Validation of Genome-Wide Association Studies as a Tool to Identify Virulence Factors in *Parastagonospora nodorum*

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ABSTRACT

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Parastagonospora nodorum is a necrotrophic fungal pathogen causing Septoria nodorum blotch on wheat. We have identified nine necrotrophic effector–host dominant sensitivity gene interactions, and we have cloned three of the necrotrophic effector genes, including *SnToxA*, *SnTox1*, and *SnTox3*. Because sexual populations of *P. nodorum* are difficult to develop under lab conditions, genome-wide association study (GWAS) is the best population genomic approach to identify genomic regions associated with traits using natural populations. In this article, we used a global collection of 191 *P. nodorum* isolates from which we identified 2,983 single-nucleotide polymorphism (SNP) markers and gene markers for *SnToxA* and *SnTox3* to evaluate the power of GWAS on two popular wheat breeding lines that were sensitive to *SnToxA* and *SnTox3*. Strong marker trait associations (MTA) with *P. nodorum* virulence that mapped to *SnTox3*

and *SnToxA* were first identified using the marker set described above. A novel locus in the *P. nodorum* genome associated with virulence was also identified as a result of this analysis. To evaluate whether a sufficient level of marker saturation was available, we designed a set of primers every 1 kb in the genomic regions containing *SnToxA* and *SnTox3*. Polymerase chain reaction amplification was performed across the 191 isolates and the presence/absence polymorphism was scored and used as the genotype. The marker proximity necessary to identify MTA flanking *SnToxA* and *SnTox3* ranged from 4 to 5 and 1 to 7 kb, respectively. Similar analysis was performed on the novel locus. Using a 45% missing data threshold, two more SNP were identified spanning a 4.6-kb genomic region at the novel locus. These results showed that the rate of linkage disequilibrium (LD) decay in *P. nodorum* and, likely, other fungi is high compared with plants and animals. The fast LD decay in *P. nodorum* is an advantage only if sufficient marker density is attained. Based on our results with the *SnToxA* and *SnTox3* regions, markers are needed every 9 or 8 kb, respectively, or in every gene, to guarantee that genes associated with a quantitative trait such as virulence are not missed.

Genome-wide association studies (GWAS) provide a powerful approach to analyze DNA sequence variation across a genome with the aim of identifying genetic variation associated with traits of interest in a natural population (Gibson and Muse 2009). Numerous quantitative genetic studies have been used to map quantitative trait loci (QTL) in plants (Zhao et al. 2014). QTL analysis uses designed, biparental populations that segregate for a certain trait or traits. Because GWAS uses naturally diverse populations that maintain a high frequency of recombination events over their evolutionary history, GWAS often has advantages over biparental populations in targeting complex traits because of its relatively high mapping resolution (Doerge 2002; Holland 2007).

Due to the falling cost of next-generation sequencing and other genomic technologies such as high-throughput genotyping, transcriptomics, and comparative genomics (Zhu et al. 2008), GWAS is widely used to identify gene loci for human diseases (Montgomery

2011) and plants (Zhu et al. 2008). GWAS has already been applied in maize, *Arabidopsis*, sorghum, wheat, barley, potato, and rice to genetically characterize complex traits such as flowering time, kernel composition, disease resistance, shoot branching, kernel size, and milling quality (Zhu et al. 2008).

In fungi, only a few studies using GWAS analysis have been reported. In *Saccharomyces cerevisiae*, a single-nucleotide polymorphism (SNP) associated with mtDNA copy number was identified by GWAS (Connelly and Akey 2012). Muller et al. (2011) analyzed a geographically diverse set of 44 clinical and 44 nonclinical *S. cerevisiae* strains to identify genetic loci associated with clinically relevant traits, including pseudohyphal formation, cell wall maintenance, and cellular detoxification associated with pathogenesis and virulence. Dalman et al. (2013) sequenced 23 haploid isolates of *Heterobasidion annosum*, a necrotrophic pathogen of *Picea abies* and *Pinus sylvestris*, to identify 33,018 SNP. In total, 12 SNP were associated with virulence using GWAS. Genomic regions associated with the 12 SNP contained eight candidate virulence genes with homology to genes in other fungal pathogens. Palma-Guerrero et al. (2013) used GWAS to identify a gene homolog encoding a neuronal calcium sensor associated with germling communication in *Neurospora crassa*. Talas et al. (2016) genotyped 220 German *Fusarium graminearum* isolates and identified 50, 29, and 74 quantitative trait nucleotides associated with aggressiveness, deoxynivalenol production, and propiconazole sensitivity,

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respectively. Although GWAS research in fungi remains rare compared with plants and animals, GWAS has significant potential applications in fungi because they have small genomes, often exhibit a haploid chromosomal stage, and often undergo regular sexual cycles that reduce linkage disequilibrium (LD) and result in high levels of diversity among individuals drawn from natural populations (Dalman et al. 2013).

Parastagonospora nodorum produces necrotrophic effectors (NE) that are critical to the virulence of the pathogen. The NE produced by *P. nodorum* are recognized directly or indirectly by dominant wheat sensitivity genes that confer susceptibility. To date, we have cloned three NE genes, including *SnToxA*, *SnTox1*, and *SnTox3* (Friesen et al. 2006; Liu et al. 2009, 2012). According to previous studies for *SnToxA* and *SnTox3* presence or absence variation across seven global geographic regions (McDonald et al. 2013), the absence of *SnToxA* ranged from 3% (Australia) to 94% (China) and *SnTox3* ranged from 4% (Australia) to 60% (China). Bertucci et al. (2014) tested 33 *P. nodorum* isolates collected from the southeastern United States on 25 winter wheat cultivars, from which 25 isolates were used in this study (Supplementary Table S2). Because sexual populations of *P. nodorum* are difficult to produce under lab conditions, biparental populations needed for QTL mapping have not been used to genetically locate genes involved in virulence; namely, genes involved in NE production. An annotated reference genome sequence along with GWAS would provide an alternative way to target complex traits because the population used in GWAS contains natural genetic diversity, with high-frequency evolutionary and historical recombination.

Here, we report on a large set of SNP markers distributed across the *P. nodorum* genome that can be used in a GWAS analysis to identify genomic regions harboring virulence factors important in Septoria nodorum blotch (SNB). We first coupled an SNP marker set with an additional set of markers specifically targeting *SnToxA* and *SnTox3* to show that GWAS could identify marker trait associations (MTA) involving *SnToxA* and *SnTox3*. This analysis allowed us to determine the marker density needed to identify significant MTA in these regions on wheat lines known to harbor the corresponding NE sensitivity genes *Tsn1* (Faris et al. 2010) and *Snn3-B1* (Shi et al. 2016). Then, we used the SNP data in a GWAS analysis that led to the discovery of a novel virulence locus affecting the same wheat lines that harbored sensitivity to *SnToxA* or *SnTox3*, illustrating the potential of GWAS to identify novel determinants of virulence.

MATERIALS AND METHODS

Plant and fungal materials. The susceptible wheat ‘Sumai3’, which contains the NE sensitivity genes *Tsn1* and *Snn3-B1*, was used as a positive control for the *SnToxA* and *SnTox3* genes. The North Dakota hard red spring wheat ‘Alsen’ (*Tsn1*) was used as a positive control for the *SnToxA* as well as to identify a novel locus associated with *P. nodorum* virulence. Both wheat lines were inoculated with spores obtained from a global sample of 191 *P. nodorum* isolates. The 191 *P. nodorum* isolates originated from 13 U.S. states ($n = 94$ isolates), Australia ($n = 30$), Denmark ($n = 2$), Finland ($n = 3$), Lithuania ($n = 1$), Norway ($n = 8$), Sweden ($n = 3$), Brazil ($n = 3$), Switzerland ($n = 10$), China ($n = 18$), South Africa ($n = 5$), and Iran ($n = 14$) (Supplementary Table S1).

Phenotypic evaluation. The 191 *P. nodorum* isolates were grown on V8-potato dextrose agar for 7 days, as described by Friesen and Faris (2010). Conidia (pycnidiospores) served as inoculum and were concentrated to 1×10^6 spores/ml and sprayed onto seedlings at the two- to three-leaf stage (Liu et al. 2004b). The inoculated plants were placed at 100% relative humidity in the light for 24 h in a mist chamber, then moved to a growth chamber at 21°C with a 12-h photoperiod for six additional days. The disease on the second leaf of each inoculated plant was scored 7 days postinoculation using a 0-to-5 rating scale based on lesion type, where 0 = no visible lesions, 1 = few penetration points with lesions

consisting of flecking or small dark spots, 2 = lesions consisting of dark spots with little surrounding necrosis or chlorosis, 3 = dark lesions with size of 2 to 3 mm completely surrounded by necrosis or chlorosis, 4 = larger necrotic or chlorosis 4 mm or greater with little coalescence, and 5 = large coalescent lesions with very little green tissue remaining (Liu et al. 2004a). Three replicates were completed for each isolate–wheat line combination, with each replicate consisting of a single cone-tainer containing three seedlings that were evaluated collectively. After identifying SNP associated with a novel virulence identified through the GWAS (as described below in the “LD analysis and the initial association analysis” section), disease reactions of pools of isolates harboring alternative SNP alleles were investigated using a two-tailed *t* test in Excel to verify the significance of associations between genotypes and phenotypes using a 0.001 *P* value cutoff.

DNA extraction, library preparation, and genotyping.

DNA of the 191 *P. nodorum* isolates was extracted using a BioSprint 15 system (Qiagen). DNA of each isolate was initially genotyped using three methods. The first method used a restriction site associated DNA genotype by sequencing approach (Baird et al. 2008; Elshire et al. 2011) to identify SNP. A total of 60 ng of DNA from each isolate was digested with *ApeKI* and *HhaI* (NEB), then ligated with specific barcoded adaptors, as described by Leboldus et al. (2015). The barcoded DNA of 40 isolates were pooled into one tube, and five pooled genomic DNA libraries were made. A 3- μ l sample from each pooled library was subjected to electrophoresis on a 1% agarose gel at 100 V for 45 min. The DNA fragments between 200 and 250 bp were collected and purified using a gel extraction kit (Qiagen) followed by an additional size selection on a Pippin DNA size selection system (Sage Science) using the Pippin Prep cassette and Pippin Prep reagent kit (Sage Science). Library sequencing was performed using five Ion Torrent 318 microprocessor chips (Life Technologies), as described by Leboldus et al. (2015).

Second, the *SnToxA* and *SnTox3* genes were polymerase chain reaction (PCR) amplified from all 191 isolates using primer sets specific to *SnToxA* (Friesen et al. 2006) and *SnTox3* (Liu et al. 2009) to identify presence/absence variation in these two genes. Primers specific to these two genes can be found in Supplementary Table S1. These genes were used as positive controls to validate the phenotyping and effectiveness of GWAS analysis in *P. nodorum*.

Additionally, primers were designed to amplify 1-kb DNA fragments along the 20-kb genomic regions containing the *SnToxA* and *SnTox3* genes, and a novel virulence locus identified using the RAD-GBS markers described above. All PCR amplification followed the parameters of 94°C 4 min; 30 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 45 to 90 s according to the length of the target fragment; followed by 72°C for 10 min. The presence or absence markers in the 20-kb genomic regions containing *SnToxA*, *SnTox3*, and the novel locus were included in GWAS analysis.

Genotypic data analysis. The *P. nodorum* isolate that had the most reads was de novo assembled as the reference sequence using CLC Genomics Workbench (Qiagen). All the sequencing reads were aligned to the reference sequence using the Burrows-Wheeler Aligner ‘mem’ algorithm (Li and Durbin 2010). Sequence Alignment/Map (SAM) files generated by the Burrows-Wheeler Aligner were converted to Binary Alignment/Map files and subsequently used to identify SNP markers using SAM tools with default settings (Li and Durbin 2010). SNP with low quality (<100) were filtered from the dataset. SNP having a minor allele frequency less than 5% or missing data greater than 30% were eliminated. Presence/absence polymorphisms of *SnToxA* and *SnTox3* were identified by PCR. Isolates with positive amplification of *SnToxA* or *SnTox3* were scored as 0, and isolates without amplification were scored as 1. When evaluating the novel virulence locus associated with both Alsen and Sumai3, a 45% missing data cutoff was used to increase the number of markers in that region alone. Individual genotypes with quality >10 and minimum read depth of 3 were used. For each sequence tag, the SNP markers with the least

missing data were chosen, resulting in one SNP marker per tag being used as a genotypic marker. The chosen SNP were used for population structure analysis, relatedness analysis, and association mapping. SNP marker data were imported into Excel and annotated onto the sequence of the *P. nodorum* genome reference strain SN15 (Hane et al. 2007; Syme et al. 2013, 2016).

Population structure and relatedness analysis. The software STRUCTURE 2.3.4 (Pritchard et al. 2000) was used to analyze the population structure of the 191 *P. nodorum* isolates using an admixture model with a burn-in period of 10,000 followed by 50,000 Markov Chain Monte Carlo replications. Five runs for each cycle were performed with the cluster number between 1 and 20. The optimal value of the number of clusters (k) was decided based on the relative rate of change in the likelihood of the value (Δk) between two successive clusters. The value of Δk was calculated as described by Evanno et al. (2005) using STRUCTURE Harvester (Earl and vonHoldt 2012). Further analyses were performed from $k = 2$ to the optimal $k + 3$, with a burn-in period of 20,000 followed by 200,000 Markov Chain Monte Carlo replications with 20 iterations. The membership probability of each isolate at the optimal k was used as the Q matrix for further analysis.

Relative kinship was calculated by estimating the pairwise relatedness of the 191 isolates for the 2,983 SNP markers using JMP Genomics 6.1. The probability of any two individuals sharing the same allele was computed using Gower's Similarity Metric and a range standardization that is referred to as identity by state (IBS) across all markers (Stevens et al. 2011).

LD analysis and the initial association analysis. LD between SNP markers was estimated as the squared allele frequency correlation (r^2) and calculated using JMP Genomics 6.1. Decay of LD was calculated by fitting an equation developed by Hill and Robertson (1968) into a nonlinear regression curve using JMP Genomics 6.1. The marker saturation necessary to identify MTA was based on the average distance of paired SNP markers at LD level of r^2 value ≥ 0.8 (Bastien et al. 2014) and the LD decay at $r^2 \leq 0.1$ (Nordborg and Tavaré 2002; Palaisa et al. 2004; Remington et al. 2001). Both LD and LD decay r^2 thresholds indicate a pair of markers are linked. Association analysis was performed in JMP Genomics 6.1 using SNP and *SnToxA* and *SnTox3* gene markers. The SNP with a $-\log_{10}(P \text{ value})$ or adjusted $-\log_{10}(P \text{ value})$ greater than 4.5 ($P < 0.0000316$) were considered significant. The naïve model (Pasam et al. 2012) and three mixed models, including population structure (Q model), kinship (IBS model) and population structure + kinship (Q+IBS model) (Listgarten et al. 2012; Yu et al. 2005), were used to identify associations between SNP and phenotypes. A false discovery rate method was used to reduce false positives (type I error) (Benjamini and Hochberg 1995). To test the four models, each model was evaluated using a quantile-quantile plot (testing the fit of the observed P value versus the expected P value of each SNP) and mean square deviation (MSD) in JMP Genomics 6.1. (Gurung et al. 2014; Kertho et al. 2015; Mamidi et al. 2011; Tamang et al. 2015).

RESULTS

Generation of genomic data sets. Genotyping of the 191 *P. nodorum* isolates was performed using the RAD-GBS technique (Baird et al. 2008) and amplification of *SnToxA* and *SnTox3*. Using RAD-GBS technique, single reads from a size-selected library of all the isolates were obtained and, in total, 4,019 Mb with a sequence mean read length of 148 bp were obtained from the Ion Torrent PGM sequencing platform. Isolate Nor-5 (ID: 143) was chosen as the reference genotype because it had the highest number of reads (343,439). Isolates having the same nucleotide as the reference isolate at a given genomic position were scored as 0 and alternate alleles were scored as 1. Sequence tags of Nor-5 were mapped to the reference isolate SN15 (Hane et al. 2007; Syme et al. 2013, 2016). After screening out sequence tags with low quality, with less than

5% allele frequency, or missing data in more than 30% of the isolates, 2,983 SNP markers were identified. Genotypes of all 191 isolates with SNP were supplemented with the presence/absence polymorphism of *SnToxA* and *SnTox3* and then used for analysis of population structure, kinship estimates, and association analyses. The average marker density was approximately one marker every 18 kb across the 37-Mb *P. nodorum* genome (Hane et al. 2007).

Population structure and kinship in the panel of the 191 isolates. Population clustering was inferred for the 191 isolates using the 2,983 markers with the software STRUCTURE. The inferred population structure identified three populations. Using a membership probability cutoff of 0.6, 45 isolates collected from North Dakota and Iran were clustered into population 1. Population 3 included 30 Australian and three South African isolates, while population 2 included a total of 78 isolates, with 63 coming from the United States, 1 from Lithuania, 5 from Switzerland, and 9 from China. The remaining 35 isolates had lower membership probabilities and were not placed into any of these three populations (Supplementary Fig. S1). The membership probability of each isolate based on $k = 3$ clusters was used to form the Q matrix in additional analyses.

The pairwise kinship value was calculated based on the similarities between each pair of isolates in JMP Genomics 6.1. The average kinship value was 0.852. The minimum kinship value of 0.8102 indicated that all 191 isolates had more than 80% similarity, with the majority having kinship values in a range between 0.8102 and 0.9709 (Supplementary Fig. S2), indicating a high degree of kinship among the 191 isolates.

LD and LD decay. Pairwise LD was calculated using all 2,983 SNP markers across all 191 isolates. The average r^2 of all the SNP pairs was 0.0432, consistent with a low LD level among the 191 isolates. LD decay was estimated by regressing the r^2 value of all SNP marker pairs on the physical distance using the nonlinear regression model. The average distance between pairwise SNP at LD $r^2 \geq 0.8$ is 25,234 bp, indicating that, on average, SNP separated by approximately 25 kb will be correlated through linkage at least 80% of the time. The average distance between SNP pairs at LD $r^2 \geq 0.9$ is 7,083 bp, indicating that, on average, SNP separated by approximately 7 kb are correlated through linkage at least 90% of the time. Additionally, the genome-wide LD decay defined by $r^2 = 0.1$ (Supplementary Fig. S3) was approximately 10 kb, indicating that, on average, markers separated by more than 10 kb will lose association with an adjacent marker.

GWAS model evaluation and identification of marker trait association. To identify specific NE and other virulence factors important in SNB disease, four models (Q, IBS, Q+IBS, and a naïve model) were used for Alsen (harbors *Tsn1* and at least one other NE sensitivity gene) and Sumai3 (harbors *Tsn1*, *Snn3-B1*, and at least one other NE sensitivity gene) wheat. Each model was evaluated using quantile-quantile plots (Supplementary Fig. S4) and estimation of MSD (Supplementary Table S3) to identify the model with the best fit of the observed P value to the expected P value and lowest MSD. For both Alsen and Sumai3, the Q model and the naïve model showed a better fit for the observed P value to the expected P value than either the IBS or Q+IBS models. The MSD of the naïve and Q models also showed much lower values than either the IBS or Q+IBS models, indicating that the Q and naïve models performed better than the IBS and Q+IBS models.

Alsen and Sumai3 both harbor *Tsn1*, the host sensitivity gene corresponding to *SnToxA*, whereas only Sumai3 harbors *Snn3-B1*, the host sensitivity gene corresponding to *SnTox3*. An MTA between the *P. nodorum* NE gene *SnToxA* and the disease phenotype was identified in Alsen with high levels of significance ($-\log_{10}[P \text{ value}] = 13.01$ to 17.41) using both the naïve and Q models (Fig. 1; Supplementary Table S4). *SnToxA* was also significantly associated with the disease phenotype on Sumai3 using both the naïve and Q models (Fig. 1). The $-\log_{10}(P \text{ value})$ was lower than that for Alsen but still highly significant ($-\log[P \text{ value}] = 4.61$ to 6.10). *SnToxA* was

not found to be significant using either the IBS or Q+IBS models. An MTA between the *P. nodorum* NE gene *SnTox3* and the disease phenotype was identified only in Sumai3 (Fig. 1) with a high level of significance ($-\log_{10} [P \text{ value}] = 9.10\text{--}16.85$) using all four models. This was expected because Sumai3 and not Alsen harbors the *SnTox3* sensitivity gene *Snn3-B1*.

SNPAlsen_1 and SNPAlsen_2 were identified with a high level of association with disease on Alsen ($-\log_{10} [P \text{ value}] = 16.71$ to 21.13) and were also highly associated with disease on Sumai3 ($-\log_{10} [P \text{ value}] = 6.75$ to 8.59) using the naïve and Q models (Fig. 1). SNP having >30% but <45% missing data were used to increase marker saturation and add markers that flanked the novel region. Two other SNP, SNPAlsen_3 ($-\log_{10} [P \text{ value}] = 8.01$ to 11.04) and SNPAlsen_4 ($-\log_{10} [P \text{ value}] = 12.44$ to 15.72), were also associated with the Alsen disease phenotypes using both the naïve and Q models, and were also associated with the Sumai3 phenotypes ($-\log_{10} [P \text{ value}] = 5.51$ to 5.23) using the naïve model (Fig. 2). Given their physical proximity (4.6 kb), all four significant SNP (SNPAlsen_1 to SNPAlsen_4) are likely associated with the same virulence locus that interacts specifically with Alsen and Sumai3.

We investigated the genotypes of all 191 isolates for SNPAlsen_1, SNPAlsen_2, SNPAlsen_3, and SNPAlsen_4. Most of the isolates having the SNP genotype matching the Nor-5 reference sequence for SNPAlsen_1 to SNPAlsen_4 were more virulent on Alsen; likewise, isolates having the alternative SNP allele had reduced virulence on Alsen. To show the phenotypic response of spore inoculations on Alsen, eight isolates from Scandinavian populations, including four isolates (Nor-3, Nor-9, Nor-5, and Fin-5) containing the reference allele for SNPAlsen_1 to SNPAlsen_4 and four isolates (Den-1, Swe-4, Nor-6, and Nor-1) harboring the alternate alleles at these same loci, were inoculated in a new experiment (Fig. 3). An obvious correlation was found between genotype and phenotype on both Sumai3 and Alsen (Fig. 3). When averaging the phenotypes of the isolates harboring the reference allele compared with those isolates harboring the alternate allele at the two most significant SNP markers (SNPAlsen_1 and SNPAlsen_2), average disease reactions of 3.73 (range = 2.0 to 5.0) and 2.74 (range = 0.5 to 4.0) were found on Alsen for the reference and alternate alleles, respectively. Similarly, on Sumai3, average disease reactions of 3.43 (range = 0.67 to 4.75) and 2.59 (range = 0.17 to 4.75) were found for the reference and alternate alleles, respectively. A *t* test showed that average disease reactions

for isolates harboring the reference allele were significantly higher than for isolates harboring the alternate allele for both Alsen and Sumai3 ($P \leq 0.001$).

Marker trait associations identified by additional GWAS analysis at the *SnToxA* and *SnTox3* regions. Primers designed to amplify 1-kb fragments along a 20-kb region surrounding both *SnToxA* and *SnTox3* were used to identify presence/absence markers every 1 kb. This resulted in 10 markers upstream from *SnToxA* and *SnTox3* and 10 markers downstream. MTA were then reanalyzed using the Q model, as was done with the initial SNP marker set. Similar results came from using the naïve model but the Q model was expected to have fewer false positives because it considers population structure; therefore, we used the Q model for analysis.

In all, 8 of the 20 markers (SnToxA_8 to SnToxA_15) were identified above the $-\log_{10} (P \text{ value})$ threshold of 4.5. Scores for the eight markers ranged from 12.63 to 17.87 for Alsen and from 5.71 to 8.93 for Sumai3 (Table 1; Fig. 4). Markers flanking the significant MTA region, including SnToxA_7 and SnToxA_16, did not reach the 4.5 significance threshold but marked an 11-kb physical region, with 5 kb upstream of *SnToxA* and 6 kb downstream (Fig. 4).

For the *SnTox3* region, 6 of the 20 markers (SnTox3_11 to SnTox3_16) were identified above the $-\log_{10} (P \text{ value})$ threshold of 4.5 and were specific to Sumai3 because Sumai3 harbors the *SnTox3* sensitivity gene *Snn3-B1*. Scores for the six markers ranged from 17.90 to 26.26 (Table 1; Fig. 4). Markers flanking the significant MTA region, including SnTox3_10 and SnTox3_17, did not reach the 4.5 significance threshold but marked a 9-kb physical region, with 2 kb upstream and 7 kb downstream of *SnTox3* (Fig. 4).

These results indicate that the degree of recombination in the genomic region containing *SnToxA* was sufficient to provide highly significant MTA across an 11-kb region (5 kb upstream and 6 kb downstream), outside of which significant MTA were not detectable. Similarly, the presence/absence markers across the genomic region containing *SnTox3* provided highly significant MTA across a 9-kb region (2 kb upstream and 7 kb downstream), outside of which significant MTA were not detected.

Using similar methods to amplify DNA fragments across a 20-kb region surrounding the novel virulence locus containing SNPAlsen_1 to SNPAlsen_4, we found that there was little to no presence/absence or size polymorphism and, therefore, this method did not lead to additional knowledge regarding the structure of this locus. This indicates that, unlike the *SnToxA* and *SnTox3* regions, it

Fig. 1. Manhattan plots of marker trait association analyzed by genome-wide association study (GWAS). Single-nucleotide polymorphism (SNP) associated with *Septoria nodorum* blotch disease were analyzed by initial GWAS using spore inoculation data on wheat genotypes Alsen and Sumai3. The $-\log_{10} (P \text{ value})$ corrected by false discovery rate for known effector genes *SnToxA* and *SnTox3* and the two novel SNP (SNPAlsen_1 and SNPAlsen_2) are indicated by arrows. SNP markers are represented by black dots and are ordered along the previously assembled *P. nodorum* contigs (Syme et al. 2016) (https://github.com/robsyme/Parastagonospora_nodorum_SN15). A $-\log_{10} (P \text{ value})$ threshold of significance of 4.5 ($P < 0.0000316$) appears as the gray dashed line.

is unlikely that the differences in this novel region result from large insertions or deletions but, more likely, are due to smaller polymorphisms such as small indels or SNP that are responsible for the observed phenotypic differences. Therefore, to increase the marker set in this region, we reduced the missing data threshold from 30 to 45%. This led to a total of 11 SNP (3 markers at the 30% threshold and 8 markers at the 45% threshold) across a 50-kb region, with 4 of these SNP having significance above the $-\log_{10}$ (P value) threshold of 4.5. The closest markers flanking this region did not reach the 4.5 threshold but spanned a physical interval of 20 kb that provides an interval to begin evaluating candidate genes. A detailed sequence analysis of this region for all 191 isolates will be needed to identify small indels or SNP markers more tightly associated with the novel virulence phenotype.

DISCUSSION

Though *P. nodorum* is found in wheat fields around the world, this pathogen varies widely in the amount of damage it causes, with SNB being a major problem in some areas and a relatively minor problem in others. The differences in local disease impact reflect the effectiveness of host resistance or susceptibility in local cultivars, the virulence spectrum of the local pathogen population (McDonald

et al. 2013), and local environmental factors that contribute to a compatible host–pathogen interaction. Since the first report of NE produced by *P. nodorum* (Liu et al. 2004b) and the dominant susceptibility genes that correspond to those effectors (Liu et al. 2004a), eight NE–host gene interactions that lead to NE-triggered susceptibility have been described, with three of the NE genes being cloned and functionally characterized, including *SnToxA* (Friesen et al. 2006), *SnTox1* (Liu et al. 2012), and *SnTox3* (Liu et al. 2009). Additionally, the host gene *Tsn1*, which confers sensitivity to *SnToxA*, has also been cloned and functionally characterized (Faris et al. 2010), providing a working model to characterize this interaction at the molecular level. Our working model indicates that *P. nodorum* uses NE to trigger defense response pathways, including an oxidative burst and programmed cell death to kill host cells, extract nutrients, colonize, and sporulate, resulting in the completion of its life cycle. *P. nodorum*, similar to other pathogens classified as necrotrophs, has evolved the ability to use NE to induce “host recognition” but has also evolved the ability to modulate the negative effects of the defense response that is so effective against other pathogens.

Previously, several techniques have been used to identify NE. *ToxA* was first identified in *Pyrenophora tritici-repentis* (Ballance et al. 1996; Ciuffetti et al. 1997), and we first identified *SnToxA* in *Parastagonospora nodorum* (Friesen et al. 2006) using the newly

Fig. 2. Marker trait associations of markers in a 50,000-bp genomic region containing the novel locus associated with virulence phenotypes on the wheat cultivars Alsen and Sumai3. Single-nucleotide polymorphism (SNP) with significant marker trait associations (MTA) (open circles) were identified by a genome-wide association study using spore inoculation on Alsen (top) and Sumai3 (bottom). SNP including markers with a significant MTA (open circles), SNP flanking the significant markers (black circles), and other SNP without MTA significance (gray circles) are plotted according to their genomic positions (x-axis) in the 50,000-bp genomic region. Statistic probabilities of the markers are indicated by $-\log_{10}$ (P value) corrected by false discovery rate (y-axis). A $-\log_{10}$ (P value) threshold of significance of 4.5 ($P < 0.000316$) appears as the gray dashed line.

released *P. nodorum* genome sequence (Hane et al. 2007). We identified *SnTox3* and *SnTox3* using a reverse genetics approach (Liu et al. 2009) and *SnTox1* using a prioritized candidate gene approach (Liu et al. 2012). However, due to the quantitative nature of NE–host gene interactions and the lack of classical genetic support, the validation of novel candidate NE mentioned above is difficult and labor intensive. Several factors led us to pursue a GWAS approach in the SNB system. First and foremost, although the sexual cycle is prevalent in the field, it has been difficult to produce *P. nodorum* sexual populations in the laboratory; therefore, QTL mapping cannot be used. Fortunately, natural populations are highly recombined and composed of many genotypes and, thus, GWAS provides a powerful alternative to biparental mapping populations (Grünwald et al. in press). Additionally, once a marker set is developed in a *P. nodorum* collection, any trait that can be phenotyped can be genetically characterized. These traits can include virulence on additional host genotypes as well as traits not directly associated with virulence such as fungicide sensitivity, sensitivity to reactive oxygen, and sensitivity to pathogenesis-related proteins. The genetic mapping resolution of GWAS has also been shown to be higher in natural populations compared with biparental populations (Leboldus et al. 2015), allowing us to quickly narrow a candidate gene region.

Fig. 3. Phenotypes of **A**, Alsen and **B**, Sumai3 inoculated by spores of *Parastagonospora nodorum* isolates harboring reference or alternative single-nucleotide polymorphism (SNP) genotypes for both SNPAlsen_1 and SNPAlsen_2. Two-week-old secondary leaves of Alsen and Sumai3 were inoculated with eight isolates from Scandinavian populations. Of the eight isolates, four isolates harbored the reference SNPAlsen_1 and SNPAlsen_2 marker alleles associated with higher virulence (Nor-3, Nor-9, Nor-5, and Fin-5) on **A**, Alsen (top four leaves) and **B**, Sumai3 (top four leaves) whereas four other Scandinavian isolates (Den-1, Swe-4, Nor-6, and Nor-1) harbored the alternate SNPAlsen_1 and SNPAlsen_2 marker alleles associated with reduced virulence on Alsen (bottom four leaves) and Sumai3 (bottom four leaves).

The success of a GWAS analysis in any organism depends on having a marker density that is greater than the degree of LD decay so as not to miss MTA for both major and minor traits. In this study, we used disease reaction type data from 191 *P. nodorum* isolates collected on two popular wheat lines carrying known and unknown NE sensitivity genes to look for new NE or other genes associated with virulence. We included as positive controls the known NE genes *SnToxA* and *SnTox3*, which showed presence/absence polymorphism across all 191 *P. nodorum* isolates, to evaluate the degree of marker density needed to identify these genes via GWAS.

Based on the degree of LD decay measured in this population, we showed that the average LD decay and, therefore, the minimum level of marker density needed to identify markers associated with a trait should be approximately one marker every 25 kb to identify associated genes with an 80% confidence, while markers would be required every 7 kb to identify associated genes with a 90% confidence. Given the genome size of 37 Mb and an 80% confidence, we would need a minimum of approximately 1,500 markers if (i) markers were distributed evenly across all genomic regions and (ii) the rate of linkage decay was equal across the entire genome. Neither of these assumptions are likely to be correct. Using a RAD-GBS approach (Leboldus et al. 2015) to identify polymorphic SNP, we identified 2,983 high-quality SNP markers with an allele frequency $\geq 5\%$ to be used in analyzing population structure, LD, LD decay, and MTA.

In the initial GWAS results, *SnToxA* and a novel virulence locus showed a highly significant association on both Alsen and Sumai3 whereas *SnTox3* showed a highly significant association only on Sumai3. No other loci with high significance were identified on either Alsen or Sumai3. To evaluate the level of marker density needed to identify novel genes at any given locus, we used two methods including LD and LD decay. LD calculations showed that the average distance between marker pairs across the genome was 25,234 bp at an r^2 value > 0.8 , which was within the range of our marker density of one marker every 18 kb. However, LD decay analysis showed that the average marker coverage needed at $r^2 = 0.1$ was approximately 10 kb. In a recent population genomics analysis of 213 German *F. graminearum* isolates based on 1,129 SNP markers, a similar LD decay occurred, on average, over a 1-kb interval; however, there were hotspots and coldspots of recombination across the genome and differences in rates of LD decay both among chromosomes and among field populations (Talas and McDonald 2015). In this study, the SNP density on each scaffold varied. For example, on scaffold 1, the average SNP coverage was

TABLE 1. Possibility of markers associated with traits identified by additional genome-wide association study analysis using Q model

Marker ID	Marker type	Alsen	Sumai3
		$-\log_{10}(P \text{ value})$	$-\log_{10}(P \text{ value})$
<i>SnToxA</i>	Absence/presence	15.12	6.83
SnToxA_8	Absence/presence	12.63	5.71
SnToxA_9	Absence/presence	17.87	8.93
SnToxA_10	Absence/presence	15.13	7.80
SnToxA_11	Absence/presence	17.12	8.18
SnToxA_12	Absence/presence	15.13	7.97
SnToxA_13	Absence/presence	15.57	7.51
SnToxA_14	Absence/presence	17.86	8.63
SnToxA_15	Absence/presence	17.86	8.21
<i>SnTox3</i>	Absence/presence	Not significant	18.05
SnTox3_11	Absence/presence	Not significant	18.10
SnTox3_12	Absence/presence	Not significant	20.13
SnTox3_13	Absence/presence	Not significant	20.91
SnTox3_14	Absence/presence	Not significant	26.26
SnTox3_15	Absence/presence	Not significant	20.62
SnTox3_16	Absence/presence	Not significant	17.90
SNPAlsen_1	SNP	18.20	8.87
SNPAlsen_2	SNP	18.20	8.86
SNPAlsen_3	SNP	8.01	3.95
SNPAlsen_4	SNP	12.44	3.95

one marker every 8 kb but, on scaffold 49, the average was one SNP marker every 32 kb. The unequal distribution may reflect the distribution of repetitive regions, which led to difficulties in identifying polymorphic markers across isolates. However, all the large scaffolds (scaffolds 1 to 45, containing 98% of the genome) had coverage averaging at least one marker every 17 kb. This coverage satisfies the estimated average density required (one marker approximately every 25 kb) based on an LD calculation at an $r^2 > 0.8$ but not at the LD calculation at an $r^2 > 0.9$ (one marker approximately every 7 kb) or an LD decay at an $r^2 = 0.1$ (one marker approximately every 10 kb), indicating that more markers will be needed to confidently identify novel trait–marker associations.

Population structure was taken into account to reduce false positives in the GWAS resulting from population structure. The

panel of 191 isolates was divided into three populations. Population 1 contains northern U.S. (North Dakota + Minnesota) and Iranian isolates. Population 2 contains southern, eastern, and central U.S.; Chinese; and European isolates. Population 3 contains Australian and South African isolates. The separation of these populations was based solely on the genotypes of the original 2,983 SNP. In general, population 1 and population 3 isolates were more virulent on Alsen (phenotypic scores = 3 to 5, except for two isolates in population 3), compared with population 2 isolates (phenotypic scores = 0 to 3.5, except for three isolates) on Alsen. The *SnToxA* gene region was present in all of the population 1 and most of the population 3 isolates but absent in most of the population 2 isolates. This provides evidence that selection pressure for these virulences is different in the different geographical regions, consistent with an

Fig. 4. Analysis of markers with significant marker trait associations (MTA) within a 50,000-bp genomic region identified by analyzing the inoculation data on the wheat genotypes Alsen and Sumai3. To perform genome-wide association study analysis, presence/absence polymorphisms of necrotrophic effector genes *SnToxA* (black stars) and *SnTox3* (black squares) were added to the marker data, including single-nucleotide polymorphism (SNP) markers (black circles) identified by the RAD-GBS method and presence/absence polymorphism markers (diamonds) generated by polymerase chain reaction amplifying every 1 kb along a 20-kb region flanking *SnToxA* and *SnTox3*. Using the SNP markers and the *SnToxA* and *SnTox3* genes as markers, *SnToxA* showed a significant MTA, with virulence on both **A**, Alsen and **B**, Sumai3, and *SnTox3* showed a significant MTA with virulence on only **C**, Sumai3. By adding the presence/absence markers for the *SnToxA* and *SnTox3* regions, an additional eight presence/absence markers (SnToxA_8 to SnToxA_15) linked with *SnToxA* showed significant MTA with virulence on **D**, Alsen and **E**, Sumai3. An additional six presence/absence markers (SnTox3_11 to SnTox3_16) linked with *SnTox3* showed significant MTA with virulence on **F**, Sumai3. These presence/absence markers with significant MTA are represented by open diamonds. Presence/absence markers flanking the significant markers are represented by black diamonds. Other markers without MTA significance in the 50,000-bp genomic region containing *SnToxA* or *SnTox3* are represented by gray diamonds. All markers are plotted according to their physical genomic positions (x-axis) in the *Parastagonospora nodorum* contigs. Statistical probabilities of the markers are indicated by $-\log_{10}(P \text{ value})$ corrected by false discovery rate (y-axis). A $-\log_{10}(P \text{ value})$ threshold significance of 4.5 ($P < 0.000316$) appears as the gray dashed line.

earlier analysis of effector frequencies in the global population (McDonald et al. 2013).

We were not able to identify additional presence/absence markers for the region associated with a novel virulence using a separate PCR amplification. However, four SNP markers above the $-\log_{10}(P \text{ values})$ threshold of 4.5 were identified, as well as two and five markers flanking the two sides of this region, allowing us to confidently define the interval harboring the novel gene or genes associated with virulence on both Alsen and Sumai3. It is likely that the virulence conferred by this region is not due to a large insertion or deletion as seen for *SnToxA* and *SnTox3*. We consider it likely that virulence differences in this region are due, instead, to small indels or nonsynonymous SNP within the gene or genes responsible for the phenotype. A detailed analysis of the gene sequence in this region will be required to identify candidate genes responsible for this novel virulence.

Based on our calculations of LD and LD decay, more markers will be needed to conduct future GWAS analyses in this population. However, sufficient marker density was already present to identify a novel genomic region strongly associated with virulence on both Alsen and Sumai3. Sumai3 is a Chinese wheat line that harbors good levels of resistance to Fusarium head blight (FHB), a devastating disease in many wheat-growing areas of the world. Alsen also harbors FHB resistance which, interestingly, was derived from Sumai3. It is possible that, when selecting for resistance to FHB in Alsen, the *P. nodorum* susceptibility was also brought in from Sumai3. In the past decade, Alsen has been planted on several million acres in the Northern Great Plains. Based on the GWAS analysis presented here, all *P. nodorum* isolates from North Dakota and Minnesota have the marker alleles associated with virulence at the SNPAlsen_1 and SNPAlsen_2 loci. However, these virulence alleles were rarely identified in U.S. isolates collected in Oregon (western United States) and the eastern part of the United States where mostly winter wheat is grown. Similar to the North Dakota/Minnesota (north-central United States) population, the entire Australian population analyzed here also harbors the marker alleles associated with virulence at this locus. The European population, especially from the Scandinavian countries, shows segregation for this virulence (Fig. 3). Further evaluation of popular host lines in these regions will be needed to see whether they harbor the susceptibility corresponding to this virulence. We are currently evaluating host mapping populations using both Alsen and Sumai3 as parents to identify the corresponding susceptibility or resistance genes.

In this work, we used the two cloned NE genes (*SnToxA* and *SnTox3*) as positive controls to prove the utility of the GWAS approach in identifying virulence factors important in the *P. nodorum*-wheat interactions. Using these two known loci, we showed the level of marker saturation necessary to identify these virulence-associated genes. Additionally, we were able to use the SNP marker set to identify a new locus strongly associated with virulence, showing the effectiveness of this method in identifying novel genes associated with virulence. Based on the results presented here, additional marker saturation is needed to more confidently identify marker trait associations. The most efficient and cost-effective way to identify markers associated with both SNP and larger indels will be by either full-genome resequencing or deep-transcriptome sequencing. A marker set saturating the genome will facilitate the identification of genes involved in many important pathogen traits such as fungicide resistance, thermal adaptation, and novel virulence loci associated with other wheat genotypes, including both major and minor pathogen effectors involved in disease.

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