ABSTRACT

BERTUCCI, MATTHEW BENJAMIN. Genes Conferring Sensitivity to Stagonospora nodorum Necrotrophic Effectors in SNB-Susceptible Wheat Cultivars in the Southeastern United States. (Under the direction of Dr. Christina Cowger.)

*Stagonospora nodorum* is a fungal pathogen that causes *Stagonospora nodorum* blotch (SNB) of wheat, a disease of economic importance in wheat-growing regions worldwide. SNB reduces yield and quality, and can cause significant losses when weather conditions favor disease development. SNB is a polycyclic disease with multiple cycles of pathogen reproduction per crop cycle. *S. nodorum* reproduces both sexually and asexually. The asexual conidia are disseminated by rainsplash, allowing disease to travel up the canopy as the growing season progresses. The sexual ascospores are wind-dispersed and may travel long distances. The pathogen is capable of overwintering in stubble of the previous year’s wheat crop and may also infect seed, both serving as primary inoculum the following season.

As a necrotrophic pathogen, *S. nodorum* grows on dead host tissue rather than living cells. It produces a set of proteinaceous necrotrophic effectors (NEs) that elicit a hypersensitive response (HR) in the host, resulting in localized cell death. The HR is a common defense mechanism against biotrophic pathogens, but the cell death produces additional tissue for necrotrophic pathogens to colonize. This leads to increased lesion sizes. The goal of this study was to identify genes in a genetically diverse set of SNB-susceptible southeastern U.S. wheat lines that confer sensitivity to NEs. By conducting a series of infiltration experiments, it was possible to identify which sensitivity genes were most common in these cultivars, which represent the germplasm of 8 southeastern U.S. wheat
breeding programs. Additionally, using molecular techniques, the diagnostic potential was investigated of molecular markers that had been reported for the sensitivity genes of interest.
Genes Conferring Sensitivity to *Stagonospora nodorum* Necrotrophic Effectors in SNB-Susceptible Wheat Cultivars in the southeastern United States

by
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BIOGRAPHY

Matthew was born and raised in Chalmette, Louisiana. He studied marine biology at Spring Hill College in Mobile, Alabama, and graduated with a B.S. in 2009. He returned to Louisiana and worked as a research associate at the LSU Health Science Center studying Candida albicans. After reading The Triumph of the Fungi, Matthew became interested in plant pathology and fungal pathogens. He applied to North Carolina State University and was accepted as a Master’s student in the Department of Plant Pathology in 2011. Matthew selected a project centered around the fungal pathogen Stagonospora nodorum under the direction of Dr. Christina Cowger. Following completion of his Master’s Thesis, Matthew will begin work as a research associate studying viruses of cassava, common bean, and rice at CIAT in Cali, Colombia.
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1. CHAPTER I:

Literature Review

1.1 Wheat

Wheat is a flowering monocot of the family Poaceae, the grass family. Wheat was domesticated in the Fertile Crescent approximately 8,000 years ago and has a complex ancestry. A series of combination events between ancestral wheat land races has led to multiple ploidy levels among modern wheat varieties. Modern commercial wheat varieties include diploid einkorn wheat, Triticum monococcum monococcum (2n=2x=14, AA); tetraploid durum wheat, Triticum turgidum durum (2n=4x=28, AABB); tetraploid emmer wheat, Triticum turgidum dicoccum (2n=4x=28); and hexaploid common wheat or bread wheat, Triticum aestivum (2n = 6x = 42, AABBDD). In T. aestivum pairing only occurs between homologous chromosomes, so inheritance is disomic (diploid-like). In addition to being disomic, wheat is self-pollinated; so it is a relatively straightforward crop to handle for breeding purposes despite the large, hexaploid genome.

T. aestivum is grown throughout the world in high acreage. The grains are milled to produce flour for baking bread, cookies or used as feed for livestock. In 2012, wheat was planted across 5.6 billion acres and harvested from 4.9 billion acres worldwide (USDA-NASS 2013 Crop Reports). In the southeastern United States, winter wheat is planted rather than spring wheat. Winter wheat requires a vernalization period to initiate reproductive growth, so it is typically planted in rotation with other major crops such as corn, soybean or cotton. The high market value of wheat has led to an increase of 19% in acreage dedicated to soft red winter wheat production in the United States, totaling 9.67 million acres (USDA-
NASS 2013 Crop Reports). All southeastern states, with the exception of Louisiana, planted more acres than in 2011; and in North Carolina a record setting 960,000 acres have been planted for harvest in 2013 (Figure 1).

1.2 Stagonospora nodorum and Stagonospora nodorum Blotch

*Stagonospora nodorum*, teleomorph *Phaeosphaeria nodorum*, (formerly *Septoria nodorum*, teleomorph *Leptosphaeria nodorum* (14)) is a fungal pathogen causing Stagonospora nodorum blotch (SNB) on common wheat, durum wheat, and related grass species (57). Stagonospora nodorum blotch is a yield- and quality-reducing disease, especially severe in wet years (5,12). Symptoms begin as flecking at germination sites, which expand to form lens-shaped necrotic lesions, bordered by a chlorotic ring. Symptoms may arise on any aerial part of the plant, including leaves, stem, nodes, glumes (5). Asexual fruiting bodies (pycnidia) form as small brown/black specks within the lesions. As the disease progresses, lesions expand and eventually coalesce to form large necrotic patches. Typically symptoms will be more severe in the lower canopy, with milder symptoms in the upper canopy and the spikes.

Stagonospora nodorum blotch is a polycyclic disease with multiple pathogen generations per crop cycle, and *S. nodorum* reproduces both sexually and asexually. These factors contribute to a high evolutionary potential, making *S. nodorum* a pathogen of concern (42). The disease cycle begins when spores germinate on seedlings or the lower canopy. After germination appressoria penetrate directly through the cuticle, or germ tubes enter opportunistically through stomata (29,57). Penetrated cells die rapidly, and necrotic lesions begin to form around the site of infection. Under moist conditions, the mature lesions will
form pycnidia, which can exude conidia. Conidia are splashed upward by raindrops causing secondary infections throughout the growing season (7). Secondary infections are reliant on periodic rainfall: 0.7mm per hour is sufficient to spread conidia (64). With low inoculum levels, the wheat will grow faster than the latent period can be completed (70). The vertical spacing of leaves can contribute to spread: closer leaves hasten the spread of infection, particularly in the dwarf varieties (2,15). An open canopy allows for greater splash dispersal of conidia (2).

The latent period, the time from infection to sporulation, has long been known to effect the rate of epidemics (63). In *S. nodorum*, the latent period is influenced by moisture and temperature (56,70). The ideal conditions for germination are between 20° and 25°C at 98-100% relative humidity (64), with no sporulation occurring below 7°C (16). Insufficient rainfall or low temperatures can reduce SNB severity, even when all other conditions favor disease (64). Low temperatures prevent the formation of pycnidia, and lack of rainfall prevents the upward spread of conidia (64).

The environmental effects on the latent period of *S. nodorum* make disease forecasting difficult. Timing of fungicide applications cannot be based solely on the growth stage of wheat. Vereet and Hoffmann say it best: “From an epidemiological point of view, these stage-oriented treatments are randomly timed and randomly successful” (64). The rate of plant growth is also affected by temperature, so disease development is more accurately predicted using a model that accounts for time as well as temperature (70). With low inoculum levels, the wheat will grow faster than the latent period of *S. nodorum* can be
completed (70). These factors must be included in any forecasting model or in determining thresholds for fungicide application (64,70).

There are multiple sources of inoculum for *Stagonospora nodorum* epidemics. Conidia and ascospores can overwinter in wheat debris causing early infection; windborne ascospores can travel long distances and initiate infections; and infected seed carries the fungus (15,16,55). The host range of *S. nodorum* outside of wheat has not been clearly defined, though papers have mentioned alternative graminaceous hosts (55,57). These alternative hosts would present an additional source of inoculum (57). *S. nodorum* spores have been found on barley (in symptomatic lesions), but strains pathogenic to barley are not pathogenic to wheat (51). Disease reports indicate that the occurrence of *S. nodorum* on barley is mostly saprophytic (30). It has been suggested that *S. nodorum* survives on the alternative graminaceous hosts only due to artificial greenhouse conditions (66). Field experiments with SNB are often designed with barley as buffer rows, which always remain symptom free (Christina Cowger, personal observation).

Severe epidemics of SNB in wheat have been reported to cause yield losses of 31% (6) or up to 50% in susceptible varieties (15). Early in SNB resistance breeding efforts, King et al. 1983 found that foliar symptoms could be used to predict yield loss and develop SNB resistance; however, studies suggested that location and year were the best predictors of yield loss (54). A later study proposed the use of the thousand kernel weight to measure SNB resistance or tolerance, suggesting that disease severity ratings alone were not effective predictors of yield loss (28). Ultimately, it was shown that disease severity of SNB on the flag leaf (F) and penultimate leaf (F-1) at GS75 exhibits a linear relationship with yield loss,
explaining more than 80% of the variance in yield (6,69). Significant yield penalties occur when there is high humidity and rainfall during the developmental stages between anthesis and hard-dough stage (46,47). Additionally, toxin sensitivity has been shown to affect pathogenicity of isolates and ultimately the severity of SNB in the field (21,65). Therefore it is in the interest of plant breeders to select for wheat cultivars lacking sensitivity to the toxins expressed by S. nodorum and reduce yield loss to SNB.

1.3 Necrotrophic Effectors

*Stagonospora nodorum* is a necrotrophic fungal pathogen, growing on dead host tissue rather than living cells. It uses a set of necrotrophic effectors (NEs, formerly “host-selective toxins”) to elicit a hypersensitive response (HR) in the plant cell. The hypersensitive response is a form of programmed cell death (PCD) that is the result of the gene-for-gene model (20): host R-gene products recognize Avr gene products produced by the pathogen, initiate a signaling cascade, ultimately resulting in HR. The recognition of the effector by the plant and subsequent HR is a form of resistance called effector triggered immunity (ETI) (4). But the host’s HR works to the advantage of a necrotrophic pathogen, providing additional dead tissue for the pathogen to colonize, a condition labeled effector triggered susceptibility (ETS) (34). By eliciting HR in wheat, *S. nodorum* is capable of subverting the host’s defense, allowing for further infection and ultimately a reduction in yield. In contrast to Flor’s classic gene-for-gene system, the wheat-*S. nodorum* pathosystem is often described as an “inverse gene-for-gene system” (20,25,49).

Host-selective toxins (HSTs) are a set of compounds that act as virulence determinants in phytopathogenic fungi (41,67). The earliest description of a host-selective
toxin was the AK toxin produced by *Alternaria alternata* on Japanese pear (59). Many HSTs, such as AK toxin and victorin (53), are secondary metabolites; but some are proteinaceous (67), including those of *S. nodorum*. The HSTs of *S. nodorum* have been relabeled as necrotrophic effectors (NEs), a term that more accurately reflects the function of the genes and protein products in the pathosystem. A total of six NEs have been identified in the wheat-*S. nodorum* pathosystem: SnToxA, SnTox1, SnTox2, SnTox3, SnTox4, SnTox5, with NE each recognized by a corresponding sensitivity gene in the host: *Tsn1, Snn1, Snn2, Snn3, Snn4*, and *Snn5*, respectively (22,24). The NEs can act additively and epistatically, which could explain why resistance appears to be quantitatively inherited (44,45) despite the major effect of individual genes: *Tsn1* explains up to 62% of the variation in disease (37). Additionally, it is possible for a sensitivity gene to be present in multiple genomes. This has recently been shown to be the case with *Snn3* which has been mapped to the short arm of chromosome 5B and 5D (27,72).

Determining the host genes responsible for NE sensitivity required a set of lines with differential responses. A linkage map was created for the recombinant inbred lines (RILs) of the BG population, a cross of BR34 and Grandin (36). The parents are both hard red spring wheats with varying levels of resistance to SNB. BR34 shows better SNB resistance, but Grandin has better market qualities (36). The BG population produced a number of differentials for NE sensitivity. The RILs BG-261, BG-223, and BG-220 are positive controls for *Tsn1, Snn2*, and *Snn3*, respectively; Grandin contains *Tsn1, Snn2*, and *Snn3*, while BR34 contains no known sensitivity genes (25,27,37).
1.4 SnTox1-\textit{Snn}1

SnTox1 was the first discovered and characterized NE in \textit{S. nodorum} (33). \textit{SnTox1}, the gene coding for SnTox1, is present in 85\% of \textit{S. nodorum} isolates worldwide and is the most common of known effectors (35). In a study by Liu et al. (38), Sn2000 was used to produce NEs for infiltration into wheat seedlings. The \textit{S. nodorum} isolate Sn2000 was grown in culture, and partially purified SnTox1 was collected for infiltrations (for the procedure, see (23)). Treatment of the filtrate with Proteinase K stopped toxin activity, suggesting SnTox1 is proteinaceous in nature. By passing the filtrate through a series of filters (5, 10, and 30 kDa), researchers were able to determine that the NE is 10-30 kilodaltons (38). A population was prepared from a cross between SnTox1-sensitive and -insensitive parents, and mapping identified a major QTL on the short arm of chromosome 1B (38). The QTL explained 58\% of SNB variation in a population of RILs derived from a cross between the hard red spring wheat Opata 85 and the synthetic hexaploid wheat W-7984 (38). This QTL explains 58\% of the variation of SNB and contains the sensitivity gene that Liu et al. designated \textit{Snn1} (33,38).

\textit{SnTox1} has since been cloned and characterized, showing no homology to any genes in public databases (35). \textit{SnTox1} codes for a 117 amino acid protein estimated at 10.33 kDa with significant cysteine residues, which was shown to be heat- stable, maintaining full activity after 30 minutes of boiling and reduced activity after one hour of boiling (35). By transforming the yeast \textit{Pichia pastoris} to express \textit{SnTox1} in culture, infiltration experiments validated production and activity of SnTox1 (35). SnTox1 was shown to be light-dependent, with no activity when infiltrated seedlings were kept in dark (35). In the host, the toxin
played a role in penetration and caused an oxidative burst in the host, up-regulation of PR-proteins, and DNA laddering, all signatures of the HR in the host (35).

1.5 **SnToxA-Tsn1**

Certainly, the most studied and best understood NE of *S. nodorum* is SnToxA. SnToxA was first identified in the *Pyrenophora tritici-repentis*-wheat pathosystem. Two NEs with similar structure were independently discovered in *P. tritici-repentis*: Ptr necrosis toxin in 1989 (3) and Ptr toxin in 1990 (60). At the time, only one other proteinaceous fungal toxin, cerato-ulmin, was known (58). Later research revealed yet another necrosis-inducing toxin produced by *P. tritici-repentis* which was labeled ToxA (61). Molecular techniques allowed for better characterization of the proteins; and it was discovered that Ptr necrosis toxin, Ptr toxin and ToxA were all the same protein which would be designated Ptr ToxA (10,11). Genomic sequencing of the *S. nodorum* revealed a portion with very high similarity (99.7%) to ToxA in *P. tritici-repentis* (26). The sequences were analyzed for gene diversity between species and gene diversity among isolates of each species. Eleven haplotypes were discovered for *S. nodorum* ToxA, and only one was discovered for *P. tritici-repentis* ToxA, suggesting that ToxA had been present in the *S. nodorum* genome for a longer time (26). The high similarity between the toxins (99.7%) and the greater number of haplotypes in *S. nodorum* is strong evidence for an interspecific gene transfer event from *S. nodorum* to *P. tritici-repentis* (26).

SnToxA is a 13.2 kDa protein that interacts with the wheat sensitivity gene *Tsn1*, which is found on the long arm of chromosome 5B (11,17). The SnToxA-*Tsn1* interaction is substantial, accounting for 62% of the variation in disease when RILs from the BG
population were inoculated with conidial suspensions of *S. nodorum* (37) and up to 95% of the variation in disease of the inoculated durum wheat Langdon (18). The NE is present in *S. nodorum* populations worldwide: of 755 *S. nodorum* isolates sampled across nine geographical regions, 183 (24%) carry the gene for SnToxA, ranging from 100% in Australia to 5% in China (26). The SnToxA gene has been inserted into the *Pichia pastoris* and *Escherichia coli* microbial systems for NE production. The NE produced by these simpler organisms can be used for infiltration experiments to determine the presence of *Tsn1* in wheat cultivars (13,50).

The wheat sensitivity gene *Tsn1* has been closely studied as well. *Tsn1* is necessary for a sensitive reaction to both Ptr ToxA and SnToxA (26). Like the SnTox1-*Snn1* interaction, the SnToxA-*Tsn1* interaction is light-dependent (19). *Tsn1* codes for a protein similar to R gene products in plants. The protein has a NBS-LRR (nucleotide-binding site leucine rich repeat) domain as well as an S/TPK (serine/threonine protein kinase domain) (19). This is only the second gene identified to code for both an NBS-LRR and an S/TPK domain in the same transcript, the first being *Rpg5* of barley (8). And it is the first discovered to have a C-terminal NBS-LRR. The NBS-LRR domains usually determine the protein’s specificity of interaction, but *Tsn1* has been proven not to interact directly with SnToxA (19). One study suggests that *Tsn1* is involved in uptake of SnToxA into the cell (40), but Friesen et al. 2010 showed that the protein has no transmembrane domain (19). It is more likely that *Tsn1* is involved in a downstream pathway. Interestingly, the occurrence of *Tsn1* is higher in domesticated wheat varieties than in the wild types, suggesting that the *Tsn1* gene is being selected by breeders for advanced lines (19), but one study found no
significant correlation between *in vitro* ToxA production in *S. nodorum* populations and the occurrence of *Tsn1* in the cultivated wheat varieties of the same region (50). It is possible that the passive selection is due to some other beneficial trait, like resistance to a biotrophic pathogen. This was the case for the *LOV1* gene in oats, which confers resistance to crown rust caused by *Puccinia coronata* but also causes susceptibility to Victoria blight caused by *Cochliobolus victoriae* (39).

**1.6 SnTox2-Snn2**

In 2007 SnTox2, a third NE in *S. nodorum* was reported (25). SnTox2 is a 7- to 10-kDa protein. Infiltration experiments into lines from the BG population led to the discovery of a differential for sensitivity to SnTox2, BG-223 (25). The locus for sensitivity is on the short arm of chromosome 2D and is designated *Snn2*. The SnTox2-Snn2 interaction is light-dependent and acts additively with the SnToxA-Tsn1 interaction (25). Within the BG population, the SnTox2-Snn2 explains 47% of the variation in disease, the SnToxA-Tsn1 interaction explains 20% of the variation, and together they explain 66% of variation (25). The additive effect of these interactions is an important distinction from classic R-gene responses, where a single R-gene causes an incompatible response and leads to complete resistance (25, 67). The additive effect of the sensitivity genes reveals a breakdown in the “inverse gene-for-gene” analogy. As with many biological terms and models, the “inverse gene-for-gene” model conveys the general idea (recognition of NEs by the host leads to susceptibility) without capturing every nuance of the system described.
1.7 SnTox3-Snn3

In 2008 Friesen et al. (27) reported SnTox3, the fourth NE in the wheat-\textit{S. nodorum} pathosystem. SnTox3 was shown to be recognized by the host sensitivity gene \textit{Snn3}, which is located on the short arm of chromosome 5B (27). The BG population produced its third differential line for NE sensitivity, BG-220, which harbors \textit{Snn3} and has no other known sensitivities (27). In conidial inoculation trials, the SnTox3-\textit{Snn3} interaction accounted for a significant portion of variation of disease, explaining 8-13\% of the phenotypic variation (27). The \textit{Snn3} gene for sensitivity to SnTox3 was found in 90\% of the selected wheat varieties in western Australia; however, there were no significant differences in SnTox3 sensitivity among wheat varieties with different levels of SNB susceptibility (65). By comparing SnTox3 sensitivity to SNB susceptibility in the field, Waters et al. showed that sensitivity to SnTox3 has a greater impact on SNB susceptibility than does sensitivity to SnToxA (65).

In 2009, the \textit{SnTox3} gene was cloned and characterized as a 693-nucleotide sequence which codes for SnTox3, a 230 amino acid immature protein, prior to any post-translational modification (34). Transformation experiments proved that \textit{SnTox3} could be inserted into a non-pathogenic \textit{S.nodorum} strain, making it pathogenic to \textit{Snn3}-harboring lines (34). More recently, sensitivity to SnTox3 was shown to be regulated by a second locus in the wheat ancestor \textit{Aegilops tauschii} on the short arm of chromosome 5D (72). This has necessitated nomenclature to differentiate between each gene, \textit{Snn3-5B} and \textit{Snn3-5D}. Infiltration experiments showed a quantitative response to SnTox3, suggesting the possibility of additive effects between multiple sensitivity loci (65).
1.8 SnTox4-\textit{Snn4}

Using a wheat population derived from a cross between Swedish wheat varieties Arina and Forno (AF population), Abeysekara et al. (1) discovered the fifth NE (SnTox4) and sensitivity gene (\textit{Snn4}) in the wheat-\textit{S. nodorum} pathosystem. Arina is an SNB resistant cultivar, and Forno shows high susceptibility to SNB. A series of RILs was developed from the AF population. Infiltrations with NEs were conducted as well as inoculations of seedlings with conidia. Using the linkage map developed for the AF population, \textit{Snn4} was mapped to the short arm of chromosome 1A and accounted for 41\% of the phenotypic variation in disease in inoculated wheat seedlings (1). The sensitive response of \textit{Snn4} is light-dependent like \textit{Tsn1}, \textit{Snn1}, and \textit{Snn2}; but it results in mottled necrosis rather than severe necrosis of the other NEs. SnTox4 was shown to be proteinaceous and 10-30 kDa in size. Interestingly, Abeysekara et al. (1) suggest that \textit{Snn4} may be homoallelic to \textit{Snn1}. The two sensitivity genes lie on the short arm of chromosome 1 on separate genomes, suggesting the two may have a common ancestral origin.

1.9 SnTox5-\textit{Snn5}

The most recently discovered necrotrophic effector is SnTox5, recognized by the sensitivity gene \textit{Snn5} on the long arm of chromosome 4B (24). This interaction was identified using the LP749 double-haploid (DH) tetraploid wheat population, prepared from a cross of the durum wheats Lebsock and PI 94749 (8). SnTox5 is a 10- to 30-kDa protein that is heat stable, although it shows reduced activity after boiling. The interaction is light-dependent and acts additively with the SnToxA-\textit{Tsn1} interaction (24). LP749 lines were inoculated with conidia from Sn2000 and Sn2000 KO6-1. The \textit{S. nodorum} isolate Sn2000
produces both SnToxA and SnTox5, and the Sn2000 KO6-1 mutant does not produce SnToxA. In the LP749 DH population, the SnTox5-Snn5 interaction explained 63% of variation in disease of the wild-type (Sn2000) and 37% of disease in the mutant (Sn2000 KO6-1) (24).

1.10 Molecular Markers

Phenotypic response is a reliable way to identify sensitivity to the NEs of *S. nodorum*, but molecular methods reveal the genotype of the host. Molecular markers can be quickly scored and can confirm which genes are responsible for the phenotype observed. Wheat has seven known sensitivity genes that recognize six NEs produced by *S. nodorum* (22,24,72), and it is important to identify which genes are contributing to disease susceptibility in a population. Plants have shown partial resistance to SNB, but no complete resistance. Inheritance of resistance to SNB was previously thought to be polygenic, involving many genes of minor effect (44,45,49,57). More recent studies identify a number of major genes in host that explain large variations in the level of disease.

Molecular markers have been mapped across the hexaploid wheat genome, so discovery of a gene usually starts with the discovery of a quantitative trait locus (QTL) identified with markers. Rather than physical distance measured in base pairs (bp), it is more useful to measure a gene’s position in centimorgans (cM). Centimorgans measure distance relative to the rate of recombination in that area of the genome. This unit of measurement is more descriptive of the marker’s association with the gene of interest. A tightly linked marker has a low centimorgan value and is not likely to be separated from a gene as a result recombination during meiosis. As the markers are compared between members of a
population, any polymorphisms in the product can be compared to identify regions of 
interest. Markers are often evaluated for diagnostic potential on a mapping population 
derived from a single cross. These reports are useful for preliminary studies, but it is 
necessary to evaluate a marker across a broad genetic background to ensure its utility for 
breeding programs (48). If a marker is diagnostic for the presence of a gene, it can be used 
for marker-assisted selection (MAS) and early-generation testing in plant breeding programs 
(68). One goal of this project is to evaluate the diagnostic potential of molecular markers in 
the areas of the wheat sensitivity genes in a novel genetic background, providing useful 
information for small grain breeders and pathologists in the area.

The BG population has been used for mapping wheat sensitivity genes to NEs 
produced by *S. nodorum* (71). The markers have delineated the genes to very narrow ranges 
(in cM) on their respective chromosomes, but recombination events may have occurred 
despite the tight linkage. Conventional breeding methods rely on phenotypic scoring, so 
many generations are required to achieve homozygosity at multiple loci. Implemented 
correctly, MAS would reduce the number of generations required to achieve homozygosity 
for insensitivity and reduce economic input (32). MAS has been particularly useful with 
backcrosses to introgress alleles for insensitivity into advanced lines (71).

The utility of a marker for MAS depends on multiple factors, and there are practical 
issues to consider before employing this strategy in a breeding program. MAS needs to be 
genetically and economically feasible before it can be implemented. Genetic constraints on 
MAS in wheat include availability of diagnostic markers for high effect QTL, fixation of 
genes in regions surrounding the QTL of interest, and linkage drag (43). Screening varieties
for *Fhb1* for resistance to Fusarium head blight is routine for breeding programs, but it was a long time frame to advance from identification of the *Fhb1* gene to practical application of MAS (43). The estimated effect of a resistance QTL in a mapping population is often greater than its effect upon introgression into elite material (62). Cross-validation reduces error in estimation of effects for QTL identified in mapping populations, ultimately leading to better decisions when implementing MAS in a breeding program (62).

1.11 Project Objectives

As has been previously reviewed, it is clear that NEs and host sensitivity genes play a significant role in susceptibility to SNB (49). Studies have demonstrated the efficacy of infiltration with culture filtrates of *S. nodorum* to determine the presence of sensitivity genes in particular wheat cultivars (13). The goal of this study was to determine which of the reported sensitivity genes are most common among southeastern wheat cultivars that are susceptible to SNB in the field. Screening for sensitivity was achieved by performing infiltration assays with NEs produced by *S. nodorum* into wheat seedlings. Infiltrations were conducted using engineered *Pichia pastoris* yeast strains that produce SnToxA, SnTox1, and SnTox3, as well as with 3 *S. nodorum* isolates with known NE production. Additionally, a collection of 33 southeastern *S. nodorum* isolates (selected from the isolates collected by Crook et al. 2012) was used for infiltration experiments to demonstrate which NEs are present in the pathogen population in the southeastern United States. It was also be possible to report or validate any novel NEs being produced by the southeastern *S. nodorum* isolates (13).
The BG population has produced differential wheat lines for sensitivity genes \textit{Tsn1}, \textit{Snn2}, and \textit{Snn3} (25,27,37). The results of the infiltration experiments were compared to molecular marker data, using these controls as a reference. Markers have been reported for the sensitivity genes \textit{Tsn1}, \textit{Snn1}, \textit{Snn2}, and \textit{Snn3-5B} (19,27,33,52,71). The markers have been categorized as mapped, fine-mapped, or cloned (49). Previous results provide confidence in the efficacy of the perfect marker \textit{Xfcp623} as a diagnostic marker for \textit{Tsn1} (19,49), but the other 11 markers selected showed higher degrees of polymorphisms in the amplified product.

The goals of this study were to (1) determine the sensitivity genes present in SNB-susceptible southeastern wheat cultivars, (2) identify and/or confirm the NEs in southeastern \textit{S. nodorum} isolates, and (3) evaluate the diagnostic potential of molecular markers for wheat sensitivity genes \textit{Tsn1}, \textit{Snn1}, \textit{Snn2}, and \textit{Snn3-5B}. Together, this information will be useful for wheat breeders in the southeastern United States in developing a program to select for SNB resistance by breeding out sensitivity genes in their cultivars. This information will also be useful to breeders and pathologists worldwide, revealing more information about the make-up of local \textit{S. nodorum} populations that can be useful in planning future studies.


**Figure 1.1** United States winter wheat planted by state for harvest in 2013. Blue, white, and red colorings represent an increase, no change, and decrease in acreage planted, respectively. The top number in each state represents the total acreage (in thousands of acres) of wheat planted for harvest in that state, and the bottom number represents the change from last year (in thousands of acres). NC indicates no change.
2. CHAPTER II:

Genes Conferring Sensitivity to Stagonospora nodorum Necrotrophic Effectors in SNB-Susceptible Wheat Cultivars in the Southeastern United States

by

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TO BE SUBMITTED FOR PUBLICATION IN PLANT DISEASE
2.1 Abstract

*Stagonospora nodorum* is a necrotrophic fungal pathogen that causes Stagonospora nodorum blotch (SNB), a yield- and quality-reducing disease of wheat. *S. nodorum* produces a set of necrotrophic effectors (NEs) that interact with the products of host sensitivity genes to cause cell death and increased susceptibility to disease. NEs were produced in culture and used for an infiltration bioassay to determine NE sensitivity among 25 winter wheat cultivars highly susceptible to SNB and the moderately resistant cultivar NC-Neuse. Thirty-three isolates of *S. nodorum* that had been collected from seven states in the southeastern U.S were cultured for NE production, along with control strains of *Pichia pastoris* that expressed *SnToxA*, *SnTox1*, or *SnTox3*. All SNB-susceptible cultivars were sensitive to at least one NE, while NC-Neuse was insensitive to all NEs tested. Among the selected lines, 32% contained *Tsn1* and 64% contained *Snn3*. None were sensitive to SnTox1. Additionally, ten molecular markers for sensitivity genes *Tsn1*, *Snn1*, *Snn2*, and *Snn3* were evaluated for diagnostic potential. Only the marker *Xfcp623* for *Tsn1* was diagnostic, and it was in perfect agreement with the results of the infiltration bioassays.

2.2 Introduction

Stagonospora nodorum blotch (SNB) is a yield- and quality-reducing disease of wheat (*Triticum aestivum*) that occurs ubiquitously in the southeastern United States (4,26). SNB is caused by the necrotrophic fungal pathogen *Stagonospora nodorum*, a well-studied model organism for the Dothideomycetes (23). When environmental conditions favor disease, SNB can cause yield losses up to 31% in susceptible wheat varieties (4). Disease symptoms arise as small necrotic lesions surrounded by a chlorotic halo at the site of
infection (26). Symptoms are found most commonly on the leaves and glumes but can occur on any aerial part of the plant (7). As disease progresses, lesions expand to form large necrotic patches. That pathogen undergoes multiple generations in a growing season, spreading upward in the canopy via rain-splashed conidia (7).

*S. nodorum* utilizes a set of proteinaceous necrotrophic effectors (NEs), also known as host-selective toxins, that have been shown to increase severity of disease (1,10,12,14,16,21,22). The NEs interact with the host in an inverse gene-for-gene manner: products of sensitivity genes in the host recognize the effectors produced by the fungus and trigger a hypersensitive response, leading to increased susceptibility in the field (10). Each NE produced by *S. nodorum* is recognized by at least one sensitivity gene in wheat (11,12). The best-characterized sensitivity gene *Tsn1* is a nucleotide binding site–leucine-rich repeat (NBS–LRR) class of protein, typical of those encoded by resistance (R) genes in plant defense against pathogens and some pests (2).

Development of SNB is dependent on environmental factors, including temperature and moisture (3,7,29), that are unpredictable. Wheat cultivars are most reliably evaluated for SNB resistance in field nurseries with artificial conditions that enhance disease development (5). It is possible that cultivars selected in the absence of SNB pressure could have been inadvertently selected for susceptibility to SNB, due to the hypothesized pleiotropy of NE sensitivity and resistance to other pathogens or to pests (13). Indeed, many advanced experimental wheat lines show high levels of susceptibility when tested in the USDA-ARS Eastern U.S. Septoria Nursery, where SNB pressure is uniform and adequate to separate performance of varieties [C. Cowger, personal observation].
The majority of wheat grown in the Southeast is soft wheat, useful for cookies and pastries but not for bread. Recently, some southeastern U.S. wheat breeders have established hard wheat programs aimed at producing bread wheats for local markets. Lines developed in the Great Plains have been used for crossing to introgress greater hardness. For the most part, these hard wheat cultivars were not selected for SNB resistance, and many are highly susceptible to SNB in the field [C. Cowger, personal observation]. Field susceptibility to SNB has been linked to NEs (10). SnTox3 sensitivity was found in a large majority of western Australian wheat cultivars (30). It was uncertain prior to this study which NEs might be contributing to the susceptible phenotypes that have been observed in the southeastern U.S.

Because NEs are known to play a major role in disease development, this study sought to determine which sensitivity genes were most prevalent in widely planted or representative SNB-susceptible wheat germplasm in the southeastern U.S. Field susceptibility data were available on some elite experimental materials, and pedigree information was also used to identify parents common to susceptible experimental lines. By conducting infiltration experiments with multiple S. nodorum isolates collected from across the region and with NE-producing Pichia pastoris yeast controls, we sought to determine which of the reported NEs contribute to susceptibility to SNB in the southeastern U.S.

The sensitivity genes in wheat have been identified and mapped to narrow regions in the wheat genome (11,12,23). Available molecular markers for sensitivity genes were evaluated for diagnostic potential by comparing the genotype and phenotype of the selected cultivars. The markers were identified by Dr. Justin Faris and colleagues in the hard red
spring wheat BG population, and an additional goal of this research was their cross-validation in winter wheat germplasm for potential use in marker-assisted selection (MAS) in the southeastern U.S.

2.3 Materials and Methods

2.3.1 Fungal materials. In a previous study by Crook et al. (6), 39 Stagonospora nodorum isolates had been derived from wheat debris collected in seven states in the southeastern United States. Of those isolates, 33 were used for infiltrations in the present study: 5 isolates from Arkansas, 5 isolates from Georgia, 1 isolate from Maryland, 10 isolates from North Carolina, 4 isolates from South Carolina, 4 isolates from Tennessee, and 4 isolates from Virginia. Each isolate was a different genetic individual and could potentially produce a different necrotrophic effector or effectors in culture.

Additionally, three North Dakota S. nodorum isolates kindly supplied by Dr. Tim Friesen were included due to their known NE production: Sn4 (18), Sn6 (14,18), and Sn2000 (17). Sn4 reportedly produced SnToxA, SnTox1, SnTox2, and SnTox3 in culture (18); Sn6 produced SnToxA, SnTox1, SnTox2 (14), and SnTox3 (18); and Sn2000 was used as a control for production of SnToxA (15), SnTox1 (17,22), and most recently SnTox5 (12).

All isolates were maintained on V8-PDA plates at 4°C until use in infiltrations. Long-term storage of cultures was achieved by placing mycelial plugs in 80% glycerol and storing them at -80°C.

Transformed Pichia pastoris yeast strains, also supplied by Dr. Friesen, were used as controls for NE production and infiltration. These strains had been engineered for heterologous gene expression of one NE each: SnToxA (15), SnTox1 (19), or SnTox3 (18).
For long term storage, cells of \textit{P. pastoris} strains were suspended in 80\% glycerol and stored at -80°C.

\textbf{2.3.2 Plant materials.} Wheat cultivars selected for this experiment either were highly susceptible to SNB in the field or were found in the pedigree of multiple highly susceptible cultivars. A total of 32 wheat cultivars were selected for screening: 26 cultivars that were the subject of this experiment and 6 controls with known sensitivity genes (Table 2.1).

Among the tested lines were commercial soft red winter wheat varieties from the southeastern U.S., such as AGS2000, Magnolia, and Pembroke. Also included were hard red winter wheat varieties such as Jagger, Siouxland, and Trego that have been used to introgress grain hardness into mid-Atlantic U.S. bread wheat germplasm. These lines had attractive traits such as leaf rust resistance or grain hardness and bread baking quality, and each had also shown high SNB susceptibility in the field. Some of the cultivars tested are used in U.S. breeding programs for disease resistance; e.g., the CIMMYT spring wheat Pavon 753, which possesses \textit{Lr47}, a gene for resistance to leaf rust (caused by \textit{Puccinia triticina}). Altogether, the lines of interest included 14 commercial varieties and 12 elite experimental lines. All were susceptible in the field except the moderately SNB-resistant NC-Neuse, a commercial soft red winter wheat variety.

The controls included Grandin, BR34, Chinese Spring, BG-223, BG-220, and BG-261. Grandin and BR34 were susceptible and resistant to SNB, respectively (10). Grandin was sensitive to SnToxA (15), SnTox2 (14), and SnTox3(16), while BR34 was insensitive. The two had been used as parents to create the BG population (20), a series of recombinant
inbred lines (RILs) segregating for sensitivity to NEs. BG-261, BG-223, and BG-220 were three of those RILs with sensitivity to SnToxA (15), SnTox2 (14), and SnTox3 (16), respectively. Chinese Spring, a landrace from China, was sensitive to SnTox1 (22,24).

2.3.3 Seedling growth conditions. Wheat cultivars were planted in 2M lightweight mix (Conrad Fafard, Inc., Agawam, MA) in RLC4 cone-tainers. Plants were watered from the bottom by placing cone-racks in a water-filled basin that was refilled as necessary. The plants were grown in a greenhouse with an average temperature of 22°C, or in a growth chamber at 21°C with a 12 hour photoperiod, until plants reached the 2- to 3-leaf stage (14 to 17 days).

For infiltration trials, the cones were laid out using a randomized block design with three replications across a 10-cone x 10-cone rack. The experimental unit was the planting cone, and each cone contained one plant of a single cultivar. All trials included the experimental cultivars to be tested as well as the controls for sensitivity: BR34, Grandin, BG-220, BG-223, BG-261, and Chinese Spring. Each plant was infiltrated with CFs from either the P. pastoris controls or the S. nodorum isolates. Trials were repeated for seeds that failed to germinate, and also for lines that were included later in the experiment (ARS 12-533, ARS 12-534, Chinese Spring, and NC-Neuse). In repeated trials, planting cones were randomized and all controls were included.

2.3.4 NE production. Stagonospora nodorum isolates were grown for 14 days on V8-PDA plates in a growth chamber at 26°C with a 12-hour photoperiod. The plates were stored at 4°C for up to 3 months. For liquid cultures, 4 to 6 mycelial cuttings totaling ~2 cm² were added to a 250-ml Erlenmeyer flask containing 75 ml of Fries medium (17). The flasks
were wrapped in aluminum foil to shield them from light, and were shaken at 80 rpm for 48 hours. The liquid cultures were then placed in a dark drawer for three weeks of stationary growth at room temperature (20°C).

*Pichia pastoris* controls were grown on yeast peptone dextrose (YPD) agar media at 27°C for 48 to 72 hours, and stored at 4°C for up to 3 months. To prepare liquid cultures, a single colony from a YPD plate was added to 1 ml of YPD broth. The 1-ml culture was shaken at 220 rpm at 30°C for 48 hours. Two-hundred microliters of the 1-ml culture was added to a vial containing 10 ml of fresh YPD broth. The 10-ml culture was then shaken at 220 rpm at 30°C for 48 hours.

NEs were collected by passing the liquid cultures through a series of filters. Each 75-ml liquid culture was filtered through a Whatman no. 1 filter paper by vacuum filtration, using a Büchner funnel and vacuum flask. The collected filtrate was drawn into a 60-ml syringe and filtered through a 0.80-µm Luer-lock filter tip. The remaining filtrate was again drawn into a 60-ml syringe and filtered through a 0.45-µm Luer-lock filter tip. The culture filtrate (CF) was then used for infiltrations, or stored at 4°C for up to 24 hours.

For *P. pastoris* controls, the 10-ml cultures were decanted carefully, leaving behind the majority of yeast sediment, then drawn into a 30-ml syringe and passed through a 0.45-µm Luer-lock filter tip. The CF was then used for infiltrations or stored at 4°C for up to 24 hours.

### 2.3.5 Infiltrations and scoring

Seedlings were grown to the 2- to 3-leaf stage (14 to 17 days) prior to infiltration. Infiltrations were performed with a 3-ml needleless syringe. The secondary leaf was held taut between the thumb and forefinger of one hand. With the
other hand, the syringe tip was pressed against the leaf tissue and the plunger depressed until 2 to 3 cm of leaf surface was infiltrated (~50 µL). The infiltrated region was then marked using a non-toxic felt-tip pen. Infiltrated plants were placed in a Percival growth chamber at 21°C with a 12-hour photoperiod. Plants were rated for reactions at 3, 5, and 7 days post-infiltration.

Sensitivity of wheat seedlings to CFs was scored visually, using a scale including three levels: insensitive (-), chlorotic (y), and necrotic (+) (Fig. 1). Ratings applied only to the infiltrated area marked by the felt-tip pen. An insensitive rating indicated that no tissue was discolored and the leaf remained healthy. A chlorotic rating indicated yellowing of tissue within the infiltrated region with some green tissue still visible. A necrotic rating indicated complete necrosis and leaf folding within the infiltrated region. Cultivars with chlorotic or necrotic ratings were considered “sensitive” for purposes of analysis. The ratings were performed 3, 5, and 7 days post-infiltration, but the final analysis was conducted using the results from day 7. Results of each trial were recorded as the majority score of three infiltrated plants.

2.3.6 Molecular markers. DNA was extracted from leaf tissue of each wheat cultivar using a modified protocol for *T. aestivum* extraction (27). DNA was diluted with sterile H₂O to a final concentration of 40 ng/µl for use in polymerase chain reaction (PCR) amplification. The DNA was stored in sealed freezer plates at 4°C until needed. Primer sets were selected for 10 molecular markers that have been reported for *Tsn1*, *Snn1*, *Snn2*, and *Snn3* (Table 2.2). Almost all were simple-sequence repeats (SSRs) reported to be linked to
the sensitivity genes. The markers were evaluated for diagnostic potential for their respective
sensitivity genes in the germplasm chosen for the current study.

Polymerase chain reactions (PCRs) contained 1X NH₄ buffer, 2.0 mM MgCl₂, 312.5
µM dNTP, 0.3 µM forward primer, 0.3 µM reverse primer, DNA at 40 ng/µl, and 1U Biolase
Taq polymerase. Each reaction was a total of 20 µl. The number of cycles and annealing
temperatures were adjusted for optimization of each marker. One of each pair of primers was
5’ labeled with IR 700 or IR 800 fluorescent labels. The fluorescent-labeled PCR product
was visualized on 6% polyacrylamide gel using a LI-COR 4200 gel imager (LI-COR
Biotechnology, Lincoln, NE). GeneProfiler 4.05 (Scanalytics Inc.) was used to score the
digital images of the polyacrylamide gels.

2.4 Results

2.4.1 NE sensitivity of cultivars. Sensitivity genes in the experimental wheat
cultivars were detected by infiltration with CFs produced by the P. pastoris yeast strains.
The infiltration assay showed that eight (31%) of the tested cultivars exhibited a sensitive
response (chlorotic (y) or necrotic (+)) to SnToxA produced by P. pastoris; those cultivars
were therefore determined to contain the Tsn1 sensitivity gene (Table 2.3). As expected, the
Tsn1 controls Grandin and BG-261 were sensitive to CFs of the SnToxA P. pastoris strain.

None of the experimental cultivars was sensitive to SnTox1 in CFs produced by P.
pastoris (Table 2.3), indicating that Snn1 was not present in this collection. Chinese Spring
showed the expected sensitivity as the Snn1 control.

Sixteen (62%) of the cultivars were sensitive to SnTox3 in P. pastoris CFs (Table
2.3), and therefore contained the Snn3 sensitivity gene. Grandin and BG-220, both known to

34
possess Snn3, were sensitive as well. Five of the cultivars (19%) were sensitive to both SnToxA and SnTox3 in CFs produced by P. pastoris, indicating that the cultivars harbored both Tsn1 and Snn3.

The selected cultivars varied greatly in the number of sensitive responses to CFs of southeastern S. nodorum isolates, ranging from zero sensitive responses up to nineteen (56%) in ARS05-0242 (Table 2.4). Nineteen (73%) of the selected cultivars were sensitive to CFs from at least one of the southeastern S. nodorum isolates, and 24 (92%) were sensitive to CFs from at least one of the S. nodorum control isolates from North Dakota (Table 2.4). Twenty-five (96%) of the selected cultivars were sensitive to NEs produced either by S. nodorum isolates or P. pastoris controls. Only the moderately SNB-resistant NC-Neuse was insensitive to all NEs produced in the trials. Seven (27%) cultivars were sensitive to CFs from southeastern isolates that elicited no sensitive responses among controls (Table 2.3). These sensitive responses were potentially the result of novel sensitivity genes, but it is also possible that sensitivity was conferred by Snn4 (1) or Snn5 (12), which we were unable to detect.

A majority of the sensitive responses to southeastern SNB isolates were observed in SnTox3-sensitive cultivars and appear likely to be due to the SnTox3-Snn3 interaction (Table 2.3). Among SnTox3-sensitive cultivars, the number of southeastern isolates eliciting a sensitive response ranged from nine (AGS 2000) up to 19 (ARS05-0242), or 27-56% (Table 2.3). And among SnTox3-insensitive cultivars, up to four but most commonly zero southeastern isolates elicited a sensitive response.
Insensitivity to SnToxA, SnTox1, and SnTox3 was displayed by four SNB-susceptible cultivars (Table 2.3). Of these, ARS 12-532 was sensitive to four southeastern isolates; GA 001271-10-3-5 was sensitive to NC8-6 and Sn6; and Pembroke was sensitive only to Sn6. These results suggest that the sensitivity in these three cultivars is also not due to Snn2, as they lacked sensitivity to Sn4. The fourth cultivar, MD00W16-07-3, was sensitive to both Sn4 and Sn6, and thus it might possess Snn2.

Like GA 001271-10-3-5 and Pembroke, Provinciale and Recitale were sensitive only to Sn6 CFs. However, the latter two cultivars’ field response to SNB is unknown at this time; they were included in the study because they appeared in the pedigree of susceptible cutlivars.

2.4.2 NE production of S. nodorum isolates. Twenty (61%) of the southeastern U.S. S. nodorum isolates included in this study produced NEs that caused a sensitive response in at least one of the tested wheat cultivars (Table 2.3). The remaining southeastern isolates either produced no NEs in culture or none that were recognized by the wheat cultivars represented in the trials.

The Snn1 differential wheat cultivar Chinese Spring showed no sensitivity to CFs of any of the 33 experimental isolates, but was sensitive to the CF of the SnTox1 Pichia pastoris yeast control (Table 2.3). This suggests that, although 74% of southeastern S. nodorum isolates carry the SnTox1 gene as determined by a previous dot-blot assay (6), these isolates do not produce SnTox1 in culture, at least in a form that is recognizable by wheat lines containing Snn1. Chinese Spring was insensitive to the CF produced by Sn2000, which was previously reported to produce SnTox1 (17,22).
The \textit{Snn2} differential wheat cultivar BG-223 showed no sensitivity to the CFs of any of the 33 experimental isolates. BG-223 was sensitive to Sn4 and Sn6 (Table 2.3), both known to produce SnTox2 (14,18). This suggests that no southeastern isolates are producing SnTox2 in culture, at least not in a form that is recognizable by wheat lines containing \textit{Snn2}.

The \textit{Snn3} control cultivars Grandin and BG-220 were sensitive to CFs of fourteen (42\%) and twelve (36\%) isolates, respectively. This suggested that at least 12 of the southeastern \textit{S. nodorum} isolates were SnTox3 producers. The previously conducted dot-blot assay showed that fifteen (46\%) of these isolates contained the \textit{SnTox3} gene (6). Thus, three southeastern isolates contained the \textit{SnTox3} gene but did not produce SnTox3 in culture.

North Dakota isolates Sn4 and Sn6, both known to produce SnTox3 (14,18), elicited the expected sensitive response in \textit{Snn3} controls Grandin and BG-220 (Table 2.3). Among the SnTox3-sensitive cultivars, fifteen (94\%) and sixteen (100\%) were sensitive to CFs of Sn4 and Sn6, respectively (Table 2.4). AGS2000, which showed a chlorotic rather than a necrotic response to SnTox3 from the \textit{P. pastoris} strain, was the only SnTox3-sensitive cultivar that was insensitive to CFs of Sn4. The SnTox3-\textit{Snn3} interaction can cause reactions ranging from mild chlorosis to necrosis (30), which may explain the apparent insensitivity of AGS2000 to CFs of Sn4.

Grandin is known to possess \textit{Tsn1} (15), \textit{Snn2} (14), and \textit{Snn3}(16). A sensitive response in Grandin could be the result of one or all sensitivity genes. Both the \textit{Tsn1} and \textit{Snn2} differentials (BG-261 and BG-233, respectively), were insensitive to CFs from all southeastern isolates (Table 2.3). This suggests that the sensitive reactions of Grandin were due either to the \textit{Snn3}-SnTox3 interaction or to novel NE-sensitivity gene interactions.
Six (18%) southeastern isolates produced NEs that elicited sensitivity in the experimental cultivars but caused no sensitivity in the control cultivars (Table 2.3). It is possible that these sensitive responses are due to the Snn4-SnTox4 interaction (1), the Snn5-SnTox5 interaction (12), or unreported sensitivity genes and NEs. The experimental cultivar LA04041D10 was sensitive to NEs produced by isolates NC 8-3 and TN 5-1 but was insensitive to NEs from Sn2000, which is known to produce SnTox5 (12). This suggests that the sensitivity in the Louisiana experimental line caused by the two southeastern isolates was not due to SnTox5.

2.4.3 Marker data. The Xfcp623 marker amplified the 380-bp fragment associated with Tsn1 in eight of the experimental wheat cultivars and also in the two controls, BG-261 and Grandin, known to possess Tsn1 (Table 2.2, Fig. 2). The eight experimental lines were also all sensitive to SnToxA produced in CFs of P. pastoris, confirming the utility of Xfcp623 as a diagnostic marker in germplasm relevant to southeastern wheat breeding programs (8).

Other markers tested showed no consistent banding pattern with their respective sensitivity gene. When compared to the CF infiltration assay, the amplified fragments showed either non-diagnostic polymorphism or no polymorphism between sensitive and insensitive lines (data not shown).

2.5 Discussion

To our knowledge, this study is the first to use disease nursery data to investigate which NEs contribute to SNB susceptibility among a selection of highly susceptible wheat cultivars in North America. A previous study by Crook et al. (6) showed that sensitivity to
NEs was more common in SNB-susceptible cultivars than in resistant cultivars from the eastern U.S., and the present study further elucidated which of the known NEs contribute to SNB susceptibility. Evaluation of reported molecular markers for host sensitivity genes was conducted systematically to determine whether the markers could be employed in MAS in the region; while most were found not to be diagnostic, one marker was confirmed to be useful in detecting Tsn1 in a collection of southeastern U.S. germplasm.

The present set of SNB-susceptible cultivars consisted of 58% soft red winter wheat lines (commercial varieties or advanced experimental lines) adapted to the southeastern U.S. and 42% hard wheat lines from the U.S. Great Plains, Australia, or CIMMYT used to introgress traits including hardness and rust resistance. These two groups of lines were drawn from adapted germplasm and also from new germplasm being introduced to the southeastern U.S. Infiltration results indicate that Tsn1 and Snn3 were present in 31% and 62% of the wheat lines, respectively, and 19% contained both Tsn1 and Snn3.

AGS2000 and Parula each exhibited a chlorotic reaction when infiltrated with SnTox3 produced by *P. pastoris*, but all other SnTox3-sensitive cultivars had a necrotic reaction (Table 2.3). Interestingly, AGS2000 and Parula were each insensitive to CFs of three southeastern *S. nodorum* isolates that were SnTox3 producers (Table 2.3). The SnTox3-Snn3 interaction varies in severity of the sensitive response (30), potentially as a result of multiple genes that confer sensitivity to SnTox3 (32). The sensitive reactions of these two cultivars may have been due to sensitivity genes other than *Snn3-5B*, the sensitivity gene of *Snn3* differentials Grandin and BG-220 in this study. It is possible that AGS2000 and Parula possess *Snn3-5D* (32) or a novel sensitivity gene for SnTox3.
While $Tsn1$ and $Snn3$ appeared with high frequency in the selected SNB-susceptible wheat cultivars, it should be noted that 24% of the studied cultivars were insensitive to both SnToxA and SnTox3. In most cases, these SnToxA- and SnTox3-insensitive cultivars were sensitive to CFs from the North Dakota isolate Sn6. Among those in this category that are adapted to the southeastern U.S. were GA001271-10-3-5, MD00W16-07-3, and Pembroke. Other factors apparently influence disease development in these cultivars; for example, it is possible that as-yet uncharacterized pairs of sensitivity genes and NEs are playing a role. It is also possible that traits apart from this inverse gene-for-gene dynamic are at play.

The field disease resistance scores are only comparable within a test, and the tests occurred in different years; thus, it was not possible to evaluate a possible overall association of field disease severity and presence of $Snn3$ and/or $Tsn1$. However, possession of $Tsn1$ and/or $Snn3$ did not always correspond to increased susceptibility. For example, among the three sister lines ARS 12-532, -533, and -534, the most susceptible field response was in the line without either $Snn3$ or $Tsn1$ (ARS 12-532), while the least susceptible line (ARS 12-534) had both $Snn3$ and $Tsn1$.

A similar study was recently conducted in Western Australia, where SNB is a major problem (30). The researchers compared NE sensitivity with disease resistance scores in a set of 60 Australian bread wheats with varying levels of SNB susceptibility. They found that 87% of the lines were sensitive to SnTox3 and concluded the SnTox3-$Snn3$ interaction was a major contributor to SNB susceptibility (30) in the germplasm collection, although the conclusion was questioned (9). The Australian study also found that 55% of the 60 wheat lines tested were sensitive to SnToxA. Global surveys have found that 96% of $S. nodorum$
isolates in Australia contained SnTox3 (18) and 100% contained SnToxA (28). By comparison, of the 39 southeastern U.S. isolates from which the present isolates were selected, 38% possessed SnTox3 and 15% carried SnToxA (6). The importance of the SnTox3-Snn3 interaction in eastern U.S. SNB epidemics, and thus the priority of selecting against Snn3 in wheat breeding, merits further investigation.

In a recent review, Oliver et al. summarized the mapping status of each host sensitivity gene: Tsn1 as ‘cloned’, Snn1 and Snn2 as ‘fine-mapped’, and Snn3-5B as ‘mapped’ (8,16,23-25,31). The hallmark of a diagnostic molecular marker is consistent PCR-amplified products that are indicative of the presence or absence of a gene. In our study, nine of the ten investigated markers showed non-diagnostic polymorphism or showed the same product size and no common descent. Almost all of these markers were SSRs, which are highly variable regions of the wheat genome, so the amplified product can vary in fragment size even with closely linked markers. It is also possible that recombination during meiosis has occurred between the reported markers and the genes of interest. This likely accounts for the lack of agreement between the marker results and those from CF infiltrations.

Only Xfcp623, a perfect marker internal to the Tsn1 gene, gave results that matched those obtained from infiltration, providing two potential means of early-generation testing for SnToxA sensitivity. Xfcp623 is a dominant marker, so the amplified product is either present or absent with no variation in product size. Although no molecular marker was identified as diagnostic for Snn3, CF infiltration with the P. pastoris SnTox3 control strain was effective at detecting this gene. With the prevalence of Snn3 among the susceptible
cultivars, breeders seeking to eliminate sources of SNB susceptibility could include early-generation testing to screen out \textit{Snn3} by infiltration assays. Breeders could make use of infiltrations, PCR amplification with markers, or both methods to effectively remove \textit{Tsn1} from wheat cultivars. However, given the low frequency of \textit{SnToxA} in southeastern \textit{S. nodorum} isolates, it remains unclear how important \textit{Tsn1}-mediated susceptibility is in determining disease dynamics in this region.

In summary, we now know that \textit{Tsn1} and \textit{Snn3} are relatively common in southeastern U.S. wheat germplasm. We have confirmed the availability of tools to accurately screen for these sensitivity genes. Removal of host sensitivity genes from advanced cultivars should improve resistance to SNB and reduce yield loss to disease.

\textbf{2.6 Acknowledgments}

We would like to thank all those who helped with the project: Ashley (Zearfoss) Crook, Dr. Justin Faris, Dr. Timothy Friesen, Dr. Ed Vargo, Paul Labadie, and Ryan Parks.
2.7 Literature Cited


nodorum pathosystem parallels that of the wheat-tan spot system. Genome 49:1265-1273.


Table 2.1. Twenty-six wheat cultivars selected mainly due to their susceptibility to Stagonospora nodorum blotch or their presence in the pedigrees of multiple susceptible wheat cultivars, and control cultivars.

<table>
<thead>
<tr>
<th>Cultivar^1</th>
<th>ID No.^2</th>
<th>Pedigree</th>
<th>Breeder/Origin</th>
<th>Market class^3</th>
<th>Test^4</th>
<th>Rating^5</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Unknown sensitivities</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N612005</td>
<td>F612246</td>
<td>F612246</td>
<td>University of Georgia</td>
<td>SRH</td>
<td>2009-10</td>
<td>4.0</td>
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<tr>
<td>AMS 11591</td>
<td>Exp.</td>
<td>F612246</td>
<td>USDA-ARS Raleigh</td>
<td>S/H RW</td>
<td>breeder's nursery</td>
<td>Very susceptible</td>
</tr>
<tr>
<td>AMS 11591</td>
<td>Exp.</td>
<td>F612246</td>
<td>USDA-ARS Raleigh</td>
<td>S/H RW</td>
<td>breeder's nursery</td>
<td>Intermediate</td>
</tr>
<tr>
<td>AMS 11591</td>
<td>Exp.</td>
<td>F612246</td>
<td>USDA-ARS Raleigh</td>
<td>S/H RW</td>
<td>breeder's nursery</td>
<td>Blight resistant</td>
</tr>
<tr>
<td>AM50-2042</td>
<td>Exp.</td>
<td>F612246</td>
<td>University of Georgia</td>
<td>SRH</td>
<td>2007-08</td>
<td>4.7</td>
</tr>
<tr>
<td>AM50-2042</td>
<td>Exp.</td>
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<td>SRH</td>
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<td>5.2</td>
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<td>MS-00347-102-6</td>
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<td>MSW</td>
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<td>LA022716</td>
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<td>OKLAHOMA State</td>
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</tr>
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<td>LA04D1 3-10</td>
<td>Exp.</td>
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<td>NCOVT</td>
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<td>MSW</td>
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<td>NCOVT</td>
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<td>F612246</td>
<td>OKLAHOMA State</td>
<td>MSW</td>
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</tr>
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<td>MSW</td>
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<td>NCOVT</td>
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<td>F612246</td>
<td>OKLAHOMA State</td>
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<td>2009-10</td>
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<td>F612246</td>
<td>F612246</td>
<td>OKLAHOMA State</td>
<td>MSW</td>
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<td>NCOVT</td>
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<td>F612246</td>
<td>F612246</td>
<td>OKLAHOMA State</td>
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<td>2009-10</td>
<td>NCOVT</td>
</tr>
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<td>F612246</td>
<td>F612246</td>
<td>OKLAHOMA State</td>
<td>MSW</td>
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<td>NCOVT</td>
</tr>
<tr>
<td>PR-90</td>
<td>F612246</td>
<td>F612246</td>
<td>OKLAHOMA State</td>
<td>MSW</td>
<td>2009-10</td>
<td>NCOVT</td>
</tr>
</tbody>
</table>

1. Among cultivars with unknown sensitivities, NC Neuse was moderately resistant to SNB in the field; all others were known or hypothesized to be susceptible. For controls, necrotrophic effector sensitivities are in parentheses.
2. CI = Cultivar Introduction, Exp. = experimental, PI = Plant Introduction, RIL = recombinant inbred line.
3. HRS = hard red spring, HRW = hard red winter, HWS = hard white spring, HWW = hard white winter, SRW = soft red winter, S/H RW = intermediately hard red winter.
5. Numerical scores (0-9) are only comparable within a test; 0 = no disease, 9 = maximum possible disease (whole-plot visual estimate).
Table 2.2. Molecular markers evaluated for their utility in detecting wheat genes conferring sensitivity to necrotrophic effectors produced by *Stagonospora nodorum.*

<table>
<thead>
<tr>
<th>Marker</th>
<th>Type1</th>
<th>Gene</th>
<th>Primer sequence</th>
<th>Product size2 (bp)</th>
<th>Distance from gene3</th>
<th>Wheat chromosome arm</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xfcp394</td>
<td>SSR</td>
<td>Tsn1</td>
<td>5’ GTAGCCCTGAGCTAAACTGGA 3’ 5’ CAGTGTTAAGAAGTGTGTTCTGGTC 3’</td>
<td>328 – Tsn1 383 – tsn1</td>
<td>0.5 cM distal</td>
<td>5BL</td>
<td>Zhang et al. 2009</td>
</tr>
<tr>
<td>Xfcp620</td>
<td>SSR</td>
<td>Tsn1</td>
<td>5’ CATACCTCTACCGGCTTGTCCAC 3’ 5’ TATCCCGGGAGTGGGAGGG 3’</td>
<td>252 – Tsn1 226 – tsn1</td>
<td>N/A</td>
<td>5BL</td>
<td>Zhang et al. 2009</td>
</tr>
<tr>
<td>Xfcp623</td>
<td>SSR</td>
<td>Tsn1</td>
<td>5’ GTGCCGTAATCGGCTTCTCCG 3’ 5’ CCAACTGGGAAAAGATTGAGC 3’</td>
<td>380 – Tsn1</td>
<td>Internal to gene</td>
<td>5BL</td>
<td>Faris et al. 2010</td>
</tr>
<tr>
<td>Xfcp624</td>
<td>SSR</td>
<td>Snn1</td>
<td>5’ GCCGACCTGTGTCAATGGGC 3’ 5’ GATATGCGGAGATTACAGTAC 3’</td>
<td>281 – Snn1 252 – snn1</td>
<td>0.4 cM proximal</td>
<td>1BS</td>
<td>Justin Faris, personal communication</td>
</tr>
<tr>
<td>Xpsp3000</td>
<td>SSR</td>
<td>Snn1</td>
<td>5’ GAGGCTTTTCTATTGGGAGG 3’ 5’ TGATCTTTATTGCTTGGAAG 3’</td>
<td>153 + 182 – Snn2</td>
<td>0.4 cM proximal</td>
<td>2DS</td>
<td>Reddy et al. 2008</td>
</tr>
<tr>
<td>Xcfd51</td>
<td>SSR</td>
<td>Snn2</td>
<td>5’ TTCCCCCCTCCCTCCTCCTGGC 3’ 5’ CTTGTCAACCTTTTCTGTC 3’</td>
<td>221</td>
<td>7.7 cM distal</td>
<td>2DS</td>
<td>Zhang et al. 2009</td>
</tr>
<tr>
<td>Xcfd56</td>
<td>SSR</td>
<td>Snn2</td>
<td>5’ TCCGCAATGGTGCGGCTG 3’ 5’ ATCCGTAAGTTGGGAGTGGGAG 3’</td>
<td>196, 197 – Snn2</td>
<td>3.6 cM distal</td>
<td>2DS</td>
<td>Zhang et al. 2009</td>
</tr>
<tr>
<td>Xcfd20</td>
<td>SSR</td>
<td>Snn3-5B</td>
<td>5’ GAGTGGAGGAGGGTGAGTGGC 3’ 5’ ATCCAGTCTTCTCTCAGG 3’</td>
<td>296</td>
<td>1.4 cM proximal</td>
<td>5BS</td>
<td>Friesen et al. 2008</td>
</tr>
<tr>
<td>Xgwm234</td>
<td>SSR</td>
<td>Snn3-5B</td>
<td>5’ GAGTCCTGAGTGGAAGCTGGTGG 3’ 5’ CTATTGGGAGTGGTACGGT 3’</td>
<td>244 – Snn3</td>
<td>N/A</td>
<td>5BS</td>
<td>Röder et al. 1988, Justin Faris, personal communication</td>
</tr>
</tbody>
</table>

1. Markers were simple sequence repeats (SSR) or expressed-sequence tags (EST).
2. Predicted product size based on publication or Grain Genes database. The corresponding allele is indicated where information was available.
3. Distance in centimorgans of marker from the gene of interest.
Table 2.3. Results of all assays in which culture filtrates of Stagonospora nodorum isolates or Pichia strains engineered to produce specific necrotrophic effectors were infiltrated into wheat seedling leaves. Results of infiltration of culture filtrates scored as negative (-), chlorotic (y), or necrotic (+) at 7 days post-infiltration, see Fig. 1. Disagreement between replications was recorded as a “/” and missing data are indicated by “o”.

<table>
<thead>
<tr>
<th>Wheat cultivar¹</th>
<th>Southeastern isolates of S. nodorum²</th>
<th>Control isolates³</th>
<th>Pichia pastoris⁴</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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</tr>
</tbody>
</table>

Note:
1. Controls with known sensitivity genes are indicated in parenthesis.
2. Isolates named for their state of origin.
4. *Pichia pastoris* yeast strains engineered to produce NEs in culture.

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Table 2.4. Number of *Stagonospora nodorum* isolates and *Pichia pastoris* yeast strains producing sensitive responses in infiltration assay of wheat seedling leaves. Counts included cultivars with a chlorotic (y) or necrotic (+) response at 7 days post-infiltration.

<table>
<thead>
<tr>
<th>Wheat cultivar</th>
<th>Number of isolates eliciting sensitivity$^1$</th>
<th>S. <em>nodorum</em> isolates$^2$</th>
<th>S. <em>nodorum</em> controls</th>
<th>S. <em>nodorum</em> isolates + P. <em>pastoris</em> controls$^3$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Southeast isolates</td>
<td>S. <em>nodorum</em> controls</td>
<td></td>
</tr>
<tr>
<td>NC-Neuse</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Siouxland</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Pembroke</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Provinciale</td>
<td>0</td>
<td>1</td>
<td>1</td>
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</tr>
<tr>
<td>Recital</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Jagger</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>P520360 (CSP44)</td>
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<td>2</td>
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<td>GA 001271-10-3-5</td>
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<td>2</td>
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</tr>
<tr>
<td>MD00W16-07-3</td>
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<td>2</td>
<td>3</td>
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<tr>
<td>ARS 12-532</td>
<td>4</td>
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<td>5</td>
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<td>AGS 2000</td>
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<td>1</td>
<td>11</td>
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<td>ARS 12-533</td>
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<td>2</td>
<td>12</td>
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<td>Parula</td>
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<td>13</td>
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<tr>
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<td>2</td>
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<tr>
<td>Magnolia</td>
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<td>Pavon 753 (Lr47)</td>
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<td>18</td>
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<tr>
<td>Trego</td>
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<td>2</td>
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<td>Pavon76</td>
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<td>18</td>
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<tr>
<td>T177</td>
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<tr>
<td>T178</td>
<td>15</td>
<td>3</td>
<td>19</td>
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<td>WGRRC36</td>
<td>17</td>
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<td>ARS05-0242</td>
<td>19</td>
<td>3</td>
<td>23</td>
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<tr>
<td><strong>Total sensitive cultivars$^4$</strong></td>
<td><strong>19 (73%)</strong></td>
<td><strong>24 (92%)</strong></td>
<td><strong>25 (96%)</strong></td>
<td></td>
</tr>
</tbody>
</table>

1. Sensitivity determined by culture filtrate infiltration assay.
3. *P. pastoris* strains transformed for heterologous expression of SnToxA, SnTox1, and SnTox3.
4. Out of 25 tested cultivars, number and percent that showed sensitivity in at least one CF bioassay.
Figure 2.1 Rating scale for sensitivity to necrotrophic effectors produced by Stagonospora nodorum isolates in liquid culture. NEs were infiltrated into leaves of 14- to 17-day-old wheat seedlings. Sensitivity was evaluated 7 days post-infiltration and recorded as a combined score of 3 plants per trial. Insensitive (-), chlorotic (y), necrotic (+).
Figure 2.2 Digital image of 380-bp PCR product using marker Xfcp623 on experimental and control wheat cultivars. The 380-bp product indicated the presence of the dominant Tsn1 host sensitivity gene. All cultivars that produced the 380-bp product were also sensitive to SnToxA produced by P. pastoris (Table 2.3).