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RESEARCH

Effects of Three *Parastagonospora nodorum* Necrotrophic Effectors on Spring Wheat under Norwegian Field Conditions

Anja Karine Ruud, Jon Arne Dieseth, and Morten Lillemo*

ABSTRACT

The wheat (*Triticum aestivum* L.) disease Septoria nodorum blotch (SNB) is caused by the necrotrophic fungus *Parastagonospora nodorum* (Berk.) Quaedvlieg, Verkley & Crous and causes significant yield and quality losses in several wheat growing regions. The resistance mechanisms are quantitative and progress in resistance breeding has been slow. However, gene-for-gene interactions involving necrotrophic effectors (NEs) and sensitivity genes (*Snn*) are involved, providing hope for more effective breeding. Although the interactions are significant determinants of seedling SNB susceptibility, their role in adult plant leaf blotch resistance in the field is less understood. In this study, the frequency of SnTox genes was investigated in 62 *P. nodorum* isolates collected in Norway. A panel of Norwegian and international spring wheat lines and cultivars was screened under natural SNB infection in a mist-irrigated field nursery across 7 yr. The lines were infiltrated in the greenhouse with the purified NEs SnToxA, SnTox1, and SnTox3, and the prevalence of corresponding sensitivity was investigated, as well as correlation between NE sensitivity and resistance level in the field. The frequencies of *SnToxA*, *SnTox1*, and *SnTox3* in the isolates were 0.69, 0.53 and 0.76, respectively. Sensitivity to SnToxA, SnTox1, and SnTox3 was present in 45, 12, and 55% of the plant material. Sensitivity to SnToxA was associated with significantly higher disease severity in the field than insensitivity. This indicates that elimination of SnToxA sensitivity in the breeding material by effector infiltrations or marker-assisted selection can be an effective way to increase field resistance to SNB.

A.K. Ruud and M. Lillemo, Dep. of Plant Sciences, Norwegian Univ. of Life Sciences, Post Box 5003, NO-1432 Ås, Norway; J.A. Dieseth, Graminor AS, Bjørke Gård, Hommelstadvegen 60, NO-2322 Ridabu, Norway. A.K. Ruud conducted field and infiltration experiments, analyzed the data, and wrote the manuscript. J.A. Dieseth developed and provided plant material and critically reviewed the manuscript. M. Lillemo planned the field experiments, conducted field phenotyping in 2010 to 2012, critically reviewed the manuscript, supervised the work, and obtained the funding. Received 9 May 2017. Accepted 22 Sept. 2017. *Corresponding author (morten.lillemo@nmbu.no). Assigned to Associate Editor Toi Tsilo.

Abbreviations: AUD, Australian dollars; DH, days to heading; PCR, polymerase chain reaction; PH, plant height; NE, necrotrophic effector; SNB, Septoria nodorum blotch.

The necrotrophic fungus *Parastagonospora nodorum* (Berk.) Quaedvlieg, Verkley & Crous is the causal agent of Septoria nodorum blotch (SNB) in wheat (*Triticum aestivum* L.) and can cause significant yield and quality losses (Bhathal et al., 2003). It causes both leaf and glume blotch and is the dominating leaf blotch pathogen in Norwegian spring wheat (Ficke et al., 2011b; Abrahamsen et al., 2013). The disease pressure increases under reduced tillage and rainy growth seasons. Control of SNB relies on fungicides, strobilurins and azoles in particular, but increased loss of fungicide sensitivity has been observed in European *P. nodorum* isolates (Blixt et al., 2009; Ficke et al., 2011a; Abrahamsen 2013; Pereira et al., 2017). For instance, the majority of isolates collected in Sweden from 2003 to 2005 carried an amino acid substitution associated with loss of sensitivity to strobilurins (Blixt et al., 2009). Twenty-five percent of these Swedish isolates also harbored point mutations associated with reduced triazole sensitivity. Growing cultivars with durable genetic resistance is a more sustainable way to control disease. However, breeding for

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leaf blotch resistance has been difficult due to the quantitative nature of the resistance genetics.

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

Eight *P. nodorum* NEs (SnToxA, SnTox1, SnTox2, SnTox3, SnTox4, SnTox5, SnTox6, and SnTox7) and nine corresponding *Snn* genes (*Tsn1, Snn1*, *Snn2*, *Snn3-B1, Snn3-D1*, *Snn4*, *Snn5, Snn6*, and *Snn7*) have been characterized (Liu et al., 2004a, 2004b, 2006, 2009; Friesen et al., 2006, 2007, 2012; Abeysekara et al., 2009; Zhang et al., 2011; Gao et al., 2015; Shi et al., 2015). Infiltration screenings with culture filtrates from single isolates imply that there are probably several more such interactions (Crook et al., 2012; Tan et al., 2014). The interactions are usually additive in nature (Friesen and Faris, 2010). However, epistasis is also involved; for instance, the SnToxA-*Tsn1* interaction is epistatic to SnTox3-*Snn3* (Friesen et al., 2008c). The SnTox3-*Snn3* interaction is significant only in the presence of an incompatible SnTox2-*Snn2* interaction (Friesen et al., 2008c), and SnTox3 expression can be modified and suppressed by SnTox1 (Phan et al., 2016).

Three *P. nodorum* NE genes—*SnToxA*, *SnTox1*, and *SnTox3*—have been cloned into *Pichia pastoris* (Guillierm.) Phaff (Friesen et al., 2006; Liu et al., 2009, 2012) and *Escherichia coli* (Migula) Castellani & Chalmers (Tan et al., 2012) vectors. This allows for efficient screening for the corresponding sensitivity loci in wheat germplasm. The ToxA-*Tsn1* interaction confers sensitivity to both tan spot, caused by *Pyrenophora tritici-repentis* (Died.) Drechsler, and SNB (Friesen et al., 2006). *ToxA* has also been detected in other pathogens, most recently in *Bipolaris sorokiniana* (Sacc.) Shoemaker (McDonald et al., 2017), and seems to be an important virulence factor of global relevance. The prevalence of *SnToxA*, *SnTox1*, and *SnTox3* in *P. nodorum* populations varies. For instance, *SnToxA* was only present in 12% of the European isolates included in a study by McDonald et al. (2013), whereas the frequency in Australia was 97%.

In Western Australia, economic losses caused by SNB were estimated to be 108 million Australian dollars (AUD), and losses due to tan spot up to 212 million AUD (Murray and Brennan, 2009). In Australia, SnToxA has been delivered to the breeders since 2009 (Vleeshouwers and Oliver, 2014). By 2012, 30,000 doses of SnToxA and 6000 doses each of SnTox1 and SnTox3 were provided annually (Vleeshouwers and Oliver, 2014). The area of SnToxA sensitive wheat in Australia fell from 30.4% in 2009 to 2010 to 16.9% within 3 yr. The estimated economic gain was ~50 million AUD, assuming a yield loss of 0.3 t ha^{-1} in susceptible cultivars (Vleeshouwers and Oliver, 2014).

However, the effect and relative contribution of the individual NE-*Snn* interactions to disease under field conditions is not well investigated and is still disputed. In particular, the relevance of the isolates used to identify most of the NE-*Snn* interactions has been questioned (Francki, 2013). Francki (2013) also pointed out the lack of consistent effect at the adult plant stage. For instance, SnToxA-*Tsn1* was likely to underlie a significant QTL in the 05Y001 doubled-haploid mapping population in 1 yr of a field trial, but not in the subsequent year (Francki et al., 2011).

On the other hand, one field study reported significant effects of the SnToxA-*Tsn1* and SnTox2-*Snn2* interactions after spray inoculation of the flag leaf with a single *P. nodorum* isolate (Friesen et al., 2009). The difference in SNB resistance ranking between SnToxA-insensitive and -sensitive Australian lines was reportedly lower in 2011 (Waters et al., 2011) than in a study by Oliver et al. (2009). A possible explanation for this is a shift in the NE frequencies in the pathogen population (Waters et al., 2011), perhaps triggered by the reduction in SnToxA-sensitive cultivars. The mapping of *Snn3-B1* as a major susceptibility factor in the SHA3/CBRD × Naxos population in naturally infected field nurseries was the first to validate the importance of this locus in field trials (Ruud et al., 2017).

Inoculation with the same isolate or mix of isolates in both seedling and adult plant trials may give higher reproducibility and correlation between the two. However, they might not be representative for the situation in the farmers' fields with an ever-changing pathogen population. Better estimates of resistance is expected when infection in the nursery is promoted by overhead irrigation and inoculation with naturally infected straw (Fraser et al., 2003; Cowger and Murphy, 2007).

The main objectives of the present study were to investigate (i) the prevalence of sensitivity to SnToxA, SnTox1, and SnTox3 in a diverse collection of spring wheat lines, and (ii) whether sensitivity was correlated with SNB susceptibility levels in field trials at the adult plant stage. The frequencies of *SnToxA*, *SnTox1*, and *SnTox3* genes in a collection of 62 Norwegian *P. nodorum* isolates were also investigated. Ultimately, we wanted to identify good resistance sources in the Norwegian breeding material.

MATERIALS AND METHODS

Parastagonospora nodorum Isolate Characterization

Sixty-two single-spore isolates of *P. nodorum* were isolated from leaves collected from unsprayed wheat fields in Norway in 2012 to 2014. For DNA extraction, the isolates were grown in the dark on potato dextrose agar (PDA) for 1 to 2 wk, and DNA was extracted from the mycelium with the DNeasy Plant DNA extraction kit (QIAGEN). Polymerase chain reaction (PCR) screenings for *SnTox*-genes and actin were performed as described in Gao et al. (2015).

Plant Material

A total of 157 spring wheat cultivars and breeding lines were analyzed in this study. The lines were from the MASbasis collection, which includes both Norwegian and international cultivars and breeding lines (Supplemental Table S1). The majority (86) of the studied lines are Norwegian. However, 25 lines from The International Maize and Wheat Improvement Center (CIMMYT) contribute to a substantial share of the 157 lines, as do the 22 cultivars and lines originating from Swedish breeding programs. In addition, lines from several other wheat growing areas were included.

Infiltration with Purified Effectors

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

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

When the second leaves were fully expanded, 12 to 14 d after planting, they were infiltrated with purified SnToxA, SnTox1, and SnTox3 using a 1-mL syringe without a needle. The borders of the water-soaked infiltrated area were marked with a black, nontoxic permanent marker. After 5 d, the symptoms were scored according to a 0-to-3 scale where 0 is insensitive and 3 is necrosis with tissue collapse (Friesen and Faris, 2012).

Field Trials

The lines were planted in hill plot trials during the 2010 to 2016 seasons at Vollebekk Research Station, Ås, Norway. The trials were naturally infected with *P. nodorum*, enhanced by mist irrigation for 5 min every half hour at daytime, which also discouraged powdery mildew [*Blumeria graminis* (DC) Speer f. sp. *tritici* emend. É. J. Marchal] infection. The trials were irrigated until disease scoring was completed and most of the genotypes had reached 100% disease severity. From 2013, the infection was promoted by inoculating the field with infected straw harvested from the most susceptible plots of the previous season. The straw was spread when the plants were at Zadoks stage Z13/21 (Zadoks et al., 1974) approximately, at which time the mist irrigation was started. In 2015 and 2016, the trials were sprayed with the selective fungicide Forbel 750 (Bayer Crop Science, a.i.: Phenpropimorph) every 3 wk to prevent stripe rust infection. Forbel is reported not to have an effect on *P. nodorum*. The field trials were conducted in an α lattice design with two or three replicates per year.

Phenotyping

Disease severity was scored twice per season by visually estimating the percentage of diseased leaf area considering the whole canopy of each hill plot. This is a combined measure of both the disease climb up the canopy and diseased area of the remaining green leaves. The first scoring was done after the infection level had reached 60 to 70% on the most susceptible lines, and the second scoring 7 to 10 d later. It is difficult to distinguish SNB symptoms from tan spot and leaf blotch caused by *Zymoseptoria tritici* (Desm.) Quaedvlieg & Crous, and mixed infection can be common. However, quantitative PCR screenings and identification of the pathogen by microscopic evaluation of spores from leaf samples, collected from the field nursery in different years and incubated on moist filter paper, validated that *P. nodorum* was dominant in the spring wheat (data not shown).

Plant height (PH) was measured after the plants were fully developed. Heading date was scored as the day >50% of the heads had emerged. Plant height and days from sowing to heading (DH) were used in multiple regression to estimate resistance. In 2010, PH was not measured, and for this year, the PH mean from 2011 to 2016 was used in the regression analysis. Also, the DH data for 2010 were scored in a different field trial in the same location.

DNA Extraction and PCR Marker Analysis

Genomic DNA of the lines from the MASbasis collection plus differential lines (BG261/SnToxA, M6/SnTox1, BG220/SnTox3) was extracted from young leaves with the DNeasy Plant DNA extraction kit (QIAGEN). Marker analysis was performed with fluorescently labeled primers, and PCR products were separated by capillary electrophoresis on an ABI 3730 Gene Analyzer. The PCR markers were selected on the basis of reported linkage to SnToxA (Friesen et al., 2006; Zhang et al., 2009), SnTox1 (Liu et al., 2004a), and SnTox3 (Friesen et al., 2007) sensitivity.

Statistical Analysis

Analyses of variance were calculated using the PROC GLM procedure in SAS 9.4 (SAS Institute, 2013). Broad sense heritability (H^2) was estimated using the ANOVA output and the formula

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

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

The Pearson correlation coefficients were calculated in Minitab 16 (Minitab, 2009) and in RStudio 1.0.44 (RStudio Team, 2015), using the Hmisc package. Welch two-sample *t* tests and Pearson's chi-squared tests with Yates' continuity correction were conducted in R Studio 1.0.44. Corrected SNB severities were calculated in Minitab 16 by multiple regression,

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

NE	Frequency
SnToxA	0.69
SnTox1	0.53
SnTox3	0.76

Table 2. Prevalence of sensitivity to SnToxA, SnTox1, and SnTox3 in 157 lines of the MASbasis spring wheat collection.

Effector	Number of lines (sensitive/insensitive)	Frequency of sensitive lines
SnToxA	71/86	0.45
SnTox1	19/138	0.12
SnTox3	87/70	0.55

where SNB was used as the dependent variable and PH and DH as covariates. Corrected SNB severities were calculated by subtracting the fitted leaf blotch scores from the original disease scores, meaning that the corrected severity average is 0, lines that are more resistant than the average have negative corrected SNB severity values, and the more susceptible a genotype, the higher the positive corrected severity value.

RESULTS

Characterization of the Pathogen Population

All the three effector genes—*SnToxA*, *SnTox1*, and *SnTox3*—were present in >50% of the 62 Norwegian isolates, and the proportion was highest for *SnTox3* and *SnToxA* (Table 1).

Sensitivity Distribution

Sensitivity to SnToxA and SnTox3 was present in 45 and 55% of the lines, respectively, whereas sensitivity to SnTox1 was only present in 12% of the material (Table 2). Initial analysis did not show any effect of SnTox1 sensitivity, and since the frequency was so low, it was not considered in the correlation analysis.

The main subpopulations were grouped on the basis of origin (Table 3). Chi-squared tests showed that the proportion of lines sensitive to SnToxA was not significantly different between the subpopulations (p = 0.20–0.50). The frequency of SnTox3 sensitivity was similar in both Swedish and CIMMYT lines (χ^2 = 0, p = 1). The proportion of SnTox3 sensitive to insensitive lines was significantly different between the Norwegian and Swedish subpopulations (χ^2 = 4.8, p = 0.03), and between Norwegian and CIMMYT lines (χ^2 = 5.3, p = 0.02).

Fig. 1. Top: Tox3 Type 2 (chlorosis); bottom: Type 3 reaction (necrosis with tissue collapse).

Two clearly distinguishable reaction types for sensitivity to SnTox3 were observed. In some genotypes, chlorosis developed after infiltration with SnTox3, and in other genotypes, necrosis and tissue collapse developed. The reaction types were scored as Reaction Types 2 (chlorosis) and 3 (necrosis), respectively (Fig. 1). Interestingly, the Type 3 reaction type was dominating in the CIMMYT lines, with only one line, MAYOOR//TKSN1081/*Ae. tauschii* (222), showing the Type 2 reaction. The sensitive Swedish lines only expressed the Type 2 reaction. In the Norwegian material, both reaction types were present and Type 2 was the most common (Table 2).

Field Results

Table 4 shows the ANOVA output and heritability for PH, DH, and uncorrected SNB. Days to heading and SNB severity were highly negatively correlated in all years (Table 5), whereas for the confounding effects of PH and DH, we used multiple regression to obtain corrected SNB severities. In all subsequent analyses, we have used the corrected SNB severities. The average corrected SNB severities across years for the tested lines ranged from −24.5 (Milan/SHA7) to +27.5 (Brakar) (Supplemental Table S1). The resistance levels and SnTox sensitivity status of important current and historical cultivars in Norway are shown in Table 6. The current cultivars were moderately resistant to moderately susceptible to SNB, ranging from −3 (Demonstrant) to +7.2 (Bjarne) (Table 6). A similar picture is also seen for

Table 3. Prevalence of sensitivity or insensitivity to SnToxA, SnTox1, and SnTox3 by origin of the main subpopulations of MASbasis.

	Effector							
	SnToxA		SnTox1		SnTox3		Tox3 reaction types	
Origin	Insensitive	Sensitive	Insensitive	Sensitive	Insensitive	Sensitive	Type 2	Type 3
Sweden	9	13	21	1	7	15	15	0
Norway	46	40	78	12	52	34	23	11
CIMMYT	16	9	21	4	8	17	1†	16

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

Table 4. ANOVA table and heritability (H^2) for corrected Septoria nodorum blotch (SNB) severity and the confounding traits plant height (PH) and days to heading (DH) according to field data from 2010 to 2016. Plant height was not recorded for the population in 2010, and DH was recorded in a different field trial that year.

Trait	Source	df	Mean square	*F*-value	*p*-value	H^2
PH	Genotype	170	745.2	28.87	<0.0001	0.90
	Year	5	13,526.0	4,811.62	<0.0001	
	Genotype × year	761	25.8	1.96	<0.0001	
	Replication(year)	7	135.5	10.27	<0.0001	
	Block(replication)	69	17.8	1.35	0.0323	
	Error	1,369	13.2			
DH	Genotype	170	77.5	12.39	<0.0001	0.72
	Year	5	24,988.5	3,998.74	<0.0001	
	Genotype × year	761	6.3	3.15	<0.0001	
	Replication(year)	7	34.1	17.15	<0.0001	
	Block(replication)	69	3.4	1.69	0.0005	
	Error	1,382	2.0			
SNB	Genotype	174	2,755.9	10.51	<0.0001	0.70
	Year	6	20,517.9	72.22	<0.0001	
	Genotype × year	848	262.3	2.50	<0.0001	
	Replication(year)	9	1,548.4	14.92	<0.0001	
	Block(replication)	69	297.6	2.84	<0.0001	
	Error	1,475	104.9			

Table 5. Correlation between Septoria nodorum blotch (SNB) severity and the confounding traits plant height (PH) and days to heading (DH). For PH and DH, the correlation is shown against respective years (i.e. PH measured in 2011 against SNB in 2011), unless otherwise noted.

	Field SNB severity						
Trait	2010	2011	2012	2013	2014	2015	2016
PH	−0.10†	−0.08	−0.11	−0.29*	−0.25*	−0.09	−0.30**
DH	−0.31‡**	−0.60***	−0.54***	−0.70***	−0.64***	−0.47***	−0.65***
2010		0.65***	0.67***	0.77***	0.77***	0.68***	0.57***
2011			0.69***	0.80***	0.68***	0.71***	0.70***
2012				0.67***	0.56***	0.56***	0.58***
2013					0.72***	0.67***	0.72***
2014						0.72***	0.72***
2015							0.66***

*,**,*** Significant at 0.05, 0.001, and 0.0001 probability, respectively.

† Mean value based on all years' measurements.

‡ Heading data from a different experiment (weather resistance) at the same location.

Table 6. Overview of current and historically important spring wheat cultivars in Norway, release year, their origin, corrected Septoria nodorum blotch (SNB) severity and sensitivity to SnToxA, SnTox1, and SnTox3. Sensitivity was scored on the 0-to-3 scale, and cultivars were ranked as sensitive if they scored ≥2, annotated with +. Insensitive cultivars are annotated with –.

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

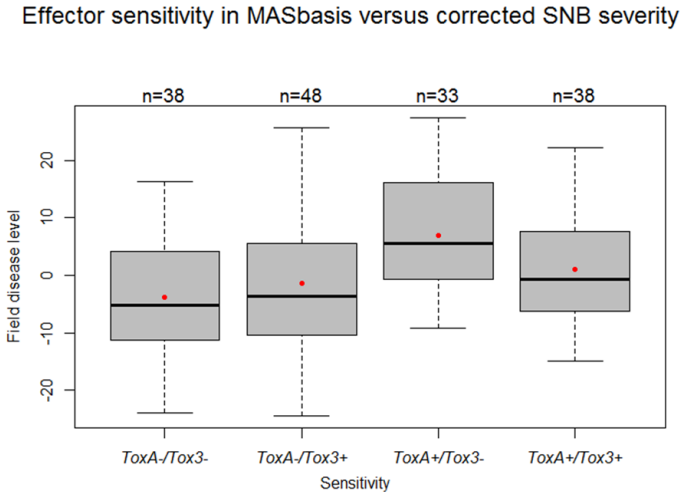

Fig. 2. Boxplots comparing corrected Septoria nodorum blotch (SNB) severity (*y*-axis) for cultivars with different sensitivity combinations (*x*-axis): ToxA, SnToxA; Tox3, SnTox3; +, sensitive; –, insensitive. Mean over 7 yr, all lines (see also Table 7). Red dot indicates mean value, black horizontal line median.

the important historical cultivars, except 'Polkka', which is highly susceptible. SnToxA sensitivity was present in 50% of these lines, whereas sensitivity to SnTox1 and SnTox3 was less common. The weaker SnTox3 Reaction Type 2 (chlorosis) was more prevalent than the Type 3 reaction, which was only present in Reno.

Figure 2 shows the relationships between corrected SNB severity and different combinations of insensitivity and sensitivity to SnToxA and SnTox3. The disease mean for lines with sensitivity to SnToxA alone was significantly higher than for lines insensitive to both effectors ($p = 1.295 \times 10^{-5}$) (Table 7). The mean for lines with sensitivity to both effectors was lower than for lines only sensitive to SnToxA (Fig. 2). Analyzed for individual years, SnToxA sensitivity was significantly correlated to increased disease severity every year (data not shown).

Sensitivity to SnTox3 alone did not have a significant effect on the field disease mean compared with double-insensitive lines (Table 7, $p = 0.29$). Year 2010 was the only year where we found significant association between SnTox3 sensitivity and field resistance levels ($p = 0.04$) compared to resistance scores for double-insensitive lines. When the exotic material (i.e., all non-European lines) was analyzed alone, the correlation was even more significant ($p = 0.01$) in 2010 and at a 0.05 level across years ($p = 0.03$) while not being significant for the European subpopulation.

Marker Correlations

Correlation of markers linked to *Tsn1* and sensitivity to SnToxA was high, with *fcp623* as the most significant marker. The markers *fcp1* and *fcp620* linked to *Tsn1* were also significantly correlated to corrected SNB severity (Table 8).

Markers linked to *Snn1* were not significantly correlated with SnTox1 sensitivity (Table 8). The marker with highest correlation to SnTox3 sensitivity was *cfd20* (a 294-bp fragment), strongly linked to reaction Type 2 (Table 8). These markers were not significantly correlated to corrected SNB severity.

DISCUSSION

SnToxA, SnTox1, and SnTox3 Characterization of Norwegian Isolates and Prevalence of Corresponding Sensitivities

Based on the screening of 62 individual *P. nodorum* isolates, we found that the *SnToxA*, *SnTox1*, and *SnTox3* genes were present in the majority of the isolates (Table 1). In particular, the frequency of *SnToxA* was significantly higher in the Norwegian isolates than reported from Switzerland, where only 12% of the isolates carried *SnToxA* (McDonald et al., 2013). Sensitivity to SnToxA was also common in the Norwegian breeding material and cultivars (45%, Table 2), and we speculate whether the high frequency of SnToxA in the isolates is an adaptation of the pathogen to the local host cultivars. More exhaustive collection and NE screening of the pathogen population should be performed to validate whether the frequencies are representative for the Norwegian *P. nodorum* population.

Prevalence of Sensitivity to SnToxA, SnTox1, and SnTox3

Sensitivity to SnTox1 was only present in 12% of the lines (Table 2). This is in the same range as the 16% of sensitive hexaploid wheat accessions reported by Shi et al. (2016b), but substantially less than in the Australian cultivars screened by Tan et al. (2014), where 33 of 46 genotypes showed moderate to strong sensitivity to SnTox1.

Table 7. Results of *t* tests comparing the effect of different combinations of sensitivity to SnToxA/SnTox3 on corrected Septoria nodorum blotch (SNB) severity, with a 95% confidence interval. The disease level is based on mean over 7 yr. – denotes insensitive and + denotes sensitive for SnToxA/SnTox3.

SnToxA/SnTox3 mean comparisons	*t*	df	*p*	95% confidence interval
–/– versus –/+	–1.04	81.29	0.29	–7.15, 2.23
–/– versus +/+	–2.18	72.21	0.03	–9.43, –0.43
± versus –/–	–4.40	68.72	3.3×10^{-5}	–15.58, –5.93
–/+ versus ±	–3.53	74.43	0.0007	–1.36, 7.31
± versus +/+	2.59	65.77	0.012	1.34, 10.31
–/+ versus +/+	–1.13	83.95	0.26	–6.80, 1.87

Table 8. Markers associated with SnTox reaction tested on the 157 genotyped lines. Correlations performed and *p* values calculated in R using the Hmisc package.

Gene	Marker	SnToxA	SnTox1	SnTox3 average	SnTox3 type 2	SnTox3 type 3	Mean corrected SNB† severity
Tsn1	fcp1	0.42***					0.26**
	fcp623	0.91***					0.27**
	fcp620	0.82***					0.29***
	fcp626	0.82***					0.20*
	fcp394	0.65***					0.16*
Snn1	fcp618		−0.16				0.11
	psp3000		−0.22*				0.09
Snn3-B1	cfd20			0.48***	0.68***	−0.02	−0.11
	gwm234 (264 bp)			0.41**	0.18*	0.24*	−0.07
	gwm234 (257 bp)			0.06	0.47***	−0.32***	0.02

*,**,*** Significant at 0.05, 0.001, and 0.0001 probability, respectively.

† SNB, Septoria nodorum blotch.

Sensitivity to SnTox3 was common and found in 55% of our material (Table 2). We observed two reaction types for sensitivity to SnTox3, one causing severe and complete necrosis (Reaction Type 3) and one causing chlorosis, but not necrosis (Reaction Type 2) (Fig. 1). This corresponds to the literature (Waters et al., 2011; Shi et al., 2016b), although it has not yet been established whether these are caused by different sensitivity loci, alleles, or downstream mechanisms. Reaction Type 2 was the only reaction type towards SnTox3 in the Swedish material we screened (Table 3), whereas Reaction Type 3 was most common in the CIMMYT material, illustrating a difference between materials of different origin.

Field Results

The heritability of SNB severity was 0.70 (Table 4) and lower than observed for many biparental mapping populations, for instance, SHA3/CBRD × Naxos (Lu and Lillemo, 2014). It was, however, higher than reported by Shankar et al. (2008) for a doubled-haploid population. As described above, the development of SNB is significantly correlated with and confounded by other traits, and the relatively lower heritability of SNB in a diverse population like MASbasis can partly be explained by the heritability of DH, which was 0.72 (Table 4). A likely contribution to the large variation in heading dates across years is the presence of *Vrn* and *Ppd* genes in the germplasm that respond differently to varying planting date and growth season temperatures of the field trials used in our study. The heritability of PH was high (0.90, Table 4), as could be expected for this trait.

The field trials were conducted under natural infection promoted by infected straw and mist irrigation. The natural population of *P. nodorum* is expected to vary over time, and thus variability in the individual NE-*Snn* interactions is expected to differ between years. However, the correlation of disease severity between years was high (Table 5). The correlation between SNB severity and DH was highly significant in all years, whereas the correlation between PH and disease varied from insignificant in 2013 to significant at a 0.01 level in 2016 (Table 5). Conidiospores of *P. nodorum* are spread upward in the canopy by rain splash, and taller plants generally show less severity if relative disease spread is not accounted for (Eyal et al., 1987; Francki, 2013). The applied mist irrigation provided a favorable environment for SNB development, but not the rain-splash effect. The correlation between PH and SNB severity varied between years. The lowest correlation between SNB severity and PH was observed in 2011 and 2015 (Table 5). In 2015, an extreme rainfall eighth of July accounted for 76 mm precipitation in 24 h (LMT, 2017). Perhaps the spores were distributed higher up in the canopy than normal due to this rain and the PH effect was minimized. In 2011, several rainfalls higher than 10 mm precipitation in July might have contributed to a similar effect.

Correlation between Effector Sensitivity and SNB Susceptibility in the Field

In most important current and historical cultivars in Norway, sensitivity to SnToxA was most prevalent (50%), whereas Reaction Type 2 for SnTox3 sensitivity was more common than Reaction Type 3 (Table 6). The presence of Reaction Type 2 in the Norwegian landrace J03 (Table 6) indicates that this trait might have been common in Scandinavian spring wheat since the onset of modern plant breeding.

We found that lines sensitive to SnToxA had a significantly higher field SNB disease mean than insensitive lines (Fig. 2, Table 7). This trend was significant in all years. The most resistant SnToxA-insensitive lines were clearly more resistant than the most resistant SnToxA-sensitive lines (Fig. 2), regardless of SnTox3 sensitivity. The SnToxA sensitive lines scored from −14.9 (Milan, Fig. 2, Table 7) compared with the most resistant insensitive lines (from −24.5, Milan/SHA7, Fig. 2, Table 7).

Interestingly, the SNB mean for lines with sensitivity to both effectors were significantly ($p < 0.05$) lower

than for lines only sensitive to SnToxA (Fig. 2, Table 7). A part of the explanation can be that SnToxA-*Tsn1* is epistatic to SnTox3-*Snn3* (Friesen et al., 2008a, 2008b), so an additive effect of double sensitivity is not expected. Although few lines were sensitive to SnTox1, the effector might be produced by the pathogen and inhibit the production of SnTox3 (Phan et al., 2016). Other NE-*Snn* interactions may also be important, as well as other resistance mechanisms.

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

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

Interestingly, in 2010 the SnTox3-*Snn3* interaction was also highly significant in a biparental mapping population, SHA3/CBRD × Naxos, evaluated in the same field nursery (Ruud et al., 2017). In this population, the parent Naxos carried the *Snn3* allele producing the most severe necrosis (i.e., a Type 3 reaction). In seedling inoculations with SnTox3-producing isolates, plants carrying the Type 3 sensitivity have been shown to develop more severe disease symptoms than plants with the Type 2 sensitivity (see Fig. 1 in Shi et al., 2016b). We speculate whether the more severe Type 3 sensitivity has a stronger association to adult plant SNB severity as well. The change in the pathogen population over time and relatively larger effect of other interactions may explain why the SnTox3-*Snn3* interaction played a minor role in MASbasis in other years.

Marker Correlations

The 157 lines were also genotyped with PCR markers known to be associated with SnToxA, SnTox1, and SnTox3. Markers *fcp1*, *fcp394*, *fcp620*, *fcp623*, and *fcp626* were all significantly correlated with sensitivity to SnToxA, with *fcp623* showing the strongest association (Pearson correlation 0.91, Table 8). The marker *fcp623* is located in an intron of *Tsn1* and is reported to cosegregate almost 100% with ToxA sensitivity (i.e., in 386 *Triticum* accessions) (Faris et al., 2010). The distance between *Tsn1* and *fcp394* is comparable with the distance between *Tsn1* and *fcp620* on the physical map, whereas *fcp1* is more distantly linked (Faris et al., 2010). However, *fcp1* had higher correlation than *fcp394* to corrected SNB disease level (Table 8).

The panel was also genotyped with markers *fcp618* and *psp3000* known to be linked to *Snn1*, but the markers only had low correlation with sensitivity to SnTox1 (Table 8) in our material. Reasons for this may be that the markers are not sufficiently closely linked to the sensitivity locus. The combination of historical recombination events in the relatively diverse population and few SnTox1-sensitive lines can explain the lack of association.

The SSR marker *cfd20* was strongly associated with SnTox3 Type 2 sensitivity, while it was not associated with reaction Type 3 (Table 8). This may support the assumption that the different reaction types are caused by different alleles of *Snn3* but does not exclude the possibility of two different loci. Marker *gwm234* amplified different fragments in different genotypes, and the 257-bp amplicon was linked to Reaction Type 2 (Table 8). The 264 bp allele is the same that is amplified in SnTox3-differential line BG220, where *Snn3* was first mapped. However, this allele was not strongly associated with SnTox3 sensitivity in MASbasis but had higher correlation with the average SnTox3 scores (Reaction Types 2 and 3).

Recommendations for Breeding

All the important current cultivars were moderately resistant to moderately susceptible to SNB, ranging from −3 (Demonstrant) to +7.2 (Bjarne) (Table 6). The range of resistance in MASbasis was much higher, from −24.5 as the most resistant (Milan/SHA7) to +27.5 (Brakar) (Supplemental Table S1) on the disease severity scale corrected for PH and DH, implying that there is great genetic potential to improve the level of resistance by breeding.

Given that field resistance to SNB is governed by many factors, sensitivity to SnToxA was not always a reliable predictor of field susceptibility. For instance, the sensitive Demonstrant performed better in the field than the SnToxA-insensitive Bjarne. However, sensitivity to SnToxA was significantly associated with higher corrected SNB severity, and given this, we suggest that screening for sensitivity either by seedling infiltrations or marker-assisted selection is a reasonable and affordable measure to improve SNB and subsequently tan spot resistance in the breeding material.

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

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

Conflict of Interest

The authors declare that there is no conflict of interest.

Supplemental Material Available

Supplemental material for this article is available online.

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