Genome-Wide Association Mapping of Resistance to Septoria Nodorum Leaf Blotch in a Nordic Spring Wheat Collection

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ABSTRACT *Parastagonospora nodorum* is the causal agent of Septoria nodorum leaf blotch (SNB) in wheat (*Triticum aestivum* L.). It is the most important leaf blotch pathogen in Norwegian spring wheat. Several quantitative trait loci (QTL) for SNB susceptibility have been identified. Some of these QTL are the result of underlying gene-for-gene interactions involving necrotrophic effectors (NEs) and corresponding sensitivity (*Snn*) genes. A collection of diverse spring wheat lines was evaluated for SNB resistance and susceptibility over seven growing seasons in the field. In addition, wheat seedlings were inoculated and infiltrated with culture filtrates (CFs) from four single spore isolates and infiltrated with semipurified NEs (SnToxA, SnTox1, and SnTox3) under greenhouse conditions. In adult plants, the most stable SNB resistance QTL were located on chromosomes 2B, 2D, 4A, 4B, 5A, 6B, 7A, and 7B. The QTL on chromosome 2D was effective most years in the field. At the seedling stage, the most significant QTL after inoculation were located on chromosomes 1A, 1B, 3A, 4B, 5B, 6B, 7A, and 7B. The QTL on chromosomes 3A and 6B were significant both after inoculation and CF infiltration, indicating the presence of novel NE–*Snn* interactions. The QTL on chromosomes 4B and 7A were significant in both seedlings and adult plants. Correlations between SnToxA sensitivity and disease severity in the field were significant. To our knowledge, this is the first genome-wide association mapping study (GWAS) to investigate SNB resistance at the adult plant stage under field conditions.

Abbreviations: AM, association mapping; CF, culture filtrate; G × E, genotype-by-environment; GLM, general linear model; GWAS, genome-wide association mapping study; *K*, kinship matrix; LD, linkage disequilibrium; MLM, mixed linear model; MTA, marker–trait association; NE, necrotrophic effector; PC, principal component; *Q*, population structure; QTL, quantitative trait loci; SNB, Septoria nodorum leaf blotch; SSR, simple-sequence repeat.

CORE IDEAS

- First genome-wide association mapping of adult plant Septoria nodorum blotch resistance.
- Some adult plant resistance loci were shared with seedling resistance loci.
- Other adult plant resistance loci were significant across environments.
- Resistant haplotypes were identified, which can be used for breeding.

Septoria nodorum leaf blotch caused by the necrotrophic ascomycete *Parastagonospora nodorum* is a major disease in many wheat growing areas such as Australia, the United States, and Norway (Ficke et al., 2011b; Francki, 2013; Friesen et al., 2006; Oliver et al., 2016; Ruud and Lillemo, 2018). In Norway, SNB is the dominant leaf blotch disease in spring wheat (Ficke et al., 2011b). Fungicides are commonly used to control SNB, but widespread use of the same active ingredients exert a high selection pressure on the pathogen to develop fungicide resistance as already demonstrated for strobilurin and azole fungicides (Abrahamsen, 2013; Blixt et al.,

Ruud, A.K., J.A. Dieseth, A. Ficke, E. Furuki, H.T.T. Phan, R.P. Oliver, K.C. Tan, and M. Lillemo. 2019. Genome-wide association mapping of resistance to Septoria nodorum leaf blotch in a Nordic spring wheat collection. Plant Genome 12:180105. doi: 10.3835/plantgenome2018.12.0105

Received 20 Dec. 2018. Accepted 6 May 2019.
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2009; Ficke et al., 2011a; Pereira et al., 2017). Integrated disease management with reduced dependency on fungicides and use of cultivars with sufficient genetic resistance is economically and environmentally beneficial. However, breeding for SNB resistance has been hampered by the complex nature of the phenotype.

It is important to note the difference between seedling resistance and adult plant resistance. Seedling resistance is evaluated on young seedlings, while adult plant resistance is evaluated on fully developed plants after the emergence of the flag leaf. For practical reasons, seedling tests are usually conducted under controlled greenhouse or growth chamber conditions and often with the use of single isolates to study isolate-specific interactions at the seedlings stage. When adult plant resistance is evaluated under similar controlled conditions and with the same isolates, correlations between resistance at the two growth stages can be moderately high (Jönsson, 1985). However, adult plant resistance is most commonly evaluated in field trials either with natural infection or with a mixed inoculum. Depending on the isolates being used, the correlation of adult plant field resistance and seedling resistance under controlled conditions can range from moderate to low (Jönsson, 1985; Ruud and Lillemo, 2018).

Both seedling and adult plant resistance to SNB is polygenic (Bostwick et al., 1993; Fried and Meister, 1987; Wicki et al., 1999) and large genotype-by-environment (G × E) interactions have been observed. Although many QTL for SNB resistance have been detected in single locations or years, only a few have proven to be stable across multiple environments (Francki, 2013). Furthermore, seedling and adult plant SNB resistance are often independently inherited (Fried and Meister, 1987; Rosielle and Brown, 1980; Shankar et al., 2008; Tommasini et al., 2007).

The dissection of the *P. nodorum*–wheat pathosystem into host-specific inverse gene-for-gene interactions has provided opportunities for more effective resistance breeding. *Parastagonospora nodorum* produces several small, secreted proteins known as NEs (previously called host-specific or host-selective toxins). *SnToxA* encodes a 13.2 kDa mature protein (SnToxA) and wheat lines that carry the sensitivity gene *Tsn1* (located on chromosome 5BL) undergo necrosis on infiltration (Faris et al., 2010; Friesen et al., 2006). Similarly *SnTox1* encodes a 10.3 kDa small cysteine-rich protein and sensitivity is conferred by *Snn1* on 1BS (Liu et al., 2012; Shi et al., 2016). *SnTox3* encodes a 17.5 kDa mature cysteine-rich protein (Liu et al., 2009) and sensitivity is conferred by *Snn3* on 5BS in bread wheat and 5DS in *Aegilops tauschii* Coss. (Zhang et al., 2011). In addition, at least five other NEs (SnTox2, SnTox4, SnTox5, SnTox6, and SnTox7) and corresponding *Snn* genes (*Snn2*, *Snn4*, *Snn5, Snn6*, *and Snn7*) have been partially characterized (Abeysekara et al., 2009; Friesen et al., 2007; Gao et al., 2015; Liu et al., 2006, 2009; Shi et al., 2015).

Biparental mapping populations and interval mapping for seedling SNB resistance have identified QTL on chromosomes 1A, 1B, 2B, 2D, 4B, 5A, 5B, 6A, 7A, and 7B (Abeysekara et al., 2009; Arseniuk et al., 2004; Czembor et al., 2003; Friesen et al., 2007, 2009; Gao et al., 2015; Gonzalez-Hernandez et al., 2009; Liu et al., 2004, 2006, 2009; Ruud et al., 2017; Shi et al., 2015). Adult plant leaf resistance has frequently been identified on chromosomes 1A, 1B, 2A, 2D, 3AS, 3B, 5A, 5B, 6BS, 7A, and 7B (Aguilar et al., 2005; Francki et al., 2011; Friesen et al., 2009; Lu and Lillemo, 2014; Phan et al., 2016; Ruud et al., 2017; Shankar et al., 2008).

The NE–*Snn* model suggests additive contributions to disease development from each compatible interaction (Friesen et al., 2007; Tan et al., 2012). However, epistasis has also been shown to be important. The SnTox3–*Snn3* interaction is only significant in the absence of compatible SnToxA–*Tsn1*, SnTox1–*Snn1*, and SnTox2–*Snn2* interactions (Friesen and Faris, 2012; Phan et al., 2016).

Mapping in biparental populations is limited in scope and the genetic resolution is relatively low because of high linkage disequilibrium (LD) (Flint-Garcia et al., 2003; Gupta et al., 2014). Therefore, validation of the QTL and identification of markers often requires study of several further populations. In addition, development of large secondary fine-mapping populations is required to look for candidate genes within a narrow genomic region.

Linkage disequilibrium–based association mapping (AM, or GWAS) is complementary to biparental interval mapping. Polymorphic markers associated with a phenotypic trait can be identified by means of LD between loci (Flint-Garcia et al., 2003; Thornsberry et al., 2001). The large number of historical recombination events in diverse AM panels allows for higher resolution than in biparental populations and more effective fine-mapping. Furthermore, an AM panel can be designed to capture much of the genetic variation available to a breeding program (Gupta et al., 2014).

Genome-wide association mapping studies have successfully been used to identify marker–trait associations (MTAs) for complex traits in crop plants including disease resistance and grain quality (Breseghello and Sorrells, 2006; Crossa et al., 2007; Ghavami et al., 2011; Perez-Lara et al., 2017; Tommasini et al., 2007). It has successfully been used to identify MTAs of adult plant resistance in polygenic plant–fungal pathosystems like fusarium head blight (Miedaner et al., 2011), septoria tritici blotch (Perez-Lara et al., 2017) and tan spot (Dinglasan et al., 2019).

Only a few GWAS have investigated SNB resistance and only on seedlings. Adhikari et al. (2011) identified QTL on chromosomes 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 chromosome 3A in 528 lines from the same collection. A QTL on chromosome 3BS was investigated in a small panel of 44 cultivars by Tommasini et al. (2007). Significant QTL were detected on chromosomes 5A, 5B, and 5D after GWAS of 70 hard red winter wheat lines after inoculation with isolate Sn4 (Liu et al., 2015). Recently, Phan et al. (2018) used a genetically diverse historical wheat collection sourced from the Vavilov Institute to look for QTL associated with SNB. Association mapping detected SNB QTL on 1BS

(*Snn1*), 5BS (*Snn3*), 2DS (*Snn2*), 3AL, 4AL, 4BS, 5AS, 2DL, and 7AL. Furthermore, cultivars that displayed strong resistance to SNB were observed from the Vavilov panel.

In this study, we investigated 121 spring wheat lines consisting of current and historical cultivars, breeding lines, and landraces of both Norwegian and international origins. The collection had already been screened for prevalence of sensitivity to SnToxA, SnTox1, and SnTox3 by Ruud et al. (2018). The results from that study 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 are investigated here. SnTox3 sensitivity was present in 55% of the genotypes, while sensitivity to SnTox1 was rare. Sensitivity to these latter two NEs was not correlated to increased SNB severity in the field. On this background, the objectives of the present study were to (i) evaluate the genetic basis of seedling and adult plant resistance to SNB infection in the collection; (ii) identify markers associated with sensitivity to SnToxA, SnTox1, and SnTox3 effectors; and (iii) investigate whether known or novel NE–*Snn* interactions underlie the MTAs related to SNB susceptibility.

MATERIALS AND METHODS

Plant Material

A total of 121 spring wheat lines were genotyped and analyzed in the GWAS. The lines are a subset of a Nordic spring wheat panel for MAS, which includes both Norwegian and international lines. The population is described in more detail in Ruud et al. (2018) and listed in Supplemental Table S1.

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 in Ås, Norway, as described in Ruud et al. (2018). Alpha lattice designs with two to three replicates per year were used. The natural infection and development of SNB was enhanced by mist irrigation 5 min every 30 min during daytime. From 2013 onward, naturally infected straw was spread in the field at Zadoks stage 13/21 (Zadoks et al., 1974) to further promote SNB infection. Leaf blotch severity was scored twice every season by estimating the percentage diseased canopy in the individual plots. Because of differences in earliness, the first score captures resistance differences among the early lines while the second score helps to differentiate resistance levels of the late lines. The mean of the first and second scoring was used for analysis. The SNB scoring can be confounded by variation in traits such as earliness and plant height. Therefore, plant height and days from sowing to heading were used as covariates in multiple regression to obtain corrected SNB severities. These were calculated by subtracting the expected disease scores based on the model with days to heading and plant height from the original disease scores. This gives negative corrected SNB severities for lines that are more resistant than the average for the given earliness and plant height and positive corrected SNB severities for lines that are more susceptible than the average. See Ruud et al. (2018) for further details.

Parastagonospora nodorum Infection Assays

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

Four previously characterized *P. nodorum* isolates differing in their presence or absence of *SnToxA, SnTox1*, and *SnTox3* genes were used (Table 1). The isolates NOR4, 201593, and 201618 were the same as those described and tested on the biparental recombinant inbred line population SHA3/CBRD × Naxos (Ruud et al., 2017), while isolate 201614 was collected from the Swiss spring wheat cultivar Quarna in Kure, Østfold, Norway (Ruud and Lillemo, 2018).

Parastagonospora nodorum isolates were grown on V8-PDA at 20 to 23°C under 24 h light (white plus near ultra-violet) for ~7 d 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 mL^{-1}. One drop of Tween 20 was added per 50 mL spore suspension to reduce surface tension. The 2-wk-old seedlings were inoculated with a spray painter until runoff, placed in a humidity chamber at 100% relative humidity, 20°C, and constant light for 24 h 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). A score of 0 indicates that no disease symptoms were observed whereas a score of 5 indicates a fully necrotic leaf. All experiments were performed in biological triplicates.

Effector and Culture Filtrate Infiltrations

SnToxA, SnTox1, and SnTox3 infiltration assays were performed as described in Ruud et al. (2018) on plants that were grown as described above but with two seeds per cone per genotype. Differential lines BG261 (*Tsn1*, *snn1*, *snn3*), M6 (*tsn1*, *Snn1*, *snn3*), and BG220 (*tsn1*, *snn1*, *Snn3*) were used as positive controls to ensure that SnToxA, SnTox1, and SnTox3 were active. Briefly, the semipurified effectors were infiltrated into the middle part of the second fully expanded leaves of 12- to 14-d-old seedlings using a needleless syringe. The sensitivity reactions were scored on a 0-to-3 scale after 5 to 7 d according to the protocol described in Friesen and Faris (2012): 0 = no reaction, 1 = mottled chlorosis, 2 = chlorosis, and 3 = necrosis and tissue collapse.

For culture filtrate (CF) infiltration, *P. nodorum* isolates were grown as described for preparation of inoculum until sporulation (5–7 d). The cultures were then flushed with distilled water and scraped with a

flame-sterilized inoculation loop to release the spores. Six microliters 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 and 27°C for 72 h before placed in darkness in an incubation chamber at 20°C for 3 wk. The cultures were then filter sterilized and a needleless syringe was used to infiltrate the CF into the second leaf of 12- to 14-d-old seedlings. The sensitivity reactions were scored according to the same scale as for semipurified effectors.

Genotyping of the Wheat Panel

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 (simple-sequence repeat [SSR]) analysis was performed with fluorescent-labeled primers and polymerase chain reaction 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 markers with minor allele frequencies below 0.05 and >10% missing data. Low-quality markers were further filtered out based on having many lines with no-call or heterozygote scores. Only unique markers, based on segregation of genotypes in the AM panel, were included. The final set for GWAS consisted of 21,475 SNP and SSR markers.

The polymorphic markers were given positions based on the consensus map developed by Wang et al. (2014). The 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 LD) with other significant SNP markers with known position.

Association Analysis

Least squares means were obtained for each trait using PROC MIXED in SAS 9.3 (SAS Institute, 2011). 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 Ruud et al. (2018).

Six different regression models were tested: Naïve (general linear model, [GLM]), GLM + population structure (Q), GLM + PC, mixed linear model (MLM) + kinship matrix (K), MLM + K + Q, and MLM + K + PC. The best model based on the generated quantile–quantile plots (Supplemental Fig. S4, S5) was the MLM + K + Q model. Genomic regions associated with the traits were identified using the weighted compressed 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 $\mathbf{y} = \mathbf{X}\beta + \mathbf{Q}\mathbf{v} + \mathbf{u} + \mathbf{e}$, where $\mathbf{y}$ is the vector of the phenotypic values (best linear unbiased predictors), $\mathbf{X}$ is the vector of SNP marker genotypes, β is the vector of marker fixed effects to be estimated, $\mathbf{Q}$ is the population structure matrix derived from structure analysis, $\mathbf{v}$ is a vector of fixed effects due to population structure, $\mathbf{u}$ is the vector of random effects, and $\mathbf{e}$ is the vector of residuals.

Linkage disequilibrium 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 >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). The 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 intrachromosomal loci against genetic distance (cM) between marker pairs. The relationship between LD decay and distance was summarized by fitting the data to a nonlinear 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.

A population structure of $K = 5$ was used, as determined by Jansen (2014).

Significance Threshold

For traits where a single gene-for-gene interaction was expected, that is, the infiltration with SnToxA, SnTox1, and SnTox3, the Bonferroni correction with $\alpha = 0.05$ was used, giving a threshold of $-\log10\ (0.05/21475) = 5.63$.

For complex traits such as disease rating or to account for the possibility of multiple effectors produced in culture filtrates, a 0.1 percentile of the distribution of p-values was used as an exploratory cutoff following the method described in Pasam et al. (2012). A rough approach to further evaluate the threshold was performed by visually inspecting the quantile–quantile plots (Supplemental Fig. S4, S5). 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 based on the TASSEL output were visualized in R Studio (RStudio Team, 2016) with the R package qqman (Turner, 2014).

Allele Stacking and Haplotype Analysis

To investigate the effect of accumulated alleles on resistance level, the wheat lines were assigned to groups according to their number of resistance alleles. The resistant allele was

determined based on the predicted effect of significant markers associated with the QTL from the field trials. Significant differences in corrected field resistance to SNB between the groups were determined by a Tukey's HSD test.

Two loci of particular interest were selected for haplotype analysis: the stable adult plant QTL on chromosome 2DL and the QTL on chromosome 5BS covering the *Snn3* locus. Markers were used for haplotype construction based on these criteria: (i) they were located near the QTL, (ii) with high LD (≥0.8), and (iii) were significant for the trait. Haplotypes were determined by R package Haplotypes, and haplotype networks based on TCS genealogy (Clement et al., 2000) were calculated using PopART (http://popart.otago.ac.nz/index.shtml) (Leigh and Bryant, 2015).

RESULTS

Characterizing the Effector Profile of the *Parastagonospora nodorum* Isolates

The effector profile of *P. nodorum* isolates grown in Fries 3 broth medium was determined by infiltrating filter-sterilized culture filtrates into 10 wheat lines that carry known differential sensitivities to SnToxA, SnTox1, SnTox2, SnTox3, SnTox4, SnTox5, and SnTox6 (Table 1).

Resulting chlorosis–necrosis reactions that were 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. Chlorotic activities were observed on wheat cultivar M6 (*Snn1*) when infiltrated with culture filtrates derived from NOR4 and 201618. Wheat lines ITMI37 (*Snn6*) and BG223 (*Snn2*) were sensitive to culture filtrates derived from all four *P. nodorum* isolates, whereas, all other remaining lines displayed differential sensitivity. We were unable to detect SnToxA activity on BG261 using culture filtrates derived from the NOR4 isolate that carries the *SnToxA* gene, indicating that this effector is not expressed in liquid culture. Overall, the results show that isolates 201593, 201614, and 201618 possess different effector profiles.

Seedling and Adult Plant Disease Data

The corrected SNB severities for the field showed continuous bell-curve distributions resembling the normal distribution (Supplemental Fig. S1). Hence, the requirement for application of linear mixed models is met. The disease severities after seedling inoculation with isolates 201593 and 201614 were also close to normally distributed (Supplemental Fig. S2). However, the wheat lines were more susceptible to isolate NOR4, and more intermediate susceptibility was observed after inoculation with isolate 201618.

Correlation Between Seedling Disease Reactions and Sensitivity Reactions

We first investigated whether disease reactions between different isolates were correlated. Based on our SNB assay, the pattern of seedling SNB on the Nordic wheat panel significantly correlated. Septoria nodorum leaf blotch caused by isolates 201593 and 201614 possessed the highest level of positive correlation ($r = 0.78$; Table 2). The correlation between reaction types after inoculation with isolate 201618 and the other isolates was lower but remained significant.

Table 1. Combinations of *SnToxA*, *SnTox1*, and *SnTox3* genes in the isolates NOR4, 201593, 201614, and 201618 based on polymerase chain reaction results. Sensitivity reactions to single isolate culture filtrate (CF) infiltration by differential lines with known, single SnTox sensitivities. Reaction scale: 0 = no reaction observed, 1 = mottled chlorosis, 2 = chlorosis, 3 = necrosis and tissue collapse.

			Presence or absence (+ or –) of ToxA, Tox1, Tox3			
			+ + +	– – +	– + +	– – –
Line	Provider	Sensitivity	NOR4	201593	201614	201618
BR34	T. Friesen	Insensitive	0	0	0	0
BG261	T. Friesen	SnToxA	0	0	0	0
M6	T. Friesen	SnTox1	1	0	0	1
BG223	T. Friesen	SnTox2	2	2	2	2
BG220	J. Faris	SnTox3	3	2	2	0
AF89	J. Faris	SnTox4	1	2	0	1
LP29	S. Xu	SnTox 5	0	0	1	0
ITMI37	T. Friesen	SnTox6	2	2	2	2

We also determined whether disease scores were correlated with CF infiltration, which would indicate that NEs expressed in culture were significant virulence determinants. The correlation between the sensitivity reaction types after infiltration with CF and disease reaction types after inoculation was significant ($p < 0.0001$) for isolates NOR4, 201593, and 201614 (Table 2). The highest correlations were observed between disease reaction types after inoculation with isolate 201593 and sensitivity reactions after infiltration with CF from isolates 201614, NOR4, and 201593. Isolate 201618 was most different from the others, and sensitivity reaction types to infiltration with this isolate did not correlate with inoculation results from the other three isolates.

The plants were also infiltrated with single, semipurified effectors. The correlation between the sensitivity reaction after these infiltrations and disease reaction type after inoculation was significant and highest between disease reactions after inoculation with isolate NOR4 (which produces SnToxA) and sensitivity reaction types after SnToxA infiltration (Table 2). The correlation between SnToxA sensitivity at the seedling stage, and corrected SNB severity from the field trials, was significant in several years. However, there was no significant correlation between the results of SnTox3 infiltration and field results (Table 3).

Examining the Correlation Between Seedling and Adult Plant Disease Severity

We then determined if seedling assays can be used as a reliable indicator for disease severity under field conditions (Table 3). We observed a positive correlation between the corrected field severity scores and the

Table 2. Pearson's correlation coefficient between the disease reaction types after inoculations with the four different isolates NOR4, 201593, 201614, and 201618 (spore concentration = 10^6 spores mL^{-1}), 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
Inoculation	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 at the 0.05 probability level.

** Significant at the 0.01 probability level.

*** Significant at the 0.001 probability level.

seedling data using isolates 201593, 201614, and NOR4. In contrast, isolate 201618, that lacked *SnToxA*, *SnTox1*, and *SnTox3*, was not significantly correlated to any year of corrected SNB severity from the field trials. The lowest correlation between the field data and the seedling inoculation results was shown in 2012.

Linkage Disequilibrium

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 (Supplemental Fig. S3). The r^2 for initial LD ($p < 0.001$) was 0.49 and varied on each subgenome 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.

Genome-Wide Association Mapping

We then investigated whether QTL corresponding to *Tsn1* and *Snn3* can be detected in the Nordic wheat panel using sensitivity responses to SnToxA and SnTox3 infiltration. For phenotypic response to SnToxA, a major QTL was located on chromosome 5BL (Fig. 1; Supplemental Fig. S6) close to the SSR markers *fcp1*, *fcp620*, and *fcp394*, which are known to be closely linked to *Tsn1* (Zhang et al., 2009). Three SNP markers (*Tdurum_contig12066_126*, *Tdurum_contig12066_247*, and *tplb0027f13_1346*, marked with and asterisk (*) in Supplemental Table S2) were assigned to chromosome 5A in the consensus map (Wang et al., 2014) but were in complete LD with the significant markers on chromosome 5B. These markers were subsequently reassigned to chromosome 5B.

The wheat panel was infiltrated with SnTox3 and sensitivity reactions scored based on two reaction types: 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. Markers associated with sensitivity to SnTox3 were observed through association mapping of different sensitivity response phenotype (Fig. 1; Supplemental Fig. S7).

The chlorotic Type 2 reaction was more associated with the SSR markers *cfd20* and *gwm234*, and SNPs assigned to chromosome 5A in the consensus map (Wang et al., 2014) (Supplemental Table S3). The necrotic Type 3 reaction mapped to chromosome 5BS, but also some markers on chromosome 2D and 2A showed associations below the Bonferroni threshold (Supplemental Table S3). Notably, the SSR markers *cfd20* and *gwm234* known to be associated with SnTox3 sensitivity from literature did not show any strong association with the Type 3 reaction, only the Type 2 reaction.

Table 3. Pearson's correlation coefficient between seedling 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 semipurified necrotrophic effectors (NEs) (SnToxA and SnTox3) 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***	0.19	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 average	0.04	–0.06	–0.10	–0.10	–0.02	–0.08	0.03	–0.019

* Significant at the 0.05 probability level.

** Significant at the 0.01 probability level.

*** Significant at the 0.001 probability level.

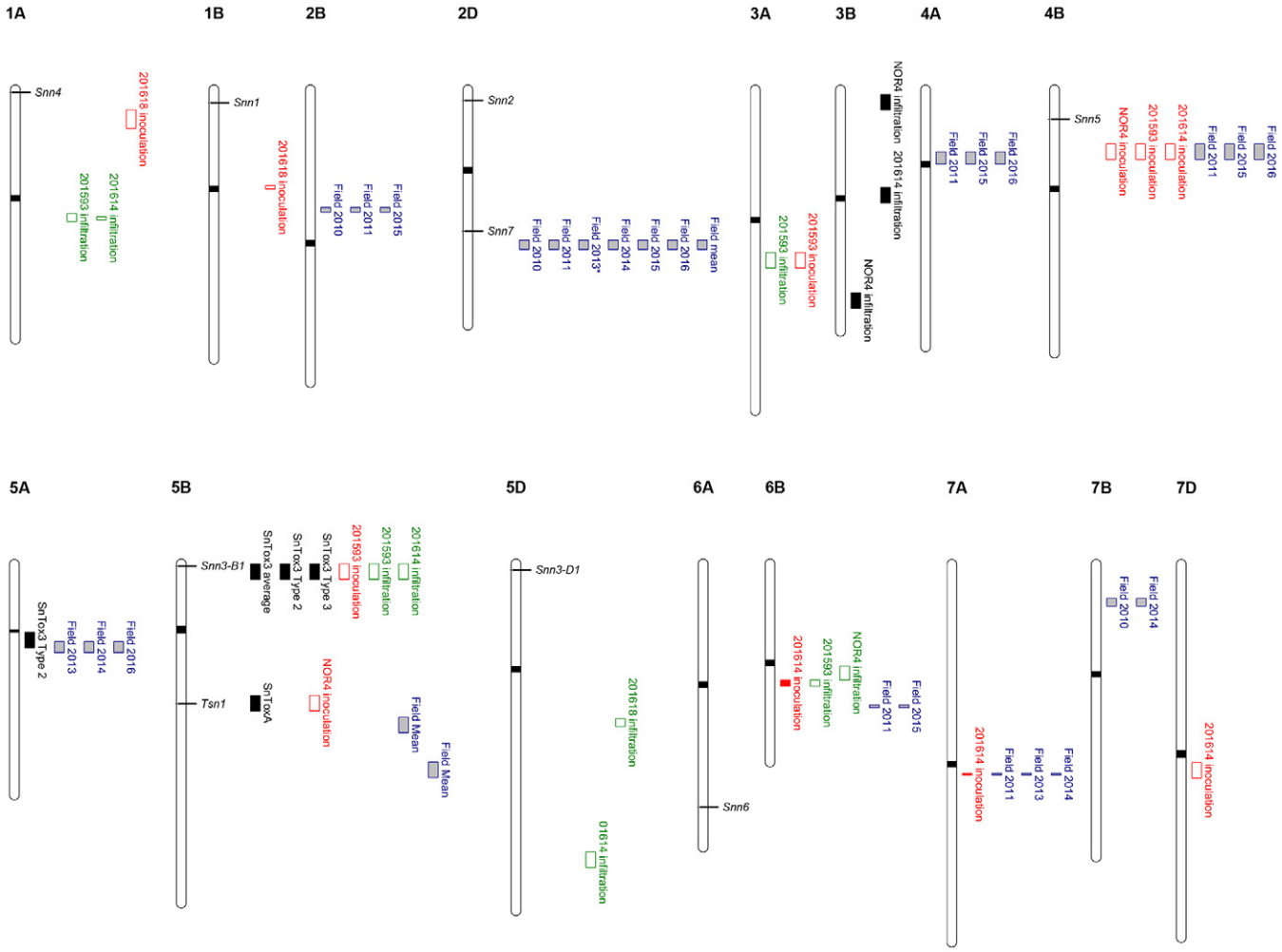

Fig. 1. Chromosome maps showing the locations of significant marker–trait associations in the present genome-wide association study (GWAS) together with the locations of known necrotrophic effector (NE) sensitivity genes (*Tsn1* and *Snn1*–*Snn7*). The quantitative trait loci (QTL) detected in the GWAS are indicated by bars on the right hand side of each chromosome. The QTL for NE sensitivity reactions are indicated in black, seedling inoculation in red, CF infiltrations in green, and adult plant field reactions in blue.

Quantitative Trait Loci Associated with Seedling Inoculation with Single *Parastagonospora nodorum* Isolates

Genome-wide association mapping was performed on the Nordic spring wheat panel inoculated at the seedling stage with individual *P. nodorum* isolates NOR4, 201593, 201614, and 201618 (see Fig. 1 for overview, more details in Supplemental Fig. S8 and Supplemental Table S4). The most significant MTAs after inoculation were detected on chromosome 5B associated to SnToxA sensitivity after inoculation with isolate NOR4 and 5BS associated with SnTox3 sensitivity after inoculation with isolate 201593, respectively.

Another QTL was detected on chromosome 4B and was significant after inoculation with NOR4, 201593, and 201614. Quantitative trait loci were also detected on chromosome 3A after inoculation with isolate 201593, on chromosomes 6B and 7A after inoculation with isolate 201614, and on chromosomes 1A and 1B after inoculation with 201618.

Quantitative Trait Loci Associated with Sensitivity to *Parastagonospora nodorum* Culture Filtrate Infiltration

Significant markers were detected on many chromosomes after infiltration with filter sterilized CF from the four isolates (see Fig. 1 for overview, more details in Supplemental Fig. S9 and Supplemental Table S5). The most significant markers were located on chromosome 5B associated with SnTox3 sensitivity and on chromosomes 6B and 1A. The QTL on chromosome 6B was significant after infiltration with NOR4 and 201593 and was in the same position as the QTL detected after inoculation with 201614. The QTL detected on chromosome 1A after infiltration with isolate 201614 was not identical with the QTL on chromosome 1A detected after inoculation with isolate 201618.

Quantitative Trait Loci Associated with Adult Plant Septoria nodorum Leaf Blotch under Field Conditions

Corrected SNB severity scores were obtained by multiple regression with plant height and days to heading as covariates (Ruud et al., 2018) and used for the GWAS of adult plant SNB resistance from the field trials. Unique

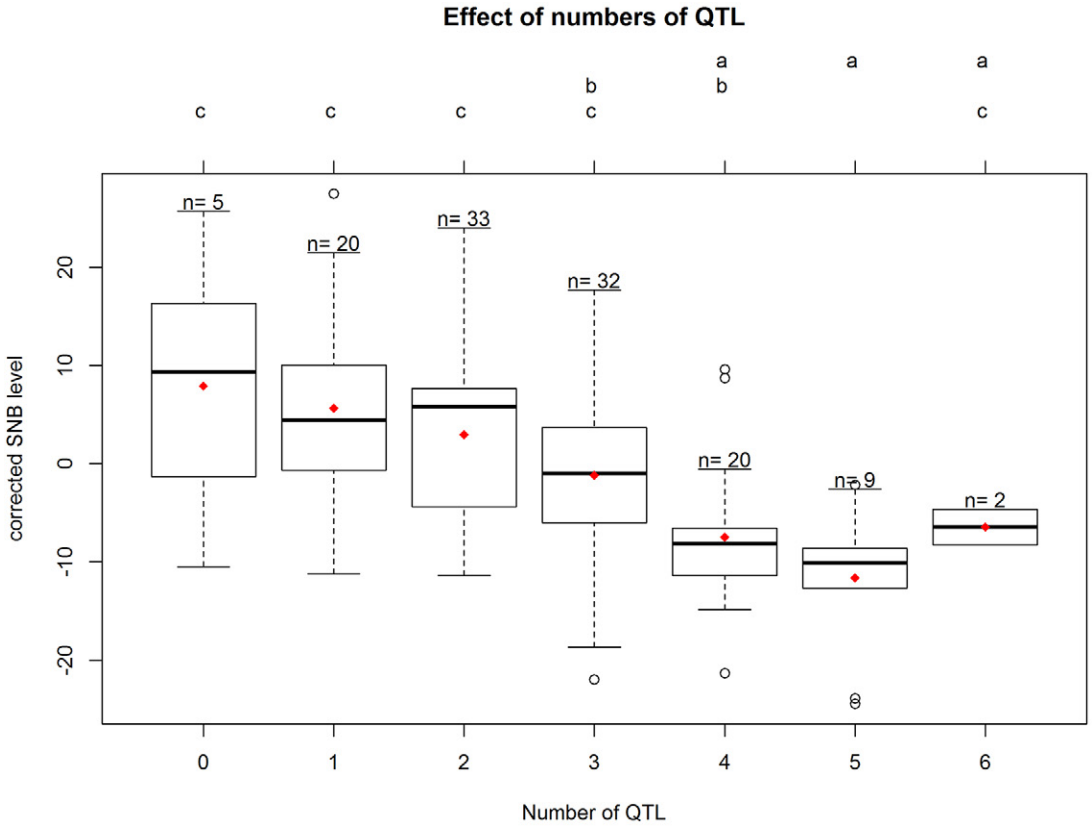

Fig. 2. Boxplot showing the effect of numbers of resistance quantitative trait loci (QTL) based on the mean corrected Septoria nodorum blotch (SNB) severity. Groups are significantly different from groups with a different letter (on top of the plot) based on Tukey's HSD test. The horizontal line in each box represents the median and the red dot represents the mean for each group.

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. As such, QTL identified on chromosomes 2B, 2D, 4A, 4B, 5A, 6B, 7A, and 7B were detected in multiple environments (shown in Fig. 1; see Supplemental Fig. S10 and Supplemental Table S6 for details).

Allele Stacking

The effect on corrected SNB severity by stacking different numbers of the robust adult plant resistance alleles was examined (Fig. 2). We observed that individual lines carried from zero to six of the eight QTL. Wheat lines that carried four or more resistance-associated alleles were significantly more resistant to SNB than lines with two or less resistance alleles (Fig. 2).

Haplotype Analysis

Seven haplotypes were identified based on haplotype analysis of five markers on chromosome 5BS significantly associated with SnTox3 sensitivity (Supplemental Table S7; Fig. 3). Haplotypes 1, 3, and 6 were predominantly connected to insensitive genotypes. Haplotypes 2 and 4 included most of the Type 2 sensitive genotypes although haplotype 2 also included several lines with Type 3 sensitivity. Haplotype 7 was dominated by Type 3 sensitive lines, and Haplotype 1 consisted of a single insensitive Norwegian breeding line. Haplotypes 2 and 4 were associated with significantly higher seedling resistance after inoculation with the Tox3-producing isolate 201593 than haplotype 7 (Fig. 3b).

Three haplotypes were identified based on haplotype construction using seven markers associated with the adult plant QTL on chromosome 2D (Supplemental Table S8; Fig. 4). Because of different allele frequencies in the subpopulations of the mapping panel, we analyzed the effect of 2D haplotype separately for the exotic vs. the adapted lines. Lines with Haplotype 1 were significantly more resistant than lines with Haplotype 2 in both subpopulations (Fig. 4b,c).

DISCUSSION

The Roles of SnToxA, SnTox1, and SnTox3 Sensitivities

The *SnToxA*, *SnTox1*, and *SnTox3* genes are present in the majority of Norwegian isolates (Ruud et al., 2018) and of the 121 lines that were genotyped and used for the present GWAS, 46, 14, and 58.5% of the lines were sensitive to SnToxA, SnTox1, and SnTox3, respectively. It seems plausible that the high frequency of *SnToxA* and *SnTox3* in the *P. nodorum* isolates collected from Norwegian wheat fields is an adaptation of the pathogen to the local host cultivars.

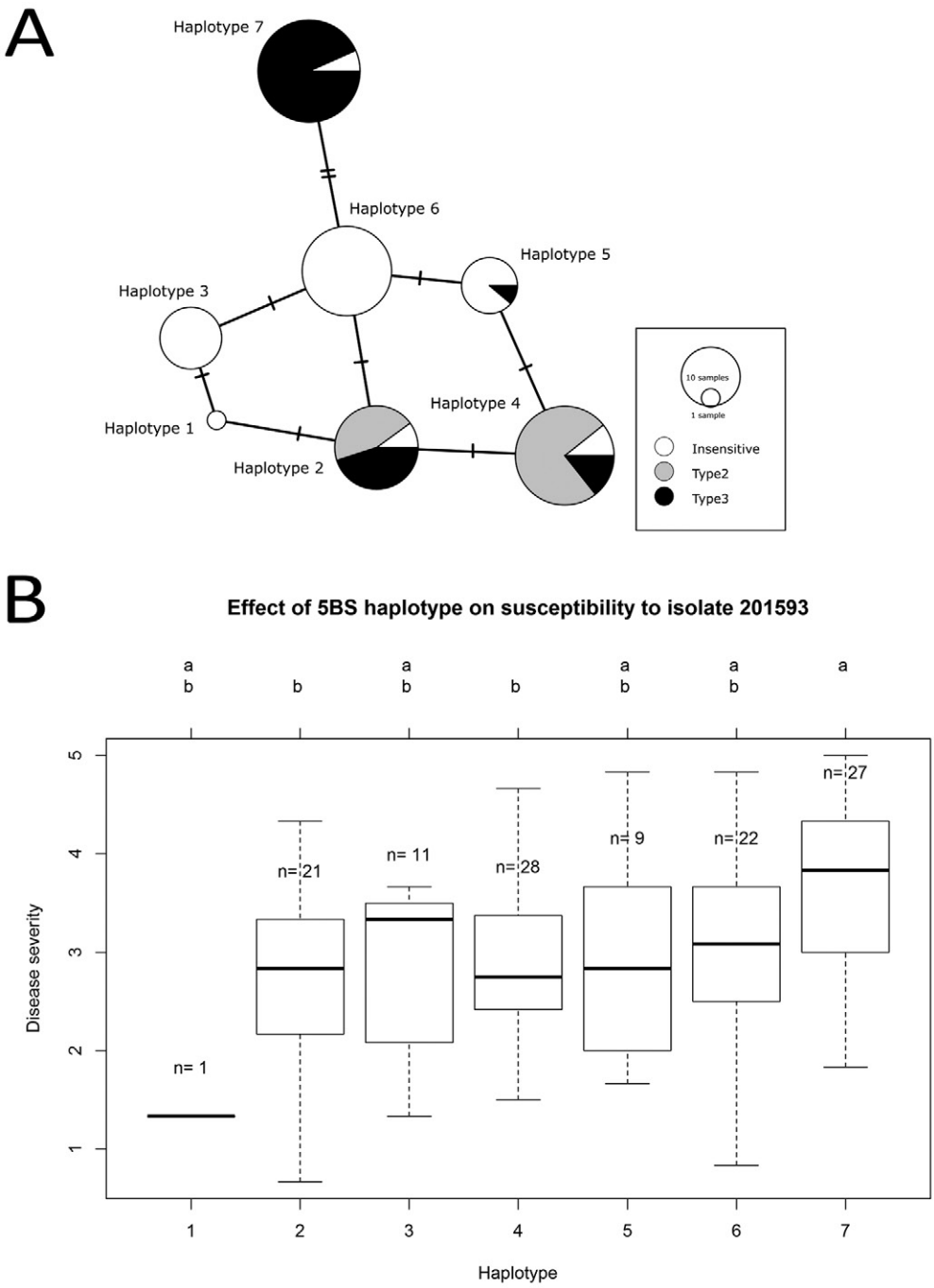

Fig. 3. Haplotype analysis of the *Snn3* locus. (A) Network of the seven haplotypes linked to *Snn3*. The size of the node is proportional to the number of genotypes with the haplotype. (B) Boxplots showing the effects of different haplotypes at the *Snn3* locus after inoculation with *Parastagonospora nodorum* isolate 201593. The haplotypes are ordered according to their mean disease severities.

Only a few lines were sensitive to SnTox1, which is in line with other screens of hexaploid wheat (Downie et al., 2018; Shi et al., 2016). It 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 and the results are not included. Insufficient linkage of the *Snn1* locus to the markers may be an additional explanation.

ToxA is a major pathogenicity factor on wheat expressing *Tsn1* (Ciuffetti et al., 2010; Friesen et al., 2006), and significant SNP and SSR markers for SnToxA-sensitivity were detected on chromosome 5BL (Fig. 1; Supplemental Fig. S6, Supplemental Table S2). Three of the markers (marked with and asterisk (*) in Supplemental Table S2) were placed on chromosome 5A in the consensus map (Wang et al., 2014) but were in complete LD with the markers on chromosome 5B. They also mapped to chromosome 5B in several biparental mapping populations (Supplemental Table S6 in Wang et al.

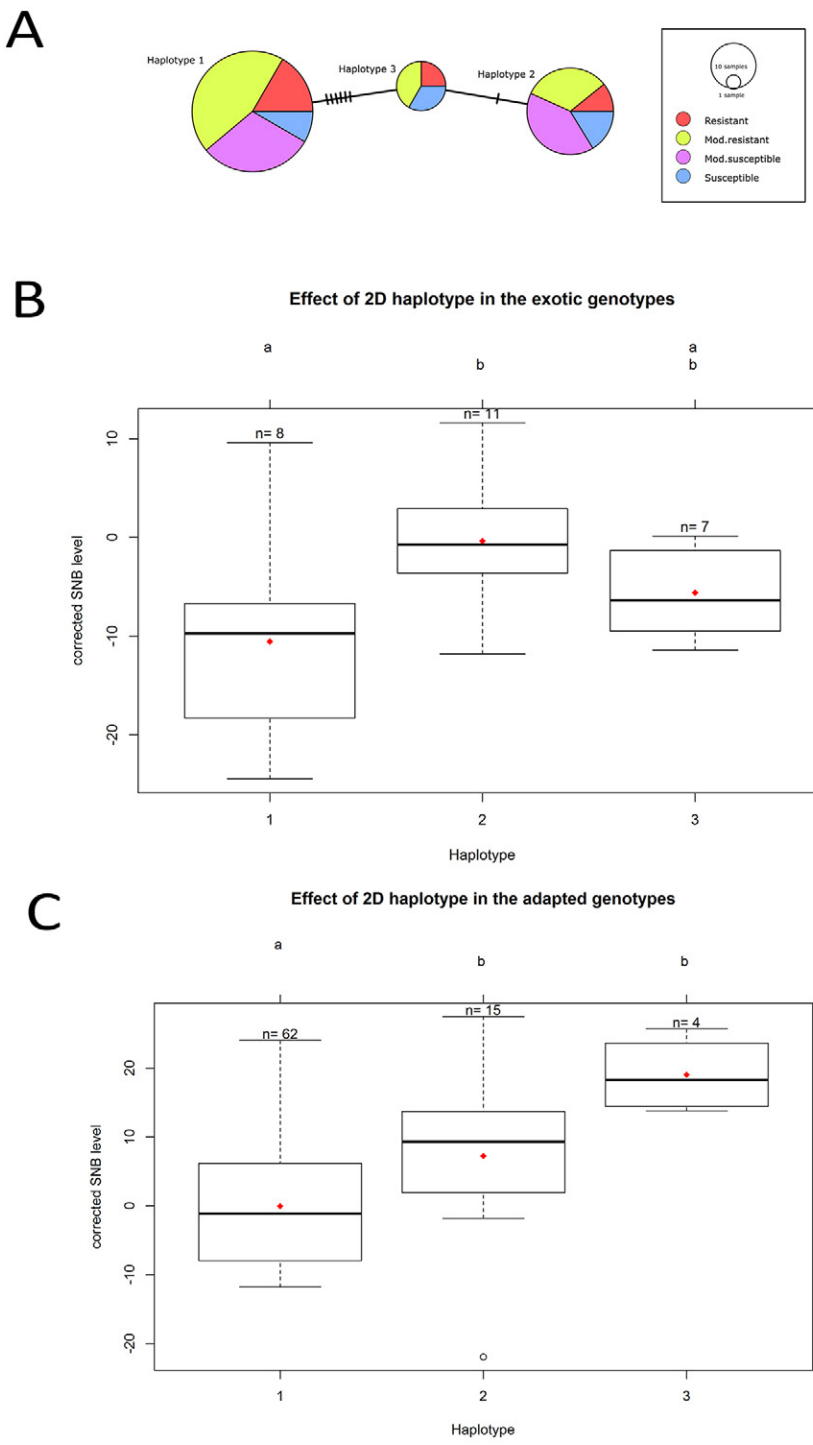

Fig. 4. Haplotype analysis of the adult plant Septoria nodorum blotch (SNB) severity quantitative trait locus (QTL) on 2DL. (A) Network of the three haplotypes linked to the QTL on 2DL. The size of the node is proportional to the number of genotypes with the haplotype. (B) Boxplots showing the effects of different haplotypes at the 2DL locus on field SNB susceptibility in the exotic lines. Significant susceptibility differences between haplotypes are indicated with different letters above the plots. (C) Boxplots showing the effects of different haplotypes at the 2DL locus on field SNB susceptibility in the adapted lines.

[2014]). These markers also corresponded to the 5A locus reported by Liu et al. (2015) on 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 chromosome 5BL. The *Tsn1* locus on 5BL was the most important QTL after inoculation with isolate NOR4 (Fig. 1). However, since SnToxA is usually not produced in liquid *P. nodorum* culture (Rybak et al., 2017; Tan et al., 2015), the QTL could not be detected after infiltration with the CF of NOR4.

We have shown before that sensitivity to SnToxA is associated with higher field disease severity in our spring wheat panel (Ruud et al., 2018). Although the *Tsn1*-linked markers were not detected above the threshold for the

field data in the present study they were the markers on chromosome 5BL with strongest association in 2012 and 2014 (Supplemental Fig. S10c,e). This illustrates the complexity of the trait and a limitation of GWAS to capture minor QTL for polygenic traits.

While the sensitivity reaction after infiltration with SnToxA appeared to be qualitative with infiltration resulting in either necrotic or insensitive leaves, the reactions to SnTox3 included a group of lines that developed chlorosis instead of necrosis. This has also been observed by others (Shi et al., 2016; Waters et al., 2011), and we showed previously that the Type 2 reaction was the only sensitivity reaction to SnTox3 in the Swedish material (Ruud et al., 2018). Here, in the GWAS we attempted to disentangle the genetics by analyzing the two reaction types separately. The SSR markers *cfd20* and *gwm234* known from literature to be associated with *Snn3* (Friesen et al., 2008) were significant for the Type 2 reaction (Supplemental Fig. S7b, Supplemental Table S3). These markers have been mapped to the distal end of chromosome 5BS (Friesen et al., 2008). Some SNP markers associated to the Type 2 reaction were also detected on chromosome 5A but below the Bonferroni thresholds for significance (Supplemental Fig. S7b, Supplemental Table S3). The markers most strongly associated with the Type 3 reaction (Supplemental Fig. S7c, Supplemental Table S3) were identical with SNP markers detected in the biparental mapping population SHA3/CBRD × Naxos (Ruud et al., 2017), in a multiparent advanced genotype cross (MAGIC population) and in a panel of 480 European wheat varieties (Downie et al., 2018). Interestingly, the SSR markers *cfd20* and *gwm234* were not associated with the Type 3 reaction in GWAS (Supplemental Fig. S7c, Supplemental Table S3) and were monomorphic in the SHA3/CBRD × Naxos population (Ruud et al., 2017).

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 whether modulating factors are involved, also need to be resolved. This seems to be consistent with what was observed in SnTox1–Snn1 interaction. Although *Snn1* has been cloned (Shi et al., 2016), phenotypic differences in response to SnTox1 could not be explained by SNP variations in *Snn1* coding regions (Shi et al., 2016).

Haplotype analysis was also conducted to investigate the background for the two sensitivity reaction types to SnTox3 (Supplemental Table S7; Fig. 3). While Haplotypes 1, 3, and 6 only included insensitive lines and could be used to breed for SnTox3-insensitive lines, the other haplotypes were more (Haplotype 2) or less (Haplotype 4, 5, and 7) mixed. Downie et al. (2018) developed a highly but not completely diagnostic marker for SnTox3 sensitivity based on *Excalibur_c47452_183*, which was also the most significant SNP marker in our spring wheat panel.

Other Important Quantitative Trait Loci at the Seedling Stage

A QTL was detected on chromosome 1AS after inoculation with isolate 201618 (Fig. 1; Supplemental Fig. S8d, Supplemental Table S4). *Snn4* is located on chromosome 1AS (Abeysekara et al., 2009). However, infiltration of the *Snn4* differential line AF 89 with CF from isolate 201618 did not produce a sensitive reaction (Table 1). Only CF from isolate 201593 induced sensitivity in the *Snn4* differential line AF89 (Table 1). Two isolates (201593 and 201614) produced seedling reactions located to chromosome 1AL, but only for infiltration with CF and not for inoculation (Fig. 1).

The QTL on 1B after inoculation with 201618 seemed to be isolate specific but was also significant in the field in one of the years (2011; Supplemental Fig. S10b; Supplemental Table S6). *Snn1* is located on chromosome 1BS (Liu et al., 2004b) but in a more distal position than the QTL detected in the present study. A QTL on chromosome 1B was also detected after inoculation with the same isolate in the SHA3/CBRD × Naxos population (Ruud et al., 2017) but similarly with a more distal position on the chromosome.

A QTL on chromosome 3A was detected after inoculation and infiltration with isolate 201593 but not the others and may be an isolate-specific, novel NE–*Snn* interaction. Two loci associated with seedling SNB resistance were detected on chromosome 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).

A significant QTL was detected on chromosome 4B after inoculation with NOR4, 201593, and 201614 (Fig. 1; Supplemental Fig. S8a,b,c, Supplemental Table S4). The locus was also significantly associated with corrected SNB severity in the field in 2011, 2015, and 2016. Several QTL for seedling SNB resistance have previously been identified on chromosome 4B (Liu et al., 2004b; Phan et al., 2016) 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. (2004) and Phan et al. (2016) were located on the long chromosome arm, while the QTL detected in the present study and *Snn5* are located on the short arm.

The significant markers on chromosome 6B were detected after inoculation with one isolate (201614) and infiltration with another isolate (201593), 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 used, also illustrating functional redundancies (Tan et al., 2015) and not always additive interactions.

Differences in QTL profiles detected by culture filtrates and inoculation assays is a common phenomenon and have been investigated in other plant pathogens such as *Leptosphaeria maculans* causing black leg of canola [*Brassica juncea* (L.) Czern. subsp. *Juncea*] (Soyer et al., 2015). In their study, differential expressions of different sets of effectors were found to be controlled by epigenetics (Soyer et al., 2015).

Adult Plant Field Resistance

Since the field trials relied on natural infection, variation was expected because of 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.

Quantitative trait loci on chromosomes 2B, 2D, 4A, 4B, 5A, 6B, 7A, and 7B were detected across at least 2 yr (Fig. 1). The QTL on chromosomes 2B, 2D, 4A, 4B, and 7A were the most stable loci, and the QTL on chromosomes 4B and 7A were detected both at the seedling and adult plant stage. The QTL on chromosome 5A conferring adult plant SNB resistance in 2013, 2014, and 2016, mapped closely to the markers associated with Type 2 reaction to SnTox3 (Fig. 1; Supplemental Table S3, S6) according to the consensus map. However, the LD between markers identified after SnTox3 infiltration and field experiments, respectively, was very low ($r^2 < 0.07$) and marker effects were specific to the traits. Therefore, we concluded that these QTL are independent.

The QTL on chromosomes 2B, 4A, 4B, 5A 6B, and 7B appeared to be novel QTL for adult plant SNB resistance, although a QTL on chromosome 2B has been described for seedling SNB resistance (Czembor et al., 2003) and the QTL on chromosome 4B was discussed above.

A Stable Adult Plant Resistance Locus on Chromosome 2DL

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 chromosome 2DL. At least two QTL for SNB flag leaf resistance have previously been described on chromosome 2D (Aguilar et al., 2005; Shankar et al., 2008): one on the long arm and the other 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 chromosome 2DL (Shi et al., 2015). The importance of the 2DL QTL was confirmed in the haplotype analysis, showing that lines with Haplotype 1 were significantly healthier in the field than lines with Haplotype 2 (Fig. 4a,b,c). The robust nature of this QTL makes it a good candidate for marker-assisted selection on the haplotype level.

Other Important Quantitative Trait Loci at the Adult Plant Stage

The QTL on chromosome 7A detected in 2011, 2013, and 2014 (Supplemental Fig. S10b,d) and after inoculation with isolate 201614 (Supplemental Fig. S8c) mapped to the same locus as the QTL reported in SHA3/CBRD × Naxos (Lu and Lillemo, 2014; Ruud et al., 2017). The QTL on chromosome 7B significant in 2010 and 2014 seemed to be unique and did not correspond to other QTL on chromosome 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 chromosome 1B after inoculation with isolate 201618 and in the field in 2011 (Supplemental Fig. S10b). The markers and positions were not identical but sufficiently close to be considered the same QTL, taking the half-decay LD distance into account.

Despite the importance for seedling reactions after infiltration and inoculation, no effect was identified for the SnTox3–*Snn3* interaction in the field. The masking effect of SnToxA-*Tsn1* and other NE–*Snn* interactions 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 (Fig. S8). Both SnToxA and SnTox3 were prevalent in the 62 genotyped Norwegian *P. nodorum* isolates, and the corresponding sensitivities were present in ~50% of the genotypes in the Nordic spring wheat collection (Ruud et al., 2018). Perhaps SnToxA-*Tsn1* has a masking or epistatic effect on SnTox3-*Snn3* also at the adult plant stage or other QTL can mask the effect.

The quantitative effects of SNB resistance could be illustrated by the fact that three resistance QTL were necessary to display an average level of resistance among the 121 lines (Fig. 2). Only two genotypes (J03 and Fram II) carried as many as six resistance alleles. With eight relatively stable QTL identified in the germplasm, there is a potential to increase the average SNB resistance level. Some of the QTL, like the ones on chromosomes 2B and 5A, were rare and mainly present in exotic material and may provide resistance sources for wheat breeding programs targeting the Nordic region.

CONCLUSIONS

This study is the first to use GWAS to investigate marker association with adult plant SNB resistance. Several novel loci were detected, and others, like the QTL on chromosome 7A in 2011, 2013, and 2014, 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 be used for marker-assisted selection. In particular, the QTL on chromosome 2DL was stable. The infiltration assays were not well correlated to adult plant resistance, but the detection of the same, novel QTL on chromosomes 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.

Supplemental Information Available

Supplemental information is available with the online version of this manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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. EF supplied the effectors. HTTP supplied the effectors, analyzed the data, and critically revised the manuscript. RPO supplied the effectors, analyzed the data, and critically revised the manuscript. KCT supplied the effectors, analyzed the data, and critically revised the manuscript. ML did planning, field experimental design, assessed field phenotypic data in 2010–2012, critically revised the manuscript, supervision, and obtained the funding.

ACKNOWLEDGMENTS

This work was funded by the Norwegian Research Council (NFR) grant 224833. The SNP genotyping was paid by Graminor AS. The authors would also like to thank Qiongxian Lu and Susanne Windju for SNP calling, Stine Jansen for the STRUCTURE analysis, Tatiana Belova for help with bioinformatics (in particular filtering of the original genotype data), Min Lin for help with validating lab results, Anne Guri Marøy for DNA extraction and SSR genotyping, Danielle Holmes for primer sequences, Yalew Tarkegne and Cecilie Yri for help with the field trials and Marit Holmøy for taking care of the plants in the greenhouse. KCT, RPO, HTTP, and EF were supported by the Centre for Crop and Disease Management, a joint initiative of Curtin University and the Grains Research and Development Corporation [research grant CUR00023 (Programme 3)]

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