GENOMIC ANALYSIS OF SEPTORIA NODORUM BLOTCH SUSCEPTIBILITY GENES *SNN1* AND *SNN2* IN WHEAT

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

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ABSTRACT

Septoria nodorum blotch is a disease of wheat caused by the necrotrophic fungus *Parastagonospora nodorum.* In the wheat-*P. nodorum* pathosystem, recognition of pathogen-produced necrotrophic effectors (NEs) by dominant host genes leads to host cell death, which allows the pathogen to gain nutrients and proliferate. To date, nine host gene-NE interactions have been reported in this pathosystem. Among them, the *Snn2*-SnTox2 interaction has shown to be important in both seedling and adult plant susceptibility. A saturated genetic linkage map was developed using a segregating population of recombinant inbred lines and a high-resolution map was then developed using F_2 plants derived from a cross between the SnTox2-insensitive wheat line BR34 and the SnTox2-sensitive line BG301. Over 10,000 gametes were screened for high-resolution mapping and the *Snn2* gene was delineated to a genetic interval of 0.10 cM that corresponds to a physical segment of approximately 0.53 Mb on the short arm of wheat chromosome 2D. A total of 27 predicted genes present in this region and thirteen of them were identified as strong candidates. Seven EMS-induced *Snn2*-insensitive mutants were generated for gene validation. Results of this study provide the foundation for cloning of *Snn2*.

The host sensitivity gene *Snn1*, which confers sensitivity to SnTox1, was previously cloned. Here, allelic diversity of *Snn1* was studied to identify causal polymorphisms, and to develop markers useful for marker assisted selection (MAS). Twenty-seven coding sequence haplotypes that correspond to 21 amino acid haplotypes were identified. Three SNPs were identified as the possible mutations that caused the insensitive allele in wild emmer to become the sensitive allele in domesticated wheat. In addition, four SNPs that changed the sensitive allele into insensitive alleles were identified. SNP-based markers that could detect three of those

SNPs were developed. Results of this study help to increase our knowledge in wheat-NE interactions and host sensitivity gene evolution.

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CHAPTER 1. GENERAL INTRODUCTION

Wheat is one of the most important staple foods of the human diet around the world. It is the leading source of plant-derived protein in food and contributes 20% of the caloric intake worldwide (Bockus et al. 2010; Curtis et al. 2002). In 2018/2019, 730.7 million tons of wheat were produced worldwide (FAO 2019). According to the Food and Agricultural Organization of the United Nations, this production is sufficient for the current global demand. However, the global population is estimated to exceed nine billion people by 2050 which is an increase of about 1.5 billion. It is estimated that the annual global wheat production must be increased to more than 900 million tons by 2050 to meet the demand of the increased world population. (Ray et al. 2013; Singh and Upadhyaya 2015). However, wheat production is under continuous threats including a decrease in the amount of arable farmland, climate change, and abiotic and biotic stresses (Figueroa et al. 2017). Despite the need to increase production, 25 to 30% of the global wheat production is lost due to abiotic and biotic stresses (Bockus et al. 2010). Also, pathogens are constantly evolving to overcome genetic resistance. Therefore, continuous identification and implementation of new sources of genetic resistance is essential.

A major biotic stress on bread wheat (*Triticum aestivum*) and durum wheat (*T. durum*) is disease caused by necrotrophic pathogens. One example is *Parastagonospora* [teleomorph: *Phaeosphaeria* (Hedjar.) syn. *Leptosphaeria nodorum* (Müll.), syn. *Septoria nodorum* (Berk.), syn. *Stagonospora nodorum* (Berk.)] *nodorum* (Berk.) Quaedvleig, Verkley & Crous, which is the causal agent of Septoria nodorum blotch (SNB). *P. nodorum* affects leaves and glumes of both bread wheat (*T. aestivum*) and durum wheat (*T. durum*) creating major yield losses and reduction in quality (Solomon et al. 2006).

The wheat-*P. nodorum* pathosytem has been used as a model to study inverse-gene-for-gene interactions. Nine NE-host sensitivity gene interactions have been identified in this pathosystem, and they include SnToxA-*Tsn1*, SnTox1-*Snn1*, SnTox2-*Snn2*, SnTox3-*Snn3-B1*, SnTox3-*Snn3-D1*, SnTox4-*Snn4*, SnTox5-*Snn5*, SnTox6-*Snn6* and SnTox7-*Snn7* (Friesen et al. 2006; Liu et al. 2006; Faris et al. 2010; Liu et al. 2004a,b, Liu et al. 2012; Reddy et al. 2008; Shi et al. 2016b; Friesen et al. 2007; Zhang et al. 2009; Friesen et al. 2008; Liu et al. 2009; Zhang et al. 2011; Abeysekara et al. 2009; Friesen et al. 2012; Gao et al. 2015; Shi et al. 2015). Three NE-encoding genes have been cloned from the pathogen including *SnToxA* (Friesen et al. 2006), *SnTox1* (Liu et al. 2012), and *SnTox3* (Liu et al. 2009). The corresponding host sensitivity genes (*Tsn1*, *Snn1* and *Snn3-D1*) have also been cloned (Faris et al. 2010, Shi et al. 2016b, Faris et al. unpublished). Further studies on this system will enhance our knowledge and understanding of host-necrotroph interactions and will be useful to control the diseases caused by necrotrophic pathogens through genetic manipulation.

In this dissertation, I report on the saturation and high-resolution mapping of the host sensitivity gene *Snn2*, and the development of markers suitable for map-based cloning and marker-assisted selection. I also report on the analysis of diversity and allelic variants of the *Snn1* gene.

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CHAPTER 2. LITERATURE REVIEW

2.1. Wheat

Wheat is one of the most important food crops that contributes to a great amount of the world's caloric intake. It is also the most important protein source among cereals. It is the most widely grown crop cultivated over 200 Mha worldwide and contributing 30% of the total world cereal production (Wheat initiative 2018). The European Union, China, India, the Russian Federation and the United States rank as the world's leading producers of wheat with an average production of 150.3, 129.2, 94.6, 70.5 and 54.6 million tons, respectively. The global wheat production in the 2018/2019 marketing year was 730.7 million metric tons, which included 51.3 million tons produced by the United States (FAO 2019, USDA Foreign agricultural service 2019). However, wheat production is needed to be increased by 60% to meet the demand of the predicted world population of 9.1 billion by 2050 (Wheat initiative 2018).

2.2. The evolution of wheat

The two fully domesticated wheats are known as common wheat (*T. aestivum* ssp. *aestivum* L.) and durum wheat (*T. turgidum* ssp. *durum* L.), which are free-threshing hexaploid and tetraploid wheats, respectively. Wild and domesticated wheat species belong to two genera, *Triticum* and *Aegilops*. It has been found that these wheat species have evolved through frequent allopolyploidization events, giving rise to many allopolyploids. The diploid progenitors and close relatives of modern wheat have evolved from a common ancestor with seven chromosomes about three million years ago (Dvorak and Zhang 1990; Chalupska et al. 2008). This gave rise to the *Triticum* and *Aegilops* taxa.

The wild A-genome diploids of the *Triticum* group consist of *T. urartu* Tumanian ex Gandylian ($2n = 2x = 14$, AA) and *T. monococcum* ssp. *aegilopoides* ($2n = 2x = 14$, A^mA^m).

There were several diploid *Aegilops* species including *Ae. tauschii* Coss. (the progenitor of the D genome, $2n = 2x = 14$, DD) and a progenitor to the *Sitopsis* section of *Aegilops* which gave rise to the S-genome containing *Aegilops* species. The only domesticated diploid wheat is *T. monococcum* ssp. *monococcum* L. ($2n = 2x = 14$, A^mA^m), which was domesticated from ssp. *aegilopoides* (Faris 2014).

The formation of the cultivated forms of polyploid wheat occurred through two basic lineages that involved two amphiploidization events. One lineage started with the hybridization of *T. urartu* and *Ae. speltoides*, or a close relative, which led to the formation of the wild wheat *T. timopheevii* ssp. *araraticum* Jakubz. ($2n = 4x = 28$, AAGG) containing a pair of A genomes from *T. urartu* and a pair of G genomes (Dvorak et al. 1993; Blake et al. 1999; Huang et al. 2002; Chalupska et al. 2008; Salse et al. 2008). The G genomes are considered as a divergent form of the S genome of the *Aegilops* progenitor. *T. timopheevii* ssp. *araraticum* had a brittle rachis. A mutation led to the domesticated form *T. timopheevii* ssp. *timopheevii* ($2n = 4x = 28$, AAGG), which has a non-brittle rachis. *T. timopheevii* was not cultivated as a significant crop (Faris 2014).

A hexaploid wheat known as *T. zhukovskyi* Menabde et Ericzjan ($2n = 6x = 42$, A^mA^m AAGG) emerged from a hybridization between *T. timopheevii* ssp. *timopheevii* and domesticated einkorn wheat. Like ssp. *timopheevii*, *T. zhukovskyi* also was not cultivated and did not contribute to the formation of economically important wheats found today (Nesbitt and Samuel 1996).

The widely cultivated allohexaploid wheat *T. aestivum* L. ($2n = 6x = 42$, AABBDD) is commonly known as common or bread wheat. It originated as the result of two separate amphiploidization events. First, a hybridization between *T. urartu* and an unidentified diploid

Aegilops species gave rise to the tetraploid wild emmer wheat *T. turgidum* ssp. *dicoccoides* (Körn. Ex Asch. & Graebner) Aarons (2*n* = 4*x* = 28, AABB) (Dvorak and Zhang 1990; Chalupska et al. 2008). The unidentified diploid species is thought to be *Aegilops speltoides* (2*n* = 2*x* = 14, SS) or a close relative as the donor of the B genome. This event has happened less than 0.5 million years ago (see Faris, 2014 for review). The second event occurred about 8,000 years ago (Huang et al. 2002), which involved a hybridization between a subspecies of *T. turgidum* and the diploid goatgrass *Ae. tauschii.* This gave rise to the wheat species *T. aestivum* ssp. *aestivum*. Hence, *T. urartu* and *Ae. tauschii* are the diploid progenitors of the A and D genomes, respectively and *Ae. speltoides* is considered as the closest living relative of the diploid B genome progenitor (McFadden and Sears 1946).

There are several other tetraploid wheat subspecies in addition to durum wheat. These include spp. *turgidum, turanicum, polonicum, carthlicum*, and other species that are quite similar to ssp. *durum*. These probably arose relatively recently through secondary hybridizations (see Faris 2014 for review).

2.3. The wheat genome

The wheat genome was once considered intractable and its genes inaccessible to cloning by traditional means due to its large genome. However, the sequencing of wheat DNA and several early map-based cloning efforts (Huang et al. 2003; Yan et al. 2003) indicated that genes in the wheat genome are accessible by positional cloning. The International Wheat Genome Sequencing Consortium (IWGSC) released an annotated reference genome of the 21 chromosomes of the hexaploid wheat cultivar Chinese Spring (IWGSC et al. 2018). This data is a tremendous resource for marker development and gene cloning. The IWGSC RefSeq v1.0 has a 94% coverage of the entire wheat genome which was estimated to be 15.4 – 15.8 Gb. The

released 14.5 Gb genome assembly had contigs, scaffolds and superscaffolds with N50 values of 52 kb, 7 Mb and 22.8 Mb, respectively. RefSeq Annotation v1.0 was developed using two independent annotation pipelines. A total of 107,891 high-confidence (HC) and 161,537 low-confidence (LC) protein-coding genes were identified. They consist of 35,345, 35,643 and 34,212 genes in the A, B and D subgenomes, respectively. In addition, 3,968,974 copies of transposable elements that belonged to 505 families were identified. They represent 85% of the genome and are responsible for the large genome size of wheat (IWGSC et al. 2018). Recently, IWGSC RefSeq v2.0 was released by improving the original IWGSC RefSeq v1.0 using whole genome optical maps and WGS PacBio SMRT reads (Eid et al. 2009). However, this version has not yet been annotated. Currently, IWGSC RefSeq v1.0 is publicly available and IWGSC RefSeq v2.0 is available for people who signed the Toronto agreement.

In 2016, the 10+ Wheat Genome Project was started to create a wheat pan-genome. The objective was to identify structural variation, copy number variation, and genes that are absent in the initial reference genome. High quality reference genomes were released for 13 cultivars under this project. NRGene's DeNovoMagicV3.0 assembler was used to create the reference genomes from the Canadian spring wheat varieties Landmark and Stanley, the USA variety Jagger, the German winter wheat variety Julius, the Swiss winter wheat variety Arina, the Australian varieties Mace and Lancer, the Japanese variety Norin61, and the variety from France known as SY_Mattis. The assembly was also done for cultivars Cadenza, Paragon, Kronos, Robigus and Claire using the W2RAP assembly algorithms developed by the Earlham Institute. The annotation data for these assemblies are yet to be released. In addition, genome assemblies were released for the wild emmer wheat accession Zavitan (Avni et al. 2017), *Ae.*

tauschii ssp. *strangulata* accession AL8/78 (Luo et al. 2017) and durum wheat cultivar Svevo (Maccaferri et al. 2019).

2.4. Plant response to biotic stresses

Biotic stresses do damage to the growth and development of plants and can cause major yield losses. FAO (2017) reports that biotic stress can cause about 20 - 40 % yield loss worldwide annually. This accounts for more than US$220 billion in losses. Plants have evolved multiple defense mechanisms to survive against the pathogens. These mechanisms predominantly include two main classes known as pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) (Zipfel 2009), and effector-triggered immunity (ETI) (Eitas and Dangl 2010).

PTI is the first layer of defense in plants that act against a wide range of pathogens. It detects the pathogens by recognizing the conserved microbe-associated, pathogen-associated or damage-associated molecular patterns (MAMPs, PAMPs, DAMPs) using cell surface-localized pattern recognition receptors (PRRs) (Monaghan and Zipfel 2012). PRRs of plants usually include receptor-like kinases (RLKs) and receptor like proteins (RLPs). RLKs are more complex than RLPs and consist of extracellular domains, transmembrane domains, and intracellular kinase domains. Recognition of MAMPs, PAMPS or DAMPs by PRRs trigger the activation of mitogen-activated protein kinases (MAPKs) which activate multiple signaling defense responses such as biosynthesis/signaling of plant stress/defense hormones, reactive oxygen species generation, stomatal closure, defense gene activation, phytoalexin biosynthesis, cell wall strengthening, and a hypersensitive response (HR) (Zhang et al. 2019). As a response to PTI, pathogens have evolved to secrete effectors to disrupt the PTI pathway. This is known as the effector-triggered susceptibility or ETS. As a response to ETS, plants have evolved a second

layer of defense involving a robust host defense response upon the recognition of effector proteins. This level of resistance is called effector-triggered immunity (ETI) (Zipfel et al. 2009). A resistance response occurs when a pathogen-produced effector is detected by the corresponding host resistance (R) gene and a susceptible (compatible) interaction occurs if either the pathogen effector or the host R gene is absent.

Most of the R genes involved in the ETI pathway include intracellular nucleotide-binding site and leucine-rich repeat (NBS-LRR or NLR) genes. NLRs are typically cytoplasmic receptor proteins and can recognize specific pathogen-secreted effector proteins and then transfer the signals to activate multiple defense responses including a hypersensitive response (HR), which is a form of programmed cell death at the site of infection (Eitas and Dangl 2010). NLR proteins in plants belong to two classes depending on the N-terminal domain which can be either a terminal Toll/interleukin-1 receptor (TIR) or coiled-coil (CC) domain. This TIR and CC domains have found to be important in signal transmission (Mukhtar et al. 2011).

Recognition of an avirulence (Avr) protein of a pathogen by a single R gene in the host plant to activate the resistant response was introduced as the gene-for-gene hypothesis by H.H. Flor in the 1950s (Flor 1956). Since then, many *R-Avr* gene combinations have been characterized (Dangl and Jones 2011). Many studies conducted over the past years have revealed that R gene products and effectors can interact both directly (the ligand- receptor model) and indirectly in multiple ways. Four models of effector recognition have been proposed so far. According to the initially proposed elicitor-receptor model, the R protein directly recognizes the corresponding Avr protein (Keen 1990; Jia et al. 2000; Dodds et al. 2006; Catanzariti et al. 2010; Steinbrenner et al. 2015). According to the guard model, the effector does not directly interact with the R protein. Instead, it has a different host target protein known as “the guardee”

and the R protein detects the modifications of the guardee caused by the pathogen's effector (Dangl and Jones 2001). The third model is called the decoy model. Here, the host plant has a protein known as "the decoy" that mimics the effector's host target protein. The effector binds to the decoy instead of the target protein and R gene monitors the modifications of the decoy and activates the defense responses protein (van der Hoorn and Kamoun 2008). The recently proposed integrated decoy model states that the decoy that mimics the effector's target is integrated into the NLR making the recognition and defense pathway activation faster (Cesari et al. 2014; Le Roux et al. 2015; Sarris et al. 2015; Petit-Houdenot et al. 2017).

2.5. Necrotrophic plant pathogens

Plant pathogens can be classified as biotrophs, hemi-biotrophs, and necrotrophs based on their lifestyles. Biotrophs are a class of pathogens that require living host cells to feed, grow, and complete their life cycles. They produce limited amounts of cell wall-degrading enzymes and effectors to suppress the host immune system (Dean et al. 2012). Necrotrophic pathogens gain their nutrients and complete their life cycles on dead or dying tissue. Hemi-biotrophs have a biotrophic phase early during infection followed by a necrotrophic phase at later stages of the lifecycle (Wang et al. 2014; Horbach et al. 2011). Both host-specific and broad host-range necrotrophic pathogens have been identified. Common host-specific necrotrophic fungal pathogens include *Cochliobolus carbonum* (causal agent of northern corn leaf spot), *C. heterostrophus* (causal agent of southern corn leaf blight), *C. victoriae* (causal agent of Victoria blight of oats), *Pyrenophora tritici-repentis* (causal agent of tan spot of wheat) and *Parastagonospora nodorum* (causal agent of Septoria nodorum blotch of wheat) (Wang et al. 2014).

Recent studies have shown that some necrotrophs have evolved mechanisms to disrupt the mechanisms that plants have evolved to fight pathogens (Faris et al. 2010). Many necrotrophs produce necrotrophic effectors (NEs), which are effectors of pathogenicity. NEs are toxic only to host genotypes that express a gene that recognizes the corresponding NE. Such host genes are called dominant disease susceptibility genes. The presence of a pathogen-produced NE and the corresponding dominant host gene for sensitivity leads to a compatible interaction and ultimately disease susceptibility. This is known as necrotrophic effector-triggered susceptibility (NETS). If either the NE or the dominant host allele is absent, a resistance response occurs. Therefore, these host-necrotroph interactions are the inverse of the classic host-biotroph gene-for-gene interactions at the host-NE interface (Wolpert et al. 2002).

Studies involving NE sensitivity genes (Lorang et al. 2007; Nagy and Bennetzen 2008; Faris et al. 2010) indicate that necrotrophic pathogens exploit ETI pathways to trigger responses that lead to death of the plant tissue creating an environment favorable for the necrotrophs, which would otherwise be unfavorable to the survival of a biotrophic pathogen (Faris et al. 2010). Therefore, it is important to study the role of resistance and/or susceptibility genes when trying to breed disease resistant crops.

2.6. Septoria nodorum blotch (SNB)

Septoria nodorum blotch (SNB) is a severe foliar and glume blotch disease that can cause about 10-20% yield losses in wheat (Bockus et al. 2010). SNB has been reported in most of the wheat-growing areas including Australia, South Asia, North Africa and Europe and North America (Oliver et al. 2012; Bearchell et al. 2005; Crook et al. 2012). SNB is caused by *Parastagonospora* [syn. anamorph: *Stagonospora*; teleomorph: *Phaeosphaeria*] *nodorum,* which is a heterothallic, necrotrophic, filamentous, ascomycete fungus pathogenic on wheat, barley and

a wide range of wild grasses. It affects both the glume and the leaf of wheat leading to yield losses and reductions in grain quality and grain weight. Initially, this fungus was classified under the genus *Stagonospora* and known as *Stagonospora nodorum* (Goodwin and Zismann 2001). Later, it was moved to the genus *Parastagonospora* based on morphology of sexual and asexual stages and sequence analysis of ribosomal DNA (Quaedvlieg et al. 2013).

Because *P. nodorum* is a necrotrophic pathogen, its lifecycle can be divided into two phases known as parasitic phase and saprophytic phase. During the parasitic phase, the pathogen can infect all the above-ground plant parts and during the saprophytic phase, the pathogen overwinters on wheat straw and stubble until the parasitic phase starts again. During the parasitic phase, sexual reproductive structures known as pseudothecia produce ascospores. They can travel long distances by wind. Generally, ascospores cause the first infection (Solomon et al. 2006; Oliver et al. 2012). Upon infection, small, water-soaked chlorotic lesions appear on the lower leaves of the plant. They later become red-brown and lens-shaped lesions with a gray-brown center (Friesen and Faris 2010). These lesions contain the asexual pycnidiospores that produce pycnidia which are dispersed primarily via rain splashes. In severe cases, the pathogen can infect the glume and cause dark brown or purple lesions and lightweight and shriveled kernels (McMullen and Adhikari 2009). This pathogen can have a polycyclic infection cycle within a single growing season increasing the chance of an epidemic (Scharen 1966). During the saprophytic phase, the pathogen overwinters on wheat straw, stubble, and other wheat residues. The ascospore-producing perithecia are present during both the parasitic and saprophytic phases. Infected seeds and ascospores are considered as the major inocula of SNB (Chooi et al. 2014).

2.7. The host-NE interactions in the wheat-*P. nodorum* pathosystem

Nine NE-host sensitivity gene interactions have been identified in the *P. nodorum*-wheat pathosystem including SnToxA-*Tsn1* (Friesen et al. 2006; Liu et al. 2006; Faris et al. 2010), SnTox1-*Snn1* (Liu et al. 2004a, b, 2012; Reddy et al. 2008), SnTox2-*Snn2* (Friesen et al. 2007; Zhang et al. 2009), SnTox3-*Snn3-B1* (Friesen et al. 2008; Liu et al. 2009), SnTox3-*Snn3-D1* (Zhang et al. 2011), SnTox4-*Snn4* (Abeysekara et al. 2009), SnTox5-*Snn5* (Friesen et al. 2012), SnTox6-*Snn6* (Gao et al. 2015), and SnTox7-*Snn7* (Shi et al. 2015). Pathogen genes encoding three of the NEs (SnToxA, SnTox1 and SnTox3) and three host sensitivity genes (*Tsn1*, *Snn1* and *Snn3-D1*) have been cloned so far.

2.7.1. SnToxA-*Tsn1*

ToxA was first purified from the wheat tan spot pathogen *Pyrenophora tritici-repentis* and named as Ptr ToxA (Tomás and Bockus 1987; Ballance et al. 1989). Later, a homologous gene that had 99.7 % similarity was found in the *P. nodorum* genome (Friesen et al. 2006) and designated as *SnToxA*. Both of these genes interact with the same host sensitivity gene *Tsn1* (Liu et al. 2006). In addition to *P. tritici-repentis* and *P. nodorum*, homologous genes of *ToxA* have been found in *Bipolaris sorokiniana,* which causes spot blotch, Helminthosporium leaf blight, and common root rot in wheat (McDonald et al. 2018). Friesen et al. (2006) identified more haplotypes of the *ToxA* gene in *P. nodorum* compared to *P. tritici-repentis*. Therefore, it was concluded that *ToxA* had been horizontally transferred to *P. tritici-repentis* from *P. nodorum*. The *ToxA* gene was found to encode a protein with a size of 13.2 kDa (Sarma et al. 2005). The genes that encode both Ptr ToxA and SnToxA have been cloned (Ballance et al. 1996; Ciuffetti et al. 1997; Friesen et al. 2006).

The host sensitivity gene *Tsn1* is located on chromosome 5BL of wheat (Faris et al. 1996). The SnToxA-*Tsn1* interaction can account for 25-95 % of the disease variation in both common and durum wheat (Faris and Friesen. 2009; Virdi et al. 2016). *Tsn1* was cloned by Faris et al. (2010) using map-based cloning. *Tsn1* is a relatively large gene with 10,581 bp and consists of eight exons that encode a protein of 1490 amino acids. It contains C-terminal NB and LRR domains and an N-terminal S/TPK domain. Analysis of mutants revealed that all three domains of the protein are required for sensitivity. Transcription analysis showed that *Tsn1* expression increases under light conditions. According to yeast two-hybrid assay experiments, *Tsn1* does not directly interact with SnToxA. Therefore, it's likely a guard in a guard-guardee model (Faris et al. 2010). Immunolocalization experiments have shown that ToxA is localized in the choloroplast after infection (Manning and Ciuffetti 2005). Several studies revealed that ToxA directly binds to a conserved plant chloroplast protein ToxABP1 (Manning et al. 2007), chloroplast-associated plastocyanin (Tai et al. 2007) and a dimeric PR-1-type pathogenesis-related protein (PR-1-5) (Lu et al. 2014).

2.7.2. SnTox1-*Snn1*

SnTox1-*Snn1* was the first NE-sensitivity gene interaction that was identified in the wheat-*P. nodorum* pathosystem (Liu et al. 2004a) and accounts for up to 58% of the disease variation (Liu et al. 2004b; Friesen et al. 2007; Chu et al. 2010; Phan et al. 2016). *SnTox1* was the third NE gene cloned from *P. nodorum*. This was done by comparing the sequence similarities between the candidate genes identified from *P. nodorum* reference genome and previously cloned NE genes and using previously identified characteristics of SnTox1 (Liu et al. 2012). This led to the identification of the gene SNOG_20078 which was later verified to be *SnTox1*. The SnTox1 protein is 10.33 kDa in size and consists of a signal peptide and a chitin-

binding domain (Liu et al. 2012, 2016). The chitin binding domain has found to be important for the protection of the fungus from plant chitinases during the initial penetration step of an infection (Liu et al. 2016). Transcriptional expression analysis revealed that *SnTox1* expression was high during early stages of infection which also suggested the importance of SnTox1 for the initial penetration step (Liu et al. 2012). An avirulent isolate was converted into a virulent isolate by co-inoculation with SnTox1. This experiment confirmed that SnTox1 is involved in the penetration process. During infection, SnTox1 is localized to the outer surface of the mycelium and allows *P. nodorum* to successfully penetrate the host tissue for colonization (Liu et al. 2016). A compatible SnTox1-*Snn1* interaction led to defense responses including an oxidative burst, up-regulation of PR-genes, and DNA laddering (Liu et al. 2012).

The host sensitivity gene *Snn1* was mapped to the chromosome arm 1BS (Liu et al. 2004a). Saturation and high-resolution mapping conducted using a population derived from a cross between Chinese Spring (CS) and a CS-*T. dicoccoides* 1B disomic chromosome substitution line delimited *Snn1* to a 0.46 cM interval (Reddy et al. 2008). Shi et al. (2016b) cloned *Snn1* by positional cloning and validated by mutagenesis and transgenesis approaches. *Snn1* is 3045 bp in length and consists of 3 exons. It encodes a protein that contains a signal sequence, a wall-associated receptor kinase galacturonan binding domain (GUB_WAK), epidermal growth factor-calcium binding domain (EGF_CA), a transmembrane domain, and a serine/threonine protein kinase (S/TPK) domain. It was predicted that GUB_WAK and EGF_CA domains are located extracellularly whereas the S/TPK domain is located intracellularly (Shi et al. 2016b). *Snn1* expression is regulated by light signals and its expression is highest at subjective dawn. A yeast two-hybrid assay revealed that SnTox1 directly binds to a region between the GUB_WAK and EGF_CA domains.

2.7.3. SnTox2-*Snn2*

The third NE identified in *P. nodorum* is SnTox2 (Friesen et al. 2007). It was partially purified from the culture filtrates of isolate Sn6. The size of the protein was estimated to be between 3 and 10 kDa (Friesen et al. 2007). The *Snn2*-SnTox2 interaction can account for 47% of the disease variation and plays a strong role in conferring disease in both seedlings and adult plants (Friesen et al. 2007). The interaction was also found to be light-dependent (Friesen et al. 2007). The corresponding dominant sensitivity gene *Snn2* was mapped to the short arm of chromosome 2D using a recombinant inbred population developed from crossing the two hard red spring wheat cultivars BR34 and Grandin. Zhang et al. (2009) developed a low-resolution map that delineated *Snn2* to a genetic interval of 4.0 cM.

2.7.4. SnTox3-*Snn3*

SnTox3-*Snn3* is the fourth interaction identified in the wheat – *P. nodorum* pathosystem (Friesen et al. 2008). The pathogen NE *SnTox3* was cloned by Liu et al. (2009). It encodes a mature protein with a size of 25.88 kDa. *SnTox3* has a higher expression during the initial days after infection, and the expression level decreases with time (Liu et al. 2009). A compatible interaction causes upregulation of PR proteins, jasmonic acid pathway proteins and phenylpropanoid pathway proteins, which are typically associated with defense responses (Winterberg et al. 2014).

SnTox3 is recognized by two homoeologous genes known as *Snn3-B1* and *Snn3-D1,* which are located on wheat chromosomes 5B and 5D, respectively (Friesen et al. 2008; Zhang et al. 2011). The SnTox3-*Snn3-D1* interaction is much more severe and epistatic to SnTox3-*Snn3-B1.* A saturated genetic map of the *Snn3-D1* region was developed using an *Aegilops tauschii* TA2377 × AL8/78 F_2 population. Subsequent high-resolution mapping delineated *Snn3-D1* to a

genetic interval of 1.38 cM (Zhang et al. 2011). Shi et al. (2016a) developed a saturation map for *Snn3-B1* using two F_2 populations, which were developed by crossing the sensitive cultivar Sumai3 to the insensitive cultivar BR34 (BS population) and by crossing Sumai3 to a Chinese Spring-*T. turgidum* ssp. *dicoccoides* chromosome 5B disomic substitution line (CS-DIC 5B) (CS population). High resolution mapping was performed using the BS population and *Snn3-B1* was delimited to a 1.5 cM genetic interval.

2.7.5. SnTox4-*Snn4*

SnTox4-*Snn4* was the fifth interaction identified in this pathosystem (Abeysekara et al. 2009). It accounts for 23-41 % of disease variation depending on the wheat background and the NE susceptibility genes present. The phenotype of this interaction is a mottled necrosis and appears to be less severe than the other interactions observed in the pathosystem. SnTox4 was partially purified from the Swiss isolate Sn99CH1A7a, which was also known as Sn99. It was expected to be a protein of the size between 10-30 kDa. The *Snn4*-SnTox4 interaction was also found to be light dependent. The corresponding dominant host sensitivity gene *Snn4* was mapped to the short arm of wheat chromosome 1A using an RI population developed by crossing the Swiss winter wheat cultivars Arina and Forno. *Snn4* was delimited to a 2.5 cM interval by Abeysekara et al. (2009).

2.7.6. SnTox5-*Snn5*

The sixth NE-host gene interaction identified in the wheat-*P. nodorum* pathosystem was SnTox5-*Snn5* (Friesen et al. 2012). It explained up to 63% of the disease variation depending on the other interactions in the background. SnTox5 was identified from the *SnToxA*-knockout Sn2000 fungal isolate Sn2000K06-1. The size of SnTox5 was estimated to be 10-30 kDa. This interaction was also identified as light-dependent. The host sensitivity gene *Snn5* was mapped to

the long arm of chromosome 4B using a tetraploid doubled haploid population derived from the North Dakota durum variety Lebsock and *T. turgidum* ssp. *carthlicum* accession PI 94749 (LP population) (Chu et al. 2010). Currently *Snn5* is delineated to a genetic distance of 2.8 cM (Sharma et al. unpublished).

2.7.7. SnTox6-*Snn6*

The SnTox6-*Snn6* interaction was characterized by Gao et al. (2015) using culture filtrates of *P. nodorum* isolate Sn6 and Sn6KOTox3.This interaction explained 27% of the disease variation also found to be light dependent. SnTox6 was estimated to be 12 kDa in size. were used to initially characterize this interaction and to map the wheat sensitivity gene *Snn6* to the distal region of the long arm of chromosome 6A. The corresponding host sensitivity gene *Snn6* was mapped to a 3.2 cM genetic distance in the International Triticeae Mapping Initiative (ITMI) population. The *Snn1*-SnTox1 and *Snn3-B1*-SnTox3 interactions did not affect the level of sensitivity of the *Snn6*-SnTox6 interaction although such multiple interactions were additive in previous studies (Gao et al. 2015).

2.7.8. SnTox7-*Snn7*

The latest NE-host gene interaction identified in the wheat-*P. nodorum* pathosystem was SnTox7-*Snn7* (Shi et al. 2015). It explained 33% of the disease variation. This interaction was not completely light-dependent. SnTox7 was identified from the *P. nodorum* isolate Sn6 and estimated to be less than 30 kDa. The corresponding host sensitivity gene *Snn7* was mapped to the long arm of chromosome 2D using a population developed from Chinese Spring and CS-Tm 2D (Chinese Spring - Timstein 2D disomic chromosome substitution line) (Shi et al. 2015).

2.8. Molecular markers and genetic mapping

Allelic variations within a genome of the same species can be classified into several major groups that include differences in the number of tandem repeats at a particular locus (microsatellites, or simple sequence repeats), insertions or deletions of a segment (InDels), and changes of a single nucleotide at a particular position of a sequence (single nucleotide polymorphisms; SNPs). In order to detect these variations in the individuals of a progeny at the DNA level, researchers develop and use molecular markers. Molecular markers are DNA sequences that are associated with a certain location on a chromosome. They do not necessarily affect the function of genes. Over the years, many different types of molecular markers were developed and used such as random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), simple sequence repeat (SSR) and SNP markers. Among them, SSRs are widely being used because of their cost effectiveness, locus specificity and high polymorphism level. Lately, SNPs have become extremely popular in plant molecular genetics. A single nucleotide base is the smallest unit of inheritance and thus SNPs are highly abundant in genomes. Therefore, they can provide the highest map resolution compared to other marker systems. Unlike earlier marker systems, SNPs made it possible to create saturated and high-resolution maps of target regions, rapidly identify marker-trait associations, and accelerated the cloning of genes of interest. SNP markers became the marker of choice especially after the development of NGS. The availability of reference genome sequences allowed the development of SNP-based markers much easier. Various means of SNP genotyping have also been developed. KASP (Kompetitive Allele Specific PCR) and STARP (semi-thermal asymmetric reverse PCR) currently appear to be the most promising SNP-based markers (Long et al. 2017).

Genetic mapping studies involve genetic linkage analyses, which are based on the concept of genetic recombination during meiosis. The chance of having a recombination event between loci that are closer to each other is lower than for the loci located far apart. This can be observed in a segregating population and used in genetic mapping to determine the distance between loci. DNA-based markers are widely used in developing genetic linkage maps. Map-based cloning has several steps that includes saturation mapping, high resolution mapping, mutagenesis and gene complementation.

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CHAPTER 3. GENOMIC ANALYSIS AND HIGH-RESOLUTION MAPPING OF THE *SNN2* GENE IN WHEAT

3.1. Abstract

Septoria nodorum blotch (SNB) of wheat is caused by the necrotrophic fungus *Parastagonospora nodorum*. The recognition of *P. nodorum*-produced necrotrophic effectors (NEs) by dominant host sensitivity genes in wheat leads to disease development according to the inverse gene-for-gene model. There have been nine host gene-NE interactions reported in the wheat-*P. nodorum* pathosystem so far, and the *Snn2*-SnTox2 interaction has been shown to be important in both seedling and adult plant susceptibility. The overall goal of this study was to develop a high-resolution map to provide a foundation for the map-based cloning and characterization of *Snn2* in wheat. A saturated genetic linkage map was developed using a segregating population of 164 $F_{7:8}$ recombinant inbred lines. A high-resolution map was then developed using F_2 plants derived from a cross between the SnTox2-insensitive wheat line BR34 and the SnTox2-sensitive line BG301. Markers were identified by SNP genotyping using the 90K iSelect wheat SNP chip and simple sequence repeat markers. New markers were developed based on whole-genome sequence scaffolds, wheat survey sequences, and the IWGSC RefSeqv1.0 wheat reference genome. Over 10,000 gametes were screened for high-resolution mapping. I have delineated the *Snn2* gene to a genetic interval of 0.10 cM that corresponds to a physical segment of approximately 0.53 Mb on the short arm of wheat chromosome 2D. A total of 27 predicted genes reside in this region, thirteen of which are considered as strong candidates for *Snn2* including four NB-ARC domain-containing genes and nine protein kinase-containing genes. I also developed seven EMS-induced *Snn2*-disrupted mutants for gene validation and functional analyses. Results of this study provide the knowledge and tools for cloning *Snn2*,

which will increase our knowledge of wheat-*P. nodorum* interactions and help to develop better host resistance in the future.

3.2. Introduction

Wheat is one of the most important food crops because it provides 20% of the world's caloric intake. However, worldwide wheat production is constantly under numerous biotic stresses. Septoria nodorum blotch (SNB), caused by the necrotrophic fungus *Parastagonospora* (syn. ana, *Stagonospora*; teleo, *Phaeosphaeria*) *nodorum* (Berk.) Quaedvleig, Verkley & Crous, is a severe foliar and glume disease that can cause yield losses up to 50% and reduction of quality in most wheat growing areas. SNB affects both common wheat (*Triticum aestivum* L.) and durum wheat (*T. turgidum* L.). SNB resistance is inherited quantitatively and many interactions show additive effects (Xu et al. 2004; Zhang et al. 2009; Friesen et al. 2012).

Biotrophs are a class of pathogens that require living host cells to feed, grow, and complete their life cycles. Plants have multiple layers of recognition to protect themselves against biotrophs. The first and basal layer involves the identification of microbe-, pathogen-, or damage-associated molecular patterns (PAMPs/MAMPs/DAMPs) using plant surface-localized pattern recognition receptors (PRRs) (Zipfel 2009; Tsuda and Katagiri 2010). This recognition leads to a resistance response by the plant which is known as PAMP-triggered immunity (PTI) (Zipfel 2009). With time, many pathogens have adapted to secrete some effector proteins to suppress PTI. Then, plants evolved a second layer of defense involving a robust host defense response upon the recognition of effector proteins. This level of resistance is called effector-triggered immunity (ETI) (Zipfel 2009). A resistance response occurs when a pathogen-produced effector is detected by the corresponding host resistance (R) gene and a susceptible (compatible) interaction occurs if either the pathogen effector or the host R gene is absent.

Unlike biotrophs, necrotrophic pathogens gain their nutrients from dead, or dying, cells. Recent studies have shown that some necrotrophs have evolved to obtain nutrients from plants by utilizing the defense mechanisms that plants have evolved to fight biotrophic pathogens (Faris et al. 2010; Lorang et al. 2007; Nagy and Bennetzen 2008; Shi et al. 2016b). Many necrotrophs produce necrotrophic effectors (NEs), which are effectors of pathogenicity (Wang et al. 2014b). NEs are toxic only to host genotypes that express a corresponding gene that recognizes the NE. Such host genes are called dominant disease susceptibility genes. In these systems, the presence of a pathogen-produced NE and the corresponding dominant host gene for sensitivity leads to a compatible interaction, and ultimately, disease susceptibility (Wang et al. 2014b). This is known as necrotorophic effector-triggered susceptibility (NETS) (Jones and Dangl 2006; Liu et al. 2009; Faris et al. 2010). If either the NE or the dominant host allele is absent, a resistance response occurs. This is the opposite of what happens in a host-biotroph interaction explained by gene-for-gene theory introduced by Flor (1956). Therefore, these host-necrotroph interactions follow an inverse gene-for-gene model (Friesen et al. 2007).

Nine NE-host sensitivity gene interactions have been identified in the *P. nodorum*-wheat pathosystem including SnToxA- *Tsn1* (Friesen et al. 2006; Liu et al. 2006; Faris et al. 2010), SnTox1-*Snn1* (Liu et al. 2004a, 2012; Reddy et al. 2008), SnTox2-*Snn2* (Friesen et al. 2007; Zhang et al. 2009), SnTox3-*Snn3-B1* (Friesen et al. 2008; Liu et al. 2009), SnTox3-*Snn3-D1* (Zhang et al. 2011), SnTox4-*Snn4* (Abeysekara et al. 2009), SnTox5-*Snn5* (Friesen et al. 2012), SnTox6-*Snn6* (Gao et al. 2015), and SnTox7-*Snn7* (Shi et al. 2015). Three NE-encoding genes have been cloned from the pathogen including *SnToxA* (Friesen et al. 2006), *SnTox1* (Liu et al. 2012), and *SnTox3* (Liu et al. 2009). The corresponding host sensitivity genes *Tsn1*, *Snn1* and *Snn3-D1* have also been cloned (Faris et al. 2010; Shi et al. 2016b; Faris et al. unpublished).

The wheat *Snn2* gene confers sensitivity to the NE SnTox2. The *Snn2*-SnTox2 interaction plays a strong role in conferring both seedling and adult plant susceptibility (Friesen et al. 2007). The objectives of this study were to build the foundation for cloning *Snn2* by developing high-resolution maps, molecular markers, and *Snn2*-disrupted mutants, and to identify candidate genes, which would significantly extend our knowledge of wheat-*P. nodorum* interactions. Results of this study will further increase our knowledge of this system which would help to obtain complete host resistance through genetic manipulation.

3.3. Materials and methods

3.3.1. Plant materials

A segregating population of recombinant inbred (RI) lines derived from a cross between the Brazilian hard red spring wheat breeding line BR34 and the North Dakota hard red spring variety Grandin were used for saturation mapping. Previous studies have indicated that Grandin is sensitive to SnTox2 whereas BR34 is insensitive (Zhang et al. 2009). This population consisting of 164 $F_{7:8}$ lines was used for saturation mapping of the *Snn2* locus. A population consisting of 5073 F_2 plants generated using a cross between BR34 and BG301 was used for high-resolution mapping. BG301 is a hexaploid SnTox2-sensitive RI line derived from the BR34 × Grandin RI population. It is not sensitive to any other NEs identified and thus used for the cross with BR34 as the resulting population segregates only for *Snn2* and no other NE sensitivity genes. The SnTox2-sensitive line BG301 was used for EMS mutagenesis as well.

3.3.2. DNA extraction

DNA from the parents and the RIL population was extracted as described by Faris et al. (2000). Young leaf tissue (5 g) was collected from each plant into separate microfuge tubes (1.5 ml) frozen in liquid nitrogen and ground into a fine powder. Sodium bisulfite (3.8 g/l) was added

to the monocot extraction buffer [500 mM NaCl, 100 mM Tris-HCl, pH 8.0, 50 mM EDTA, 0.84% (w/v) SDS], and the pH was adjusted to 8.0 by adding NaOH. Extraction buffer (600 µl) was preheated to 65 °C, added to frozen tissue, and incubated at 65 °C for 30 min. A 24:1 solution of chloroform:isoamyl alcohol was added (600 µl), mixed vigorously, and centrifuged at 7000 g for 12 min at room temperature. The supernatant was added to a new microfuge tube (1.4 ml), and the DNA was precipitated by adding 850 µl of 95% (v/v) ethanol and centrifuging at 7000 g for 30 s. The pellet was rinsed in 70% (v/v) ethanol, dried and dissolved in TE buffer (60 µl). DNA from the F_2 plants was extracted using the ammonium acetate method (Pallotta et al. 2003). Extracted DNA was quantified using a Nanodrop spectrophotometer ND-1000 (Thermo Scientific, Wilmington, DE) and diluted to approximately 150 ng/µL.

3.3.3. PCR amplification and electrophoresis

Each polymerase chain reaction (PCR) experiment was conducted using a total volume of 10 µl and consisted of 2 µl of template DNA, 1X PCR buffer, 1 mM $MgCl_2$, 0.2 mM dNTPs, 4 pmol of the primer and 0.5 units of homemade Taq DNA polymerase. The PCR conditions were initial denaturation at 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, the annealing temperature of 60.8 °C for 30 s with a 0.2 ºC decrement at every cycle, and extension at 72 °C for 90 s, followed by a final extension of 72 °C for 7 min. Amplicons were electrophoresed on 6 % polyacrylamide gels, stained with GelRed stain, and visualized with a Typhoon 9500 variable mode imager (GE Healthcare Life Sciences, Piscataway, NJ).

3.3.4. Saturated mapping and marker development

SNP genotyping was performed using the Illumina 90K iSelect wheat SNP chip (Wang et al. 2014a). The array was processed using a BeadStation and iScan instrument by Dr. Shiaoman Chao at the small grains genotyping laboratory in Fargo, ND, USA. SNP clustering was

performed with GS Polyploid Clustering v1.0 software (Illumina). The setting of the minimum number of points in a cluster was adjusted to five to minimize the merging of clusters into a single cluster when clusters were not well separated. The clustered SNPs were then filtered based on custom cluster number and call rate. The accuracy for SNP clustering was visually checked, and incorrectly clustered SNPs were manually curated. Sample cluster assignments for each SNP assay were converted to genotype calls.

The contextual sequences of SNP markers on wheat 2DS from the 90K SNP arrays were identified and used as queries in BLASTn (Altschul et al. 1997) searches of the wheat 2DS survey sequences and *Ae. tauschii* whole genome draft sequences. New PCR markers were developed based on whole-genome sequence scaffolds, wheat survey sequences, and the IWGSC RefSeqv1.0 wheat reference genome. Primers were designed using Primer3 (Rozen and Skaletsky 2000) and NCBI primer design tool and tested for polymorphism between the parents.

3.3.5. Linkage analysis

The computer program MapDisto v.1.8 (Lorieux 2012) was used for the linkage analysis using the Kosambi mapping function (Kosambi 1943) with a logarithm of odds (LOD) threshold of 3.0 and a maximum recombination frequency of 0.3. The command 'find groups' was used to verify the linkage of markers. The seriation algorithm using sum of adjacent recombination frequencies was conducted with the 'order sequence' command. The 'check inversions', 'ripple order', and 'drop locus' commands were applied for the local improvement of the order of loci.

3.3.6. High resolution mapping

All PCR-based markers developed were amplified and visualized. Co-dominant PCR markers flanking *Snn2* based on saturation mapping in the 164 RILs were subsequently used to screen the BR34 × BG301 population of 5073 F_2 plants to identify plants with putative

recombination events within the *Snn2* interval. All the recombinant plants (plants with crossovers between the markers flanking *Snn2*) were screened for reaction to an SnTox2-containing culture filtrate. Plants that were insensitive to SnTox2 were considered homozygous for a recessive *snn2* allele, and plants sensitive to SnTox2 were considered to be either homozygous for the *Snn2* allele or heterozygous. All the identified recombinants were transplanted in pots and self-pollinated to obtain F_3 seeds. Sixteen plants from each F_3 family were screened with the same flanking markers to identify homozygous recombinants. One homozygous recombinant plant was selected from each F_3 family and transplanted to increase seed to be used in further experiments. All the homozygous recombinant plants were genotyped with the markers between the flanking markers. Also, they were phenotyped for reaction to an SnTox2-containing culture filtrate. The genotypic and phenotypic data of the high-resolution population were used to develop the high-resolution map that further narrowed down the *Snn2* interval. Additional markers were develop based on the high-resolution map and were used to further narrow down the region.

3.3.7. Fungal cultures and culture filtrate production

SnTox2 was obtained from the SnTox2-expressing *P. nodorum* isolate Sn6 as described by Friesen et al. (2007). Culture filtrates of *P. nodorum* were produced by growing the fungus on V8-potato dextrose agar medium as previously described by Liu et al. (2004). Each plate was washed with 50 ml of sterile distilled water once the pycnidia begin to release spores. The spore suspension (200 µl) was added to 50 ml of liquid Fries medium (5 g of ammonium tartrate, 1.0 g of ammonium nitrate, 0.5 g of magnesium sulfate, 1.3 g of potassium phosphate [dibasic], 2.6 g of potassium phosphate [monobasic], 30.0 g of sucrose, 1.0 g of yeast extract, dissolved in 1,000 ml of water) and cultures were grown in an orbital shaker at 80 rpm at 27 °C for 48 to 72 h

followed by stationary growth at 21 °C for 3 weeks. Both growth steps were conducted in the dark. The cultures were filtered through two layers of cheese cloth followed by vacuum filtration through a Whatman No. 1 filter and a 0.45-µm Whatman cellulose nitrate filter for sterilization.

3.3.8. Phenotyping

Plants were infiltrated at the second leaf stage as described in Liu et al. (2004a). The fully expanded secondary leaves of the plants were infiltrated with 25 µl of SnTox2-containing cultures using a 1 ml syringe with the needle removed. The boundaries of the infiltration sites were marked before water-soaking disappeared. After infiltration, all plants were grown at 21°C with a 12-h photoperiod. Leaves were evaluated 5 days after infiltration and scored as insensitive or sensitive based on the presence or absence of necrosis within the infiltrated area.

3.3.9. Mutagenesis

Snn2-disrupted mutants were developed and identified using the methods described in Faris et al. (2010). Mutants were generated by treating seeds of BG301 with 0.35 % v/v ethyl methanesulfonate (EMS) as described in Williams et al. (1992). Here, seeds were presoaked for 8 h in 0.05 M phosphate buffer at pH 8 and treated by soaking at 20 °C for 16 h in a 0.35% (v/v) solution of EMS in the same buffer. The EMS solutions were aerated by gentle agitation on a shaker during treatment. Treated seeds were rinsed in running tap water for 1 min to remove EMS solution from surfaces and planted immediately. The M_1 plants were maintained at 20°C to 24 °C with a 16/8-h day/night cycle. Fourteen plants from each M_2 family at the two-leaf stage were infiltrated with SnTox2-containing cultures and plants were scored for the development of the disease.

3.3.10. Identification of candidate genes

The markers most closely flanking and cosegregating with *Snn2* on the high-resolution map were used as queries in BLAST searches of the IWGSC RefSeqv1.0 wheat reference genome. This information was used to develop additional markers for mapping and further delineation of the *Snn2* gene region. The genomic sequence corresponding to the interval between markers that flanked *Snn2* was obtained from the Chinese Spring reference sequence (https://urgi.versailles.inra.fr/download/iwgsc/IWGSC_RefSeq_Assemblies/v2.0/). The candidate genes for *Snn2* were identified by analyzing the candidate region with IWGSC RefSeq v1.0 annotation. In addition, corresponding candidate region in the sequence scaffolds of other available sequenced lines were downloaded and analyzed for putative genes using BLAST analysis against the NCBI non-redundant database (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch) and by *ab initio* gene prediction programs in Triannot (https://urgi.versailles.inra.fr/triannot/).

3.4. Results

3.4.1. Saturation mapping

All markers were tested for polymorphism between the parents BR34 and Grandin. In the previously published 2D map (Zhang et al. 2009), the markers *TC253803*, *cfd56* and *gwm614.1* mapped distal to *Snn2,* and *cfd51, barc95* and *cfd53* mapped proximal to *Snn2*. In addition to these markers, 11 new markers were added to the *Snn2* candidate region during saturation mapping. Three of them were SNP markers from the 90K SNP chip and nine were SSR markers. The SNP markers *15798* (D_contig15948_318), *18107* (D_GB5Y7FA02HSMR1_278) and *34642* (IAAV298) mapped at distances of 0.9, 5.5 and 14.2 cM distal to the *Snn2,* respectively (Figure 3.1). Contextual sequences were downloaded for these three SNP markers from the

wheat 90K consensus maps and the un-mapped SNPs in between them according to the consensus map were identified. (Wang et al. 2014a). The contextual sequences of the mapped and unmapped SNPs were subjected to BLAST searches against the Chinese Spring 2DS survey sequences to identify scaffolds spanning the *Snn2* region. Three scaffolds (29046, 5390773 and 32556) were identified and used for designing new SSR markers. This led to the development of the eight new SSR markers (Table 3.1, Figure 3.1). Among them, the markers *29046 F2R2, 29046 F3R3,* and *29046 F5R5* co-segregated at a distance of 7.5 cM distal to *Snn2*. The marker *32556 F31R31* mapped at 0.3 cM distal to *Snn2*. The markers *5390773 F5R5* and *5390773 F10R10* co-segregated with *Snn2*. The markers *32556 F36R36* and *32556 F20R20* co-segregated with each other at a distance of 1.0 cM proximal to *Snn2*. Therefore, *Snn2* was delimited to a genetic interval of 1.3 cM after saturation mapping (Figure 3.1).

3.4.2. High resolution mapping

A total of 10,146 gametes of the BR34/BG301 population were genotyped with the *Snn2* flanking markers *TC253803* and *32556 F20R20*, which flanked *Snn2* at genetic distances of 5.8 cM and 1.0 cM, respectively, in the saturation map to identify recombinants for high-resolution mapping (Figure 3.1). This screening resulted in the identification of 280 plants with recombination events between *TC253803* and *32556 F20R20*. The identified recombinant plants were self-pollinated and 16 F_3 plants from the progeny of each recombinant were genotyped with the same flanking markers to identify homozygous recombinants. Markers that mapped between *TC253803* and *32556 F20R20* in the saturation map were used to genotype the identified 280 recombinants of the BR34/BG301 population to place them on the high-resolution map. The marker *32556 F31R31* mapped at a distance of 0.10 cM distal to *Snn2* and the markers *32556 F36R36* and *32556 F20R20* mapped at distances of 0.56 and 1.06 cM proximal to *Snn2*,

respectively (Figure 3.1). The markers *5390773 F5R5* and *5390773 F10R10* still co-segregated with *Snn2*.

The positions of the markers on the genetic map were compared with those in the Chinese Spring reference genome to identify the physical location of the markers. This information was used to design new markers in the region between the flanking markers. Eight new SSR markers were developed in this way and mapped in the high-resolution population (Table 3.1). The markers *CSRef2DS F12R32* and *CSRef2DS F46R46* mapped at distances of 0.53 cM and 0.09 cM distal to *Snn2*, respectively. The markers *CSRef2DS F48R48, CSRef2DS F54R54* and *CSRef2DS F55R55* co-segregated with *Snn2* along with the previously mapped co-segregating markers *5390773 F5R5* and *5390773 F10R10*. The markers *CSRef2DS F56R56, CSRef2DS F57R57* and *CSRef2DS F64R64* mapped at distances of 0.01, 0.04 and 0.49 cM proximal to *Snn2*, respectively. These results delimited *Snn2* to a 0.10 cM interval flanked by *CSRef2DS F46R46* and *CSRef2DS F56R56*. This genetic distance corresponded to a physical distance of 526,787 bp in the IWGSC RefSeq v1.1 Chinese Spring reference genome (Figure 3.1).

3.4.3. Candidate genes

The genomic sequence corresponding to the interval between markers that flanked *Snn2* was downloaded from IWGSC RefSeq v1.1 reference genome and used to identify candidate genes using IWGSC RefSeq v1.0 annotation and the URGI TriAnnot pipeline. Twenty-seven high confidence genes were identified between the *Snn2* flanking markers *CSRef2DS F46R46* and *CSRef2DS F56R56* (Table 3.2). Among them, 13 genes were identified as most plausible candidates including four NB-ARC domain-containing genes and nine protein kinase-containing genes.

3.4.4. Mutagenesis

More than 500 M_1 plants were generated. Thirteen SnTox2-insensitive mutants belonging to 10 different M_2 families were identified by the initial infiltration with a SnTox2-containing culture filtrate. Among them, seven independent mutants were confirmed as real mutants by phenotyping M_3 plants.

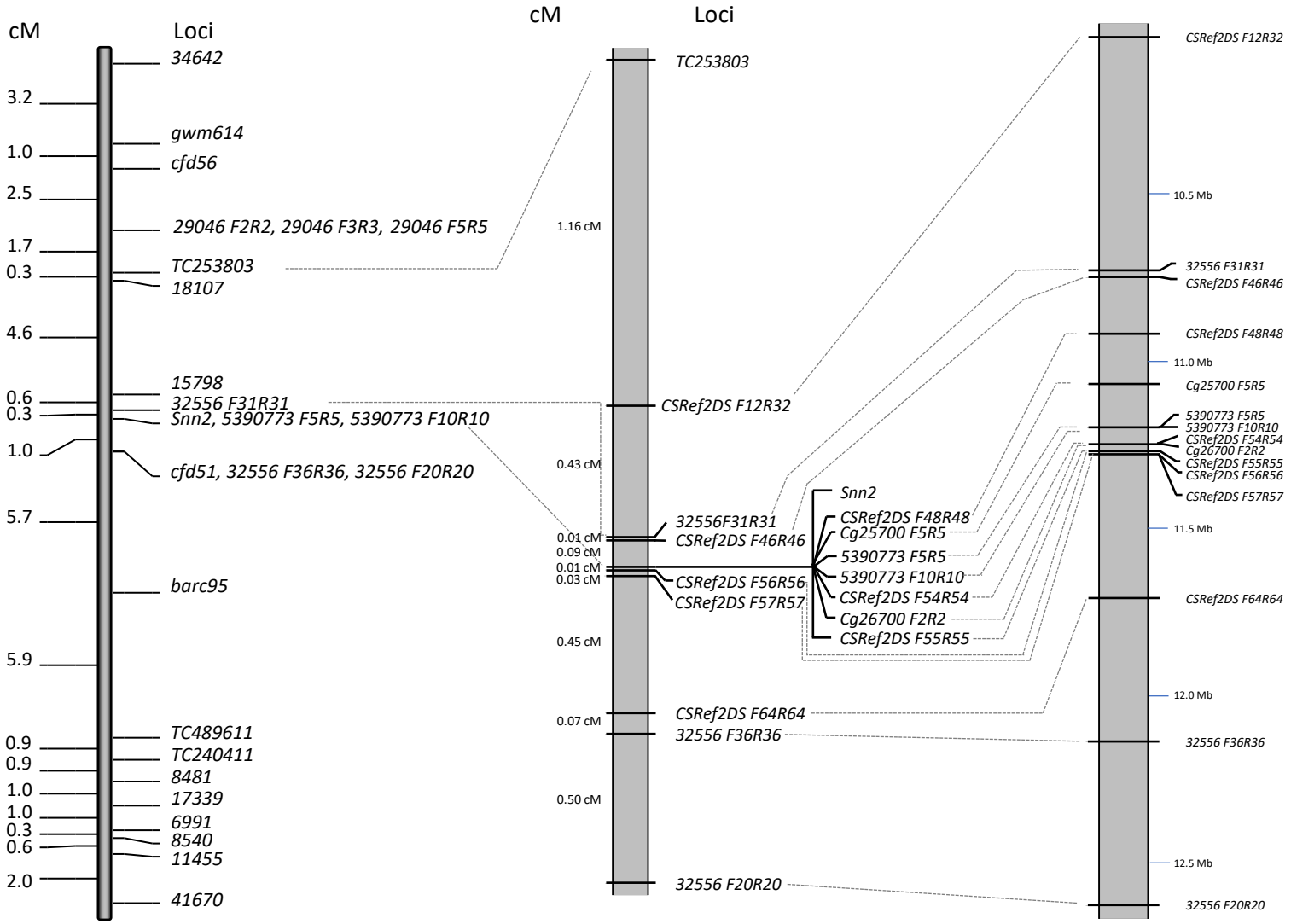

Figure 3.1. Map-based analysis of *Snn2* region. Left: Saturation map constructed from 164 $F_{7:8}$ RIL population. Center: High-resolution map constructed from 5073 F2 plants from BR34/BG301 population. Right: Physical map constructed using IWGSC RefSeq v1.1 reference genome. The dash lines connect the same marker mapped in different maps.

Table 3.1. Markers developed for molecular mapping of *Snn2*.

Marker designation	Marker type	Forward primer	Reverse primer	Start position [a]	End position [b]	Polymorphism	Motif [c]
29046 F5R5	SSR	TCTGGTTTATTGACCAAACTG	GTGTGGCAAAAATTAGAGATG	7219210	7219374	Co-dominant	(TC)26
29046 F3R3	SSR	GTGCGTTGATTTTCTGTTATC	GAGACATGAAAGGTTTTCACC	7644602	7644752	Co-dominant	(AT)22
29046 F2R2	SSR	GACAAGAATACGACCAATGTC	GTTGCCTAGATTATTGTCACG	7698555	7698704	Co-dominant	(AC)26
CSRef2DS F12R32	SSR	AGAGAAAAACCATGGTGAAGT	GGCTCTGATATCAATTGTTGG	10025895	10026046	Co-dominant	(GA)17
32556 F31R31	SSR	CATGATGTGTTTGTCATGTGT	AGGCTGCTCTTCATTTGTACT	10729838	10729995	Co-dominant	(AAC)35
CSRef2DS F46R46	SSR	TACATACTGTCGACAACCACA	GCTCAATCATGCTTTAATTTG	10748550	10748717	Co-dominant	(TC)17
CSRef2DS F48R48	SSR	ATCAGTCTGGACATGATATGC	TGTGATACCGACATCTCTAGC	10914483	10914637	Co-dominant	(GA)19
Cg25700 F5R5	InDel	AATCGGGTTGGCTCATTGTT	CATCAGGAGCGTGGAGTT	11065247	11066175	Dominant	
5390773 F5R5	SSR	GCTTCCTCTTCTAAAGCAAAT	TATACATCCTGTTCAGCCACT	11192553	11192694	Dominant	(AGA)4
5390773 F10R10	SSR	TGTATCCAAACTAGCATCCTG	TATACCTCTTCCACGGTCCT	11198053	11198202	Dominant	(TTTC)5
CSRef2DS F54R54	SSR	CATAGGTACAGCCGCAATCT	CGGGAACACATTCTACTCTCA	11243928	11244075	Dominant	(GA)21
Cg26700 F2R2	InDel	CATATTCCACGCTGCTCCTT	ACGCCTCAGAACCATTCATC	11248733	11249437	Dominant	
CSRef2DS F55R55	SSR	GCACCCGAGTGAATATGAGAT	TTTTCAAGTGGGTCATTGTTC	11262021	11262175	Dominant	(AG)29
CSRef2DS F56R56	SSR	CTATGGGTAGGGAGGTGGTAG	GCCAACTAGGCATGTTGAGTA	11275504	11275690	Dominant	(AG)18
CSRef2DS F57R57	SSR	GGACGAGCAATCTATTTTCCA	CCCGCTTCTAAACTAACTCCA	11278393	11278543	Dominant	(TA)37
CSRef2DS F64R64	SSR	ACAAAAGGCCCTTACAAACTG	GGGCTGTCTTCTTCTTCTTCT	11710547	11710680	Co-dominant	(AGA)15
32556 F36R36	SSR	ACTAGAACAAGGAGGCTTCTC	CCATATTGCGCAGTAGTATTC	12134664	12134802	Co-dominant	(GA)22
32556 F20R20	SSR	GTTTCATTTAAAAGCGGATCT	TGAAACAACCAAGAAGAACAT	12625514	12625662	Co-dominant	(TAA)26

[a, b] The start and end position are in respect to the IWGSC RefSeqv1.0 reference genome.

[c] The repeat motif is shown if the marker is an SSR

Table 3.2. Candidate genes for *Snn2* based on IWGSC RefSeq v1.1. Genes present between the flanking markers *CSRef2DS F46R46* and *CSRef2DS F56R56*. The most plausible candidates based on putative function are highlighted in green.

	Gene ID	Position in IWGSC RefSeq v1.1	Length (bp)	Function description
1	*TraesCS2D02G024100*	chr2D:10748558..10750844 (- strand)	2,287	Cytochrome P450
2	*TraesCS2D02G024200*	chr2D:10798665..10800402 (- strand)	1,738	Cytochrome P450
3	*TraesCS2D02G024300*	chr2D:10816958..10819230 (- strand)	2,273	Kinase, putative
4	*TraesCS2D02G024400*	chr2D:10827275..10831015 (- strand)	3,741	Heavy metal transport/detoxification superfamily protein
5	*TraesCS2D02G024500*	chr2D:10831623..10835466 (- strand)	3,844	Disease resistance protein (NBS-LRR class) family
6	*TraesCS2D02G024600*	chr2D:10867514..10872335 (+ strand)	4,822	NBS-LRR-like resistance protein
7	*TraesCS2D02G024700*	chr2D:10892973..10901915 (- strand)	8,943	Receptor kinase 1
8	*TraesCS2D02G024800*	chr2D:10905482..10906797 (- strand)	1,316	Jasmonate-induced protein
9	*TraesCS2D02G024900*	chr2D:10915163..10917127 (+ strand)	1,965	Receptor-like protein kinase
10	*TraesCS2D02G025000*	chr2D:10964738..10966700 (- strand)	1,963	receptor kinase 1
11	*TraesCS2D02G025100*	chr2D:10979371..10981010 (- strand)	1,640	Glycosyltransferase
12	*TraesCS2D02G025200*	chr2D:10986657..10987050 (+ strand)	394	Defensin
13	*TraesCS2D02G025300*	chr2D:11030130..11030556 (+ strand)	427	Defensin
14	*TraesCS2D02G025400*	chr2D:11037567..11040831 (+ strand)	3,265	Receptor kinase 1
15	*TraesCS2D02G025500*	chr2D:11044131..11049743 (+ strand)	5,613	Receptor-like protein kinase
16	*TraesCS2D02G025600*	chr2D:11057629..11061196 (+ strand)	3,568	Glutamate receptor
17	*TraesCS2D02G025700*	chr2D:11062302..11068423 (- strand)	6,122	Disease resistance protein RPM1
18	*TraesCS2D02G025800*	chr2D:11081912..11083979 (+ strand)	2,068	GDSL esterase/lipase
19	*TraesCS2D02G025900*	chr2D:11087178..11088316 (- strand)	1,139	Endonuclease 8-like 3
20	*TraesCS2D02G026000*	chr2D:11094325..11095952 (- strand)	1,628	Cytochrome P450
21	*TraesCS2D02G026100*	chr2D:11127563..11127862 (+ strand)	300	FORMS APLOID AND BINUCLEATE CELLS 1A
22	*TraesCS2D02G026200*	chr2D:11138562..11149777 (- strand)	11,216	NBS-LRR disease resistance protein homologue
23	*TraesCS2D02G026300*	chr2D:11167498..11169390 (- strand)	1,893	Glutamyl-tRNA (Gln) amidotransferase subunit A
24	*TraesCS2D02G026400*	chr2D:11194726..11198310 (- strand)	3,585	Carboxyl methyltransferase
25	*TraesCS2D02G026500*	chr2D:11212998..11216043 (+ strand)	3,046	Protein kinase family protein
26	*TraesCS2D02G026600*	chr2D:11218167..11221640 (+ strand)	3,474	Protein kinase family protein
27	*TraesCS2D02G026700*	chr2D:11245171..11259790 (+ strand)	14,620	Protein kinase family protein

3.5. Discussion

Although nine host sensitivity gene-NE interactions have been identified in the wheat-*P. nodorum* pathosystem, *Tsn1*, *Snn1* and *Snn3-D1* are the only wheat host sensitivity genes cloned so far (Friesen et al. 2006; Liu et al. 2006; Faris et al. 2010; Liu et al. 2004, 2012; Reddy et al. 2008; Friesen et al. 2007; Zhang et al. 2009; Friesen et al. 2008; Liu et al. 2009; Zhang et al. 2011; Abeysekara et al. 2009; Friesen et al. 2012; Gao et al. 2015; Shi et al. 2015, Shi et al. 2016b, Faris et al. unpublished). It is important to clone host sensitivity genes as well as NE genes in order to understand the molecular mechanisms underlying interactions in this system. Saturated and high-resolution genetic linkage maps of the *Snn2* locus were developed in this study for map-based cloning of *Snn2*.

Friesen et al. (2007) mapped *Snn2* to the short arm of chromosome 2D in a genetic interval of 13.5 cM flanked by the markers *Xgwm614* and *Xbarc95*. Later, Zhang et al. (2009) added additional PCR-based markers that delineated the *Snn2* interval to 4.0 cM. The new markers added in this study further reduced the *Snn2* interval to a genetic distance of 0.10 cM which corresponds to a physical distance of 526,787 bp. The candidate genes for *Snn2* were identified based on the IWGSC RefSeq v1.1 wheat reference genome which was developed using the landrace Chinese Spring. Having a reference genome has made gene cloning much easier than traditional chromosome walking using BACs. However, the infiltration of Chinese Spring with SnTox2-containing culture filtrates showed that it does not have a functional copy of *Snn2*. Recently, several other sequenced wheat genomes also became available under the 10+ wheat genome project. Infiltration of these sequenced wheat lines including Arina, Cadenza, Claire, Jagger, Landmark, Norin61, Paragon, Robigus, Soissons and Stanley revealed that none of them contains a functional copy of *Snn2*. This suggests *Snn2* might be somewhat rare and

more difficult to clone despite having a reference genome. The IWGSC Chinese Spring reference genome was used in this study for both marker development and candidate gene identification because it is the most comprehensive and contiguous assembly of the hexaploid wheat genome that was available at the time this study was conducted.

A total of 27 genes were found between the newly identified flanking markers *CSRef2DS F46R46* and *CSRef2DS F56R56*. Among them, 13 were identified as plausible candidates because they had domains that are commonly found in disease resistance genes. All the host sensitivity genes cloned in the wheat - *P. nodorum* pathosystem have domains typically found in common plant disease resistance genes. *Tsn1*, *Snn1* and *Snn3-D1* all harbor a serine/threonine protein kinase domain (Faris et al. 2010; Shi et al. 2016b; Faris et al. unpublished). Protein kinases have been found to be important for signaling during pathogen recognition and the subsequent activation of plant defense mechanisms. For example, the resistance genes *Pto* in tomato and *Xa21* in rice each possess a serine/threonine protein kinase domain and confer race-specific resistance against *Pseudomonas syringae* pv. *tomato* and *Xanthomonas oryzae* pv. *oryzae,* respectively (Martin et al. 1993; Song et al. 1995). In addition, *Tsn1*, the first host sensitivity gene cloned in the wheat - *P. nodorum* pathosystem, contains NB and LRR domains, which are also commonly found in plant disease resistance genes. The NLR class is considered as the most prevalent type among the R genes identified in plants (Meyers et al. 2003). Plant NLR gene products can recognize pathogen-encoded effectors directly or indirectly to initiate defense responses. For example, *Pita* is an NLR gene in rice that acts against the effector AVR-Pita from *Magnaporthe grisea* through direct recognition (Jia et al. 2000). On the contrary, the Arabidopsis *RPS2* gene recognizes *AvrRpt2* from *Pseudomonas syringae* in an indirect manner through a guardee protein known as RIN4 (Axtell and Staskawicz 2003; Mackey et al.

2002; Kim et al. 2005). Nine protein kinase-containing genes and four NLR genes were found in the *Snn2* candidate region. None of the genes contain a protein kinase in addition to NB and LRR domains. However, two of the protein kinases (*TraesCS2D02G024900* and *TraesCS2D02G025400*) have a major sperm protein domain in addition to the kinase domain and therefore resemble the structure of *Snn3-D1* (Faris et al. unpublished). The candidate genes with the protein kinase domains may be considered strong candidates for *Snn2* as all the previously cloned SNB susceptibility genes have this domain. However, the other genes within the region should not be completely ignored.

One of the biggest challenges of this study was to develop polymorphic markers for genetic mapping. Only 18 markers were found to be polymorphic out of the 184 markers designed in this study. A majority of the markers designed amplified fragments within the target region and were specific to 2DS, but they were not polymorphic between the two parents. This low-level polymorphism has been observed throughout the wheat D genome in many different studies. The number of SNPs mapped in the D genome has been about five-fold lower than that of A and B genome in a consensus map built using six biparental populations and one four-parent multiparent advanced generation intercross (MAGIC) population (Cavanagh et al. 2013). Only 15% of markers in the wheat 90K SNP array were mapped to the D genome in eight double-haploid mapping populations (Wang et al. 2014a). The wheat 90K SNP array was also used in this current study during saturation mapping. However, only 406 SNPs were mapped to the 2D chromosome and only three SNPS (*15798*, *18107* and *34642*) were mapped close to *Snn2*. Sixteen of the eighteen markers developed were SSRs and among them, six were dominant markers. The remaining two markers were gene-specific markers designed for the candidate genes *TraesCS2D02G025700* and *TraesCS2D02G026200*. Both of those markers were also

dominant markers. Also, six of the seven markers that co-segregated with *Snn2* (*Cg25700 F5R5, 5390773 F5R5, 5390773 F10R10, CSRef2DS F54R54, Cg26700* and *CSRef2DS F55R55*) were dominant markers in which the amplicon was present in BG301 and absent in BR34. This suggests that there might be one or more deletions in BR34 within the *Snn2* candidate region.

Genetic linkage mapping and map-based cloning largely depends on the frequency of recombination events between loci. However, the recombination frequency is not equal along a chromosome. It has been found that recombination frequency is higher towards the ends of a chromosome and in regions close to or within genes in wheat (Darrier et al. 2017; Gardnier et al. 2019). The physical to genetic ratio of the *Snn2* candidate region was 5,268 kb/cM which shows a very low recombination frequency. Therefore, screening of more gametes will be needed to resolve the co-segregating markers in the candidate region.

Currently, the identified candidate genes are being sequenced from the BG301 parent line and the *Snn2*-disrupted EMS-mutants for gene validation. Putative genes will be eliminated as candidates if there are no difference in the sequence for a given gene in BG301 and the mutants. This method would work if Chinese Spring has at least a non-functional copy of *Snn2*. However, if Chinese Spring does not carry the gene, it is possible for none of these candidates to be *Snn2*. In that case, alternative approaches will have to be taken for *Snn2* cloning. New techniques such as MutRenSeq (Steuernagel et al. 2017) and AgRenSeq (Arora et al. 2019) are emerging for rapid cloning of resistance genes. However, both these methods have a bias towards NLR genes and can still be dependent on the reference genome. The traditional chromosome walking approach using a BAC library of BG301 will also be a possible option. A gene complementation study will be conducted to validate the gene function after identifying the most promising candidate gene using the mutant analysis. This information will be useful to increase our

understanding of the function, evolution and the molecular mechanisms underlying the wheat – *P. nodorum* interactions. Transcriptional expression and phylogenetic analyses will be possible after the gene validation.

In addition to map-based cloning approach, 120 *Aegilops tauschii* accessions were phenotyped with a SnTox2 containing culture filtrate to be used in a genome wide association study (GWAS). This study would help to narrow down the *Snn2* candidate region to a great extent as the GWAS panel has a higher recombination rate than the bi-parental population used in the current study.

3.6. References

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CHAPTER 4. CHARACTERIZATION OF ALLELIC VARIATION IN THE *SNN1* GENE

4.1. Abstract

Septoria nodorum blotch (SNB) is a severe foliar and glume disease of durum and common wheat. Disease is initiated by the interaction of necrotrophic effectors (NE) produced by the fungus *Parastagonospora nodorum* and host sensitivity genes in an inverse gene-for-gene manner. To date, nine NE sensitivity genes have been identified in the wheat-*P. nodorum* pathosystem. Among them, the SnTox1-*Snn1* interaction accounts for up to 58% of the disease variation. *Snn1* is a member of the wall-associated kinase (WAK) class of plant receptor kinases. The objectives of this study were to analyze *Snn1* allelic diversity, identify causal polymorphisms, and to develop markers useful for marker assisted selection (MAS). The full length *Snn1* gene was sequenced from 70 accessions including diploid, tetraploid and hexaploid wheat species. Multiple sequence alignments revealed 27 haplotypes based on the coding sequence of the gene. Three SNPs were identified as the possible mutations that changed the primitive insensitive allele in wild emmer into the sensitive allele in domesticated wheat. Four subsequent and independent SNPs that caused the sensitive allele to form insensitive alleles were also identified. SNP-based markers were developed for three of these mutations. Results of this study help to develop more specific markers to be used in marker-assisted selection and increase our understanding of the NE sensitivity gene evolution.

4.2. Introduction

Wheat is a major source of sustenance throughout the world and possesses a large market share (FAO 2019). Therefore, factors that affect wheat yield and quality have a significant impact on the agricultural economy. Studies have indicated that global climate change leading to increased ozone levels could promote the colonizing of plants by necrotrophs at an accelerated

rate (Manning et al. 1995). Therefore, it is important to improve our knowledge of host-necrotroph interactions.

Many necrotrophs produce necrotrophic effectors (NEs), which are effectors of pathogenicity (Wang et al. 2014b). NEs are toxic only to host genotypes that express a corresponding gene that recognizes the NE. Such host genes operate as dominant disease susceptibility genes. In these systems, the presence of a pathogen-produced NE and the corresponding dominant host gene for sensitivity leads to a compatible interaction, and ultimately, disease susceptibility (Wang et al. 2014; Oliver et al. 2012). This is the inverse of the classic gene-for-gene interaction commonly observed in plant-biotroph interactions (Friesen and Faris 2010).

A total of nine sensitivity genes that interact with NEs produced by *P. nodorum* have been identified (Friesen et al. 2006; Liu et al. 2006; Faris et al. 2010; Liu et al. 2004a, 2012; Reddy et al. 2008; Friesen et al. 2007; Zhang et al. 2009; Friesen et al. 2008; Liu et al. 2009; Zhang et al. 2011; Abeysekara et al. 2009; Friesen et al. 2012; Gao et al. 2015; Shi et al. 2015). However, only three of them (*Tsn1*, *Snn1*, *Snn3-D1*) have been cloned so far (Faris et al. 2010; Shi et al. 2016b; Faris et al. unpublished). All three genes have characteristics typically found in genes that typically govern disease resistance. SnTox1-*Snn1* was the first NE-sensitivity gene interaction that was identified and *Snn1* was the second host sensitivity gene cloned in the wheat-*P. nodorum* pathosystem (Liu et al. 2004a; Shi et al. 2016b). A compatible SnTox1-*Snn1* interaction leads to defense responses including an oxidative burst, up-regulation of PR-genes, and DNA laddering (Liu et al. 2012). Transcription expression analysis revealed that the *Snn1*-SnTox1 interaction activated TaMAPK3, which belonged to the family of MAPK genes that are typically associated with the PTI pathway (Couto and Zipfel 2016; Shi et al. 2016b).

The host sensitivity gene *Snn1* was mapped to chromosome arm 1BS using the ITMI population (Liu et al. 2004a). Saturation and high-resolution mapping performed using a population derived from a cross between Chinese Spring (CS) and a CS-*T. dicoccoides* chromosome 1B disomic substitution line (Reddy et al. 2008). Shi et al (2016b) cloned *Snn1* using the same population by positional cloning and validated by mutagenesis and transgenesis approaches. *Snn1* is 3045 bp in length and consists of 3 exons and a coding region of 2145 bp. It encodes a protein that contains a signal sequence, a wall-associated receptor kinase galacturonan binding domain (GUB_WAK), epidermal growth factor-calcium binding domain (EGF_CA) a transmembrane domain and a serine/threonine protein kinase (S/TPK) domain and is therefore a member of the wall-associated kinase (WAK) class of receptor kinase genes, which typically act as pathogen recognition receptors (PRRs) for damage-associated molecular patterns (DAMPs) and activate PAMP-triggered immunity (PTI) (Brutus et al. 2010; Delteil et al. 2016). A yeast two-hybrid assay revealed that SnTox1 directly binds to a region between the GUB_WAK and EGF_CA domains. To date, this is the only direct interaction observed in this pathosystem (Shi et al. 2016b, Chapter 2).

Multiple studies have been done to discover resistance gene variants to be used in breeding programs through allele mining. Allele mining is a process of identification and isolation of alleles of a gene responsible for a given trait(s) and their variants in other genotypes (Thakur et al. 2013). It is important to understand the evolutionary forces of selection to allow breeders to directly access key alleles that confer resistance. In this study, my objectives were to determine the level of natural and induced allelic variation in the *Snn1* gene, identify natural and induced mutations that affect a compatible SnTox1-*Snn1* interaction, and developed allele-specific markers to be used in marker-assisted selection.

4.3. Materials and methods

4.3.1. Plant materials

A total of 70 wheat accessions that included ten accessions of *Triticum aestivum* L. ssp. *aestivum*, 42 accessions of *T. turgidum* ssp. *durum,* two accessions of *T. aestivum* ssp. *macha,* two accessions of *T. aestivum* ssp. *compactum,* ten accessions of *T. turgidum* ssp. *dicoccum,* three accessions of *T. turgidum* ssp. *dicoccoides* and one accession of *Aegilops speltoides* ssp. *ligustica* were used for the haplotype analysis (Appendix A). Shi et al. (2016b) found that the functional SnTox1-sensitivity allele is more prevalent in domesticated durum wheat (*T. turgidum* ssp. *drurum)* with 65.91% of the evaluated accessions being sensitive as opposed to only 9.86% of common wheat (*T. aestivum* ssp. *aestivum*) accessions being sensitive (Appendix B). Therefore, more durum wheat lines (42) were included in this study to increase the identification of different haplotypes of *Snn1*. Also, accessions were selected from different parts of the world to increase the diversity of the population.

A total of 69 ethyl methanesulfonate (EMS)-mutants from the targeting induced local lesions in genomes (TILLING) population of *T. turgidum* ssp. *durum* cv. Kronos (Krasileva et al. 2017) were evaluated to determine the effects of mutations on reaction to SnTox1. To identify the mutants for analysis, the *Snn1* genomic sequence was subjected to BLAST searches against the Kronos TILLING database at http://dubcovskylab.ucdavis.edu/wheat_blast, and the inbuilt Jbrowse was used to identify the available mutants with mutations in the target region. The mutants were evaluated as described below.

4.3.2. Fungal culture production

A *Pichia pastoris* culture expressing SnTox1 was started in yeast extract-peptone-dextrose (YPD, 2 mL) medium from a frozen stock and incubated at 30^{o}C for 48 hours with

vigorous shaking. The resulting culture was diluted 1:100 and sub-cultured in new YPD medium (250 mL). It was then incubated at 30°C for another 48 hours with vigorous shaking. The SnTox1-containing cultures were harvested by subjecting the culture to centrifugation at 4000 g for ten minutes followed by filtration through a 0.45-µm Whatman cellulose nitrate filter.

4.3.3. Phenotyping and statistical analysis

A total of six replicates were grown in a completely randomized design (CRD). Each replicate consisted of plants grown in small plastic cones with three plants per cone to represent a single experimental unit. Plants were infiltrated with SnTox1 at the second leaf stage as described in Liu et al. (2004b). The fully expanded secondary leaves of the plants were infiltrated with 25 µl of the SnTox1 culture using a 1 mL syringe with the needle removed. The boundaries of the infiltration sites were marked before water-soaking disappeared. After infiltration, all plants were grown at 21 °C with a 12-h photoperiod. Leaves were evaluated 5 days after infiltration and scored for severity of necrosis within the infiltrated area.

An expanded scoring scale, which included seven categories (0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0), was developed for rating SnTox1 sensitivity levels based on the one defined by Zhang et al. (2011) (Figure 4.1). The scores 0, 1.0, 2.0 and 3.0 were same as the ones defined by Zhang et al. (2011) where 0 = no visible necrosis; 1.0 = mottled necrosis extending to the boundaries of the infiltrated area; 2.0 = highly visible necrosis with little mottling extending to the boundaries of the infiltrated area without complete tissue collapse and little or no shriveling or narrowing of the leaf within the infiltrated region; and 3.0 = extensive and severe necrosis throughout the entire infiltrated area with complete tissue collapse and shriveling or narrowing of the leaf within the infiltrated region. Scores of 0.5, 1.5 and 2.5 were newly added to the scale to represent sensitivity levels that were intermediate between 0 – 1.0, 1.0 – 2.0 and 2.0 – 3.0, respectively. A

score ≤ 1.0 was considered as an insensitive phenotype and a score > 1.0 was considered as a sensitive phenotype.

Bartlett's test for homogeneity of variances (Snedecor and Cochran 1989) and analysis of variance was conducted using RStudio Version 1.1.442 (RStudio). Fisher's protected least significant difference (LSD) was also calculated at an α level of 0.05 to identify significant differences of the phenotypic scores among lines.

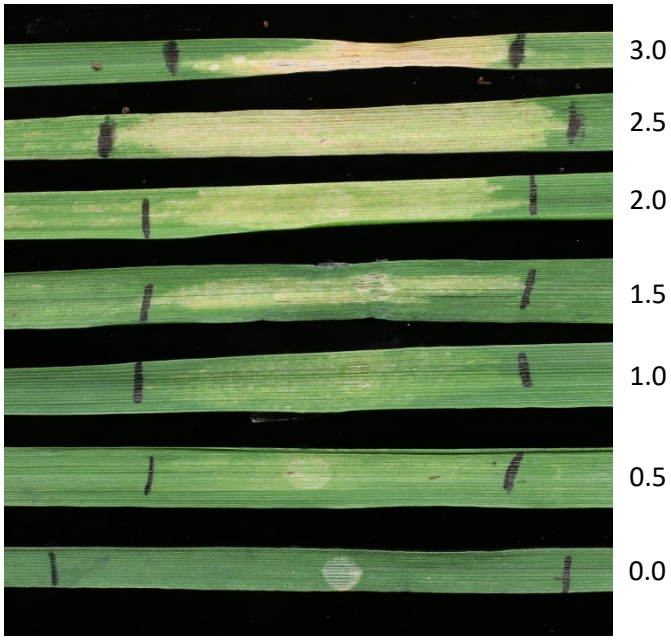


Figure 4.1. The scoring scale used for phenotyping wheat leaves after infiltration with SnTox1. A score of 0 indicates an insensitive response and a score of 3.0 indicates a severely sensitive response.

4.3.4. Identification of lines with two copies of *Snn1*

In work previously done by Shi et al. (unpublished), sequencing of *Snn1* and analysis of the sequencing reads revealed that some lines contained double peaks in chromatograms of sequence reads, mostly at one position (127). This indicated that two copies of the gene existed and were being amplified. A 4-bp cutter restriction enzyme (*Hpy*CH4IV) that detected the SNP (G127A) was identified. It was used to develop a CAPS marker for *Snn1* to test all lines to be sequenced in an effort to identify lines with two copies of the gene (Shi et al. unpublished). This

CAPS marker was designated as *fcp667* and was amplified using primers *3476283F9* and *3476283R3* reported in Shi et al. (2016b).

Each polymerase chain reaction (PCR) was conducted using a total volume of 10 µl consisting of 2 µl of template DNA, 1X PCR buffer, 1 mM $MgCl_2$, 0.2 mM dNTPs, 4 pmol of the primers and 0.5 units of homemade Taq DNA polymerase. The PCR conditions were initial denaturation at 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, an annealing temperature for 30 s started at 60.8 °C with 0.2 ºC decrements at each cycle, and extension at 72 °C for 90 s, followed by a final extension of 72 °C for 7 min. The restriction enzyme digestion was conducted in a total volume of 17 µl consisting of 10 units of the restriction enzyme, 2 µl of 10X CutSmart buffer (New England BioLabs Incorporation) and 10 µl of the PCR product. Digested product was electrophoresed on 1.5 % agarose gels for 60 minutes at 90 V and the banding pattern was used to identify the lines with two copies of *Snn1* (Figure 4.2). It was later found that although most lines with two copies of *Snn1* had the SNP at position 127 bp, there were others with two copies that did not differ at position 127 bp and instead had SNPs at other positions. Therefore, *fcp667* was not completely diagnostic for the presence of two copies of *Snn1*, and some additional sequencing was needed to characterize the gene in two-copy accessions not harboring the SNP at 127 bp.

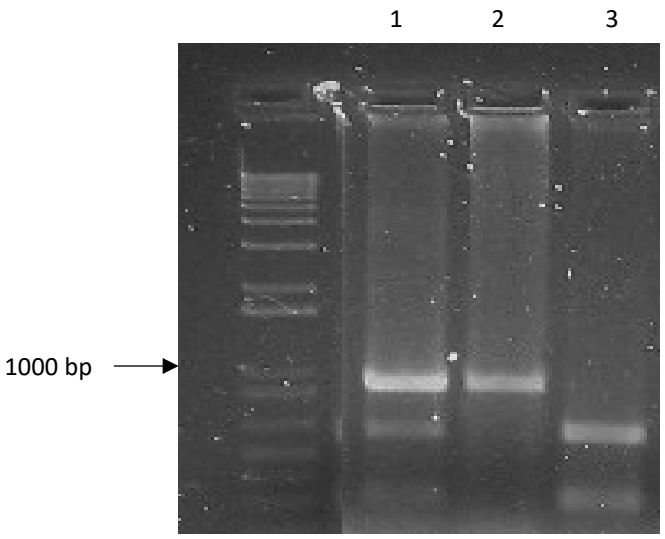


Figure 4.2. Restriction enzyme digestion profiles of PCR products amplified from genomic DNA using the CAPs marker *fcp667*. Fragments are separated on a 1.5% agarose gel. Lanes 1, 2 and 3 contain digested PCR products from an accession with two copies of *Snn1*, an accession with copy 1 of *Snn1,* and an accession with copy 2 of *Snn1*, respectively.

4.3.5. Polymerase chain reaction and sequencing

4.3.5.1. Sequencing the lines with two copies of the gene

If *fcp667* did reveal the presence of two copies through cleavage of the amplicon, we did the following to separately sequence the two copies. The full-length genomic sequence of *Snn1* was amplified from the identified lines using the primers *3476283R3* and *3UTRF4* reported in Shi et al. (2016b). PCR was done for a total volume of 30 µl consisting of 2.5 µl of template DNA, 1X PCR buffer, 1 mM $MgCl_2$, 0.2 mM dNTPs, 4 pmol of the primers and 0.5 units of Biolase Taq DNA polymerase (Bioline USA Inc.). The PCR reaction had two phases. In phase 1, the conditions were initial denaturation at 94 °C for 5 min, followed by 5 cycles of 94 °C for 30 s, annealing temperature of 58 °C for 30 s, and extension at 72 °C for 90 s. Then it was immediately followed by phase two with 30 cycles of 94 °C for 30 s, the annealing temperature started at 60.8 °C for 30 s with 0.2 ^{o}C decrements at each cycle, and extension at 72 °C for 90 s, followed by a final extension of 72 °C for 7 min.

At least two independent PCR reactions for each fragment were performed to identify PCR errors. PCR amplicons were electrophoresed on 1.5 % agarose gels for 60 minutes at 90 V. The amplified fragment was excised from the gel and purified using the Wizard ® SV gel and PCR Clean Up System from Promega (Madison, WI). Each purified product was ligated to the pDrive cloning vector from QIAGEN PCR cloning kit (QIAGEN, Hilden, Germany) and transformed into NEB 5-α competent *E. coli* cells (New England Biolabs, Ipswich, MA). Blue-white selection with X-gal was used to identify positive colonies that contained the ligated vector. Colonies containing copy 1 and colonies with copy 2 were differentiated by PCR followed by restriction digestion for five colonies for each cloning reaction. At least two colonies from each type were then cultured in 2.5 mL liquid LB media with carbenicillin for 12-16 hours. Plasmid DNA was extracted from cells using the QIAprep spin Miniprep kit (QIAGEN, Hilden, Germany) and sent to Eurofins MWG Operon (Louisville, KY) for Sanger sequencing. Each clone was sequenced using four primer pairs that amplified overlapping fragments as reported in Shi et al. (2016b).

Total RNA was isolated from leaf tissue of the lines with two copies and used for cDNA synthesis as described by Faris et al. (2010). The cDNA was amplified using the primers reported in Shi et al. (2016b). The amplicons were sequenced and compared with the corresponding genomic sequence to identify the expressed copy.

4.3.5.2. Sequencing the lines with one copy of the gene

If *fcp667* indicated that a line carried a single copy of *Snn1*, i.e. it did not cleave the PCR product, it was sequenced as follows. Four overlapping fragments representing the complete gene were amplified from the lines with only one copy using the primers reported in Shi et al. (2016b). At least two independent PCR reactions for each fragment were sequenced to eliminate

PCR and sequencing errors. The chromatograms of the resulting sequence reads were checked for the presence of double peaks to identify lines with two copies that could not be identified by *fcp667*. If none were observed, then we assumed that there was a single copy present.

4.3.5.3. Sequencing the lines with two copies of the gene that were not identified by the CAPs marker

If double peaks were observed in sequencing reads of a line that was not revealed to have two copies based on the *fcp667* CAPs marker, the two copies were sequenced as follows. The full-length genomic sequence of *Snn1* was amplified and cloned in *E. coli* cells (New England Biolabs, Ipswich, MA) from the identified lines as described in 4.3.5.1. Blue-white selection with X-gal was used to identify positive colonies that contained the ligated vector. Multiple positive colonies were cultured separately in liquid LB medium (2.5 mL) with carbenicillin for 12-16 hours. Plasmid DNA was extracted from cells using the QIAprep spin Miniprep kit (QIAGEN, Hilden, Germany) and sent to Eurofins MWG Operon (Louisville, KY) for Sanger sequencing. Resulting sequencing reads were compared with those obtained before cloning (See 4.3.5.2) and the nucleotides at the positions where double peaks were observed were used to separate the two copies. Plasmid DNA was extracted and sequenced from multiple colonies until three replicates of each *Snn1* copy were obtained.

4.3.6. Sequence data analysis

Sequence reads were assembled into contigs for each accession using the software CodonCode V7.1.2 (CodonCode corporation). The predicted amino acid sequences were generated from the contigs using ExPASy translation tool (Swiss Institute of Bioinformatics). Multiple sequence alignments were generated using MUSCLE (Edgar 2004) in MEGA v7.0 (Kumar et al. 2016) for both genomic and deduced amino acid sequences. The phylogenetic tree

was constructed using the Neighbor Joining method using the p distance model with pairwise-deletion option in MEGA v7.0. Confidence values for branch nodes were calculated using 1000 bootstraps. Haplotype analysis, nucleotide diversity analysis (Nei 1987), Tajima (Tajima 1989) and Fu and Li (Fu and Li 1993) neutrality tests were performed using DnaSP v5 (Librado and Rozas 2009) (Significance $P < 0.05$). SIFT scores of the critical SNPs were calculated using the Ensemble Variant Effect Predictor (McLaren et al. 2016).

4.3.7. Marker development

Diagnostic dCAPS markers were developed for the identified critical SNPs using dCAPS Finder 2.0 (Neff et al. 2002). Primers containing one or more mismatches to the template DNA were designed in a way that they introduce or disrupt a restriction enzyme recognition site at the target site. PCR and restriction enzyme digestion conditions were as described in 4.3.4. The resulting products were subjected to electrophoresis on 6 % polyacrylamide gels for 1 hour, stained with GelRed stain, and visualized with a Typhoon 9500 variable mode imager (GE Healthcare Life Sciences, Piscataway, NJ).

4.3.8. Analysis of Kronos mutants

Three plants from each mutant line were used to confirm the presence of the expected mutation. This was done by amplification and sequencing of fragments expected to have mutations. Chromatograms of the sequence reads were studied to identify the lines with mutations that were homozygous at the target site using the software Sequencher v4.8 (Gene Codes Corporation). Mutants with homozygous mutations were self-pollinated to increase seeds and the seeds from the progenies were used in the phenotypic analysis. A total of six replicates were grown in a completely randomized design. Each replicate consisted of plants grown in small plastic cones with three plants per cone to represent a single experimental unit. Plants were

infiltrated with a purified SnTox1 at the second leaf stage as described in Liu et al. (2004b) and scored according to the scoring scale developed in this study.

4.4. Results

4.4.1. Reaction of the 70 wheat lines to SnTox1

Screening of the wheat accessions with SnTox1 showed that different lines had different levels of sensitivity to SnTox1. Bartlett's Chi-squared test for homogeneity of variances showed that the variance among the six replicates was not significantly different ($\chi^2_{df=5} = 6.8537$, $P = 0.2318$). Therefore, means were combined for further analysis. According to the analysis of variance, mean phenotypic scores were significantly different among the accessions [Pr (>F) = $2.2e^{-16}$]. LSD data are shown in Table 4.1. A majority of the 70 accessions had phenotypic scores between 0.0 and 0.5 or between 2.1 and 2.5 (Figure 4.3). Lines with scores ≤ 1.0 were considered as insensitive and lines with scores > 1.0 were considered as a sensitive.

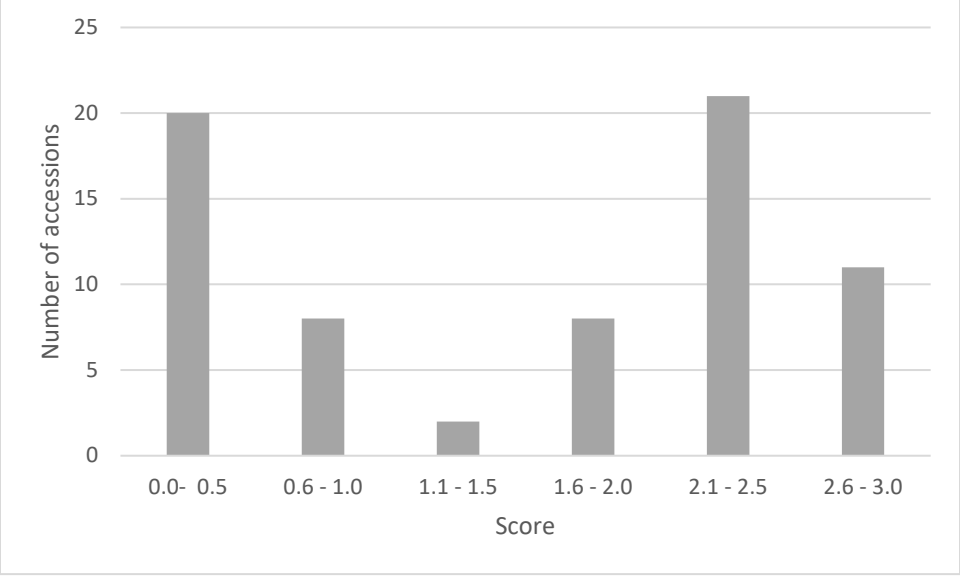


Figure 4.3. Number of accessions present in each phenotypic group. A score ≤ 1.0 was considered as insensitive and a score > 1.0 was considered as sensitive.

4.4.2. Analysis of *Snn1* copy number and expression in the 70 wheat lines

Among the 70 lines studied, 46 had only one copy and 24 had two copies of *Snn1*. Two SNPs that were different in the two copies of most lines were identified by sequence alignment (G127A and A264G). Both are located in the coding region of the gene. G127A encodes a missense variant V43I, and A264G is a synonymous mutation. These two SNPs were used to differentiate the two copies as copy 1 and copy 2 if the mutation was present. Copy 1 had an A at position 127 and a G at position 264. Copy 2 had a G at position 127 and an A at position 264. Five lines in which both copies had A at position 127 and G at 264 were observed (Opata85, Renan, Langdon, N-85, *T. dicoccum* 42). These were the lines where the presence of two copies could not be identified using the marker *fcp667*. For those lines in which SNP G127A was absent, each copy was assigned as copy 1 or copy 2 arbitrarily.

Sequencing of cDNA revealed that when two copies were present only one copy was expressed. Except for *T. dicoccum* 58, all the lines contained two copies had copy 1 expressed. Among lines that possessed only one copy, either copy 1 or copy 2 was expressed depending on which copy was present. Nineteen lines with copy 1 and 27 lines with copy 2 were found to be expressed among the lines with one copy of *Snn1* (Table 4.1).

Table 4.1. *Snn1* copy number and expression.

Accession	Number of copies	Expressed copy	Average phenotypic score[a]	Amino acid haplotype
Faia	2	Copy 1	3.00	AA_Hap 14
T. dicoccum 178	2	Copy 1	2.94	AA_Hap 14
Trinakria	1	Copy 2	2.86	AA_Hap 8
CHEN 7	1	Copy 2	2.83	AA_Hap 8
Cappelli	1	Copy 2	2.79	AA_Hap 8
Mocho de Espiga Quadrada	1	Copy 1	2.79	AA_Hap 14
Altar 84	1	Copy 2	2.67	AA_Hap 7
Durati	1	Copy 2	2.67	AA_Hap 8
Red Egyptian	2	Copy 1	2.67	AA_Hap 14
Agamia	1	Copy 2	2.58	AA_Hap 8
Biskri Glabre RP 2	1	Copy 2	2.54	AA_Hap 8
12:61-8T-5T-2aT-2B-2T	1	Copy 2	2.50	AA_Hap 8
Rurik	2	Copy 1	2.50	AA_Hap 14
Vallelunga Glabra	1	Copy 1	2.50	AA_Hap 14
T. dicoccum 135	2	Copy 1	2.46	AA_Hap 14
Sora	1	Copy 2	2.43	AA_Hap 8
Rieti	2	Copy 1	2.42	AA_Hap 14
Croc 1	1	Copy 2	2.39	AA_Hap 8
Iumillo	2	Copy 1	2.39	AA_Hap 14
Golden Ball	2	Copy 1	2.36	AA_Hap 14
Bidi 17	1	Copy 2	2.33	AA_Hap 8
Mahmoudi Ag	1	Copy 2	2.32	AA_Hap 8
Indian Runner	1	Copy 1	2.25	AA_Hap 21
Mountrail	1	Copy 2	2.25	AA_Hap 8
Souri	1	Copy 1	2.25	AA_Hap 21
Wales	1	Copy 2	2.17	AA_Hap 8
Svevo	1	Copy 2	2.14	AA_Hap 8
Ble Dur	1	Copy 1	2.08	AA_Hap 21
T. dicoccum 63	2	Copy 1	2.08	AA_Hap 14
Kronos	1	Copy 2	2.06	AA_Hap 8
Chinese Spring	1	Copy 1	2.03	AA_Hap 14
Laidley	1	Copy 2	2.03	AA_Hap 8
Maier	1	Copy 2	2.00	AA_Hap 8
Yar	1	Copy 2	2.00	AA_Hap 8
Adjini AC 1	2	Copy 1	1.94	AA_Hap 14
DN-2378	1	Copy 2	1.93	AA_Hap 8
Dverd 2	1	Copy 2	1.92	AA_Hap 8
Volo	2	Copy 1	1.82	AA_Hap 14
G532	1	Copy 2	1.67	AA_Hap 8
Termok	2	Copy 1	1.58	AA_Hap 14
Falcin 1	1	Copy 2	1.50	AA_Hap 8
Botno	2	Copy 1	1.10	AA_Hap 20
Kubanka	2	Copy 1	0.97	AA_Hap 20
T. dicoccum 177	1	Copy 1	0.78	AA_Hap 13
Grandin	1	Copy 2	0.72	AA_Hap 9
Ben	1	Copy 1	0.71	AA_Hap 12
Cotrone	1	Copy 1	0.69	AA_Hap 17
Divide	2	Copy 1	0.60	AA_Hap 12
Amery	1	Copy 2	0.54	AA_Hap 10
Hope	1	Copy 2	0.53	AA_Hap 9
Langdon[b]	2	Copy 1	0.44	AA_Hap 12

Table 4.1. *Snn1* copy number and expression (continued).

Accession	Number of copies	Expressed copy	Average phenotypic score[a]	Amino acid haplotype
Ajaia 9	2	Copy 1	0.42	AA_Hap 15
Timstein	1	Copy 2	0.42	AA_Hap 9
T. dicoccum 119	1	Copy 1	0.39	AA_Hap 13
T. dicoccum 120	1	Copy 1	0.36	AA_Hap 13
Renan[b]	2	Copy 1	0.31	AA_Hap 12
T. dicoccum 36	1	Copy 1	0.29	AA_Hap 13
T. dicoccum 42[b]	2	Copy 1	0.28	AA_Hap 6
T. dicoccum 58	2	Copy 2	0.28	AA_Hap 5
T. dicoccum 10	1	Copy 1	0.25	AA_Hap 13
FHB4512	2	Copy 1	0.22	AA_Hap 15
Negro	1	Copy 1	0.22	AA_Hap 16
T. dicoccoides 10	1	Copy 1	0.08	AA_Hap 2
Kahla	1	Copy 1	0.04	AA_Hap 19
Ae speltoides 92	1	Copy 1	0.00	AA_Hap 1
N-85[b]	2	Copy 1	0.00	AA_Hap 15
Novo	2	Copy 1	0.00	AA_Hap 11
Opata 85[b]	2	Copy 1	0.00	AA_Hap 18
T. dicoccoides 13	1	Copy 1	0.00	AA_Hap 4
T. dicoccoides 27	1	Copy 1	0.00	AA_Hap 3

[a] Fisher's least significant difference (LSD) of average phenotypic scores was 0.469 at the 0.05 level of probability.
[b] Copy 1 and copy 2 were assigned arbitrarily because the SNP G127A was absent.

4.4.3. Nucleotide diversity and haplotype analysis

Analysis of the full-length coding sequence revealed 144 SNPs defining 38 haplotypes with a haplotype diversity (Hd) = 0.919 (Table 4.2). The alignments of the coding region of the expressed copies of the gene identified 68 SNPs that correspond to 42 amino acid changes. These polymorphisms represent 27 coding sequence haplotypes (CDS_Haps) (Figure 4.4) based on the nucleotide sequences with a haplotype diversity of 0.905 (Table 4.1). This corresponds to 21 haplotypes based on the predicted amino acid sequences (Figure 4.5). The most frequent haplotype, CDS_ Hap8, was identified in 19 out of the 70 accessions. According to the reaction to SnTox1, nine haplotypes were associated with the sensitive phenotype and 18 haplotypes were associated with the insensitive phenotype.

A comparative analysis revealed high levels of nucleotide diversity in *Snn1*. The average number of nucleotide differences (K) was 9.04679 (Table 4.2). The overall nucleotide diversity

(π) was 0.00298, and the overall nucleotide polymorphism (θw) was 0.00942. The highest level of nucleotide diversity was in the first intron, which had a value of 0.0091. This nucleotide polymorphism level in intron 1 was more than threefold higher than the level observed in the other regions. In addition, the genetic variability of the *Snn1* gene was evaluated using the Tajima's D and the Fu and Li neutrality tests to determine if the target *Snn1* gene sequence fits the neutrality model of evolution. According to the results, the Tajima's D and Fu and Li's D and F values for the full genomic region of *Snn1* were negative. However, neutrality tests were not significant ($P < 0.05$) for exon 2.

Table 4.2. Nucleotide diversity and neutrality test of different regions along the *Snn1* gene.

Region	Fragment length	π	θw	Tajima' D	Fu and Li's D	Fu and Li's F	K	HD
Full gene	3045	0.00298	0.00942	-2.37537*	-6.57698*	-5.86209*	9.04679	0.919
Coding region	2145	0.00237	0.00658	-2.15187*	-5.40327*	-4.94689*	5.08447	0.905
Exon1	991	0.00331	0.00713	-1.72179	-4.56544*	-4.17623*	3.27619	0.856
Intron1	93	0.00910	0.06158	-2.73140*	-7.03900*	-6.48974*	0.82774	0.084
Exon2	165	0.00169	0.00629	-1.65756	-0.96041	-1.38980	0.27909	0.239
Intron2	807	0.00390	0.01110	-2.12419*	-5.58455*	-5.09799*	3.13458	0.828
Exon3	989	0.00155	0.00609	-2.36229*	-5.50172*	-5.17345*	1.52919	0.631

π nucleotide diversity (Nei and Li 1979); θw per-site estimates of diversity by Watterson's theta; K average nucleotide difference.
* Significant at $p < 0.05$

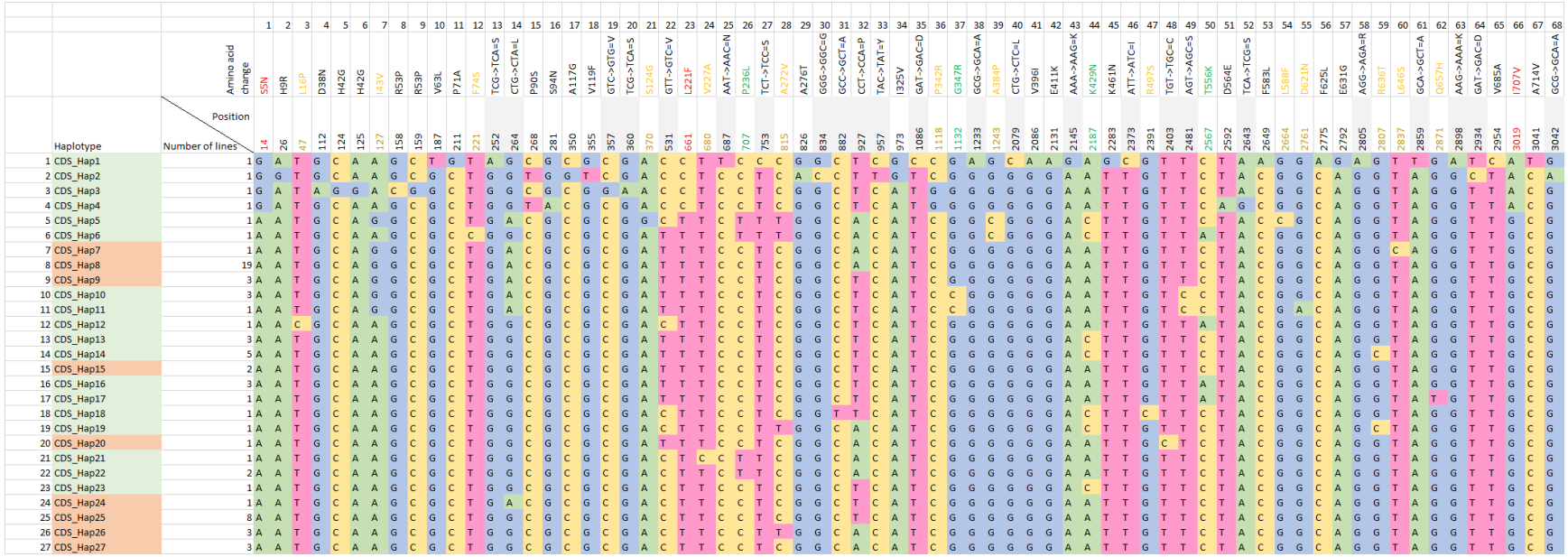
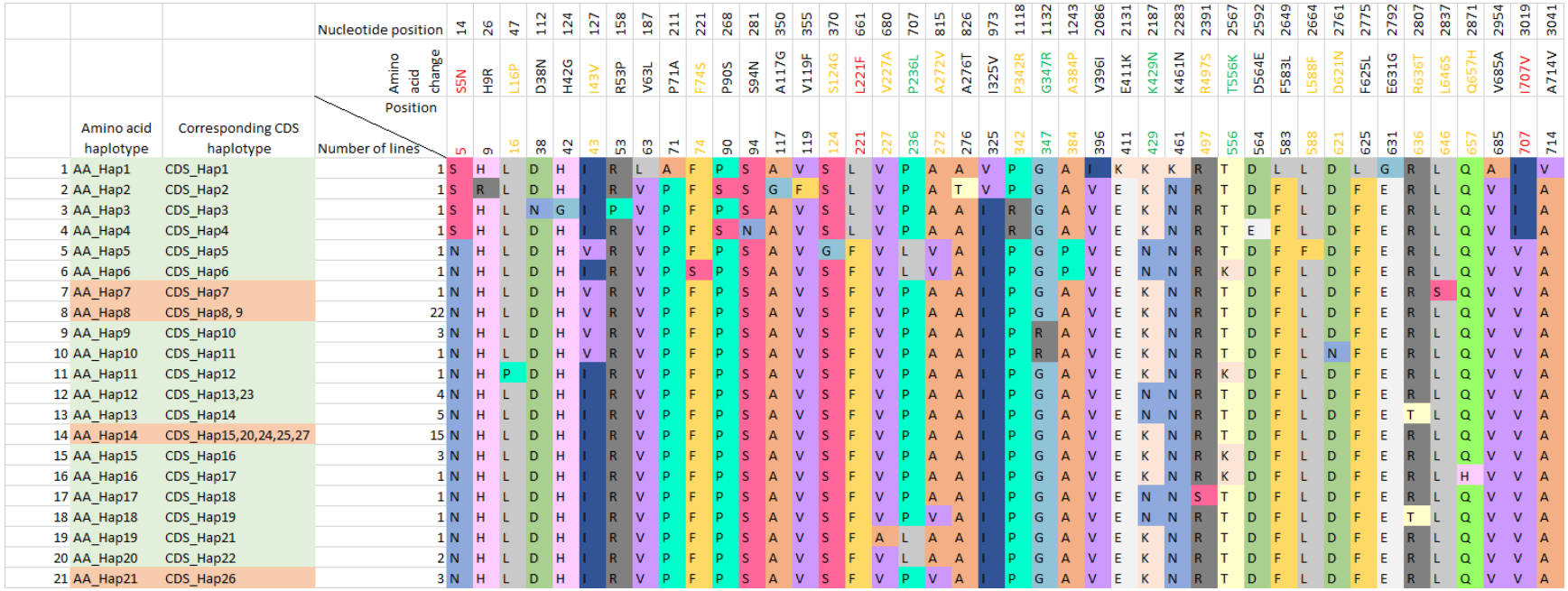


Figure 4.4. Haplotypes identified based on the coding sequences. Green shade: Insensitive haplotype, Tan shade: sensitive haplotype, Red font: change from insensitive to sensitive, Green font: change from sensitive to insensitive, Tan font: no change in phenotype, Grey shade: synonymous mutation



	Amino acid haplotype	Corresponding CDS haplotype	Nucleotide position	14	26	47	112	124	127	158	187	211	221	268	281	350	355	370	661	680	707	815	826	973	1118	1132	1243	2086	2131	2187	2283	2391	2567	2592	2649	2664	2761	2775	2792	2807	2837	2871	2954	3019	3041
			Amino acid change	S5N	H9R	L16P	D38N	H42G	I43V	R53P	V63L	P71A	F74S	P90S	S94N	A117G	V119F	S124G	L221F	V227A	P236L	A272V	A276T	I325V	P342R	G347R	A384P	V396I	E411K	K429N	K461N	R497S	T556K	D564E	F583L	L588F	D621N	F625L	E631G	R636T	L646S	Q657H	V685A	I707V	A714V
			Position	5	9	16	38	42	43	53	63	71	74	90	94	117	119	124	221	227	236	272	276	325	342	347	384	396	411	429	461	497	556	564	583	588	621	625	631	636	646	657	685	707	714
1	AA_Hap1	CDS_Hap1	Number of lines: 1	S	H	L	D	H	I	R	L	A	F	P	S	A	V	S	L	V	P	A	A	V	P	G	A	I	K	K	K	R	T	D	L	L	D	L	G	R	L	Q	A	I	V
2	AA_Hap2	CDS_Hap2	1	S	R	L	D	H	I	R	V	P	F	S	S	G	F	S	L	V	P	A	T	V	P	G	A	V	E	K	N	R	T	D	F	L	D	F	E	R	L	Q	V	I	A
3	AA_Hap3	CDS_Hap3	1	S	H	L	N	G	I	P	V	P	F	P	S	A	V	S	L	V	P	A	A	I	R	G	A	V	E	K	N	R	T	D	F	L	D	F	E	R	L	Q	V	I	A
4	AA_Hap4	CDS_Hap4	1	S	H	L	D	H	I	R	V	P	F	S	N	A	V	S	L	V	P	A	A	I	R	G	A	V	E	K	N	R	T	E	F	L	D	F	E	R	L	Q	V	I	A
5	AA_Hap5	CDS_Hap5	1	N	H	L	D	H	V	R	V	P	F	P	S	A	V	G	F	V	L	V	A	I	P	G	P	V	E	N	N	R	T	D	F	F	D	F	E	R	L	Q	V	V	A
6	AA_Hap6	CDS_Hap6	1	N	H	L	D	H	I	R	V	P	S	P	S	A	V	S	F	V	L	V	A	I	P	G	P	V	E	N	N	R	K	D	F	L	D	F	E	R	L	Q	V	V	A
7	AA_Hap7	CDS_Hap7	1	N	H	L	D	H	V	R	V	P	F	P	S	A	V	S	F	V	P	A	A	I	P	G	A	V	E	K	N	R	T	D	F	L	D	F	E	R	S	Q	V	V	A
8	AA_Hap8	CDS_Hap8, 9	22	N	H	L	D	H	V	R	V	P	F	P	S	A	V	S	F	V	P	A	A	I	P	G	A	V	E	K	N	R	T	D	F	L	D	F	E	R	L	Q	V	V	A
9	AA_Hap9	CDS_Hap10	3	N	H	L	D	H	V	R	V	P	F	P	S	A	V	S	F	V	P	A	A	I	P	R	A	V	E	K	N	R	T	D	F	L	D	F	E	R	L	Q	V	V	A
10	AA_Hap10	CDS_Hap11	1	N	H	L	D	H	V	R	V	P	F	P	S	A	V	S	F	V	P	A	A	I	P	R	A	V	E	K	N	R	T	D	F	L	N	F	E	R	L	Q	V	V	A
11	AA_Hap11	CDS_Hap12	1	N	H	P	D	H	I	R	V	P	F	P	S	A	V	S	F	V	P	A	A	I	P	G	A	V	E	K	N	R	K	D	F	L	D	F	E	R	L	Q	V	V	A
12	AA_Hap12	CDS_Hap13,23	4	N	H	L	D	H	I	R	V	P	F	P	S	A	V	S	F	V	P	A	A	I	P	G	A	V	E	N	N	R	T	D	F	L	D	F	E	R	L	Q	V	V	A
13	AA_Hap13	CDS_Hap14	5	N	H	L	D	H	I	R	V	P	F	P	S	A	V	S	F	V	P	A	A	I	P	G	A	V	E	N	N	R	T	D	F	L	D	F	E	T	L	Q	V	V	A
14	AA_Hap14	CDS_Hap15,20,24,25,27	15	N	H	L	D	H	I	R	V	P	F	P	S	A	V	S	F	V	P	A	A	I	P	G	A	V	E	K	N	R	T	D	F	L	D	F	E	R	L	Q	V	V	A
15	AA_Hap15	CDS_Hap16	3	N	H	L	D	H	I	R	V	P	F	P	S	A	V	S	F	V	P	A	A	I	P	G	A	V	E	K	N	R	K	D	F	L	D	F	E	R	L	Q	V	V	A
16	AA_Hap16	CDS_Hap17	1	N	H	L	D	H	I	R	V	P	F	P	S	A	V	S	F	V	P	A	A	I	P	G	A	V	E	K	N	R	K	D	F	L	D	F	E	R	L	H	V	V	A
17	AA_Hap17	CDS_Hap18	1	N	H	L	D	H	I	R	V	P	F	P	S	A	V	S	F	V	P	A	A	I	P	G	A	V	E	N	N	S	T	D	F	L	D	F	E	R	L	Q	V	V	A
18	AA_Hap18	CDS_Hap19	1	N	H	L	D	H	I	R	V	P	F	P	S	A	V	S	F	V	P	V	A	I	P	G	A	V	E	N	N	R	T	D	F	L	D	F	E	T	L	Q	V	V	A
19	AA_Hap19	CDS_Hap21	1	N	H	L	D	H	I	R	V	P	F	P	S	A	V	S	F	A	L	A	A	I	P	G	A	V	E	K	N	R	T	D	F	L	D	F	E	R	L	Q	V	V	A
20	AA_Hap20	CDS_Hap22	2	N	H	L	D	H	I	R	V	P	F	P	S	A	V	S	F	V	L	A	A	I	P	G	A	V	E	K	N	R	T	D	F	L	D	F	E	R	L	Q	V	V	A
21	AA_Hap21	CDS_Hap26	3	N	H	L	D	H	I	R	V	P	F	P	S	A	V	S	F	V	P	V	A	I	P	G	A	V	E	K	N	R	T	D	F	L	D	F	E	R	L	Q	V	V	A

Figure 4.5. Haplotypes identified based on the deduced amino acid sequences. Green shade: Insensitive haplotype, Tan shade: sensitive haplotype, Red font: change from insensitive to sensitive, Green font: change from sensitive to insensitive, Tan font: no change in phenotype, Grey shade: synonymous mutation

4.4.4. Identification of critical SNPs

According to the phylogenetic analysis, there were two major clades of sensitive wheat lines (Figure 4.6). These two clades were different from a single missense mutation (I43V). In addition, there was another sensitive clade that included three lines which was separated from the missense mutation A272V. These three clades represent the haplotypes AA_Hap 8, AA_Hap 14 and AA_Hap 21. Although they belong to three clades, their phenotypic scores were not significantly different from each other (Table 4.1). All the insensitive lines made different clades that were clearly separated from sensitive clades (Figure 4.6). Phenotypic scores of insensitive clades are significantly different from those of sensitive clades. The line Botno considered as sensitive but belong to a clade with the insensitive line Kubanka. However, their phenotypic scores are not significantly different according to LSD analysis.

Phylogenetic analysis of the sequenced lines identified four SNPs (C707T, G1132C, A2187C, C2567A) that change the sensitive phenotype to insensitive phenotype. All four SNPs cause missense mutations that cause amino acid changes P236L, G347R, K429N and T556K, respectively (Figure 4.5). All these changes have SIFT scores < 0.05 that indicate deleterious mutations (Table 4.3). The four SNPs represent all three exons of the gene showing the importance of all domains for the SnTox1 sensitivity. In addition, three SNPs were identified as possible SNPs that can change the insensitive phenotype into a sensitive phenotype (Table 4.3). They are located at nucleotide positions G14A, C661T and A3019G. All three of them cause missense mutations and the amino acid changes are S5N, L221F and I707V, respectively. It is possible that one or more of these three mutations caused the change of insensitive allele in *T. dicoccoides* to the sensitive allele in durum and common wheat. Locations of the identified critical mutations are shown in the Figure 4.7.

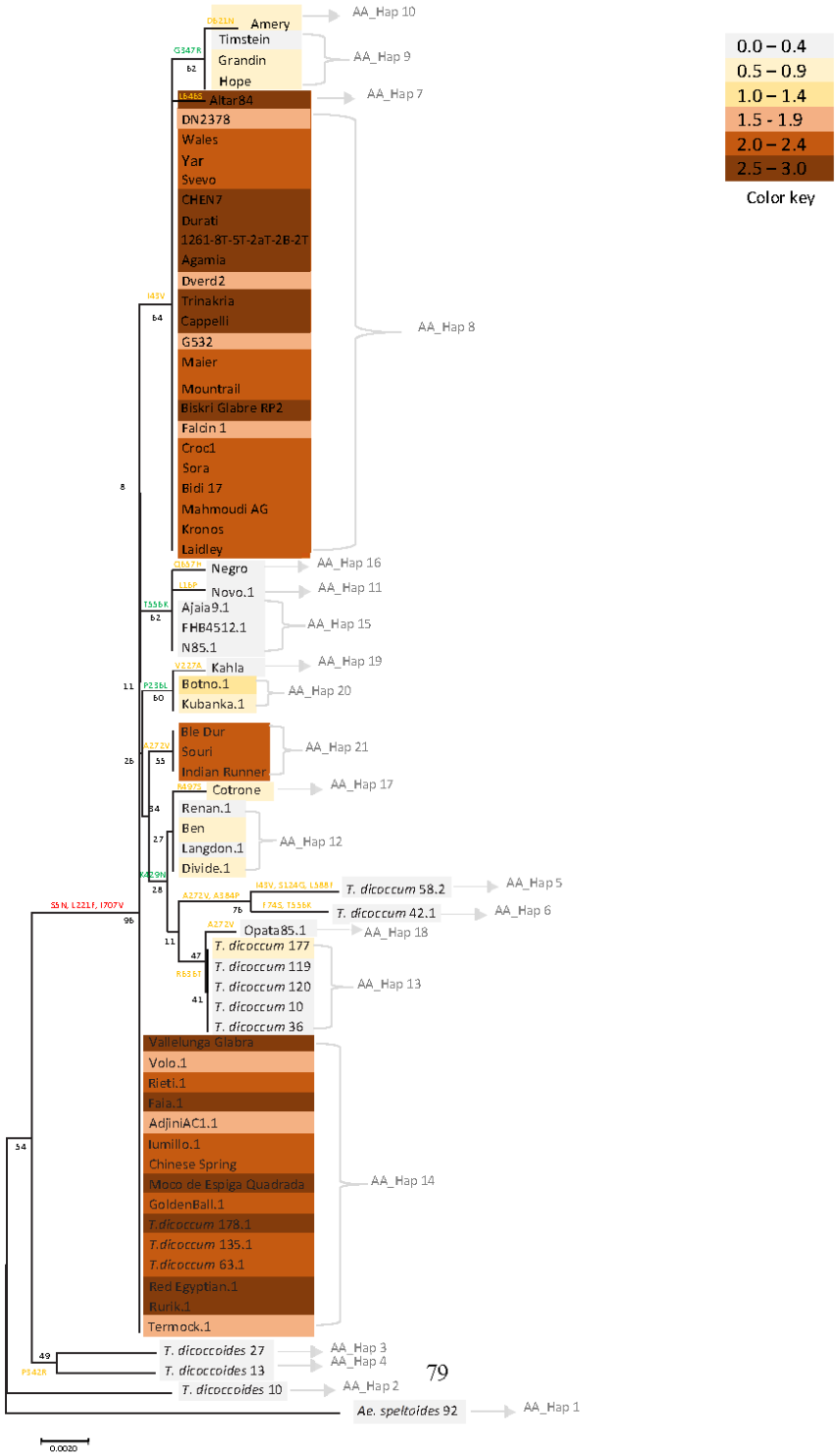


Figure 4.6. Phylogenetic tree based on the deduced amino acid sequences. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test are shown in black next to the branches. The color scheme of the heat map is based on the average phenotypic score of each accession. Critical mutations are shown in red (insensitve to sensitive), green (sensitve to insensitive) and tan (no change in phenotype) colors. Corresponding amino acid haplotypes are shown in gray next to the accession names

Table 4.3. Descriptions of critical natural mutations identified within the *Snn1* gene.

SNP ID	Existing variation	Exon	Amino acid position	Major domain	Amino acid change	SIFT score	Expected impact
G14A	CDS_Hap1, 2, 3, 4	1	5	Signal peptide	N->S	1.00	Tolerated_Low_Confidence
C661T	CDS_Hap1, 2, 3, 4	1	221	None	F->L	0.39	Tolerated
C707T	CDS_Hap5, 6, 21, 22	1	236	None	P->L	0.00	Deleterious
G1132C	CDS_Hap10, 11	2	347	EGF_CA	G->R	0.05	Deleterious
A2187C	CDS_Hap5, 6, 13,14, 18, 19, 23	3	429	None	K->N	0.00	Deleterious
C2567A	CDS_Hap6, 12, 16, 17	3	556	PKc	T->K	0.00	Deleterious
A3019G	CDS_Hap1, 2, 3, 4	3	707	None	V->I	1.00	Tolerated

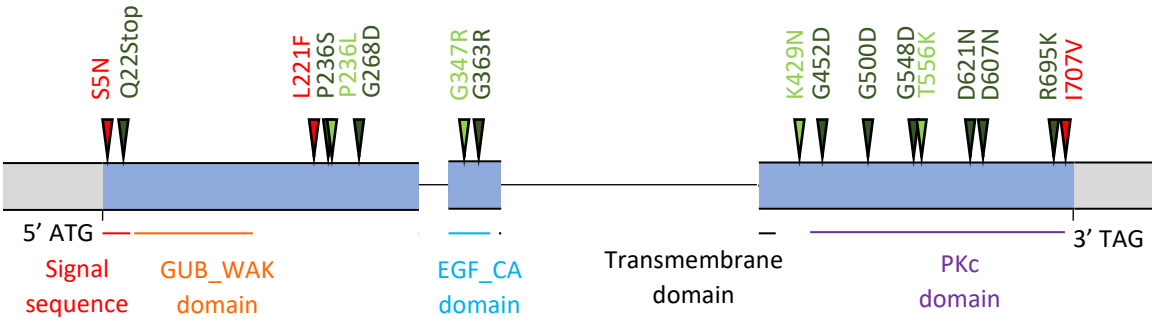


Figure 4.7. Locations of critical mutations in the *Snn1* gene. SNPs indicated in red are natural mutations associated with the evolutionary change from insensitivity to sensitivity, but it is not known if only one or more of these changes is causal to the phenotype. SNPs indicated in light green are natural mutations that change sensitive phenotype to insensitive phenotype. SNPs indicated in dark green are induced mutations that changed sensitive phenotype to insensitive phenotype.

4.4.5. Marker development

Three dCAPs markers were developed for three of the four SNPs identified as responsible for generating SnTox1-insensitive alleles. The resulting gel image pattern generated after restriction enzyme digestion was used to identify the presence of absence of the target SNP (Figure 4.8). Three dCAPs markers were developed that could successfully differentiate the SNPs C707T, G1132C and A2187C, and they were designated as *Snn1*dCAPs707, *Snn1*dCAPs1132, and *Snn1*dCAPs2187, respectively (Table 4.4). The marker *Snn1*dCAPs707 creates a 156 bp fragment when the corresponding SNP is present and a single 138 bp fragment when the SNP is absent. The marker *Snn1*dCAPs 1132 creates a 104 bp fragment when the corresponding SNP is present and a 128 bp fragment when the SNP is absent. The *Snn1*dCAPs2187 creates a 313 bp fragment when the SNP is present and a single 291 bp fragment when the corresponding SNP is absent. Attempts to develop a marker for the fourth critical SNP (C2567A), were unsuccessful.

Table 4.4. Markers develop to identify lines with critical SNPs.

SNP	Marker name	Sequence (5'->3')	Annealing temperature (°C)	Product size (bp)	Restriction enzyme	Recognition sequence
C707T	*Snn1*dCAPs707-F3	AGCATGGCGTAGGAGCCC	61.87	156	*Sma*I	CCGG
	*Snn1*dCAPs707-R3	AAGTGTGACCCCAACAAACG	58.9			
G1132C	*Snn1*CAPs1232-F1	GCATGTGTATCCCTGCTCA	57.21	268	*Hae*III	GGCC
	*Snn1*CAPs1232-R1	CACACCCGAAGGGTTTTGAT	58.38			
A2187C	*Snn1*dCAPs2187-F1	TTAGAGAAGGCAGATGTCTTTA	54.29	313	*Dra*I	TTTAAA
	*Snn1*dCAPs2187-R1	TGTGAAGGATGTCAAGGAGG	56.84			

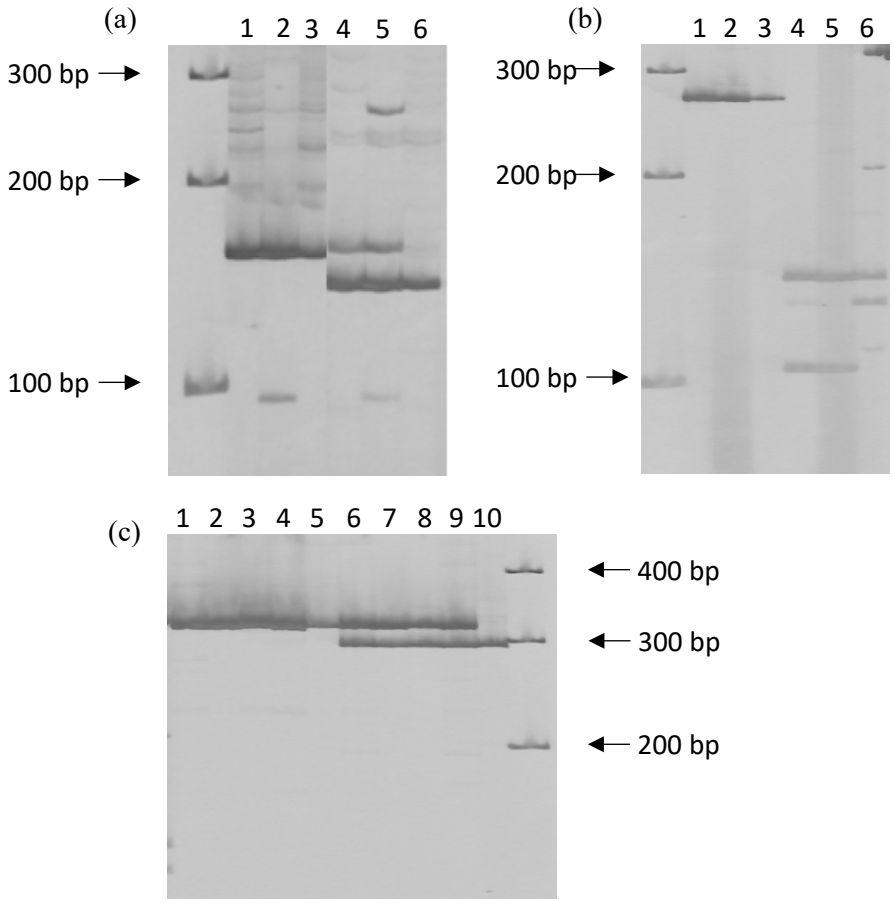


Figure 4.8. Restriction enzyme digestion profiles of PCR products amplified from genomic DNA using dCAPs markers. Fragments are separated on 6% polyacrylamide gels. (a) PCR with *dCAPs707F3R3* followed by digestion with *SmaI* for the SNP C707T. Lanes 1, 2 and 3 contain undigested PCR products from Kahla, PI41025 and Kronos, respectively. Lane 4, 5 and 6 contain the same PCR products after digestion with *Sma*I. (b) PCR with *dCAPs1132F1R1* followed by digestion with *Hae*III for the SNP G1132C. Lanes 1, 2 and 3 contain undigested PCR products from Amery, Grandin and Kronos, respectively. Lane 4, 5 and 6 contain the same PCR products after digestion with *Hae*II. (c) PCR with *dCAPs2187F1R1* followed by digestion with *Dra*I for the SNP A2187C. Lanes 1, 2, 3, 4 and 5 contain undigested PCR products from Divide, Ben, Cotrone, Langdon and Kronos, respectively. Lane 6, 7, 8, 9 and 10 contain the same PCR products after digestion with *Dra*I.

4.4.6. Analysis of Kronos EMS-mutants

Forty-five of the 69 Kronos mutants analyzed had the expected mutations (Appendix C). Thirty-eight of these had missense mutations, six had intron variants, and one had a mutation that caused a premature stop codon. Ten of the mutants had critical mutations that changed the sensitive phenotype to an insensitive phenotype (Table 4.5). Among them, six mutations occurred in the protein kinase domain, one was in the EGF_CA domain, one was in the signaling domain and two were in the region between GUB_WAK domain and EGF_CA domain (Table 4.5, Figure 4.9). Among the 45 mutations analyzed, 17 caused amino acid substitutions that were known to have similar properties. The majority of the mutations in the protein kinase domain that were expected to cause deleterious amino acid substitutions did affect the phenotype as expected (Figure 4.9). However, there were 15 mutations that were expected to be deleterious and did not affect the function of the gene. The majority of such mutations were present in exons 1 and 2.

Table 4.5. Descriptions of critical induced mutations identified within the *Snn1* gene.

SNP ID	Mutant ID	Exon	Amino acid position	Major domain	Amino acid change	SIFT score	Expected impact
C64T	Kronos3810	1	22	Signal peptide	Q->Stop	-	Deleterious
C706T	Kronos1070	1	236	None	P->S	0.09	Tolerated
G803A	Kronos3588	1	268	None	G->D	0.01	Deleterious
G1180A	Kronos2958	2	363	EGF_CA	G->R	0.06	Tolerated
G2255A	Kronos4218	3	452	PKc	G->D	0.00	Deleterious
G2399A	Kronos2581	3	500	PKc	G->D	0.00	Deleterious
G2543A	Kronos2088	3	548	PKc	G->D	0.00	Deleterious
G2719A	Kronos683	3	607	PKc	D->N	0.00	Deleterious
G2761A	Kronos4432	3	621	PKc	D->N	0.00	Deleterious
G2984A	Kronos2928	3	695	PKc	R->K	0.00	Deleterious

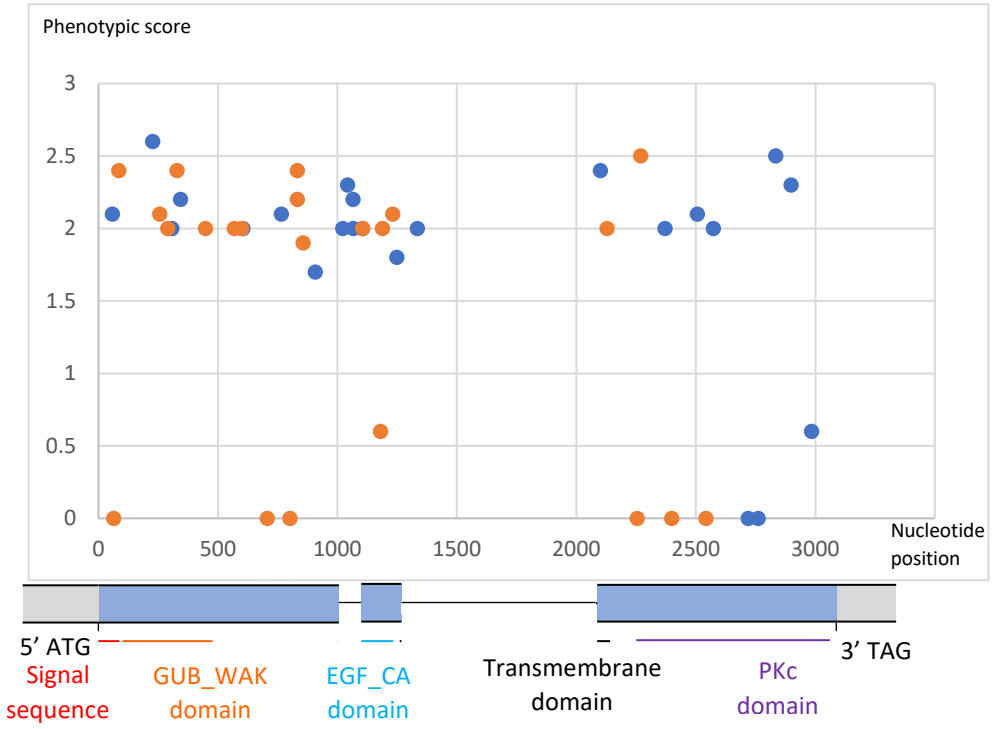


Figure 4.9. Positions and the phenotypic effects of mutations in Kronos population. Mutations that were expected to be deleterious based on the corresponding amino acid substitutions are shown in orange dots. Mutations that were expected to be less deleterious are shown in blue dots.

4.5. Discussion

The use of more durum lines in which *Snn1* was prevalent and the high diversity gained using accessions from different parts of the world allowed the identification of 27 coding sequence haplotypes of *Snn1*. Among the haplotypes identified, the wild wheat relatives *Ae. speltoides* and *T. turgidum* ssp. *dicoccoides* had the most divergent haplotypes as expected (CDS_Hap1, CDS_Hap2, CDS_Hap3, CDS_Hap4). Although only nine of the 27 haplotypes were associated with SnTox1 sensitivity, they included 41 of the analyzed accessions. This included 3 of the 10 common wheat varieties and 31 of the 42 durum wheat varieties analyzed, which agrees with Shi et al. (2016b) that *Snn1* alleles that confer sensitivity to SnTox1 are frequent among durum varieties. A relatively high frequency of sensitive alleles in durum wheat varieties compared to a much lower frequency among hexaploid common wheat varieties

suggests that *Snn1* might provide a secondary function that may be more important in durum than common wheat and perhaps compensated for by factor(s) on the D genome in the latter.

Hexaploid bread wheat, durum wheat and cultivated emmer have lower overall nucleotide diversity than wild emmer (Haudry et al. 2007). Initial diversity of wild emmer was reduced by 69% in *T. aestivum* and 84% in *T. durum.* This reduction of nucleotide diversity in wheat is lower than that of most other crop species. The average overall nucleotide diversity of *T. aestivum* A and B genomes was reported to be 0.00059 and 0.0008 in two separate studies (Akhunov et al. 2010, Haudry et al. 2007). The nucleotide diversity of *Snn1* was 0.00298. High polymorphism levels in R genes facilitate rapid evolution in response to pathogen evolution (Kuang et al. 2004; Allen et al. 2004; Yang et al. 2007). Genes with different nucleotide diversity levels have been observed in previous studies. For example, the nucleotide diversity π of the NLR gene *BPH9* in rice was found to be 0.04421 (Zhao et al. 2016) and that of the wheat transcription factor gene *DREB*, which is important for abiotic stress tolerance, was 0.180 (Edae et al. 2013). As would be expected, the nucleotide diversity of the coding region of *Snn1* was lower than that of the non-coding region. The highest π was observed in intron 1 (0.00910) and the lowest π was observed in exon 3 (0.00155). The conservation of exon 3, which predominantly contains the protein kinase domain, demonstrates the importance of this domain to the functionality of *Snn1*.

The theory of neutral molecular evolution (Kimura 1983) states that the majority of DNA polymorphisms are selectively neutral in a population. Therefore, the diversity in a population is due to the introduction or loss of polymorphism by mutations and genetic drift. The standard statistics Tajima's D (Tajima 1989), Fu and Li's D, and Fu and Li's F (Fu and Li 1993) estimate the deviation from the neutral model expectation. A value of zero for Tajima's D indicates a

neutral locus and a positive value reveals the predominance of rare alleles indicating a negative selection. A negative Tajima's D value indicates the predominance of intermediate frequency alleles and is associated with positive selection pressure (Akhunov et al. 2010). According to the results, the Tajima's D and Fu and Li's D and F values for the entire genomic region of *Snn1* gene were negative. This suggests that this gene may have been under positive selection during evolution. If *Snn1* in fact possesses a secondary function as suggested earlier, it might be the reason for this positive selection observed. However, neutrality tests were not significant ($P < 0.05$) for some regions and thus this observation needs further validation.

Three SNPs (G14A, C661T and A3019G) were identified as possible mutations that changed the insensitive *Snn1* allele in *Ae. speltoides* and wild emmer to a sensitive allele found in cultivated emmer, durum and common wheat. The expected impacts of these changes are not expected to be deleterious, and instead, all are expected to be tolerated. However, the SNPs C661T and A3019G do not encode amino acids that belong to any of the conserved functional domains of *Snn1*, whereas the SNP G14A is located at the beginning of the gene and encodes an amino acid in the signal peptide domain. Therefore, it may be that the G14A SNP causal mutation that change the insensitive allele to the sensitive allele during the domestication of wheat, but further investigation is needed to confirm this.

Sequence analysis of the 70 lines also revealed four SNPs (C707T, G1132C, A2187C, and C2567A) that generated insensitive alleles from sensitive alleles. Two of these SNPs (G1132C and A2187) were previously reported by Shi et al. (2016b). All four SNPs cause missense mutations and have SIFT scores < 0.05 indicating deleterious effects. These mutations likely occurred relatively recently within cultivated wheat giving rise to new insensitive alleles. It therefore appears that the evolution of the *Snn1* gene has gone from insensitive to sensitive and

back to insensitive. In other words, the wild progenitors of domesticated wheat were insensitive to SnTox1 but mutation G14A, C661T, or A3019G gave rise to an allele that allowed *Snn1* to be recognized by SnTox1 leading to the induction of NETS and the development of SNB. Subsequent mutations within the SnTox1-sensitive allele(s) such as C707T, G1132C, A2187C, and C2567A led to the formation of insensitive alleles, which confer SNB resistance.

The four SNPs C707T, G1132C, A2187C, and C2567A were targeted for the development of diagnostic markers to differentiate sensitive and insensitive *Snn1* alleles among durum and common wheat varieties. Attempts to convert the identified critical SNPs to STARP markers (Long et al. 2017) were unsuccessful (data not shown). Therefore, dCAPs markers were developed for three of the four SNPs identified. The dCAPS markers were introduced (Michaels and Amasino 1998; Neff et al. 1998) as a modification of CAPS markers (Konieczny and Ausubel 1993). The CAPS method uses amplification of a target region followed by restriction digestion of the amplified product at the target SNP. However, the usage of this method is limited because it requires the target SNP to disrupt a naturally occurring restriction enzyme recognition site. The dCAPS method uses primers that contain one or more mismatches that can create or destroy a restriction enzyme recognition site at the target SNP during PCR. The PCR product is then digested with the corresponding restriction enzyme and the resulting profile on the gel is used to determine the presence or absence of the SNP. These markers can be used in marker-assisted selection after validation using a large set of wheat accessions. However, it is possible that other naturally occurring mutations that gave rise to insensitive alleles exist and have yet to be identified. In that case, additional markers will be needed.

Although some of the sensitive lines had two copies of *Snn1*, cDNA sequencing showed that only one of the copies was expressed. Two SNPs that differentiated the two copies in some

of the two-copy lines (G127A and A264G) are located in the coding region of the gene. G127A encodes a missense variant V43I, and A264G is a synonymous mutation. However, V43I was a common variant observed in most of the expressed sensitive accessions. Therefore, it is likely that neither of these two SNPs are responsible for the differences in the expression of the two copies. Shi et al. (2016b) showed that the durum variety Lebsock was not expressed even though it had the same sequence as several other SnTox1-sensitive lines. Therefore, the differences in expression are likely due to differences in the promoter region. BLAST searches of *Snn1* gene sequence against wheat reference genomes that recently became available including Chinese Spring, Arina, Cadenza, Claire, Jagger, Julius Mace, Lancer, Landmark, Norin61, Paragon, Robigus, Soissons, Stanley, SY_Mattis and Zavitan revealed that two of the sequenced lines (Stanley and Norin61) contains two copies of *Snn1*. The two copies in Stanley could be differentiated by the SNP G127A. In Stanley, copy 2 was present in chromosome 1B as expected, but copy 1 was located in a chromosome assembly designated as 'unknown.' Similarly, the two copies in Norin61 were also located in chromosome 1B and an assembly designated as 'unknown.' However, the two copies of *Snn1* in Norin61were identical to each other, which raises the question whether the previously identified lines with single copy may be lines with two identical copies. More work is needed to determine the physical positions of the two copies relative to each other and the reason for the copy 1-containing contig not assembling with the rest of chromosome 1B in Stanley and Norin61.

The *T. turgidum* cv. Kronos TILLING resource was used to identify additional amino acids important for *Snn1* functionality. Among the mutants that are insensitive to SnTox1, six had mutations in the protein kinase domain, one had the mutation in the EGF_CA and one had a mutation in the signal peptide domain. Also, two had mutations in the region between the

GUB_WAK and EGF_CA domains which can be explained by the fact that SnTox1 directly binds to a region between the GUB_WAK and EGF_CA domains (Shi et al. 2016b). Seven out of the ten critical mutations were observed in the protein kinase domain. This suggests that the protein kinase domain is very important for the proper function of *Snn1*. The majority of the critical mutations completely knocked down the *Snn1* gene. The mutation in EGF_CA domain (G363R) and one mutation in protein kinase domain (R695K) showed an average sensitivity score of 0.6.

Among the 45 mutants used, 15 had C to T transitions and 29 had G to A transitions. EMS is known to induce predominantly G to A and C to T transitions because it preferentially induces alkylation of G residues (Henry et al. 2014; Greene et al. 2003; Till et al. 2007). Although there were 45 different mutants, only ten of them caused the insensitive phenotype. The effect of an amino acid substitution on a protein depends on both the position and the degree of similarity between the new and the original amino acid (Goldberg and Wittes 1966). Among the 35 mutants that remained sensitive to SnTox1, 16 had mutations in introns or regions between the major conserved domains. It can be assumed that they did not alter function of the protein because they did not disrupt the major domains. However, the remaining 19 SnTox1-sensitive Kronos mutants had missense mutations within one of the major domains. It is possible these amino acid substitutions had less impact due to their physical properties. Except Tyr, hydrophobic amino acids (Phe, Ile, Leu, Met and V) can substitute for each other without affecting protein function. Similarly, polar or hydrophilic amino acids can substitute for amino acids in the same group. The hydrophilic amino acids can be divided into two subgroups known as polar-charged amino acids (Glu, Asp, Lys, Arg, Hys) and polar-partially-charged amino acids (Tyr, Gln, Asn, Thr, Ser). Substitution of an amino acid by another in the same subgroup would

have less impact on the protein. Some amino acids have been identified as more interchangeable both within and across their corresponding groups while retaining protein function. They include Ala for Val, Ala for Phe, Val for Ile or Leu, Leu for Met, Phe or Ile, Gly for Ala, Glu for Asp, Arg for Lys, Ser for Thr, Gln for Asn or Glu, Asn for Asp and Phe for Ile (Castro-Chavez 2010; Goldberg and Wittes 1966). Eleven of the 19 missense mutations that did not affect *Snn1* function were in this group of interchangeable amino acids (V20I, A76V, L103F, I491L, D536N) or had an exchange with another amino acid in the same polarity group (V115M, P338L, S558N, S645N, E667K) (Appendix C).

4.6. Conclusions

One of the major goals of cloning resistance genes is to develop diagnostic markers to be used in MAS. To date, we were unable to develop diagnostic markers for *Snn1* even though the gene was cloned. Current study revealed that this was because of the presence of multiple haplotypes that could confer SnTox1 sensitivity. Analysis of allelic diversity of *Snn1* revealed 27 haplotypes based on the coding sequence of the gene. Three SNPs were identified as the possible mutations that changed the insensitive allele in wild emmer into the sensitive allele in domesticated wheat. Four independent SNPs that most likely occurred later and changed the sensitive allele to insensitive alleles in domesticated wheat were also identified. It was also found that some of the wheat lines carry two copies of *Snn1*. However, the location of the second copy and the factors governing the expression of the different copies are yet to be identified. SNP-based markers were developed for three of the mutations that caused insensitive alleles. More gene-specific markers for *Snn1* can be developed based on the results of this study to be used in MAS. The results also help to increase our understanding of the NE sensitivity gene evolution.

This knowledge will be useful to obtain better host resistance against necrotrophic pathogens in the future through genetic manipulation.

4.7. References

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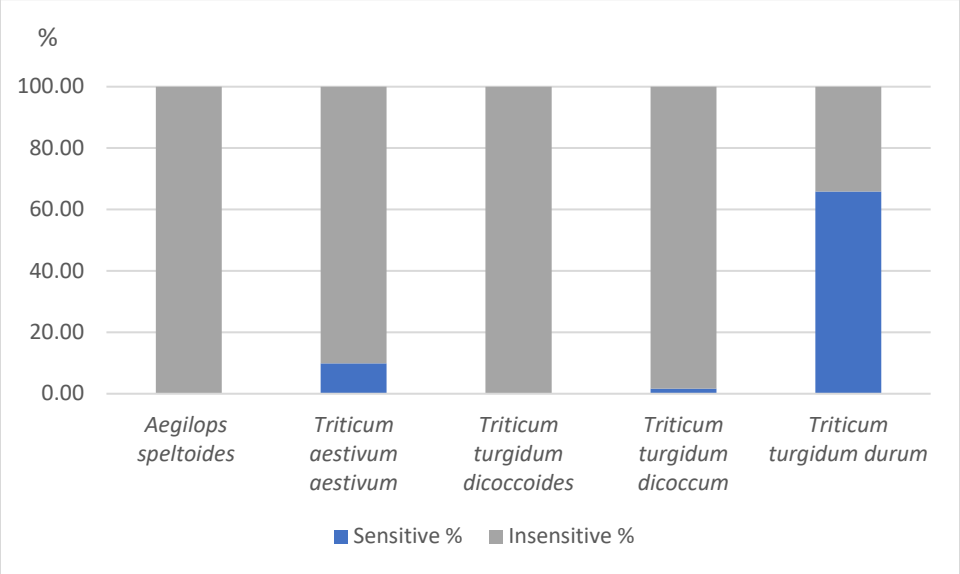
APPENDIX A. ACCESSIONS OF WHEAT SPECIES DEPLOYED IN THE PHYLOGENETIC INTERACTION STUDY

Accession	PI number	Ploidy level	Genus	Species	Subspecies	Origin
Amery	N/A	6	*Triticum*	*aestivum*	*aestivum*	Austalia
Chinese Spring	CItr 14108	6	*Triticum*	*aestivum*	*aestivum*	China
Grandin	PI 531005	6	*Triticum*	*aestivum*	*aestivum*	US North Dakota
Hope	CItr 8178	6	*Triticum*	*aestivum*	*aestivum*	US South Dakota
Novo	N/A	6	*Triticum*	*aestivum*	*aestivum*	India
Opata 85	PI 591776	6	*Triticum*	*aestivum*	*aestivum*	CIMMYT
Red Egyptian	PI45403	6	*Triticum*	*aestivum*	*aestivum*	South Africa
Renan	PI 564569	6	*Triticum*	*aestivum*	*aestivum*	France
Rurik	N/A	6	*Triticum*	*aestivum*	*aestivum*	France
Timstein	PI 168688	6	*Triticum*	*aestivum*	*aestivum*	US Minnesota
Mocho de Espiga Quadrada	PI56213	6	*Triticum*	*aestivum*	*compactum*	Portugal
Termok	PI41023	6	*Triticum*	*aestivum*	*compactum*	Kyrgyzstan
DN-2378	PI361862	6	*Triticum*	*aestivum*	*macha*	Denmark
G532	PI428146	6	*Triticum*	*aestivum*	*macha*	Sweden
12:61-8T-5T-2aT-2B-2T	PI324929	4	*Triticum*	*turgidum*	*durum*	Australia
Adjini AC 1	CItr 3137	4	*Triticum*	*turgidum*	*durum*	Tunisia
Agamia	TA4154-29	4	*Triticum*	*turgidum*	*durum*	Mexico
Ajaia 9	TA4154-40	4	*Triticum*	*turgidum*	*durum*	CIMMYT
Altar 84	TA4154-4	4	*Triticum*	*turgidum*	*durum*	CIMMYT
Ben	PI596557	4	*Triticum*	*turgidum*	*durum*	US-ND
Bidi 17	PI306641	4	*Triticum*	*turgidum*	*durum*	France
Biskri Glabre RP 2	CItr 3180	4	*Triticum*	*turgidum*	*durum*	Tunisia
Ble Dur	CItr 1471	4	*Triticum*	*turgidum*	*durum*	Algeria
Botno	TA4154-35	4	*Triticum*	*turgidum*	*durum*	United States
Cappelli	PI264949	4	*Triticum*	*turgidum*	*durum*	Italy
CHEN 7	TA4154-48	4	*Triticum*	*turgidum*	*durum*	CIMMYT
Cotrone	PI157975	4	*Triticum*	*turgidum*	*durum*	Italy
Croc 1	TA4154-1	4	*Triticum*	*turgidum*	*durum*	CIMMYT
Divide	PI642021	4	*Triticum*	*turgidum*	*durum*	US-ND
Durati	PI434645	4	*Triticum*	*turgidum*	*durum*	Australia
Dverd 2	TA4154-5	4	*Triticum*	*turgidum*	*durum*	CIMMYT
Faia	PI 584835	4	*Triticum*	*turgidum*	*durum*	Portugal
Falcin 1	TA4154-43	4	*Triticum*	*turgidum*	*durum*	CIMMYT
FHB4512	Cltr5094	4	*Triticum*	*turgidum*	*durum*	China
Golden Ball	CItr 11477	4	*Triticum*	*turgidum*	*durum*	United States, North Dakota
Indian Runner	CItr 5136	4	*Triticum*	*turgidum*	*durum*	Victoria Australia
Iumillo	PI210973	4	*Triticum*	*turgidum*	*durum*	Italy
Kahla	PI 7794	4	*Triticum*	*turgidum*	*durum*	Setif Algeria

Accession	PI number	Ploidy level	Genus	Species	Subspecies	Origin
Kronos	N/A	4	*Triticum*	*turgidum*	*durum*	United Kingdom, England
Kubanka	PI 2758	4	*Triticum*	*turgidum*	*durum*	Russian Federation, Samara
Laidley	PI67342	4	*Triticum*	*turgidum*	*durum*	Australia
Langdon	CItr13165	4	*Triticum*	*turgidum*	*durum*	US-ND
Mahmoudi Ag	PI41046	4	*Triticum*	*turgidum*	*durum*	Tunisia
Maier	PI607531	4	*Triticum*	*turgidum*	*durum*	US-ND
Mountrail	PI607530	4	*Triticum*	*turgidum*	*durum*	US-ND
N-85	PI79900	4	*Triticum*	*turgidum*	*durum*	China
Negro	PI 7425	4	*Triticum*	*turgidum*	*durum*	Cordoba Spain
Rieti	CItr 2793	4	*Triticum*	*turgidum*	*durum*	Leiria Portugal
Sora	TA4154-16	4	*Triticum*	*turgidum*	*durum*	CIMMYT
Souri	PI41051	4	*Triticum*	*turgidum*	*durum*	Tunisia
Svevo		4	*Triticum*	*turgidum*	*durum*	
Trinakria	TA4154-38	4	*Triticum*	*turgidum*	*durum*	Italy
Vallelunga Glabra	PI157979	4	*Triticum*	*turgidum*	*durum*	Italy
Volo	CItr 2462	4	*Triticum*	*turgidum*	*durum*	Germany
Wales	N/A	4	*Triticum*	*turgidum*	*durum*	US-ND
Yar	TA4154-31	4	*Triticum*	*turgidum*	*durum*	CIMMYT
T. dicoccum 42	PI 41025	4	*Triticum*	*turgidum*	*dicoccum*	Russian Federation, Samarskaja oblast'
T. dicoccum 10	PI 12214	4	*Triticum*	*turgidum*	*dicoccum*	
T. dicoccum 119	PI 164582	4	*Triticum*	*turgidum*	*dicoccum*	India, Tamil Nadu
T. dicoccum 120	PI 168673	4	*Triticum*	*turgidum*	*dicoccum*	United States, Wisconsin
T. dicoccum 135	PI 193641	4	*Triticum*	*turgidum*	*dicoccum*	Ethiopia, Ādīs Ābeba
T. dicoccum 177	PI 217640	4	*Triticum*	*turgidum*	*dicoccum*	India, Tamil Nadu
T. dicoccum 178	PI 221400	4	*Triticum*	*turgidum*	*dicoccum*	Serbia
T. dicoccum 36	PI 14919	4	*Triticum*	*turgidum*	*dicoccum*	
T. dicoccum 58	PI 94617	4	*Triticum*	*turgidum*	*dicoccum*	Russian Federation, Dagestan, Respublika
T. dicoccum 63	PI 94624	4	*Triticum*	*turgidum*	*dicoccum*	Iran
T. dicoccoides 10	Td G -11	4	*Triticum*	*turgidum*	*dicoccoides*	
T. dicoccoides 27	Td 582	4	*Triticum*	*turgidum*	*dicoccoides*	
T. dicoccoides 13	Td B - 6	4	*Triticum*	*turgidum*	*dicoccoides*	
Ae speltoides 92	PI 542256	2	*Aegilops*	*speltoides*	*ligustica*	Turkey, Adıyaman

APPENDIX B. COMPARISON OF SNTOX1-SENSITIVITY LEVELS AMONG DIFFERENT WHEAT SUBSPECIES EVALUATED



(Data were taken from Shi et al. 2016b)

APPENDIX C. DETAILS OF THE KRONOS MUTANTS USED IN THE STUDY

ID	Nucleotide change	Nucleotide position	Amino acid change	Amino acid position	Mutation effect	Average phenotypic score
Kronos873	G > A	58	V > I	20	Missense variant	2.1
Kronos3810	C > T	64			Stop codon	0.0
Kronos826	G > A	85	A > T	29	Missense variant	2.4
Kronos2019	C > T	227	A > V	76	Missense variant	2.6
Kronos4539	C > T	257	S > F	86	Missense variant	2.1
Kronos3842	C > T	290	S > F	97	Missense variant	2.0
Kronos771	C > T	307	L > F	103	Missense variant	2.0
Kronos3011	C > T	329	T > M	110	Missense variant	2.4
Kronos4256	G > A	343	V > M	115	Missense variant	2.2
Kronos1257	C > T	448	R > C	150	Missense variant	2.5
Kronos3870	C > T	568	P > S	190	Missense variant	2.5
Kronos1400	C > T	599	T > M	200	Missense variant	2.0
Kronos2379	G > A	605	R > K	202	Missense variant	2.0
Kronos1070	C > T	706	P > S	236	Missense variant	0.0
Kronos2622	G > A	766	E > K	256	Missense variant	2.1
Kronos3588	G > A	803	G > D	268	Missense variant	0.0
Kronos2431	G > A	832	G > R	278	Missense variant	2.2
Kronos2594	G > A	833	G > E	278	Missense variant	2.4
Kronos2282	G > A	856	G > R	286	Missense variant	1.9
Kronos798	C > T	908	A > V	303	Missense variant	1.7
Kronos4688	G > A	1023			Intron variant	2.0
Kronos1361	G > A	1043			Intron variant	2.3
Kronos3737	G > A	1066			Intron variant	2.2
Kronos2425	G > A	1067			Intron variant	2.0
Kronos3934	C > T	1105	P > S	338	Missense variant	2.0
Kronos2434	C > T	1106	P > L	338	Missense variant	2.0
Kronos2958	G > A	1180	G > R	363	Missense variant	0.6
Kronos1270	G > A	1189	G > S	366	Missense variant	2.0
Kronos2032	G > A	1231	A > T	380	Missense variant	2.1
Kronos594	G > A	1249			Intron variant	1.8
Kronos4683	G > A	1334			Intron variant	2.0
Kronos3352	C > T	2101	L > F	401	Missense variant	2.4
Kronos2306	C > T	2129	T > M	410	Missense variant	2.0
Kronos4218	G > A	2255	G > D	452	Missense variant	0.0
Kronos3657	G > A	2269	G > S	457	Missense variant	2.5
Kronos2261	A > C	2371	I > L	491	Missense variant	2.0
Kronos2581	G > A	2399	G > D	500	Missense variant	0.0
Kronos590	G > A	2506	D > N	536	Missense variant	2.1
Kronos2088	G > A	2543	G > D	548	Missense variant	0.0

ID	Nucleotide change	Nucleotide position	Amino acid change	Amino acid position	Mutation effect	Average phenotypic score
Kronos929	G > A	2573	S > N	558	Missense variant	2.0
Kronos683	G > A	2719	D > N	607	Missense variant	0.0
Kronos4432	G > A	2761	D > N	621	Missense variant	0.0
Kronos3633	G > A	2834	S > N	645	Missense variant	2.5
Kronos3669	G > A	2899	E > K	667	Missense variant	2.3
Kronos2928	G > A	2984	R > K	695	Missense variant	0.6