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Philosophiae Doctor (PhD), Thesis 2017:63

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Norwegian University of Life Sciences
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Ås, June 2017

Anja

Summary

Wheat is one of the most important food crops worldwide. In normal years, the proportion of food quality wheat grown in Norway exceeds 50 %. However, the quality and yield can be significantly challenged by unfavorable weather and disease epidemics. Septoria leaf blotch (SNB) is one of the most important diseases in Norwegian spring wheat, and is caused by the ascomycete *Parastagonospora nodorum*. Breeding for resistance to SNB has been hampered due to the polygenic and quantitative nature of the genetic resistance, and the farmers often have to rely on fungicides to control the disease.

In recent years, substantial progress has been made in understanding the *P. nodorum*-wheat pathosystem. Several host-specific interactions between necrotrophic effectors (NEs) and host sensitivity (*Snn*) genes have been identified and play major roles in SNB seedling resistance. Some of the NEs have been cloned and can be used to screen breeding material at the seedling stage. The effects of these host-specific interactions have been less investigated at the adult plant stage under field conditions.

In this PhD project, we used spring wheat populations that segregated for susceptibility and resistance to SNB. The plants were screened for adult plant resistance to SNB under natural infection in mist irrigated field trials. To investigate seedling resistance, seedling plants were inoculated with *P. nodorum* isolates in the greenhouse, infiltrated with culture filtrate from the isolates and with semi-purified necrotrophic effectors SnToxA, SnTox1 and SnTox3.

We found that sensitivity to the two major necrotrophic effectors SnToxA and SnTox3 can contribute significantly to increased disease severities at the adult plant stage in the field. Sensitivities to the necrotrophic effectors SnToxA and SnTox3 were common in the Nordic breeding material, and the effector genes seem prevalent in the Norwegian *P. nodorum* pathogen population. The effect of other host-specific interactions at the adult plant stage could not be validated in this study. The genetic analysis revealed that several quantitative trait loci (QTL) for SNB resistance were significant at both the seedling and adult plant stage. Some of these loci were stable across several years in the field. In addition, several stable loci were identified as significant only in the field at the adult plant stage and could also be interesting for breeding.

Sammendrag

Hvete er en av de viktigste matplantene på verdensbasis. I gjennomsnittsår er mer enn 50 % av hveten som konsumeres i Norge produsert innenlands. Hveteaksprikk forårsaket av soppen *Parastagonospora nodorum* er en av de viktigste sykdommene i vårhvete i Norge. Foredling for resistens mot denne sykdommen er krevende, blant annet fordi den genetiske resistensen består av mange gener, hvert med relativt liten effekt. Bøndene er derfor ofte avhengige av å sprøyte med fungicider.

I senere tid har forståelsen av mekanismene bak samspillet mellom *P. nodorum* og hvete økt betraktelig. Flere vertsspesifikke interaksjoner er involvert, mellom nekrotrofe effektorer (NE) som produseres av soppen, og korresponderende sensitivitets-gen (*Snn*) i planten. Noen av de nekrotrofe effektorene har blitt klonet og kan brukes til å teste foredlingsmateriale for sensitivitet på småplantestadiet. Effekten av disse interaksjonene på resistens hos voksne planter under feltforhold har blitt mindre undersøkt.

I dette prosjektet studerte vi ulike vårhvetepopulasjoner som segregerte for mottagelighet og resistens mot hveteaksprikk. Plantene ble testet for voksenplanteresistens under naturlig smitte i dusjvannede feltforsøk. For å undersøke småplanteresistens, ble småplanter inokulert med *P. nodorum*-isolater i veksthus, infiltrert med kulturfiltrat fra isolatene og med enkelt-effektorene SnToxA, SnTox1 og SnTox3.

Vi fant at sensitivitet for de to nekrotrofe effektorene SnToxA og SnTox3 kunne bidra til signifikant høyere mottagelighet for hveteaksprikk under feltforhold. Sensitivitet for SnToxA og SnTox3 var utbredt i det norske vårhvetematerialet, og det så ut til at disse effektorgenene var vanlige i den norske *P. nodorum*-populasjonen. Effekten av andre vertsspesifikke samspill på voksenplantestadiet kunne ikke bli bekreftet i denne studien. De genetiske analysene viste at flere loci for kvantitativ hveteaksprikk-resistens var felles for både småplante- og voksenplanteresistens. Flere av disse hadde stabil effekt over flere år i felt, noe som gjør dem til gode kandidater for markørassistert seleksjon. I tillegg var hadde flere loci stabil effekt bare på voksenplantestadiet, og kan også være interessante for foredling.

Abbreviations

AFLP	Amplified fragment length polymorphism
AM	Association mapping
Avr	Avirulence
BC	Backcross
DArT	Diversity array technology
DH	Doubled haploid
ETI	Effector triggered immunity
G × E	Genotype × Environment
GBS	Genotyping-by-sequencing
GWAS	Genome wide association mapping
HR	Hyper sensitive response
HST	Host specific toxin
LD	Linkage disequilibrium
LOD	Logarithm of odds
MAF	Minor allele frequency
MAS	Marker-assisted selection
MAT	Marker trait association
NE	Necrotrophic effector
NBS-LRR	Nucleotide-binding site-leucine rich repeats
PAMP	Pathogen associated molecular pattern
QTL	Quantitative trait loci
RFLP	Restriction fragment length polymorphism
R-gene	Resistance-gene
RIL	Recombinant inbred line
SM	Secondary metabolite
SNB	Septoria nodorum leaf blotch
Snn	Sensitivity locus
SNP	Single nucleotide polymorphism
SSR	Short sequence repeat (microsatellite marker)

List of papers

I. **Mapping of SnTox3-*Snn3* as a major determinant of field susceptibility to Septoria nodorum leaf blotch in the SHA3/CBRD x Naxos population** (Ruud, A.K., Windju, S., Belova, T., Friesen, T.L., Lillemo, M. Theoretical and Applied Genetics (2017) 130: 1361)

II. **Effects of three *Parastagonospora nodorum* necrotrophic effectors in Norwegian spring wheat** (Ruud, A.K., Dieseth, J.A., Lillemo, M. Manuscript)

III. **Genome wide association mapping of seedling and adult plant resistance to Septoria nodorum leaf blotch in a Nordic spring wheat collection** (Ruud, A.K., Dieseth, J.A., Ficke, A., Lillemo, M. Manuscript)

1. Introduction

1.1. Wheat

The global production of wheat was approximately 729 million tons in 2014 and around 70 % is consumed as food (FAO 2017), making it one of the largest food crops in the world. Due to its adaptability, wheat is grown in a wide range of climates. Bread wheat (*Triticum aestivum,* L.) accounts for roughly 95 % of the wheat production, durum (*T. durum*, L.) for the remaining 5 %.

Bread wheat is an allohexaploid (AABBDD) species derived from two hybridization events between different species. The first allopolyploidization happened around 500 000 years ago between einkorn (*T. urartu*, AA) and an unknown, close relative of *Aegilops speltoides* (BB), forming tetraploid wheat (*T. turgidum*, AABB). A hybridization event between *T. turgidum* and *Ae. tauschii* (Tausch's goatgrass, DD) resulted in the hexaploid *T. aestivum.* Recent research by Marcussen et al. (2014) have suggested that the D genome evolved after hybridization between A and B genome ancestors ~5.5 million years ago. Based on archeological evidence and the absence of wild hexaploid wheat, the last polyploidization has been assumed to have happened ~10 000 years ago (Salamini et al. 2002) and coincided the domestication of wheat and the rise of agriculture in the fertile crescent (Tanno and Willcox 2006). The polyploidy provides a large extent of genome plasticity and facilitates adaptability to different environments. Due to the polyploid nature, genes can be present in duplicates or triplicates across the subgenomes. Changes in one copy of a gene can result in subtle dosage effects, upon which selection can work (Dubcovsky and Dvorak 2007).

Modern wheat breeding originated in the 19th century, when crosses were made between plants with reciprocal traits and offspring carrying both traits were selected. With the discovery of evolutionary and genetic theory in the 20th and 21th centuries progress was also made in technology development to improve breeding. The most significant genetic improvements of wheat were done during The Green Revolution between the 1930s and 1960s, when a series of actions were initiated to increase agricultural production. The wheat breeding program was led by Norman Borlaug at the International Maize and Wheat Improvement Centre (CIMMYT) in Mexico. From 1966 to 1999 the global production of wheat increased with 91 % with only a marginal increase of harvested area (Khush 2001), due to the combination of industrial agricultural methods and new adapted cultivars, i.e. semi-dwarfs that could tolerate higher

levels of fertilization than tall landraces. By the early 1990s, more than 90 % of all wheat varieties released in developing countries were semi-dwarfs and the majority have CIMMYT germplasm in their pedigrees (Byerlee and Moya 1993). The Green Revolution breeders were also early advocates of incorporating general resistance against diseases (Niederhauser et al. 1954; Borlaug 1966; Caldwell 1968), as a more durable strategy than race-specific resistance. Examples of general, durable resistance are the broad spectrum loci *Lr34, Lr46* and *Lr67*, conferring resistance to stripe and leaf rust (caused by *Puccinia striiformis* f.sp. *tritici* and *P. triticina,* respectively) and powdery mildew (caused by *Blumeria graminis* f.sp. *tritici*) (Lillemo et al. 2008; Moore et al. 2015). Selection for quantitative, durable resistance has been successful for instance for powdery mildew (Miedaner and Flath 2007; Singh et al. 2011).

In Norway, the most damaging wheat diseases are caused by fungal pathogens. Powdery mildew, leaf blotch diseases (where Septoria nodorum leaf blotch is most prevalent) and Fusarium head blight are the most important (Lillemo and Dieseth 2011; Moore et al. 2015), although stripe rust has caused severe epidemics in unsprayed fields since 2014 (Abrahamsen et al. 2017).

1.2. The leaf blotch disease complex

The "*Septoria* leaf blotch disease complex" includes Septoria nodorum leaf and glume blotch (SNB) caused by *Parastagonospora nodorum*, *Zymoseptoria tritici* leaf blotch, tan spot caused by *Pyrenophora tritici-repentis* and, of less importance, *P. avenae* blotch. In Western Australia SNB can be responsible for yield losses up to 31 % (Bhathal et al. 2003). Reliable identification of the pathogen should be done under microscope or by polymerase chain reaction (PCR).

1.3. The pathogen – *Parastagonospora nodorum*

Parastagonospora nodorum [teleomorph: *Phaeosphaeria* (Hedjar.) syn. *Leptosphaeria nodorum* (Müll.), syn. *Septoria nodorum* (Berk.), syn. *Stagonospora nodorum* (Berk.)] is a filamentous Ascomycete and member of the Dothideomycetes class, which includes several phytopathogens (Murray and Brennan 2009; Crook et al. 2012; Quaedvlieg et al. 2013; Stergiopoulos et al. 2013).

P. nodorum is the causal agent of Septoria nodorum leaf blotch (SNB, also called Stagonospora nodorum leaf blotch) in spring wheat in Norway, although tan spot is also common in some areas. *Z. tritici* has become more common in recent years, but is mainly found in winter wheat (Ficke et al. 2011a; Abrahamsen et al. 2013).

In Norwegian trials, the estimated yield loss due to SNB in the susceptible cultivar Bjarne was calculated to be on average almost 25 %, based on data from 2009 to 2012 and a mean SNB severity of 20 % (Abrahamsen 2013). Quality measures like thousand kernel weight, hectoliter weight and grain filling were also well correlated with fungicide treatment. Interestingly, the gain of fungicide treatment was lower in some of the most resistant cultivars like Zebra and Mirakel. For these cultivars the yield was high also in the untreated plots, illustrating the potential of cultivars with genetic resistance to reduce the need for fungicide spraying (Abrahamsen 2013). It is also worth noting that in some areas the disease severity and actual losses can be significantly higher than the reported average used in the yield calculations, for instance SNB severity in some areas was reported up to 70 % in 2012 (Abrahamsen 2013).

1.3.1. Life cycle and epidemiology

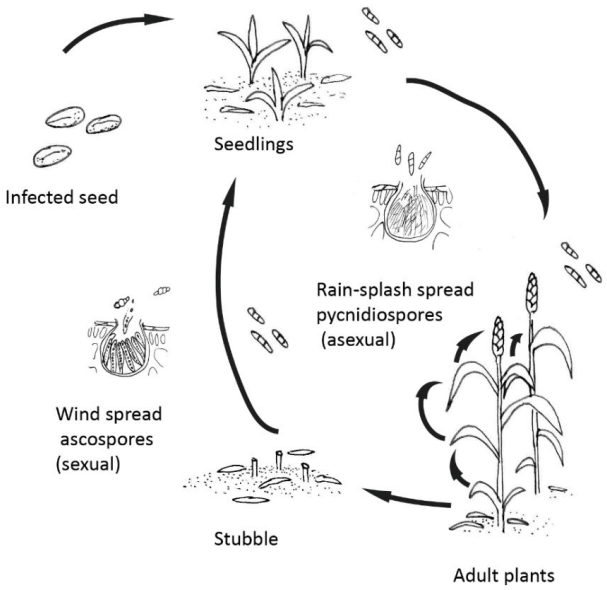

Figure 1 The life cycle of *P. nodorum.* Drawing by A. Ruud, adapted from Sommerhalder et al. (2011).

P. nodorum has a mixed reproduction system. The fungus is heterothallic with two mating types. Both mating types have to be present for sexual recombination to occur (Halama and Lacoste 1991). The sexual fruiting bodies, pseudothecia (Figure 1), contains numerous asci which release ascospores. These ascospores are wind borne over short and long distances (Bathgate and Loughman 2001). The sexual stage is known from most areas where SNB is

significant, including Norway (Bathgate and Loughman 2001; Cowger and Silva-Rojas 2006; Blixt et al. 2008; Ficke et al. 2011a). Asexual fruiting bodies, so-called pycnidia, produce pycnidiospores which are splash dispersed within the canopy during rain events (Figure 1) (Eyal et al. 1987; Solomon et al. 2006; Sommerhalder et al. 2011).

The mixed reproduction system provides both increased diversity through genetic recombination, and fast clonal reproduction of favorable genotypes. Selection in different environments has likely given rise to high levels of variation in aggressiveness and as far as it has been investigated, no single *P. nodorum* genotype dominates in any environment (Engle et al. 2006; Stukenbrock et al. 2006; Ali and Adhikari 2008; Blixt et al. 2008; Francki 2013).

The pathogen survives on infected seeds and wheat stubble which serve as primary inoculum sources (Figure 1). Formation of pseudothecia and sexual reproduction occur the whole growth season (Blixt et al. 2008; Sommerhalder et al. 2010). Wind borne ascospores are released from pseudothecia on stubble. The ascospore release is often assumed to be most important during fall and spring, coinciding with the emergence of wheat seedlings (Mittelstädt and Fehrmann 1987; Bathgate and Loughman 2001; Bennett et al. 2007). Rain-splash dispersed asexual pycnidiospores produced on the infected plants serve as primary and secondary inoculum (Eyal et al. 1987). In order for efficient splash dispersal to occur, at least 5 mm rainfall and temperature >10 ° C, followed by at least 10 mm rainfall within the next 48 hours is necessary (Eyal et al. 1987) although dew and mist is sufficient to promote spore release (Bathgate and Loughman 2001).

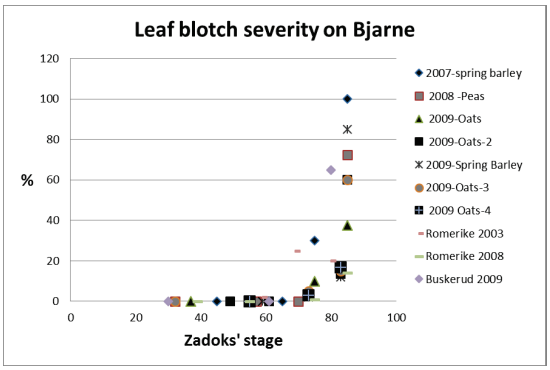

Figure 2 Percentage of Septoria nodorum leaf blotch (SNB) on the Norwegian spring wheat cultivar Bjarne after various pre-crops. The disease develops exponentially from Zadoks' stage 70-75 (Zadoks et al. 1974). Adapted from Ficke et al. (2011a).

The disease progresses exponentially after Zadoks' stage 70-75 (Zadoks et al. 1974) when the plant approaches maturity (Figure 2), but with different slope depending on factors like pre-crop, inoculum pressure, cultivar resistance and environmental conditions.

1.3.2. Symptoms

The *P. nodorum* germ tubes penetrate the leaf either directly through the cuticle or through open or closed stomata. Chlorosis at the infection site expands into oval lesions, often accompanied by necrosis. Pycnidia can form in the infected tissue within a week under optimal conditions (Solomon et al. 2006).

Figure 3 Left: Leaf blotch symptoms in the field. Top right: Pycnidia developing in the necrotic lesion. Bottom right: Necrotic lesions and chlorosis on a flag leaf. (Photos: Anja K. Ruud)

In the field (Figure 3), symptoms of SNB first develop on the lower leaves and progress to the upper leaves through rain splash dispersal. Under sufficiently long growth season and favorable weather conditions the pathogen will eventually reach the glumes and cause glume blotch (Eyal et al. 1987; Solomon et al. 2006; McMullen and Adhikari 2009).

1.4. Disease management

The Septoria leaf blotch diseases, including SNB, have only been recognized as major diseases since the introduction of high yielding, semi-dwarf cultivars (King et al. 1983; Scharen 1999). SNB can be controlled through appropriate application of agricultural practices like proper crop rotation and tillage, fungicides and by using resistant cultivars. In later years, the recommended agricultural practice of reduced tillage to prevent soil erosion lead to increased disease pressure. The plant residues (Figure 1) serve as primary inoculum in the subsequent growth season (McMullen and Adhikari 2009; Lillemo and Dieseth 2011).

1.4.1. Agricultural practice

Cultural practices have always been used to control disease pressure and optimize the growth conditions for the crop. Rotation with crops that are non-hosts to *P. nodorum* is advised, since the pathogen survives on plant residues from the previous wheat crop. For instance, oil seed rape or potatoes would be suitable pre-crops (Lillemo and Dieseth 2011). Rotations with resistant wheat cultivars may also reduce the disease pressure since less inoculum is carried over from these (Krupinsky 1999). Crop rotation is most effective to control diseases disseminated over short distances, like *P. nodorum* (Cunfer 1998). However, political and economic incentives affect whether crop rotation is implemented by the farmer. In Norway, farmers often grow wheat after wheat since this is economically more attractive than the rotational crops (Lillemo and Dieseth 2011). *P. nodorum* can be further promoted if direct seeding or minimum tillage practices are applied (Sutton and Vyn 1990; Krupinsky 1999). In order to reduce soil erosion, reduced tillage including light spring harrowing, chisel plowing and spring plowing, is common in Norway (Lillemo and Dieseth 2011).

1.4.2. Fungicide control

Leaf blotch diseases are mainly controlled by application of fungicides at the heading stage (Lillemo and Dieseth 2011). The main fungicide groups are strobilurins and triazoles.

Strobilurins inhibits fungal respiration by binding to the cytochrome *b* complex III at the Q_0 site in the mitochondrial electron transport chain (Bartlett et al. 2002). Thus, strobilurins have a very specific target or mode-of-action, which also make them susceptible to fungal resistance development. Loss of strobilurin sensitivity is associated with a mutation in the pathogen's mitochondrial genome leading to an amino acid change in cytochrome *b* (Gisi et al. 2002). This mutation is common both in European *Z. tritici* isolates (Leroux et al. 2007) and *P. nodorum*,

including the majority of Swedish *P. nodorum* isolates collected between 2003 and 2005 (Blixt et al. 2009).

Azoles, including triazoles, are also called sterol demethylation inhibitors (DMIs). They target CYP51, a cytochrome 450 enzyme responsible for the 14-α-demethylation of ergosterol (Siegel 1981; Bossche et al. 1995). The consequence is ergosterol depletion, altered permeability of the fungal cell wall, and affected cell wall synthesis. Reduced sensitivity to azoles can be caused by three mechanisms: Point mutation in the target gene *CYP51*, overexpression of CYP51 and up-regulation of efflux proteins leading to reduced accumulation of the fungicide inside the cells (Leroux et al. 2007).

Up to 30 non-synonymous *CYP51* point mutations have been identified in *Z. tritici* associated with increased fungicide resistance (Leroux and Walker 2011; Cools and Fraaije 2013), the first identified already in 1993. The higher number of mutations in *Z. tritici CYP51* indicates that reduced azole sensitivity developed earlier in *Z. tritici* than *P. nodorum*, and the effectiveness of all classes of this fungicide group is threatened (Cools et al. 2011), while still mostly effective against *P. nodorum* (Pereira et al. 2016). But two non-synonymous amino acid substitutions in CYP51 associated with reduced azole sensitivity were recently reported in European and Chinese *P. nodorum* isolates (Pereira et al. 2016), including 25 % of Swedish isolates.

Perhaps the advantage of *Z. tritici* to *P. nodorum* in areas where triazoles are applied can explain some of the shift in importance between the two pathogens in many European countries in recent years (McDonald, B, personal communication). In Norway, *P. nodorum* remains the major leaf blotch pathogen in spring wheat. But also Norwegian *P. nodorum* isolates have been reported with resistance to strobilurins and with reduced sensitivity to triazoles (Ficke et al. 2011b; Abrahamsen et al. 2013). The potential loss of effective fungicides underlines the need to control the disease by other and more sustainable methods.

1.4.3. Genetic resistance

Breeding for resistance to SNB is challenged by the lack of major resistance genes. The inheritance of resistance is complex (Scharen and Krupinsky 1978; Mullaney et al. 1982) and strong genotype × environment (G×E) interactions can mask the relatively small contributions of the individual genes. Plant height and maturity are also associated with the development of the disease (Rosielle and Brown 1980; Scott et al. 1982; Francki 2013). However, significant

residual resistance that is not associated with the confounding traits is also observed (Scott et al. 1982).

1.4.3.1. The gene-for-gene models

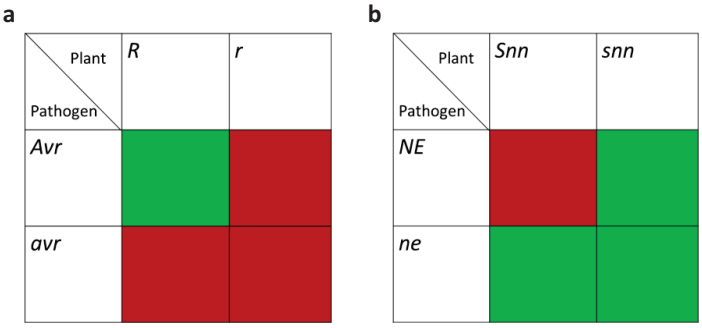

Figure 4 a. The classical gene-for-gene model adapted from Flor (1971). In a biotrophic system, resistance is conferred when the product of a resistance (*R*) gene in the host recognizes a avirulence (*Avr*) gene product secreted by the pathogen. **b.** The "inverse" model adapted from Friesen et al. (2007). In a necrotrophic system, HR is induced upon recognition of a necrotrophic effector (NE) by the product of a sensitivity (*Snn*) gene, and leads to increased susceptibility.

The inheritance of pathogenicity and resistance in a gene-for-gene manner was investigated already in the 1940s (Flor 1942). Flor's studies of flax rust (*Melampsora lini*) on flax (*Linum marginale*) led to the classical gene-for-gene model (Figure 4a) of resistance (Flor 1956; Flor 1971). The resistance is conferred when the product of an avirulence gene (*Avr*) from the pathogen is recognized by a resistance (*R*) gene in the host and hypersensitive response (HR) and programmed cell death is initiated (Figure 4a). Many such *Avr*-gene products, or effectors, with a demonstrated effect on virulence are known in biotrophic pathosystems. They generally lack structural similarities (van't Slot and Knogge 2002; van't Slot et al. 2003), but share a general function in optimizing the pathogens' development on the host (van't Slot et al. 2003).

The first host-specific toxins (HSTs) were also discovered in the 1930-40s, for instance AK toxin produced by *Alternaria alternata* (Tanaka 1933) and victorin produced by *Cochliobolus victoriae* (Meehan and Murphy 1947). While resistance genes in the classical model are dominant (Figure 4a), susceptibility is usually caused by a dominant *susceptibility* gene and is referred to as an inverse or mirror model (Figure 4b) (Wolpert et al. 2002; Friesen et al. 2007)

HSTs produced by fungi are, like *Avr*-gene products in biotrophic systems, also diverse in structure and biosynthetic mechanisms (Wolpert et al. 2002). Some peptide HSTs act as effectors by inducing HR response (Faris et al. 2010; Oliver and Solomon 2010) and are called necrotrophic effectors (NEs).

1.4.3.2. NE and sensitivity gene interactions in the P. nodorum – wheat pathosystem

It has been known for almost 40 years that *P. nodorum* produces phytotoxic compounds inducing plant cell death prior to hyphal growth (Bird and Ride 1981). However, the role of these phytotoxins in relation to resistance was not understood by the end of the last century (Cunfer 1999).

Liu et al. (2004a) characterized the first host-specific protein secreted by *P. nodorum*, and named it SnTox1. Earlier, Tomas and Bockus (1987) had described that the causal agent of tan spot, *P. tritici-repentis,* secretes a host-specific toxin, Ptr toxin, later renamed ToxA (Tomas et al. 1990). The corresponding sensitivity locus *Tsn1/tsn1* was mapped to chromosome 5BL and reported as a dominant susceptibility locus (Faris et al. 1996). Friesen et al. (2006) discovered a *P. nodorum* gene that shared 99.7 % sequence similarity to the *ToxA* in *P. tritici-repentis* described above, and the sensitivity also mapped to *Tsn1* (Liu et al. 2006). It was estimated that the *ToxA*-gene was introduced from *P. nodorum* into *P. tritici-repentis* through horizontal gene transfer before 1941 when *P. tritici-repentis* emerged as a pathogen on wheat (Friesen et al. 2006). The *ToxA*-gene is also present in *P. avenaria* f.sp. *tritici*, closely related to *P. nodorum* (McDonald et al. 2012; McDonald et al. 2013). Recently, the *ToxA*-gene was also discovered in *Bipolaris sorokiniana*, the causal agent of spot blotch in wheat (McDonald et al. 2017). The *ToxA* region in *B. sorokiniana* showed more similarities with *P. tritici-repentis* than *P. nodorum*.

The characterized *P. nodorum* NEs are small, secreted proteins, and virulence factors rather than true pathogenicity factors (Friesen et al. 2007), i.e. they affect the degree of disease in the host. So far, at least eight NEs (SnToxA, SnTox1, SnTox2, SnTox3, SnTox4, SnTox5, SnTox6 and SnTox7) and nine corresponding *Snn*-genes (*Tsn1*, *Snn1*, *Snn2*, *Snn3-B1, Snn3-D1*, *Snn4*, *Snn5, Snn6 and Snn7*) have been characterized (Friesen et al. 2006; Liu et al. 2006; Friesen et al. 2007; Abeysekara et al. 2009; Liu et al. 2009; Gao et al. 2015; Shi et al. 2015).

The NE-*Snn* interactions are usually additive in nature, but epistatic and modifying effects are also known. For instance, the presence of SnToxA-*Tsn1* (Friesen et al. 2008c; Friesen et al.

2008b) is epistatic to the SnTox3-*Snn3* and SnTox1 modifies the expression of SnTox3 (Phan et al. 2016).

P. nodorum is a good model organism for genomic studies. It grows willingly on artificial media, it is important as a crop pathogen and has a relatively small sized genome (≈ 37 Mb). The first *P. nodorum* reference genome was published by Hane et al. (2007), and it has later been re-sequenced (Syme et al. 2013) using isolates with different effector profiles and annotated (Syme et al. 2016). Bioinformatic tools have been used to search for novel effector candidate genes and were successful in identifying the *SnTox1* gene (Liu et al. 2012). The criteria used to predict candidate effector genes are 1) small secreted protein (< 30 kDa), 2) cysteine rich, 3) located near repetitive DNA regions or scaffold ends, 4) no blast matches. In addition, criteria like presence/absence of genes in virulent versus non-virulent isolates and evidence of positive selection can be applied when isolates with known differences in pathogenicity are compared (Syme et al. 2013). By applying these criteria on genomic data from three isolates (SN15, Sn4 and Sn79 (non-virulent)), a candidate list of 159 potential effector genes was the result (Syme et al. 2013). Although two of the known effectors ranked top of the list of predicted candidate genes, the large number of genes that would need further experimental investigation is somewhat discouraging. Also, since few structural similarities have been identified between effectors, the prediction criteria may not capture all the real candidate genes. Although acknowledging that effector genes are often located in repetitive regions (criterion 3), such regions have presented technical challenges and been filtered out in many next-generation-sequencing studies (Alkan et al. 2011), which means that many true candidate genes may be missed (Treangen and Salzberg 2011). More recently, technologies that also capture these regions, i.e. sequences longer continuous pieces of DNA, like PacBio (Pacific Biosciences), have been developed (Goodwin et al. 2016).

The relative contributions of NE-*Snn* interactions to disease under field conditions are still discussed (Francki 2013), although more and more evidence supports that at least some of the interactions are important. In Australia SnToxA has been delivered to the breeders since 2009 (Vleeshouwers and Oliver 2014). By 2012, 30 000 doses of SnToxA and 6 000 doses each of SnTox1 and SnTox3 were provided annually (Vleeshouwers and Oliver 2014). The area of SnToxA sensitive wheat in Australia fell from 30.4 % in 2009-2010 to 16.9 % within three years. The estimated economic gain was approximately 50 million AUD, assuming a yield loss of 0.3 tons per hectare in susceptible cultivars (Vleeshouwers and Oliver 2014). SnToxA-*Tsn1* and SnTox2-*Snn2* were identified after spray inoculation of the flag leaves with a single *P.*

nodorum isolate in the field (Friesen et al. 2009). The effect of SnToxA-*Tsn1* was also likely to underlie a significant QTL in the 05Y001 doubled haploid (DH) mapping population one year, but not in the subsequent trial (Francki et al. 2011). Waters et al. (2011) found a lower difference in resistance rating between SnToxA insensitive and sensitive cultivars than Oliver et al. (2009) and suggested that reduction in SnToxA sensitive cultivars could have triggered a shift in the NE frequencies in the pathogen population. Waters et al. (2011) also found a low, but significant correlation between sensitivity to SnTox3 and disease resistance ratings in Australian wheat cultivars. The genetic mapping of the SnTox3-*Snn3* interaction as a major determinant of SNB susceptibility in the field was the first validation of the importance of this locus (Ruud et al. 2017). This work will be discussed later.

1.4.3.3. The nature of resistance and sensitivity genes

Most of the R-genes encode proteins with a nucleotide binding site (NBS) and leucine-rich repeats (LRRs). Upon direct or indirect recognition of a pathogen effector (i.e. the product of an *Avr*-gene), the NBS-LRR initiates signaling pathways, in most cases leading to HR and cell death (Jones and Jones 1997; van't Slot et al. 2003).

Less is known about the genes conferring susceptibility to NEs. However, the molecular cloning of a number of sensitivity genes including *Tsn1* involved in ToxA sensitivity, have showed that they often have NBS and LRR domains associated with effector triggered immunity (ETI) (Lorang et al. 2007; Nagy and Bennetzen 2008; Faris et al. 2010). Recently, *Snn1* conferring sensitivity to SnTox1 was cloned and shown to encode a wall-associated kinase (WAK) (Shi et al. 2016a). Receptor kinases are usually pattern recognition receptors (PRR) involved in pathogen-associated molecular patterns (PAMP) triggered immunity. Responses to ETI and PAMP overlap, including the HR response (Dodds and Rathjen 2010).

These gene-for-gene interactions, inducing HR upon recognition, forms the framework for identifying resistance and sensitivity to SNB at the seedling stage. Single *P. nodorum* isolates can be grown in liquid culture, and are expected to secrete NEs into the medium.

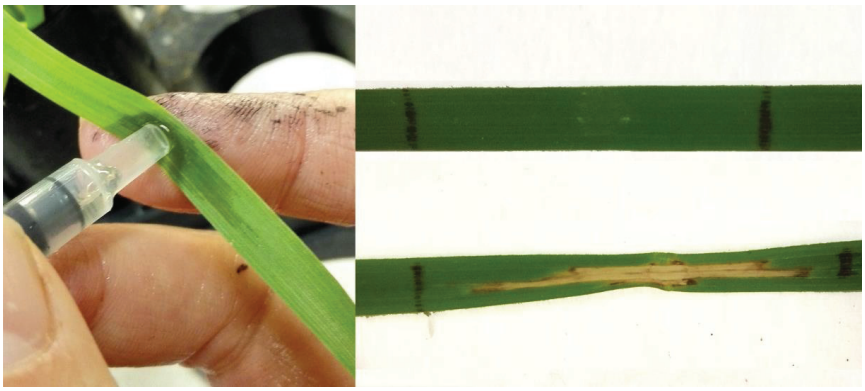

Figure 5 Left: Infiltration of seedling leaf with culture filtrate, using a needleless syringe. Right, top: SnToxA-insensitive leaf 5 days post inoculation (d.p.i). Right, bottom: Necrotic tissue developed in the infiltrated area in a SnToxA-sensitive leaf 5 d.p.i. (Photos: Left: Anja K. Ruud. Right: Min Lin).

When seedling leaves are infiltrated with filter sterilized culture filtrate (CF) (Figure 5), sensitive lines develop chlorosis or necrosis, while resistant lines remain healthy. The reaction types are usually scored on a 0-3 scale (Friesen and Faris 2012) and the sensitivity locus can be genetically mapped if a segregating mapping population is used. Typically, symptoms develop after 3 to 5 days in the greenhouse.

Since the NE is a virulence factor, it should also have an effect on disease development after inoculation with a conidiospore suspension. Historically, different methods have been used to evaluate the role of different components of resistance at the seedling stage. The methods include latency period, lesion expansion and development and number of pycnidia in the lesions (Eyal and Scharen 1977; Eyal et al. 1987; Czembor et al. 2003). Quantitative measurements have also been used, for instance in Eyal and Scharen (1977), Karjalainen (1985) and Jönsson (1985). However, a reaction type scale from 0 to 5 (Liu et al. 2004b) emphasizing the extent of chlorosis and necrosis associated with the lesions is now commonly applied. This method is believed to be accurate in capturing the effect of potential NE-*Snn* interactions (Friesen and Faris 2012).

1.4.3.4. General resistance

Adult plant resistance to SNB is mainly quantitative and additive (Fried and Meister 1987; Bostwick et al. 1993; Wicki et al. 1999). Dominant SNB resistance is also observed, and the segregation patterns of intermediate reactions can indicate the presence of modifier genes

(Kleijer et al. 1977; Ma 1993; Ma and Hughes 1993, 1995). General mechanisms like cell wall degrading enzymes produced by the pathogen (Magro 1984; Lehtinen 1993), host lignification and papilla formation to reduce hyphae penetration (Bird and Ride 1981) also explain variation in resistance.

1.4.3.5. Secondary metabolites

Metabolomics studies have identified several secondary metabolites produced by *P. nodorum*, for instance mellein, septorines and mycophenolic acids as summarized by Chooi et al. (2014). However, their roles as pathogenicity or virulence factors are not well understood. Many secondary metabolite pathways are not active unless under certain environmental stresses (Brakhage and Schroeckh 2011).

Genomic studies have provided further insight in the range of secondary metabolite biosynthesis genes that the fungus possesses (Chooi et al. 2014). Among the most likely candidates are polyketide kinases with significant homology to polyketide kinases in secondary metabolite producing fungi like *Aspergillus* and motifs similar to what is found in different plant pathogens like *Fusarium*, *Alternaria* and *Botrytis* (Chooi et al. 2014). Other potential secondary metabolite synthesizing genes are terpene transferases and prenyltransferases. In other fungi these gene families are known to be involved in mycotoxin and phytotoxin production, for instance ergot alkaloids (Chooi et al. 2014). Still, the identity of the gene products is unknown, and only a few secondary metabolites have been identified in *P. nodorum*. However, the genetic potential for secondary metabolite biosynthesis is there.

1.4.4. Escape mechanisms

Morphological traits that reduce the contact between the pathogen and the plant can be identified as disease escapes (Parlevliet 1977), and often lead to misinterpretation of true association with resistance. These include variation in plant height and timing of heading (earliness) (Scott et al. 1982). Taller plants may escape from rain driven spread of disease in the canopy. Early maturing plants may escape the highest disease pressure (Francki 2013), but on the other hand, later plants may appear more resistant at the time of disease scoring since the disease develops faster in more mature plants.

1.4.5. Environmental factors

The development of disease is affected by weather conditions like temperature, rainfall and humidity. Variation in these factors within and between growth seasons can have a strong effect on the relative resistance rankings (Kim and Bockus 2003).

1.5. Genetic mapping of resistance

1.5.1. Molecular markers

Breeding for improvement of polygenic, quantitative traits is complicated. The contribution of each individual locus is moderate and can be masked by other, dominant loci or epistatic effects. However, the development of molecular markers could provide a help in overcoming some of these difficulties. Co-dominant markers can distinguish between all genotypes (Tanksley 1983). Markers can be either hybridization or polymerase chain reaction (PCR) based. In the first case, a probe is hybridized to the DNA. In PCR based systems small fragments of DNA are amplified with polymerase enzymes.

The first markers were hybridization based restriction fragment length polymorphism (RFLP) markers (Botstein et al. 1980). Amplified fragment length polymorphism (AFLP) combines the strength of RFLP with PCR flexibility and have a high selectivity (Vos et al. 1995). DArT (Diversity Array Technology) markers have the advantage of being open source and allowing many polymorphisms along the genome to be discovered simultaneously (Jaccoud et al. 2001; Semagn et al. 2006a). DArT marker systems are now mostly replaced with single nucleotide polymorphism (SNP) and genotyping-by-sequencing platforms (GBS or next-generation sequencing, NGS).

Simple sequence repeats (SSRs), microsatellites or short tandem repeats (STRs) belong to the smallest class of simple repetitive DNA sequences (Akkaya et al. 1992). The definition varies, as reviewed by Semagn et al. (2014), but Chambers and MacAvoy (2000) suggested to follow the original definition that the repeats are between 2-6 base pairs (bp) long. SSR markers have many advantages. They can be non-anonymous, abundant, reproducible and show a high degree of inter- and intra-specific polymorphism (Mammadov et al. 2012; Semagn et al. 2014). Microsatellites originate from regions of the DNA where repeated motifs are already overrepresented (Tautz et al. 1986). The main mutational mechanism behind SSRs is ‘slipped-strand-mispairing’, resulting in gain or loss of one or more repeats (Levinson and Gutman 1987).

Single nucleotide polymorphisms (SNPs) are the most abundant of molecular markers, estimated to occur for every 100-300 bp in any genome (Gupta et al. 2001). In particular, the availability of expressed sequence tags (ESTs) have facilitated the development of SNP markers. While most SSR markers are located in inter-genetic repeat regions, the EST based SNP markers are located in expressed regions which allows for a higher potential for candidate

gene targeting (Gupta et al. 2001). This method works best for crops with a reference genome sequence or large transcriptome database.

SNP identification in allopolyploids like wheat is extra challenging. Genetic similarities between the three subgenomes (AA, BB and DD, presented in 1.1.) in wheat makes it difficult to distinguish within and between subgenomes (Ganal et al. 2009). While homologue differences refer to within subgenome, allelic SNPs – which are useful since they provide information about variation at the same locus, homeologous differences are polymorphisms occurring between subgenomes. Since they point to different loci, they are not informative as SNPs.

The wheat genome is also rich in noncoding repetitive DNA, and these regions are not covered by the EST approach (Mammadov et al. 2012). It is also worth noting that while the EST based SNPs target mutations within expressed genes and can be used to identify causal mutations, QTL are often located in noncoding, regulatory regions (Mammadov et al. 2012). Software for genotype calling (i.e. the identification of sequence variations where genotypes vary by a single nucleotide) is often compromised in polyploids. The reason for this is that the allelic variant ratio differs from diploid species, which makes genotype cluster plots difficult to analyze without manual scoring or specialized software (Wang et al. 2014).

Chip based SNP platforms are oligonucleotide based DNA microarrays and cover many more genes than *in silico* analysis of ESTs (Ganal et al. 2009; Mammadov et al. 2012). Chip platforms still have relatively high cost per sample and are less suitable for studies requiring lower numbers of markers than in the multiplex chip arrays, like quality control (Semagn et al. 2012) and marker assisted selection (MAS) (Semagn et al. 2014). For such applications, uniplex (single-plex) platforms like KASP are more suitable. KASP, or Kompetitive Allele Specific PCR, is a method of SNP genotyping developed by KBioscience, now LGC Genomics (http://www.lgcgroup.com/). KASP is a fluorescence based technology that uses allele-specific oligo extension and fluorescence resonance energy transfer (FRET) to generate signals (Semagn et al. 2014). SNPs are quite easily transferred from one platform to another, and compared to other uniplex systems, KASP markers are less expensive, have greater flexibility and higher conversion rate than alternative platforms (USDA 2012; Semagn et al. 2014).

Since SSRs are usually located in inter-genetic regions the selection pressure is lower than for SNPs within genes. Also, SNPS are bi-allelic, which means that the maximum heterozygosity is 0.5. In contrary, the number of new SSR alleles that can be generated through slippage is

unlimited, the mutation rate is higher and heterozygosity approaches 1 (Hamblin et al. 2007). While singleton SNPs can be discovered in genotyping, they are removed in the ascertainment process. Selection of SNPs is thus skewed towards intermediate frequencies, while SSRs are biased towards rare alleles (Hamblin et al. 2007). For germplasm characterization studies it has been shown that a higher number of SNPs than SSRs are needed to obtain similar resolution for diversity estimates and for assigning individuals to populations (Hamblin et al. 2007; Moragues et al. 2010; Emanuelli et al. 2013). However, for mapping purposes, this difference between SSRs and SNPs is not important.

Genotyping-by-sequencing (GBS, also called next generation sequencing, NGS) can be an alternative to chip based arrays. Briefly, the steps in GBS are template preparation, sequencing and imaging and data analysis. Template preparation generally includes randomly breaking the DNA, and for complex genomes also reduction of complexity by using restriction enzymes (Metzker 2010; Elshire et al. 2011). GBS is particularly suitable for projects where the genomes of several specimens are sequenced to discover large numbers of single nucleotide polymorphisms (SNPs). No prior knowledge of the genome is necessary and the cost is lower than for chip based arrays, but GBS usually produces more missing data.

The populations used in this thesis were genotyped both with SSR and DArT markers and with the Illumina iSelect 90K wheat SNP Chip (Wang et al. 2014). The 90 K chip design is based on gene-associated SNPs (from RNAseq) corresponding to reference genome contigs from the chromosome survey sequencing (CSS) project (http://wheat-urgi.versailles.inra.fr/Seq-Repository).

1.5.2. Linkage QTL mapping

Linkage mapping is the most widely used method to dissect complex traits and identify markers linked to them. Many important traits are controlled by multiple genes, and are impossible to identify only by phenotypic evaluation. The genomic regions with genes associated with such traits are called quantitative trait loci (QTL). The development of molecular marker technologies in the 1980s facilitated the construction of genetic linkage maps (Collard et al. 2005) and complex traits could be separated into discrete QTL (Paterson et al. 1988).

The basic principle behind linkage mapping is that recombination occurs during meiosis (Tanksley 1993). Maps can be constructed for specific, segregating populations. Preferably, recombinant inbred lines (RILs) or doubled haploids (DHs) are used since they are homozygous and can be maintained and reproduced forever (Collard and Mackill 2008).

However, for some purposes and in outbreeding crops where inbreeding causes loss of vigor, other populations can be more suitable, like F_2, F_3 or backcrosses (BC). Logarithm of odds (LOD) is often used as the statistical test for linkage. To identify QTL, the population is phenotyped for the traits of interest, and the phenotypic and genotypic data is analyzed to uncover linkage between a certain phenotype and genetic regions.

After the initial detection, additional steps to confirm a QTL and validate associated markers are usually required (Langridge et al. 2001). The effect and position of a QTL can be inaccurate due to sampling bias (Melchinger et al. 1998) and flanking markers may not be polymorphic in other genotypes.

QTL mapping utilizes linkage disequilibrium (LD), the non-random association of alleles. The power of QTL mapping is statistical rather than biological, i.e. it has not generated much new understanding of the underlying genes (Bernardo 2016). Due to strong LD and limited number of recombination events, fine-mapping of a QTL in a bi-parental population is difficult (Flint-Garcia et al. 2003; Gupta et al. 2014). Utilization of QTL mapping in breeding works best when there are major QTL that can easily be introgressed (Bernardo 2008).

Table 1 Overview of quantitative trait loci (QTL) for seedling and flag leaf resistance, including necrotrophic effector-sensitivity (NE-*Snn*) gene interactions. Adapted from Francki (2013). *T. aestivum* unless otherwise noted below. Flag leaf resistance is only listed if it has been reported significant in at least two environments (years, locations).

Plant tissue	Population	NE-*Snn*	QTL, chromosome	Markers	Reference
Seedling	Liwilla × Begra, DH		*QSnl.ihar-2B*	*gwm501 – gwm410*	(Czembor et al. 2003)
			QSnl.ihar-5B	*barc32 – gwm499*	
			QSnl.ihar-5D	*gwm205 - gwm212*	
	W7984 × Opata85	SnTox1-*Snn1*	1B	*mwg938 – snn1 fcp618, psp3000*	(Liu et al. 2004b)
			4B	*cdo1312*	
	Alba × Begra		*QSnl.ihar-6A*	*gwm570 – mwg934*	(Arseniuk et al. 2004)
	BR34 × Grandin	SnToxA-*Tsn1*	5BL	*fcp1, fcp2, fcp394, fcp620*	(Friesen et al. 2006)
	BR34 × Grandin	SnTox2-*Snn2*	2DS	*TC253803, cfd51*	(Friesen et al. 2007)
		SnTox3-*Snn3-B1*	5BS	*gwm234, cfd20*	
	LDN × LDN (DIC-1B) (*T. turgidum*)	(SnToxA-*Tsn1*)	5BL	*bcd9 – fbb237*	(Gonzalez-Hernandez et al. 2009)
	Arina × Forno	SnTox4-*Snn4*	1AS	*BG262267, BG26975, cfd58*	(Abeysekara et al. 2009)
Seedling and flag leaf	BR34 × Grandin	SnTox2-*Snn2*	*QSnb.fcu-2DS*	*gwm614 – cfd53*	(Friesen et al. 2009)
			QSnb.fcu-5AL	*barc151 – fcp13*	
		SnToxA-*Tsn1*	*QSnb.fcu-5BL*	*barc1116 – barc43*	
Seedling	*Aegilops tauschii*	SnTox3-*Snn3-D1*	*5D*	*cfd18 - hbg337*	(Zhang et al. 2011)
	Lebsock × PI 94749 (*T. turgidum* subsp. *durum* × *T. turgidum* subsp. *carthlicum)*	SnTox5-*Snn5*	4BL	*wmc349 - cfd22, barc163*	(Friesen et al. 2012)
	W7984 × Opata85	SnTox6-*Snn6*	6AL	*BE424987 - BE403326*	(Gao et al. 2015)
	Chinese Spring × Timstein	SnTox7-*Snn7*	2D	*cfd44 – gwm311*	(Shi et al. 2015)
Flag leaf	Forno x Oberkulmer		*QSnl.eth-2D*	*psr932 – psr331a*	(Aguilar et al. 2005)
			QSnl.eth-4B	*glk348 – psr921*	
			QSnl.eth-7B	*mwg710a – glk576*	
	WAWHT2074 x 6HRWSN125		*QSnl.daw-2D*	*cfd11 – gwm30*	(Shankar et al. 2008)
	BR34 x Grandin		*QSnb.fcu-1BS*	*fcp267 – barc240*	(Friesen et al. 2009)
	P92201D5 x P91193D1		*QSnl.daw-2A*	*gwm614a– wPt-7056*	(Francki et al. 2011)
	EGA Blanco x Millewa		*QSnl.daw-1B*	*wPt-8949 – wPt-2575*	
			QSnl.daw-5B	*wPt-3457 – wPt-0935*	
	SHA3/CBRD x Naxos		*1B*	*wmc619*	(Lu and Lillemo 2014)
			3AS	*gwm2*	
			3B	*wPt-4127*	
			3BL	*wPt-4933*	
			5BS	*wPt-5346*	
			5BL	*fcp1*	
			7A	*wmc603*	
			7B	*wPt-0963*	

Table 1 shows an overview of SNB resistance QTL detected in linkage QTL mapping studies. Only QTL significant in at least two environments are included. In addition, multiple QTL have been identified in several studies as significant in only one environment, but were not included here.

1.5.3. Association mapping

Association mapping (AM, also called genome wide association mapping, GWAS) emerged in the early 2000s as an alternative to biparental linkage mapping (Gupta et al. 2014). One advantage of this approach is that the time-consuming development of inbred or double haploid lines of a bi-parental mapping population is avoided (Crossa et al. 2007). Unlike in linkage mapping where the individuals are derived from two or a few parents, an AM panel can be constructed with unrelated individuals. Polymorphic markers associated with a phenotypic trait can be identified by means of linkage disequilibrium (LD) between loci (Thornsberry et al. 2001; Flint-Garcia et al. 2003). Since the number of historic recombination events is usually higher in a diverse panel of genotypes than in a biparental population where the genetic variation is limited to what is present in the two parents, the resolution is expected to be higher in AM. Also, an association mapping panel can be designed to capture most of the available variation. Breeding populations are suitable for AM for several reasons. They generate QTL directly relevant for the breeding program and extensive sources of phenotypic data are available, since the plants are routinely screened for agronomically important traits (Gupta et al. 2014).

Statistical errors can be categorized as Type I and Type II error. Type I error is the incorrect rejection of a true null hypothesis – or the risk of reporting "false positive" results. Type II error is incorrect retaining of a false null hypothesis, i.e. the risk of reporting "false negative" results. The risk of Type I and Type II error is higher in AM than biparental QTL mapping. False positive associations or Type I error can arise from population structure not accounted for (Pritchard et al. 2000). Population stratification and relatedness between the genotypes in the AM panel will often cause false associations, i.e. the associations are not caused by actual genetic linkage. This will influence the LD and thus the marker-trait association (Gupta et al. 2014). Different methods have been developed to deal with the effect of population structure. Mixed linear models (MLM) are considered better than generalized linear models (GLM), and can be combined with Bayesian analysis to determine population structure by assigning

individuals to subpopulations (Q) and kinship (K) matrices to account for relatedness (Yu et al. 2006).

Reduced power or Type II error ("false negatives") of the AM compared to biparental analysis can be caused by lower correlation between markers and traits because LD usually decays faster in diverse AM panels. Also, unbalanced presence of different alleles and a multiple-testing problem leads to very strict significance thresholds due to the relative independence of testing positions (Carlson et al. 2004; Breseghello and Sorrells 2006).

A major disadvantage with AM is that it fails to detect rare alleles (Gupta et al. 2014; Bernardo 2016). Firstly, alleles with minor allele frequency (MAF) ≤ 5 % are generally excluded from the analysis. Secondly, rare variants often fail detection due to "missing heritability". Loci detected by GWAS almost without exception explain only a small minority of the inferred variation. However, while the frequency of any rare allele is low, the sum of rare alleles associated with a trait can make them quite common (Gupta et al. 2014). The "common disease, rare variant" hypothesis (Reich and Lander 2001) suggests that there should be many moderate to large effect rare alleles controlling one complex trait. Case studies have shown that the effects of rare variants are generally higher than for common variants (Gibson 2012). In other words, identification of rare variants is important, but the methodology to handle this in GWAS is still developing.

Only a handful of GWAS studies have investigated SNB resistance. AM was used to fine map a region on 3BS associated with Septoria nodorum glume blotch in 44 European winter wheat varieties (Tommasini et al. 2007). Adhikari et al. (2011) detected unique SNB seedling resistance QTL on 6A and 7A in a GWAS panel consisting of 576 land races from the USDA Small Grains Collection. A novel QTL on 3A was identified in a set of 528 spring wheat landraces from the same USDA Small Grains Collection in a study by Gurung et al. (2014), while two other QTL on 2D and 5B were described previously by Adhikari et al. (2011). Also, Liu et al. (2015) identified seedling QTL on 5A, 5B and 5D in a GWAS panel of 70 hard red winter wheats. To our knowledge no GWAS study of flag leaf resistance has been published. However, GWAS has been successfully used to detect markers associated with complex disease resistance based on field studies. One example is resistance to adult plant leaf rust (Caused by *Puccinia striiformis* f.sp. *tritici*) and yellow rust (Caused by *Puccinia graminis* f.sp. *tritici*), common bunt (Caused by *Tilletia caries* and *T. laevis*) and tan spot in Canadian winter wheat (Perez-Lara et al. 2017).

1.5.4. Marker assisted selection and resistance breeding

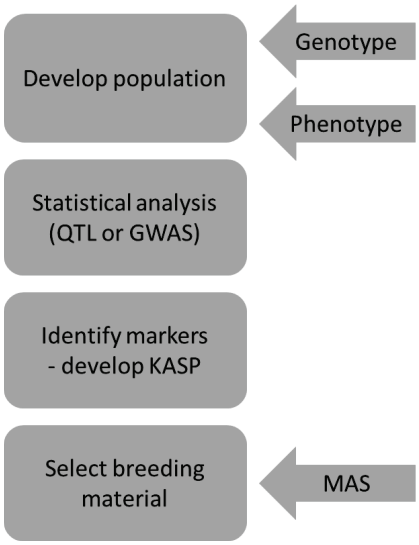

Figure 6 Typical workflow of a genetic mapping study with marker assisted selection (MAS) as the goal.

Figure 6 shows a simplified workflow for a genetic mapping study where the aim is to identify markers for marker assisted selection (MAS). A suitable population is genotyped with genetic markers (usually SNPs or SSRs), and phenotyped for the traits of interest. QTL or GWAS mapping is performed to identify significant marker trait associations (MAT). The most promising markers can then be validated in other populations, converted to economical KASP markers and used to screen and select breeding material.

Compared to phenotypic selection at the adult plant stage, marker assisted selection (MAS) can provide higher accuracy and save time in the breeding cycle. Ideally, the markers should be diagnostic or "perfect" markers, i.e. situated within the causal gene. MAS can be particularly useful for 1) traits that are difficult to phenotype and not expressed at the seedling stage, 2) traits that do not show effect in all environments, 3) maintenance of recessive alleles and to speed up backcrossing 4) pyramiding of several monogenic traits or QTL for a single resistance, and for genetic studies.

MAS can be a useful tool to improve breeding for quantitative resistance traits. For instance, selection for partial, polygenic resistance to biotrophs have proven durable and effective over time (Miedaner and Flath 2007). MAS has also been applied to stack QTL with large effects,

for instance *Fhb1* + *Qfhs.ifa-5A* conferring resistance to Fusarium head blight. The best strategy is suggested to be MAS followed by phenotypic selection. The subsequent step of phenotypic selection allows the inclusion of minor QTL (Miedaner and Korzun 2012).

Economic and practical constraints decide whether MAS is feasible in a resistance breeding program. MAS is an alternative to phenotyping for resistance that is not expressed at the seedling stage. It must be noted that strictly selecting for one single locus in a breeding program will lead to high selection pressure on the pathogen. Selection increases the risk that the pathogen overcomes the resistance. Also, the chromosomal region flanking the QTL will be fixed. Simulation studies have shown that more than 30 cM introgressed regions can be present at the 6th backcross generation (Stam and Zeven 1981). In conclusion, several unwanted genes can be linked to the introgressed QTL and contribute to reduced fitness, for instance yield penalty.

Before applying MAS in the breeding program, an important step is validation of the markers initially detected in biparental or GWAS studies. Often, markers are not useful in different genetic backgrounds or in different environments. This can be due to QTL background effects, caused by linkage, epistasis and G × E interactions (Miedaner and Korzun 2012). Ideally, the markers should be tested on relevant breeding populations.

A QTL should explain at least 10 to 20 % of the phenotypic variation in order to be considered for MAS (Kover and Caicedo 2001). Broad spectrum resistance loci conferring protection against several pathogens are desirable. The *tsn1*-locus involved in ToxA-*Tsn1* interaction can be considered a broad spectrum locus, conferring resistance against several related pathogens (*P. nodorum, P. tritici-repentis, B. sorokiniana*). Screening and selection against this sensitivity locus can be performed either through seedling infiltration assays or MAS.

1.5.5. Genomic prediction and selection

Genomic prediction and selection (Meuwissen et al. 2001) is an approach that predicts the best individuals based on genetic values. Compared to traditional models where only markers significantly linked to the trait are considered, genomic selection considers ALL markers across the genome to predict breeding values. Known QTL with large effects can be included in the genomic prediction models as fixed effects and further increase the prediction accuracy (Bernardo 2014). The genomic selection is performed on a different population than the reference (training) set on which the genetic marker effects were calculated. Although initially used to predict breeding values of animals, genomic selection also has the potential to improve

genetic gain in crops like wheat (Crossa et al. 2010; Ornella et al. 2012; Storlie and Charmet 2013). Juliana et al. (2017) found that for prediction of breeding values for SNB seedling resistance, genomic prediction models performed better than a least squares approach, which only considered markers significantly associated with the trait.

2. The thesis

2.1. Background and motivation

Until the 1970s Norway was entirely dependent on import of food quality wheat, mainly from USA and Canada (Lillemo and Dieseth 2011). Due to political decisions and successful efforts to breed adapted cultivars, wheat has since become a major food crop in Norway. In good years as much as 75 % of wheat for human consumption can be provided by domestically grown wheat (LD 2017). However, unfavorable weather, disease epidemics and pre-harvest sprouting can cause severe drops in the domestic proportion of food quality wheat, with less than 20 % in 2012 as an extreme (LD 2017).

SNB is one of the most important diseases in spring wheat in Norway (Ficke et al. 2011a; Lillemo and Dieseth 2011). The disease is mainly controlled by fungicides since the use of other measures like crop rotation and autumn ploughing is limited (see 1.4.). The pathogen's potential to develop resistance to the fungicides (Ficke et al. 2011b; Abrahamsen 2013; Pereira et al. 2016) and concern about health and environmental risks related to fungicide spraying, underlines the need for more sustainable control. Growing plants with durable genetic resistance against SNB is both sustainable and economic, but the increased benefit from breeding for resistance to SNB has not been realized in Norway.

However, great progress has been made in understanding the *P. nodorum*-wheat pathosystem. The identification of multiple NE-*Snn* interactions explained by the inverse gene-for-gene model and the use of *SnToxA*, *SnTox1* and *SnTox3* in Australian breeding programs, served as motivation for this PhD project.

The main objectives of the project were to

I) Investigate to what extent differences in SNB resistance under Norwegian field conditions could be explained by NE-*Snn* interactions

II) Evaluate the extent of shared components of seedling and adult plant SNB resistance

III) Identify robust resistance sources in the Norwegian spring wheat germplasm, and genetic markers closely linked to these QTL, to be utilized in marker assisted selection

2.2. Overview of methods

To identify genetic SNB resistance, the first steps are to collect and screen diverse germplasm in the field and in the greenhouse. In the field, one can either rely on natural infection by the ambient pathogen population, or inoculate with single isolates or a mixture of isolates. The reproducibility across locations or seasons may be higher when the same isolates are used. On the other hand, one or a few isolates may not be representative of the situation in the farmers' fields. Breeders usually rely on natural infection in the field for evaluation of leaf blotch resistance (Cowger and Murphy 2007). Fraser et al. (2003) suggested that promotion of infection by natural inoculum gives a better estimate of host resistance under natural epidemics than inoculation of the nurseries with selected isolates. In the field trials evaluated in this thesis, we relied on natural infection promoted by mist irrigation and naturally infected straw.

Development of SNB at the adult plant stage in the field is influenced by variation in plant height and earliness (timing of heading). It is important to account for the effects of these traits. This can be done in several ways:

1) Score the plants and/or spray inoculate the flag leaves at the same developmental stage, in the greenhouse or in a tunnel to avoid effects of plant height by rain-splash spread spores. These are good measures, but very labor intensive and time-consuming when large populations are screened
2) Choose or develop mapping populations with little variation in earliness and height.
3) Score all traits of interest separately and only consider QTL for SNB resistance that does not co-locate with QTL for the confounding traits. However, true resistance QTL under the threshold can go undetected by this method
4) Include the confounding traits as covariates in a regression model with SNB severity as the dependent trait. This is the method we used. The QTL detected when the corrected values are analyzed are assumed to capture the true residual, genetic resistance to SNB. The corrected resistance was annotated as "corrected SNB severity" in the discussion and papers

The effect of plant height on SNB development varied from year to year. As described in 1.3.1., the pathogen has certain rainfall and relative humidity requirements in order to sporulate, spread by rain-splash and successfully infect new leaves (Eyal et al. 1987). In years with moderate rainfalls during mid-June to July, the effect of plant height on disease severity was usually significant in our field trials. In years with low correlation between plant height and

SNB development, extreme rainfalls (i.e. 76 mm in 24 h in 2015) seemed to reduce the differences between tall and short plants, by spreading the spores to the flag leaf also in tall cultivars.

Temperature affects plant development, like the timing of heading. Although spring wheat normally does not require vernalization, i.e. a cooler period after germination to induce heading and flowering, some of our germplasm harbor the *Vrn-A1* gene (Yan et al. 2003). This gives them a weak vernalization requirement. In particular, this applies to several lines and cultivars originating from CIMMYT. When the spring is warm, the requirement may not be met, and the induction of heading and flowering is significantly delayed. Thus, the effects of the individual vernalization genes vary between years, depending on temperature. In 2013 the temperatures in early spring were higher than usual, mainly due to late sowing after a wet early spring. The effect could be observed by much higher variation in days to heading in 2013 than in other years.

While field experiments are more relevant for the situation in the farmers' fields, experiments in controlled environments are useful for more accurate dissection of underlying mechanisms. Seedling and adult plant resistance are at least partly independently inherited (Rosielle and Brown 1980; Fried and Meister 1987; Shankar et al. 2008), but we also wanted to investigate to what extent resistance components were shared between the developmental stages, and if host-specific interactions were important at both stages. The seedlings were evaluated in the greenhouse at the 2 to 3 leaf stage. The secondary leaves were infiltrated with the individual, semi-purified SnToxA, SnTox1 and SnTox3 produced in transformed *Escherichia coli* and yeast (*Pichia pastoris*), in order to identify corresponding sensitivity loci in the plants. *P. nodorum* isolates were collected from unsprayed fields in the major Norwegian wheat growing areas. The isolates used for seedling inoculations were selected based on variation in virulence on differential lines and subsets of the mapping populations, and *SnToxA*, *SnTox1* and *SnTox3*-profile based on PCR screenings. We also used the North Dakotan isolate Sn4, which has been sequenced (Syme et al. 2013) and used in previous NE-*Snn*-characterization studies (Zhang et al. 2011; Liu et al. 2015). For inoculation assays, the isolates were grown on V8-PDA agar until sporulation, and the seedlings were inoculated with a standardized spore suspension. The isolates were also grown in liquid culture, and the plants were infiltrated with culture filtrates (CF) from these to unveil the contribution of NE-*Snn* interactions. To further characterize a potential new NE-*Snn* interaction, a rough size-selection of filter sterilized CF was done by ultracentrifugation. During the ultracentrifugation, filters with different pore sizes separate the

molecular components of the CF in fraction based on molecular mass (measured in kilo Dalton, kDa). The different fractions were infiltrated into sensitive lines to determine the approximate size. In addition, an F_2 population from a cross between sensitive and insensitive lines was infiltrated with the CF. If a dominant susceptibility locus is involved, a 3:1 segregation in sensitive to insensitive lines is expected in this generation.

Two populations developed from biparental crosses and a diverse collection of Nordic and exotic spring wheats (unofficially called MASbasis) were evaluated for SNB resistance. Most of the plants had previously been genotyped with SSR and DArT markers, but to increase the marker density and cover larger regions of the chromosomes, the populations were also genotyped with the Illumina iSelect 90K wheat SNP Chip (Wang et al. 2014) described in 1.5.1. An integrated genetic map with SNP, SSR and DArT markers was constructed for SHA3/CBRD × Naxos, and QTL analysis performed. Genome wide association mapping (GWAS) was used to analyze the marker-trait associations in the diverse Nordic collection.

2.3. Main results

In **Paper I**, the role of the SnTox3-*Snn3* interaction under Norwegian field conditions was investigated in the SHA3/CBRD × Naxos population. A genetic map with 567 SSR and DArT markers was available, and the population had previously been evaluated for field resistance to SNB (Lu and Lillemo 2014). While the population segregated for sensitivity to SnTox3 when infiltrated with the purified NE, the *Snn3* locus could not be mapped with significant linkage to the SSR or DArT markers. The population was genotyped with the Illumina iSelect 90K wheat SNP Chip (Wang et al. 2014) and we constructed an integrated SNP, SSR and DArT map with 4177 markers. With the new map, the *Snn3* locus could be mapped with tight linkage to SNPs at the telomeric end of 5BS. The SNB severity data from the field trials was re-analyzed with the new map, and the SnTox3-*Snn3* interaction was identified as a major determinant of susceptibility in the field, explaining up to 24 % of the phenotypic variation. This is the first report of the significance of the SnTox3-*Snn3* interaction in the field. The population was also inoculated with four *P. nodorum* isolates at the seedling stage and infiltrated with filter sterilized culture filtrate (CF) from the same isolates. The SnTox3-*Snn3* interaction was highly significant at the seedling stage after inoculation, explaining up to 51 % of the phenotypic variation, and was also the major determinant of sensitivity after infiltration with CF.

In **Paper II** the relationship between sensitivity to the cloned effectors SnToxA, SnTox1 and SnTox3 and their potential effect on disease severity in the field were investigated. A Nordic collection of spring wheat cultivars, landraces and breeding lines was used for this purpose. This collection is also called MASbasis. The plants were screened in mist irrigated field trials from 2010 to 2016, and phenotyped for plant height, earliness (days from sowing to heading) and % leaf blotch. Sensitivity to SnToxA, SnTox1 and SnTox3 was investigated in greenhouse experiments where seedlings were infiltrated with the purified NEs. Sensitivity to SnToxA and SnTox3 was common (45 and 55 % respectively) while sensitivity to SnTox1 was only present in 12 % of the genotypes. Sensitivity to SnToxA was significantly correlated to higher SNB severity in the field, while sensitivities to SnTox1 and SnTox3 were not significant in the field in this material.

In **Paper III,** 121 lines from the Nordic spring wheat collection **(Paper II)** were genotyped with the Illumina iSelect 90K wheat SNP Chip (Wang et al. 2014) and SSR markers. A total of 22 031 polymorphic markers were included. In addition to performing GWAS of the corrected SNB severities from the field experiments and SnToxA, SnTox1 and SnTox3 infiltration data from **Paper II**, the plants were inoculated with four *P. nodorum* isolates and infiltrated with CF from the same isolates. One aim was to investigate whether NE-*Snn* interactions could be detected with significant marker-trait associations (MTAs) in genome wide association (GWAS) analysis. Another aim was to explore whether the NE-*Snn* interactions were significant after both seedling inoculation and in adult plant resistance. We also wanted to identify markers associated with stable adult plant resistance to SNB, and investigate to which degree seedling and adult plant resistance overlapped (i.e. the same significant markers could be identified at both stages).

Markers associated with *Tsn1* conferring sensitivity to SnToxA were highly significant at the seedling stage, but only detected below the significance threshold at the adult plant stage. Significant QTL for seedling SNB resistance were located on 1A, 1B, 3A, 4B, 5B, 6B, 7A and 7B. At the adult plant stage the most robust QTL were located on 2B, 2D, 4A, 4B, 5A, 6B, 7A and 7B. The most stable QTL in the field was located on 2DL and was significant in all years except 2012. QTL on 4B and 7A were significant both after seedling inoculations in the greenhouse and at the adult plant stage in the field. The QTL on 7A could be validated in SHA3/CBRD × Naxos (**Paper I**). Several significant QTL were identified after infiltration with CF from the four isolates. Only three corresponded to QTL detected after inoculation, indicating they may be NE-*Snn* interactions. These were the locus associated to *Snn3* on 5BS,

and QTL on 3A and 6B. None of the QTL detected after CF infiltration were significant in the field.

Additional work not included in manuscripts:

Investigation of a potential new NE-*Snn* interaction We also tried to characterize a potential novel NE-*Snn* interaction on chromosome 7B. This interaction was initially detected after infiltration of SHA3/CBRD × Naxos with CF from isolate 201618 (reported in **Paper I**). A rough size-selection of the filter sterilized CF was done by ultracentrifugation and the different fractions were infiltrated into sensitive lines. The size of the secreted molecule causing sensitivity was estimated to 10-30 kDa, since this was the fraction causing sensitivity on the lines. 83 lines of an F_2 population derived from a cross between the SHA3/CBRD × Naxos RILs S×N-082 and S×N-065, segregating for the 7B QTL, were infiltrated with the CF. We found that the lines segregated in a ratio of 56 sensitive:27 insensitive, which is not different from a 3:1 segregation (χ^2= 0.73, p = 0.39). This is indicative of dominant sensitivity, which is a hallmark of a NE-*Snn* interaction. But since the interaction did not significantly affect the disease development after inoculation with the same isolate, it could not be defined as a NE-*Snn* interaction.

Resistance to SNB in Arina × NK936934 The doubled haploid (DH) population Arina × NK93604 (Semagn et al. 2006b) was evaluated for field and seedling resistance to SNB, and for sensitivity to SnToxA, SnTox1 and SnTox3. The population was genotyped with SSR, AFLP and DArT markers and the original map by Semagn et al. (2006b) was improved with new markers. This work was a continuation of the M.Sc. thesis by Ruud (2013).

Table 2 Pearson's correlation between corrected SNB from field and disease reactions after inoculation with three isolates in the Arina × NK93604 population.

Isolate	Year			
	2010	2011	2012	2013
Voll73_3	0.12	0.25	0.36	0.20
NOR4	0.19	0.25	0.27	0.22
Sn4	0.20	0.23	0.25	0.14

The correlation between seedling and adult plant resistance was low (Table 2) in this population, which was particularly difficult to evaluate in the field. Firstly, all the DH lines

developed some extent of leaf tip necrosis (LTN) associated with the slow rusting gene *Lr46* inherited from both parents. Many lines developed even stronger LTN due to *Lr34* and possibly other genetic factors inherited from NK93604. The leaf tip necrosis complicates the visual assessment of disease. Secondly, the population showed large variation in earliness, since it is a cross between winter and spring wheat although true winter wheat lines of course had been excluded.

Table 3 QTL for adult plant resistance in the Arina × NK93604 population, based on field trials at Vollebekk, Norway. Plant height † and heading were included as covariates in the multiple regression model. QTL are listed if LOD value is > 2.0 for at least one year or mean over years. Interval mapping in MapQTL 6.

		corrected SNB severity ‡					
Chr.	Close marker	2010	2011	2012	2013	Mean	Source
1AL	*barc17*	5.0	6.8	2.0		4.0	Arina
1DS	*wmc147*	3.4	4.5	4.0	2.0	5.3	Arina
2BS	*wPt-7995*			6.6		2.8	Arina
4B	*wmc349*	5.6	5.8	2.5		3.1	NK
4DL	*gwm624a*	4.1		5.4		5.4	NK
6AS	*P7M62-214*	3.4			3.6	2.3	Arina
6AL	*cfa2114*	6.2	5.8	2.5		3.0	Arina
6BL	*gwm58*		3.5	5.0	3.9	3.4	Arina
6DL	*gwm325*			6.4			Arina
7DS	*gwm473*	3.6	4.8	4.3	3.2	4.6	NK

†) mean 2011-13

‡) percent phenotypic variation explained. Underscored when LOD ≥ 3.0.

Table 4 QTL for seedling resistance after inoculation and infiltration with NOR 4 and Sn4 (MQM mapping), and inoculation with isolate Voll73_3 (V73_inoc). Infiltration with Voll73_3 showed no sensitive reactions.

		SNB disease reaction †					
Chr.	Marker	V73_inoc	Nor 4 inoc	Nor 4 inf.	Sn4 inoc	Sn4 inf.	Source
1B	*P5M47-178*		9.6	13.2	6.7	2.9	Arina
7B	*wpt-1553*	19.4	15.4	20.6	27.2	24.9	Arina

\†) percent phenotypic variation explained. Underscored when LOD ≥ 3.0.

In individual years, only a single QTL for SNB resistance was found with LOD > 3 (Table 3), and two for the mean of years at the adult plant stage. At the seedling stage, only two significant QTL (LOD > 3) were detected (Table 4), and they did not correspond to adult plant QTL. While we cannot rule out that QTL were missed due to low marker density (662 markers), these results also illustrate some of the challenges associated with evaluation of SNB.

2.4. Discussion

2.4.1. Did NE-*Snn* interactions influence SNB susceptibility at the adult plant stage?

In **Paper I** we showed that the *Snn3* locus explained up to 24 % of the phenotypic variation in SNB susceptibility in the field and 51 % in seedling experiments in SHA3/CBRD × Naxos (Ruud et al. 2017). The effect varied between environments (years), and was highly significant in 2010 and 2011. The sensitivity was inherited from Naxos. This was the first validation of the importance of this sensitivity locus under field conditions. The population was insensitive to SnToxA and SnTox1 which otherwise could have masked the effect of the SnTox3-*Snn3* interaction.

On the other hand, we could not find a significant effect of SnTox3 sensitivity on SNB susceptibility at the adult plant stage in the Nordic spring wheat collection (**Paper II** and **III**). Screenings with purified SnTox3 revealed that sensitivity to SnTox3 was common in this collection, with 55 % sensitive lines (**Paper II**), and PCR indicated that the frequency of *SnTox3* in Norwegian *P. nodorum* isolates (N = 62) was also high (76 %) (**Paper III**). Many lines (45 %) were sensitive to SnToxA, and the majority (69 %) of the Norwegian *P. nodorum* isolates investigated harbored the *SnToxA* gene (**Paper III**). The SnToxA-*Tsn1* interaction masks the SnTox3-*Snn3* interaction at the seedling stage (Friesen et al. 2008c; Friesen et al. 2008b). If this is true also at the adult plant stage, the SnToxA-*Tsn1* interaction may have contributed to the lack of significance of SnTox3-sensitivity in the Nordic collection. Although sensitivity to SnTox3 was not important under field conditions in the Nordic collection, the interaction was significant at the seedling stage after inoculation with SnTox3-producing isolates that lacked SnToxA (**Paper III**).

The frequency of *SnToxA* was much higher in the Norwegian *P. nodorum* isolates than in Swiss isolates; 69 % versus 10 % (McDonald et al. 2013). This may be an adaptation to the corresponding high frequency of SnToxA sensitive Norwegian wheat. In **Paper II**, we found that SnToxA sensitive lines in the Nordic spring wheat collection had significantly higher corrected SNB severity mean than insensitive lines. This trend was significant in all individual seasons, although the contribution varied from year to year. The most resistant SnToxA-sensitive lines were also clearly more susceptible than the most resistant SnToxA-insensitive lines. Markers associated with *Tsn1* were the most significant markers on 5B based on corrected SNB severities from the field trials in some years (**Paper III**), but below the significance threshold set for the GWAS analysis.

While SnToxA and SnTox3 sensitivity were common in the Nordic spring wheat collection SnTox1 sensitivity was only found in 12 % of the lines (**Paper II**). The SnTox1 sensitivity locus *Snn1* seems to be rare in hexaploid wheat, as was previously reported by Shi et al. (2016a). Due to the low frequency of SnTox1-sensitive lines, and perhaps insufficient linkage of the locus to associated markers, the potential effect of SnTox1 sensitivity could not be detected in the GWAS mapping.

One of the hopes for this PhD project was that it would be possible to explain most variation in SNB resistance in the field with NE-*Snn* interactions. If so, selection of resistant cultivars could be done at the seedling stage after infiltrations with purified NEs or CF from suitable isolates. However, CF infiltration assays were not very useful to predict SNB susceptibility in the field. One exception was the major interaction SnTox3-*Snn3* in SHA3/CBRD × Naxos at both seedling and adult plant stage, since SnTox3 was reliably produced by the isolates in liquid culture (**Paper I**). It is already established that *P. nodorum* does not necessarily produce SnToxA in liquid culture (Shi et al. 2015), although the interaction is significant upon inoculation with the same isolate. We also observed this after CF infiltration with the isolate NOR4, where the SnToxA-*Tsn1* interaction was significant after inoculation of sensitive lines, but absent after infiltration. This may also be the case for other NEs. Screening for and validation of new NE-*Snn* interactions seem to require both a suitable mapping population and efficient knock out of other effectors. By now, targeted knock out is only possible of *SnToxA*, *SnTox1* and *SnTox3* where the genetic sequence is known. In the future, effective knock out of other NEs may be an option. Perhaps optimization of the liquid media also can induce secretion of other NEs. The importance of using suitable mapping populations and relevant isolates, can be illustrated by the lack of significant correlation between seedling and adult plant resistance in Arina × NK93604, presented above.

2.4.2. What about NE-*Snn* interactions at the seedling stage?

The role of NE-*Snn* interactions has mainly been investigated at the seedling stage. At this stage, NEs can be major determinants of susceptibility after inoculation. The SnTox3-*Snn3* interaction was always detected after infiltration with CF from *SnTox3*-harboring isolates (**Paper I** and **III**). Apart from this, only a few QTL were significant at the seedling stage after both inoculation and infiltration with CF from single isolates. In the Nordic collection, (**Paper III**), two QTL on 3A and 6B were significant after both inoculation and infiltration. The QTL on 3A may represent a novel NE-*Snn* interaction specific for isolate 201593. The markers on 6B were significant after inoculation with two isolates and infiltration with a third isolate,

highlighting the relative influence of other effectors and mechanisms present in the individual isolates. To further investigate the QTL on 3A and 6B, genotypes with single sensitivity to each of the interactions could be crossed with insensitive lines to develop mapping populations.

In the Nordic spring wheat collection the SnToxA-*Tsn1* interaction was highly significant after inoculation with isolate NOR4, and SnTox3-*Snn3* was most significant after inoculation with isolate 201593 (**Paper III**). Although infiltration with purified SnToxA and SnTox3 will identify directly the sensitive genotypes, inoculation of seedlings with isolates that produce SnToxA and SnTox3 will also identify *Tsn1* and *Snn3* as major susceptibility loci.

Two reaction types to SnTox3 sensitivity could be observed after infiltration of the plants in the Nordic collection with purified SnTox3 (**Paper II**). Different reaction types have also been reported after infiltration with SnTox3 in other studies (Waters et al. 2011; Shi et al. 2016b). Some lines responded with chlorosis without tissue collapse (Type 2 reaction). In other genotypes, complete necrosis with tissue collapse was induced (Type 3 reaction). In the GWAS performed on the subset of the Nordic collection (**Paper III**), the Type 2 reaction seemed to map to a locus on chromosome 5A, but was also linked to *cfd20* and *gwm234*. The SSR markers have previously been reported as linked to *Snn3* and are most likely located on 5BS (Friesen et al. 2008a). The markers associated with SnTox3-sensitivity after seedling inoculation and with the strongest reaction type (Type 3) after infiltration, corresponded to the markers identified in the SHA3/CBRD × Naxos population (**Paper I**). The Type 3 reaction was not significantly linked to the SSR markers *cfd20* and *gwm234*.

2.4.3. How can we use the results to select resistant genotypes?

To be relevant for breeding, the genetic resistance must act at the adult plant stage in the field. However, SNB evaluation in the field is resource demanding, complicated by G × E interactions and by confounding traits like plant height and earliness. Thus, time and money could be saved if seedling experiments could replace the field trials. We compared the results of the seedling and field experiments to investigate the extent of shared components of resistance.

The correlation between seedling disease reaction types after inoculation and corrected SNB severity at the adult plant stage in the field, could be highly significant with Pearson's correlation coefficients up to 0.5 and 0.6 (Table 4 in **Paper III**, Table 4 in **Paper I**). However, strong correlation required that representative isolates were used for the seedling inoculations, i.e. isolates that reproduced mechanisms important both at the seedling and adult plant stage.

Inoculation with isolate 201618 which did not carry SnToxA, SnToxA1 or SnToxA3, did not have significant correlation with SNB severity in the field.

Both SnToxA and SnToxA3 were significant virulence factors at the seedling stage if the corresponding sensitivity locus was present. Screening for sensitivity to SnToxA and SnToxA3 with purified NEs is recommended, based on the effects of these NE-*Snn* interactions on disease severity in the field (**Paper I** for SnToxA3, **II** and **III** for SnToxA). However, the correlation between corrected SNB severity from field trials, and disease reactions from seedling inoculations, was usually higher than the correlation between corrected SNB severity and sensitivity reactions from infiltration with purified SnToxA or SnToxA3 (Table 4 in **Paper I**, Table 4 in **Paper III**). These results indicate that inoculation capture a larger fraction of the resistance components relevant also under field conditions, than the single sensitivities against each effector.

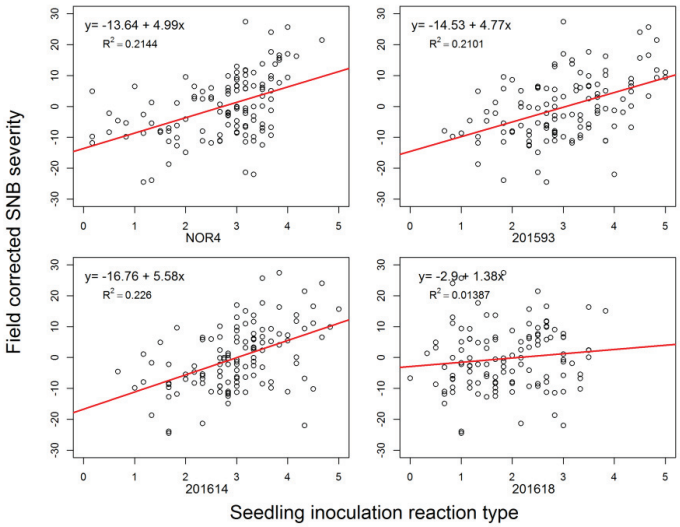

Figure 7 Correlation between seedling inoculation disease reaction type and the mean corrected SNB severity from seven years of field trials in the Nordic spring wheat collection (**Paper II** and **III**).

The scatter plots in Figure 7 show the correlation between seedling inoculation disease reaction types and the mean corrected SNB severity based on seven years of field trials. Although the correlation coefficients were significant, we can see that only the few most susceptible lines

based on seedling inoculation with isolates NOR4, 201593 and 201614 correspond to the most susceptible lines at the adult plant stage. Only these lines could be confidently discarded based on seedling screenings alone. The most resistant lines based on seedling inoculations were not the most resistant in the field, but usually better than the average. The information gained from seedling inoculations alone, without genetic analysis, is probably not sufficient for breeders to evaluate their breeding material.

Some QTL for SNB resistance were significant at both the seedling and adult plant stage (**Paper I** and **III**). In particular, the *Snn3* locus in SHA3/CBRD × Naxos and the QTL on 4B and 7A identified in the GWAS panel were robust. The QTL on 7A also corresponded to a QTL for adult plant SNB resistance in SHA3/CBRD Naxos (**Paper I**). Based on the analyses in **Paper II** and the effect of *Tsn1*-linked markers in **Paper III**, we also conclude that the *Tsn1* locus has an effect across developmental stages.

2.4.4. Advantages and limitations of QTL mapping and GWAS

Genetic analysis could identify the components of resistance that were shared between the seedling and adult plant stage. The QTL associated to *Snn3* in the biparental population (**Paper I**) validated the significance of an NE-*Snn* interaction under field conditions, and in the GWAS panel three QTL were identified at both the adult plant and seedling stage. QTL detected in seedling screenings tended to be more accurate than the QTL based on field data, and provided a better source for marker selection. The LOD/-log10(*p*) scores of the QTL based on field data were lower than seedling QTL. Here, the seedling results were useful in several ways:

1) when the same markers were detected both at the seedling and adult plant stage, we could conclude that a QTL was present, even though we initially had doubts regarding the field QTL due to overall lower LOD/-log10(*p*) values
2) The thresholds for QTL detected only in the field could be adjusted based on the thresholds for shared QTL
3) QTL that were shared were also significant in more than one year in the field, which indicate they are robust

As discussed in 1.5.3. GWAS has the disadvantage that the effects of rare alleles are missed. One example from the GWAS analysis in the Nordic collection is the effect of the semi-dwarfing gene *Rht-D1* located on 4D (Sourdille et al. 1998). 121 lines from the Nordic collection were genotyped with a diagnostic KASP marker for this gene. The minor allele frequency (MAF) was 0.19 (23 lines carried the allele contributing to reduced height). When

GWAS was performed for plant height, no significant association was found to *Rht-D1*. Although the effect of *Rht-D1* on plant height is reported to be stronger than the effect of the semi-dwarfing gene *Rht-B1* on 4B (Börner et al. 1993; Sourdille et al. 1998), only *Rht-B1* could be identified in GWAS (data not shown). The minor and major allele frequencies for this gene were more balanced (0.37 and 0.58, respectively). It is likely that other associations also went undetected due to this lack of statistical power, including associations to SNB susceptibility.

2.5. Conclusions

This study provides insight in the potential of improved SNB resistance breeding in Norwegian spring wheat. We could show that sensitivity to the two major NEs SnToxA and SnTox3 can contribute significantly to increased disease severities also at the adult plant stage in the field. Sensitivities to SnToxA and SnTox3 are common in the Nordic breeding material, and the effector genes seem prevalent in the Norwegian *P. nodorum* pathogen population. Based on these results elimination of these loci in the breeding programs is recommended. The effect and detection of the *Snn3* locus depend on presence of other sensitivity loci in the mapping population, or the presence of other NEs in the pathogen. The modifying or epistatic effects of some NE-*Snn* interactions on SnTox3-*Snn3* were known from seedling experiments, but may also be important at the adult plant stage in the field. The effect of other NE-*Snn* interactions at the adult plant stage could not be validated in this study. Infiltration with CF from single isolates grown in liquid cultures is hypothesized to predict the presence of NEs that also play a role in disease development after inoculation. But with few exceptions, the infiltrations based on CF were not reliable predictors of SNB susceptibility even at the seedling stage. Unless the methodology can be improved so other NEs can be cloned or otherwise reliably produced in liquid culture, this method is not recommended for resistance screening purposes, and should always be validated with inoculation. The genetic analysis revealed that several QTL for SNB resistance were significant at both the seedling and adult plant stage. Some of these QTL were stable across multiple years in the field, which makes them good candidates for MAS. The QTL detected at the seedling stage were often more accurate and with higher significance values than the corresponding QTL identified in the field. Therefore, the seedling inoculation experiments can be useful for selecting which markers to use in MAS. The field screenings identified additional, robust QTL that would have been missed if only seedling screenings were considered. These QTL are also good candidates for MAS, and reasons for why field screenings for adult plant resistance to SNB are essential.

Future work would include validation of significant markers in different wheat germplasm, since markers detected in one population are sometimes not polymorphic or associated with the trait in other populations. The background for the two reaction types for SnTox3 sensitivity observed in the Nordic spring wheat collection, should also be further investigated. One way to do this is to develop mapping populations segregating for the two different reaction types. Sequencing of the *Snn3*-region in genotypes with different reaction types could also suggest an answer to this puzzle. In order to characterize the potential new NE-*Snn* interactions on 3A and 6B, mapping populations segregating for single sensitivities to these QTL should also be developed. Additionally, it would be interesting to investigate the genetic mechanisms underlying QTL that did not seem associated with NE-*Snn* interactions. The genetic regions of interest can be narrowed down through fine-mapping. The putative function of candidate genes in the region can be identified by exploring the newly released, improved version of the wheat genome (Clavijo et al. 2017), and validated through knockout experiments. Comparison of the potential of MAS based on our results, and accuracy of genomic prediction and selection methods for the same traits, could also be relevant both for breeders and crop scientists.

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Paper I

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ORIGINAL ARTICLE

Mapping of SnTox3–*Snn3* as a major determinant of field susceptibility to Septoria nodorum leaf blotch in the SHA3/CBRD × Naxos population

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Abstract

Key message **The effect of the SnTox3–*Snn3* interaction was documented for the first time under natural infection at the adult plant stage in the field. Co-segregating SNP markers were identified.**

Abstract *Parastagonospora nodorum* is a necrotrophic pathogen of wheat, causing Septoria nodorum blotch (SNB) affecting both the leaf and glume. *P. nodorum* is the major leaf blotch pathogen on spring wheat in Norway. Resistance to the disease is quantitative, but several host-specific interactions between necrotrophic effectors (NEs) and host sensitivity (*Snn*) genes have been identified, playing a major role at the seedling stage. However, the effect of these interactions in the field under natural infection has not been investigated. In the present study, we saturated the genetic map of the recombinant inbred (RI) population SHA3/CBRD × Naxos using the Illumina 90 K SNP chip. The population had previously been evaluated for segregation of SNB susceptibility in field trials. Here, we infiltrated the population with the purified NEs SnToxA, SnTox1 and SnTox3, and mapped the *Snn3* locus on 5BS based on sensitivity segregation and SNP marker data. We also conducted inoculation and culture filtrate (CF) infiltration experiments on the population with four selected *P. nodorum* isolates from Norway and North America. Remapping of quantitative trait loci (QTL) for field resistance showed that the SnTox3–*Snn3* interaction could explain 24% of the phenotypic variation in the field, and more than 51% of the variation in seedling inoculations. To our knowledge, this is the first time the effect of this interaction has been documented at the adult plant stage under natural infection in the field.

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Introduction

Parastagonospora (syn. *Septoria*, syn. ana *Stagonospora*) *nodorum* (Berk.) (Quaedvlieg et al. 2013) [teleomorph:*Phaeosphaeria* (syn. *Leptosphaeria*) *nodorum* (Müll), Hedjar.] is the causal agent of Septoria nodorum leaf and glume blotch (SNB), a disease that can cause yield losses of up to 31% (Bhathal et al. 2003). The main hosts of *P. nodorum* are bread wheat (*Triticum aestivum*), durum wheat (*T. durum*) and triticale but also other cereals and a range of wild grasses. The pathogen is common in major geographical regions where wheat is grown, including the USA, Australia and Europe (Solomon et al. 2006; Francki 2013), particularly in rainy climates, and is the major leaf blotch pathogen in Norwegian spring wheat.

QTL for flag leaf resistance have consistently been detected on chromosomes 1A, 1B, 2A, 2D, 3AS, 3B, 5A, 5B, 7A and 7B (Aguilar et al. 2005; Shankar et al. 2008; Friesen et al. 2009; Francki et al. 2011; Lu and Lillemo

2014). Most of the QTL explain less than 20% of the phenotypic variation, as reviewed by Francki (2013).

Lately, it has been shown that host-specific interactions play an important role in this pathosystem, at least at the seedling stage (Oliver and Solomon 2010). The necrotroph and the host interact in an inverse gene-for-gene manner based on necrotrophic effectors (NEs) and corresponding sensitivity loci (*Snn*) in the host (Friesen and Faris 2012). The effect of each SnTox-*Snn*-interaction is incomplete and usually additive in nature (Friesen and Faris 2010). However, epistatic interactions are also involved, affecting toxin expression, host gene action and cross talk between pathways (Friesen et al. 2008b). At least eight NE (SnToxA, SnTox1, SnTox2, SnTox3, SnTox4, SnTox5, SnTox6 and SnTox7) and nine corresponding *Snn* genes (*Tsn1, Snn1, Snn2, Snn3-5B, Snn3-5D, Snn4, Snn5, Snn6 and Snn7*) have been characterized (Friesen et al. 2006, 2007; Liu et al. 2006, 2009; Abeysekara et al. 2009; Gao et al. 2015; Shi et al. 2015). *SnToxA, SnTox1* and *SnTox3* have been cloned into *Pichia pastoris*, and the purified effectors are being used for seedling screenings (Friesen et al. 2006; Liu et al. 2009, 2012). In Australia, screenings with NEs have been implemented in wheat breeding programs (Tan et al. 2014). Two of the sensitivity genes have been cloned. *Tsn1* encodes a protein with N-terminal nucleotide binding site, leucine-rich repeats (NBS-LRR) and a C-terminal serine/threonine protein kinase (S/TPK) (Faris et al. 2010)—representing a minor class of the classical NBS-LRR resistance genes typically conferring race-specific resistance to biotrophs. The recent positional cloning of *Snn1* identified a wall-associated kinase class of receptor, which is also associated with resistance to biotrophic pathogens (Shi et al. 2016b), supporting the hypothesis that the necrotrophic pathogens hijack biotrophic resistance pathways.

SnTox3–*Snn3* was the fourth NE–*Snn* interaction to be identified (Friesen et al. 2008a) and *SnTox3* the second necrotrophic effector from *P. nodorum* to be cloned (Liu et al. 2009). The gene encodes for a 693 bp small secreted protein with no known homology to other proteins (Liu et al. 2009), and at least 11 haplotypes are known (McDonald et al. 2013). The SnTox3–*Snn3* interaction was first described by Friesen et al. (2008b), and the sensitivity locus mapped to the distal end of 5BS, with *cfd20* as the closest marker, but almost 30 cM from the next linked markers. In the BR34×Grandin population the interaction explained up to 17% of the phenotypic variation in disease after inoculation at the seedling stage. Recently, a saturated map covering the *Snn3-B1* region was also published, delineating the gene to a 1.5 cM interval (Shi et al. 2016a). At least two NB-LRR-like genes were linked to markers (*fcp652* and *fcp665, fcp666*) within this interval.

The SnTox3–*Snn3* interaction has been reported to be significant only in the presence of incompatible SnTox2–*Snn2* interaction, the SnToxA-*Tsn1* interaction is epistatic to SnTox3–*Snn3* (Friesen et al. 2008b; Cockram et al. 2015) and SnTox1 can suppress the expression of SnTox3 (Phan et al. 2016). A low, but significant negative correlation between sensitivity to SnTox3 and lower disease resistance ratings in Australian wheat cultivars has been reported (Waters et al. 2011; Francki 2013), indicating, but not confirming, that the interaction probably is significant in disease development also in the field.

Leaf infiltrations with single effectors have uncovered gene-for-gene-interactions, but the interactions are not always additive and the relative importance of each effector in a mixed natural pathogen population might change over time. Thus, it is necessary to investigate the relationships further. One study showed the significant effect of the SnToxA–*Tsn1* and SnTox2–*Snn2* interactions on adult plants in the field after inoculation with a single isolate (Friesen et al. 2009). An experimental design with naturally infected plants better explains the relationship between the natural pathogen population and the host. However, such a study is more complex and one can run the risk of not finding consistent effects across years due to fluctuations in the pathogen populations.

The damaging effect of SNB is largest in moist periods when the pathogen infects the flag and sub-ultimate leaf during grain filling (Francki 2013) and the milk stage in particular (Bhathal et al. 2003). Evaluation and genetic analysis of adult plants under field conditions are therefore of great importance, but also challenging. Considerable genotype×environment (G×E) interaction is expected, and many QTL have been detected in only one environment. To be relevant for breeders, the QTL should be consistent in several environments (Francki 2013).

Breeders usually rely on natural infection in the field for evaluation of leaf blotch resistance (Cowger and Murphy 2007). Fraser et al. (2003) suggested that promotion of infection by natural inoculum, by overhead irrigation and/or inoculation with naturally infected straw gives a better estimate of host resistance under natural epidemics than inoculation of the nurseries with selected isolates.

The recombinant inbred line (RIL) population SHA3/CBRD×Naxos was previously analyzed for leaf blotch susceptibility (Lu and Lillemo 2014). Screenings with the cloned effectors showed that it most likely segregated for *Snn3*, but the sensitivity locus did not map to any linkage group, the population was monomorphic to linked markers *cfd20* and *gwm234*, and the effect of the interaction in the field could not be verified. To improve the map resolution, SHA3/CBRD×Naxos was genotyped with the Illumina iSelect 90 K wheat SNP Chip (Wang et al. 2014) and QTL mapping was performed again on the field data. The population was also inoculated and infiltrated at the seedling stage with four *P. nodorum* isolates with different

effector profiles (Table 1). This mapping revealed that the SnTox3–*Snn3* interaction indeed could explain a major proportion of the variation in resistance between genotypes. To our knowledge, this is the first time the effect of SnTox3 has been mapped under natural infection in the field.

The objectives of this study were to (1) perform new and more precise QTL mapping of the field data with high-density SNP marker maps and (2) investigate to what degree these field QTL can be explained by seedling reactions to single isolates and infiltration with purified effectors.

Materials and methods

Plant material and foregoing field study

The development and field evaluation of Shanghai3/Catbird (SHA3/CBRD) × Naxos are described by Lu and Lillemo (2014). Briefly, it is an F_6-derived RIL population that segregates for SNB resistance in the field. The CIMMYT line SHA3/CBRD is highly resistant while the German spring wheat parent Naxos is susceptible. The main conclusion from Lu and Lillemo (2014) was that the field resistance was based on many minor effect genes. Although the population segregated for SnTox3 sensitivity, the position or any clear effect of the interaction in the field could not be mapped or verified in the study, which used a set of 564 SSR and DArT markers.

Linkage mapping

A total of 166 individuals from the SHA3/CBRD × Naxos RIL population were genotyped with the Illumina iSelect 90 K wheat SNP Chip (Wang et al. 2014). Analyzing and scoring of the genotype results were performed manually for every SNP marker with the software Genome Studio Genotyping Module v1.0 from Illumina.

Markers scored as polymorphic were used for constructing linkage groups and genetic linkage maps. The markers were sorted in linkage groups with MSTmap (Wu et al. 2008). The linkage groups were assigned to chromosomes based on the best BLASTn hit from a comparison of SNP-flanking sequences with the Chinese Spring chromosome survey sequences (http://wheat-urgi.versailles.inra.fr/Seq-Repository). Previously developed SSR and DArT marker data in the population (Lu et al. 2012) were added to the SNP marker data.

Markers belonging to linkage groups assigned to the same chromosomes based on the BLASTn search were loaded into Join Map v. 4.0 (Van Ooijen 2006), and the linkage groups were refined using the maximum likelihood mapping algorithm. The genetic distances between markers were calculated by converting recombination fractions into map distances (cM) based on the Kosambi mapping function with minimum LOD score of 3.0 (Kosambi 1943).

QTL analysis

QTL analysis was performed using the software MapQTL6 (van Ooijen 2011). Multiple QTL mapping (MQM) was used, based on cofactors for major QTL initially detected with interval mapping (IM). The LOD significance threshold was set to 3.0. The software MapChart, v.2.2 was used to draw the genetic maps and LOD curves. For analysis of field resistance, the confounding traits plant height, heading date and maturity were used as covariates to disease score in MapQTL6 as described by Lu and Lillemo (2014).

P. nodorum isolates: DNA extraction and screening for *SnTox* genes

Four isolates of *P. nodorum* were selected for the study (Table 1). Sn4 is a North American isolate known to produce SnToxA, SnTox1, SnTox2 and SnTox3, as described by Faris et al. (2011) and Crook et al. (2012). NOR4 was collected in Romerike, Akershus, Norway in 2011, from the spring wheat variety Zebra. Isolate 201593 was collected from the leaf blotch field trials at Vollebekk, Ås, Norway in 2014 from the Norwegian spring wheat cultivar Demonstrant (sensitive to SnTox3). Isolate 201618 was collected in Øsaker, Østfold in 2012 from the cultivar Quarna. The three Norwegian isolates were collected from leaves with visible leaf blotch symptoms, and grown on V8-PDA in 24h light (white + near ultraviolet (NUV)) to enhance sporulation before mycelial plugs were harvested with a cork borer and dried before storage at −80 °C. For

Table 1 List of isolates included in the study, with SnTox-profile (presence/absence based on PCR) and disease range and mean in the RIL population

Isolate	Presence (+) or absence (−) of SnToxA, SnTox1 and SnTox3, respectively	Disease range in the RILs	Population mean reaction
Sn4	+++	0.17–3.83	2.23
NOR4	+++	0.00–4.00	2.13
201593	−−+	0.00–5.00	3.37
201618	−−−	0.00–4.80	2.7

DNA extraction, the isolates NOR4, 201593 and 201618 were grown in the dark on PDA for 1–2 weeks and DNA extracted from the mycelium with the DNEasy plant kit (Qiagen). PCR screenings for *SnTox* genes and actin were performed as described in Gao et al. (2015).

Inoculum preparation and seedling inoculation

Dried plugs of the *P. nodorum* isolates were plated on V8-PDA agar and grown for approximately one week in incubation chambers with constant light (white fluorescent + NUV) and temperature around 21 °C until sporulation. The plates were flooded with distilled water and scraped with a sterilized inoculation loop to release pycnidiospores, and the final concentration of spores was adjusted to 1×10^6 spores/ml. One drop of Tween 20 (polyoxy-ethylene-20-sorbitan monolaureate) was added per 50 ml inoculum to reduce surface tension.

Seeds of the mapping population were planted in plastic cone-tainers (Stuewe and sons, Tangent, Orlando, USA), with potting mixture (peat soil with clay and sand, Gartnerjord, Tjerbo, Norway), and grown in the greenhouse under 18 °C day/15 °C night temperature and 16 h light cycle until the second leaf was fully expanded—approximately 14 days after planting. Three seeds were planted per cone. The susceptible cultivar Brakar was used as a border to reduce edge effect.

The 14-day-old plants were spray inoculated with a paint sprayer until runoff, placed in a mist chamber with 100% RH for 24 h in constant light before they were returned to the greenhouse. Seven days after inoculation, the second leaf of each plant in the accessions was evaluated for disease reactions on a scale from 0 to 5 (Liu et al. 2004), where 0 is highly resistant and 5 is highly susceptible.

Infiltrations

Two seeds per RIL were planted in individual cones in racks fitting 98 cones and grown in the greenhouse under similar conditions as for the inoculation experiments. The experiments were repeated three times.

Liquid cultures of the isolates were produced in Fries 3 medium as described in Friesen and Faris (2012). After three weeks in stationary phase, the cultures were filter sterilized and infiltrated into the fully expanded second leaf of 12–14-day-old seedlings, using a 1-mL needleless syringe. The infiltrated areas were marked with a non-toxic felt marker. After five days, the reactions were scored according to a 0–3 scale (Friesen and Faris 2012). These experiments were repeated three times with two infiltrated plants per genotype in each replicate.

Infiltration with purified SnToxA, SnTox1 and SnTox3

With partly purified SnToxA, SnTox1 and SnTox3, 12–14-day-old lines of the population were infiltrated. Approximately 25 µL of the partly purified NE was infiltrated into the fully expanded secondary leaf using a needleless syringe. The infiltrations were done in Fargo, North Dakota in 2013 with effectors produced by *Pichia pastoris* using the pGAPzA expression vector (Liu et al. 2009), and repeated in Ås, Norway with effectors provided by Dr. Richard Oliver. SnToxA from Dr. Oliver was expressed in *Escherichia coli* BL21E using the pET21a expression vector (Tan et al. 2012), while SnTox1 and SnTox3 were produced as above. All protein preparations containing the expressed effectors were desalted (Waters et al. 2011) prior to infiltration (Liu et al. 2009). The plants were evaluated after 3 to 5 days and scored on a 0–3 scale (Friesen and Faris 2012).

Gene annotations

The contextual sequences of the SNP markers with the closest linkage to *Snn3* were downloaded from https://triticeaetoolbox.org/ and BLASTED at http://plants.ensembl.org/Multi/Tools/Blast and https://urgi.versailles.inra.fr/Tools/BLAST. Annotated genes were identified, and the sequences were aligned against rice orthologues available through the rice genome annotation project http://rice.plantbiology.msu.edu/ in order to compare the results with previously reported genes in Shi et al. (2016a).

Results

Seedling inoculations and infiltrations

The frequency distribution histograms (Fig. 1) show that inoculation with isolate 201593 produced more severe necrosis (reaction type 5) than inoculation with the other isolates. Correlations between the SnTox3-positive isolates were highly significant after inoculation (Pearson's correlations 0.623–0.785, $P < 0.0001$, Table 2), while correlations between the SnTox3-negative isolate 201618 and the others were lower, but still significant. Also, the correlation between seedling inoculations and sensitivity data based on purified SnTox3 infiltration was high except for the SnTox3-negative isolate, as expected (Table 2).

Correlation between infiltration experiments with different isolates indicated that SnTox3 was the single effector produced in liquid culture by SnTox3-positive isolates causing sensitivity in the SHA3/CBRD × Naxos population (Table 3). Based on reactions on differential lines, we assume that Sn4 and NOR4 also produced SnTox1 and

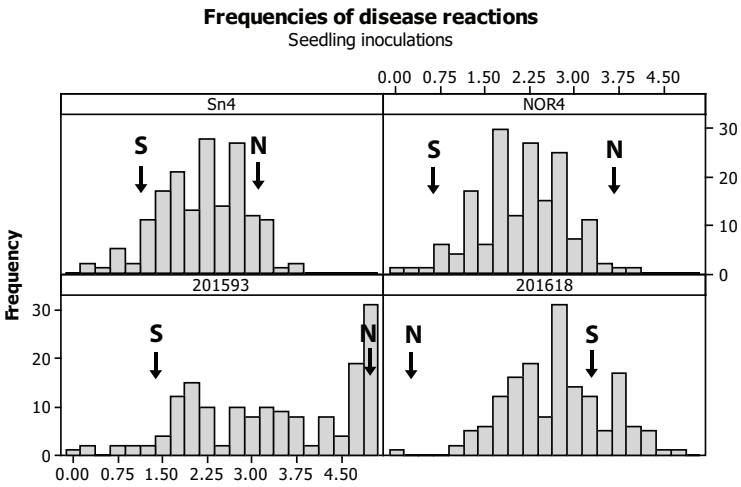


Fig. 1 Frequency distributions of disease reaction types for the SHA3/CBRD × Naxos RIL, after seedling inoculations. Parental phenotypes are indicated by *arrows*

Table 2 Pearson's correlation coefficients between single isolate inoculations at the seedling stage and correlation with reaction to purified SnTox3

	NOR4	201593	Sn4	SnTox3
201618	0.260**	0.300***	0.325***	0.062
Sn4	0.785***	0.623***		0.559***
201593	0.670***			0.741***
NOR4				0.626***

***<0.0001, **<0.001, *<0.01

Table 3 Pearson's correlation coefficients between sensitivity scores after single isolate culture filtrate (CF) infiltration and correlation between CF reactions and reactions to purified SnTox3 infiltration

	NOR4	201593	Sn4	SnTox3
201618	0.012	−0.097	−0.002	−0.07
Sn4	0.924***	0.863***		0.912***
201593	0.890 ***			0.952***
NOR4				0.935***

***<0.0001, **<0.001, *<0.01

SnTox2 and 201593 and 201618 produced SnTox2 and SnTox6 (data not shown) as well as unpublished effectors, but the population did not segregate for sensitivity to these.

Correlation between adult plant and seedling stage results

The correlation was highly significant ($P < 0.0001$) between disease reaction scores based on single isolate inoculations with SnTox3 positive isolates NOR4, Sn4 and 201593 and field disease severities in 2010 and 2011 and for the mean over years (Table 4). The correlation was lower between these isolates and field scores for 2012 and 2013. The correlation between field scores and the North American isolate Sn4 was as significant as the Norwegian isolates except for 2012. Correlation between isolate 201618 and field scores was only significant in 2010.

Table 4 Pearson's correlation coefficients between corrected leaf blotch severities in the field trials (years, 2010–2013 and mean) and disease reactions after seedling inoculations with single isolates, and infiltration with purified SnTox3

Year	Inoculation with single spore isolates				SnTox3
	NOR4	Sn4	201593	201618	
2010	0.486***	0.519***	0.615***	0.335***	0.486***
2011	0.344***	0.360***	0.291***	0.092	0.222**
2012	0.262**	0.182	0.243*	0.036	0.080
2013	0.235*	0.264**	0.334***	0.161	0.205**
mean	0.387***	0.366***	0.432***	0.154	0.262**

***<0.0001, **<0.001, *<0.01

Frequency distribution and mapping of *Snn3*

The RILs segregated for SnTox3 sensitivity as either completely sensitive (reaction type 3) or insensitive (reaction type 0), with 75 insensitive to 82 sensitive, which is not significantly different from 1:1 ($\chi^2 = 0.312$, $P = 0.576$). Eleven lines (of 168) were coded as missing, due to inconsistent reactions, to avoid misclassification of the alleles. The susceptibility was inherited from parent Naxos.

The phenotypic scores for SnTox3 sensitivity were used to infer allele variants (a and b for parent SHA3/CBRD and Naxos, respectively) and the position of the sensitivity locus mapped with linkage analysis (Fig. 2). The locus could not previously be mapped with SSR markers polymorphic in the population (Lu and Lillemo 2014). Only with the improved resolution and coverage provided by the SNP markers, the locus could be mapped as Fig. 2 shows. The population was insensitive to SnToxA and SnTox1.

QTL—seedling resistance

The major QTL at the *Snn3* locus on 5BS explained up to 51.8% of the phenotypic variation when the population was inoculated with SnTox3-positive, SnTox1-negative isolate 201593, and was also highly significant after inoculation with SnTox1-positive Sn4 and NOR4 (Table 2; Fig. 3) where suppressed expression of SnTox3 was expected according to the literature (Phan et al. 2016). The QTL on 5BS was the only significant genomic region after inoculation with isolates NOR4 and 201593 (Table 5; Fig. 3). After inoculation with Sn4, a QTL on 7B was also detected, but not after infiltration. After inoculation with 201618, QTL were detected on 1A, 1B and 2D. However, all three had only moderate or minor effects and did not correspond to the adult plant QTL on 1A and 1B (Table 7; Figure S1). Interestingly, the QTL showing significance on 7B after Sn4-inoculation corresponded to the only significant QTL after infiltration with 201618 (Tables 5, 6).

QTL—adult plant resistance

Seven significant and one putative QTL for adult plant resistance to SNB were previously reported in the population, based on the field evaluations from 2010 to 2013 (Lu and Lillemo 2014). The major QTL was found on 3BL flanked by

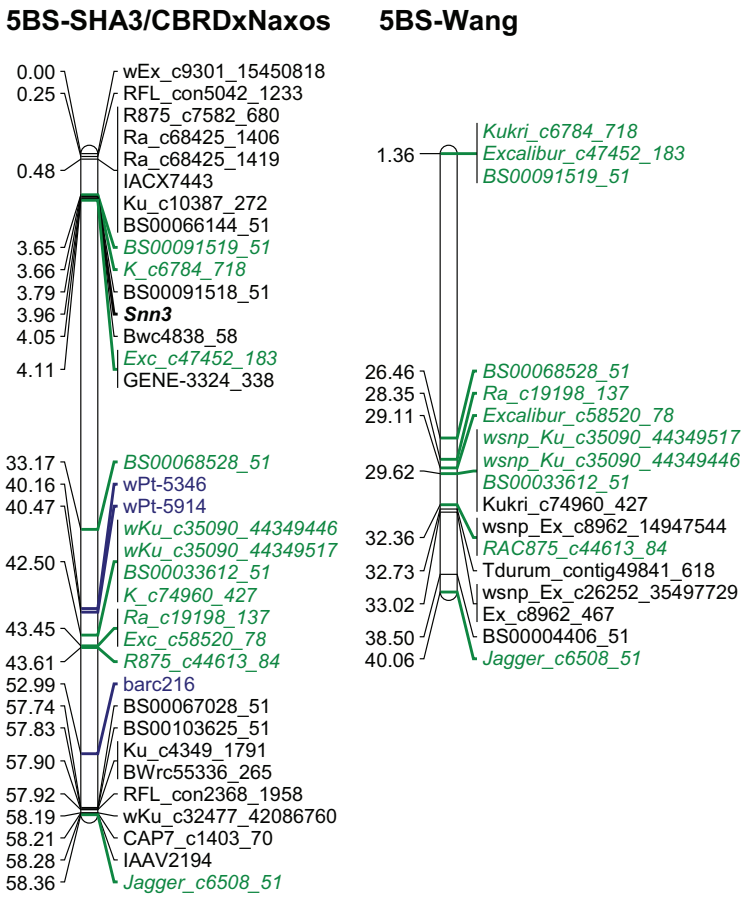


Fig. 2 *Left* Mapping of the *Snn3* locus on chromosome 5BS in SHA3/CBRD × Naxos based on segregation of SnTox3-sensitivity. *Right* region of 5BS in the Wang et al. (2014) consensus map covered by polymorphic SNPs in SHA3/CBRD × Naxos. Common markers are indicated in *italics*. The maps are drawn in Mapchart v. 2.2 (Voorrips 2002)

wpt-4933. However, improved map resolution and reanalysis of QTL captured a total of 11 significant QTL, with four being new (Table 7, Figure S1).

The QTL explaining most of the variation in any environment was located on the telomeric end of 5BS (Table 7; Fig. 4), not mapped with the initial set of SSR and DaRT markers in the study by Lu and Lillemo (2014). This QTL is located at the *Snn3* locus (Fig. 2) and explained as much as 24.0 and 9.0% of the phenotypic variation in 2010 and 2011, respectively. It was also significant across years (mean) and had an effect in 2013. However, in 2012 the *Snn3* region was not significant in QTL analysis. These results are also reflected by the correlations between infiltration with purified SnTox3 and field trials (Table 4), where the correlation is highly significant ($p < 0.0001$) between SnTox3-sensitivity for 2010 and across years, and significant at $p < 0.05$ in 2011, but not significant for 2012.

A novel QTL was detected on 1A in 2012 (Table 7). Higher map resolution and MQM mapping also revealed that 3A harbors at least two QTL (3AS.1 and 3AS.2), the most significant QTL in 2013. The 3AS.2 QTL was also significant in 2011 and across years (mean). The region covering 3AS.2 was not well covered in the SSR/DArT map.

The originally putative QTL on 3BS, important in 2013 (3BS.1) and 2013 (3BS.2), respectively, appear to be two distinct QTL although located approximately 8 cM apart. The QTL on 3BL was highly significant in 2011 and marker *wPt-4933* showed an effect in all years except 2012. In addition to the major QTL explained by *Snn3*, the QTL on 5B flanked by *wPt-5346* detected before, was also significant in 2013.

Gene annotations

Most of the SNPs co-segregating with *Snn3* could be matched to genes on scaffold TGACv1_scaffold_423631_5BS (Table 8). Although *Traes_6DL_388658304.1* was reported to be located on 6DL and *Traes_5AS_905D6F817.1:1* on 5AS, our mapping results as well as Wang et al. (2014) indicate that they are located on 5BS. Some of the genes share hallmarks of R-genes, i.e., coiled-coil (CC) (*Traes_5BS_C460CEDFB*), leucine-rich repeats (LRR) (*Traes_5BS_E0680D15E.2.path1*) and nucleotide binding sites (NBS) (*Traes_5BS_C460CEDFB, Traes_5AS_905D6F817.1:1*) domains (Table 8).

Discussion

General

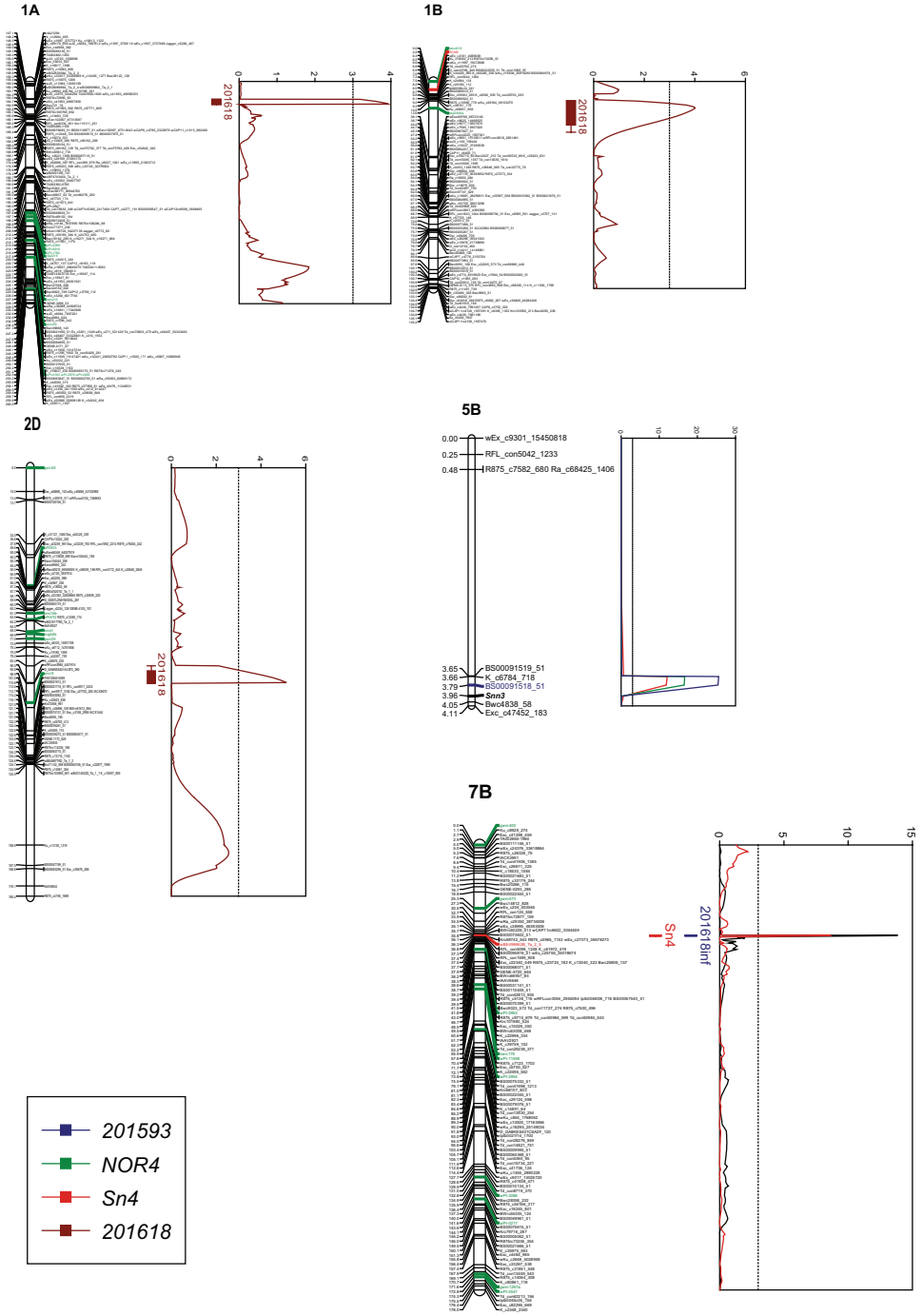
In this study, we mapped the *Snn3* locus (Fig. 2) in the SHA3/CBRD × Naxos population and identified it as a major determinant of susceptibility to SNB both under natural field infection at the adult stage and single spore isolate inoculations of seedlings (Tables 5, 6, 7; Figs. 3, 4). In the previous study by Lu and Lillemo (2014), the effect of this interaction was not identified, due to lack of segregating SSR and DART markers in the chromosome area. Although the locus has been mapped in other populations, this is, to our knowledge, the first time the effect of the SnTox3–*Snn3* interaction has been detected under natural infection in the field (Table 7; Fig. 4). We also identified SNP markers tightly linked to *Snn3*, some of which are located within putative NBS-LRR genes (Table 8).

Seedling QTL

The most significant interaction after seedling inoculation was SnTox3–*Snn3*, explaining as much as 51.8% of the phenotypic variation (Table 5) and producing strong necrosis on the leaves of susceptible lines after inoculation with SnTox3-positive isolates. Prior to screening the entire population, a selection of differential lines from SHA3/CBRD × Naxos, segregating for single field resistance QTL, were screened with several locally collected isolates to test for differential segregation (data not shown). However, very few isolates produced higher reaction scores than 2.5 on the lines unless they were also SnTox3-positive. One exception was isolate 201618 which was selected to possibly capture different QTL than the one explained by *Snn3*. QTL on 1A, 1B and 2D were detected after inoculation with 201618 (Table 5; Fig. 3). The QTL on 1A overlap partly with the QTL on 1A detected in 2012 (Table 7), but the resistance source was opposite. The QTL on 1B also seems to be specific to this particular isolate. After infiltration, a QTL on 7B corresponding to the QTL detected after inoculation with Sn4 was discovered, indicating a putative new NE/*Snn* interaction that will be investigated in further studies.

Of the three major interactions SnToxA/*Tsn1*, SnTox1/*Snn1* and SnTox3–*Snn3*, SHA3/CBRD × Naxos only segregated for *Snn3*. The limited number of genes segregating in a two-parent cross is a limitation to the range of the results, and several important interactions may not be detected due to monomorphism in the population. On the other hand, it also allows better investigation of interactions that may be statistically undetectable in the presence of other genes and epistatic interactions.

It has been suggested that presence of SnTox1 suppresses SnTox3 production (Phan et al. 2016). We found that the SnTox3–*Snn3* interaction was highly significant in all relevant inoculation experiments, and that infiltration with CF with SnTox3 positive isolates produced the same necrotic symptoms regardless of SnTox1 presence. However, the frequency of RIL with reaction type 5 was


1A
1B
2D
5B
7B
201618
201618
201618
Sn4
201618int
201593
NOR4
Sn4
201618

◂**Fig. 3** From top: QTL detected on 1A, 1B and 2D after inoculation with 201618. QTL on 5B after inoculation with NOR4, Sn4 and 201593. QTL on 7B detected after infiltration with 201618 and inoculation with Sn4. Genetic distances are shown in centimorgans to the left of the chromosomes. A threshold of 3.0 is indicated by a *dashed vertical line* in the LOD graphs. The maps are drawn in Mapchart v.2.2 (Voorrips 2002). (Color figure online)

much higher after inoculation with the SnTox1-negative isolate 201593 (Fig. 1).

Effect of *Snn3* in the field

Saturation of the genetic map with the 90 K SNP chip showed that *Snn3* can explain up to 24% of the phenotypic variation in the field (Table 7; Fig. 4: 2010). The results favor the hypothesis that host-specific interactions also play a role in adult plant susceptibility to *P. nodorum* leaf blotch. It also serves as a confirmation that the multiple regression approach where confounding traits (plant height, heading date and maturity) are included as covariates, works well. However, the SnTox3–*Snn3* interaction was only significant in two out of four years of field trials—illustrating the complexity of the disease. One definition of a robust QTL is that it is significant in two or more environments (Francki 2013). Under this definition, selection against lines carrying *Snn3* would be recommended based on our findings.

Since the field experiments depended on natural infection, the results capture a more realistic picture of the situation in farmers' fields rather than after artificial inoculation with single isolates. Nevertheless, very few QTL studies rely on natural inoculum, where one takes a higher risk of large variability between environments.

Mapping of other QTL for field resistance

The fine mapping improved the coverage of the chromosomes and led to the discovery of a significant novel QTL for field resistance on 3A (3A.2, Table 7; Figure S1). Lu and Lillemo (2014) reported that MQM or CIM mapping did not improve the results for the field resistance QTL. However, with the new maps, we found that the significance and precision increased with MQM mapping for several field QTL [1B, 3A, 3BL, 5B (Table 7; Fig. 4; Figure S1)], although different cofactors were used for different years. In 2012, the use of cofactors did not improve the results. Improved coverage of the chromosomes also revealed that some QTL are probably linked and that different underlying genes may be involved in different years, for instance the two on 3BS (Table 7; Figure S1). The novel QTL detected on 1A (Table 7) was below significance threshold when mapped on the original SSR and DArT map.

Although the effect of SnTox3–*Snn3* was highly significant in 2010 and in 2011, the variation between years shown both in correlation coefficients and relative importance of individual QTL, also emphasizes the need to screen the plants in multiple environments and/or locations as discussed by Francki (2013), before selecting genotypes or markers for marker-assisted selection (MAS). The variation illustrates the complexity of the trait and diversity of the natural pathogen population. For some QTL, the % explained variation was lower with the new maps.

Correlation field—seedling trials

A main objective of this study was to investigate the correlation between seedling and adult plant resistance to SNB. Based on the Pearson correlation coefficients between field

Table 5 Significant QTL (LOD > 3.0) for seedling resistance to SNB in inoculation experiments with single isolates, after MQM mapping

Chromosome	Markers (cofactors)	Isolate				R-source
		Sn4	NOR4	201593	201618	
1A	*RAC875_c10083_800*				**11.7**	Naxos
1B	*psp3000*				**10.4**	SHA3/CBRD
2D	*wsnp_RFL_Contig3960_4401914*				**11.1**	Naxos
5B (*Snn3*)	*BS00091518_51*	**27.5**	**35.4**	**51.8**		SHA3/CBRD
7B	*wsnp_BE498662B_Ta_2_5*	**15.5**				Naxos

% phenotypic variance (PEV) explained for significant QTL is listed

Table 6 Marker correlations after infiltration with culture filtrate from single isolates

Chromosome	Markers	Isolate				R-source
		Sn4	NOR4	201593	201618	
5BS (*Snn3*)	*BS00091518_51*	**82.7**	**87.2**	**73.4**		SHA3/CBRD
7B	*wsnp_BE498662B_Ta_2_5*				**32.6**	Naxos

The % phenotypic variance (R^2 values) is listed for the significant interactions

Table 7 List of significant QTL with close markers based on 4 years and the mean of field scorings at Vollebekk, Norway

Chr.	Markers	2010	2011	2012	2013	Mean	R-source
1A	*wsnp_Ex_ c25734_34995416*		2.4	10.3		3.0	SHA3/CBRD
1B.1RS	*SCM9*		5.2		**8.1**	**7.7**	Naxos
3AS.1	*gwm2 IAAV6676*	6.5			**11.5**	3.7	Naxos
3AS.2	*Ku_c41007_116 Excalibur_ c52446_519*		6.6		**9.4**	2.2	SHA3/CBRD
3BS.1	*BS00030534_51*				**5.7**		SHA3/CBRD
3BS.2	*wBE445348B_ Ta_2_1*	**6.9**					
3BL	*wPt-4933*	4.6	**11.2**		3.5	3.9	Naxos
5BS	*BS00091518_51*	**24.0**	**9.0**		4.7	**9.9**	SHA3/CBRD
5B.2	*wPt-5914*	4.8	3.4		**5.6**	2.4	SHA3/CBRD
7 A	*RAC875_ c14195_1155*	2.9	4.1	3.4	**6.5**	**6.2**	Naxos
7B	*BobWhite_rep_ c50229_413*			**8.4**		2.7	Naxos

The % explained phenotypic variation (R^2) is listed if above the LOD threshold of 3 in at least one environment. QTL detected above the LOD threshold in the corresponding environment are indicated in bold. The phenotypic data are identical to the dataset used for the analysis published by Lu and Lillemo (2014)

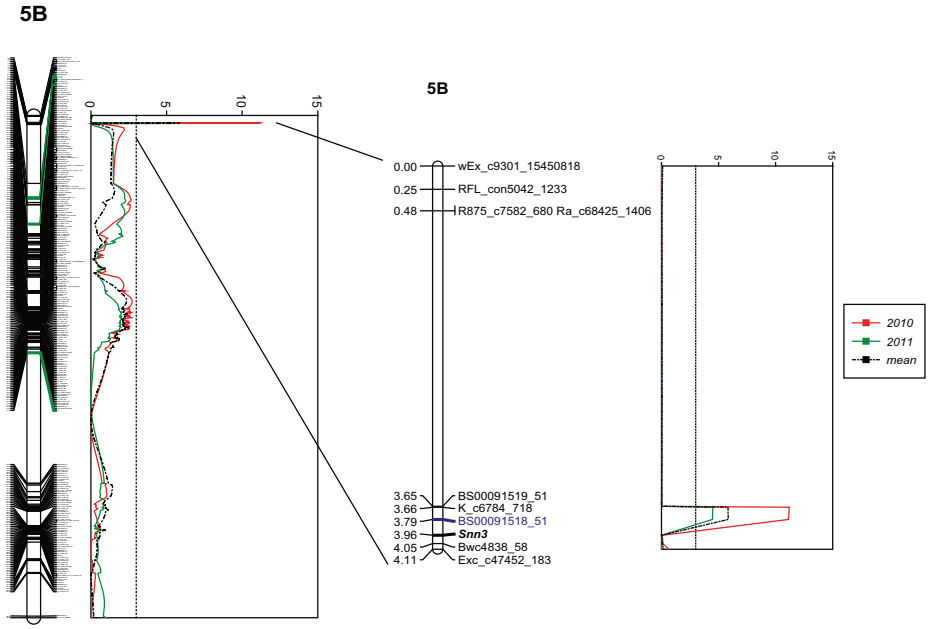


Fig. 4 Linkage group 5B with LOD curves for the major QTL for field susceptibility to SNB at the *Snn3* locus detected in the field trials at Vollebekk, Ås, Norway in 2010, 2011 and across years (mean). Genetic distances are shown in centimorgans to the left of the chromosomes. A threshold of 3.0 is indicated by a dashed vertical line in the LOD graphs. The maps are drawn in Mapchart v.2.2 (Voorrips 2002). (Color figure online)

Table 8 List of SNPs tightly linked to *Snn3* in the SHA3/CBRD × Naxos population and gene annotations based on the draft genome sequence (Mayer et al. 2014) unless otherwise noted (in hexaploid wheat within scaffold TGACv1_scaffold_423631_5BS)

SNP marker	NCBI *Triticum aestivum* gene	Rice orthologue	Function	Reference
BS00091519_51	*Traes_5BS_C460CEDFB*	*Os06g30380.1*	P-loop containing nucleoside triphosphate hydrolases superfamily protein GTP-binding domain GTPase	http://plants.ensembl.org/ (Kersey et al. 2016) http://www.uniprot.org/uniprot/Q656A4
Excalibur_c47452_183	*Traes_5BS_E0680D15E.2.path1* *TRIAE_CS42_5BS_TGACv14236631_AA1380950.1*	*Os12g44000*	Ubiquitin-conjugating enzyme 15-like Panther: Leucine-rich repeat-containing protein (PTHR23155) (Traes_5BS_E0680D15E.2.path1)	http://plants.ensembl.org/ (Kersey et al. 2016) https://urgi.versailles.inra.fr http://www.uniprot.org/ http://www.pantherdb.org/ (Mi et al. 2016)
Kukri_c6784_718	*Traes_6DL_388658304.1*	*Os05g05354*	Trypsin-like cysteine/serine peptidase domain superfamily	http://plants.ensembl.org/ (Kersey et al. 2016)
BS00091518_51	*Traes_5BS_C460CEDFB*	*Os06g30380.1*	P-loop containing nucleoside triphosphate hydrolases superfamily protein	http://plants.ensembl.org/ (Kersey et al. 2016)
BobWhite_c4838_58	100% BLAST match to *Traes_5BS_C460CEDFB*	*Os12g44000 (MSU)* *Os06g30380.1 (IRGSP)*	Coiled-coil superfamily (based on *Arabidopsis thaliana* match)	http://plants.ensembl.org/ (Kersey et al. 2016) http://rice.plantbiology.msu.edu/ http://rgp.dna.affrc.go.jp/IRGSP/
GENE-3324_338	*Traes_5AS_905D6F817.1:1*	*Os06g30380.1*	Nontranslating coding sequence (CDS) GTP-binding domain P-loop NTPase	https://urgi.versailles.inra.fr/ http://www.uniprot.org/

years and single isolates (Table 4), the correlation seems to be highest between SnTox3-producing isolates and years where *Snn3* was significant (2010, 2011 and mean). However, correlation was also significant between the SnTox3-negative isolate 201618 and the field scores in 2010, indicating that other infection mechanisms or effectors may also play a role. Interestingly, the correlation between this isolate and field resistance was negligible for all other years. Although the correlation between 201593 and 2013 was significant ($p < 0.0001$), no significant QTL were shared between the field and seedling resistance. In other words, correlation alone is a fairly rough mean to compare experiments compared to genetic analysis. Interestingly, the correlation between the North American isolate and the field trials conducted in Norway was as high as for Norwegian isolates, illustrating the global relevance of the disease and host resistance mechanisms.

Genetic mapping of *Snn3*

The markers linked to *Snn3* mapped to the telomeric end of 5BS, about 30 cM from the nearest markers in SHA3/CBRD × Naxos (Figs. 2, 4). In the consensus map (Wang et al. 2014), several markers that clustered in this distal group were not assigned to any chromosome, or mapped to different chromosomes (like *Kukri_c6784_718*, assigned to 6DL) in the different populations used to build the consensus map. The high recombination frequency in this region challenges the mapping algorithms, and we want to underline the importance of including unassigned and unmapped markers in the analysis (i.e., association mapping or linkage maps) before filtering.

We did not observe recombination between *Snn3* and the markers *BS00091518_51, BS00091519_51, BobWhite_c4838_58, Excalibur_c47452_183* or *GENE-3324_338* in the RIL lines. However, a small number of missing data points contributed to the minor distances between the markers in the map (Figs. 2, 3, 4).

Gene annotations

The SNP markers *BS00091518_51* and *BS00091519_51* are located 20 bp apart from each other in an exon of a P-loop containing nucleoside triphosphate hydrolases superfamily protein (Table 8, *Traes_5BS_C460CEDFB*, https://triticeaetoolbox.org/jbrowse). The P-loop is a common motif

in NTP-binding proteins including NBS-LRRs (Marone et al. 2013). *Excalibur_ c47452_183* is located within a gene (*Traes_5BS_E0680D15E.2.path1*) expressing a protein with leucine-rich repeats (LRR, Table 8), also a feature of the classical R-genes. The genes in which *Excalibur_ c47452_183* and *BobWhite_c4838_58* are located, corresponded to rice orthologue *Os12g44000* (http://rice.plantbiology.msu.edu/) (Table 8). This rice gene was also reported by Shi et al. (2016a). Indeed, the sequence for marker XTC266536 (Table 1) in Shi et al. (2016a) corresponded to the same gene, *TRIAE_CS42_5BS_TGACv14236631_AA1380950.1*, as *Excalibur_c47452_183* and *BobWhite_c4838_58*. Interestingly, this gene has been annotated both as an NBS-LRR (PTHR23155) and ubiquitin-conjugating enzyme.

In the case of *BobWhite_c4838_58*, the rice orthologue is identified as *Os06g30380.1* by the International Rice Sequencing Project (IRGSP) (http://rgp.dna.affrc.go.jp/IRGSP/), which corresponds to the gene in which SNPs *BS00091518_51, BS00091519_51* and possibly *GENE-3324_338* are located (Table 8). We speculate whether the orthologues in reality correspond to different motifs in the same gene, allelic or splice variants or if more than one gene belonging to the same gene family are clustered within the scaffold.

The markers *Excalibur_c47452_183, Kukri_c6784_718, BobWhite_c4838_58* and *GENE-3324_338* also co-segregate with the loose smut resistance gene *UtBW278*, conferring resistance to *Ustilago tritici* race T9 (Kassa et al. 2015). Since the *Snn* genes confer dominant susceptibility and the NE-*Snn*-interactions are described as hijacking traditional R-genes to biotrophs, it has been speculated that they may counteract with these. However, SnTox3-resistant cultivars like BR34 are also resistant to T9 (Kassa et al. 2015), while T9-susceptible lines like Sumai3 and Grandin also carry *Snn3*. Clustering of NBS-LRR genes after duplications and the following evolution through local rearrangements and gene conversions is common, as is the irregular distribution of the gene family across chromosomes (Marone et al. 2013). Screening of SnTox3-sensitivity in a wide association mapping panel of spring wheat (MASbasis) revealed that the markers are not diagnostic or that there may be more than one sensitivity locus present (data not shown). Hence, it is likely that several NBS-LRR-like genes, including *UtBW278, Traes_5BS_C460CEDFB* and *Traes_5BS_E0680D15E.2.path1* are clustered within scaffold TGACv1_scaffold_423631_5BS, and further work is needed to identify *Snn3*, potential splice variants, allelic variants and other genes within its proximity.

Author contribution statement AKR conducted seedling inoculation, culture filtrate infiltrations and validation of infiltration with purified effectors, analyzed the data from seedling experiments, refined linkage mapping of chromosome 5B in JoinMap, performed QTL mapping, reanalyzed the field data and wrote the manuscript. SW analyzed and scored the SNP genotyping results in Genome Studio and performed linkage mapping in JoinMap. TB performed linkage mapping in MSTmap and assigned linkage groups to chromosomes based on BLASTn hits. TF was responsible for seedling inoculations and infiltrations with isolate Sn4 and NOR4 and screening with purified SnToxA, SnTox1 and SnTox3. ML obtained the funding, supervised the work and edited the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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Paper II

Effects of three *Parastagonospora nodorum* necrotrophic effectors on spring wheat under Norwegian field conditions

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Author contributions

AKR Conducted field and infiltration experiments, analyzed the data, wrote the manuscript. JAD Developed and provided plant material, critically reviewed the manuscript. ML Planned the field experiments, conducted field phenotyping in 2010-2012, critically reviewed the manuscript, supervised the work and obtained the funding.

Abstract

The wheat (*Triticum aestivum* L.) disease Septoria nodorum leaf and glume blotch (SNB) is caused by the necrotrophic fungus *Parastagonospora nodorum* and causes significant yield and quality losses in several wheat growing regions. The resistance mechanisms are quantitative and progress in resistance breeding has been slow. However, gene-for-gene interactions involving necrotrophic effectors (NEs) and sensitivity genes (*Snn*) are involved, providing hope for more efficient breeding. Although the interactions are significant determinants of seedling SNB susceptibility, their role in adult plant resistance in the field is less understood. In this study, we screened a panel of Norwegian and international spring wheat lines and cultivars under natural SNB infection in a mist irrigated field nursery across seven years. We also infiltrated the lines with the purified NEs SnToxA, SnTox1 and SnTox3 and investigated the prevalence of corresponding sensitivity in the germplasm, and correlation between NE sensitivity and resistance level in the field. Sensitivity to SnToxA, SnTox1 and SnTox3 was present in 45, 12 and 55 % of the material, respectively. Sensitivity to SnToxA was associated with significantly higher disease severity in the field than insensitivity. This indicates that elimination of SnToxA sensitivity in the breeding material by effector infiltrations or marker assisted selection can be an efficient way to increase field resistance to SNB.

Introduction

The necrotrophic fungus *Parastagonospora nodorum* is the causal agent of Septoria nodorum leaf and glume blotch (SNB) in wheat, and can cause significant yield and quality losses (Bhathal et al. 2003). It is the dominating leaf blotch pathogen in Norwegian spring wheat (Ficke et al. 2011; Abrahamsen et al. 2013), and the disease pressure increases under reduced tillage and rainy growth seasons. Control of SNB relies on fungicides, but loss of fungicide sensitivity is often observed. For instance, the majority of isolates collected in Sweden between 2003-2005 carried an amino acid substitution associated with loss of sensitivity to strobilurins (Blixt et al. 2009). Growing cultivars with durable genetic resistance is a more sustainable way to control disease. However, breeding for leaf blotch resistance has been difficult due to the quantitative nature of the resistance genetics.

It has been shown that host-specific interactions play important roles in this pathosystem (Friesen et al. 2006; Oliver and Solomon 2010). The pathogen excretes small proteins, necrotrophic effectors (NEs) which interact with corresponding sensitivity loci (*Snn*) in the host in an inverse gene-for-gene manner (Friesen and Faris 2012). The sensitive plant responds to NE recognition by inducing hypersensitive response and programmed cell death (Friesen et al. 2007). This is advantageous for the necrotrophic pathogen which feeds on the dead tissue. The cloning of several sensitivity genes has shown that they often feature classic resistance gene characteristics (Lorang et al. 2007; Nagy and Bennetzen 2008; Faris et al. 2010; Shi et al. 2016a), which supports the hypothesis that the necrotrophs hijack pathways involved in resistance to biotrophs (Friesen and Faris 2010).

Eight *P. nodorum* NEs (*SnToxA*, *SnTox1*, *SnTox2*, *SnTox3*, *SnTox4, SnTox5, SnTox6* and *SnTox7*) and nine corresponding *Snn* genes (*Tsn1, Snn1*, *Snn2*, *Snn3-5B, Snn3-5D*, *Snn4*, *Snn5, Snn6 and Snn7*) have been characterized (Friesen et al. 2006; Liu et al. 2006; Friesen et al. 2007; Abeysekara et al. 2009; Liu et al. 2009; Friesen et al. 2012; Gao et al. 2015; Shi et al. 2015). Infiltration screenings with culture filtrates from single isolates imply that there are probably several more such interactions (Crook et al. 2012; Tan et al. 2014). The interactions are usually additive in nature (Friesen and Faris 2010). However, epistasis is also involved, for instance is the SnToxA-*Tsn1* interaction epistatic to SnTox3-*Snn3* (Friesen et al. 2008a). The SnTox3-*Snn3* interaction is significant only in the presence of an incompatible SnTox2-*Snn2* interaction (Friesen et al. 2008a) and SnTox3 expression can be modified and suppressed by SnTox1 (Phan et al. 2016).

Three *P. nodorum* NEs – SnToxA, SnTox1 and SnTox3 – have been cloned into *Pichia pastoris* (Friesen et al. 2006; Liu et al. 2009; Liu et al. 2012) and *Escherichia coli* (Tan et al. 2012) vectors. This allows for efficient screening for the corresponding sensitivity loci in wheat germplasm.

The SnToxA-*Tsn1* interaction confers sensitivity to both tan spot caused by *Pyrenophora tritici-repentis* and SNB (Friesen et al. 2006). In Western Australia, economic losses caused by SNB were estimated to be 108 million Australian $ (AUD), and losses due to tan spot up to 212 million AUD (Murray and Brennan 2009). In Australia SnToxA has been delivered to the breeders since 2009 (Vleeshouwers and Oliver 2014). By 2012, 30 000 doses of SnToxA and 6 000 doses each of SnTox1 and SnTox3 were provided annually (Vleeshouwers and Oliver 2014). The area of SnToxA sensitive wheat in Australia fell from 30.4 % in 2009-2010 to 16.9 % within three years. The estimated economic gain was approximately 50 million AUD, assuming a yield loss of 0.3 tons per hectare in susceptible cultivars (Vleeshouwers and Oliver 2014).

However, the effect and relative contribution of the individual NE-*Snn* interactions to disease under field conditions is not well investigated and is still disputed. In particular, the relevance of the isolates used to identify most of the NE-*Snn* interactions have been questioned by (Francki 2013). Francki (2013) also pointed out the lack of consistent effect at the adult plant stage. For instance, SnToxA-*Tsn1* was likely to underlie a significant QTL in the 05Y001 doubled haploid mapping population in one year of a field trial, but not in the subsequent year (Francki et al. 2011).

On the other hand, one field study reported significant effect of the SnToxA-*Tsn1* and SnTox2-*Snn2* interactions after spray inoculation of the flag leaf with a single *P. nodorum* isolate (Friesen et al. 2009). The difference in SNB resistance ranking between SnToxA-insensitive and sensitive Australian lines was reportedly lower in 2011 (Waters et al. 2011) than in a study by Oliver et al. (2009). A possible explanation for this is a shift in the NE frequencies in the pathogen population (Waters et al. 2011), perhaps triggered by the reduction in SnToxA-sensitive cultivars. The mapping of *Snn3* as a major susceptibility factor in the SHA3/CBRD × Naxos population in naturally infected field nurseries was the first to validate the importance of this locus in field trials (Ruud et al. 2017). ToxA has also been detected in other pathogens, most recently in *Bipolaris sorokiniana* (McDonald et al. 2017), and seems to be an important virulence factor of global relevance.

Inoculation with the same isolate or mix of isolates in both seedling and adult plant trials may give higher reproducibility and correlation between the two. However, they might not be representative for the situation in the farmers' fields with an ever-changing pathogen population. Better estimates of resistance is expected when infection in the nursery is promoted by overhead irrigation and inoculation with naturally infected straw (Fraser et al. 2003).

The objectives of the present study were to investigate 1) the prevalence of sensitivity to *SnToxA*, *SnTox1* and *SnTox3* in a diverse collection of spring wheat lines and 2) whether sensitivity was correlated with SNB susceptibility levels in field trials at the adult plant stage. Ultimately, we wanted to identify good resistance sources in the Norwegian breeding material.

Materials and methods

Plant material

A total of 157 spring wheat cultivars and breeding lines were analyzed in this study. The lines were from the MASbasis collection which includes both Norwegian and international spring and winter wheat cultivars and breeding lines (Supplementary table 1). The majority, 85 of the studied lines, are Norwegian. However, 25 lines from The International Maize and Wheat Improvement Center (CIMMYT) contribute to a substantial part of the population, as do the 22 cultivars and lines originating from Swedish breeding programs. In addition, lines from several other wheat growing areas were included.

Field trials

The lines were planted in hill plot trials during the 2010-2016 seasons at Vollebekk Research Station, Ås, Norway. The trials were naturally infected with *P. nodorum*, enhanced by mist irrigation 5 minutes every half hour at daytime, which also discouraged powdery mildew (*Blumeria graminis* f.sp. *tritici*) infection. From 2013, the infection was promoted by inoculating the field with infected straw harvested from the most susceptible plots the previous season. The straw was spread when the plants were at Zadoks stage Z13/21 (Zadoks et al. 1974) approximately, at which time the mist irrigation was started. In 2015 and 2016 the trials were sprayed with the selective fungicide Forbel® 750 (Bayer Crop Science, active ingredient: Phenpropimorph) every 3 weeks to prevent stripe rust infection. The field trials were conducted in an alpha lattice design with 2 or 3 replicates per year.

Phenotyping

Disease severity was scored twice per season by visually estimating the percentage of diseased canopy in each hill plot. The first scoring was done after the infection level had reached 60-70 % on the most susceptible lines, and the second scoring 7-10 days later. It is difficult to distinguish SNB symptoms from tan spot and leaf blotch caused by *Zymoseptoria tritici* and mixed infection can be common. However, PCR screenings and microscopic evaluation of leaf samples collected from the field nursery in different years, validated that *P. nodorum* was dominant in the spring wheat and the most dominating leaf blotch pathogen on the examined leaves every year (data not shown).

Plant height was measured after the plants were fully developed. Heading date was scored as the day most of the heads had emerged. Plant height and days from sowing to heading were used in multiple regression to estimate resistance.

Infiltration with purified effectors

Two seeds per genotype were planted in plastic conetainers in racks fitting 98 cones (Stuewe and sons, Tangent, Orlando, USA), with potting mixture (peat soil with clay and sand, Gartnerjord, Tjerbo, Norway). The plants were grown in the greenhouse with 20 ° C day/16 ° C night temperature, 16 h light cycle and 65 % relative humidity. All experiments were repeated three times with two replicates per repetition.

Partially purified SnTox1 and SnTox3 were produced in *P. pastoris* using the pGAPzA expression vector (Liu et al. 2009). SnToxA was produced in *E.coli* BL21E using the pET21a expression vector (Tan et al. 2012). Before infiltration, the protein preparations with the effectors were desalted with 10 mM sodium phosphate buffer with pH 7.0.

When the second leaves were fully expanded, 12-14 days after planting, they were infiltrated with purified SnToxA, SnTox1 and SnTox3 using a 1 mL syringe without a needle. The borders of the water soaked infiltrated area were marked with a black, non-toxic permanent marker. After five days the symptoms were scored according to a 0-3 scale where 0 is insensitive and 3 is necrosis with tissue collapse (Friesen and Faris 2012).

DNA extraction

Genomic DNA of 129 lines from the MASbasis collection plus differential lines (BG261/SnToxA, M6/SnTox1, BG220/SnTox3) was extracted from young leaves with the DNeasy Plant DNA extraction kit (QIAGEN). Microsatellite (SSR) analysis was performed

with fluorescently labeled primers and PCR products were separated by capillary electrophoresis on an ABI 3730 Gene Analyzer.

Statistical analysis

Analyses of variance (ANOVA) were calculated using the PROC GLM procedure in SAS v. 9.4 (SAS Institute Inc.). Broad sense heritability (h^2) was estimated using the ANOVA output and the formula

$$h^2 = \sigma^2_g / (\sigma^2_g + \sigma^2_{(g \times y)} / y + \sigma^2_E / ry)$$

Where σ^2_g is genetic variance, $\sigma^2_{(g \times y)}$ is genotype-by-year interaction, σ^2_E is error variance, y is the number of years and r is the number of replicates.

The Pearson correlation coefficients were calculated in Minitab and in R Studio v 1.0.44, using the Hmisc package. Welch two sample *t*-tests and Pearson's χ^2 tests with Yates' continuity correction were conducted in R Studio v 1.0.44. The multiple regression expressions for corrected SNB severity were calculated in Minitab v. 16 by regression and corrected SNB severities were calculated by subtracting the fitted leaf blotch scores from the original disease scores.

Results

Sensitivity distribution

Table 1 shows that sensitivity to SnToxA and SnTox3 was present in 45 and 55 % of the lines, respectively, while sensitivity to SnTox1 was only present in 12 % of the material. Initial analysis did not show any effect of SnTox1 sensitivity, and since the frequency was so low it was not considered in the correlation analysis.

The main subpopulations were grouped based on origin (Table 2). χ^2 tests showed that the proportion of lines sensitive to SnToxA was not significantly different between the subpopulations (*p*-values between 0.20-0.50). The frequency of SnTox3 sensitivity was similar in both Swedish and CIMMYT lines ($\chi^2 = 0$, $p = 1$). The proportion of SnTox3 sensitive to insensitive lines was significantly different between the Norwegian and Swedish subpopulations ($\chi^2 = 4.8$, $p = 0.03$), and between Norwegian and CIMMYT lines ($\chi^2 = 5.3$, $p = 0.02$).

Two clearly distinguishable reaction types for sensitivity to SnTox3 were observed– scored as reaction type 2 and 3. Interestingly, the Type 3 reaction type was dominating in the CIMMYT lines, with only one line, MAYOOR//TKSN1081/*Ae. tauschii* (222), showing the Type 2 reaction. The sensitive Swedish lines only expressed the Type 2 reaction. In the Norwegian material both reaction types were present, and Type 2 was the most common (Table 2).

Field results

Table 3 shows the ANOVA output and heritability for PH, DH and uncorrected SNB.

Table 4 shows the correlation among uncorrected SNB severities per year and the correlation between SNB severity and the confounding traits plant height and days to heading. Days to heading and disease severity was highly negatively correlated in all years, while the correlation between disease severity and plant height was weaker and varied between years. To minimize the confounding effects of PH and DH, we used multiple regression to obtain corrected SNB severities. In all subsequent analyses, we have used the corrected SNB severities.

Figure 1 shows the relationships between corrected SNB severity and different combinations of insensitivity/sensitivity to SnToxA and SnTox3.

The disease mean for lines with sensitivity to SnToxA alone was significantly higher than for lines insensitive to both effectors (p = 1.295e-05). The mean for lines with sensitivity to both effectors was lower than for lines only sensitive to SnToxA (Figure 1). Analyzed for individual years, SnToxA sensitivity was significantly correlated to increased disease severity every year (data not shown).

Sensitivity to SnTox3 alone did not have a significant effect on the mean corrected SNB severity from the field trials, compared to double insensitive lines (Table S2, p = 0.2185). 2010 was the only year where we found significant association between SnTox3 sensitivity and corrected SNB severity from field trials (p = 0.041) and only when compared to resistance scores for double insensitive lines. When the exotic material, i.e. all non-European lines, was analyzed alone, the correlation was even more significant (p = 0.008) in 2010 and at a 0.05 level across years (p = 0.032), while not significant for the European subpopulation.

Table 5 shows an overview of important current and historical cultivars in Norway and the corrected field SNB severity and SnTox-sensitivity status. Zebra is a Swedish cultivar. The landrace J03 was used in early Norwegian spring wheat breeding as a powdery mildew resistance source (Vik 1937). All the cultivars range among the moderately susceptible to

moderately resistant to SNB, except Polkka which is very susceptible. SnToxA sensitivity was present in 50 % of these lines, while sensitivity to SnTox1 and SnTox3 was rarer. The weaker SnTox3 reaction type 2 (chlorosis) was more prevalent than the Type 3 reaction which was only present in Reno.

Marker correlations

Table 6 shows the most significant correlations between markers associated with SnToxA, SnTox1 and SnTox3 sensitivity, respectively, and sensitivity results after infiltration of MASbasis with the purified NEs. Correlation to markers linked to *Tsn1* and sensitivity to SnToxA was high, with *fcp620* as the most significant marker. *fcp1* and *fcp623* linked to *Tsn1* were also significantly correlated to corrected SNB severity (Table 6).

Markers linked to *Snn1* were not significantly correlated with SnTox1 sensitivity (Table 6). The marker with highest correlation to SnTox3 sensitivity was *cfd20* (a 294 bp fragment), strongly linked to reaction Type 2 (Table 6). These markers were not correlated to corrected SNB severity.

Discussion

Prevalence of sensitivity to SnToxA, SnTox1 and SnTox3

A main objective of our study was to investigate the prevalence of sensitivity to the three cloned NEs SnToxA, SnTox1 and SnTox3. We found that sensitivity to SnToxA and SnTox3 was present in 46 and 56 % of our material, respectively (Table 1). Sensitivity to SnTox1 was only present in 12 % of the lines. This is in the same range as the 16 % of sensitive hexaploid wheat accessions reported by Shi et al. (2016a), but substantially less than in the Australian cultivars screened by Tan et al. (2014), where 33 of 46 genotypes showed moderate to strong sensitivity to SnTox1.

We observed two reaction types for sensitivity to SnTox3, one causing severe and complete necrosis (Reaction Type 3) and one causing chlorosis, but not necrosis (Reaction Type 2). This corresponds to literature (Waters et al. 2011; Shi et al. 2016a) although it has not yet been established whether these are caused by different sensitivity loci, alleles or downstream mechanisms. Reaction Type 2 was the only reaction type towards SnTox3 in the Swedish material we screened (Table 2), while reaction Type 3 was most common in the CIMMYT material, illustrating the differences between materials of different origin. The proportion of

sensitive to insensitive lines for each effector was only significantly different for SnTox3 between Norwegian and Swedish and Norwegian and CIMMYT lines (Table 2).

Field results

The heritability of SNB severity was 0.70 (Table 3) and lower than observed for many bi-parental mapping populations, for instance SHA3/CBRD × Naxos (Lu and Lillemo 2014). It was, however, higher than reported by Shankar et al. (2008) for a doubled haploid population. As described above, the development of SNB is significantly correlated with and confounded by other traits, and the relatively lower heritability of SNB in a diverse population like MASbasis can partly be explained by the heritability of days to heading. The heritability of earliness (days to heading) was 0.72 (Table 3). A likely contribution to the large variation in heading dates across years is the presence of *Vrn*- and *Ppd*-genes in the germplasm, that respond differently to varying planting date and growth season temperatures of the field trials used in our study. The heritability of plant height was high (0.90, Table 3), as could be expected for this trait.

The field trials were conducted under natural infection promoted by infected straw and mist irrigation. The natural population of *P. nodorum* is expected to vary over time, and thus variability in the individual NE-*Snn* interactions is expected to differ between years. However, the correlation of disease severity between years was high (Table 4). The correlation between SNB severity and days to heading was highly significant in all years, while the correlation between plant height and disease varied from insignificant in 2013 to significant at a 0.01 level in 2016 (Table 4). Conidiospores of *P. nodorum* are spread upwards in the canopy by rain splash and taller plants generally show less severity if relative disease spread is not accounted for (Eyal et al. 1987; Francki 2013). The applied mist irrigation provided a favorable environment for SNB development, but not the rain splash-effect. The correlation between plant height and SNB severity varied between years. The lowest correlation between SNB severity and plant height was observed in 2011 and 2015 (Table 4). In 2015, an extreme rainfall 8th of July accounted for 76 mm precipitation in 24 hours (historical data from http://lmt.bioforsk.no/). Perhaps the spores were distributed higher up in the canopy than normal due to this rain and the plant height effect was minimized. In 2011 several rainfalls higher than 10 mm precipitation in July might have contributed to a similar effect.

Correlation between effector sensitivity and SNB susceptibility in the field

We found that lines sensitive to SnToxA had a significantly higher field SNB disease mean than insensitive lines (Figure 1, Table S2). This trend was significant in all years. The most resistant SnToxA-insensitive lines were clearly more resistant than the most resistant SnToxA-sensitive lines (Figure 1) regardless of SnTox3-sensitivity. The SnToxA-sensitive lines scored from -14.9 (Milan, Figure 1, Table S2) compared to the most resistant insensitive lines (from -24.5, Milan/SHA7, Figure 1, Table S2).

Interestingly, the SNB mean for lines with sensitivity to both effectors was significantly ($p < 0.05$) lower than for lines only sensitive to SnToxA (Figure 1, Table S2). A part of the explanation can be that SnToxA-*Tsn1* is epistatic to SnTox3-*Snn3* (Friesen et al. 2008b; Friesen et al. 2008c), so an additive effect of double sensitivity is not expected. Although few lines were sensitive to SnTox1, the effector might be produced by the pathogen and inhibit the production of SnTox3 (Phan et al. 2016). Other NE-*Snn*-interactions may also be important, as can other resistance mechanisms.

In contrast to Waters et al. (2011) we did not find any significant correlation between SnTox3 sensitivity and field susceptibility in MASbasis. The exception was in 2010 ($p = 0.041$) and only when compared to double insensitive lines. The correlation between SnTox3-sensitivity and SNB susceptibility across years was significant ($p = 0.032$) when the exotic material was analyzed separately, but not in the European material.

When the results for the exotic (non-European) material was analyzed separately for 2010, we found that lines with single sensitivity to SnTox3 were significantly more susceptible than double insensitive lines ($p = 0.008$). In the European material, SnTox3 sensitivity was not significantly associated to disease in this or any other year. In the Exotic material, the most severe Type 3 reaction was predominant (28, compared to 2 producing the Type 2 reaction). In the European material, the less severe Type 2 reaction was more common (41 Type 2 compared to 16 Type 3).

Interestingly, in 2010 the SnTox3-*Snn3*-interaction was also highly significant in a bi-parental mapping population, SHA3/CBRD × Naxos, evaluated in the same field nursery (Ruud et al. 2017). In this population, the parent Naxos carried the *Snn3*-allele producing the most severe necrosis, i.e. a Type 3 reaction. In seedling inoculations with SnTox3 producing isolates, plants carrying the Type 3 sensitivity have been shown to develop more severe disease symptoms than plants with the Type 2 sensitivity, see Figure 1 in Shi et al. (2016a). We speculate whether

the more severe sensitivity type has a stronger association to adult plant SNB severity as well. The change in the pathogen population over time and relatively larger effect of other interactions may explain why the SnTox3-*Snn3*-interaction played a minor role in MASbasis other years.

All the important current cultivars grown in Norway ranked around average for SNB resistance in the field (Table 5). This was regardless of sensitivity combination. Polkka was the only very susceptible historically important cultivars (Table 5). In most important current and historical cultivars in Norway, sensitivity to SnToxA was most prevalent (50 %), while Type 2 reaction type for SnTox3 sensitivity was more common than reaction type 3 (Table 5). The presence of reaction Type 2 in the Norwegian landrace J03 (Table 5) indicates that this trait has been common in Scandinavian spring wheat since the onset of modern plant breeding.

Marker correlations

129 of the lines were tested with SSR markers known to be associated with SnToxA, SnTox1 and SnTox3. Markers *fcp1*, *fcp620, fcp623, fcp626* and *fcp394* were all significantly correlated with sensitivity to SnToxA, with *fcp620* showing the strongest association (Pearson correlation 0.85, Table 6). The distance between *Tsn1* and *fcp394* is comparable to the distance between *Tsn1* and *fcp620* on the physical map, while *fcp1* is more distantly linked (Faris et al. 2010). However, *fcp1* had higher correlation corrected SNB disease level (Table 6). The marker *fcp623* located in an intron of *Tsn1* and which is reported to cosegregate almost 100 % with ToxA-sensitivity (i.e. in 386 *Triticum* accessions) (Faris et al. 2010), was not diagnostic in our material (Table 6). The marker is dominant which complicates the scoring and we cannot rule out that failed amplification in some of the samples can have been misinterpreted as absence of the dominant allele.

The SSR marker *cfd20* was strongly associated with SnTox3 Type 2 sensitivity, while not associated with reaction Type 3 (Table 6). Marker *gwm234* amplified different fragments in different genotypes, and the 257 bp amplicon was linked to reaction Type 2 (Table 6).The 264 bp allele is the same that is amplified in SnTox3-differential line BG220 where *Snn3* was first mapped. However, this allele was not strongly associated with SnTox3 sensitivity in MASbasis, but had higher correlation with the average SnTox3 scores (Reaction Type 2 + 3).

Recommendations for breeding

All the important current cultivars were moderately resistant to moderately susceptible to SNB, ranging from -3 (Demonstrant) to +7.2 (Bjarne) (Table 5). The range of resistance in MASbasis

was from -24.5 as the most resistant (Milan/SHA7) to +27.5 (Brakar) (Table S1) on the disease severity scale corrected for PH and DH, implying that there is great genetic potential to improve the level of resistance by breeding.

Sensitivity to SnToxA was not a 1:1 predictor of field susceptibility, for instance does the sensitive Demonstrant perform better in the field than SnToxA-insensitive Bjarne. However, sensitivity to SnToxA was consistently correlated with higher corrected SNB severity, and based on this we suggest that screening for sensitivity either by seedling infiltrations or marker assisted selection is a reasonable and affordable measure to improve SNB and subsequently tan spot resistance in the breeding material.

SnTox3 sensitivity had a weak to no correlation with disease. This was in contrast to one previous study (Waters et al. 2011). However, our work support other studies that there are at least two different *Snn3* alleles or loci causing different levels of sensitivity to purified SnTox3 (Waters et al. 2011; Tan et al. 2014; Shi et al. 2016b) and susceptibility levels in seedling inoculations (Shi et al. 2016b). In another study it was shown that "*Snn3*-Type 3" had a significant effect in the field in a bi-parental population (Ruud et al. 2017). Based on these results we also recommend elimination of this susceptibility allele from the breeding material.

To further investigate the importance of the individual NEs and the correlation between seedling and adult plant resistance to SNB, seedling inoculation and infiltration experiments with representative *P. nodorum* isolates should be performed.

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Conflict of interest

The authors declare no conflict of interest.

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Figure captions

Figure 1 Boxplots comparing corrected Septoria nodorum blotch (SNB) severity (y-axis) for cultivars with different sensitivity combinations (x-axis): ToxA = SnToxA, Tox3 = SnTox3, + = sensitive, - = insensitive. Mean over 7 years, all lines (see also Table S2). Red dot indicates mean value, black horizontal line median.

Tables

Table 1 Prevalence of sensitivity to SnToxA, SnTox1 and SnTox3 in 157 lines of the MASbasis spring wheat collection.

Effector	Number of lines (Sensitive/Insensitive)	Frequency of sensitive lines
SnToxA	71/86	0.45
SnTox1	19/134	0.12
SnTox3	86/70	0.55

Table 2 Prevalence of sensitivity/insensitivity to SnToxA, SnTox1 and SnTox3 by origin of the main subpopulations of the Nordic spring wheat collection.

Effector	SnToxA		SnTox1		SnTox3		Tox3 reaction types	
Origin	Insensitive	Sensitive	Insensitive	Sensitive	Insensitive	Sensitive	Type 2	Type 3
Sweden	9	13	21	1	7	15	15	0
Norway	44	40	77	12	51	33	21	12
CIMMYT	16	9	20	4	8	17	1[†)]	16

[†)] MAYOOR//TKSN1081/*Ae. tauschii* (222)

Table 3 ANOVA table and heritability (h^2) for corrected Septoria nodorum blotch (SNB) severity and the confounding traits plant height (PH) and days to heading (DH) based on field data from 2010 to 2016. Plant height was not recorded for the population in 2010, and days to heading was recorded in a different field trial that year.

Trait	Source	DF	MS	F value	P value	h^2
PH	Genotype	170	745.2	28.87	<.0001	0.90
	Year	5	13526.0	4811.62	<.0001	
	Genotype*Year	761	25.8	1.96	<.0001	
	Rep(Year)	7	135.5	10.27	<.0001	
	Block(Rep)	69	17.8	1.35	0.0323	
	Error	1369	13.2			
DH	Genotype	170	77.5	12.39	<.0001	0.72
	Year	5	24988.5	3998.74	<.0001	
	Genotype*Year	761	6.3	3.15	<.0001	
	Rep(Year)	7	34.1	17.15	<.0001	
	Block(Rep)	69	3.4	1.69	0.0005	
	Error	1382	2.0			
SNB	Genotype	174	2755.9	10.51	<.0001	0.70
	Year	6	20517.9	72.22	<.0001	
	Genotype*Year	848	262.3	2.50	<.0001	
	Rep(Year)	9	1548.4	14.92	<.0001	
	Block(Rep)	69	297.6	2.84	<.0001	
	Error	1475	104.9			

Table 4 Correlation between Septoria nodorum blotch (SNB) severity and the confounding traits plant height (PH) and days to heading (DH). For PH and DH the correlation is shown against respective years (i.e. PH measured in 2011 against SNB 2011) unless otherwise noted.

Trait	Field SNB severity						
	2010	2011	2012	2013	2014	2015	2016
PH	-0.10[†)]	-0.08	-0.11	-0.29*	-0.25*	-0.09	-0.30**
DH	-0.31[‡)]**	-0.6***	-0.54***	-0.70***	-0.64***	-0.47***	-0.65***
2010		0.65***	0.67***	0.77***	0.77***	0.68***	0.57***
2011			0.69***	0.80***	0.68***	0.71***	0.70***
2012				0.67***	0.56***	0.56***	0.58***
2013					0.72***	0.67***	0.72***
2014						0.72***	0.72***
2015							0.66***

[†)]Mean value based on all years' measurements

[‡)] Heading data from a different experiment (weather resistance), same location

*,**,*** significant at p <0.05, 0,001, 0.0001, respectively

Table 5 Overview of current and historically important spring wheat cultivars in Norway, release year, their origin, corrected SNB severity and sensitivity to SnToxA, SnTox1 and SnTox3. Sensitivity was scored on the 0-3 scale, and cultivars were ranked as sensitive if they scored ≥2, annotated with "+". Insensitive cultivars are annotated "-".

Cultivar	corrected SNB severity	Release year	Origin (country)	SnToxA	SnTox1	SnTox3 sensitivity	SnTox3 reaction Type 2	SnTox3 reaction Type 3
Current								
Zebra	-8.32	2001	Sweden	-	-	-	-	-
Bjarne	7.21	2002	Norway	-	-	-	-	-
Demonstrant	-9.27	2008	Norway	+	+	-	-	-
Krabat	-1.69	2010	Norway	+	-	-	-	-
Mirakel	-5.32	2012	Norway	+	-	-	-	-
Rabagast	-5.95	2013	Norway	-	-	+	+	-
Historical								
Fram II	-8.29	1940	Norway	-	+	-	-	-
Norrøna	-5.34	1952	Norway	-	-	-	-	-
Rollo	-4.11	1963	Norway	+	+	-	-	-
Møystad	3.36	1966	Norway	+	-	-	-	-
Runar	2.33	1972	Norway	+	-	-	-	-
Reno	3.84	1975	Norway	-	-	+	-	+
Tjalve	6.11	1987	Sweden	+	-	-	-	-
Bastian	5.21	1989	Norway	-	-	-	-	-
Polkka	22.14	1992	Sweden	+	-	+	+	-
Avle	7.71	1996	Sweden	+	-	+	+	-
J03	-4.68	Landrace	Norway	-	-	+	+	-

Table 6 Markers associated to SnTox reaction tested on 129 genotyped lines. Correlations performed and *p* values calculated in R using the Hmisc package.

Gene	Marker	SnToxA	SnTox1	SnTox3 average	SnTox3 Type 2	SnTox3 type 3	Mean corrected SNB severity
Tsn1	*fcp1*	0,48***					0.24*
	fcp623	0.62***					0.31**
	fcp620	0.85***					0.21
	fcp626	0.82***					0.20
	fcp394	0.68***					0.07
Snn1	*fcp618*		-0.15				-0.07
	psp3000		-0.05				0.09
Snn3-5B	*cfd20*			0.36**	0.69***	-0.12	-0.02
	gwm234 (264 bp)			0.32**	0.22*	0.21*	-0.04
	gwm234 (257 bp)			0.03	0.46***	-0.33*	0.03

* $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$

Figures

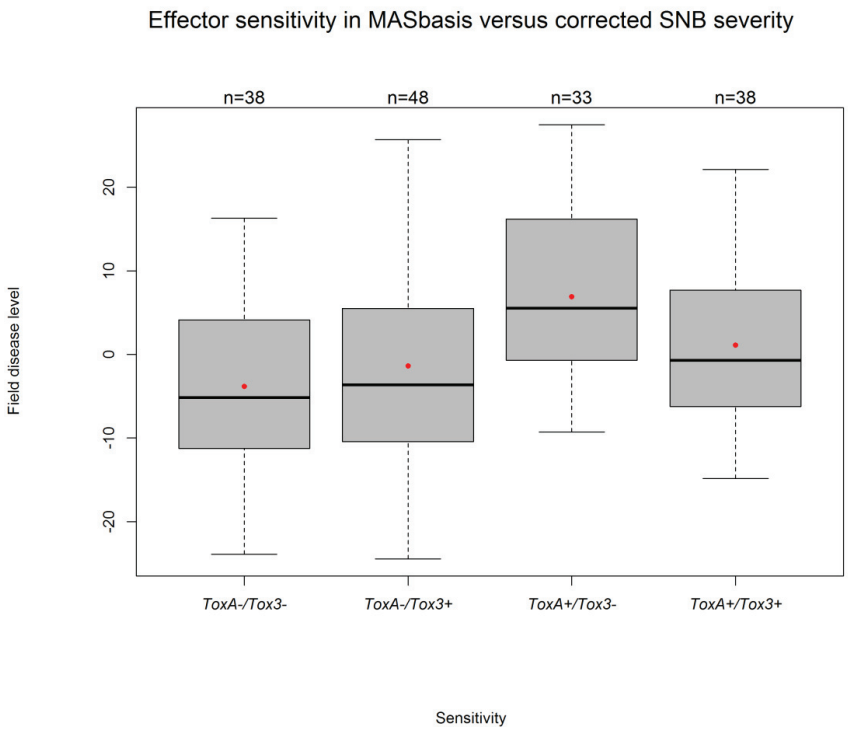

Figure 1 Boxplots comparing corrected Septoria nodorum blotch (SNB) severity (y-axis) for cultivars with different sensitivity combinations (x-axis): ToxA = SnToxA, Tox3 = SnTox3, + = sensitive, - = insensitive. Mean over 7 years, all lines (see also Table S2). Red dot indicates mean value, black horizontal line median.

Supplementary material

Table S1 List of lines included in the analysis and their sensitivity status towards SnToxA, SnTox1 and SnTox3. The Septoria nodorum blotch (SNB) severity ranking is corrected for plant height (PH) and days to heading (DH). SnTox-positive lines are marked with +, insensitive lines with -. The varieties and lines are ranked according to the corrected SNB susceptibility severities, from resistant to susceptible. Generic line names have been created for some of the Swedish breeding lines included.

NAME	Corrected SNB severity	SnToxA	SnTox1	SnTox3 average	SnTox3 Type 2	SnTox3 Type 3	Origin
MILAN/SHA7	-24.45	-	-	+	-	+	CIMMYT
ALTAR84/*Ae.tauschii*(224)//ESDA	-23.9	-	-	-	-	-	CIMMYT
GN07581	-21.97	-	-	+	+	-	Norway
CJ9306	-21.32	-	-	-	-	-	China
DH49-18 Bastian/Adder	-20.41	-	-	-	-	-	Norway
GN07560	-19.12	-	-	-	-	-	Norway
SHA3/CBRD	-18.7	-	-	-	-	-	CIMMYT
BJY/COC//CLMS/GEN	-15.4	-	-	+	-	+	CIMMYT
Milan	-14.86	+	+	+	-	+	CIMMYT
Pfau/Milan	-12.73	-	-	-	-	-	CIMMYT
Chara	-12.7	+	-	+	-	+	Australia
Kariega	-12.45	-	-	+	-	+	South Africa
GN07548	-12.18	-	-	+	+	-	Norway
CBRD/KAUZ	-11.81	-	-	+	-	+	CIMMYT
GN03531	-11.79	-	-	-	-	-	Norway
GN10521	-11.47	-	-	-	-	-	Norway
Ning8343	-11.43	-	-	-	-	-	China
GN10512	-11.39	-	-	+	+	-	Norway
Breeding line 5	-11.26	-	+	+	+	-	Sweden
Breeding line 7	-11.24	-	-	+	+	-	Sweden
DH20070	-11.23	-	-	-	-	-	Norway
Breeding line 3	-11.14	-	-	+	+	-	Sweden
Bombona	-11.1	+	-	+	+	-	Sweden
Nobeokabouzu	-11.02	-	-	+	-	+	Japan
Croc_1/*Ae.tauschii*(205)//Kauz	-11.01	-	-	+	-	+	CIMMYT
GN07580	-10.42	+	-	+	+	-	Norway
C80.1/3*QT4522//2*ATTILA	-10.15	+	-	+	-	+	CIMMYT
Bjarne/LW91W86	-9.83	-	-	+	-	+	Norway
QUARNA	-9.78	-	-	+	+	-	Switzerland
Amulett	-9.78	-	-	-	-	-	Sweden
NK01565	-9.69	-	-	-	-	-	Norway
Tom	-9.31	+	-	+	-	+	USA
Demonstrant	-9.27	+	+	-	-	-	Norway
ALTAR 84/*Ae.tauschii* (224)//2*YACO/3/KAUZ	-9.21	-	-	-	-	-	CIMMYT
GN04526	-8.65	-	-	-	-	-	Norway

GN08588	-8.52	-	-	+	+	-	Norway
Zebra	-8.32	-	-	-	-	-	Sweden
Fram II	-8.29	-	-	-	-	-	Norway
Catbird	-8.28	-	-	+	-	+	CIMMYT
GN03503	-7.99	-	-	-	-	-	Norway
GN03597	-7.98	-	-	+	+	-	Norway
MAYOOR//TKSN1081/*Ae.tauschii*(222)	-7.93	-	-	+	+	-	CIMMYT
Avocet-YrA	-7.87	+	+	+	-	+	Australia
GN08557	-7.03	-	NA	-	-	-	Norway
Sport	-6.88	+	-	-	-	-	Sweden
AC Somerset	-6.75	+	-	+	-	+	Canada
Nanjing 7840	-6.67	-	-	+	-	+	China
R37/GHL121//KAL/BB/3/JUP/MUS/4/2*YMI#6/5/CBRD	-6.37	+	-	+	-	+	CIMMYT
Breeding line 6	-6.25	+	-	+	+	-	Sweden
Saar	-6.12	-	NA	+	-	+	CIMMYT
Rabagast	-5.95	-	-	+	+	-	Norway
CJ9403	-5.88	-	-	+	-	+	China
GN08595	-5.73	+	-	+	+	-	Norway
GN08647	-5.41	-	-	+	+	-	Norway
Norrøna	-5.34	-	+	-	-	-	Norway
Mirakel	-5.32	+	-	-	-	-	Norway
GN09584	-4.94	-	-	-	-	-	Norway
Laban	-4.74	+	-	+	+	-	Norway
J03	-4.68	-	-	+	+	-	Norway
GN08568	-4.56	-	-	-	-	-	Norway
RB07	-4.42	+	-	-	-	-	USA
GN08554	-4.12	+	-	+	+	-	Norway
Rollo	-4.11	+	+	-	-	-	Norway
GN08597	-3.07	+	+	+	+	-	Norway
Breeding line 9	-2.93	+	-	+	+	-	Sweden
GUAM92//PSN/BOW	-2.79	+	-	+	-	+	CIMMYT
BCN*2//CROC_1/*Ae.tauschii*(886)	-2.62	-	-	+	-	+	CIMMYT
Altar84/*Ae.tauschii*(219)//2*Seri/3/Avle	-2.2	-	-	-	-	-	Norway
GN08596	-2.2	+	-	+	+	-	Norway
Gondo	-2.2	+	+	+	-	+	CIMMYT
BAJASS	-2.19	-	-	-	-	-	Norway
DH20097	-1.83	-	-	-	-	-	Norway
Krabat	-1.69	+	-	-	-	-	Norway
NG8675/CBRD	-1.34	+	-	-	-	-	CIMMYT
Sabin	-1.29	+	-	+	-	+	USA
GN08530	-1.22	+	-	-	-	-	Norway
C80.1/3*QT4522//2*PASTOR	-0.72	+	-	-	-	-	CIMMYT

T9040	-0.55	+	+	-	-	-	Norway
Dragon	-0.45	-	-	+	+	-	Sweden
Paros/NK93602	-0.23	-	-	+	+	-	Norway
Paros/T9040	-0.14	+	-	+	+	-	Norway
GN05580	-0.02	+	-	-	-	-	Norway
Naxos/2*Saar	-0.02	-	-	+	+	-	Norway
GN10510	-0.01	-	-	+	+	-	Norway
Sumai#3-(12SRSN)	0.12	+	-	+	-	+	China
Frontana	0.73	-	-	-	-	-	Brazil
GN03529	1.12	-	-	-	-	-	Norway
GN04528	1.33	-	-	-	-	-	Norway
Runar	2.33	+	-	-	-	-	Norway
GN09572	2.38	+	-	-	-	-	Norway
Scirocco	2.4	-	-	+	-	+	Germany
T9040/Paros	2.41	+	-	-	-	-	Norway
Breeding line 2	2.52	-	-	+	+	-	Sweden
TJALVE/Purpurseed	3.01	+	-	-	-	-	Norway
Møystad	3.36	+	-	-	-	-	Norway
Reno	3.84	-	-	+	-	+	Norway
GN05507	4.09	-	-	-	-	-	Norway
GN10524	4.14	-	-	-	-	-	Norway
Arabella	4.59	-	-	+	+	-	Poland
Dulus	4.86	-	-	+	-	+	CIMMYT
T9040(1995)	4.92	-	-	+	+	-	Norway
Aino	5.04	-	+	+	-	+	Finland
Granary	5.1	+	+	+	-	+	Great Britain
Bastian	5.21	-	-	-	-	-	Norway
GN08504	5.54	+	-	-	-	-	Norway
Breeding line 8	5.8	+	-	+	+	-	Sweden
Breeding line 4	5.84	+	-	+	+	-	Sweden
T7347	5.93	+	-	-	-	-	Norway
Altar84/*Ae.tauschii*(219)//2*Seri	6	-	-	+	-	+	CIMMYT
Tjalve	6.11	+	-	-	-	-	Sweden
Breeding line 1	6.18	+	-	+	+	-	Sweden
Breeding line 10	6.5	-	-	-	-	-	Sweden
GN06557	6.55	+	-	+	+	-	Norway
Filin	6.57	-	-	+	-	+	CIMMYT
Vinjett	6.68	+	-	+	+	-	Sweden
Paros	6.75	+	-	-	-	-	Norway
Bjarne	7.21	-	-	-	-	-	Norway
GN08533	7.64	+	-	+	-	+	Norway
Avle	7.71	+	-	+	+	-	Sweden

GN06573	8.7	+	-	+	+	-	Norway
Sumai3(18.)	9.02	-	-	+	-	+	China
Avans	9.27	-	-	-	-	-	Sweden
GN08541	9.33	+	-	+	-	+	Norway
GN05589	9.37	-	-	+	-	+	Norway
CD87	9.61	-	-	+	+	-	Australia
GN04537	9.9	+	-	+	-	+	Norway
Soru#1	9.92	-	-	+	-	+	CIMMYT
Senorita	10.11	+	-	-	-	-	Norway
MS273-150	10.13	+	-	-	-	-	Norway
512-21	10.97	-	-	-	-	-	Norway
GN08581	11.06	-	-	-	-	-	Norway
Naxos	11.09	-	-	+	-	+	Germany
Breeding line 11	11.1	+	-	+	+	-	Sweden
Kukri	11.62	+	+	+	-	+	Australia
GN08534	11.78	+	-	+	-	+	Norway
T2038	11.78	+	+	-	-	-	Norway
GN08564	13.01	+	+	+	-	+	Norway
GN08531	13.73	-	-	-	-	-	Norway
512-87	13.8	+	-	-	-	-	Norway
512-50	15.08	-	+	-	-	-	Norway
NK93602	15.68	-	-	+	+	-	Norway
Berlock	16.2	+	-	-	-	-	Sweden
NK93604	16.32	-	-	-	-	-	Norway
GN06578	16.59	-	-	+	-	+	Norway
512-70	17.01	+	+	-	-	-	Norway
Berserk	17.68	+	+	+	+	-	Norway
HAHN/PRL//AUS1408	17.94	+	-	-	-	-	CIMMYT
NK00521	18.08	+	-	-	-	-	Norway
Kruunu	18.52	-	-	+	+	-	Finland
TUI/RL4137	18.58	+	-	-	-	-	CIMMYT
GN07525	21.02	+	-	-	-	-	Norway
512-54	21.5	+	+	-	-	-	Norway
Polkka	22.14	+	-	+	+	-	Sweden
NK01513	22.73	+	-	-	-	-	Norway
GN05551	24.03	-	-	+	-	+	Norway
T10014	25.69	-	-	+	-	+	Norway
Brakar	27.47	+	+	-	-	-	Norway

Table S2 Results of *t*-tests comparing the effect of different combinations of sensitivity to SnToxA/SnTox3 on corrected SNB severity, with 95 % confidence interval. Degrees of freedom (df) are also given in the table. The disease level is based on mean over 7 years. A "-" sign denotes insensitive and "+" sensitive, for SnToxA/SnTox3, respectively.

SnToxA/SnTox3 mean comparisons	*t*	df	*p*	95 % confidence interval
-/- versus -/+	-1.04	81.29	0.29	-7.15, 2.23
-/- versus +/+	-2.18	72.21	0.03	-9.43 , -0.43
+/- versus -/-	-4.40	68.72	3.3e-05	-15.58, -5.93
-/+ versus +/-	-3.53	74.43	0.0007	-1.36, 7.31
+/- versus +/+	2.59	65.77	0.012	1.34, 10.31
-/+ versus +/+	-1.13	83.95	0.26	-6.80, 1.87

Paper III

Genome wide association mapping of seedling and adult plant resistance to Septoria nodorum leaf blotch in a Nordic spring wheat collection

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Author contributions

AKR wrote the manuscript, planned and conducted the experiments, analyzed the data. JAD developed and contributed plant material, critically revised the manuscript, supervision. AF participated in planning of experiments, critically revised the manuscript, supervision. ML Planning, field experimental design, assessed field phenotypic data in 2010-2012, critically revised the manuscript, supervision, obtained the funding.

Abstract

Parastagonospora nodorum is the causal agent of Septoria nodorum leaf blotch (SNB) in wheat. It is the most important leaf blotch pathogen in Norwegian spring wheat, causing significant yield and quality losses in years of epidemics. Several quantitative trait loci (QTL) for SNB resistance have been identified. Often, underlying gene-for-gene interactions involving necrotrophic effectors (NEs) and corresponding sensitivity (*Snn*) genes are involved. Here, we report the first genome wide association mapping study (GWAS) investigating the marker trait associations for SNB susceptibility at the adult plant stage under field conditions. A collection of diverse spring wheat lines was evaluated over seven growth seasons in the field. In addition, wheat seedlings were inoculated and infiltrated with four single spore isolates in the greenhouse, and infiltrated with semi-purified NEs. At the adult plant stage the most stable QTL were located on 2B, 2D, 4A, 4B, 5A, 6B, 7A and 7B. The QTL on 2D was significant all years in the field, except 2012. At the seedling stage, the most significant quantitative trait loci (QTL) were located on 1A, 1B, 3A, 4B, 5B, 6B, 7A and 7B. QTL on 3A and 6B were significant both after inoculation and infiltration, which are indicative of novel NE-*Snn* interactions. The QTL on 4B and 7A were significant at both the seedling and adult plant stages. Correlations between SnToxA sensitivity and disease severity in the field were often

significant, but markers linked to the sensitivity locus *Tsn1* were only detected below the significance threshold in GWAS.

Introduction

Wheat is one of the most important food crops in the world, with a production of 729 million tons in 2014 (FAO 2017). Hexaploid bread wheat (*Triticum aestivum,* L.) accounts for roughly 95 % of the wheat production, durum (*T. durum*, L.) for the remaining 5 %. Due to its adaptability, wheat is grown in a wide range of climates. In Norway, more than 50 % of the bread wheat for human consumption is grown domestically in an average season (LD 2017). This is possible due to political incentives and systematic breeding efforts since the 1970s, which has resulted in adapted, high yielding cultivars (Lillemo and Dieseth 2011). Nevertheless, the proportion of harvested food quality wheat in Norway varies significantly between years, mostly due to problems with pre-harvest sprouting and disease epidemics (Lillemo and Dieseth 2011).

Septoria nodorum leaf blotch (SNB) caused by the necrotrophic ascomycete *Parastagonospora nodorum* is a major disease in many areas where wheat is grown, including Australia, USA and Norway (Solomon et al. 2006; Ficke et al. 2011a; Francki 2013). In Norway, SNB is the dominating leaf blotch disease in spring wheat (Ficke et al. 2011a). The disease is often controlled by fungicides, but increased loss of fungicide sensitivity has been documented (Blixt et al. 2009; Ficke et al. 2011b; Abrahamsen 2013; Pereira et al. 2016), and more sustainable solutions are needed. Growing cultivars with sufficient genetic resistance is economically and environmentally sustainable, but breeding for SNB resistance has been hampered due to the complex and usually quantitative nature of the genetic resistance. Resistance to SNB is polygenic (Fried and Meister 1987; Bostwick et al. 1993; Wicki et al. 1999) and large genotype × environment (G × E) interactions are expected. Although many quantitative trait loci (QTL) for SNB resistance at the adult plant stage have been detected in single locations or years, only a few have proven to be stable across environments (Francki 2013). Often, seedling and adult plant SNB resistance are independently inherited, and most resistance QTL are unique for one developmental stage (Rosielle and Brown 1980; Fried and Meister 1987; Shankar et al. 2008).

The dissection of the *P. nodorum*-wheat pathosystem into host-specific gene-for-gene interactions, has provided hope for more effective resistance breeding. *P. nodorum* produces small, secreted proteins known as necrotrophic effectors (NEs, previously called host-specific toxins, HSTs). These NEs can be recognized by the gene product of a corresponding sensitivity

(*Snn*) gene in the host. Recognition triggers hyper sensitive response (HR) and cell death, upon which the necrotrophic pathogen will thrive. A compatible NE-*Snn* interactions leads to more disease development.

Biparental mapping populations segregating for susceptibility and resistance to SNB are often used to identify QTL for resistance. Biparental interval mapping for seedling SNB resistance has identified QTL on chromosomes 1A, 1B, 2B, 2D, 4B, 5A, 5B, 6A, 7A and 7B (Czembor et al. 2003; Arseniuk et al. 2004; Liu et al. 2004b; Friesen et al. 2006; Liu et al. 2006; Friesen et al. 2007; Abeysekara et al. 2009; Friesen et al. 2009; Gonzalez-Hernandez et al. 2009; Liu et al. 2009; Gao et al. 2015; Shi et al. 2015; Ruud et al. 2017). At least eight NEs (SnToxA, SnTox1, SnTox2, SnTox3, SnTox4, SnTox5, SnTox6 and SnTox7) and nine corresponding *Snn* genes (*Tsn1*, *Snn1*, *Snn2*, *Snn3-B1, Snn3-D1*, *Snn4*, *Snn5, Snn6 and Snn7*) have been characterized (Friesen et al. 2006; Liu et al. 2006; Friesen et al. 2007; Abeysekara et al. 2009; Liu et al. 2009; Gao et al. 2015; Shi et al. 2015). These interactions play a significant role in SNB seedling susceptibility. Consistent flag leaf resistance has been identified on chromosomes 1A, 1B, 2A, 2D, 3AS, 3B, 5A, 5B, 7A and 7B in biparental QTL studies (Aguilar et al. 2005; Shankar et al. 2008; Friesen et al. 2009; Francki et al. 2011; Lu and Lillemo 2014; Ruud et al. 2017).

However, genetic variation in a biparental population is limited to what is present in the two parents and the genetic resolution is relatively low due to high linkage disequilibrium (LD) (Flint-Garcia et al. 2003; Gupta et al. 2014). Therefore, validation of the QTL effects and the markers flanking the QTL in other populations is often necessary. In addition, further development of large secondary fine-mapping populations is required to look for candidate genes within a narrow genomic region.

LD-based association mapping (AM, or genome-wide association mapping, GWAS) is an alternative to biparental interval mapping. Polymorphic markers associated with a phenotypic trait can be identified by means of linkage disequilibrium (LD) between loci (Thornsberry et al. 2001; Flint-Garcia et al. 2003). One advantage of this approach is that the time consuming development of inbred or doubled haploid lines of a bi-parental mapping population is avoided (Crossa et al. 2007). The larger number of historical recombination events in diverse AM panels allows for higher resolution than biparental populations and more effective fine-mapping. Also, QTL discovered in a bi-parental population are relevant only for breeding programs where lines segregate for those QTL, while an AM panel can be designed to capture

most of the available genetic variation (Gupta et al. 2014). However, one drawback of AM is the reduced ability to capture rare alleles, while in a biparental population the allele frequencies are approximately 50/50 (Gupta et al. 2014). In addition, the risk of both reporting "false positive" (Type I error) and "false negative" (Type II error) results is higher in AM mapping than in biparental population. Unless population structure and relatedness is accounted for, false associations (Type I error) can occur that are due to relatedness between the individuals rather than genetic linkage. Type II error leads to reduced power of the AM. In this case, true associations are discarded. In AM, many markers are usually used, and multiple tests are applied to test the marker-trait associations. If the threshold is calculated on the assumption that all these tests are independent, it may be too strict, since many markers are genetically linked and thus not independent.

GWAS has been used successfully to identify marker trait associations (MATs) in complex traits in crop plants including disease resistance genes and quality traits (Breseghello and Sorrells 2006; Crossa et al. 2007; Tommasini et al. 2007; Ghavami et al. 2011; Perez-Lara et al. 2017). Only a few GWAS studies have investigated SNB resistance, and only at the seedling stage. In addition to the QTL detected in the biparental studies above, Adhikari et al. (2011) identified QTL on 6A and 7A in a panel of 567 spring wheat landraces from the USDA Small Grains Collection. Gurung et al. (2014) identified a novel QTL on 3A in 528 lines from the same collection. Also, a QTL on 3BS was investigated in a small panel of 44 cultivars by Tommasini et al. (2007) and QTL were detected on 5A, 5B and 5D after GWAS of 70 hard red winter wheat lines after inoculation with isolate Sn4 (Liu et al. 2015). While no GWAS study has been performed to identify SNB adult plant resistance, the method has successfully been used to identify marker-trait associations of adult plant resistance in comparably polygenic pathosystems, like Fusarium head blight (Miedaner et al. 2011) and Septoria tritici blotch caused by *Zymoseptoria tritici* (Perez-Lara et al. 2017).

In this study, we investigated 121 spring wheat lines with relevance for Norwegian wheat breeders. Most of the lines in the collection were Norwegian cultivars, breeding lines and landraces, but also international lines, mainly from Sweden and the International Maize and Wheat Improvement Center (CIMMYT) in Mexico. Introgression from CIMMYT and Swedish lines has influenced the Norwegian breeding programs, and some Swedish cultivars are also grown commercially in Norway. The objectives of the study were to 1) evaluate the genetic basis of seedling and adult plant resistance to SNB infection in the spring wheat collection, 2) identify markers associated with sensitivity to the purified effectors SnToxA, SnTox1 and

SnTox3 and 3) investigate whether known or novel NE-*Snn* interactions underlie the marker-trait associations (MTAs) related to SNB susceptibility. The wheat lines and cultivars used in this study represent a subset of 121 genotyped lines from the collection studied in **Paper II**. In **Paper II**, the prevalence of sensitivity to SnToxA, SnTox1 and SnTox3 in the material was investigated. The results from **Paper II** showed, in brief, that sensitivity to SnToxA was present in 45 % of 157 lines. Sensitivity to SnToxA was significantly associated with higher SNB disease severity in the field, and the MTAs will be investigated here. SnTox3 sensitivity was present in 55 % of the genotypes, while sensitivity to SnTox1 was rare. Sensitivity to these NEs was not correlated to increased SNB severity in the field.

Materials and methods

Plant material

A total of 121 spring wheat lines were genotyped and analyzed in the GWAS study. The lines are a subset of a Nordic spring wheat GWAS mapping panel for marker assisted selection ("MASbasis"), and includes both Norwegian and international lines. The population is described in more detail in **Paper II.**

Field data

The 121 lines from the Nordic spring wheat collection were planted in hill plot trials during the 2010 to 2016 field seasons at Vollebekk Research Station, Ås, Norway, as described in Paper II. The natural infection and development of SNB was enhanced by mist irrigation five minutes every half hour during daytime. From 2013 naturally infected straw was spread in the field at Zadoks' stage Z13/21 (Zadoks et al. 1974) to further promote SNB infection. The plants were sown in hill plots in an alpha lattice design with 2 to 3 replicates per year. Leaf blotch severity was scored twice every season by estimating the percent diseased canopy in the individual plots. Plant height and days from sowing to heading were used as covariates in multiple regression to obtain a more correct resistance score. See **Paper II** for further details.

P. nodorum isolates

62 single spore isolates of *P. nodorum* were isolated from leaves collected from unsprayed wheat fields in Norway in 2012 to 2014. For DNA extraction, the isolates were grown in the dark on PDA for 1-2 weeks. and DNA was extracted from the mycelium with the DNeasy Plant DNA extraction kit (QIAGEN). PCR screenings for *SnTox*-genes and actin were performed as described in Gao et al. (2015). Four isolates were selected for the full inoculation and

infiltration assays based on their different SnToxA/SnTox1/SnTox3 profile obtained from the PCR results. Additional isolate selection was based on variation in virulence after inoculation and infiltration with culture filtrate (CF) of a subset of differential lines and important lines from the Nordic spring wheat GWAS panel.

The isolates NOR4, 201593 and 201618 have been described and tested on the biparental RIL population SHA3/CBRD × Naxos (Ruud et al. 2017). Isolate 201614 was collected from the Swiss cultivar Quarna in Kure, Østfold, Norway, and it was selected since it lack SnToxA which masks or is epistatic to the SnTox3-*Snn3* interaction (Friesen et al. 2008b; Friesen et al. 2008c), but harbored SnTox1 and SnTox3. Infiltration with purified effectors was described in **Paper II**.

Inoculation experiments

Three seeds per genotype were planted in a randomized design in plastic cone-tainers in racks fitting 98 cones (Stuewe and sons, Tangent, Orlando, USA), with potting mixture soil (Gartnerjord, Tjerbo, Norway). The susceptible cultivar Brakar was used as a border to avoid border effects. The plants were grown in the greenhouse with 20 ° C day/16 ° C night temperature, 16 h light cycle and 65 % relative humidity.

The *P. nodorum* isolates were grown on V8-PDA at 20-23 ° C under 24 hour light (white plus near ultra-violet, NUV) for approximately seven days or until sporulation. The cultures were flushed with distilled water and scraped with an inoculation loop to release spores. The spore concentration was measured with a hemocytometer and adjusted to a final concentration of 1 × 10^6 spores per mL. One drop of Tween 20™ was added per 50 mL spore suspension to reduce surface tension. The two weeks old seedlings were inoculated with a spray painter until runoff, placed in a humidity chamber at 100 % relative humidity and constant light for 24 hours before returned to the greenhouse. Seven days after inoculation the disease reactions were scored on the 0 to 5 scale described by Liu et al. (2004b). All experiments were repeated three times.

Infiltration experiments

The infiltration with semi-purified effectors was described in **Paper II**.

The plants were grown as described for inoculation experiments, but with two seeds per cone per genotype. The *P. nodorum* isolates were grown as described for preparation of inoculum until sporulation (5-7 days). The cultures were then flushed with distilled water and scraped with a flame sterilized inoculation loop to release the spores. 6 µL of the spore solution was

inoculated into 60 mL liquid Fries 3 medium in 250 mL Erlenmeyer flasks. The liquid cultures were placed on a rotary shaker at 100 rounds per minute (RPM) and 27 ° C for 72 hours before placed in darkness in an incubation chamber at 20 ° C for three weeks. The cultures were then filter sterilized and a needleless syringe was used to infiltrate the CF into the second leaf of 12-14 days old seedlings. The sensitivity reactions were scored on a 0-3 scale after 5-7 days according to the protocol described in Friesen and Faris (2012).

Genotyping

Genomic DNA was extracted from young leaves with the DNeasy Plant DNA extraction kit (QIAGEN). The 121 lines from the Nordic spring wheat collection were genotyped with the Illumina iSelect 90K wheat SNP Chip (Wang et al. 2014). Analyzing and scoring of the genotype results was performed manually for every SNP marker with the software Genome Studio Genotyping Module v1.0 from Illumina. Microsatellite (SSR) analysis was performed with fluorescently labeled primers and PCR products were separated by capillary electrophoresis on an ABI 3730 Gene Analyzer. The polymorphic SSR markers were converted to biallelic states. The fragment lengths of significant SSR alleles are given in base pairs (bp) next to the marker name in the results.

The genotype data was filtered to remove minor allele frequencies (MAF) at a ≤ 0.05 threshold. Markers were considered good if more than 90% of the lines had a genotype for the particular marker, and a minor allele frequency $\geq 5\%$. Low quality markers were filtered out based on markers being “no call” or had many lines with many heterozygotes. In addition, a final filtration was performed where the markers having less than 5 lines with the most rare allele were removed. Only unique markers, based on segregation of genotypes in the AM panel, were included. The final set for GWAS consisted of 22 031 SNP and SSR markers.

The polymorphic markers were given positions based on the consensus map developed by Wang et al. (2014). SSR markers significantly associated with the traits were placed on the map with approximate positions based on information from the integrated maps by Li et al. (2015) (hexaploid) and Maccaferri et al. (2015) (tetraploid, durum), the consensus map by Somers et al. (2004), and linkage (measured as linkage disequilibrium) with other significant SNP markers with known position.

Population structure

Population structure was calculated using a subset of 338 SNP markers chosen with 5 cM intervals based on the consensus map (Wang et al. 2014). The population structure and

underlying number of subgroups (K) were estimated with a Bayesian clustering method in STRUCTURE v.2.3.4 (Pritchard and Falush 2007). The analysis was performed with K = 10, 5000 burnin length and 50 000 reps over 3 iterations. For estimated K, the STRUCTURE results were run in Structure Harvester (Earl and vonHoldt 2012). Also, the relationships between the genotypes were calculated in TASSEL v.5.0 (Bradbury et al. 2007) to produce a kinship matrix (*K*) using the centered identity-by-state IBS (Endelman - previously Scaled_IBS) method, which produces a kinship matrix that is scaled to give a reasonable estimate of additive genetic variance. Principal component analysis (PCA) with five components was also calculated in TASSEL from the filtered genotypic data and used as a Q-matrix.

Linkage disequilibrium (LD)

LD was calculated across the genome using the locations of the SNPs on the consensus map from Wang et al. (2014). Only SNPs with minor allele frequencies (MAF) > 0.05 were included in the calculations. The pairwise LD was measured in TASSEL v.5.0 (Bradbury et al. 2007), using the squared allele frequency correlation r^2 (Hill and Weir 1988). *p*-values for each r^2 estimate were calculated using 1000 permutations and Fisher's exact test in TASSEL. The loci were considered to be in significant LD when $p < 0.001$.The rate of LD decay and LD levels were assessed by plotting the r^2 values for significant intra-chromosomal loci against genetic distance (cM) between marker pairs. The relationship between LD decay and distance was summarized by fitting the data to a non-linear model as described in Marroni et al. (2011). The estimated maximum value of LD was used to calculate the half decay distance. Significant LD was also measured across each subgenome.

Association analysis

Least squares means (LSM) were obtained for each trait using PROC MIXED in SAS 9.3 (SAS Institute, Inc.). Replications and environments (years) were treated as random effects, while genotypes were considered fixed effects. Pearson correlation coefficients were computed in R Studio (RStudio Team 2016) using the Hmisc package. Heritabilities and ANOVA results for the field data can be found in **Paper II**.

Six different regression models were tested: Naïve (General linearized model, GLM), GLM + Q (Population structure), GLM + PC (Principal components), MLM + K (mixed linear model + kinship matrix), MLM + K+ Q and MLM + K + PC. The best model based on the generated

qq-plots (Figures S1 and S2) was the MLM + K + Q model. Genomic regions associated with the traits were identified using the weighted compressed mixed linear model (MLM) in TASSEL v.5.0 (Bradbury et al. 2007). A *p*-value was generated by fitting each SNP marker into the MLM that has the form $y = X\beta + Qv + u + e$, where y is the vector of the phenotypic values (best linearized unbiased predictors, BLUPs), X is the vector of SNP marker genotypes, β is the vector of marker fixed effects to be estimated, Q is the population structure matrix derived from Structure analysis, v is a vector of fixed effects due to population structure, u is the vector of random effects and e is the vector of residuals.

Selection of significance threshold

A threshold where *p*-values ≤ 0.001 are considered significant has been used in several comparable studies (Kollers et al. 2013; Gurung et al. 2014; Kollers et al. 2014; Singh et al. 2016). Pasam et al. (2012) suggested that the bottom 0.1 percentile of the distribution of *p*-values obtained can be considered as significant and we followed this method. A rough approach to further evaluate the threshold was performed by visually inspecting the qq-plots (Figures S1 and S2). True association between marker and trait is expected where the line deviates in a flat pattern from the expected/observed line. In general, this deviation was observed at a slightly higher threshold than the 0.1 percentile. To provide a complementary summary of declared putative QTL, Manhattan plots were generated in TASSEL v 5.0 and visualized in R Studio (RStudio Team 2016) with the R package qqman (Turner 2014).

BLAST

SNPs associated with *Tsn1* were blasted using https://triticeaetoolbox.org/ and the TAGCv1 wheat genome assembly by Clavijo et al. (2017) at http://plants.ensembl.org/ to investigate whether they were located within the NBS-LRR gene or in closely linked genes (Faris et al. 2010).

Results

Pathogen characterization

Table 1 shows the frequencies of SnToxA, SnTox1 and SnTox3, respectively, in 62 Norwegian isolates collected between 2012 and 2014, based on PCR screenings. All the three effector genes were present in more than 50 % of the isolates, and the proportion was highest for SnTox3 and SnToxA.

Table 2 shows the reactions of the 10 differential lines after infiltration with filter sterilized CF from single isolates. Reactions scored as 2 and 3 indicate presence of the NE causing sensitivity in the differential line, although other, unknown NE-*Snn* interactions can also underlie the reaction.

Correlation between seedling disease reactions and sensitivity reactions

Table 3 shows the correlation coefficients between the disease reaction types after inoculation and sensitivity reaction types after infiltration with culture filtrate (CF) with the four different isolates. The correlation between disease reactions after inoculation with the single isolates was significant and in some cases very high – i.e. the correlation between isolate 201593 and 201614 (0.77) and NOR4, 201593 and 201614. The correlation between reaction types after inoculation with isolate 201618 and the other isolates was lower, but still. The correlation between the sensitivity reaction types after infiltration with CF and disease reaction types after inoculation was significant for isolates NOR4, 201593 and 201614. The highest correlation was observed between disease reaction types after inoculation with isolate 201593 and sensitivity reactions after infiltration with CF from isolate 201614. The correlation between disease reaction type after inoculation and sensitivity reaction after infiltration with isolate 201618 was weak, but significant. Sensitivity reaction types to infiltration with isolate 201618 were not significantly correlated with inoculation results from the other three isolates. The correlation between disease reaction type after inoculation with single isolates and sensitivity reaction after infiltration with single effectors was moderate, and highest between disease reactions after inoculation with isolate NOR4 (which produces SnToxA) and sensitivity reaction types after SnToxA infiltration.

Figure 1 shows the histograms of the corrected SNB severity distribution, based on the field data. The histograms confirms that the severity follows the normal distribution. Hence, the requirement for application of linear mixed models is met.

Figure 2 shows histograms for the distribution of disease reaction scored after seedling inoculation experiments with the four isolates. While the normal distribution is met with isolates 201593 and 201614, the distribution of isolate NOR4 is skewed to the right, and is also different for isolate 201618. However, transformation did not improve the data, so the untransformed data was used in the subsequent analyses.

Correlation between seedling disease reactions and field disease severity

The correlation between disease reactions after seedling inoculations and corrected SNB severity from field trials, ranged between -0.005 (isolate 201618 and year 2012) and 0.54 (isolate 201593 and year 2014) (Table 4). In general the results from the seedling inoculations with isolates NOR4, 201593 and 201614 showed significant correlation to corrected SNB severity in the field, although the correlation varied between individual years. 2012 was the year with lowest correlation between the field data and the seedling inoculation results. The results from inoculation with isolate 201618, which does not contain SnToxA, SnTox1 or SnTox3, were not significantly correlated to any year of corrected SNB severity from the field trials. The correlation between sensitivity reaction type after SnToxA infiltration at the seedling stage, and corrected SNB severity from the field trials, was significant in several years. There was no correlation between the results of SnTox3-infiltration and results from the field experiments.

The correlation between sensitivity reaction type after CF infiltration experiments and corrected SNB severity from the field was not significant. Only correlation between corrected SNB severities, SnToxA and SnTox3 infiltrations, and seedling inoculation experiments, are included in Table 4.

Population structure and linkage disequilibrium

STRUCTURE was run with K from 1 to 10 for the 121 spring wheat lines, and the estimated ΔK gave K = 5 subpopulations. The population structure has been described in more detail in the master thesis by Jansen (2014). The estimated r^2 for half decay was 0.23 (critical value of r^2) and the genome wide half decay distance was 4 cM (Figure 3). The r^2 for initial LD ($p <$ 0.001) was 0.49 and varied on each sub-genome between 0.47 (A-genome) and 0.63 (D-genome). The LD decayed to 50 % of the initial value at 3 cM in both the A and B genome, and 6 cM in the D-genome.

Association mapping

SnToxA and SnTox3 infiltrations

The Manhattan plot in Figure 4 shows the markers associated with SnToxA sensitivity. The significant markers were located on 5BL and the SSR markers (*fcp1, fcp620, fcp394*) are known to be closely linked to *Tsn1*. Three SNPs (*Tdurum_contig12066_126, Tdurum_contig12066_247* and *tplb0027f13_1346*, marked * in Table 5) were assigned to 5A

in the consensus map (Wang et al. 2014) but were in complete LD with the significant markers on 5B, so they were placed in the same position as the other significant markers on 5B.

BLAST searches using https://triticeaetoolbox.org/wheat/ and http://plants.ensembl.org/ showed that most of the detected SNPs were located in a zinc finger domain (*wsnp_Ku_c40334_48581010*) and a potassium transporter (*Tdurum_contig12066_126, Tdurum_contig12066_247, BobWhite_c48435_165*). *Excalibur_c37642_1416)* was located in a P-loop containing nucleoside triphosphate hydrolase.

The Manhattan plots in Figure 5 show markers associated with sensitivity to SnTox3. Sensitivity to SnTox3 was expressed as chlorosis (Type 2 reaction) in some genotypes, and a strong necrosis with tissue collapse (Type 3) in other genotypes. The Type 2 reaction was mores associated with the SSR markers *cfd20* and *gwm234*, and SNPs assigned to 5A in the consensus map (Wang et al. 2014). The Type 3 reaction mapped to 5B, but some markers were also significant on 2D and 2A. Also, the SSR markers *cfd20* and *gwm234* known to be associated with SnTox3 sensitivity from literature seemed to be more associated with the Type 2 reaction, not the Type 3 reaction.

Seedling inoculation with single isolates

The most significant MTAs after inoculation were detected on 5B associated to SnToxA after inoculation with isolate NOR4 (Figure 6 A, Table 7) and SnTox3 sensitivity after inoculation with isolate 201593, respectively (Figure 6 B, Table 7). Another QTL was detected on 4B and was significant after inoculation with NOR4, 201593 and 201614. QTL were also detected on 3A after inoculation with isolate 201593 (Figure 6 B, Table 7), on 6B and 7A after inoculation with isolate 201614 (Figure 6 C, Table 7), and on 1A and 1B after inoculation with 201618 (Figure 6 D, Table 7).

Infiltration

Significant markers were detected on many chromosomes after infiltration with filter sterilized CF from the four isolates (Figure 7, Table 8). The most significant markers were located on 5B associated with SnTox3-sensitivity (Figure 7 A, B, C, Table 8), and chromosomes 6B and 1A. The QTL on 6B was significant after infiltration with NOR4 and 201593, and was in the same position as the QTL detected after inoculation with 201614. The two QTL detected on 1A after infiltration with isolate 201614 were not identical with the QTL on 1A detected after inoculation with isolate 201618.

Field experiments

Corrected SNB severity scores obtained by multiple regression with plant height and days to heading as covariates, were used for the GWAS of adult plant SNB resistance from the field trials. Unique QTL for adult plant SNB resistance were detected on almost all chromosomes in at least one environment (year). However, to be considered robust, a QTL should be significant across at least two environments. The most consistent QTL were identified on 2B, 2D, 4A, 4B, 5A, 6B, 7A and 7B and highlighted in Figure 8.

Discussion

SnToxA, SnTox1 and SnTox3 characterization of Norwegian isolates

Based on the screening of 62 individual *P. nodorum* isolates, we found that the *SnToxA*, *SnTox1* and *SnTox3* genes were present in the majority of the isolates (Table 1). In particular, the frequency of *SnToxA* was significantly higher in the Norwegian isolates than reported from Switzerland, where only 10 % of the isolates carried *SnToxA* (McDonald et al. 2013). Sensitivity to SnToxA was also common in the Norwegian breeding material and cultivars (45 %, **Paper II**), and we speculate whether the high frequency of SnToxA in the isolates is an adaptation of the pathogen to the local host cultivars. More exhaustive collection and NE-screening of the pathogen population should be performed to validate whether the frequencies are representative for the Norwegian *P. nodorum* population.

Correlation between seedling disease reaction and sensitivity reaction

The isolates used for inoculation and infiltrations were chosen based on different presence of the main effectors SnToxA, SnTox1 and SnTox3, and for showing sufficient virulence levels in pre-screenings on selected lines (Table 2). The correlations between disease reactions after inoculations with the four single isolates (Table 3) were generally high, indicating some shared infection and resistance mechanisms. This may also include other NEs than SnToxA and SnTox3. Table 4 showed that the correlation between corrected SNB severity from field trials and the results from seedling inoculation was higher than correlation between corrected SNB severity and purified NE (SnToxA and SnTox3) infiltration. The higher correlation between the results from seedling inoculations and field trials supports the assumption that other mechanisms and NEs contribute to disease development, in addition to SnToxA and SnTox3.

Population structure and linkage disequilibrium (LD)

The population split into five distinguishable subpopulations. The grouping in subpopulations can be justified based on origin, since the material grouped as mainly: CIMMYT, CIMMYT and Chinese lines from Fusarium Head Blight (FHB) resistance breeding programs, Swedish, Norwegian and European other than Scandinavian. The genome wide LD is expected to be high due to homozygosity in the inbreeding species, and the relatively narrow germplasm represented in mainly elite germplasm (Breseghello and Sorrells 2006). The genome wide LD decayed to 50 % at ca 4 cM. This is comparable to what was found in the mainly current breeding material and cultivars investigated by Chao et al. (2010), but 1/10 of the CIMMYT historical bread wheat panel (Crossa et al. 2007). An reason why the LD decay is slower in the CIMMYT material is probably the use of synthetic lines and introgression of haplotypes from divergent populations (here: species) which can increase LD extent (Chao et al. 2010).

Model testing for association mapping

False positive associations is a limitation to GWAS. This may be accounted for by including population structure (Q) and kinship (K) in the linear models. Based on evaluation of the qq-plots (Figures S1 and S2), we found that overall, MLM models were better than GLM, and that the best model included both a population structure matrix (Q) from STRUCTURE (Pritchard and Falush 2007) and a centered IBS kinship matrix (K) from TASSEL (Bradbury et al. 2007). However, results between MLM models with PCA + K or Q + K were almost identical (Figures S1 and S2). Also, MLM + K models were very similar to MLM + K + Q and MLM + K + PC models (Figures S1 and S2), indicating that familial relatedness accounts for most of the false positive marker trait associations. Including the Q matrix in a GLM model (GLM + Q) had an effect compared to the Naïve model (GLM with no Q matrix) (Figure S1 and S2). In general, the GLM models were inflated, overestimated the significance of the observed associations and strict correction for false positive association would be necessary. Although the $-\log10(p)$ values were lower in the MLM models, these models compensated better for relatedness, since both kinship and population structure matrices were included in the analysis. Jiang et al. (2015) found that within an investigated range of p-values, increasing the power of QTL detection with a more relaxed significance threshold was more relevant than increasing the risk to detect false-positive QTL. Relatedness can be exploited better in GWAS and contributes to the accuracy of QTL detection, and can be portrayed more precisely with more relaxed significance

thresholds. While the effect of population structure seemed to be accounted for by the K matrix, we also included the Q matrix in our model.

Association mapping

Infiltration with purified SnToxA, SnTox1 and SnTox3

As shown in **Paper II**, 45, 12 and 55 % of the 157 genotypes in the Nordic spring wheat collection were sensitive to SnToxA, SnTox1 and SnTox3 respectively. Of the 121 lines that were genotyped and used for the GWAS in the present study, 46, 14 and 58.5 % of the lines were sensitive to SnToxA, SnTox1 and SnTox3. The low frequency of lines sensitive to SnTox1 was in line with other screenings of hexaploid wheat (Shi et al. 2016a), but also highlights a limitation for GWAS: Rare allele frequencies may lead to exclusion of potentially associated markers, and rare genetic variants often escape detection, contributing to the phenomenon called "missing heritability" (Gupta et al. 2014). No significant association between markers and SnTox1-sensitivity was found in the GWAS analysis and the results are not included. Insufficient linkage of the *Snn1* locus to the markers may be an additional explanation.

Highly significant SNP and SSR markers for SnToxA-sensitivity were detected on 5B (Figure 4, Table 5). Three of the markers (Marked with * in Table 5) were placed on 5A in the consensus map (Wang et al. 2014), but were in complete LD with the markers on 5B. They also mapped to 5B in several bi-parental mapping populations (Table S6, Wang et al. (2014)). These markers also corresponded to the 5A locus reported by Liu et al. (2015) upon inoculation of a GWAS panel with a SnToxA producing *P. nodorum* isolate, but we suggest that these markers are located close to the *Tsn1* locus on 5B. The BLAST searches supported that the significant SNPs were located in genes previously identified close to *Tsn1* (Faris et al. 2010), for instance a zinc finger and a potassium transporter. One SNP (*Excalibur_c37642_1416)* was also located in a P-loop containing nucleoside triphosphate hydrolase, which is a nucleotide binding domain. However, this SNP was not tightly linked to SnToxA sensitivity, and we could not find SNPs unambiguously associated to a NBS-LRR gene like *Tsn1*. This may be explained by the fact that Chinese Spring which was the source of the reference genome to which the genes are annotated, is insensitive to SnToxA and this insensitivity is usually caused by gene loss (Faris et al. 2010). The identified SSR markers were known from the literature to be linked to *Tsn1* (Faris et al. 2010).

While the sensitivity reaction after infiltration with SnToxA appeared to be qualitative with infiltration resulting in either necrotic or insensitive leaves, the reaction to SnTox3 was quantitative. Some genotypes developed chlorosis, but not necrosis, after infiltration with SnTox3. In other genotypes SnTox3 induced a strong necrosis. This has also been observed by others (Waters et al. 2011; Shi et al. 2016b). The reaction types could be coded and analyzed separately. The SSR markers *cfd20* and *gwm234* known from literature to be associated with *Snn3* (Friesen et al. 2008a), were significant for the Type 2 reaction (Figure 5B, Table 6 B). These markers have been mapped to the distal end of chromosome 5BS (Friesen et al. 2008a). SNP markers significantly associated to the Type 2 reaction were also detected on chromosome 5A (Figure 5 B, Table 6 B). The markers most strongly associated with the Type 3 reaction (Figure 5 C, Table 6 C) were identical with markers detected in the biparental mapping population SHA3/CBRD × Naxos (Ruud et al. 2017). Interestingly, the SSR markers *cfd20* and *gwm234* were not associated with the Type 3 reaction type in GWAS (Figure 5 C, Table 6 C), and were monomorphic in the biparental population.

Only Type 2 and Type 3 reaction were considered as sensitive reactions. While Type 1 reaction (mottled chlorosis) is considered as insensitive here, this reaction type may also be indicative of a quantitative, stepwise sensitivity. Both linkage to markers and to different reaction types seemed to be dependent on the origin of the wheat material. For instance, we showed in **Paper II** that the Type 2 reaction was the only sensitivity reaction to SnTox3 in the Swedish material. These relationships need to be investigated in more detail. Whether the stronger reaction is caused by for instance stronger affinity of the NE to one allelic variant of *Snn3*, or if modulating factors are involved, also need to be resolved. The QTL on 2D detected when the Type 3 reaction is analyzed alone (Table 6 C) may be indicative of a regulatory factor. However, the qq-plot (Figure S1) indicated that the markers on 5BS might be the only true associations. Further studies with suitable mapping populations should be carried out to answer these questions.

Seedling inoculations

The most significant QTL detected after seedling inoculations with single isolates were located on 5B (Figure 6, Table 7). The SnToxA-*Tsn1* interaction on 5BL was the most important after inoculation with isolate NOR4 (Figure 6A). As expected based on epistasis between SnToxA and SnTox3 (Friesen et al. 2008a), the SnTox3-*Snn3* interaction was not significant although isolate NOR4 also harbors *SnTox3*. After inoculation with isolate 201593 which is *SnToxA* negative, the SnTox3-*Snn3* interaction was significant, and the MATs corresponded to the

Type 3 sensitivity reaction. Although isolate 201614 also carries *SnTox3* and not *SnToxA,* the SnTox3-*Snn3* interaction was not significant after inoculation, but the markers were detected below the significance threshold. It has been reported before that presence of SnTox1 modulates the production of SnTox3 (Phan et al. 2016). *SnTox1* was present in roughly 50 % of the investigated isolates (Table 1), including isolate 201614, and may play a role in reducing the effect of SnTox3.

QTL were detected on 1AS and 1B after inoculation with isolate 201618 (Figure 6 D, Table 7). The QTL on 1AS was not significant after inoculation with the other isolates, but the markers could be detected under the threshold (Figure 6 A, B, C). *Snn4* is located on 1AS (Abeysekara et al. 2009). However, infiltration of the *Snn4* differential line AF 89 with CF from the isolate 201618, did not produce a sensitive reaction (Table 2).

The QTL on 1B seemed to be isolate specific, but was also significant in the field (2011, Figure 8 B; Table 9). *Snn1* is located on 1BS (Liu et al. 2004a), but in a more distal position than the QTL detected in the present study. A QTL on 1B was also detected after inoculation with the same isolate of the SHA3/CBRD × Naxos population (Ruud et al. 2017), but also had a more distal position on the chromosome.

A QTL on 3A was detected after inoculation with isolate 201593, but not the others, and may be specific to this isolate. This QTL was also detected after infiltration with the same isolate (Table 8, Figure 7 B) and may be a novel NE/*Snn* interaction. Two loci associated with seedling SNB resistance were detected on 3A by Gurung et al. (2014), but not in close proximity to the locus identified here when the marker positions for the loci were compared on the consensus map by Wang et al. (2014). Two QTL for SNB resistance were also identified on 3A at the adult plant stage in SHA3/CBRD × Naxos (Ruud et al. 2017). One of these (3AS.2) corresponded to one of the QTL identified by Gurung et al. (2014), but none with the QTL detected in the present study.

A significant QTL was detected on 4B after inoculation with NOR4, 201593 and 201614 (Table 6, Figure 7 A, B, C). The locus was also significantly associated with corrected SNB severity in the field in 2011 and 2014 (Table 9). At least two QTL for seedling SNB resistance have previously been identified on 4B (Liu et al. 2004b), including *Snn5* (Friesen et al. 2012). The use of different markers makes it difficult to compare the QTL, but the QTL described by Liu et al. (2004b) was located on the long chromosome arm, while the QTL detected in the present study and *Snn5* are located on the short arm. The QTL was, however, not detected after

infiltration (Table 7, Figure 8), which can indicate that the underlying factor was not an NE-*Snn* interaction. The interaction could also have been masked by SnTox3-*Snn3* or the NE is not consistently produced in liquid culture.

Infiltrations with CF from single isolates

SnToxA is a major pathogenicity factor when SnToxA-producing *P. nodorum* isolates are inoculated on *Tsn1*-harboring wheat genotypes. However, SnToxA is usually not produced in liquid *P. nodorum* culture. As a consequence we could not find the effect of SnToxA-*Tsn1* after infiltrating the GWAS panel with CF of NOR4 (Figure 8 A, Table 7). Significant markers were found on 5B associated to *Snn3*, 1B and 6B after NOR4 CF infiltration. The QTL on 6B was also detected after inoculation with isolate 201614, and infiltration with isolate 201593. Interestingly, considering the result from inoculation, the QTL was not significant after infiltration with 201614. This can illustrate the relative effect of individual interactions depending on presence of other NEs or infection mechanisms, or that the NE was not reliably released in liquid culture. As far as we know, no sensitivity locus has been detected on 6B in other studies. Further evaluation, including testing of segregating F_2 lines from biparental crosses would be necessary to investigate whether this is a novel NE-*Snn*-interaction.

After infiltration with isolates 201593 and 201614 the SnTox3-*Snn3* interaction was the most significant (Figure 8 B, C, Table 7). A QTL was also detected on 1AS, but was not the same QTL as the one identified after inoculation. *Snn4* is located on 1AS, but we could not validate that *Snn4* was underlying the QTL we detected, due to different markers used in the studies. Only CF from isolate 201593 induced sensitivity in the *Snn4* differential line AF89 (Table 2).

The most significant markers after infiltration with 201618 were located on 5A, 4D and 5D. Other significant markers were found on 2B and 7B. The QTL on 3A was not the same as detected after inoculation and infiltration with isolate 201593.

To summarize, we could validate that SnTox3 was a major pathogenicity factor in the infiltration assays in all SnTox3-harboring isolates, even when the interaction is not important after inoculation. Other NEs or other secreted molecules (enzymes, secondary metabolites) seemed to play a role as well, given the number of significant QTL detected after infiltration. Only a few of these were significant after both inoculation and infiltration, namely 3A and 6B, Although detected at a relatively low threshold, the significant markers on 3A may identify a novel NE/*Snn* interaction specific for isolate 201593. The markers on 6B were significant after inoculation with two isolates and infiltration with another isolate, highlighting the relative

influence of other effectors and mechanisms present in the individual isolates. In many studies where NE/*Snn*-interactions have been characterized, knock-out mutants of the isolates have been utilized, also illustrating the potential problems with multiple and not always additive interactions. Either the suite of NEs produced by the pathogen or the corresponding *Snn* genes in the host have to be compatible in a way that allows us to study just the interactions of interest and not mask them. To further investigate the QTL on 3A and 6B, genotypes with single sensitivity reactions could be crossed with insensitive lines to develop mapping populations.

Field experiments

QTL were detected at least once on almost every chromosome. Only the QTL identified in two or more years and/or in both seedling and inoculation experiments were considered as robust and promising for breeding. Consistent QTL were detected on 2B, 2D, 4A, 4B, 5A, 6B, 7A and 7B across at least two years (Figure 8, Table 9). The QTL on 2B, 2D, 4A and 7A were the most stable loci. Since the field trials relied on natural infection, variation was expected due to fluctuations in the pathogen population. This was also supported by the findings that no single QTL was significant in every year in the field trials, and that the importance of individual QTL varied between years. Additionally, other G × E interactions contributed to the variation between the individual years of field trials.

The QTL on 2B seemed to be novel, although a QTL has been described for seedling SNB resistance (Czembor et al. 2003). The most consistent QTL in the spring wheat panel was detected in all years except 2012 and was also significant for the mean across years, and was located on 2DL. At least two QTL for SNB flag leaf resistance have been described on 2D earlier (Aguilar et al. 2005; Shankar et al. 2008) – one on the long arm and one on the short arm. The short arm QTL was located in the same region as *Snn2* (Francki 2013) Since different markers were used in these studies and the marker resolution is low on the D genome, it is difficult to compare the results, but the QTL identified in our study could be the same as described by Aguilar et al. (2005). If so, it confirms the importance and robustness of this locus. The *Snn7* locus is also situated on 2DL (Shi et al. 2015). The QTL on 4A which was significant in 2011, 2015, 2016 and across years (Figure 8 B, F, G, H, Table 9) appeared to be novel. The QTL on 4B was described above, as it was also detected in the seedling inoculation assays (Table 7). QTL for adult plant resistance to SNB on 5A has not been described earlier to our knowledge. Hence, the QTL significant in 2014 and 2016 were probably novel. No QTL has been described for SNB resistance on 6B either. The QTL on 6B significant in 2011 and 2014 (Figure 8 B, E) was different than the QTL detected on 6B in seedling screenings. The QTL on

7A detected in 2011 and 2013 (Figure 8 B, D) and after inoculation with isolate 201614 (Figure 6 C), mapped to the same locus as the QTL reported in SHA3/CBRD × Naxos (Lu and Lillemo 2014; Ruud et al. 2017). The QTL significant in 2010 and 2014 seemed to be unique and did not correspond to other QTL on 7B detected at the seedling stage or in SHA3/CBRD × Naxos (Lu and Lillemo 2014; Ruud et al. 2017). A QTL was also detected on 1B after inoculation with isolate 201618 and in the field in 2011 (Figure 8 B). The markers and positions were not identical, but sufficiently close to be considered the same QTL, taking the half decay LD distance into account.

Effect of SnToxA-Tsn1 in the field

We have shown before (**Paper II**) that sensitivity to SnToxA is associated with higher disease severity in our spring wheat panel. Although the *Tsn1*-linked markers were not detected above the threshold they were the most significant markers on 5B in 2012 and 2014 (Figure 8 C, E). This illustrates the complexity of the trait and a limitation of GWAS to capture minor QTL in polygenic traits.

No effect was identified for the SnTox3-*Snn3* interaction. The masking effect of SnToxA-*Tsn1* over SnTox3-*Snn3* after inoculation at the seedling stage is well known. This effect was also demonstrated in the seedling experiments conducted here, after inoculation with NOR4 which produces both SnToxA and SnTox3 (Figure, Table). Both SnToxA and SnTox3 were prevalent in the 62 genotyped Norwegian *P. nodorum* isolates, and the corresponding sensitivities were present in approximately 50 % of the genotypes in the Nordic spring wheat collection. Perhaps SnToxA-*Tsn1* has a masking or epistatic effect on SnTox3-*Snn3* also at the adult plant stage.

Conclusions

This study is the first to use GWAS to investigate association of markers to adult plant resistance to SNB. Several novel loci were detected, and other, like the QTL on 7A in 2011 and 2013, could validate QTL from other studies. This validation is important for breeding purposes. The proportion of shared genetic basis between seedling and adult plant resistance was studied, and we could confirm that at least three QTL were important both at the seedling and adult plant stage, which make them interesting for breeding purposes. Although many QTL were detected in only one environment in the field trials, several stable QTL were also identified, and can also be used for marker assisted selection. In particular the QTL on 2DL was stable. The infiltration assays were not well correlated to adult plant resistance, but the detection of the same, novel QTL on 3A and 6B after both infiltration and seedling inoculation

could be investigated further with proper mapping populations. We are also investigating further the genetic basis for the two different reaction types to SnTox3, by developing mapping populations using parents differing in sensitivity reaction.

Conflicts of interest

The authors declare no conflict of interest.

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Figure captions

Figure 1 Histograms of the distribution of corrected Septoria nodorum blotch (SNB) severities from seven years of field trials (2010 to 2016) and the mean, in the Nordic spring wheat collection.

Figure 2 Histograms showing the distribution of disease reaction scores (0-5) after inoculation of two weeks old seedlings with the four *P. nodorum* isolates NOR4, 201593, 201614 and 201618.

Figure 3 Genome wide LD decay plot based on pairwise comparisons of loci. The red line shows the estimated LD points. The horizontal line represent the critical value for LD significance based on the estimated LD value for half decay, 0.23, calculated as described by Marroni et al. (2011).

Figure 4 Manhattan plot showing the significant SNP and SSR markers associated with reactions to SnToxA infiltration.

Figure 5 Manhattan plots showing significant SNP and SSR markers associated with A) average SnTox3 reaction, i.e. both chlorotic and necrotic reaction type, to infiltration with purified SnTox3, B) Type 2 (chlorotic) reaction to SnTox3 infiltration and C) Type 3 (necrotic) reaction to SnTox3. Note that the markers associated with the Type 2 reaction on 5B are SSR markers (*gwm234* and *cfd20*), and no SNP markers on 5B. For the Type 3 reaction only SNP markers on 5B are associated, not the SSR markers.

Figure 6 Manhattan plots with markers associated with SNB after inoculation with isolate A) NOR4, B) 201593, C) 201614 and D) 201618. Red rectangle: Markers associated with SnTox3 reaction. Blue rectangles: Markers associated with SnToxA reaction. Purple rectangles highlight SNPs with significance -log10(p) > 3, while the blue horizontal line represents the 0.1 percentile threshold.

Figure 7 Manhattan plots showing the SNPs significantly associated with reaction to culture filtrate (CF) infiltration with four Norwegian isolates A) NOR 4, B) 201593, C) 201614 and D) 201618. The colored rectangles indicate marker-trait associations that are significant at –log(p)>3, while the horizontal line represents the significance threshold at the 0.1 % percentile. Red rectangles are associated with SnTox3-sensitivity.

Figure 8 Manhattan plots of the corrected SNB severities per year: A) 2010, B) 2011, C) 2012, D) 2013, E) 2014, F) 2015), G) 2016, H) Mean. The horizontal blue line is based on the 0.1 percentile thresholds. The scale of the y-axis is different between plots. Purple rectangles highlight QTL that are significant in more one year in the field. Green rectangles highlight QTL that were significant both in inoculation experiments with single isolates and at least one year in the field. Blue rectangles highlight *Tsn1*-linked markers in years where they were the most significant 5B markers (although under LOD threshold).

Tables

Table 1 Frequencies of necrotrophic effectors (NE) in 62 Norwegian *P. nodorum* isolates based on PCR screening

NE	Frequency
SnToxA	0.69
SnTox1	0.53
SnTox3	0.76

Table 2 Sensitivity reaction to single isolate culture filtrate (CF) infiltration by differential lines with known, single SnTox-sensitivities. Reaction scale: 0 = Insensitive, no reaction, 1 = Weak, mottled chlorosis (insensitive), 2 = Sensitive, chlorosis, 3 = Sensitive, necrosis and tissue collapse.

Line	Provider	Sensitivity	NOR4	201593	201614	201618
BG261	T. Friesen	SnToxA	0	0	0	0
M6	T. Friesen	SnTox1	1	0	0	1
BG220	J. Faris	SnTox3	3	2	2	0
BG223	T. Friesen	SnTox2	2	2	2	2
LP29	S. Xu	Tox 4Bb (unpubl)	0	0	1	0
ITMI44	T. Friesen	Tox4Ba (unpubl)	2	0	3	2
ITMI37	T. Friesen	SnTox6	2	2	2	2
BR34	T. Friesen	Insensitive	0	0	0	0
CS(DIC1)	J. Faris	Tox5D (unpubl)	2	0	1	1
AF89	J. Faris	SnTox4	1	2	0	1

Table 3 Pearson's correlation coefficient between the disease reaction types after single spore inoculations with the four different isolates NOR4, 201593, 201614 and 201618, between disease reaction after inoculations and sensitivity reactions after infiltrations and between sensitivity reactions from infiltration.

		Inoculation			Infiltration				purified NE	
	Isolate	NOR4	201593	201614	NOR4	201593	201614	201618	SnToxA	SnTox3
Inoculati	NOR4				0.41***	0.21	0.24***	0.17	0.41***	0.17
	201593	0.66***			0.43***	0.41***	0.44***	0.16	0.28*	0.21
	201614	0.66***	0.78***		0.41***	0.35***	0.37***	0.23	0.21	0.24*
	201618	0.32***	0.25*	0.29**	0.097	-0.079	-0.009	0.24*	0.15	-0.06
Infiltration	NOR4					0.70***	0.71***	0.51***		
	201593						0.85***	0.41***		
	201614							0.34***		

*Significant differences at $p \leq p < 0.01$, 0,001, 0,0001, are indicated by *, **, *** , respectively*

Table 4 Pearson's correlation coefficient between disease reaction types from inoculation with single isolates and corrected Septoria nodorum blotch (SNB) severity from each year of the field trials, and correlation between sensitivity reaction types after infiltration with semi-purified NEs (SnToxA and SnToxA3) and corrected SNB severity from each year of field trials.

	Year							
Isolate	2010	2011	2012	2013	2014	2015	2016	mean
NOR4	0.45***	0.33**	0.31**	0.44***	0.51***	0.406***	0.189	0.45***
201593	0.44***	0.27*	0.27*	0.38***	0.54***	0.403***	0.27*	0.46***
201614	0.49***	0.37***	0.23	0.46***	0.53***	0.430***	0.28 *	0.47***
201618	0.11	0.12	-0.14	0.06	0.20	0.17	0.12	0.12
SnToxA	0.16	0.33***	0.26**	0.24*	0.29***	0.23*	0.13	0.21
SnTox3	0.04	-0.06	-0.10	-0.10	-0.02	-0.08	0.03	-0.019

*, **, *** - significant at the 0.01, 0.001 and 0.0001 level respectively

Table 5 Significant markers associated with SnToxA-sensitivity. Positions and rescaled positions in centiMorgan (cM) based on the consensus map by Wang et al. (2014). SSR markers are placed putatively on the map.

SNP	Chr.	Position, cM	Rescaled, cM	-log10(*p*)	R^2
Tdurum_contig12066_126	5B*	280.68	90.35	13.8	0.71
Tdurum_contig12066_247	5B*	280.68	90.35	13.8	0.71
BobWhite_c48435_165	5B	280.68	90.35	13.8	0.71
tplb0027f13_1346	5B*	280.68	90.35	13.6	0.71
IACX9261	5B	280.68	90.35	13.3	0.63
Tdurum_contig25513_123	5B	280.68	90.35	13.2	0.67
Tdurum_contig25513_195	5B	280.68	90.35	13.0	0.67
tplb0027f13_1493	5B	280.68	90.35	13.0	0.67
wsnp_Ku_c40334_48581010	5B	280.68	90.35	13.0	0.67
BS00010590_51	5B	280.68	90.35	12.3	0.61
fcp620 (274 bp)	5B	280.68	90.35	12.2	0.61
fcp394 (345 bp)	5B	280.68	90.35	9.0	0.43
fcp394 (392 bp)	5B	280.68	90.35	9.0	0.43
fcp620 (247 bp)	5B	280.68	90.35	8.2	0.37
fcp1 (410 bp)	5B	280.68	90.35	5.8	0.22
fcp1 (408 bp)	5B	280.68	90.35	5.2	0.20
Excalibur_c37642_1416	5B	280.68	90.35	3.8	0.13

*) Originally placed on 5A, in the consensus map by Wang et al. (2014)
Positions of SSRs extrapolated from position of linked SNPs in Wang et al. (2014)

Table 6 Significant markers associated with SnTox3-reactions; A) average (sensitive reactions of Type 2 (chlorosis) and Type 3 (necrosis) combined), B) Type 2 reaction – chlorosis without tissue collapse and C) Type 3 (necrotic) reaction analyzed separately. Positions and rescaled positions in centiMorgan (cM) based on the consensus map by Wang et al. (2014). SSR markers are placed putatively on the map.

SnTox3-reaction	SNP	Chr.	Position, cM	Rescaled, cM	-log10(*p*)	R^2
A) Average	*gwm234 (260 bp)*	5B	0	0	6.0	0.23
	Excalibur_c47452_183	5B	4.22	1.36	4.9	0.18
	BS00023070_51	5A	639.0	127.63	4.5	0.15
	BS00091519_51	5B	4.22	1.36	4.5	0.16
	Ku_c5969_1667	7B	456.82	142.69	3.4	0.11
	wsnp_BM140362A_Ta_2_2	1A	260.38	84.33	3.3	0.11
	BS00069829_51	1B	311.35	97.71	3.3	0.10
B) Type 2	*cfd20 (294 bp –sensitivity)*	5B	0	0	6.9	0.38
	cfd20 (0-allele, resistance)	5B	0	0	6.9	0.38
	gwm234 (257 bp)	5B	0	0	5.5	0.19
	gwm234 (260 bp)	5B	0	0	5	0.17
	BS00036907_51	5A	247.28	49.39	4.5	0.15
	BobWhite_c4336_127	5A	247.28	49.39	4.5	0.15
	Excalibur_c38185_633	5A	247.28	49.39	4.2	0.14
	Kukri_c36747_195	5A	246.48	49.39	3.7	0.12
	wsnp_Ex_rep_c101757_87064771	5A	246.48	49.39	3.7	0.12
	wsnp_Ex_rep_c101757_87065032	5A	247.28	49.39	3.7	0.12
	wsnp_Ex_rep_c101757_87065169	5A	247.28	49.39	3.7	0.12
	BS00022500_51	5A	247.28	49.39	3.6	0.11
C) Type 3	*Excalibur_c47452_183*	5B	4.22	1.36	6.9	0.26
	BS00091519_51	5B	4.22	1.36	6.7	0.26
	Kukri_rep_c69087_153	2D	137.8	51.31	3.8	0.13
	Kukri_rep_c110868_147	2D	126.51	47.11	3.7	0.12
	wsnp_Ex_c14107_22021215	2D	126.51	47.11	3.7	0.12
	wsnp_Ku_c14251_22503965	2D	126.51	47.11	3.7	0.12
	RFL_Contig5334_831	1A	334.02	108.17	3.6	0.12
	D_GB5Y7FA01EHPZX_186	2D	136.5	50.83	3.4	0.11
	Tdurum_contig75762_377	1A	342.49	110.92	3.4	0.11
	Kukri_c27874_515	4A	257.94	65.95	3.3	0.10
	RAC875_c60162_129	1A	342.49	110.92	3.3	0.10

Table 7 Markers significantly associated with disease after inoculation with isolates NOR4, 201593, 201614 and 201618. Positions and rescaled positions in centiMorgan (cM) based on the consensus map by Wang et al. (2014). SSR markers are placed putatively on the map.

Isolate	SNP	Chr.	Position, cM	Rescaled, cM	-log10(*p*)	R^2
NOR4	*Tdurum_contig12066_126*	5B*	280.68	90.35	4.4	0.15
	Tdurum_contig12066_247	5B*	280.68	90.35	4.4	0.15
	BobWhite_c48435_165	5B	280.68	90.35	4.4	0.15
	tplb0027f13_1346	5B*	280.68	90.35	4.3	0.15
	Tdurum_contig25513_195	5B	280.68	90.35	4.2	0.14
	tplb0027f13_1493	5B	280.68	90.35	4.2	0.14
	wsnp_Ku_c40334_48581010	5B	280.68	90.35	4.2	0.14
	Tdurum_contig25513_123	5B	280.68	90.35	4.2	0.14
	BS00011510_51	4B	112.7	38.62	3.5	0.11
	wsnp_Ex_c37502_45236634	4B	111.08	38.30	3.4	0.11
	Kukri_c29267_215	5B	252.96	81.43	3.3	0.11
	fcp620 (247 bp)	5B	280.68	90.35	3.2	0.11
	BS00010590_51	5B	280.68	90.35	3.0	0.10
	IACX9261	5B	280.68	90.35	3.0	0.10
201593	*Excalibur_c47452_183*	5B	4.22	1.36	4.7	0.19
	BS00091519_51	5B	4.22	1.36	4.2	0.17
	BS00011510_51	4B	112.7	38.62	3.6	0.12
	wsnp_Ex_c37502_45236634	4B	111.08	38.30	3.6	0.12
	BS00064703_51	3A	350.21	109.95	3.2	0.11
	BS00065840_51	3A	350.21	109.95	3.2	0.11
	BS00106932_51	3A	350.21	109.95	3.2	0.11
201614	*BS00011510_51*	4B	112.7	38.62	4.4	0.16
	wsnp_Ex_c37502_45236634	4B	111.08	38.30	4.2	0.15
	Tdurum_contig76997_462	6B	257.73	75.47	3.6	0.12
	wsnp_Ex_c16389_24884851	4B	107.31	36.78	3.6	0.12
	Tdurum_contig76997_244	6B	257.73	75.47	3.6	0.12
	Ku_c71122_384	4B	107.31	36.78	3.6	0.12
	RAC875_c27160_307	4B	107.31	36.78	3.6	0.12
	RAC875_rep_c71114_699	4B	107.31	36.78	3.6	0.12
	wsnp_Ra_rep_c71114_69138821	4B	107.31	36.78	3.6	0.12
	Ex_c9556_2547	7A	398.79	135.81	3.4	0.11
	wsnp_CAP11_c2839_1425826	7D	294.97	133.18	3.4	0.11
	RFL_Contig2647_624	7B	534.63	166.99	3.1	0.10
	wsnp_CAP12_c455_248396	2A	157.96	47.22	3.0	0.10
	wsnp_Ku_c10355_17149304	7B	248.86	77.73	2.9	0.10
	RAC875_c9309_145	7A	458.76	156.23	2.8	0.09
201618	*BS00012321_51*	1B	201.25	62.58	4.4	0.16
	RAC875_c30657_82	1A	78.25	25.34	4.0	0.14
	Ku_c28007_1398	1A	66.54	21.55	3.7	0.13
	cnl137a (0-allele)	1A	66.54	21.55	3.7	0.13

	BS00033750_51	1A	42.4	13.73	3.5	0.13
	BS00082566_51	1B	206.01	64.10	3.4	0.12
	BS00023201_51	1A	66.54	21.55	3.4	0.12
	RAC875_c38756_141	1A	66.54	21.55	3.4	0.12
	RAC875_c42700_264	1A	42.4	13.73	3.4	0.12
	Ku_c8810_903	1B	142.58	43.86	3.1	0.11
	Excalibur_c35316_154	1A	51.48	16.67	3.1	0.10
	GENE-1118_58	1A	78.25	25.34	2.8	0.09

Table 8 Markers significantly associated with sensitivity reaction after infiltration with the isolates NOR4, 201593, 201614 and 201618. Positions and rescaled positions in centiMorgan (cM) based on the consensus map by Wang et al. (2014). SSR markers are placed putatively on the map.

	SNP	Chr.	Position, cM	Rescaled, cM	-log10(*p*)	R2
NOR4	*Tdurum_contig76997_462*	6B	257.73	75.47	4.4	0.15
	Tdurum_contig76997_244	6B	257.73	75.47	4.4	0.15
	gwm234 (260 bp)	5B	0	0	3.5	0.11
	Tdurum_contig76997_664	6B	258.7	75.75	3.3	0.10
	BS00104265_51	6B	258.7	75.75	3.3	0.10
	RAC875_rep_c81781_202	6B	226.76	66.40	3.1	0.10
	Kukri_rep_c69810_502	1B	226.76	70.78	3.0	0.09
	VP1_B2 (559 bp)	3B	545.36	136.36	2.9	0.12
	Kukri_rep_c80051_93	4B	182.55	62.56	2.8	0.08
	BS00091262_51	6B	226.76	66.36	2.8	0.09
	Tdurum_contig8348_831	5A	709.71	141.75	2.7	0.08
	Kukri_c32803_150	3B	34.37	8.59	2.7	0.08
	Excalibur_c11601_231	3B	33.84	8.46	2.6	0.09
	BS00023050_51	6B	258.7	75.75	2.6	0.07
201593	*Excalibur_c47452_183*	5B	4.22	1.36	4.5	0.17
	BS00094553_51	1A	256.0	82.91	4.2	0.14
	BS00091519_51	5B	4.22	1.36	3.9	0.14
	Kukri_rep_c105589_73	1A	261.31	84.63	3.8	0.13
	Kukri_rep_c109167_89	4A	392.59	100.38	3.3	0.11
	BS00074487_51	6A	73.95	30.73	3.2	0.12
	wsnp_Ex_c3475_6362087	1A	261.32	84.63	3.2	0.11
	CAP12_c475_289	6B	269.77	78.99	3.1	0.09
	wsnp_BF474862A_Ta_2_1	4A	368.07	94.11	3.1	0.10
	wsnp_Ex_c28728_37832012	4A	368.07	94.11	3.1	0.10
	wsnp_Ex_rep_c106527_90571247	4A	372.59	95.27	3.1	0.10
	RAC875_c95081_166	2B	458.55	142.99	3.1	0.10
	IAAV1502	2B	450.5	140.48	3.0	0.10
	wsnp_Ku_c10292_17066821	1A	261.32	84.63	3.0	0.10
	IAAV7930	3A	350.21	109.95	2.8	0.09
	Kukri_c4324_74	3A	350.21	109.95	2.8	0.09

	GENE-4221_519	6B	269.77	78.99	2.7	0.08
	IACX1609	6B	269.77	78.99	2.7	0.08
201614	*Excalibur_c47452_183*	5B	4.22	1.36	5.6	0.21
	BS00091519_51	5B	4.22	1.36	4.7	0.18
	RAC875_c29540_391	1A	256.0	82.91	3.7	0.12
	wsnp_BG274584A_Ta_2_4	2A	385.17	115.14	3.6	0.13
	RAC875_c50787_146	3B	271.1	67.78	3.6	0.12
	Tdurum_contig76595_208	2A	86.88	25.97	3.5	0.11
	wsnp_Ex_c20489_29564938	1A	260.68	84.42	3.3	0.11
	gwm234 (260)	5B	0	0	3.3	0.11
	wsnp_Ex_c23618_32855041	5D	489.95	190.80	3.1	0.11
	wsnp_Ra_c33025_41968284	1A	255.26	82.67	3.0	0.09
	RAC875_c56535_256	4A	356.65	91.19	3.0	0.09
	Kukri_c31891_1355	1A	260.68	84.42	2.9	0.09
	wsnp_Ra_c9209_15425473	1A	258.46	83.70	2.7	0.09
	Excalibur_c1604_2710	2A	388.71	116.19	2.7	0.09
	Kukri_c62142_683	2A	388.71	116.19	2.7	0.09
	RAC875_c68530_59	5B	117.45	23.46	2.7	0.09
201618	*wsnp_Ex_c9301_15450818*	5A	79.39	15.86	4.0	0.13
	BobWhite_c20689_427	4D	176.42	101.98	3.6	0.12
	BS00066144_51	5D	264.26	102.91	3.2	0.11
	Kukri_c73802_205	6D	50.3	22.92	3.0	0.09
	RAC875_c39430_181	5D	264.26	103.16	3.0	0.11
	Ex_c52711_584	2B	458.55	142.99	3.0	0.09
	RAC875_c19685_944	2B	464.94	144.98	2.9	0.09
	BS00101087_51	7B	379.94	118.67	2.8	0.09
	wsnp_Ex_rep_c66685_65003254	3A	291.96	91.66	2.8	0.08
	BS00035267_51	1B	388.66	122.38	2.8	0.08
	RAC875_c25848_122	2A	469.34	140.30	2.7	0.08
	RAC875_c75448_80	3A	311.56	97.81	2.7	0.08
	GENE-0875_887	2D	134.59	50.12	2.7	0.09
	D_GA8KES401AVZF3_380	2D	136.5	50.83	2.7	0.08
	GENE-0687_448	2D	136.5	50.83	2.7	0.08
	GENE-0875_506	2D	136.5	50.83	2.7	0.08
	GENE-0875_620	2D	136.5	50.83	2.7	0.08
	Tdurum_contig49841_618	5B	101.68	32.73	2.7	0.08

Table 9 Markers significantly associated with corrected SNB severity at the adult plant stage in the field in 2010 to 2016 and the mean of the seven years. Positions and rescaled positions in centiMorgan (cM) based on the consensus map by Wang et al. (2014). SSR markers are placed putatively on the map.

2010	SNP	Chr.	Position, cM	Rescaled, cM	-log10(*p*)	R^2
	RAC875_c36670_72	7B	70.0	21.86	3.1	0.15
	Tdurum_contig85266_280	7B	70.0	21.86	3.1	0.15

	Tdurum_contig30677_55	7B	87.3	27.27	3.1	0.15
	gwm293 (217 bp)	5A	314.02	62.72	3.1	0.16
	Excalibur_c50044_749	7B	14.89	4.65	3.1	0.15
	wsnp_BE497845D_Ta_1_1	7D	326.45	147.40	3.0	0.15
	BS00009514_51	6A	40.8	16.96	3.0	0.14
	Excalibur_c11798_2274	2D	150.78	56.15	2.9	0.14
	Excalibur_c3423_994	7B	77.0	24.05	2.8	0.14
	BS00007384_51	2B	498.37	155.41	2.7	0.13
	Excalibur_c33525_279	2B	498.37	155.41	2.7	0.13
	GENE-0977_215	2A	157.96	47.22	2.6	0.14
	BobWhite_c31129_60	2B	253.32	78.99	2.6	0.12
	RFL_Contig4626_873	6D	180.23	82.14	2.6	0.12
	BS00070695_51	1A	462.61	149.82	2.5	0.12
	BS00070991_51	1A	462.61	149.82	2.5	0.12
2011	*gwm301 (237 bp)*	2D	260.85	97.14	2.8	0.19
	gwm894 (142 bp)	4A	272.18	69.6	2.7	0.09
	BobWhite_c22266_315	1B	195.12	60.62	2.7	0.09
	RAC875_c14195_1155	7A	398.79	135.81	2.6	0.09
	RAC875_c42756_168	4A	191.56	48.98	2.6	0.09
	Ku_c665_985	7A	186.24	58.17	2.6	0.09
	Excalibur_s111479_146	6B	168.21	49.25	2.6	0.09
	BS00022499_51	6B	80.92	23.69	2.6	0.09
	Tdurum_contig81683_217	7A	186.24	58.17	2.6	0.09
	wsnp_Ex_c6590_11419735	7A	186.24	58.17	2.6	0.09
	BobWhite_c31129_60	2B	253.32	78.99	2.6	0.09
	BS00023222_51	3A	475.61	149.31	2.5	0.09
	BS00011510_51	4B	112.7	38.62	2.5	0.09
	Excalibur_rep_c115852_82	5B	214.95	69.19	2.5	0.09
	RFL_Contig799_2152	6B	317.66	93.01	2.5	0.09
	RFL_Contig799_2434	6B	317.66	93.01	2.5	0.09
	IACX473	7A	249.05	103.50	2.5	0.09
	Tdurum_contig11028_236	7B	186.24	58.17	2.5	0.09
	Kukri_c2706_1424	4A	237.61	60.76	2.5	0.09
	BS00067590_51	6B	168.21	49.47	2.5	0.09
	GENE-0221_350	6B	168.21	49.25	2.5	0.09
	GENE-0221_721	6B	168.21	49.25	2.5	0.09
	Kukri_c31032_897	6B	168.21	49.47	2.5	0.09
	Kukri_c32307_481	6B	168.21	49.47	2.5	0.09
	RAC875_c10650_90	6B	168.21	49.47	2.5	0.09
	RAC875_rep_c116755_285	6B	168.21	49.47	2.5	0.09
	RFL_Contig2024_600	6B	168.21	49.47	2.5	0.09
	TA005332-1378	6B	168.21	49.47	2.5	0.09
	BS00067630_51	6A	32.37	13.45	2.5	0.09
	BS00047044_51	6B	168.21	49.47	2.5	0.09

	BobWhite_c10832_972	6B	388.21	113.67	2.5	0.08
	Kukri_c15310_755	7A	360.88	122.90	2.5	0.08
2012	*BS00101408_51*	7B	329.1	102.79	4.1	0.11
	TA006077-0786	7B	319.23	99.71	3.9	0.10
	Kukri_c100592_82	7B	321.13	100.30	3.9	0.10
	RAC875_c33564_238	7B	321.13	100.30	3.9	0.10
	RAC875_c33564_454	7B	321.13	100.30	3.9	0.10
	RAC875_c37552_149	7B	321.13	100.30	3.9	0.10
	GENE-4996_592	7B	329.1	102.79	3.9	0.10
	wsnp_BE605194B_Ta_2_1	7B	329.1	102.79	3.9	0.10
	BS00064146_51	7B	329.1	102.79	3.7	0.10
	RAC875_c33564_120	7B	321.13	100.30	3.6	0.10
	Excalibur_c74925_338	2B	555.91	173.35	3.6	0.09
	wsnp_BE498985A_Ta_2_1	7B	328.43	102.58	3.6	0.09
	BS00039118_51	7B	329.1	102.88	3.6	0.09
	Tdurum_contig10677_529	7B	329.1	102.88	3.6	0.09
	wsnp_Ex_c10231_16783750	5A	217.19	43.38	3.4	0.09
	wsnp_BE605194B_Ta_2_7	7B	329.1	102.79	3.4	0.09
	RAC875_c48766_224	7B	315.52	98.55	3.2	0.08
	Kukri_c12901_706	7B	316.07	98.72	3.2	0.08
	Tdurum_contig81911_179	7B	316.07	98.72	3.2	0.09
	wsnp_Ex_c10193_16730126	7B	323.95	101.18	3.2	0.08
	wsnp_Ex_c10193_16730348	7B	323.95	101.18	3.0	0.08
	BS00080621_51	7B	337.35	105.37	3.0	0.08
	BS00027054_51	7B	329.1	102.79	3.0	0.08
2013	*RAC875_c1828_1130*	4B	215.45	73.84	3.6	0.12
	IAAV558	4B	215.45	73.84	3.6	0.12
	Ku_c700_2585	4B	215.45	73.84	3.6	0.12
	BS00021722_51	4B	215.45	73.84	3.6	0.12
	Ra_c32919_1154	4B	215.45	73.84	3.6	0.12
	TA004905-0613	4B	215.45	73.84	3.6	0.12
	Kukri_c57086_133	7A	458.76	156.23	3.5	0.12
	wsnp_Ku_c21665_31431143	7A	458.76	156.23	3.5	0.12
	Excalibur_c12996_775	7A	400.14	136.27	3.4	0.11
	BobWhite_c47283_127	7A	400.14	136.27	3.4	0.11
	Ex_c25467_796	4B	215.45	73.84	3.4	0.11
	Ex_c25467_851	4B	215.45	73.84	3.4	0.11
	Ex_c9296_605	4B	215.45	73.84	3.4	0.11
	Ex_c9296_858	4B	215.45	73.84	3.4	0.11
	Ku_c48056_436	4B	215.45	73.84	3.4	0.11
	Ra_c32919_1289	4B	215.45	73.84	3.4	0.11
	wsnp_Ex_c107075_90880218	4B	215.45	73.84	3.4	0.11
	wsnp_Ex_c22785_31991891	4B	215.45	73.84	3.4	0.11
	Ku_c14007_1088	4B	215.45	73.84	3.4	0.11
	BS00009926_51	7A	448.64	152.78	3.3	0.11

	BS00070857_51	7A	400.61	136.43	3.3	0.11
	wsnp_Ex_c7830_13323473	5A	252.25	50.38	3.2	0.10
	Excalibur_c11258_1700	4A	51.66	16.73	3.1	0.10
	BS00109319_51	7A	400.14	136.27	3.1	0.10
2014	*BobWhite_c9000_114*	3D	338.33	116.11	4.2	0.15
	gwm133 (136 bp)	2B	320.05	NA	3.8	0.18
	IAAV6032	2D	264.77	98.59	3.5	0.12
	Excalibur_rep_c67599_2154	2D	260.85	97.14	3.5	0.12
	BS00015680_51	2D	277.48	103.33	3.5	0.12
	Excalibur_rep_c67599_242	2D	277.48	103.33	3.5	0.12
	IAAV1322	2D	277.48	103.33	3.4	0.12
	RAC875_c14195_1155	7B	398.79	135.81	3.3	0.11
	wsnp_RFL_Contig4134_4692458	2D	136.5	50.83	3.3	0.11
	Excalibur_c31806_912	2D	277.48	103.33	3.3	0.12
	IACX8002	5A	282.76	56.47	3.3	0.11
	Tdurum_contig63196_123	2A	86.88	25.97	3.3	0.11
	Tdurum_contig30677_55	7B	87.3	27.27	3.2	0.11
	Excalibur_c3423_994	7B	77.0	24.05	3.1	0.11
	wsnp_Ex_c16090_24522660	6B	1.28	0.37	3.1	0.10
	Kukri_c542_1538	7B	0	0.00	2.9	0.10
	BS00011510_51	4B	112.7	38.62	2.9	0.09
	Ex_c14898_287	5A	266.62	53.25	2.9	0.10
	wsnp_Ku_c6319_11093041	5A	266.62	53.25	2.9	0.10
	GENE-1298_29	2A	86.88	25.97	2.9	0.10
	CAP8_c4697_108	3A	276.05	86.66	2.9	0.11
	RAC875_c36670_72	7B	70.0	21.86	2.8	0.09
	Tdurum_contig85266_280	7B	70.0	21.86	2.8	0.09
	Excalibur_c50044_749	7B	14.89	4.65	2.8	0.09
	Ku_c24324_850	5A	266.62	53.25	2.8	0.09
	Ku_c6319_201	5A	266.62	53.25	2.8	0.09
	BS00004089_51	2A	86.88	25.97	2.8	0.09
	GENE-0918_140	2D	277.48	103.33	2.8	0.09
2015	*BS00104199_51*	1D	215.43	133.99	3.5	0.11
	BS00023049_51	1D	215.43	133.99	3.3	0.10
	BS00067983_51	6B	312.5	91.50	3.3	0.10
	RAC875_c1188_531	1B	273.29	85.57	3.2	0.09
	CAP11_rep_c6465_98	1B	142.58	43.86	3.1	0.10
	Excalibur_rep_c67599_2154	2D	260.85	97.14	3.1	0.09
	BS00015680_51	2D	277.48	103.33	3.1	0.09
	Excalibur_rep_c67599_242	2D	277.48	103.33	3.1	0.09
	RAC875_c19690_358	2B	245.97	76.70	3.1	0.09
	RAC875_c2040_564	1B	141.97	43.66	3.1	0.10
	Ra_c19690_1998	2B	245.97	76.70	3.1	0.09
	Excalibur_c31806_912	2D	277.48	103.33	3.1	0.09
	IAAV1322	2D	277.48	103.33	3.1	0.09

	IAAV6032	2D	264.77	98.59	3.1	0.09
	Excalibur_c35316_154	1A	51.48	16.67	3.0	0.09
	CAP8_c1305_148	1D	71.85	44.69	3.0	0.09
	Ku_c33271_432	1B	273.29	85.57	3.0	0.09
	Kukri_rep_c70501_255	1B	273.29	85.57	3.0	0.09
	Ra_c35710_395	1B	273.29	85.57	3.0	0.09
	Kukri_c46169_294	1D	71.85	44.69	3.0	0.09
	GENE-4918_283	3B	228.92	57.24	2.9	0.09
	RFL_Contig2647_624	7B	534.63	166.99	2.9	0.08
	BobWhite_c5633_59	4A	157.49	40.27	2.9	0.08
	BobWhite_rep_c66057_98	4A	157.49	40.27	2.9	0.08
	IAAV3697	4A	157.49	40.27	2.9	0.08
	CAP7_c3847_204	1B	142.58	43.86	2.9	0.08
2016	*Excalibur_c31806_912*	2D	277.48	103.33	3.6	0.11
	IAAV1322	2D	277.48	103.33	3.6	0.11
	BS00011060_51	4A	156.52	40.02	3.5	0.11
	Excalibur_rep_c67599_2154	2D	260.85	97.14	3.4	0.10
	BS00015680_51	2D	277.48	103.33	3.4	0.10
	Excalibur_rep_c67599_242	2D	277.48	103.33	3.4	0.10
	Kukri_c22231_87	4A	157.49	40.27	3.4	0.10
	wmc552 (184 bp)	3D	304.73	104.58	3.3	0.11
	IAAV6032	2D	264.77	98.59	3.3	0.10
	Kukri_c2326_1037	5A	267.69	53.47	3.3	0.10
	wsnp_Ex_c28957_38032895	5A	267.69	53.47	3.3	0.10
	Kukri_c2326_659	5A	267.69	53.47	3.3	0.10
	Kukri_c2326_995	5A	267.69	53.47	3.3	0.10
	CAP8_c8516_542	2B	417	130.29	3.3	0.10
	gwm33b (188 bp)	1A	42.4	13.73	3.2	0.11
	BS00067797_51	5A	267.69	53.47	3.1	0.09
	BS00069980_51	5A	267.69	53.47	3.1	0.09
	BobWhite_c10901_240	5A	267.69	53.47	3.1	0.09
	BobWhite_c10901_578	5A	267.69	53.47	3.1	0.09
	BobWhite_c46338_76	5A	267.69	53.47	3.1	0.09
	Excalibur_c63344_424	5A	267.69	53.47	3.1	0.09
	GENE-3455_115	5A	267.69	53.47	3.1	0.09
	IACX12578	5A	267.69	53.47	3.1	0.09
	IACX3152	5A	267.69	53.47	3.1	0.09
	IACX3154	5A	267.69	53.47	3.1	0.09
	Kukri_c24787_51	5A	267.69	53.47	3.1	0.09
	Kukri_c75644_104	5A	267.69	53.47	3.1	0.09
	RAC875_c79944_269	5A	267.69	53.47	3.1	0.09
	RAC875_rep_c110032_317	5A	267.69	53.47	3.1	0.09
	RAC875_rep_c110032_448	5A	267.69	53.47	3.1	0.09
	Tdurum_contig49751_2541	5A	267.69	53.47	3.1	0.09
	Tdurum_contig49751_2646	5A	267.69	53.47	3.1	0.09

	Tdurum_contig57742_400	5A	267.69	53.47	3.1	0.09
	Tdurum_contig94007_225	5A	267.69	53.47	3.1	0.09
	wsnp_Ex_rep_c102281_87481676	5A	267.69	53.47	3.1	0.09
	wsnp_Ex_c18883_27772081	3A	169.84	53.32	3.1	0.10
	wsnp_Ex_c54453_57331510	4A	156.21	39.94	3.1	0.10
	Kukri_c30875_283	5A	267.69	53.47	3.1	0.09
	RAC875_c17455_152	2B	365.88	114.09	3.1	0.09
	wsnp_Ku_c9901_16493072	2B	365.88	113.86	3.1	0.09
	wsnp_Ex_c5123_9087869	2B	365.88	114.09	3.1	0.09
	wsnp_Ex_c5123_9089025	2B	365.88	113.86	3.1	0.09
mean	*Excalibur_c31806_912*	2D	277.48	103.33	3.8	0.05
	IAAV1322	2D	277.48	103.33	3.7	0.05
	Excalibur_rep_c67599_2154	2D	260.85	97.14	3.6	0.12
	BS00015680_51	2D	277.48	103.33	3.6	0.12
	Excalibur_rep_c67599_242	2D	277.48	103.33	3.6	0.12
	IAAV6032	2D	264.77	98.59	3.6	0.12
	wsnp_Ex_c97184_84339976	5B	412.97	132.93	3.5	0.05
	BobWhite_c39214_164	5B	412.97	132.93	3.4	0.04
	wsnp_Ex_c53170_56501500	5B	412.97	132.93	3.4	0.04
	Excalibur_c29304_176	5B	323.81	104.23	3.1	0.04
	IACX6034	5B	323.81	104.23	3.1	0.04
	wsnp_Ex_c29304_38355434	5B	323.81	104.23	3.1	0.04
	BS00010136_51	3A	313.46	98.41	3.1	0.04
	wsnp_Ex_c18223_27035083	3A	313.46	98.41	3.1	0.04
	wsnp_Ex_c21733_30892583	3A	314.37	98.69	3.1	0.04
	wsnp_Ex_c11297_18254062	3A	312.73	98.18	3.1	0.04
	BobWhite_rep_c64211_305	3A	313.46	98.41	3.1	0.04
	Ra_c8717_520	3A	313.46	98.41	3.1	0.04
	BS00070856_51	6D	335.87	153.08	3	0.04
	Ku_c61039_131	3A	316.88	99.48	3	0.04
	GENE-0918_140	2D	277.48	103.33	3	0.04
	Kukri_c82145_51	5B	412.97	132.93	3	0.04
	Tdurum_contig22253_104	3A	276.91	86.93	3	0.04
	GENE-0977_215	2A	157.96	47.22	2.9	0.04
	Tdurum_contig10979_1523	3A	271.98	85.39	2.9	0.03
	Ex_c24992_1659	3A	314.37	98.69	2.9	0.03
	Ku_c14982_168	3A	276.05	86.66	2.9	0.03
	RFL_Contig102_119	3A	276.05	86.66	2.9	0.03
	RFL_Contig4399_956	3A	276.05	86.66	2.9	0.03
	wsnp_Ex_c11397_18400400	3A	276.05	86.66	2.9	0.03

Figures

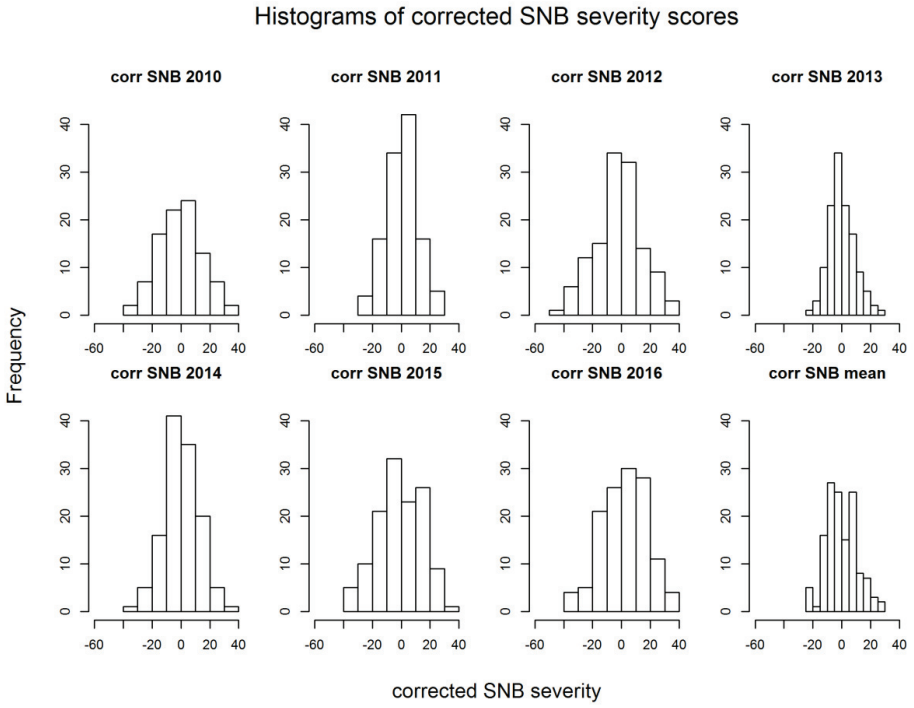

Figure 1 Histograms of the distribution of corrected Septoria nodorum blotch (SNB) severities from seven years of field trials (2010 to 2016) and the mean, in the Nordic spring wheat collection.

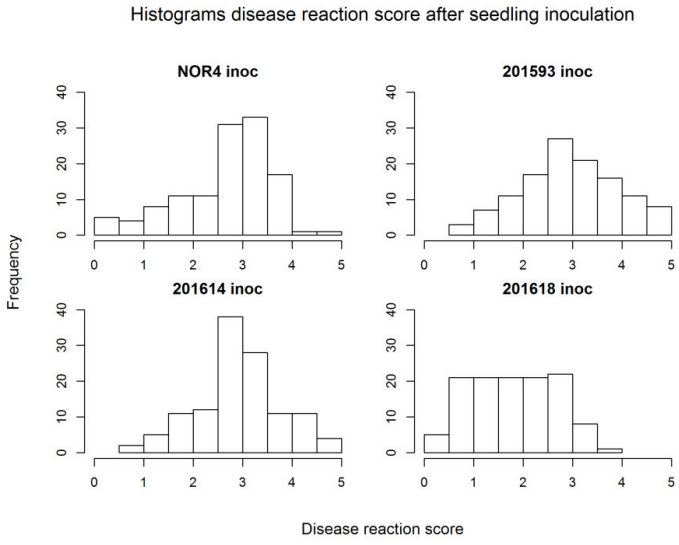

Figure 2 Histograms showing the distribution of disease reaction scores (0-5) after inoculation of two weeks old seedlings with the four *P. nodorum* isolates NOR4, 201593, 201614 and 201618.

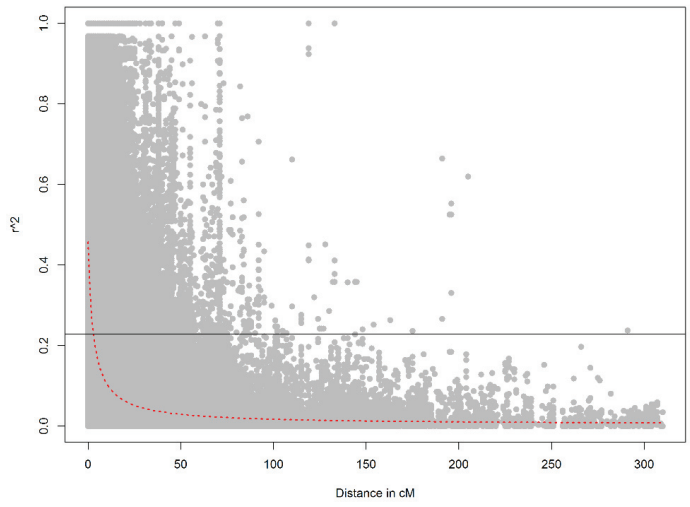

Figure 3 Genome wide LD decay plot based on pairwise comparisons of loci. The red line shows the estimated LD points. The horizontal line represent the critical value for LD significance based on the estimated LD value for half decay, 0.23, calculated as described by Marroni et al. (2011).

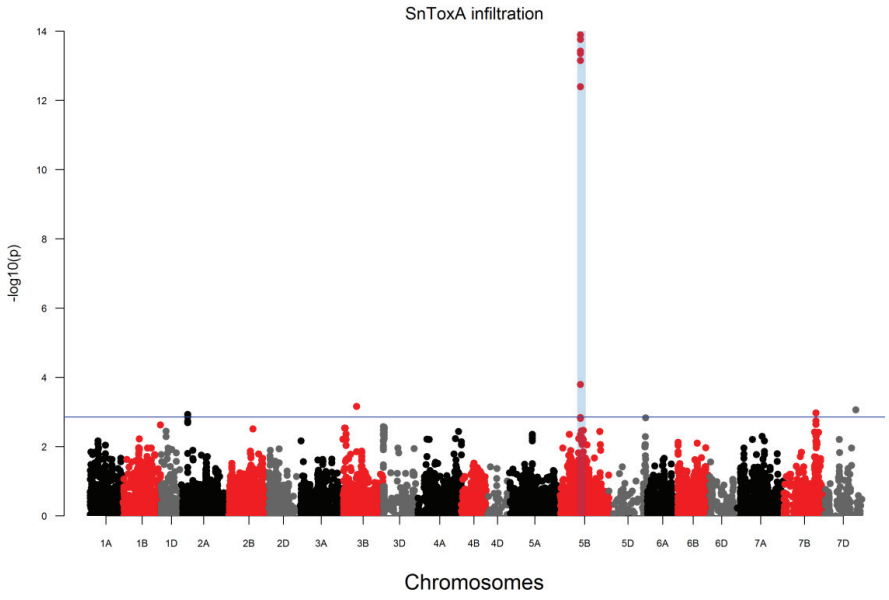

Figure 4 Manhattan plot showing the significant SNP and SSR markers associated with reactions to SnToxA infiltration.

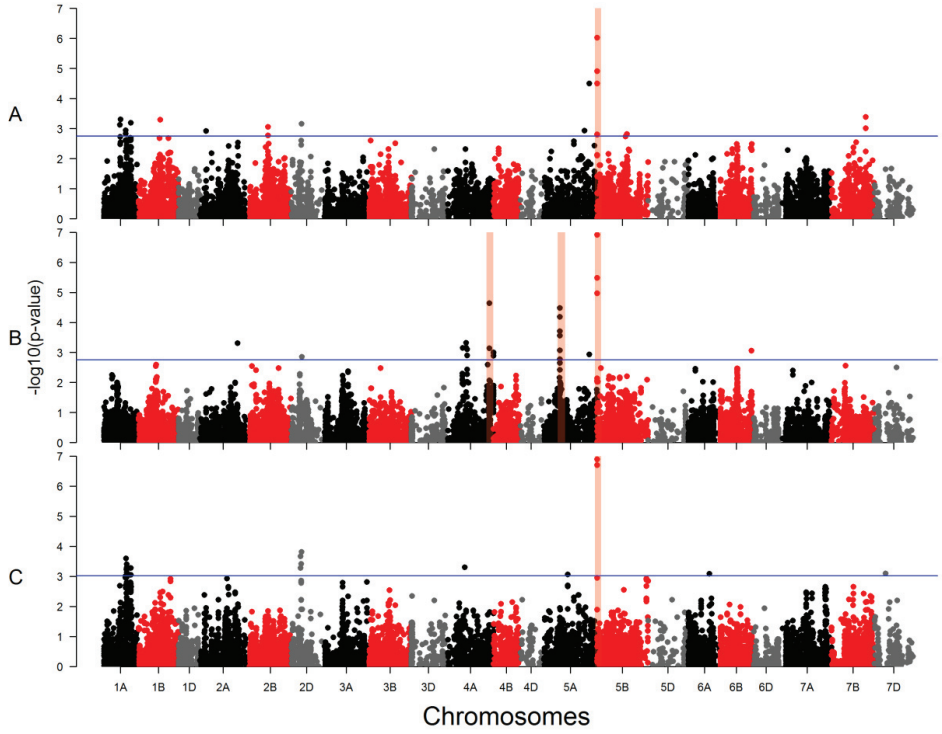

Figure 5 Manhattan plots showing significant SNP and SSR markers associated with A) average SnTox3 reaction, i.e. both chlorotic and necrotic reaction type, to infiltration with purified SnTox3, B) Type 2 (chlorotic) reaction to SnTox3 infiltration and C) Type 3 (necrotic) reaction to SnTox3. Note that the markers associated with the Type 2 reaction on 5B are SSR markers (*gwm234* and *cfd20*), and no SNP markers on 5B. For the Type 3 reaction only SNP markers on 5B are associated, not the SSR markers.

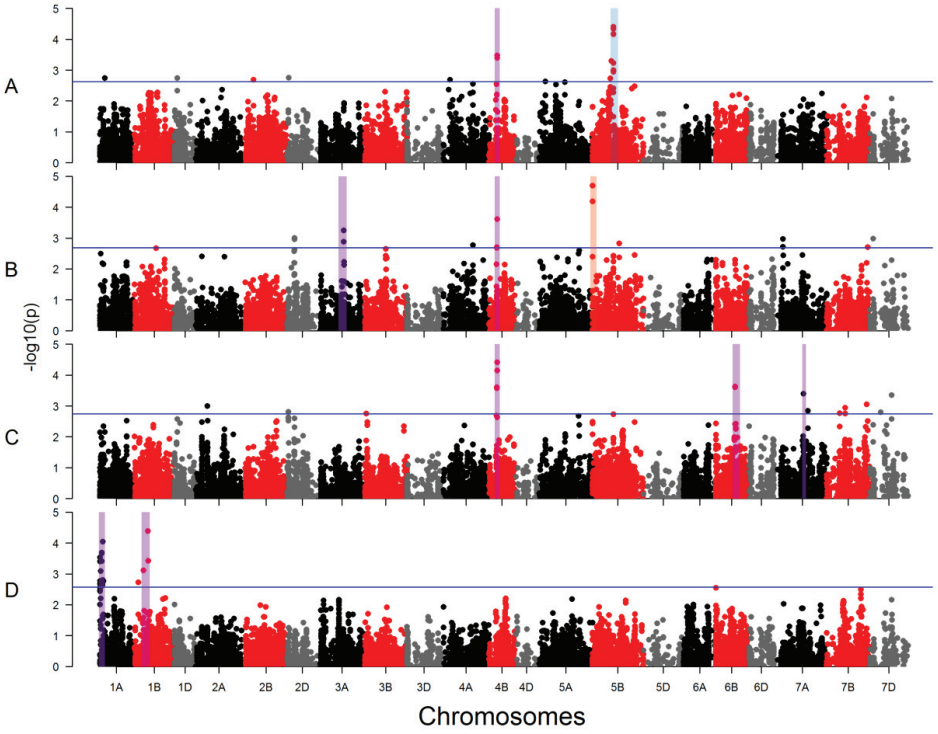

Figure 6 Manhattan plots with markers associated with SNB after inoculation with isolate A) NOR4, B) 201593, C) 201614 and D) 201618. Red rectangle: Markers associated with SnTox3 reaction. Blue rectangles: Markers associated with SnToxA reaction. Purple rectangles highlight SNPs with significance -log10(p) > 3, while the blue horizontal line represents the 0.1 percentile threshold.

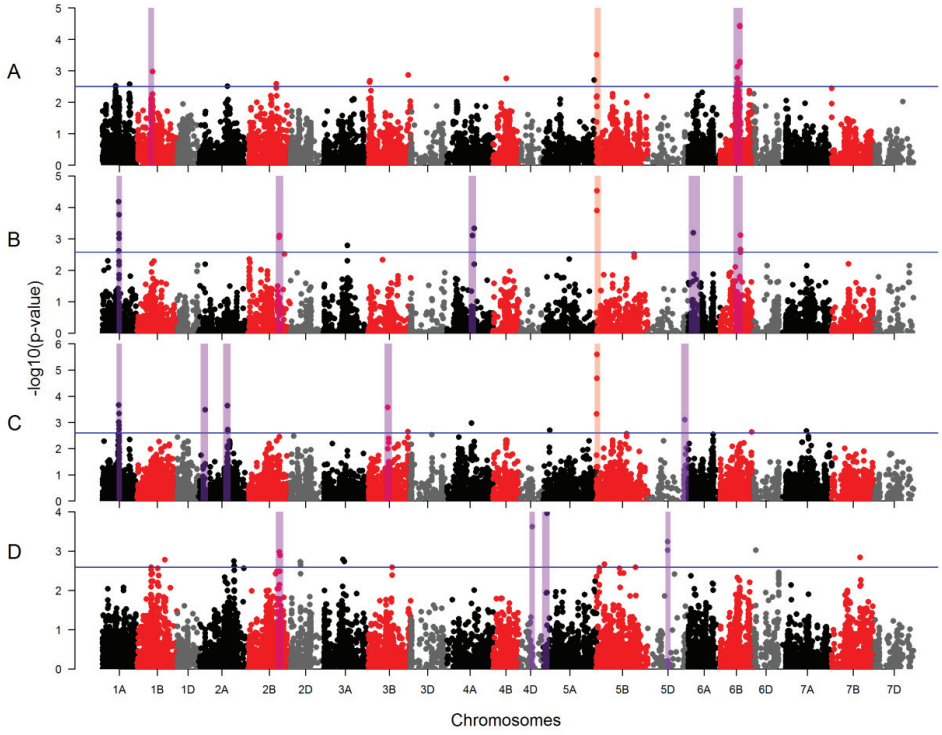

Figure 7 Manhattan plots showing the SNPs significantly associated with reaction to culture filtrate (CF) infiltration with four Norwegian isolates A) NOR 4, B) 201593, C) 201614 and D) 201618. The colored rectangles indicate marker-trait associations that are significant at –log(p)>3, while the horizontal line represents the significance threshold at the 0.1 % percentile. Red rectangles are associated with SnTox3-sensitivity.

Figure 8 Manhattan plots of the corrected SNB severities per year: A) 2010, B) 2011, C) 2012, D) 2013, E) 2014, F) 2015), G) 2016, H) Mean. The horizontal blue line is based on the 0.1 percentile thresholds. The scale of the y-axis is different between plots. Purple rectangles highlight QTL that are significant in more one year in the field. Green rectangles highlight QTL that were significant both in inoculation experiments with single isolates and at least one year in the field. Blue rectangles highlight *Tsn1*-linked markers in years where they were the most significant 5B markers (although under LOD threshold).

Supplementary material

Table S1 List of the 121 spring wheat genotypes included in the genome wide association analysis (GWAS). Some breeding lines have been anonymized and named Breeding line #.

Name	**Origin**
512-21	Norway
512-50	Norway
512-54	Norway
512-70	Norway
512-87	Norway
AC Somerset	Canada
Altar84/*Ae.tauschii*(219)//2*Seri	CIMMYT
Altar84/*Ae.tauschii*(219)//2*Seri/3/Avle	Norway
ALTAR84/*Ae.tauschii*(224)//ESDA	CIMMYT
Amulett	Sweden
Avle	Sweden
Avocet-YrA	Australia
BAJASS	Norway
Bastian	Norway
BCN*2//CROC_1/*Ae.tauschii*(886)	CIMMYT
Berserk	Norway
Bjarne	Norway
Bombona	Sweden
Brakar	Norway
Breeding line 1	Sweden
Breeding line 10	Sweden
Breeding line 2	Sweden
Breeding line 3	Sweden
Breeding line 4	Sweden
Breeding line 5	Sweden
Breeding line 6	Sweden
Breeding line 7	Sweden
Breeding line 8	Sweden
Breeding line 9	Sweden
C80.1/3*QT4522//2*ATTILA	CIMMYT
C80.1/3*QT4522//2*PASTOR	CIMMYT
Catbird	CIMMYT
CBRD/KAUZ	CIMMYT
CD87	Australia
Chara	Australia
CJ9306	China
CJ9403	China

Croc_1/*Ae.tauschii*(205)//Kauz	CIMMYT
Demonstrant	Norway
DH20070	Norway
DH20097	Norway
Dulus	CIMMYT
Filin	CIMMYT
Fram II	Norway
Frontana	Brazil
GN03503	Norway
GN03529	Norway
GN03531	Norway
GN03597	Norway
GN04526	Norway
GN04528	Norway
GN04537	Norway
GN05507	Norway
GN05551	Norway
GN05580	Norway
GN05589	Norway
GN06557	Norway
GN06573	Norway
GN06578	Norway
GN07581	Norway
GN08504	Norway
GN08531	Norway
GN08533	Norway
GN08534	Norway
GN08541	Norway
GN08554	Norway
GN08557	Norway
GN08564	Norway
GN08568	Norway
GN08588	Norway
GN08595	Norway
GN08596	Norway
GN08597	Norway
GN08647	Norway
Gondo	CIMMYT
Granary	UK
GUAM92//PSN/BOW	CIMMYT
J03	Norway
Kariega	South Africa

Krabat	Norway
Kukri	Australia
MAYOOR//TKSN1081/*Ae.tauschii*(222)	CIMMYT
Milan	CIMMYT
MILAN/SHA7	CIMMYT
MS273-150	Norway
Møystad	Norway
Nanjing 7840	China
Naxos	Germany
Naxos/2*Saar	Norway
NG8675/CBRD	CIMMYT
Ning8343	China
NK00521	Norway
NK01565	Norway
NK93602	Norway
NK93604	Norway
Nobeokabouzu	Japan
Norrøna	Norway
Paros	Norway
Paros/NK93602	Norway
Paros/T9040	Norway
QUARNA	Switzerland
R37/GHL121//KAL/BB/3/JUP/MUS/4/2*YMI#6/5/CBRD	CIMMYT
RB07	USA
Runar	Norway
Saar	CIMMYT
Sabin	USA
SHA3/CBRD	CIMMYT
Soru#1	CIMMYT
Sport	Sweden
Sumai#3-(12SRSN)	China
Sumai3(18.)	China
T10014	Norway
T2038	Norway
T9040	Norway
T9040(1995)	Norway
T9040/Paros	Norway
Tjalve	Sweden
TJALVE/Purpurseed	Norway
Tom	USA
Vinjett	Sweden
Zebra	Sweden

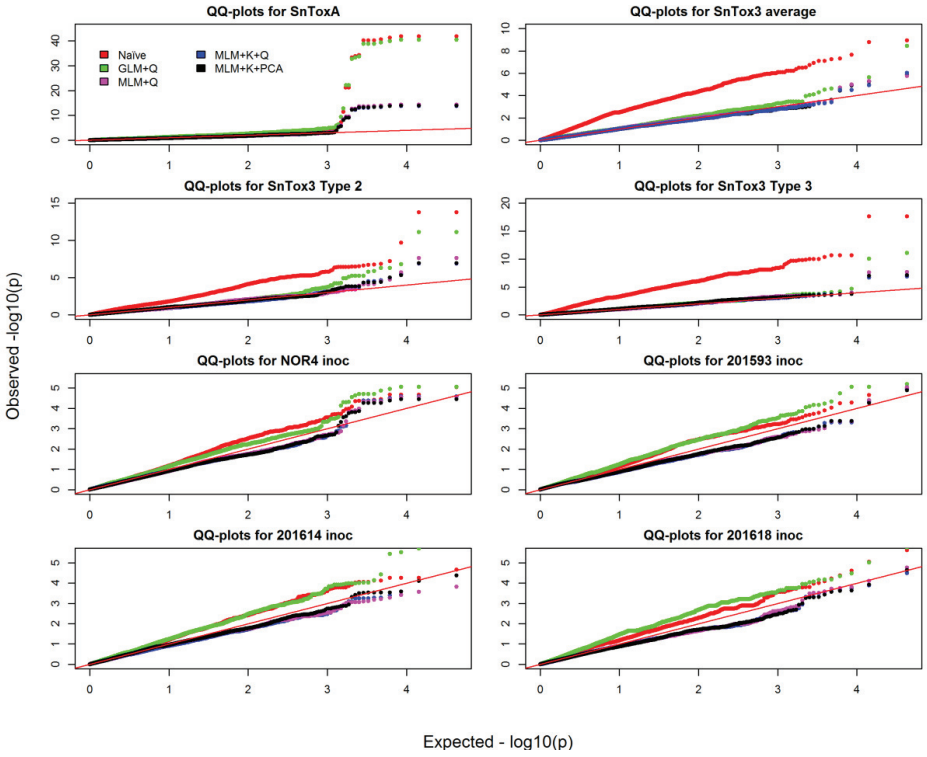

Figure S1 qq-plots for different models for marker-trait association, after infiltration with purified SnToxA and SnTox3, and inoculations (inoc) with single spore isolates NOR4, 201593, 201614 and 201618

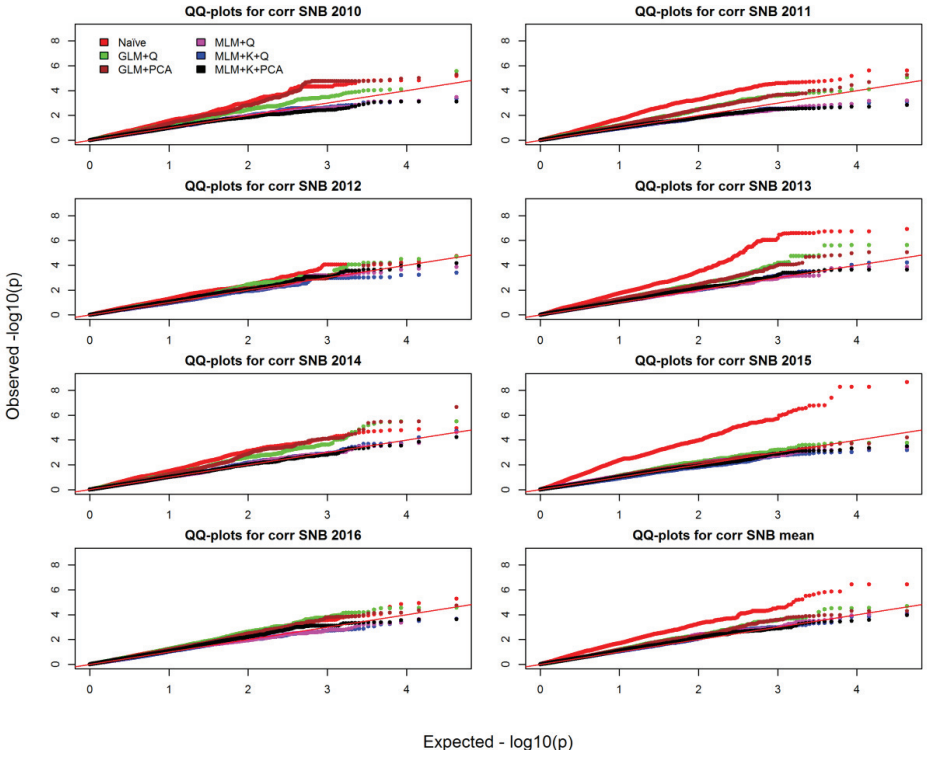

Figure S2 qq-plots for different models for marker-trait association, based on analysis of corrected SNB severities from the field trials at Vollebekk, Ås, Norway, from 2010 to 2016.

Errata

Page number	Paragraph	Changed from	Changed to
ii	L 9	*P.nodorum*	*P. nodorum*
5	L 1	stage 65-70	stage 70-75
7	L 4	target the CYP51 gene,	target CYP51,
7	L 7	target gene CYP51	target gene *CYP51*
7	L 10-15	CYP51	*CYP51*
11	L 11	a *Avr*-gene	an *Avr*-gene
15	L 25	(http://www.lcgroup.com/)	(http://www.lcgroup.com/)
16	L 31	double haploids	doubled haploids
20	L 30	f.sp. *tritici*)	f.sp. *tritici*)
23	L 2	(Juliana et al. 2017) found	Juliana et al. (2017) found
26	L 26	SnToxA, SnTox1 and SnTox3 profile	*SnToxA*, *SnTox1* and *SnTox3* profile
27	L 25	SnTox3-*Snn3* locus	SnTox3-*Snn3* interaction
29	L 20		Add sentence: This work was a continuation of the M.Sc. thesis by Ruud (2013). The reference should be: Ruud, A.K. QTL for leaf blotch resistance in spring wheat, and a method to inoculate wheat seedlings with Stagonospora nodorum. Master thesis. UMB, 2013.
Paper II, p. 5	L 30	BG261/SnTox3	BG220/SnTox3
Paper II, p. 9	L19	Conidospores	Conidiospores
Paper II, p. 9	L 29	Correlation between effector sensitivity and SNB susceptibility in the field	**Correlation between effector sensitivity and SNB susceptibility in the field**
Paper II, p. 11	L 28	Table 3	Table 5
Paper II	Table S1	Heading of column 7: SnTox3 Type2	Heading of column 7: SnTox3 Type3
Paper III, p. 3	L 28	double haploid	doubled haploid
Paper III, p. 9	L 15	True associations between marker and trait is	True association between marker and trait is
Paper III, p. 12	L 28	ignificant	significant
Paper III, p. 16	L 33	which does is *SnToxA*	which is *SnToxA*
Paper III, p. 17	L 1	*SnTox*	*SnTox3*