (Ballance et al., 1989; Tomas, 1990; Tuori, 1995; Zhang, Francl, Jordahl, and Meinhardt, 1997). Therefore, insensitivity of Tris-tricine gels to the low concentration of protein seems to be less plausible explanation for the absence of SnToxA in Tris-tricine gel analysis in this study. Moreover, this suggests that the HST sensitivity at *Tsn1* locus observed in this study is either due to a novel toxin interacting with *Tsn1* or a tightly linked gene.

Although the chromosome 5BS linkage group was incomplete, *Snn3* is likely responsible for the QTL for reaction to Swift Current culture filtrate that was detected on the distal end of chromosome 5BS in the Laura/86ISMN 2137 DH population. The Swift Current culture filtrate contained SnTox3 based on the results in the Kenyon/86ISMN 2137 mapping population, and Laura and 86ISMN 2137 are known to be sensitive and insensitive to SnTox3, respectively. Therefore, the QTL observed on chromosome 5BS in Laura/86ISMN 2137 DH population is likely due to the segregation of *Snn3*. It still may be possible to develop a linkage map of the *Snn3* region with the use of single nucleotide polymorphism (SNP) markers. An improved linkage map would more accurately determine the location of the Swift Current culture filtrate QTL in the Laura/86ISMN 2137 population.

In the light of the observations made in this study, it is evident that in-vitro production of HSTs results in complex mixtures of proteins which require meticulous purification and separation in order to facilitate their study in isolation. This study used protein purification methods and genetics approach to identify new HSTs. A variety of HSTs were successfully

produced in vitro, however, the activity of two of the purified HSTs resembled previously identified HSTs SnToxA and SnTox3. The corresponding sensitivity genes of these purified toxins were successfully mapped on to *Tsn1* and *Snn3* loci. Although, the presence of SnTox3 in the Swift Current culture filtrate wasn't initially considered, it was soon apparent that one of the HSTs interacting with the wheat varieties “Kenyon” and "Laura" was in fact SnTox3. The presence of HSTs other than the already known ones could not be verified based solely on the currently available plant populations. Therefore, additional growth conditions for production of additional HSTs in liquid medium could be explored. Mapping populations segregating for different HST sensitivities could be developed in order to ensure a successful identification and purification of a novel HST(s). Ideally, these populations would be insensitive to known HSTs.

4. IDENTIFICATION OF A PUTATIVE *STAGONOSPORA NODORUM* TOXIN AND HETEROLOGOUS EXPRESSION IN *PICHIA PASTORIS*.

4.1. Abstract

Stagonospora nodorum is an important member of the wheat leaf spot complex. The diseases caused by *S nodorum* are referred to as Stagonospora nodorum leaf blotch (SNB) and Stagonospora nodorum glume blotch (SGB). *S. nodorum* has multiple hosts which include wheat, barley and wild grass species. *S. nodorum* is prevalent in regions of Australia, the United States of America, Canada, Asia, Europe and the Middle East. A multitude of proteinaceous host selective toxins (HSTs) is produced by *S. nodorum*. The HST-sensitivity gene interactions follow inverse gene-for-gene theory. The recognition of a HST by its corresponding sensitivity gene in wheat causes a susceptible reaction in wheat. A total of six different HST-host sensitivity gene interactions have been characterized. SnToxA-*Tsn1*, SnTox1-*Snn1*, SnTox2-*Snn2*, SnTox3-*Snn3*, SnTox4-*Snn4* and SnTox5-*Snn5*. In this study, novel HST-host sensitivity gene interactions were investigated and a putative HST-producing gene in *S. nodorum* was cloned and heterologously expressed. The novel HST was purified and identified using cation exchange chromatography and mass spectrometry respectively. On the basis of differential line reactions, the purified fractions from cation exchange chromatography consisted of HST(s) interacting with the *Tsn1* locus. Therefore, the purified HST in the fractions was first believed to be SnToxA. However, no detectable amount of SnToxA was observed on the Tris-tricine gel electrophoresis. The mass spectrometry analysis of the candidate protein (P-NEC) for novel HST revealed partial sequence match of the P-NEC with SNOT_15679 (translated sequence of SNOG_15679). The complete nucleotide sequence of SNOG_15679 was obtained from the Broad Institute *S. nodorum*

database, which was used to design primers and amplify the same gene in the isolates used in this study. SNOG_15679 was successfully amplified from isolate Langham with 100% nucleotide sequence similarity. The amplicon was cloned and heterologously expressed in *Pichia pastoris*. The heterologously expressed product of SNOG_15679 produced mild chlorosis on Kenyon (susceptible parent) after seven days post-infiltration and the 86ISMN 2137 (resistant parent) remained unaffected. The failure of heterologously expressed product of SNOG_15679 to produce stronger bioactivity was attributed to the concentration of the expressed protein. Additionally, the use of stronger promoter systems for heterologous expression and use of protein tags in order to purify the expressed protein could potentially improve the final protein concentration. In conclusion, isolate Langham likely produces a novel necrosis toxin that interacts with *Tsn1* or a tightly linked gene.

4.2. Introduction

Stagonospora nodorum causes Stagonospora nodorum blotch disease in wheat. *S. nodorum* infected wheat develop characteristic necrotic lesions which progressively coalesce into a large mass of necrotized tissue in most severe cases. Although, *S. nodorum* and other pathogens (biotrophic and necrotrophic) have existed for a long time, the underlying mechanisms of pathogenesis in these pathogens were not understood. The major breakthrough in the study of host-pathogen interaction was achieved when the Gene-for-Gene theory was proposed by Flor in 1956. The gene-for-gene theory states that for every dominant virulent gene in the pathogen, there is a corresponding dominant avirulence gene in the host which leads to resistant reaction. This theory successfully explained the host-pathogen interaction in the case of "biotrophs", where the pathogen is strictly interested in keeping the host alive. Unlike biotrophs, necrotrophs including *S. nodorum* readily infect and devour the host tissue while causing necrotic lesions on the host. Thus the classical gene-for-gene model was unable to explain the mechanism of infection by necrotrophs.

The involvement of effectors or host specific toxins (HSTs) was suspected in the case of *Pyrenophora tritici-repentis* (etiological agent of tan spot of wheat) based upon wheat cultivar-specific toxic components that reproduced the tan spot symptoms in wheat (Lamari and Bernier, 1989; Tomas and Bockus, 1987). PtrToxA was the first host selective toxin (HST) to be purified from *Pyrenophora tritici-repentis* (Ptr), which confirmed the involvement of HST in the pathogenicity of necrotrophs (Ballance et al. 1989; Tomas et al. 1990; Tuori & Ciuffetti 1995; Zhang et al. 1997). The underlying mechanism in Ptr-wheat interaction seemed to follow the gene-for-gene theory but with an opposite phenotypic manifestation. Therefore, when PtrToxA was recognized by a specific gene in a sensitive wheat cultivar, the wheat cultivar essentially

became susceptible to the tan spot, thereby confirming the inverse gene-for-gene relationship (Wolpert et al. 2002; Lamari et al. 2003).

HSTs were later studied in *S. nodorum* and SnTox1 was the first HST to be purified in *S. nodorum* (Liu, Faris, et al. 2004). Likewise, SnToxA was identified in *S. nodorum* and was found to be identical with PtrToxA (Friesen et al. 2006). Liu et al. (2006) demonstrated the interaction of the *Tsn1* gene with both PtrToxA and SnToxA. Also, evidence suggested that SnToxA was laterally transferred from *S. nodorum* to *P. tritici-repentis* (Friesen et al. 2006). Therefore, a general term ToxA is used to address PtrToxA from *P. tritici-repentis* and SnToxA from *S. nodorum*. To date, many other *S. nodorum* HSTs have been purified, which includes SnTox2 (Friesen, Meinhardt, and Faris, 2007), SnTox3 (Friesen, Zhang, et al. 2008; Liu et al. 2009), SnTox4 (Abeysekara et al. 2009) and SnTox5 (Friesen et al. 2012). These are also known to follow an inverse gene-for-gene relationship with their corresponding sensitivity genes in the host.

The purification of HSTs along with identification of its corresponding sensitivity gene in the host plant (wheat) is an important step in understanding resistance to Stagonospora nodorum blotch (SNB). Once insensitivity genes against *S. nodorum* HSTs are identified, resistance against SNB can be achieved by introgressing these genes into elite wheat breeding lines in order to develop a resistant cultivar. Additionally, wheat cultivars and breeding lines can be screened for identified HST sensitivity loci in order to improve resistance.

The specific objectives of this study were 1) identification and purification of a putative HST, and 2) production of the putative HST with the purpose of identifying the corresponding sensitivity locus

4.3. Materials and methods

Plant material

Kenyon (Neepawa*5/Buck Manantial) is a wheat cultivar highly susceptible to SNB, whereas 86ISMN 2137 (Unknown Pedigree) is an inbred wheat line highly resistant to SNB. A F_9-derived recombinant inbred line (RI) population was developed from the cross Kenyon/86ISMN 2137 using the single-seed descent method. This RI population was used in linkage and QTL mapping. The parents Kenyon (SNB susceptible) and 86ISMN 2137 (SNB resistant), along with a subset of the Kenyon/86ISMN 2137 RIL population and the differential lines were used in the bioassays to test culture filtrate and purified HST(s) (Table 4.1). Two seeds of each line were planted in a cone-tainer cell (Ray Leach cone-tainers, Tangent, OR). A total of two cone-tainer cells were used for planting four seeds from each line using the potting mix Sunshine Mix #5 (Sun Gro Horticulture Canada Ltd.).

Bioassay

The bioassays were conducted in order to test culture filtrates, purified HST(s) and heterologously expressed protein. During a bioassay, the second leaf of a wheat seedling was placed on the index finger and infiltrated with the help of a needle-less syringe. The infiltrated seedlings from the bioassays were incubated for three days in a growth cabinet maintained at 21/19°C (day/night) with 16 hour photoperiod. After three days, the infiltrated plants were rated using a scale of 0 to 3 where 0 indicates insensitive reaction, 1 indicates faint chlorosis, 2 indicates necrosis and/or chlorosis and 3 indicates severe necrosis. The rating scale of 0 to 3 was developed in Dr. Tim Friesen's lab (personal communication).

Toxin production

Isolates of *S. nodorum* were cultured on 2X V8 agar plates (314ml V8 juice, 3 g $CaCO_3$, 15 g Agar in 686ml distilled water). Each isolate was used to inoculate 125ml of Fries medium in a Corning 175 cm^2 angled neck flask (Corning Incorporated, Corning, NY). Fries medium was composed of 5 g $(NH_4)_2C_4H_4O_6$, 1 g NH_4NO_3, 0.5 g $MgSO_4.7H_2O$, 1.3 g KH_2PO_4, 2.6 g K_2HPO_4, 30 g sucrose, 1 g yeast extract and 2 ml trace elements (167 mg LiCl, 107 mg CuCl-H_2O, 34 mg H_2MoO_4, 72 mg $MnCl_2$-$4H_2O$, 80 mg $CoCl_2$-$4H_2O$ in 1000ml water) in 1000 ml distilled water. These inoculated flasks were incubated in the dark at room temperature with shaking at 80 rpm for three days. The incubation was continued in the dark at 21°C without shaking for an additional six days. A bioassay was performed nine days post Fries media inoculation to test the culture for HST production. A small aliquot of nine day old culture was filtered through two layers of Miracloth (Merck KGaA Darmstadt, Germany) and used for bioassay.

Protein purification

The Fries medium cultures of *S. nodorum* that tested positive in the bioassay were then purified using cation exchange chromatography. The culture was first filtered through two layers of miracloth and then through 0.45 µm Millipore Durapore membrane (EMD Millipore, Billerica, MA). After multiple filtrations, the filtrate was dialyzed overnight against approximately 3 liters of cold Mili-Q purified water. Forty ml of this dialyzed filtrate was then purified using 5 ml SP XL Sepharose column using ÄKTA Prime Fast Protein Liquid Chromatography (FPLC) unit (AktaPrime: GE Healthcare, UK) and 40 fractions were collected. The fractions were eluted using a gradual increase in sodium chloride concentration from 0 to

300 mM. Another bioassay was performed to test the bioactivity of putative HST(s) purified by liquid chromatography. The fractions that tested positive in this bioassay along with PtrToxA (control) were then analyzed using Tris-tricine gel electrophoresis.

Polyacrylamide gels were casted by pouring 12 % separating and 3 % stacking gel into the Mini-PROTEAN Tetra Cell system (Bio-Rad Hercules, CA). Running buffer for the electrophoresis consisted of 1X Cathode buffer (From 10X stock: 1 M Tris pH 8.25, 1 M Tricine and 1 % w/v SDS) and 1X Anode buffer (From 10X stock: 2 M Tris pH 8.9). The 15 µl of each sample was mixed with 6 µl of 6X sample buffer (1 M Tris pH 6.8, 10.28% (w/v) SDS, 9.3% (w/v) Dithiothreitol (DTT), 36 % (v/v) glycerol and 0.012 % (w/v) bromophenol blue) and were incubated in a boiling water bath for exactly 5 min. The samples were then loaded onto the casted polyacrylamide gels and electrophoresed for three hours using constant voltage of 80 V. The candidate protein bands that separated on the Tris-tricine gel along with PtrToxA band were alkylated and digested with trypsin using the method described by Rampitsch and Bykova (2009). The digested protein was then identified by mass spectrometry. The MS/MS spectra were then queried to database search against *Stagonospora nodorum* database [*Stagonospora nodorum* sequencing project, Broad Institute of Harvard and MIT (http://www.broadinstitute.org/) (12,379 sequences)] using Mascot (v2.4).

Table 4.1. List of wheat lines with differential activity to a known HST or postulated sensitivity loci

Wheat lines	Type of wheat line	Differential activity/Postulated sensitivity loci
Kenyon	susceptible parent	*
86ISMN 2137	resistant parent	*
Line 28	subset (Kenyon/86ISMN 2137 RIL)	2A.2 / 2D (postulated)
Line 44	subset (Kenyon/86ISMN 2137 RIL)	2D/ 5B (postulated)
Line 50	subset (Kenyon/86ISMN 2137 RIL)	6D (postulated)
Line 52	subset (Kenyon/86ISMN 2137 RIL)	2A.2 / 5B (postulated)
Line 64	subset (Kenyon/86ISMN 2137 RIL)	2A.1/ 5B (postulated)
Line 83	subset (Kenyon/86ISMN 2137 RIL)	*
Line 85	subset (Kenyon/86ISMN 2137 RIL)	2D/ 6D (postulated)
Line 88	subset (Kenyon/86ISMN 2137 RIL)	2A.1/ 2D (postulated)
BG261	Differential	ToxA sensitive
BG220	Differential	SnTox3 sensitive
BG 223	Differential	SnTox2 sensitive
M6	Differential	SnTox1 sensitive
Laura	*	ToxA insensitive
Superb	*	ToxA sensitive
CDC Alsask	*	*
BW 880	*	*
Erik	*	ToxA insensitive
Amazon	*	ToxA insensitive
Glenlea	*	ToxA sensitive

Note: Wheat lines in Table 4.1 were used in bioassay of culture filtrate and purified HSTs

Statistical analysis

Least squares means were calculated for the toxin infiltration data (0-3 scale) with JMP Genomics 6.0 (SAS Institute Inc.) using a mixed model where wheat lines were considered fixed effects and replicate was considered a random effect.

The χ^2 analysis was conducted for the phenotypic data obtained from the Langham culture filtrate infiltration in the Kenyon/86ISMN 2137 RI population. The Kenyon/86ISMN 2137 RI population was phenotyped based on a HST sensitivity scale of 0 to 3, where 0 refers to no visible necrosis/ chlorosis and 3 refers to severe necrosis. RILs with a mean ranging from 0-

0.5 were considered insensitive to the HST and RILs with a mean > 0.5 was considered sensitive. A χ^2 test was used to determine if the observed segregation ratio fit tested Mendelian segregation ratios. The χ^2 value was calculated as follows:

$$\chi 2 = \frac{\Sigma[(\text{observed number} - \text{expected number}) - 1/2]\text{^}2}{\text{expected number}}$$

Where, reduction of 1/2 from the absolute value of difference between observed and expected number is the Yates correction term (Strickberger 1985), which is used when only two phenotypic classes are observed.

The significance of calculated χ^2 value was calculated at 5% confidence limit and 1 degree of freedom (df).

Linkage and QTL mapping

Ninety-seven RILs of the Kenyon/86ISMN 2137 population were previously genotyped with a combination of simple sequence repeat (SSR), diversity array technology (DArT), and single nucleotide polymorphism (SNP) markers. DNA was extracted from freeze-dried seedling leaf tissue with the Qiagen DNeasy® 96 Plant Kit (Qiagen, Mississauga, ON). Hoechst 33258 stain was used to quantify DNA by fluorimetry. DArT markers were assayed by Diversity Arrays Technology Pty. Ltd (Yarralumla, Australia). The SNP markers were genotyped using the Illumina Infinium 9K wheat SNP beadchip, as per manufacturer's instructions. Each SSR PCR reaction was 10 µl in volume and was composed of 25 ng of template DNA, 1x PCR buffer (Applied Biosystems, Foster City, CA), 1.5 mM MgCl2, 0.5 U of Taq DNA polymerase (Gibco/BRL, Mississauga, ON), 200 µM each dNTP, 20 µM forward primer, 180 µM 6-

FAM/HEX/NED-labelled M13 primer (5’ – 3’ CACGACGTTGTAAAACGAC; Applied Biosystems, Foster City, CA) and 200 µM reverse primer. SSR PCR reactions were performed in 384 well PCR plates. A 5’, 19 nucleotide M13 tail (5’ - 3’, CACGACGTTGTAAAACGAC) (Schuelke 2000) modification was added to all forward microsatellite primers. The denaturation of the reaction mixture was performed at 94°C for 2 min, followed by 30 cycles of 95°C for 1 min, 49/58°C for 50 s, 73°C for 1 min, with a final extension at 73°C for 5 min. An ABI Prism 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA) was used to resolve microsatellite PCR amplicons. To this genetic map, the *Snn3* and *Tsn1* loci were added with the toxin reaction data from PtrToxA and SnTox3, respectively. The linkage map was developed with MapDisto version 1.7.5 (Lorieux, 2012).

QTL analysis was conducted with QGene version 4.3.10 (Joehanes & Nelson 2008). Simple interval mapping was conducted with the single-trait composite interval mapping (CIM) maximum likelihood (MLE) QTL analysis method with no co-factors selected. A total of 10,000 permutation tests were conducted to determine the appropriate LOD significance threshold at Pr = 0.05 (Churchill & Doerge 1994).

Cloning and heterologous expression

DNA extraction

Isolates 06SN002 and Langham were used for DNA extraction because both isolates were known to produce the candidate protein. Isolate 06SN010 was used as a negative control because isolate 06SN010 was not producing any toxin *in vitro*. All three isolates (06SN002, Langham and 06SN010) were cultured in Potato Dextrose broth and incubated at room

temperature with shaking for 3-4 days until enough mycelia was formed. The mycelium was then filtered through sterilized Whatman® qualitative filter paper, grade 1 (Sigma-Aldrich St. Louis, MO). Prior to DNA extraction, the filtered mycelium was lyophilized and was later crushed into a powder in presence of liquid nitrogen. DNA was extracted using the protocol for fungal DNA extraction outlined in DNeasy® 96 Plant Kit (QIAGEN).

Cloning in PYES2.1

Two forward primers (*F-SNOG-47* and *F-SNOG-17*) and a reverse primer (*R-SNOG-698*) were designed flanking the open reading frame of the SNOG_15679 gene (Table 4.2). The primer pairs (*F-SNOG-47/R-SNOG-698* and *F-SNOG-17/R-SNOG-698*) were used to obtain the full length amplification of accession *SNOG_15679* by polymerase chain reaction (PCR) in the isolates 06SN002, Langham and 06SN010. Each 25 µl PCR reaction comprised of 30 ng genomic DNA, 10 pmol of each primer, 1.5 mM $MgCl_2$, 0.8 mM total dNTPs, 0.1 µl of 10X Bovine albumin serum and 0.3 µl of Taq DNA polymerase (5 units/µl). The PCR reactions were first subjected to denaturation at 94ºC for 5 min which was followed by 35 cycles of denaturation at 94ºC for 30 sec, annealing at 54ºC for 30 sec, 1 min extension at 72ºC and 10 min final extension at 72ºC. Following visualization on 1 % agarose gel, the PCR products were cleaned according to manufacturer's instructions for $Multiscreen_{384}$- PCR Plate (Millipore Billericia, MD). The DNA sequencing reaction for cleaned PCR product was set up as mentioned in (Huang & Cloutier 2007) and Big Dye Terminator chemistry (v3.1) using primers *F-SNOG-47*, *F-SNOG-17* and *R-SNOG-698*. Each sequencing reaction consisted of 0.4 µl of Big Dye v3.1 and 5.2 pmol/µl of primer in a 6 µl reaction. An ABI 3130xl Genetic Analyzer (Applied Biosystems

Foster City, CA) was used for sequencing. The sequence obtained was analyzed using SOOMOS v0.6 (Banks, personal communication) software and aligned with available sequence of accession *SNOG_15679* [*Stagonospora nodorum* Sequencing Project, Broad Institute of Harvard and MIT (http://www.broadinstitute.org/)] using DNAMAN v3.2 (Lynnon Corp., Vaudreuil-Dorion, QC) software in order to ensure 100 % sequence match before cloning and transformation.

Table 4.2. List of primers used for PCR amplification, colony PCR and sequencing

Primer name	Primer sequence (5'-3')	Remarks
F-SNOG-17	ACTGCAATTCCACTCAATACATC	PCR, Colony PCR & Sequencing
F-SNOG-47	TAGTAGAACAACAGTCATTCGAG	PCR & sequencing
R-SNOG-698	GCTGTTCAACTACTTGCTACG	PCR & sequencing
GAL1-F	AATATACCTCTATACTTTAACGTC	Sequencing
V5C-term-R	ACCGAGGAGAGGGTTAGGGAT	Sequencing
KpnI Forward	ACGGGTACCATGTACTTCGCAACA	PCR
XbaI Rev	GCGTCTAGATTACACTTTGCTCGGC	PCR
Seq_GapZAA_607F	ATCGGTTACTCAGATTTAGAAG	Sequencing
Seq_GapZAA_325F	ATACTTCAAAGGCGAAACC	Sequencing
Seq_GapZAA_974R	CTCTTGATTAGAATCTAGCAAGAC	Sequencing
SeqGapZAA_450R	TTCAGGTTGAAGTCTCCC	Sequencing
pGAP Forward	GTCCCTATTTCAATCAATTGAA	Colony PCR
3´ AOX1	GCAAATGGCATTCTGACATCC	Colony PCR

Since *SNOG_15679* has one intron, cDNA synthesis was necessary before proceeding for ligation and transformation. Preceding cDNA synthesis, RNA was extracted from isolate Langham. For RNA extraction, isolate Langham was cultured in Fries medium at room temperature with shaking. The mycelium for RNA extraction was harvested after 3, 5, 7 and 9 days post inoculation and saved in 1 ml RNA later for extraction. Total RNA was extracted from

100 mg of mycelium as per the procedure for fungal RNA extraction outlined by manufacturer for RNeasy® Mini Kit (QIAGEN). Extracted total RNA was visualized on a 1 % Agarose gel and TurboDNase® (Ambion, Austin, TX) was used for the DNase treatment of RNA samples before proceeding with cDNA synthesis. The cDNA was synthesized using First-strand cDNA synthesis method outlined by manufacturer for Superscript™ II RT (Invitrogen, Carlsbad, CA). The cDNA synthesized from total RNA obtained from 5 day old mycelia was then subjected to PCR amplification using primers *F-SNOG-17* and *R-SNOG-698*. Each 25 µl PCR reaction consisted of 30 ng of cDNA, 10 pmol of each primer, 1.5 mM $MgCl_2$, 0.8 mM total dNTPs, 0.1 µl of 10X Bovine albumin serum and 0.3 µl of Taq DNA polymerase (5 units/µl). The PCR reactions involved denaturation at 94ºC for 5 min, which was followed by 35 cycles of denaturation at 94ºC for 30 sec, annealing at 53ºC for 30 sec, 1 min extension at 72ºC and 10 min final extension at 72ºC to allow formation of 3'-A overhangs. Four individual PCR reactions were set up and later pooled. Two wells in a 1 % Agarose gel were each loaded with 30 µl aliquot of this pooled reaction and electrophoresed at 80 volts for one hour. After gel electrophoresis, the pooled PCR product was subjected to QIAEX II gel extraction protocol as described by the manufacturer (QIAGEN). 2µl of this QIAEX II purified PCR product was used for the cloning reaction.

The pYES2.1 TOPO® TA expression kit (Invitrogen) was used for initial cloning. After 30 min incubation at room temperature, purified PCR product was ligated to pYES2.1/V5-His-TOPO vector with the aid of 3'-A overhang and topoisomerase. 1 µl of ligated product was eventually transformed into 20 µl of OneShot TOP10 electrocompetent *E. coli* cells (Invitrogen) as per the manufacturer's instruction. A four-fold dilution of the salt solution was prepared prior to transformation because electrocompetent cells were used instead of chemical competent cells

that were provided with the pYES2.1 TOPO® TA expression kit. After transformation, 250 µl of S.O.C media (2 % tryptone, 0.5 % yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, and 20 mM glucose) was added to the transformed cells which were then incubated at 37˚C, 200 rpm. Following incubation, 2 µl, 5 µl, 10 µl and 25 µl of the transformed culture was plated onto Luria-Bertani (LB) agar plates (1% Tryptone, 0.5% Yeast extract, 1 % NaCl and 15 gm Agar in 1000 ml water, pH adjusted to 7) with Carbenicillin (50 µg/ml) previously layered with 20 µl of X-Gal (50 mg/ml) in dark and incubated at 37˚C for 1hour. The inoculated plates were then incubated at 37˚C overnight.

A total of 48 colonies were picked from all the cultured plates the following day. Colony PCR was used to screen these colonies for presence and correct orientation of the insert (*SNOG_15679*). Template for colony PCR was prepared by pricking each colony with a pipette tip and suspending the pipette tip in 50 µl of sterile distilled water aliquoted in first 48 wells of a 96-well PCR plate. The colony suspension was then boiled at 100˚C for 10 mins. A 5 µl aliquot of this boiled suspension was then used as a PCR template for the colony PCR using 10 pmol/µl of each of the primers *F-SNOG-F17* and *V5 C-terminal* reverse primer. Other components of PCR reaction included 1.5 mM $MgCl_2$, 0.8 mM total dNTPs, 0.1 µl of 10X Bovine serum albumin and 0.3 µl of Taq DNA polymerase (5 units/µl). The PCR reactions involved denaturation at 94ºC for 4 min which was followed by 35 cycles of denaturation at 94ºC for 30 sec, annealing at 53ºC for 30 sec, 1 min extension at 72ºC and 10 min final extension at 72ºC. The PCR products from all 48 colonies were visualized on 1 % agarose gel. Positive clones were identified from the gel and then subjected to single colony streak on LB-Carbenicillin (50 µg/ml) plates which were incubated at 37˚C overnight. The single colony streak of the each positive clone was used to start individual 1.25 ml LB-Carbenicillin (50 µg/ml) liquid culture of each

clone which was incubated at 37°C overnight at 280 rpm. The overnight liquid culture was used for plasmid preparation for downstream application.

Plasmid DNA was extracted using Eppendorf Perfectprep®96 Vac, Direct Bind Kit (Eppendorf, Hamburg, Germany). The extracted plasmid DNA (250 ng) was used as a template for sequencing. Sequencing reactions for all positive clones were set up as described by Huang and Cloutier (2007) using Big Dye (v3.1) Terminator chemistry. Each positive clone was sequenced using four different primers: *F-SNOG-17*, *R-SNOG-698*, *GAL1* forward primer and *V5 C-terminal* reverse primer. The sequencing reactions were successively cleaned using 95 % ethanol-water mixture (4:1) and 70 % ethanol which were eventually denatured using Hi-Di formamide and sequenced using an ABI 3130xl Genetic Analyzer (Applied Biosystems). The sequencing data was analyzed and the sequences were aligned using SOOMOS v0.6 (Banks, personal communication) software. DNAMAN v3.2 (Lynnon Corp., Vaudreuil-Dorion, QC) software was used to translate the sequences that aligned 100 % with the sequence of accession *SNOG_15679*. The accession *SNOG_15679* was originally maintained in pYES2.1 for sub-cloning and expression in *Saccharomyces cerevisiae*. Sub-cloning in *S. cerevisiae* was discontinued due to inefficient expression (data not shown). Instead the accession was maintained in pGAPZαA and sub-cloned and expressed in the *Pichia pastoris* expression system.

Cloning in pGAPZαA

The *SNOG_15679* construct already maintained in pYES2.1 was PCR amplified using primers *KpnI-forward* and *XbaI-Rev* such that the amplicon had *Kpn*I restriction site on 5'end and XbaI restriction site on 3'end. Each 25 µl PCR reaction was composed of 150 ng of plasmid

DNA from pYES2.1 transformant, 10 pmol/µl *KpnI-forward primer*, 10 µl of *XbaI-Rev primer*, 1.5 mM $MgCl_2$, 0.8 mM total dNTPs, 0.1 µl of 10X Bovine albumin serum and 0.3 µl of Taq DNA polymerase (5 units/µl). Initial denaturation of the PCR reaction was at 94ºC for 4 min which was followed by 35 cycles of denaturation at 94ºC for 30 sec, annealing at 60ºC for 30 sec, 40 sec extension at 72ºC and 7 min final extension at 72ºC. The amplicon was visualized on 1% agarose gel to confirm the expected size of the amplicon. This amplicon with restriction sites and the vector pGAPZαA (Invitrogen) were each subjected to separate double digestions using *Kpn*I fast digest enzyme (Thermo Scientific, Ottawa, ON) and XbaI fast digest enzyme (Thermo Scientific). Six individual digestion reactions for the pGAPZαA were prepared such that each reaction contained 200 ng of vector DNA. Likewise, six individual digestion reactions for amplicon with *Kpn*I and *Xba*I restriction sites were also prepared; each with 200 ng of vector DNA. All double digestion reactions were incubated at 37°C for a maximum of 15 hours followed by heat inactivation of enzymes for 5 min at 80°C. The digested products of pGAPZαA vector and the amplicon were resolved by gel electrophoresis on 0.8 % agarose gel. The digested product of both amplicon and vector were gel purified using QIAEX II gel extraction protocol as described by the manufacturer (QIAGEN).

After gel purification, 11 ng of the digested product of *SNOG_15679* amplicon was ligated to 20 ng of the digested pGapZαA vector in presence of T4 DNA Ligase (Invitrogen). A 5X dilution of ligation reaction was prepared prior to transformation into OneShot TOP10 electrocompetent *E. coli* cells (Invitrogen). After transformation, 250 µl of low salt Luria-Bertani (LB) medium (1% Tryptone, 0.5 % Yeast extract and 0.5 % NaCl in 1000 ml water, pH adjusted to 7.5) with Zeocin (25 µg/ml) was added to the transformed cells and incubated for 1 hour at 37°C while shaking horizontally at 180 rpm. After incubation, 10 µl, 50 µl and 100 µl of

transformation reaction was plated onto low salt LB medium amended with Zeocin (25 µg/ml). The plates were then incubated overnight in dark at 37°C. Forty-one colonies were randomly selected and screened for presence/absence of construct along with proper orientation using colony PCR. A template of each colony for PCR was prepared by pricking the colony with a pipette tip and washing the tip in 50 µl of water which was then boiled at 100°C for 10 min. An individual PCR reaction was prepared using 5 µl of boiled colony preparation from each colony as a template, 10 pmol/µl *KpnI-forward primer*, 10 µl of *XbaI-Rev primer*, 1.5 mM $MgCl_2$, 0.8 mM total dNTPs, 0.1 µl of 10X Bovine albumin serum and 0.3 µl of Taq DNA polymerase (5 units/µl). Colony PCR included initial denaturation at 94ºC for 4 min followed by 35 cycles of denaturation at 94ºC for 30 sec, annealing at 60ºC for 30 sec, 1 min extension at 72ºC and 10 min final extension at 72ºC.

The identified positive clones were then cultured in 5ml of low salt Luria-Bertani (LB) medium (1 % Tryptone, 0.5 % Yeast extract and 0.5 % NaCl in 1000 ml water, pH adjusted to 7.5) with Zeocin (25 µg/ml) and incubated overnight in dark at 37°C with shaking at 250 rpm. The overnight culture was then used for plasmid preparation by the method outlined in Plasmid Mini Kit I (Omega Bio-Tek Norcross, GA). The extracted plasmid was used for sequencing as per the method described by Huang & Cloutier (2007) using Big Dye (v3.1) Terminator chemistry. *Seq_GapZAA_607F, Seq_GapZAA_325F, Seq_GapZAA_974R* and *SeqGapZAA_450R* (Table 4.2) were the sequencing primers used to set up sequencing reactions for each positive clone. The clones were sequenced in ABI 3130xl Genetic Analyzer (Applied Biosystems) and the sequences were analyzed in SOOMOS v0.6 (Banks, personal communication) software followed by alignment in DNAMAN v3.2 (Lynnon Corp., Vaudreuil-Dorion, QC) software. After the sequencing, plasmid DNA was re-extracted from these clones

using manufacturer's protocol for PureLink® HiPure Plasmid Maxiprep Kit (Invitrogen) in order to have enough plasmid DNA for the transformation reaction in *Pichia pastoris*. A total of 12 µg of extracted plasmid DNA was linearized using *Bsp*HI enzyme (Thermo Scientific) in twelve 20 µl reactions such that each digestion reaction contained a maximum of 1 µg plasmid DNA. The digestion reactions were incubated at 37°C for a maximum of 15 hours followed by inactivation of *Bsp*HI at 80°C for 20 min. After *Bsp*HI inactivation, all reactions were pooled in one micro-centrifuge tube and vacuum dried until the volume was reduced to 5 µl.

Transformation in *Pichia pastoris*

Preparation of competent cells and transformation was carried out using *Pichia* EasyComp™ Kit (Invitrogen). Prior to transformation, *Pichia pastoris* strain X33 competent cells were prepared according to the manufacturer's instructions. Transformation reaction was prepared using manufacturer's protocol. Each transformation reaction was prepared by adding 60 µl of the competent cells to 5 µl of linearized plasmid DNA followed by incubation in a 35°C water bath for 1 hour and heat shock in a 42°C heat block. The entire transformation reaction was plated on Yeast Extract Peptone Dextrose Sorbitol (YPDS) medium (1 % Yeast Extract, 2 % peptone, 2 % Dextrose, 1 M sorbitol) with 100 µg/ml Zeocin (Invitrogen). The plates were incubated in dark at 30˚C for 2-4 days. The colonies visible on YPDS plate with 100 µg/ml zeocin after 2-4 days were sub-cultured on a fresh YPDS plate with zeocin. The identification of the sub-cultured transformed colony was achieved based on colony PCR. Template for *Pichia* colony PCR was prepared by pricking the transformed *Pichia* colony with a pipette tip and suspending it in 10 µl of sterile water. This colony suspension was incubated at -80°C for 10 min

followed by heating in a microwave for 1min. Then the suspension was transferred back to -80°C for 10 min and finally heated in a microwave for 30 sec. Five µl of the prepared template was used in each 25 µl colony PCR reaction. Other components of this PCR reaction included 10pmol/µl *pGapForward primer* (Invitrogen), 10 pmol/µl of *3'AOX1 primer* (Invitrogen), 1.5 mM $MgCl_2$, 0.8 mM total dNTPs, 0.1 µl of 10X Bovine albumin serum and 0.3 µl of Taq DNA polymerase (5 units/µl). For the colony PCR, initial denaturation was done at 94ºC for 4 min followed by 35 cycles of denaturation at 94ºC for 30 sec, annealing at 57ºC for 30 sec, 1 min extension at 72ºC, and 10 min final extension at 72ºC. PCR products were visualized on 0.8 % agarose gel in order to confirm presence or absence of the desired insert.

Heterologous expression

The positive *Pichia pastoris* clone was used to inoculate 10 ml of Yeast Extract Peptone Dextrose (YPD) medium (1 % Yeast Extract, 2 % Peptone and 2 % Dextrose). The inoculated culture was incubated overnight in a shaking incubator at 30˚C, 280 rpm. In a 250 ml flask, 50 ml of YPD medium was inoculated the following day with 0.1 ml of overnight culture. Time points measuring 2 ml each were collected 0 hrs, 24 hrs, 48 hrs, 72 hrs and 96 hrs post inoculation. Since, the protein was expected to be secreted outside the cell, the collected time points were centrifuged at 6000 rpm for 2 min and supernatant was removed into fresh tubes. In order to detect the expression of recombinant protein, an aliquot of each time point was visualized on Tris-tricine gel as mentioned in the protein purification section. The identity of expressed protein was also confirmed with mass spectrometry analysis as mentioned in the protein purification section. The activity of the expressed protein was confirmed by a bioassay.

In order to confirm the bioactivity of the expressed protein, the supernatant obtained from each time point (0 hr, 24 hr, 48 hr, 72 hr and 96 hr) was infiltrated into Kenyon and 86ISMN 2137. If all collected time points of expressed protein were initially found to be inactive, a 10 ml fraction of supernatant from 96 hr time point was evaporated in SpeedVac until dry and re-suspended in 1 ml of water which was used to re-infiltrate Kenyon and 86ISMN2137.

4.4. Results

Toxin production and purification

A bioassay was performed in order to identify toxin producing *S. nodorum* isolates under the liquid culture conditions. Among all the isolates tested for toxin production, only 06SN002, Langham and Swift Current were found to produce toxin in Fries medium culture (Chapter 3). According to differential set infiltration during the bioassay, isolates 06SN002 and Langham produced sensitive reactions on the same differential lines. The differential lines that tested sensitive to culture filtrates of 06SN002 included BG261 and the Kenyon / 86ISMN 2137 RILs 44, 52, and 64. All differential lines, with the exception of line 44 were sensitive to HST(s) present in the culture filtrates of both isolates 06SN002 and Langham. Although, lines 44, 52 and 64 were sensitive to the HST(s) produced in the culture filtrates of 06SN002, the sensitivity to these lines and some other lines, such as lines 28, 83 and 85, were variable with different batches of culture filtrate (Table 4.3). A similar observation was also made in the case of the culture filtrate of isolate Langham (Table 4.3). Therefore, the results from differential line infiltration for both isolates were inconclusive at this stage even though some batches indicated presence of HST interacting with *Tsn1*.

Table 4.3. The differential lines showing variable reactions in response to HST(s) produced in culture filtrates of *S. nodorum* isolates 06SN002 and Langham

Wheat lines	Sensitivity of known toxin and postulated sensitivity loci	Isolate 06SN002			Isolate Langham			
		9/2/12 Batch 1	9/6/12 Batch 2	12/2/12 Batch 3	12/2/12 Batch 1	5/19/13 Batch 2	5/19/13 Batch 3	12/19/13 Batch 4
Kenyon	*	3	3	3	3	2.5	2	3
86ISMN 2137	*	0	0	0	0	0	0	0
Line 28	2A.2 / 2D	0	3	0	0	0	0	0.75
Line 44	2D/ 5B	3	1	*	*	0	0	1
Line 50	6D	0	0	0	0	0	0	0
Line 52	2A.2 / 5B	3	1	3	3	1.5	1.5	3
Line 64	2A.1/ 5B	2	0	3	3	0.75	0.25	3
Line 83	*	0	2	0	0	0	0	0
Line 85	2D/ 6D	0	3	*	*	0	0	3
Line 88	2A.1/ 2D	0	0	0	0	0	0	0
BG261	ToxA	3	1	3	3	3	3	2
BG220	SnTox3	1	1	3	3	0	0	1
BG 223	SnTox2	0	1	0	0	0	0	0
M6	SnTox1	0	0	0	0	0	0	0
Laura	*Tsn1 Ø*	*	*	0	0	0	0	0
Superb	*Tsn1+*	*	*	3	3	3	2.5	2
CDC Alsask	*	*	*	0	0	0	0	0
BW 880	*	*	*	0	0	0	0	0
Erik	*Tsn1 Ø*	*	*	0	0	0	0	0
Amazon	*Tsn1 Ø*	*	*	0	0	0	0	0
Glenlea	*Tsn1+*	*	*	3	3	1.5	2	3

The toxic culture filtrates produced multiple active elution time points for each isolate when purified by chromatography (Figs. 4.1 and 4.2). The culture filtrate of isolate 06SN002 resolved into a total of five active fractions ranging from fractions S5-S9. Likewise, the culture filtrate of isolate Langham resolved into a total of four active fractions ranging from fractions L4-L7. The active elution time points of both the isolates when analyzed by Tris-tricine gel

electrophoresis showed similar protein profiles for both isolates 06SN002 and Langham (Figs. 4.3 and 4.4). A prominent protein band was visible at a relative molecular weight (M_r) range of 20-25 in all active fractions for both the isolates. This protein band was the candidate protein and will be referred as "P-NEC" from this point. The potential match to P-NEC identified based on MS/MS MASCOT search was only considered if the minimum number of significant sequence was at least two. Sequence matches that did not meet this criterion were eliminated (Table 4.4). The mass spectrometry analysis of P-NEC from fractions of both the isolates and the query against *S. nodorum* database indicated similarity with *SNOG_15679*, a hypothetical protein in *S. nodorum* (Table 4.4, Figs. 4.7A and 4.7B). PtrToxA was also processed similarly and was identified as SNOG_16571 in *S. nodorum* (Table 4.4 and Fig. 4.7C).

In contrast to the MS/MS MASCOT search results that identified P-NEC as SNOG_15679 (a hypothetical protein; 192 amino acid in length), the results of the bioassay (Fig. 4.6) for the fractions obtained from chromatography of culture filtrate of both the isolates placed the sensitivity gene on chromosome 5B at or near the *Tsn1* locus. This suggested that the purified protein was SnToxA. However, SnToxA was not identified in the Tris-tricine gel electrophoresis of individual chromatography fractions of both the isolates (Figs. 4.3 and 4.4) and also in the similar gel electrophoresis of pooled active fractions (Fig. 4.5).

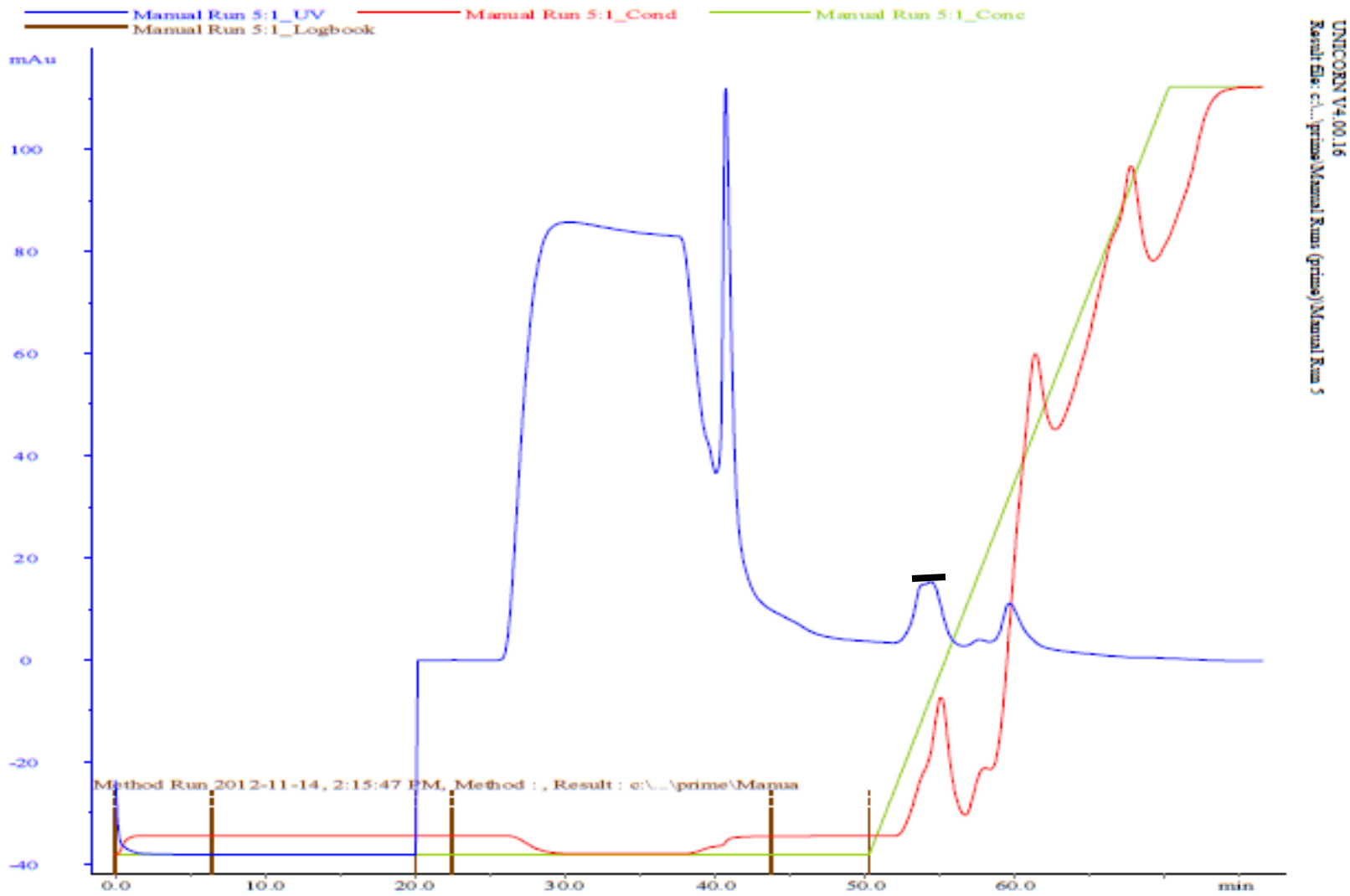

Fig. 4.1. A chromatogram produced after cation exchange chromatography of 06SN002 culture filtrate (Batch1) showing the resolution of two distinct peaks. The first peak corresponds to the active fractions (#S5- #S9) identified in the bioassay. The peak representing active fraction is indicated in the chromatogram with a bar.

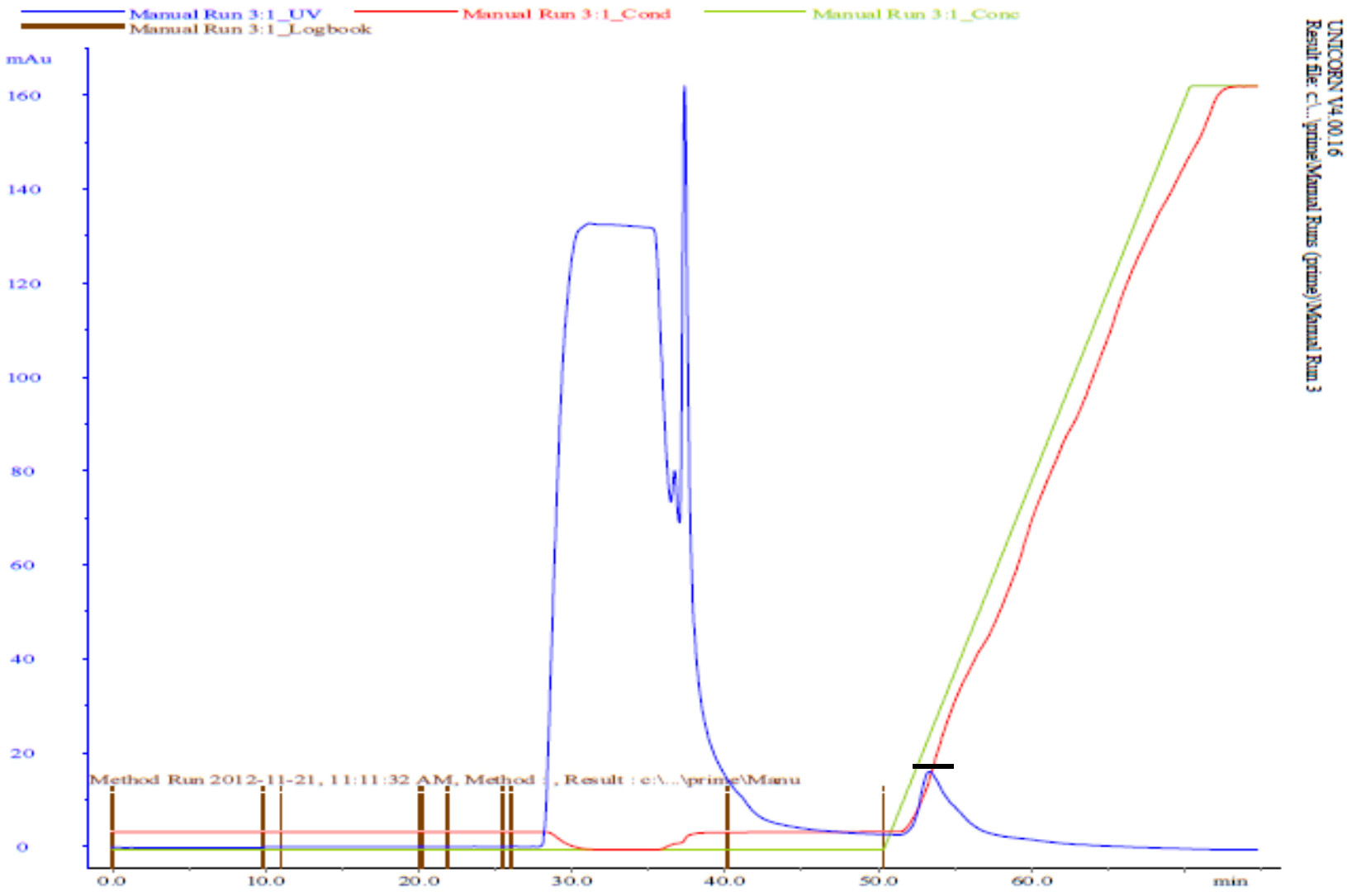

Fig. 4.2. A chromatogram produced after cation exchange chromatography of Langham culture filtrate (Batch1) showing the resolution of a distinct peak corresponding to the active fractions (#L4- #L7) identified in the bioassay. The peak representing active fraction is indicated in the chromatogram with a bar.

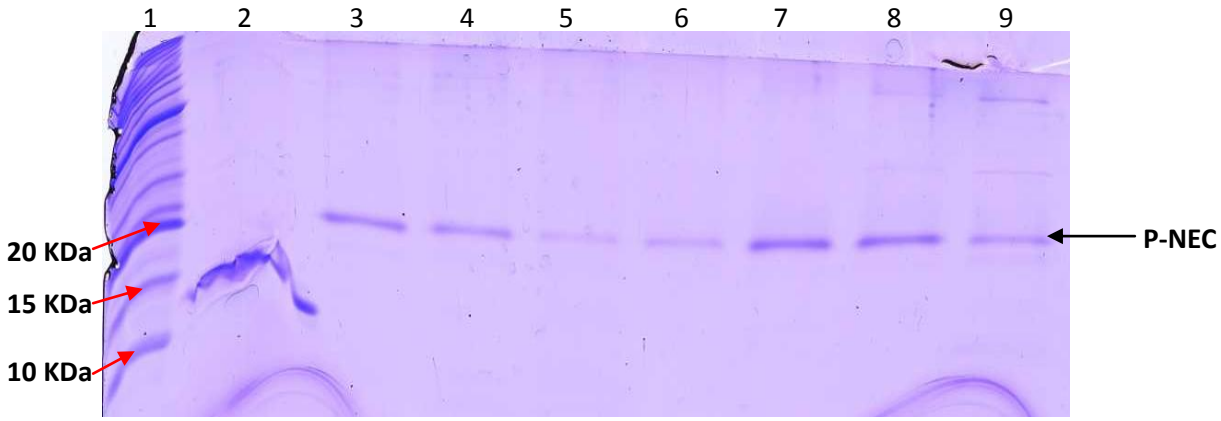

Fig. 4.3. Tris-tricine gel analysis for fractions obtained from cation exchange chromatography of 06SN002 and Langham Culture filtrate. Lane1, Benchmark protein ladder (Invitrogen); Lane 2, PtrToxA; Lane 3, fraction S7; Lane 4, fraction S8; Lane 5, fraction S9; Lane 6, fraction L4; Lane 7, fraction L5; Lane 8, fraction L6; Lane 9, fraction L7. Fractions S7-S9 are active fractions from isolate 06SN002 and fractions L4-L7 are active fractions from isolate Langham.

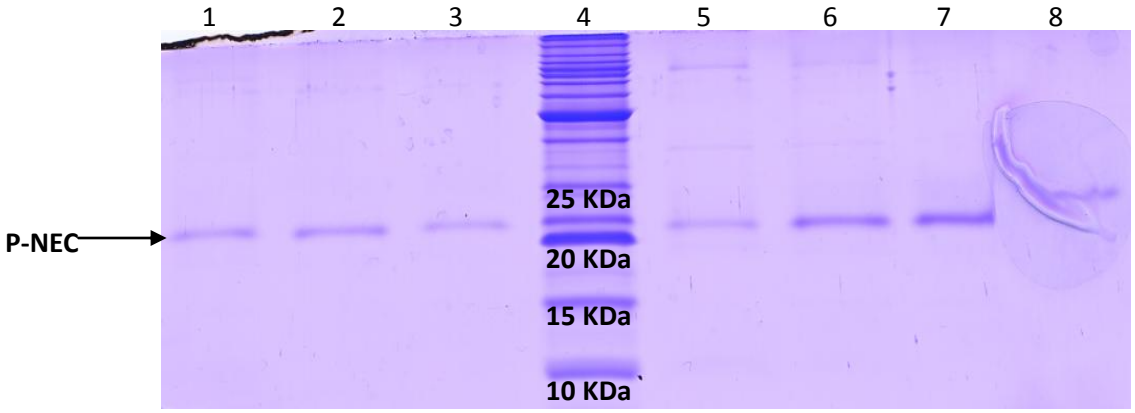

Fig. 4.4. Tris-tricine gel analysis for fractions obtained from cation exchange chromatography of 06SN002 and Langham Culture filtrate. Lane 1, fraction S7; Lane 2, fraction S6; Lane 3, fraction S5; Lane 4, Benchmark protein ladder (Invitrogen); Lane 5, fraction L7; Lane 6, fraction L6; Lane 7, fraction L5; Lane 8, fraction L4. Fractions S5-S7 are active fractions from isolate 06SN002 and fractions L4-L7 are active fractions from isolate Langham.

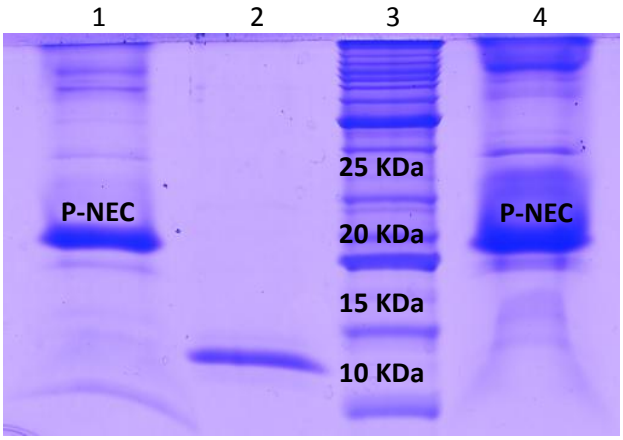

Fig. 4.5. Tris-tricine gel analysis for pooled chromatography fractions of 06SN002 and Langham Culture filtrate showing resolved P-NEC. Lane 1, an aliquot of pooled fractions S5-S9; Lane 2, PtrToxA; Lane 3, Benchmark protein ladder (Invitrogen); Lane 4, an aliquot of pooled fractions L4-L7. Fractions S5-S7 are active fractions from isolate 06SN002 and fractions L4-L7 are active fractions from isolate Langham.

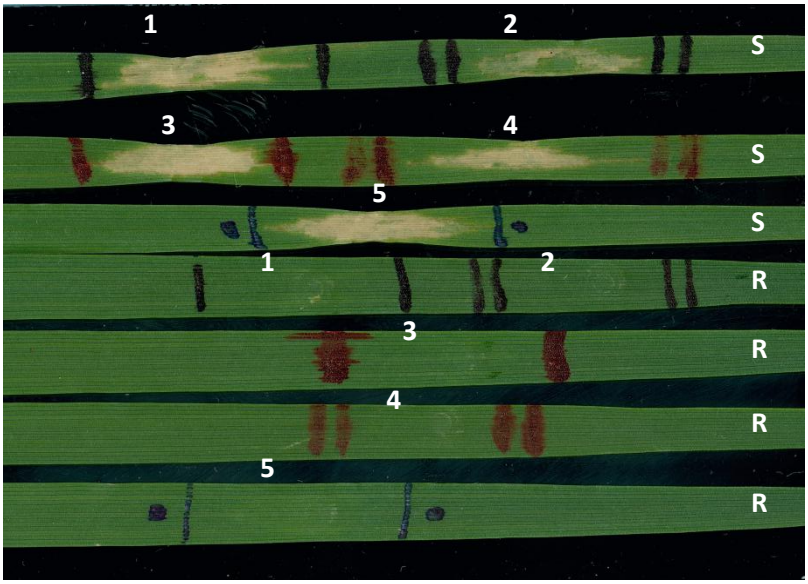

Fig. 4.6. A photograph demonstrating localized necrosis on susceptible parent and absence of necrosis in resistant parent as a result of infiltration of pooled chromatography fractions containing P-NEC obtained from different isolates. "S" indicates susceptible parent Kenyon and "R" indicates resistant parent 86ISMN2137. "1, 2 and 5" represent pooled fractions of isolate 06SN002 from Batch 1, Batch 2 and Batch 3 respectively. "3 and 4" represent pooled fractions of isolate Langham from Batch 1 and Batch 2 respectively.

Table 4.4. Sequence identification of P-NEC protein (obtained from isolates 06SN002 and Langham) and PtrToxA control using MASCOT identification with MS/MS spectra

Sample	Database	Accession	Score	Mass	Number of significant sequences	emPAI	Description
P-NEC (Isolate 06SN002)	*S. nodorum*	*SNOG_15679*	1123	20441	7	2.94	Hypothetical protein (192 Amino acids)
	S. nodorum	*SNOG_10694*	86	23826	2	0.3	Hypothetical protein (219 Amino acids)
P-NEC (Isolate Langham)	*S. nodorum*	*SNOG_15679*	674	20441	7	2.94	Hypothetical protein (192 Amino acids)
	S. nodorum	*SNOG_10694*	226	23826	3	0.48	Hypothetical protein (219 Amino acids)
PtrToxA	*S. nodorum*	*SNOG_16571*	1012	19752	4	3.12	Hypothetical protein (179 Amino acids)

Note: emPAI = Exponentially modified protein abundance index

1	MYFATPTIAA	VMAFTSFAAA	TPLQARQDTL	QDWQVTSVNS	HTPSGRPGSY
51	PWSSLSANIT	DPNTINLGTS	DSDGTSVIVP	AGSQGINCEA	K**YFKGETPLG**
101	**R**TWPCDAITG	GYWTMQVLAG	SSGQYSSGDF	NLK**FRHVPDV**	**LYRGAQYTAT**
151	**FEATGHFAIG**	**DNLKGTCGGS**	**GVCNWGLAAE**	**KKPVLIKPSK**	**V**

Fig. 4.7A. The protein view of SNOG_15679 *Phaeosphaeria nodorum* hypothetical protein (obtained as a match with P-NEC produced by isolate 06SN002) showing matched peptides in **bold red** after MS/MS MASCOT identification and a database search. The matched peptides have protein sequence coverage of 35%

1	MYFATPTIAA	VMAFTSFAAA	TPLQARQDTL	QDWQVTSVNS	HTPSGRPGSY
51	PWSSLSANIT	DPNTINLGTS	DSDGTSVIVP	AGSQGINCEA	KYFKGETPLG
101	R**TWPCDAITG**	**GYWTMQVLAG**	**SSGQYSSGDF**	**NLKFRHVPDV**	**LYRGAQYTAT**
151	**FEATGHFAIG**	**DNLKGTCGGS**	**GVCNWGLAAE**	**KKPVLIKPSK**	**V**

Fig. 4.7B. The protein view of SNOG_15679 *Phaeosphaeria nodorum* hypothetical protein (obtained as a match with P-NEC produced by isolate Langham) showing matched peptides in **bold red** after MS/MS MASCOT identification and a database search. The matched peptides have protein sequence coverage of 47%

1	MRSILVLLFS	AAAVLAAPTP	EADPGYEIVK	LFEAANSSEL	DARGLSLDWT
51	LKPRGLLQER	QGSCMSITIN	PSRPSVNNIG	QVDIDSVILG	RPGAIGSWEL
101	NNFVTIGLNR	**VNANTVR**VNI	NNTGRTNR**LI**	**ITQWDNTLTR**	**GDVYELFGDY**
151	**ALIQGRGSFC**	**LNIR**SDSGRE	NWRMQLEN		

Fig. 4.7C. The protein view of SNOG_16571 *Phaeosphaeria nodorum* hypothetical protein (obtained as a match with PtrToxA control) showing matched peptides in **bold red** after MS/MS MASCOT identification and a database search. The matched peptides have protein sequence coverage of 24%

Segregation of mapping population to Langham culture filtrate

The Kenyon/86ISMN 2137 RI population segregated in a 64:61 sensitive: insensitive ratio in response to infiltration of Langham culture filtrate (Fig. 4.8). The observed ratio of 64:61 fit the expected 1:1 single gene segregation ratio, which was supported by a non-significant χ^2 value (χ^2= 0.032, df=1).

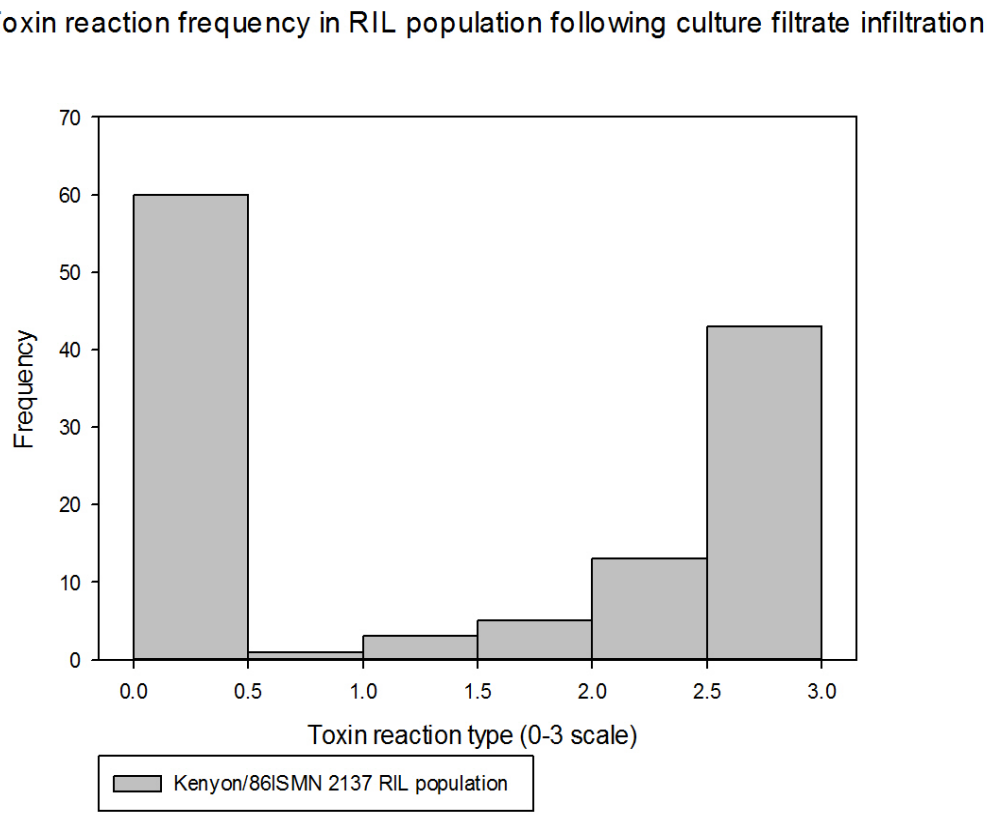

Fig. 4.8. A histogram showing frequency of sensitive and insensitive RI lines in response to the infiltration of Langham culture filtrate in Kenyon/86ISMN 2137 RI population. The sensitive parent (Kenyon) and insensitive parent (86ISMN 2137) showed type 3 and type 0 toxin reaction respectively.

Linkage mapping

The Kenyon/86ISMN 2137 linkage map was 2,655 cM in length and consisted of 25 linkage groups and 3,087 loci. Most of the wheat genome was covered by 22 linkage groups, with chromosome 1A consisting of two linkage groups. The three remaining linkage groups were small (7, 13, and 15 cM) and were not assigned to chromosomes.

Langham QTL mapping

Simple interval mapping identified a single major QTL controlling reaction to the Langham culture filtrate in the Kenyon / 86ISMN 2137 RI population (Table 4.5). This QTL was located on chromosome 5B at the *Tsn1* locus (Fig. 4.9). The LOD score was 38.7 and the additive effect was 1.3 on the 0-3 scale used to evaluate to HST sensitivity. RIs homozygous for the 86ISMN 2137 allele at this QTL were 2.5 units lower on the 0-3 scale than RIs homozygous for the Kenyon allele. This QTL explained 84.1% of the phenotypic variation for response to the Langham culture filtrate. No other QTL were identified for reaction to the Langham culture filtrate. This result indicated that *Tsn1*, or a linked gene, was interacting with a HST in the Langham culture filtrate. This result is consistent with the presence of SnToxA in the Langham culture filtrate.

Table 4.5. QTL statistics for QTL analysis using Kenyon/86ISMN 2137 RIL population infiltrated with Langham culture filtrate

Population	Chromosome	Map Position (cM)	LOD	Additive Effect[a]	R	LOD Threshold[b]
Kenyon / 86ISMN 2137	5B	66.0	38.73	1.27	84.1	19.70

[a]Relative effect of Kenyon alleles on the trait mean [b]10,000 permutations

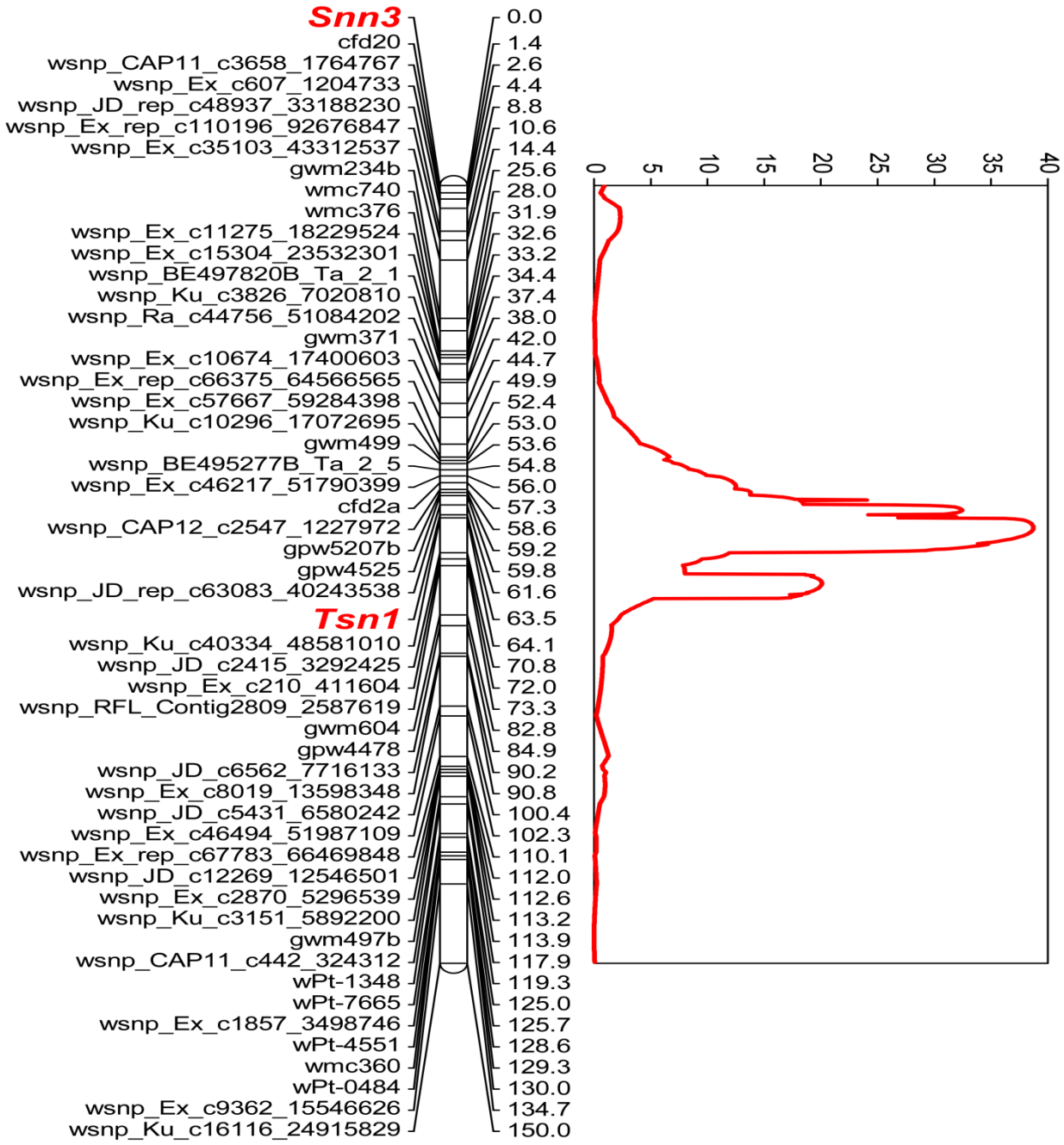

Fig. 4.9. LOD score diagram showing a significant QTL at *Tsn1* locus in response to Langham culture filtrate infiltration in Kenyon/86ISMN 2137 RI population

Cloning and heterologous expression

The mass spectrometry sequence coverage of P-NEC was restricted to C-terminal end. Consequently, the identification of the P-NEC related gene was not possible based on the available mass spectrometry data. However, *SNOG_15679* was revealed to be the closest match to P-NEC by database query of the MS spectra. Therefore, primer pairs (*F-SNOG-47*/*R-SNOG-698* and *F-SNOG*-17/*R-SNOG-698*) designed based on *SNOG_15679* produced amplicons of comparable size (coding sequence of *SNOG_15679* is 984 bps including a single intron) in isolate Langham. The amplification using both primer pairs *F-SNOG-47*/*R-SNOG-698* and *F-SNOG*-17/*R-SNOG-698* failed in isolates 06SN002 and 06SN010 (Fig. 4.10). The sequencing of the cDNA from isolate Langham confirmed the presence of *SNOG_15679* gene (Fig. 4.11). The first successful cloning of *SNOG_15679* was achieved in PYES2.1/V5-His-TOPO vector using OneShot TOP10 electrocompetent *E. coli* cells. Among 48 colonies screened, 20 positive clones with the correct insert orientation were identified by colony PCR. A total of fifteen clones were used for plasmid preparation and sequencing. Among the fifteen clones used, only nine clones were found to be an exact match to *SNOG_15679* sequence. The translated sequence of the nine confirmed positive clones evidently had exact amino acid sequence as translated *SNOG_15679* sequence (*SNOT_15679*). The other clones were ruled out based on incorrect nucleotide and amino acid sequence (Fig. 4.12). The plasmid DNA from clone #18 was used for downstream cloning applications.

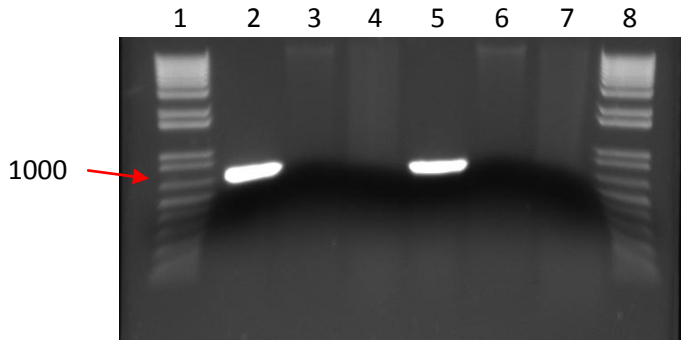

Fig. 4.10. PCR amplification of SNOG_15679 in isolates Langham, 06SN002 and 06SN010 on 0.8% Agarose gel. Lane 1 and 8; 1Kb-Plus DNA ladder (Invitrogen), Lane 2 and 5; amplification in isolate Langham, Lane 3 and 6; amplification in isolate 06SN010, Lane 4 and 7; amplification in isolate 06SN002. Lanes 2, 4 and 6 represent result of *F-SNOG*-17/*R-SNOG-698* PCR amplification and Lanes 4. 5 and 7 represent result of *F-SNOG-47*/*R-SNOG-698* PCR amplification

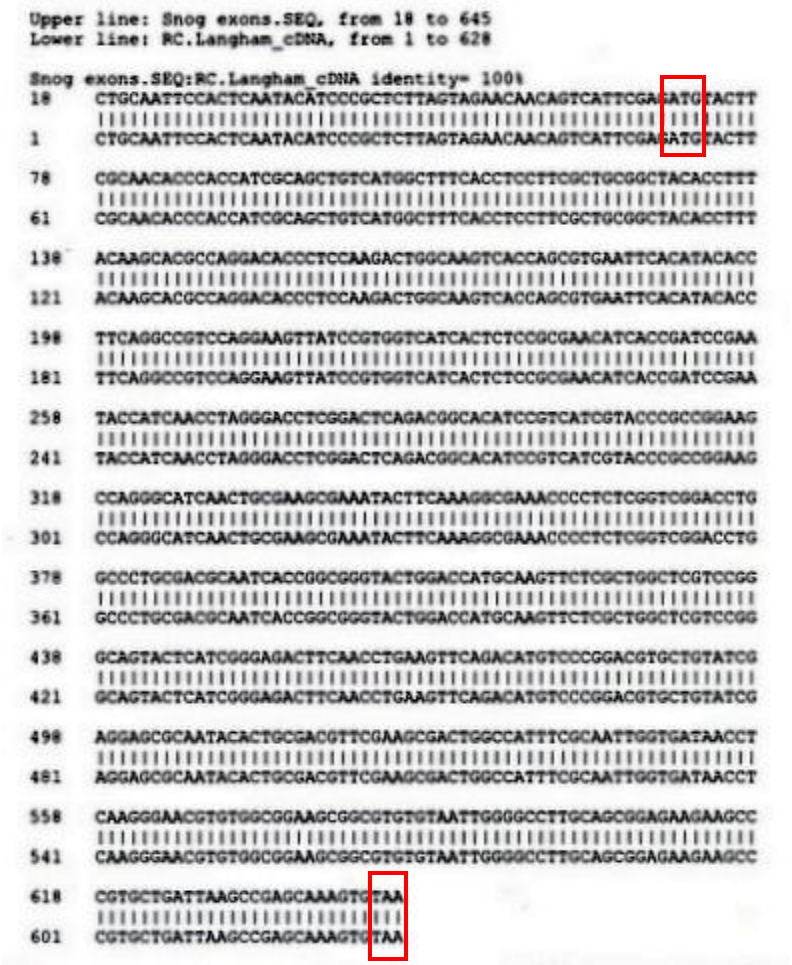

Fig. 4.11. Fast alignment of DNA sequences using DNAMan showing 100% sequence similarity between cDNA sequence obtained from isolate Langham and the sequence of exons in SNOG_15679 gene obtained from the Broad Institute *S. nodorum* database. The upper line represents the sequence on exons in SNOG_15679 gene and the lower line represents cDNA sequence obtained from isolate Langham. The start (ATG) and stop (TAA) codons are indicated by the areas enclosed in the box.

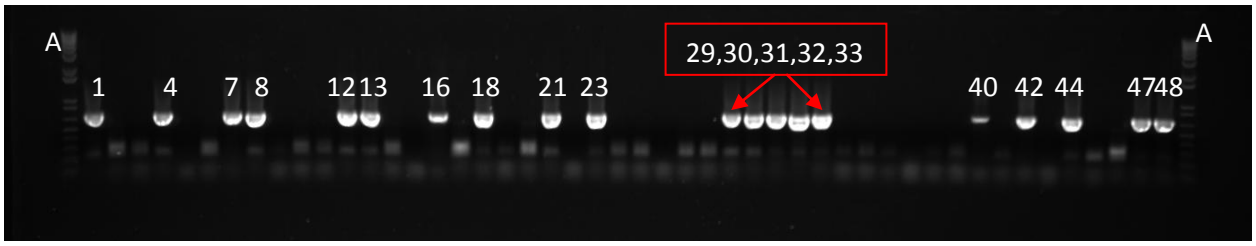

Fig. 4.12. Agarose gel (0.8%) electrophoresis of colony PCR for PYES2.1 clones where positive clones are indicated by numbered lanes. The number on each lane also corresponds to clone number (i.e. 1- 48 represents clones C1-C48). Lanes marked "A" represent 1KbPlus DNA marker (Invitrogen). Note: Missing successive numbers represent negative clones.

Since the cloning construct was already maintained in PYES2.1/V5-His-TOPO vector in the form of plasmid DNA from clone# C18, the construct for cloning in pGAPZαA was prepared by direct amplification using plasmid DNA from clone# C18 as a template. The direct amplification from clone# C18 was designed to add *Kpn*I restriction site at 5' end and *Xba*I restriction site at 3' end which appeared on 1% agarose gel as bright band near 650bp marker on 1Kb-plus DNA ladder and above 564 bp marker on lambda *Eco*RI *Hind*III DNA ladder (Fig. 4.13). The electroporation of ligated construct-vector preparation into OneShot TOP10 electrocompetent cells produced numerous colonies on LB plates with zeocin but only forty-one of these colonies were screened for positive transformants using colony PCR (Figs. 4.14A and 4.14B). The colony PCR of all forty-one clones using *KpnI-forward* primer/*XbaI-Rev* primer pair indicated twelve positive clones. After sequencing, four clones (T10, T17, T31 and T34) with 100% sequence similarity to *SNOG_15679* were identified.

The transformation of linearized plasmid DNA from clone# T10 produced two colonies on YPDS medium with zeocin as a selectable marker. After a subculture on YPDS medium with Zeocin, only one colony remained viable. The positive transformant was expected to produce a

DNA band of approximately 1122 bps after a colony PCR using *pGapForward/3'AOX1* primer pair. As expected, the multiple colony PCRs of the single viable clone produced a thick DNA band slightly above 1000 bps marker on a 1Kb-Plus ladder (Fig. 4.15).

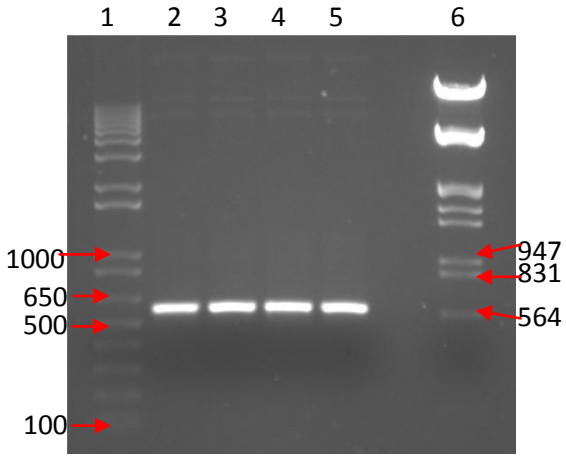

Fig: 4.13. Agarose gel (0.8%) electrophoresis showing amplicon with 5' *Kpn*I and 3' *Xba*I restriction sites produced as a result of PCR amplifcation using *KpnI-forward* primer/*XbaI-Rev* primer pair. Lane1; 1Kb-Plus DNA marker, Lanes 2-5; replicate PCR reactions containing amplicon, Lane 6; Lamda EcoRI HindIII marker.

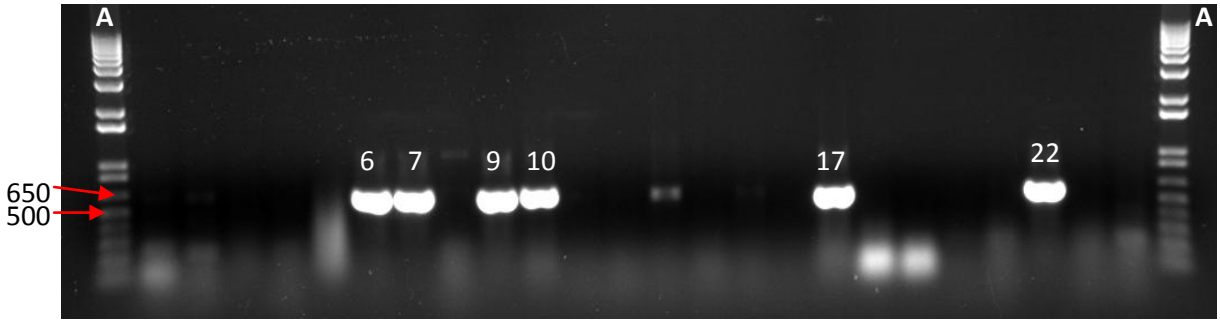

Fig. 4.14A. Agarose gel (0.8%) electrophoresis of colony PCR for pGAPZαA clones where positive clones are indicated by numbered lanes. The number on each lane also corresponds to clone number (i.e. 1- 24 represents clones T1-T24). Lanes marked "A" represent 1Kb-Plus DNA marker (Invitrogen). Note: Missing successive numbers represent negative clones.

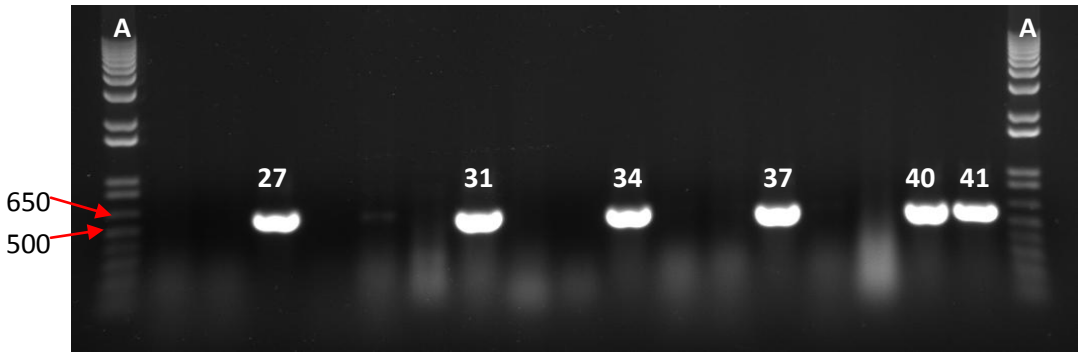

Fig. 4.14B. Agarose gel (0.8%) electrophoresis of colony PCR for pGAPZαA clones where positive clones are indicated by numbered lanes. The number on each lane also corresponds to clone number (i.e. 25- 41 represents clones T25-T41). Lanes marked "A" represent 1Kb-Plus DNA marker (Invitrogen). Note: Missing successive numbers represent negative clones.

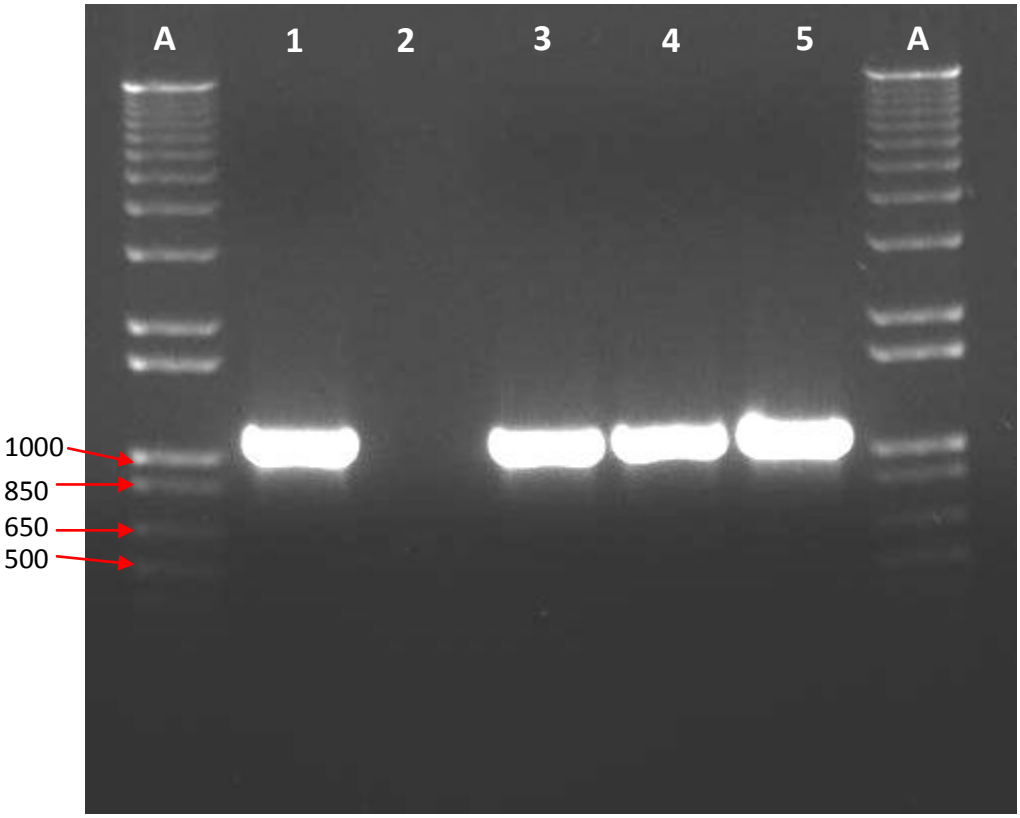

Fig. 4.15. Agarose gel (0.8%) electrophoresis of colony PCR for *Pichia pastoris* clones showing positive clones with >1000 bps band. Lanes marked "A" represent 1Kb-Plus DNA marker (Invitrogen). Lanes 1 and 3-5; replicate colony PCR reaction from single viable *P. pastoris* clone, Lane 2; Empty.

The expressed recombinant protein from the viable positive *P. pastoris* clone was collected in the form of supernatant from 0 hr, 24 hr, 48 hr, 72 hr and 96 hr time points. An aliquot of supernatant from each time point when analyzed by Tris-tricine gel electrophoresis demonstrated a clear pattern of protein expression. There was no expression of the protein at zero hour and the expression gradually increased from 24 hr to 96 hr. The expressed protein seemed

to resolve a little above 25 KDa and under 30 KDa size range (Fig. 4.16). The identification of expressed protein by MS/MS MASCOT analysis indicated a probable match to SNOG_15679. The identified protein sequence had a MASCOT score of 1318 and six significant peptide sequences were found in the search. The probable protein match was only considered if the number of significant peptide sequence was ≥ 2. Following the confirmation of expression of the recombinant protein, the supernatant from each time point 0 hr, 24 hr, 48 hr, 72 hr and 96 hr was injected into Kenyon (sensitive) and 86ISMN 2137 (resistant) parent in order to confirm bioactivity. The injected supernatant from all time points failed to produce any visible chlorosis and/or necrosis in the sensitive parent. The infiltration of concentrated expressed protein sample (96hr time point) into Kenyon produced mild chlorosis after 7days and 86ISMN 2137 remained unaffected (Fig. 4.17).

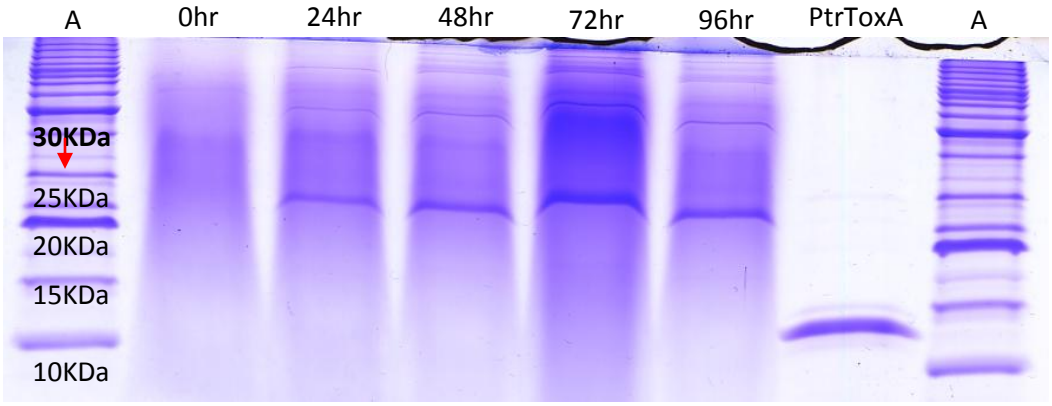

Fig. 4.16. Tris-tricine gel electrophoresis showing expression of recombinant protein at different time points. "A" indicates Benchmark protein ladder (Invitrogen).

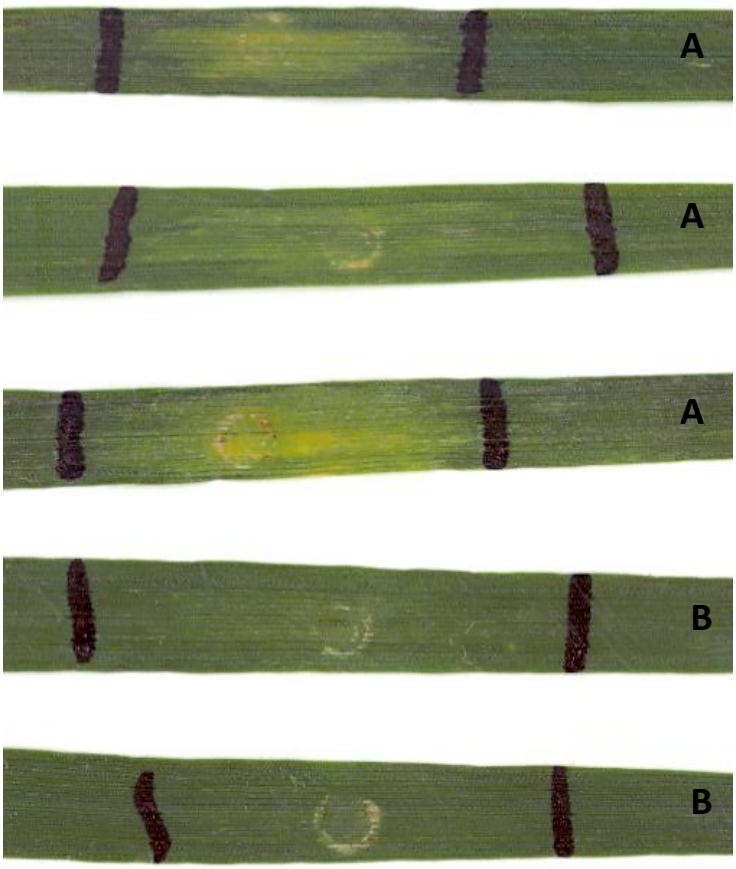

Fig. 4.17. Picture showing mild chlorosis within the marked area on Kenyon leaves and absence of sensitivity within the marked area on 86ISMN 2137 leaves as a result of infiltration of concentrated recombinant protein. "A" indicates Kenyon (sensitive parent) and "B" indicates 86ISMN 2137 (resistant parent).

4.5. Discussion

In this study, we attempted to identify and purify a novel HST and identify its corresponding sensitivity gene. In order to achieve these goals, the initial step was to purify HSTs using chromatography. During the purification of HST, only candidate protein "P-NEC"

was visible on the gel resulting from Tris-tricine gel electrophoresis for active fractions identified by bioassay. However, from the results of infiltration of the differential set with the chromatographically purified fractions, the corresponding sensitivity gene was consistent with the *Tsn1* locus which is the known sensitivity locus to ToxA. At this point, the potential presence of ToxA in the fractions was a concern but ToxA could not be identified on Tris-tricine gels even after the protein concentration attempts. The lack of visibility of proteins on a Tris-tricine gel due to the low concentration could be the simplest possible explanation to the absence of ToxA band observed in our experiments. However, PtrToxA as low as 2 µg has been demonstrated on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) (Tomas et al. 1990). This suggests that the lack of visible ToxA band on a Tris-tricine gel in this study was not a mere concentration issue. In fact, Tuori & Ciuffetti (1995) successfully demonstrated the presence of protein band corresponding to the PtrToxA size range when electrophoresis of concentrated crude culture filtrate of *Pyrenophora tritici-repentis* was carried out on SDS-PAGE gel. In the same study, the PtrToxA band from concentrated active fractions of Sephadex G50 column chromatography was demonstrated on SDS-PAGE gel, which was seemingly more purified and concentrated. Therefore, this suggests that the bioactivity observed on the sensitive leaves was likely due to a novel necrotic toxin(s).

The possibility of a novel necrotic toxin is not unlikely as evidence of necrotic components other than ToxA have been previously reported in *P. tritici-repentis*. Tuori & Ciuffetti (1995) reported the presence of necrotic components in two different fractions other than fraction containing PtrToxA. According to Tuori & Ciuffetti (1995), one of the necrotic components was non-cationic as it was collected in the flow through of a cation exchange chromatography but the second necrotic fraction was cationic and was collected following the

elution of PtrToxA. Also, in a separate study by Friesen et al. (2002) where the effect of Race 1 *P. tritici-repentis* on PtrToxA mutant wheat lines was being studied, necrotic lesions were observed on the mutant wheat lines when inoculated by Race 1 *P. tritici-repentis* suggesting the presence of necrotic toxin other than PtrToxA. Therefore, the evidence of necrotic components other than PtrToxA in *P. tritici-repentis* reinforces the possibility of presence of novel necrotic toxin. Although these non-ToxA components have only been observed in *P. tritici-repentis* to date, the presence of novel necrosis toxin in *S. nodorum* is also equally likely because all other previously identified *S. nodorum* toxins (SnTox1, SnTox2, SnTox3, SnTox4 and SnTox5) are necrosis toxins. Additionally, ToxA gene was found to be laterally transferred from *S. nodorum* to *P. tritici-repentis* (Friesen et al. 2006). Therefore, this phenomenon opens the possibility for exchange of other necrotic and/or chlorotic toxin(s) between *S. nodorum* and *P. tritici- repentis* or among other members of Pleosporales. Additional HSTs are expected given the complexity of the host-pathogen interactions in the wheat-*S. nodorum* pathosystem (Matlock et al. 2012). Besides, a recent study of the *S. nodorum* effector sensitivity genes in winter wheat germplasm by Crook et al. (2012) found that 50% of the studied cultivars had sensitivities to novel effector(s) whose corresponding sensitivity gene(s) is yet to be identified. Although this study did not elaborate on the chlorotic or necrotic nature of the potential novel effectors, this study acknowledges the presence of other unidentified toxins/effectors.

The candidate protein P-NEC was produced by both isolates (Langham and 06SN002) and was determined to have sequence similarity with a hypothetical protein SNOG_15679 in *S. nodorum.* Therefore, in presence of evidence of sequence similarity with SNOG_15679 and in lack of any other candidate protein band on Tris-tricine gel, P-NEC was assumed to be SNOG_15679. A successful amplification in isolate Langham based on SNOG_15679-derived

primer pairs and the sequence confirmation of amplicon demonstrated the presence of SNOG_15679 in Langham. However, the failure of SNOG_15679 amplification from isolate 06SN002 could have been the result of DNA sequence differences at a PCR primer site(s) or the absence of SNOG_15679. The possible priming site incompatibility issue could have been solved by attempting amplification from a different set of PCR primers but this avenue was not pursued during the course of this study. The theory that P-NEC is SNOG_15679 does not agree with the failure of SNOG_15679 amplification from 06SN002 (considering presence of P-NEC in both Langham and 06SN002). A possible explanation for this could be the fact that P-NEC from Langham and P-NEC from 06SN002 are potentially only similar proteins but not the same because only partial sequence of P-NEC was obtained from MS/MS Mascot data (in the case of both isolates). The P-NEC partial sequences obtained from MS/MS Mascot data for both isolates when compared to *S. nodorum* database showed sequence similarity with SNOG_15679. Additionally, P-NEC from Langham was still considered to be SNOG_15679 as a successful amplification was obtained in this isolate and upon sequencing, the amplicon was found to have 100% sequence similarity with SNOG_15679. The amplification of SNOG_15679 in Langham was particularly interesting because Langham was initially found to be a ToxA non-producer (based on marker data) but a significant SNB resistance QTL mapping to the *Tsn1* locus (ToxA sensitivity locus) on chromosome 5B was observed during conidia inoculation studies using isolate Langham (McCartney, unpublished data). In light of all the evidence of necrotic component we found in this study and above peculiar observations previously made with isolate Langham, it is likely that Langham produces a novel necrosis toxin. Moreover, the hypothetical protein SNOG_15679 with the help of protein-protein blast was found to have 89% similarity with Cell Death in Tomato 1 (CDiT1) protein from *Pyrenochaeta lycopersici*, a tomato pathogen

(data not shown). The necrotic nature of CDiT1 protein has been proven on tomato leaves (Clergeot et al. 2012). Therefore, this further suggests that SNOG_15679 is responsible for the necrotic symptoms in the bioassays using Langham culture filtrates

The results of bioassay and preliminary gene mapping obtained during the course of this study suggested that the HST sensitivity gene mapped to the location of *Tsn1* on chromosome 5B. Although, the novel necrotic toxin appears to interact with *Tsn1*, that may or may not be accurate. It is possible that the novel necrosis inducing HST is interacting with the novel sensitivity gene that is tightly linked to *Tsn1*. The size of the Kenyon/86ISMN 2137 RIL population will not distinguish the tight linkage from pleiotropy. Testing the sensitivity of ToxA knockout mutants could resolve this issue. Alternatively, an extensive number of closely linked markers would have to be tested around *Tsn1* region and a fine mapping population with abundant marker information would have to be developed. Therefore, even with the identification of a novel necrotic toxin, additional work may be required to correctly locate the corresponding sensitivity gene.

In the course of this study, the ToxA and *Tsn1* activity proved to be an interfering factor. Therefore, in an attempt to distinguish the activity of the novel necrotic toxin from that of ToxA, cloning and heterologous expression of SNOG_15679 in the *Pichia pastoris* system was attempted. If successful heterologous expression and bioactivity was obtained, this would prove that SNOG_15679 is a gene coding a novel necrosis HST. Despite successful heterologous expression, only a faint chlorotic reaction during bioassay was observed even after attempting to concentrate the expressed protein by evaporation. Therefore, additional attempts will be necessary to prove SNOG_15679 as a toxin producing gene. The failure of the bioassay could be due to low expressed protein level even after attempts to concentrate the protein by evaporation

or that the expressed protein did not fold properly. The expressed protein concentration could probably be improved by using a vector system with stronger promoter activity. Also, cloning and expression can be designed such that a purification tag like His-tag or GST fusion tag could be added to the expressed protein which would allow the concentration of expressed protein without concentrating the unwanted contaminants from the *P. pastoris* culture. Transformation of a non-ToxA-producing *S. nodorum* isolate could be an option to resolve a potential protein folding problem.

In conclusion, the *S. nodorum* isolate Langham likely produces necrosis toxin that interacts with *Tsn1* that is not ToxA. Although many HSTs have been identified to date, there is evidence of more HSTs yet to be discovered. Therefore, the purification of novel HST(s) and identification of the corresponding sensitivity gene(s) is a crucial step in unlocking the sophisticated mechanisms of host-pathogen interactions in the wheat-*S. nodorum* pathosystem.

5. GENERAL DISCUSSION AND CONCLUSIONS

The contribution of host-selective toxins (HSTs) in the wheat-*Stagonospora nodorum* pathosystem has been well established. The potential of each purified HST to reproduce Stagonospora nodorum blotch disease symptoms when infiltrated into sensitive wheat varieties has been documented (Liu, Faris, et al. 2004; Friesen et al. 2006; Friesen et al. 2007; Liu et al. 2009; Abeysekara et al. 2009; Friesen et al. 2012). Among six purified *S. nodorum* HSTs, only three HST producing genes for ToxA (Ballance et al. 1989; Tomas et al. 1990; Tuori & Ciuffetti 1995; Zhang et al. 1997; Tuori et al. 2000; Friesen et al. 2006; Liu et al. 2006), SnTox1 (Liu et al. 2009; Liu et al. 2012) and SnTox3 (Liu et al. 2009) have been cloned and heterologously expressed. These three toxins have been well studied. However, many studies suggest that there may be many other HSTs produced by *S. nodorum* that are yet to be discovered. In this study, we attempted to purify any novel HST produced by Canadian *S. nodorum* isolates and to characterize their interactions with the host. The interaction of existing HSTs with Canadian wheat varieties was also investigated.

The identification and purification of HSTs is an important strategy in SNB resistance breeding because in the case of SNB, resistance in the host is an outcome of unsuccessful host-pathogen interactions involving HSTs. Recently, it has been suggested that *S. nodorum* via interspecific gene transfer passed down the ToxA gene to another leaf spotting pathogen, *Pyrenophora tritici-repentis*. Such exchange of genetic information between different species could potentially lead to the introduction of novel diseases in the long run. The sole strategy to manage this problem along with SNB resistance is by developing full understanding of the pathosystem and the first step is HST identification, purification and characterization.

ToxA is the most extensively studied HST of the known *S. nodorum* HSTs [SnTox1 (Liu, Faris, et al. 2004), ToxA (Ballance et al. 1989; Tuori & Ciuffetti 1995; Friesen et al. 2006), SnTox2 (Friesen et al. 2007), SnTox3 (Liu et al. 2009), SnTox4 (Abeysekara et al. 2009) and SnTox5 (Friesen et al. 2012)]. According to different studies, ToxA interacts with an Arginyl-glycyl-aspartic acid (RGD) motif (Tuori et al. 2000; Meinhardt et al. 2002; Manning et al. 2004; Sarma et al. 2005), ToxA binding protein 1 (ToxABP1) (Manning & Ciuffetti 2005; Manning et al. 2007), plastocyanin (Tai et al. 2007) and PR-I type protein (Lu et al. 2014) of wheat. The results from different studies on ToxA binding suggests that ToxA interaction with wheat may require more than one protein-protein interaction in order to cause necrosis. Each protein-protein interaction characterized to date may be a preliminary reaction or part of the cascade of reactions launched in response to ToxA. Additionally, *Tsn1* has been reported to have NBS-LRR and STPK domains typical of a resistance gene and to indirectly interact with ToxA (Faris et al. 2010). Faris et al. (2010) further suggested that proteins other than Tsn1 protein might interact directly with ToxA leading to disease symptoms. Likewise, from this study we found evidence to suggest that a novel necrotic HST is interacting with *Tsn1*. Therefore, the complicated interactions of ToxA with compounds in the host cell suggest that wheat-*S. nodorum* pathosystem is a sophisticated system involving numerous proteins from the host that interact with a multitude of HSTs produced by the pathogen. However, the significance of each identified HST, including ToxA in the wheat- *S. nodorum* pathosystem and a hierarchy of HST deployment during the process of *S. nodorum* infection in wheat is not fully understood.

The purification and identification of HSTs had many challenges in this study. Among the two proteins studied in this research, one was identified as SnTox3 whereas the other seemed to be a novel toxin interacting with *Tsn1*. The limitations of protein purification techniques were

low resolution in gel purification of proteins, which made it difficult to conclusively tie the resolved protein with its activity. Also, failure to completely eliminate/nullify the activity of all known HSTs posed a challenge in trying to characterize the activity of the novel toxin. The presence of SnToxA in the culture filtrates was of concern as it is one of the common HSTs produced by *S. nodorum* isolates. In addition, it was necessary to nullify the activity of SnToxA because the novel toxin P-NEC seemed to be interacting with *Tsn1* which is also the sensitivity gene for SnToxA. Another challenge was posed by the host mapping populations. None of the available host populations segregate for reaction to the novel HST and are homozygous for insensitivity to SnToxA. A similar issue was addressed in the identification of SnTox3 from Swift Current culture by the use of SnToxA insensitive Laura/86ISMN 2137 DH population. Heterologous expression was pursued to eliminate the presence of other *S. nodorum* HSTs.

The evaluation of host populations with the purified HST is an important tool for studying toxin sensitivity. During this study, HST sensitivity determined from QTL analysis of seedling inoculation data was often found unreliable. This could be explained by the failure of *S. nodorum* isolates to produce a particular HST under the conditions of that study or due to the epistatic effect of a stronger toxin present in the culture filtrate. Initially, based on QTL analysis of the *S. nodorum* inoculated wheat varieties it was believed that the Kenyon/86ISMN 2137 population did not have sensitivity to SnTox1 and SnTox3. However, the infiltration studies involving direct HST infiltration into wheat leaves demonstrated that Kenyon/86ISMN 2137 RI population was insensitive to SnTox1. Surprisingly, SnTox3 was present in Canadian *S. nodorum* isolates and segregated in Kenyon/86ISMN 2137 population. This information was valuable for breeding purposes because relying on previous findings only could have mislead the breeders in terms of significance of SnTox3 sensitivity in the case of Canadian wheat lines.

Consequently, SnTox3 sensitivity loci would have been ignored during breeding for resistance. Perhaps for this reason and the ease of use, direct HST infiltration in screening tests for SNB resistance breeding has been tried in Australia (Vleeshouwers & Oliver 2014). Therefore, identification and purification of HSTs, is an excellent tool for achieving SNB resistance.

Future prospects of this study include full characterization of the purified novel toxin and establishment of its significance in the *S. nodorum*-wheat pathosystem. As a long term goal for this project, efforts should be focused on developing additional mapping populations with insensitivity to already known toxins. Also, development of a fine mapping population and/or use of point mutation wheat lines for known HST sensitivities could nullify the effect of any known HST and increase the chance of identification of a tightly linked HST sensitivity gene. Perhaps genome editing could be used to knockout the function of known HST sensitivity genes in the host in the future. The site specific nucleases like Zinc fingers nucleases (ZFN) (Tovkach et al. 2009) and Transcription activator-like effector nucleases **(**TALENS) (Chen & Gao 2013) have been suggested for targeted genome editing in plants. Another microbial nuclease system, Clustered regularly interspaced short palindromic repeats (CRISPR)-CRISPR associated proteins (Cas), has also been suggested as a site specific genome editing tool in plants (Feng et al. 2013). Recently, the use of CRISPR-Cas system as genome editing tool has been demonstrated in *Arabidopsis thaliana* (Fauser et al. 2014) and also in rice and wheat (Shan et al. 2014). Currently, ethyl methanesulfonate (EMS) can be used to generate point mutations, however, these mutations are random throughout the genome. Additionally, different culture conditions including new liquid media compositions and different pH conditions may be attempted to induce production of other novel HSTs in liquid cultures. Although protein purification and identification from a crude mixture of proteins seems daunting, advancement in protein

purification and identification techniques may hold a brighter future for research in this pathosystem.

6. LITERATURE CITED

Abeysekara NS, Friesen TL, Keller B, Faris JD. 2009. Identification and characterization of a novel host–toxin interaction in the wheat–*Stagonospora nodorum* pathosystem. *Theor Appl Genet.* 120:117–126.

Adhikari TB, Ali S, Burlakoti RR, Singh PK, Mergoum M, Goodwin SB. 2007. Genetic Structure of *Phaeosphaeria nodorum* Populations in the North-Central and Midwestern United States. *Phytopathology*. 98:101–107.

Aguilar V, Stamp P, Winzeler M, Winzeler H, Schachermayr G, Keller B, Zanetti S, Messmer MM. 2005. Inheritance of field resistance to *Stagonospora nodorum* leaf and glume blotch and correlations with other morphological traits in hexaploid wheat (*Triticum aestivum* L.). *Theor Appl Genet.* 111:325–336.

Ali S, Adhikari TB. 2008. Variation in Aggressiveness of *Stagonospora nodorum* Isolates in North Dakota. *J Phytopathol.* 156:140–145.

Arseniuk E, Czembor PC, Czaplicki A, Song Q, Cregan PB, Hoffman DL, Ueng PP. 2004. QTL controlling partial resistance to *Stagonospora nodorum* leaf blotch in winter wheat cultivar Alba. *Euphytica.* 137:225–231.

Bailey KL. 1996. Diseases under conservation tillage systems. *Can J Plant Sci.* 76:635–639.

Ballance GM, Lamari L, Bernier CC. 1989. Purification and characterization of a host-selective necrosis toxin from *Pyrenophora tritici-repentis*. *Physiol Mol Plant Pathol.* 35:203–213.

Ballance G, Lamari L, Kowatsch, R, Bernier, C. 1996. Cloning, expression and occurrence of the gene encoding the Ptr necrosis toxin from *Pyrenophora tritici-repentis*. [Internet]. [cited Jan 2015 9]. Available from: http://www.bspp.org.uk/mppol/1996/1209ballance.

Bathgate JA, Loughman R. 2001. Ascospores are a source of inoculum of *Phaeosphaeria nodorum*, *P. avenaria* f. sp. *avenaria* and *Mycosphaerella graminicola* in Western Australia. *Australas Plant Pathol*. 30:317–322.

Bhathal JS, Loughman R, Speijers J. 2003. Yield Reduction in Wheat in Relation to Leaf Disease From Yellow (tan) Spot and *Septoria Nodorum* Blotch. *Eur J Plant Pathol*. 109:435–443.

Bird PM, Ride JP. 1981. The resistance of wheat to *Septoria nodorum*: fungal development in relation to host lignification. *Physiol Plant Pathol.* 19:289–299.

Blixt E, Djurle A, Yuen J, Olson Å. 2009. Fungicide sensitivity in Swedish isolates of *Phaeosphaeria nodorum*. *Plant Pathol.* 58:655–664.

Blixt E, Olson Å, Högberg N, Djurle A, Yuen J. 2008. Mating type distribution and genetic structure are consistent with sexual recombination in the Swedish population of *Phaeosphaeria nodorum*. *Plant Pathol.* 57:634–641.

Bronson CR. 1991. The genetics of phytotoxin production by plant pathogenic fungi. *Experientia*. 47:771–776.

Carver, Brett F, editor. 2009. Wheat science and trade. Ames: Wiley-Blackwell.

Chen K, Gao C. 2013. TALENs: Customizable Molecular DNA Scissors for Genome Engineering of Plants. *J Genet Genomics.* 40:271–279.

Chisholm ST, Coaker G, Day B, Staskawicz BJ. 2006. Host-Microbe Interactions: Shaping the Evolution of the Plant Immune Response. *Cell.* 124:803–814.

Churchill GA, Doerge RW. 1994. Emperical threshold values for quantitative trait mapping. *Genetics*, *138*,963-971.

Ciuffetti LM, Manning VA, Pandelova I, Betts MF, Martinez JP. 2010. Host-selective toxins, Ptr ToxA and Ptr ToxB, as necrotrophic effectors in the *Pyrenophora tritici-repentis*–wheat interaction. *New Phytol.* 187:911–919.

Ciuffetti LM, Tuori RP. 1999. Advances in the Characterization of the *Pyrenophora tritici-repentis*—Wheat Interaction. *Phytopathology*. 89:444–449.

Ciuffetti LM, Tuori RP, Gaventa JM. 1997. A single gene encodes a selective toxin causal to the development of tan spot of wheat. *Plant Cell Online*. 9:135–144.

Clergeot P-H, Schuler H, Mortz E, Brus M, Vintila S, Ekengren S. 2012. The Corky root rot pathogen *Pyrenochaeta lycopersici* secretes a proteinaceous inducer of cell death affecting host plants differentially. *Phytopathology*. 102:878-891.

Crook AD, Friesen TL, Liu ZH, Ojiambo PS, Cowger C. 2012. Novel necrotrophic effectors from *Stagonospora nodorum* and corresponding host sensitivities in winter wheat germplasm in the Southeastern United States. *Phytopathology*. 102:498–505.

Cunfer BM, Ueng PP. 1999. Taxonomy and Identification of *Septoria* and *Stagonospora* Species on Small-Grain Cereals1. *Annu Rev Phytopathol.* 37:267–284.

Czembor PC, Arseniuk E, Czaplicki A, Song Q, Cregan PB, Ueng PP. 2003. QTL mapping of partial resistance in winter wheat to *Stagonospora nodorum* blotch. *Genome*. 46:546–554.

Du Fall LA, Solomon PS. 2013. The necrotrophic effector SnToxA induces the synthesis of a novel phytoalexin in wheat. *New Phytol.* 200:185–200.

Effertz RJ, Meinhardt SW, Anderson JA, Jordahl JG, Francl LJ. 2002. Identification of a chlorosis-inducing toxin from *Pyrenophora tritici-repentis* and the chromosomal location of an insensitivity locus in wheat. *Phytopathology*. 92:527–533.

Eyal Z. 1999. The *Septoria tritici* and *Stagonospora nodorum* blotch diseases of wheat. *Eur J Plant Pathol.* 105:629–641.

Faris J, Anderson JA, Francl LJ, Jordahl JG. 1996. Chromosomal location of a gene conditioning insensitivity in wheat to a necrosis-inducing culture filtrate from *Pyrenophora tritici-repentis*. *Phytopathology*. 86:459-463.

Faris JD, Zhang Z, Lu H, Lu S, Reddy L, Cloutier S, Fellers JP, Meinhardt SW, Rasmussen JB, Xu SS, et al. 2010. A unique wheat disease resistance-like gene governs effector-triggered susceptibility to necrotrophic pathogens. *Proc Natl Acad Sci.*107:13544-13549.

Fauser F, Schiml S, Puchta H. 2014. Both CRISPR/Cas-based nucleases and nickases can be used efficiently for genome engineering in Arabidopsis thaliana. *Plant J.* 79:348–359.

Feng Z, Zhang B, Ding W, Liu X, Yang D-L, Wei P, Cao F, Zhu S, Zhang F, Mao Y, Zhu J-K. 2013. Efficient genome editing in plants using a CRISPR/Cas system. *Cell Res.* 23:1229–1232.

Fernandez MR, Dusabenyagasani M, Pearse PG, Holzgang G. 2008. Leaf spotting diseases of common and durum wheat in Saskatchewan in 2007. *Can. Plant Dis. Surv.* 88:80–82.

Fernandez MR, Hughes GR, Pearse PG. 2002. Leaf spot diseases of durum wheat in Saskatchewan in 2001. *Can. Plant Dis. Surv.*82:34–35.

Flor HH. 1956. The complementary genetic systems in flax and flax rust. *Adv. Genet.,* 8: 29–54.

Francki MG, Shankar M, Walker E, Loughman R, Golzar H, Ohm H. 2011. New quantitative trait loci in wheat for flag leaf resistance to *Stagonospora nodorum* blotch. *Phytopathology*. 101:1278–1284.

Friesen T l., Chu C, Xu SS, Faris JD. 2012. SnTox5–Snn5: a novel *Stagonospora nodorum* effector–wheat gene interaction and its relationship with the SnToxA–Tsn1 and SnTox3–Snn3–B1 interactions. *Mol Plant Pathol*. 13:1101–1109.

Friesen TL, Faris JD. 2004. Molecular mapping of resistance to *Pyrenophora tritici-repentis* race 5 and sensitivity to Ptr ToxB in wheat. *Theor Appl Genet*. 109:464–471.

Friesen TL, Faris JD. 2010. Characterization of the wheat-*Stagonospora nodorum* disease system: what is the molecular basis of this quantitative necrotrophic disease interaction?. *Can J Plant Pathol.* 32:20–28.

Friesen TL, Faris JD, Solomon PS, Oliver RP. 2008. Host-specific toxins: effectors of necrotrophic pathogenicity. *Cell Microbiol.* 10:1421–1428.

Friesen TL, Meinhardt SW, Faris JD. 2007. The *Stagonospora nodorum*-wheat pathosystem involves multiple proteinaceous host-selective toxins and corresponding host sensitivity genes that interact in an inverse gene-for-gene manner. *Plant J.* 51:681–692.

Friesen TL, Rasmussen JB, Kwon CY, Francl LJ, Meinhardt SW. 2002. Reaction of Ptr ToxA-insensitive wheat mutants to *Pyrenophora tritici-repentis* race 1. *Phytopathology*. 92:38–42.

Friesen TL, Stukenbrock EH, Liu Z, Meinhardt S, Ling H, Faris JD, Rasmussen JB, Solomon PS, McDonald BA, Oliver RP. 2006. Emergence of a new disease as a result of interspecific virulence gene transfer. *Nat Genet.* 38:953–956.

Friesen TL, Zhang Z, Solomon PS, Oliver RP, Faris JD. 2008. Characterization of the Interaction of a novel *Stagonospora nodorum* host-selective toxin with a wheat susceptibility gene. *Plant Physiol.* 146:682–693.

Gamba FM, Lamari L, Brülé-Babel AL. 1998. Inheritance of race-specific necrotic and chlorotic reactions induced by *Pyrenophora tritici-repentis* in hexaploid wheats. *Can J Plant Pathol.* 20:401–407.

Gilbert J, Woods SM. 2001. Leaf spot diseases of spring wheat in southern Manitoba farm fields under conventional and conservation tillage. *Can J Plant Sci.* 81:551–559.

Gilbert J, Woods SM, Tekauz A. 1998. Relationship between environmental variables and the prevalence and isolation frequency of leaf-spotting pathogens in spring wheat. *Can J Plant Pathol.* 20:158–164.

Gilchrist DG, Bostock RM, Wang H. 1995. Sphingosine-related mycotoxins in plant and animal diseases. *Can J Bot*. 73:459–467.

Gonzalez-Hernandez JL, Singh PK, Mergoum M, Adhikari TB, Kianian SF, Simsek S, Elias EM. 2009. A quantitative trait locus on chromosome 5B controls resistance of *Triticum turgidum* (L.) var. *diccocoides* to *Stagonospora nodorum* blotch. *Euphytica.* 166:199–206.

Gustafson P, Raskina O, Ma X, Nevo E. 2009. Wheat evolution, domestication, and improvement. In: Carver, B. F. editor. Wheat science and trade. Ames: Wiley-Blackwell. p. 5-30.

Haen KM, Lu H, Friesen TL, Faris JD. 2004. Genomics, molecular genetics & biotechnology - Genomic targeting and high-resolution mapping of the *Tsn1* gene in wheat. *Crop Sci.* 44:951–962.

Hammond-Kosack KE, Rudd JJ. 2008. Plant resistance signalling hijacked by a necrotrophic fungal pathogen. *Plant Signal Behav.* 3:993–995.

Horbach R, Navarro-Quesada AR, Knogge W, Deising HB. 2011. When and how to kill a plant cell: Infection strategies of plant pathogenic fungi. *J Plant Physiol*. 168:51–62.

Huang X-Q, Cloutier S. 2007. Hemi-nested touchdown PCR combined with primer-template mismatch PCR for rapid isolation and sequencing of low molecular weight glutenin subunit gene family from a hexaploid wheat BAC library. *BMC Genet.* 8:18.

Joehanes R, Nelson JC. 2008. QGene 4.0, an extensible Java QTL-analysis platform. *Bioinformatics*, 24:2788-2789.

Karjalainen R, Lounatmaa K. 1986. Ultrastructure of penetration and colonization of wheat leaves by *Septoria nodorum*. *Physiol Mol Plant Pathol*. 29:263–270.

Keller B, Winzeler H, Winzeler M, Fried PM. 1994. Differential Sensitivity of Wheat Embryos against Extracts Containing Toxins of *Septoria nodorum*: First Steps towards in vitro Selection. *J Phytopathol*. 141:233–240.

Keller SM, McDermott JM, Pettway RE, Wolfe MS, McDonald BA. 1997. Gene Flow and Sexual Reproduction in the Wheat Glume Blotch Pathogen *Phaeosphaeria nodorum* (Anamorph *Stagonospora nodorum*). *Phytopathology.* 87:353–358.

Kim YM, Strelkov SE. 2007. Heterologous expression and activity of Ptr ToxB from virulent and avirulent isolates of *Pyrenophora tritici-repentis*. *Can J Plant Pathol.* 29:232–242.

King JE, Cook RJ, Melville SC. 1983. A review of *Septoria* diseases of wheat and barley. *Ann Appl Biol.* 103:345–373.

Kodama M, Rose MS, Yang G, Yun SH, Yoder OC, Turgeon BG. 1999. The translocation-associated tox1 locus of *Cochliobolus heterostrophus* is two genetic elements on two different chromosomes. *Genetics.* 151:585–596.

Kono Y, Kinoshita T, Takeuchi S, Daly JM. 1986. Structure of HV-toxin M, a host specific toxin-related compound produced by *Helminthosporium victoriae*. *Agric Biol Chem*. 50:2689–2691.

Krupinsky JM. 1982. Comparative Pathogenicity of *Septoria nodorum* Isolated from *Triticum aestivum* and *Agropyron* Species. *Phytopathology*. 72:660-661.

Krupinsky JM. 1986. Virulence on wheat of *Leptosphaeria nodorum* isolates from *Bromus inermis*. *Can J Plant Pathol.* 8:201–207.

Krupinsky JM. 1997. Aggressiveness of *Stagonospora nodorum* isolates from perennial grasses on wheat. *Plant Dis*. 81:1032–1036.

Lamari L. 1991. Genetics of tan necrosis and extensive chlorosis in tan spot of wheat caused by *Pyrenophora tritici-repentis*. *Phytopathology*. 81:1092-1095.

Lamari L, Bernier CC. 1989. Toxin of *Pyrenophora tritici-repentis* : Host-specificity, significance in disease, and inheritance of host reaction. *Phytopathology*. 79:740-744.

Lamari L, Strelkov SE. 2010. Minireview/ Minisynthèse The wheat/*Pyrenophora tritici-repentis* interaction: progress towards an understanding of tan spot disease . *Can J Plant Pathol*. 32:4–10.

Lamari L, Strelkov SE, Yahyaoui A, Orabi J, Smith RB. 2003. The identification of two new races of *Pyrenophora tritici-repentis* from the host center of diversity confirms a one-to-one relationship in tan spot of wheat. *Phytopathology*. 93:391–396.

Liu Z, Faris JD, Oliver RP, Tan K-C, Solomon PS, McDonald MC, McDonald BA, Nunez A, Lu S, Rasmussen JB, Friesen TL. 2009. SnTox3 Acts in effector triggered susceptibility to induce disease on wheat carrying the *Snn3* gene. *PLoS Pathog*. 5:e1000581.

Liu Z, Friesen TL, Ling H, Meinhardt SW, Oliver RP, Rasmussen JB, Faris JD. 2006. The Tsn1–ToxA interaction in the wheat–*Stagonospora nodorum* pathosystem parallels that of the wheat–tan spot system. *Genome.* 49:1265–1273.

Liu ZH, Faris JD, Meinhardt SW, Ali S, Rasmussen JB, Friesen TL. 2004. Genetic and physical mapping of a gene conditioning sensitivity in wheat to a partially purified host-selective toxin produced by *Stagonospora nodorum*. *Phytopathology*. 94:1056–1060.

Liu ZH, Friesen TL, Rasmussen JB, Ali S, Meinhardt SW, Faris JD. 2004. Quantitative trait loci analysis and mapping of seedling resistance to *Stagonospora nodorum* leaf blotch in wheat. *Phytopathology*. 94:1061–1067.

Liu Z, Zhang Z, Faris JD, Oliver RP, Syme R, McDonald MC, McDonald BA, Solomon PS, Lu S, Shelver WL, et al. 2012. The cysteine rich necrotrophic effector SnTox1 produced by *Stagonospora nodorum* triggers susceptibility of wheat lines harboring Snn1. *PLoS Pathog.* 8:e1002467.

Lorang JM, Sweat TA, Wolpert TJ. 2007. Plant disease susceptibility conferred by a "resistance" gene. *Proc Natl Acad Sci U S A.* 104:14861–14866.

Lorieux M. 2012 MapDisto: Fast and efficient computation of genetic linkage maps. *Mol Breed*. 30:1231-1235.

Lu S, Faris JD, Sherwood R, Friesen TL, Edwards MC. 2014. A dimeric PR-1-type pathogenesis-related protein interacts with ToxA and potentially mediates ToxA-induced necrosis in sensitive wheat. *Mol Plant Pathol*. 15:650–663.

Ma H, Hughes GR. 1993. Resistance to *Septoria nodorum* blotch in several *Triticum* species. *Euphytica.* 70:151–157.

Manning VA, Andrie RM, Trippe AF, Ciuffetti LM. 2004. Ptr ToxA requires multiple motifs for complete activity. *Mol Plant Microbe Interact.* 17:491–501.

Manning VA, Chu AL, Steeves JE, Wolpert TJ, Ciuffetti LM. 2009. A host-selective toxin of *Pyrenophora tritici-repentis*, PtrToxA, induces photosystem changes and reactive oxygen species accumulation in sensitive wheat. *Mol Plant Microbe Interact.* 22:665–676.

Manning VA, Ciuffetti LM. 2005. Localization of PtrToxA produced by *Pyrenophora tritici-repentis* reveals protein import into wheat mesophyll cells. *Plant Cell Online.* 17:3203–3212.

Manning VA, Hardison LK, Ciuffetti LM. 2007. PtrToxA interacts with a chloroplast-localized protein. *Mol Plant Microbe Interact*. 20:168–177.

Markham JE, Hille J. 2001. Host-selective toxins as agents of cell death in plant–fungus interactions. *Mol Plant Pathol.* 2:229–239.

Martinez JP, Ottum SA, Ali S, Francl LJ, Ciuffetti LM. 2001. Characterization of the ToxB gene from *Pyrenophora tritici-repentis*. *Mol Plant Microbe Interact.* 14:675–677.

Matlock ED, McCartney CA, Gilbert J. 2012. Physiological specialization in the western Canadian population of *Phaeosphaeria nodorum*. *Can J Plant Pathol.* 34:75–82.

McDonald BA. 1994. Genetic variability in nuclear DNA in field populations of Stagonospora nodorum. *Phytopathology*. 84:250-255.

McDonald MC, Razavi M, Friesen TL, Brunner PC, McDonald BA. 2012. Phylogenetic and population genetic analyses of *Phaeosphaeria nodorum* and its close relatives indicate cryptic species and an origin in the Fertile Crescent. *Fungal Genet Biol.* 49:882–895.

Meehan F, Murphy HC. 1946. A new *Helminthosporium* blight of oats. *Science.* 104:413.

Meehan F, Murphy HC. 1947. Differential phytotoxicity of metabolic by-products of *Helminthosporium victoriae*. *Science*. 106:270–271.

Meinhardt SW, Cheng W, Kwon CY, Donohue CM, Rasmussen JB. 2002. Role of the arginyl-glycyl-aspartic motif in the action of PtrToxA produced by *Pyrenophora tritici-repentis*. *Plant Physiol.* 130:1545–1551.

Menzies J, Gilbert J. 2003. Diseases of wheat. In: Bailey KL, Gossen BD, Gugel RK, Morrall RAA. editors. Diseases of field crops in Canada. Saskatoon: Houghton Boston; p. 94-128.

Miedaner T, Risser P, Paillard S, Schnurbusch T, Keller B, Hartl L, Holzapfel J, Korzun V, Ebmeyer E, Utz HF. 2012. Broad-spectrum resistance loci for three quantitatively inherited diseases in two winter wheat populations. *Mol Breed*. 29:731–742.

Murphy NE, Loughman R, Appels R, Lagudah ES, Jones MGK. 2000. Genetic variability in a collection of *Stagonospora nodorum* isolates from Western Australia. *Aust J Agric Res*. 51:679–684.

Murray GM, Brennan JP. 2009. Estimating disease losses to the Australian wheat industry. *Australas Plant Pathol.* 38:558–570.

Navarre DA, Lorang JM, Moore DL, Wolpert TJ. 1995. Molecular interactions of victorin and oats. *Can J Bot.* 73:475–482.

Newey LJ, Caten CE, Green JR. 2007. Rapid adhesion of *Stagonospora nodorum* spores to a hydrophobic surface requires pre-formed cell surface glycoproteins. *Mycol Res.* 111:1255–1267.

Nolan S, Cooke BM. 2000. Control of *Stagonospora nodorum* and *Septoria tritici* in wheat by pre-treatment with *Drechslera teres*, a non-host pathogen. *Eur J Plant Pathol*. 106:203–207.

Nürnberger T, Brunner F, Kemmerling B, Piater L. 2004. Innate immunity in plants and animals: striking similarities and obvious differences. *Immunol Rev.* 198:249–266.

Oliver RP, Friesen TL, Faris JD, Solomon PS. 2012. *Stagonospora nodorum*: From pathology to genomics and host resistance. *Annu Rev Phytopathol.* 50:23–43.

Oliver RP, Rybak K, Solomon PS, Ferguson-Hunt M. 2009. Prevalence of ToxA-sensitive alleles of the wheat gene *Tsn1* in Australian and Chinese wheat cultivars. *Crop Pasture Sci.* 60:348–352.

Oliver RP, Solomon PS. 2010. New developments in pathogenicity and virulence of necrotrophs. *Curr Opin Plant Biol.* 13:415–419.

Orolaza NP. 1995. Evidence of a host-specific chlorosis toxin from *Pyrenophora tritici-repentis*, the causal agent of tan spot of wheat. *Phytopathology*. 85:1282-1287.

Otani H, Kohmoto K, Kodama M. 1995. Alternaria toxins and their effects on host plants. *Can J Bot.* 73:453–458.

Quaedvlieg W, Verkley GJM, Shin H-D, Barreto RW, Alfenas AC, Swart WJ, Groenewald JZ, Crous PW. 2013. Sizing up *Septoria*. *Stud Mycol.* 75:307–390.

Rampitsch C, Bykova NV. 2009. Methods for functional proteomic analyses. In: Somers DJ, Langridge P, Gustafson JP. editors. Plant genomics methods and protocols. New York: Humana press, c/o Springer Science and Business media; p. 93-110.

Sarma GN, Manning VA, Ciuffetti LM, Karplus PA. 2005. Structure of Ptr ToxA: An RGD-containing host-selective toxin from *Pyrenophora tritici-repentis*. *Plant Cell Online*. 17:3190–3202.

Sacco MA, Moffett P. 2009. Disease resistance genes: Form and function. In: Bouarab K, Brisson N, Daayf F. editors. Molecular plant-microbe interactions. Bodmin: MPG books group; p. 94-141.

Schnurbusch T, Paillard S, Fossati D, Messmer M, Schachermayr G, Winzeler M, Keller B. 2003. Detection of QTL for *Stagonospora* glume blotch resistance in Swiss winter wheat. *Theor Appl Genet.* 107:1226–1234.

Schuelke M. 2000. An economic method for the fluorescent labeling of PCR fragments. *Nat. Biotechnol.* 18:233–234.

Shah DA, Bergstrom GC, Ueng PP. 2001. Foci of *Stagonospora nodorum* Blotch in winter wheat before canopy development. *Phytopathology*. 91:642–647.

Shan Q, Wang Y, Li J, Gao C. 2014. Genome editing in rice and wheat using the CRISPR/Cas system. *Nat Protoc.* 9:2395–2410.

Shankar M, Walker E, Golzar H, Loughman R, Wilson RE, Francki MG. 2008. Quantitative trait loci for seedling and adult plant resistance to *Stagonospora nodorum* in wheat. *Phytopathology*. 98:886–893.

Shipton WA, Boyd WRJ, Rosielle AA, Shearer BI. 1971. The common *Septoria* diseases of wheat. *Bot Rev.* 37:231–262.

Sleper DA, Poehlman JM. 2006. Breeding field crops. Ames: Blackwell publishing. Chapter 4, Quantitative inheritance in plant breeding; p. 53-70.

Solomon PS, Lowe RGT, Tan K-C, Waters ODC, Oliver RP. 2006a. *Stagonospora nodorum*: Cause of *Stagonospora nodorum* blotch of wheat. *Mol Plant Pathol*. 7:147–156.

Solomon PS, Parker K, Loughman R, Oliver RP. 2004. Both mating types of *Phaeosphaeria* (anamorph *Stagonospora*) *nodorum* are present in western Australia. *Eur J Plant Pathol.* 110:763–766.

Solomon PS, Wilson TJG, Rybak K, Parker K, Lowe RGT, Oliver RP. 2006b. Structural characterisation of the interaction between *Triticum aestivum* and the dothideomycete pathogen *Stagonospora nodorum*. *Eur J Plant Pathol*. 114:275–282.

Somers DJ, Isaac P, Edwards K. 2004. A high-density microsatellite consensus map for bread wheat (*Triticum aestivum* L.). *Theor. Appl. Genet.* 109:1105-1114.

Sommerhalder RJ, McDonald BA, Mascher F, Zhan J. 2010. Sexual recombinants make a significant contribution to epidemics caused by the wheat pathogen *Phaeosphaeria nodorum*. *Phytopathology*. 100:855–862.

Sommerhalder RJ, McDonald BA, Zhan J. 2006. The frequencies and spatial distribution of mating types in *Stagonospora nodorum* are consistent with recurring sexual reproduction. *Phytopathology*. 96:234–239.

Stock WS, Brûlé-Babel AL, Penner GA. 1996. A gene for resistance to a necrosis-inducing isolate of *Pyrenophora tritici-repentis* located on 5BL of *Triticum aestivum* cv. Chinese Spring. *Genome*. 39:598–604.

Strelkov SE, Lamari L, Ballance GM. 1999. Characterization of a host-specific protein toxin (Ptr ToxB) from *Pyrenophora tritici-repentis*. *Mol Plant Microbe Interact*. 12:728–732.

Strelkov S, Lamari L, Ballance GM, Orolaza NP. 1998. Isolation and mode of action of Ptr chlorosis toxin from *Pyrenophora Tritici-Repentis*. In: Kohmoto K, Yoder OC, editors. Mol genetics of host-specific toxins in plant disease [Internet]. [cited 2014 Aug 29]. Available from: http://link.springer.com/chapter/10.1007/978-94-011-5218-1_15

Strickberger MW. 1985. Genetics. 3rd ed. Macmillan publishing company, New York, NY. p. 842

Stukenbrock EH, Banke S, McDONALD BA. 2006. Global migration patterns in the fungal wheat pathogen *Phaeosphaeria nodorum*. *Mol Ecol*. 15:2895–2904.

Stukenbrock EH, Mcdonald BA. 2007. Geographical variation and positive diversifying selection in the host-specific toxin SnToxA. *Mol Plant Pathol.* 8:321–332.

Tai Y-S, Bragg J, Meinhardt SW. 2007. Functional characterization of ToxA and molecular identification of its intracellular targeting protein in wheat. *Am J Plant Physiol.* 2:76–89.

Tomas A, Bockus WW. 1987. Cultivar-specific toxicity of culture filtrates of *Pyrenophora tritici-repentis*. *Phytopathology*. 77:1337-1340.

Tomas A, Feng GH, Reeck GR, Bockus WW, Leach JE. 1990. Purification of a cultivar-specific toxin from *Pyrenophora tritici-repentis*, causal agent of tan spot of wheat. *Mol Plant Microbe Interact*. 3:221-224.

Tovkach A, Zeevi V, Tzfira T. 2009. A toolbox and procedural notes for characterizing novel zinc finger nucleases for genome editing in plant cells. *Plant J.* 57:747–757.

Tuori RP, Ciuffetti LM. 1995. Purification and immunological characterization of toxic components from cultures of *Pyrenophora tritici-repentis*. *Mol Plant Microbe Interact.* 8:41-48.

Tuori RP, Wolpert TJ, Ciuffetti LM. 2000. Heterologous expression of functional Ptr ToxA. *Mol Plant Microbe Interact.* 13:456–464.

Uphaus J, Walker E, Shankar M, Golzar H, Loughman R, Francki M, Ohm H. 2007. Quantitative trait loci identified for resistance to *Stagonospora* glume blotch in wheat in the USA and Australia. *Crop Sci*. 47:1813-1822.

Vergnes DM, Zhanarbekova A, Renard M-E, Duveiller E, Maraite H. 2006. Mating types of *Phaeosphaeria nodorum* (anamorph *Stagonospora nodorum*) from central Asia. *J Phytopathol.* 154:317–319.

Vincent D, Du Fall LA, Livk A, Mathesius U, Lipscombe RJ, Oliver RP, Friesen TL, Solomon PS. 2012. A functional genomics approach to dissect the mode of action of the *Stagonospora nodorum* effector protein SnToxA in wheat. *Mol Plant Pathol.* 13:467–482.

Vleeshouwers VGAA, Oliver RP. 2014. Effectors as tools in disease resistance breeding against biotrophic, hemibiotrophic, and necrotrophic plant pathogens. *Mol Plant Microbe Interact.* 27:196–206.

Voorrips RE. 2002. Mapchart: Software for the graphical presentation of linkage maps and QTLs. *J Hered*. 93:77-78

Walton JD. 1996. Host-selective toxins: agents of compatibility. *Plant Cell Online*. 8:1723–1733.

Walton JD, Panaccione DG. 1993. Host-selective toxins and disease specificity: Perspectives and progress. *Annu Rev Phytopathol.* 31:275–303.

Wicki W, Messmer M, Winzeler M, Stamp P, Schmid JE. 1999. In vitro screening for resistance against *Septoria nodorum* blotch in wheat. *Theor Appl Genet.* 99:1273–1280.

Wolpert TJ, Dunkle LD, Ciuffetti LM. 2002. Host-selective toxins and avirulence determinants: What's in a name?. *Annu Rev Phytopathol.* 40:251–285.

Zhang H-F, Francl LJ, Jordahl JG, Meinhardt SW. 1997. Structural and physical properties of a necrosis-inducing toxin from *Pyrenophora tritici-repentis*. *Phytopathology*. 87:154–160.

Zhang Z, Friesen TL, Xu SS, Shi G, Liu Z, Rasmussen JB, Faris JD. 2011. Two putatively homoeologous wheat genes mediate recognition of SnTox3 to confer effector-triggered susceptibility to *Stagonospora nodorum*. *Plant J*. 65:27–38.