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SnTox5–*Snn5*: a novel *Stagonospora nodorum* effector–wheat gene interaction and its relationship with the SnToxA–*Tsn1* and SnTox3–*Snn3–B1* interactions

TIMOTHY L. FRIESEN[1,2,*], CHENGGEN CHU[2,†], STEVEN S. XU[1] AND JUSTIN D. FARIS[1]

[1]*Biosciences Research Laboratory, USDA-ARS, 1605 Albrecht Blvd N., Fargo, ND 58102, USA*
[2]*Department of Plant Pathology, North Dakota State University, Walster Hall, Fargo, ND 58102, USA*

SUMMARY

The *Stagonospora nodorum*–wheat interaction involves multiple pathogen-produced necrotrophic effectors that interact directly or indirectly with specific host gene products to induce the disease Stagonospora nodorum blotch (SNB). Here, we used a tetraploid wheat mapping population to identify and characterize a sixth effector–host gene interaction in the wheat–*S. nodorum* system. Initial characterization of the effector SnTox5 indicated that it is a proteinaceous necrotrophic effector that induces necrosis on host lines harbouring the *Snn5* sensitivity gene, which was mapped to the long arm of wheat chromosome 4B. On the basis of ultrafiltration, SnTox5 is probably in the size range 10–30 kDa. Analysis of SNB development in the mapping population indicated that the SnTox5–*Snn5* interaction explains 37%–63% of the variation, demonstrating that this interaction plays a significant role in disease development. When the SnTox5–*Snn5* and SnToxA–*Tsn1* interactions occurred together, the level of SNB was increased significantly. Similar to several other interactions in this system, the SnTox5–*Snn5* interaction is light dependent, suggesting that multiple interactions may exploit the same pathways to cause disease.

INTRODUCTION

Phaeosphaeria nodorum (E. Mull.) Hedjar (anamorph: *Stagonospora nodorum*), causal agent of Stagonospora nodorum blotch (SNB), is a major problem for wheat producers throughout the world. Resistance to SNB has been shown to be a complex quantitatively inherited trait (reviewed in Xu *et al*., 2004) and, until recently, the underlying mechanism of virulence and resistance was not understood. However, numerous recent studies to dissect wheat–*S. nodorum* interactions have elucidated the genetic basis of this system at the classical and molecular levels (reviewed in Friesen and Faris, 2010; Friesen *et al*., 2008a).

**Correspondence*: Email: timothy.friesen@ars.usda.gov
†*Present address*: Heartland Plant Innovations, 3302 Throckmorton Hall, Kansas State University, Manhattan, KS 66503, USA.

Several economically important necrotrophic effectors (formerly referred to as host-selective toxins) are produced by necrotrophic fungi, especially those in the Dothideomycete class of the Ascomycetes (Wolpert *et al*., 2002). Most of the necrotrophic effectors described initially were defined as secondary metabolites, but several proteinaceous necrotrophic effectors have been identified recently (Friesen and Faris, 2010; Wolpert *et al*., 2002), ToxA being the best characterized. Nearly identical *ToxA* genes have been identified in *Pyrenophora tritici-repentis* (Ballance *et al*., 1996; Ciuffetti *et al*., 1997) and *S. nodorum* (Friesen *et al*., 2006), and the ToxA protein serves as an effector of pathogenicity for both pathogens (Liu *et al*., 2006). Recent work has shown that *Tsn1*, the sensitivity gene associated with ToxA, produced by both *S. nodorum* (SnToxA) and *P. tritici-repentis* (Ptr ToxA), contains resistance gene-like features. For example, *Tsn1* contains nucleotide-binding (NB) and leucine-rich repeat (LRR) domains, but also a serine/threonine protein kinase domain (Faris *et al*., 2010). The presence of a dominant susceptibility gene belonging to the NB-LRR class, which is typically found to be associated with resistance, provides evidence that *S. nodorum* and *P. tritici-repentis*, as well as other necrotrophic fungi (Lorang *et al*., 2007; Nagy and Bennetzen, 2008), can subvert host resistance mechanisms to induce disease.

To date, we have identified five necrotrophic effector–host gene interactions that all play significant roles in SNB disease development, accounting for a range of 18%–95% of disease development in various segregating host populations (reviewed in Friesen and Faris, 2010; Friesen *et al*., 2008a). Here, we characterized a sixth interaction, including the partial purification and initial characterization of the pathogen-produced necrotrophic effector (SnTox5), the identification and mapping of the corresponding host gene (*Snn5*) and the evaluation of the effects of a compatible SnTox5–*Snn5* interaction in causing disease. The current work adds another significant piece of evidence that the *S. nodorum*–wheat system involves pathogen-produced necrotrophic effector proteins that act in effector-triggered susceptibility to induce disease. In addition, we describe the genetic relationship of the SnTox5–*Snn5* interaction with the SnToxA–*Tsn1* and SnTox3–*Snn3–B1* interactions, giving insight into the disease manifestation of this complex necrotrophic host–pathogen system.

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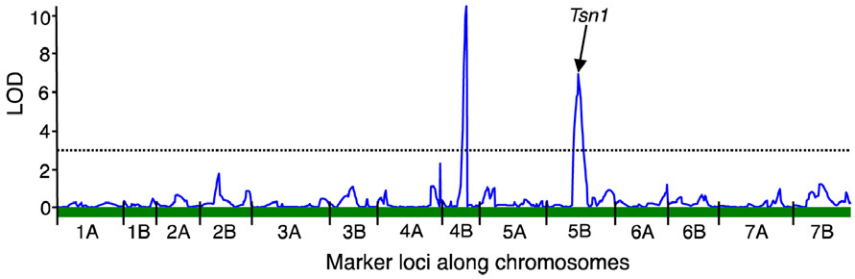

Fig. 1 A genomic scan of marker loci in the LP749 population associated with reaction to Sn2000 culture filtrates (containing both SnToxA and SnTox5) using simple linear regression. Logarithm of the odds (LOD) values are plotted along the *y* axis and marker loci on the 14 chromosomes are arranged end-to-end along the *x* axis. The significant LOD threshold of 3.1 is indicated by the dotted line. The significant peak on chromosome 5B is caused by the *Tsn1* locus and the peak on 4B is the SnTox5 necrotrophic effector sensitivity locus identified in this research, designated as *Snn5*.

RESULTS

Identification, genetic analysis and map location of *Snn5*, the SnTox5 sensitivity gene

Evaluation of the LP749 population for reaction to Sn2000 culture filtrates indicated that the *Tsn1* locus was a significant factor in conferring sensitivity to the necrosis-inducing components of the cultures, which was expected as Sn2000 is known to produce SnToxA (Fig. 1). However, in addition to *Tsn1*, a locus on chromosome 4B was also found to govern reaction to Sn2000 culture filtrates and was more significant than *Tsn1* (Fig. 1). This indicated that an effector other than SnToxA was present in the culture filtrates, and, because the host sensitivity locus was on chromosome 4B, and no *S. nodorum* effector sensitivity has yet been described on 4B, this other effector was newly identified. Therefore, we propose to designate this effector as SnTox5 and the corresponding host sensitivity gene on wheat chromosome 4B as *Snn5*.

The LP749 parental line Lebsock harbours sensitivity to SnToxA, SnTox3 and SnTox5. Therefore, we used the fungal strain Sn2000KO6-1 for SnTox5 production and genetic mapping of the *Snn5* locus because this strain does not produce SnToxA or SnTox3 (Friesen *et al*., 2006). When infiltrated with culture filtrates of Sn2000KO6-1, Lebsock and 64 of the 120 doubled haploid (DH) lines showed necrosis within the infiltrated area, 3 days after infiltration, whereas PI94749 and the remaining 56 DH lines showed no necrosis. Therefore, the segregation ratio of the LP749 population for reaction to Sn2000KO6-1 was not significantly different from 1:1 ($\chi^2 = 0.533$, $P = 0.465$), indicating that a single gene, *Snn5*, was responsible for sensitivity to SnTox5 (Table 1).

F_2 individuals of a cross between Lebsock and PI 94749 were used to evaluate the dominance of *Snn5*. A total of 32 F_2 individuals was infiltrated with Sn2000KO6-1 culture filtrates; they segregated in a ratio of 24:8 (sensitive : insensitive), which perfectly fits a 3:1 ratio ($\chi^2 = 0.0$, $P = 1.0$) (Table 1), indicating that *Snn5* functions in a dominant manner to confer sensitivity to SnTox5, similar to all the other necrotrophic effector sensitivity genes in the *S. nodorum*–wheat system.

Table 1 Genetic analysis of *Snn5* in the Lebsock × PI94749 F_2 population and of *Snn5*, *Tsn1* and *Snn3* in the LP749 doubled haploid population.

Population	Genotype	Observed	Expected	χ^2	*P*
LP F_2	*Snn5_/snn5snn5*	24:8	3:1	0	1.00
LP749	*Snn5Snn5/snn5snn5*	64:56	1:1	0.533	0.465
LP749	*Tsn1Tsn1/tsn1tsn1*	63:57	1:1	0.3	0.584
LP749	*Snn3Snn3/snn3snn3*	51:69	1:1	2.7	0.100

The phenotypic scores of the LP749 DH population for reaction to Sn2000KO6-1 culture filtrates were used to infer *Snn5* genotypes, and subsequent linkage analysis indicated that *Snn5* was located on the long arm of chromosome 4B (Fig. 3). Three additional simple sequence repeat (SSR) markers, *Xcfa2149*, *Xcfd22* and *Xwmc349*, were also placed on this linkage group. The *Snn5* locus mapped 2.8 cM proximal to the marker *Xwmc349* and 13.3 cM distal to markers *Xbarc163* and *Xcfd22*.

Markers along the genetic linkage map of chromosome 4B were compared with the deletion-based physical map of chromosome 4B constructed by Sourdille *et al*. (2004) to determine the deletion interval encompassing the *Snn5* locus (Fig. 3). Markers *Xgwm251*, *Xgwm149*, *Xgwm375*, *Xbarc163*, *Xcfd22* and *Xcfa2149* were located distal to the 4BL-5 deletion breakpoint by Sourdille *et al*. (2004). Therefore, all markers distal to *Xbarc227*, including *Snn5*, are probably located in the most distal deletion bin of 4BL, which accounts for approximately 15% of the chromosome arm.

Separation parameters and initial characterization of SnTox5

Dialysed culture filtrates containing SnTox5 were subjected to cation exchange chromatography using a 5-mL SPXL cation exchange column (GE Healthcare, Waukesha, WI, USA). The strongest SnTox5 activity eluted between 60 and 110 mM NaCl (data not shown). Size estimation was performed by ultrafiltration using 3-, 10- and 30-kDa molecular weight cut-off (MWCO) Centricon ultrafiltration filters. SnTox5 was identified in filtrates of the 30-kDa filter, but not in the filtrates of the 3- or 10-kDa filters. This result indicates that SnTox5 is between 10 and 30 kDa in size (Fig. 2a–g).

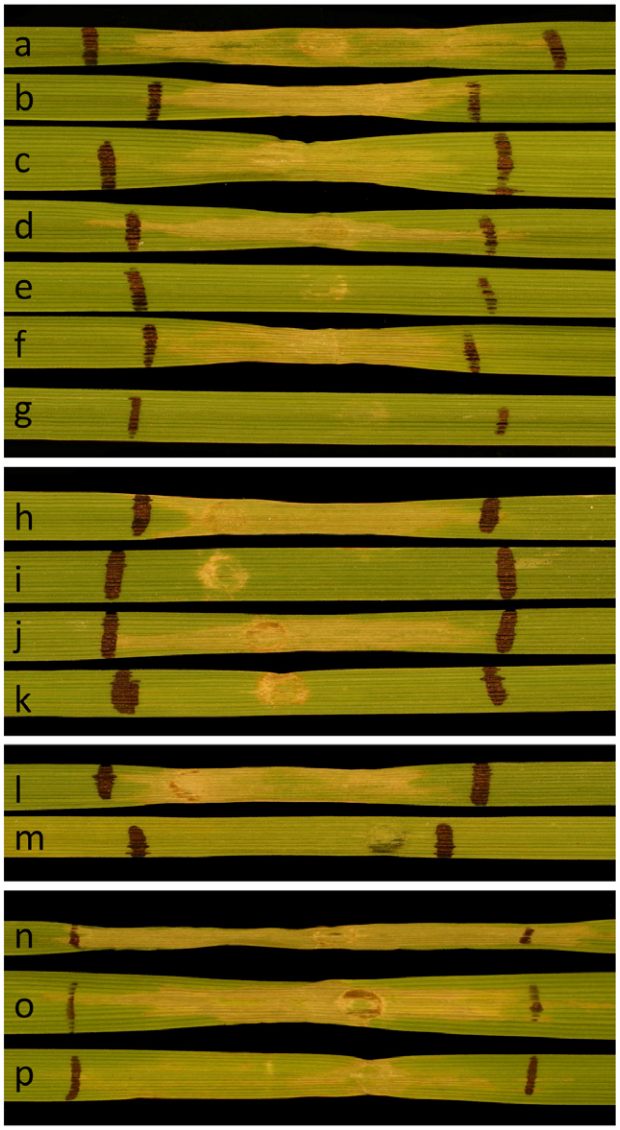

Fig. 2 Characterization of SnTox5 using culture filtrates of Sn2000KO6-1, an isolate that produces SnTox5, but does not produce SnTox3 or SnToxA. All reactions are on the *Snn5* differential line LP749-29. Size estimation of SnTox5 using molecular weight cut-off filters (a–g): (a) culture filtrate reaction; (b) 30-kDa concentrate; (c) 30-kDa filtrate; (d) 10-kDa concentrate; (e) 10-kDa filtrate; (f) 3-kDa concentrate; (g) 3-kDa filtrate. Sensitivity to Pronase (h–k): (h) culture filtrate alone; (i) Pronase alone (1 mg/mL); (j) culture filtrate diluted to the same concentration as leaf (k) with water alone (5% dilution); (k) culture filtrate with Pronase at 1 mg/mL final concentration. Evaluation of light dependence (l, m): (l) 12-h photoperiod for 48 h after infiltration ; (m) 48 h of darkness after infiltration. Evaluation of SnTox5 sensitivity to heat treatment (n–p): (n) culture filtrate unboiled; (o) culture filtrate subjected to boiling water for 30 min; (p) culture filtrate subjected to boiling water for 60 min.

Pronase, a combination of exo- and endoproteases, was used to verify the proteinaceous nature of SnTox5. Culture filtrates containing active SnTox5 subjected to Pronase treatment at a final concentration of 1 mg/mL showed no signs of necrosis production on LP749-29 after 48 h. However, the same culture filtrate diluted to the same concentration in water without Pronase showed complete necrosis after the same time period. Pronase alone showed no reaction on LP749-29 (Fig. 2h–k).

Because other effector–host interactions have been shown to be dependent on or influenced by light, including SnToxA–*Tsn1*, SnTox1–*Snn1*, SnTox2–*Snn2* and SnTox4–*Snn4*, light dependence was evaluated. The SnTox5 differential line LP749-29 was used in light dependence experiments because it is sensitive to SnTox5, but not SnToxA or SnTox3. Culture filtrates of Sn2000 KO6-1 containing active SnTox5 were infiltrated into secondary leaves of LP749-29 that had been kept in the dark for 12 h. After infiltration, plants were either placed in the dark for 48 h or under a 12-h photoperiod for 48 h. Plants treated in the dark for 48 h did not develop necrosis, whereas plants subjected to a 12-h photoperiod developed visible necrosis at 48 h, indicating that the ability of a compatible SnTox5–*Snn5* interaction to cause cell death is dependent on light (Fig. 2l–m).

Heat stability was evaluated by boiling Sn2000 KO6-1 culture filtrates for 30 and 60 min, followed by infiltration into leaves of LP749-29 (Fig. 2n–p). Both the 30- and 60-min treatments maintained activity on LP749-29, although a significant reduction in activity was visible in the 60-min treatment (Fig. 2p).

Characterization of the role of the SnTox5–*Snn5* interaction and its relationship with the SnToxA–*Tsn1* and SnTox3–*Snn3*–B1 interactions in disease development

Analysis of the mean disease reaction types conferred by *S. nodorum* isolate Sn2000 revealed quantitative trait loci (QTLs) on chromosome arms 4BL and 5BL, corresponding to the *Snn5* and *Tsn1* loci, respectively, which were significantly associated with disease (Fig. 3). Susceptibility was conferred by *Snn5* and *Tsn1* alleles contributed by Lebsock, which individually accounted for 37% and 31% of the variation, respectively (Table 2); together, they accounted for 60% of the variation (data not shown). This result was not unexpected, given the fact that Sn2000 produces the effectors SnTox5 and SnToxA. It also indicates that the effects of the SnTox5–*Snn5* and SnToxA–*Tsn1* interactions are working together and independently to play prominent roles in conferring SNB susceptibility.

Further analysis of Sn2000 phenotypic data (Table 3) showed that genotypes harbouring either susceptibility allele (*Snn5Snn5/tsn1tsn1* or *snn5snn5/Tsn1Tsn1* genotype) or both alleles (*Snn5Snn5/Tsn1Tsn1*) gave a significantly higher disease reaction than did genotypes harbouring neither allele (*snn5snn5/tsn1tsn1*).

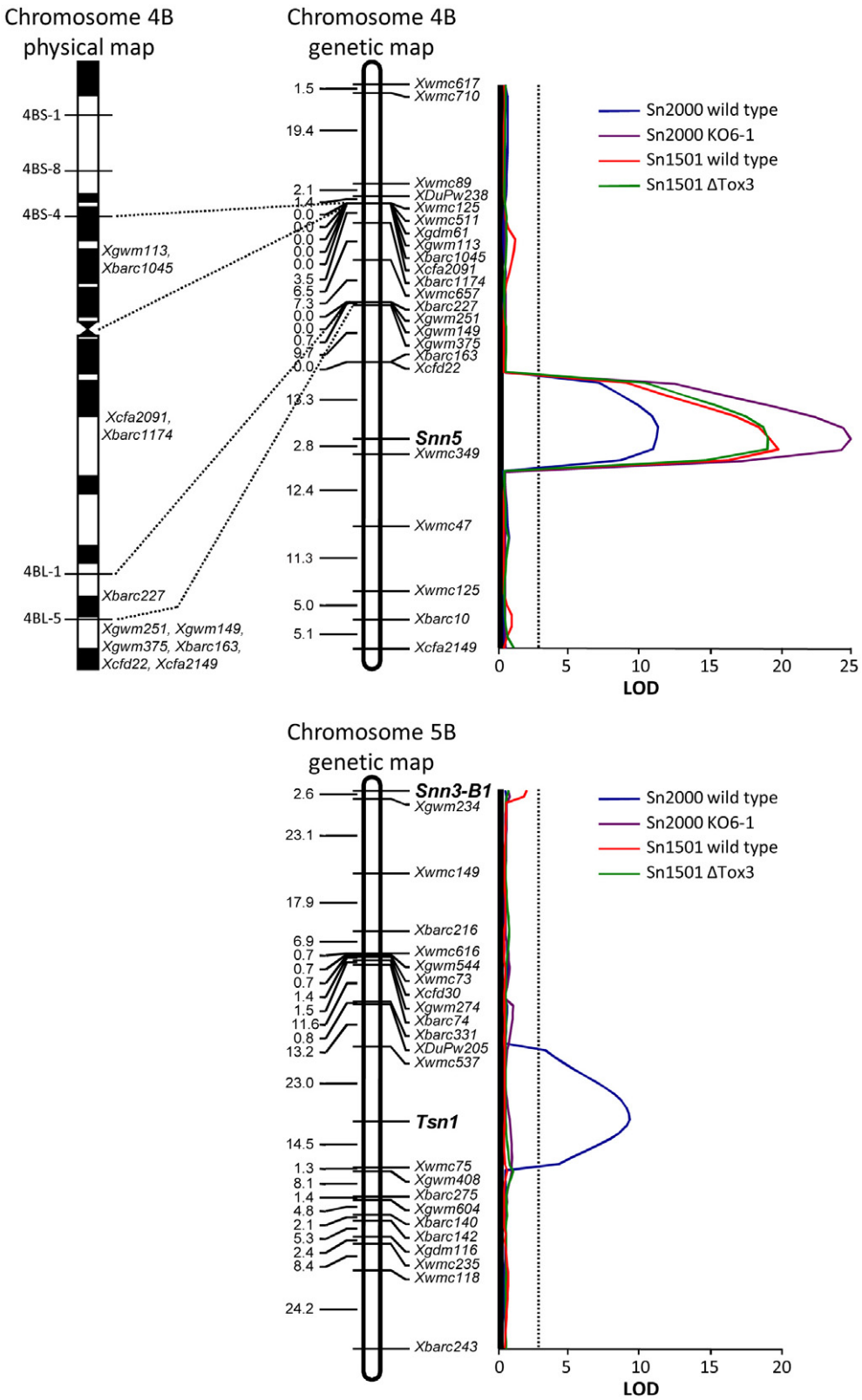

Fig. 3 Genetic mapping and interval regression analysis of the *Snn5*, *Tsn1* and *Snn3–B1* loci. Genetic linkage maps of chromosomes 4B and 5B developed in the LP749 population are in the middle. Marker loci are shown along the right of the maps and centiMorgan distances along the left. The *Snn5*, *Tsn1* and *Snn3–B1* loci are indicated in bold. The deletion-based physical map of chromosome 4B (Sourdille *et al*., 2004) is shown to the left of the 4B linkage map. Comparisons of common markers demonstrate that the *Snn5* locus is distal to the 4BL-5 breakpoint and therefore lies in the distal 15% of the chromosome arm. Results of the interval regression analysis using SNB data obtained from inoculation of the LP749 population with *Stagonospora nodorum* isolates Sn2000 (SnToxA + SnTox5), Sn2000KO6-1 (SnTox5), Sn1501 (SnTox3 + SnTox5) and Sn1501ΔTox3 (SnTox5) are shown to the right of the linkage maps. Logarithm of the odds (LOD) values are plotted along the *x* axis and the significance threshold of 3.1 is indicated by the dotted line.

Table 2 Quantitative trait locus (QTL) analysis of effector sensitivity loci in the LP749 population on inoculation of the population with *Stagonospora nodorum* strains Sn2000 (SnToxA + SnTox5), Sn2000KO6-1 (SnTox5), Sn1501 (SnTox3 + SnTox5) and Sn1501ΔSnTox3 (SnTox5).

Locus	Chromosome arm	Sn2000			Sn2000KO6-1			Sn1501			Sn1501ΔSnTox3		
		LOD	R^2	Add. effects	LOD	R^2	Add. effects	LOD	R^2	Add. effects	LOD	R^2	Add. effects
Snn3-B1	5BS	–*	–	–	–	–	–	2.0	0.03	–0.17	NS	NS	NS
Snn5	4BL	11.6	0.37	–0.57	25.0	0.63	–0.79	19.8	0.53	–0.74	18.5	0.51	–0.71
Tsn1	5BL	9.1	0.31	–0.51	NS	NS	NS	–	–	–	–	–	–

NS, not significant.

*–, the effector recognized by the corresponding sensitivity locus is not produced by the given isolates; therefore, the interaction is not relevant.

Table 3 Average disease reaction types of parents and doubled haploid (DH) lines of the LP749 population for the allelic state combinations for *Snn5* and *Tsn1* after inoculation with conidia of *Stagonospora nodorum* isolates Sn2000 (SnToxA + SnTox5) and Sn2000KO6-1 (SnTox5).

Genotype	Means		Mean difference§
	Sn2000‡	Sn2000KO6-1‡	
Lebsock	3.33	2.83	
PI94749	0.67	0.50	
All LP progeny	2.25	1.85	0.40*
snn5snn5/tsn1tsn1	1.09a	1.12a	0.03NS
snn5snn5/Tsn1Tsn1	2.41b	0.89a	1.52†
Snn5Snn5/tsn1tsn1	2.46b	2.65b	0.19NS
Snn5Snn5/Tsn1Tsn1	2.94c	2.47b	0.47*
$LSD_{0.05}$	0.31	0.33	

LSD, least-significant difference; NS, not significant.

*Significance at the probability level of $P < 0.01$.

†Significance at the probability level of $P < 0.001$.

‡Numbers followed by the same letter are not significantly different at the 0.05 level of probability.

§Mean differences between the same genotypic class inoculated with different strains of the pathogen.

In the case of Sn2000, the SnTox5–*Snn5* and SnToxA–*Tsn1* interactions induced similar amounts of disease, and lines harbouring *Snn5* alone (*Snn5Snn5*/*tsn1tsn1*) and lines harbouring *Tsn1* alone (*snn5snn5*/*Tsn1Tsn1*) were not significantly different in their average disease reaction types (Table 3, Fig. 4A).

Evaluation of the LP749 population for SNB produced by *S. nodorum* strain Sn2000KO6-1, which is a strain of Sn2000 with a disrupted *SnToxA* gene (Friesen *et al*., 2006), allowed further comparisons of the effects of the SnTox5–*Snn5* and SnToxA–*Tsn1* interactions. QTL analysis indicated that only the *Snn5* locus was significantly associated with SNB (Table 3). As expected, the *Tsn1* locus was not associated with disease because of a lack of SnToxA production by Sn2000KO6-1 (Fig. 4B, Table 3). Although the *Snn5* locus explained 63% of the variation in SNB caused by Sn2000KO6-1, as opposed to 37% for SNB caused by the wild-type strain Sn2000 (Table 2), the mean disease reaction type for the whole population decreased significantly from 2.25 to 1.85 (Table 3). In addition, significant differences were found between inoculations of Sn2000 and Sn2000KO6-1 for the genotypes *snn5snn5*/*Tsn1Tsn1* and *Snn5Snn5*/*Tsn1Tsn1*, because of the lack of SnToxA production by Sn2000KO6-1 (Table 3).

Analysis of SNB in the LP749 population caused by *S. nodorum* isolate Sn1501, which produces SnTox5 and SnTox3, but not SnToxA, revealed a significant QTL at the *Snn5* locus that explained 53% of the variation (Fig. 3, Table 2). This was the only locus significantly associated with SNB caused by Sn1501 at the logarithm of the odds (LOD) threshold of 3.1. However, the *Snn3–B1* locus was significant at LOD = 2.0 and explained about 3% of the variation (Fig. 3, Table 2). Together, *Snn5* and *Snn3–B1* explained 56% of the variation in SNB caused by Sn1501 in a multiple regression model (data not shown). When comparing the average disease reaction types of the four genotypic classes involving *Snn3–B1* and *Snn5*, significant differences were identified between lines harbouring both sensitivity alleles at both loci (*Snn5Snn5*/*Snn3Snn3*) and lines harbouring neither sensitivity allele (*snn5snn5*/*snn3snn3*), as well as between lines differing for SnTox5 sensitivity (*Snn5* or *snn5*) (Table 4, Fig. 4C,D). Lines differing for *Snn3–B1* were only significantly different in the absence of the *Snn5* allele, suggesting that a compatible *Snn5*–SnTox5 interaction masks the effects of the *Snn3–B1*–SnTox3 interaction in this population because of the more severe levels of disease contributed by the former.

Inoculation of the LP749 population with strain Sn1501ΔTox3, which is the same as Sn1501, except that it does not produce SnTox3, indicated that the *Snn5* locus accounted for 51% of the variation in SNB (Fig. 3, Table 2), and the average disease reaction

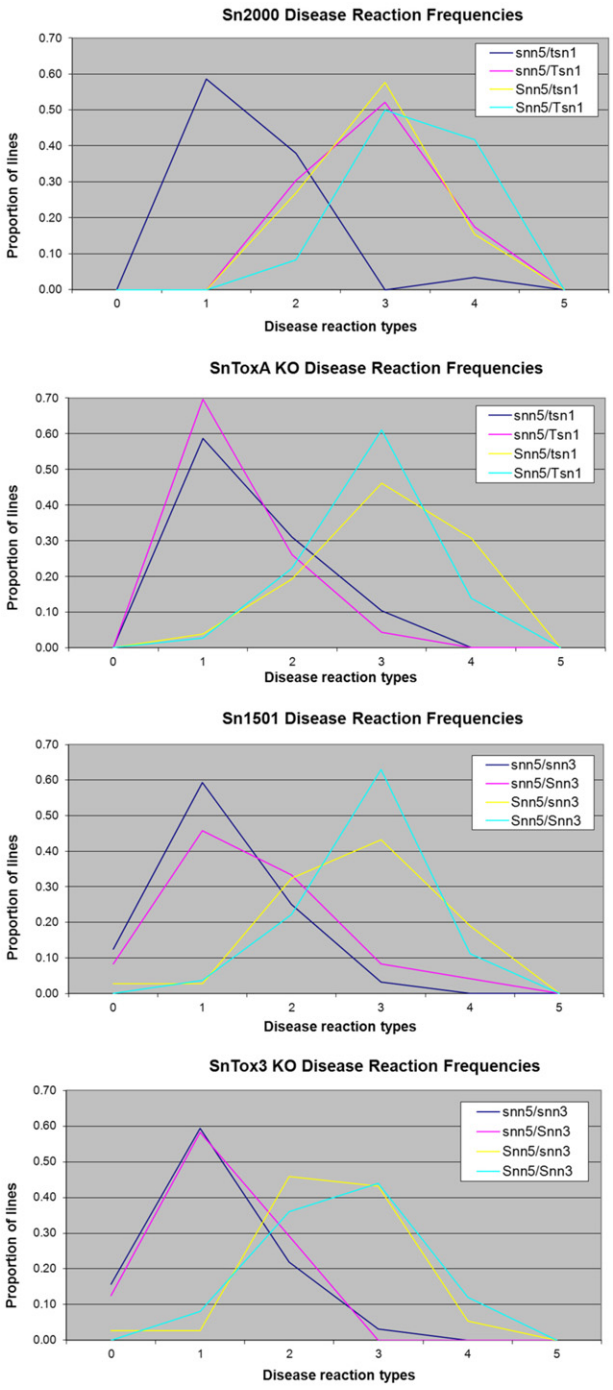

Fig. 4 Average disease reaction types by genotype caused by *Stagonospora nodorum* isolates Sn2000 (a), the corresponding *SnToxA* gene knockout (KO) strain Sn2000KO6-1 (b), Sn1501 (c) and its corresponding SnTox3 KO strain Sn1501ΔTox3 (d).

type observed for the whole population was not significantly different from that observed for the wild-type strain Sn1501 (Table 4). In addition, no significant differences were observed when comparing the four genotypic classes involving *Snn5* and *Snn3–B1* between Sn1501 and Sn1501ΔTox3 at the 0.05 level of probability (Table 4, Fig. 4C,D). However, it is noteworthy that the difference between the two strains for the *snn5snn5/Snn3Snn3* class was nearly significant ($P = 0.08$), suggesting that SnTox3–*Snn3–B1* contributes to the development of SNB in the absence of a compatible *Snn5*–SnTox5 interaction.

Table 4 Average disease reaction types of parents and doubled haploid (DH) lines of the LP749 population for the allelic state combinations for *Snn5* and *Snn3* after inoculation with conidia of *Stagonospora nodorum* isolates Sn1501 and Sn1501ΔTox3.

Genotype	Means		Mean difference†
	Sn1501*	Sn1501ΔTox3*	
Lebsock	2.33	2.5	
PI94749	0.17	0.67	
All LP progeny	1.67	1.47	0.20NS
snn5snn5/snn3snn3	0.71a	0.77a	0.06NS
snn5snn5/Snn3Snn3	1.12b	0.76a	0.36NS
Snn5Snn5/snn3snn3	2.32c	2.09b	0.23NS
Snn5Snn5/Snn3Snn3	2.40c	2.12b	0.28NS
$LSD_{0.05}$	0.36	0.36	

LSD, least-significant difference; NS, not significant.
*Numbers followed by the same letter are not significantly different at the 0.05 level of probability.
†Mean differences between the same genotypic class inoculated with different strains of the pathogen.

DISCUSSION

Until recently, SNB was thought to be quantitatively controlled and a complicated disease because little was known regarding the mechanisms underlying *S. nodorum*–wheat interactions at the molecular level. We have now identified and characterized six *S. nodorum* effector–wheat gene interactions, with several more yet to be published, making this the most extensively characterized system of inverse gene-for-gene interactions known in a necrotrophic fungal pathosystem. This system has a complex necrotrophic effector-based virulence system that is critical for disease induction, and it relies on the host (wheat) to harbour the corresponding effector sensitivity genes, which recognize the effectors. Effector recognition leads to successful elicitation of necrosis, thereby providing an area of dead tissue and nutrients for sporulation and the initiation of secondary disease cycles.

The recent cloning of the *Tsn1* gene showed that it contained hallmark features of genes that confer resistance to biotrophic pathogens, including serine/threonine protein kinase, NB and LRR domains. However, instead of conferring resistance to a biotroph, *Tsn1* confers susceptibility to SnToxA-producing strains of *S. nodorum*, suggesting that *S. nodorum* subverts resistance mechanisms to cause disease (Faris *et al*., 2010). It is possible that many, if not all, of the wheat *S. nodorum* effector sensitivity genes, including *Snn5*, are actually classic biotroph resistance-like genes

whose functions are being exploited by *S. nodorum*, a necrotrophic pathogen, to trigger programmed cell death. The recognition of necrotrophic effectors by resistance-like genes to induce disease has been shown in three necrotrophic plant–pathogen systems (Faris *et al*., 2010; Lorang *et al*., 2007; Nagy and Bennetzen, 2008), with each interaction showing hallmarks of the induction of programmed cell death in response to the molecule associated with the resistance-like gene (Dunkle and Macko, 1995; Lorang *et al*., 2007; Manning *et al*., 2009; Rasmussen *et al*., 2004).

Properties of SnTox5 and the SnTox5–*Snn5* interaction are similar to those reported previously in the SNB system. On the basis of ultrafiltration, SnTox5 is between 10 and 30 kDa, which is typical for a small secreted protein, and is in the same size range as SnToxA (Friesen *et al*., 2006), SnTox3 (Liu *et al*., 2009) and SnTox1 (Liu *et al*., 2012). Although the SnTox5–*Snn5* interaction is unique, it is dependent on light, and is therefore similar to several of the other necrotrophic effector–host gene interactions in the wheat–*S. nodorum* system (reviewed in Friesen and Faris, 2010; Friesen *et al*., 2008a). It is possible that all the light-dependent interactions, which include SnToxA–*Tsn1*, SnTox1–*Snn1*, SnTox2–*Snn2*, SnTox4–*Snn4* and now SnTox5–*Snn5*, may lead to the subversion of the same downstream pathway(s) to manifest disease. SnTox5 was also shown to be fairly stable after heat treatment. A similar level of heat stability was also shown for SnTox1 (Liu *et al*., 2012), a highly cysteine-rich protein.

SnTox5–*Snn5* is a strong interaction that accounts for as much as 63% of the disease variation in the LP749 population when it is the only interaction segregating in the population. In the presence of the SnToxA–*Tsn1* interaction, the SnTox5–*Snn5* interaction accounts for relatively less, but, when the SnToxA–*Tsn1* and SnTox5–*Snn5* interactions are present together, the disease reaction is significantly higher. The additive nature of compatible interactions is common in this system (Chu *et al*., 2010b; Friesen *et al*., 2007, 2008b), reinforcing the idea that the production of multiple effectors by the pathogen leads to compounding effects when in the presence of the corresponding host sensitivity/susceptibility genes. In the case of the combinations of SnTox5–*Snn5* and SnToxA–*Tsn1* or SnTox5–*Snn5* and SnTox3–*Snn3–B1*, the presence of two interactions leads to more severe reaction types (larger lesions) than a single interaction, and therefore the pathogen can colonize a greater area for spore production. Because SNB is a polycyclic disease and the pathogen moves up the plant by splashed spore dispersal during the growing season, increased spore production will increase the rate and significance of an epidemic in the field.

Liu *et al*. (2004b) reported a QTL on chromosome arm 4BL in the International Triticeae mapping Initiative (ITMI) wheat mapping population that accounted for 9% of the variation in SNB caused by isolate Sn2000. However, no necrotrophic effector–wheat gene interaction was reported to be associated with this QTL. Because both *Snn5* in the LP749 population and the minor QTL reported in the ITMI population were located to the same chromosome arm, and both were detected using the same *S. nodorum* isolate, it is possible that the underlying gene in the ITMI population is actually *Snn5*. However, further comparative mapping analysis and evaluation of the ITMI population with SnTox5-containing cultures are needed to determine whether the *Snn5*–SnTox5 interaction is responsible for the effects of the 4BL QTL in the ITMI population.

A large body of evidence demonstrating that the *S. nodorum*–wheat system consists of an arsenal of pathogen-produced necrotrophic effectors that target corresponding host sensitivity/susceptibility gene products to cause disease is now available. Therefore, it would be beneficial for breeders to identify and remove host sensitivity/susceptibility genes from their breeding lines to develop varieties with high levels of SNB resistance. This can be accomplished through the use of effector-containing culture filtrates, purified necrotrophic effectors or molecular markers tightly linked to wheat genes that govern effector sensitivity. The use of these bioassays and molecular genetics tools to characterize lines and select against effector sensitivity genes may greatly expedite the development of SNB-resistant germplasm. In areas in which SNB is a problem, the elimination of effector sensitivities will lead to decreased pathogen colonization of the host, resulting in increased yield and quality. The current work led to the identification and genetic characterization of the SnTox5–*Snn5* interaction, the sixth in a growing list of necrotrophic effector–host sensitivity gene interactions in the wheat–*S. nodorum* system.

EXPERIMENTAL PROCEDURES

Plant materials

A tetraploid wheat population (hereafter referred to as the LP749 population), consisting of 120 DH lines derived from a cross between the North Dakota durum wheat variety 'Lebsock' and the *T. turgidum* ssp. *carthlicum* ($2n = 4x = 28$, AABB) accession PI 94749 (Chu *et al*., 2010a), was used for molecular mapping and genetic analyses of reactions to SNB and *S. nodorum*-produced effectors (see below). Molecular linkage maps consisting of 280 SSR markers spanning 2034.1 cM and covering all 14 chromosomes have been developed previously (Chu *et al*., 2010a). Lebsock is susceptible to SNB because it harbours several effector sensitivity genes, including *Tsn1*, *Snn3-B1* and *Snn5*. PI94749 is moderately resistant to SNB and is insensitive to the currently known *S. nodorum*-produced necrotrophic effectors.

Culture filtrate production, effector bioassays and identification of a novel effector–host gene interaction

The LP749 population was screened with culture filtrates of isolates Sn2000 (Liu *et al*., 2004a, b) and Sn1501 (Friesen *et al*., 2008b; Zhang *et al*., 2011) to determine whether the population segregated for sensitivity to any previously unidentified *S. nodorum* effectors. Sn2000

produces SnToxA, but not SnTox3, because of the absence of the *SnTox3* gene, whereas Sn1501 is deficient in SnToxA production because of the absence of *SnToxA*, but does produce SnTox3 (Liu *et al*., 2009). Culture filtrates of the isolates were produced and assayed as described in Friesen and Faris (2012). Approximately 50 μL of culture filtrate were infiltrated into the second leaf of seedling plants (approximately 14 days old) of the entire LP749 population using a 1-mL syringe with the needle removed. Areas of infiltration were marked with a felt pen. Three days after infiltration, the reactions to the culture filtrates were scored using the 0–3 scale described by Friesen and Faris (2012). At least three replications were completed for each isolate used. The phenotypic data were then subjected to simple linear regression against the entire marker dataset for the LP749 population to identify marker loci associated with effector sensitivity, as described in Friesen *et al*. (2008b).

Partial purification of a newly identified effector

The DH line LP749-29 from the LP749 population was selected for use as a differential line for subsequent experiments because it was found to be sensitive to a newly identified effector, but insensitive to SnToxA and SnTox3. Culture filtrates of Sn2000, Sn1501, and also Sn2000KO6-1 and Sn1501ΔTox3, which contain site-directed disruptions of *SnToxA* (Friesen *et al*., 2006) and *SnTox3* (Liu *et al*., 2009), respectively, were grown as described previously (Liu *et al*., 2004a, 2009) and used for partial effector purification. Briefly, culture filtrates were dialysed for 4 h to overnight against water using 3500 MWCO dialysis tubing (Fisher Scientific, Pittsburgh, PA, USA). Dialysed culture filtrates were subjected to low-pressure cation exchange chromatography using a 5-mL SPXL cation exchange column (GE Healthcare). A 0–300 mM NaCl linear gradient in a running buffer of 20 mM sodium acetate, pH 5.2, was used to elute 1-mL fractions from the column at 1 mL/min. Fractions were assayed on the differential line LP749-29 to evaluate when the effector was eluting, and active fractions were then used in further experiments.

Effector characterization

Protease sensitivity was assayed as described by Friesen and Faris (2012). Active fractions were treated for 4 h at room temperature with Pronase (EMD Biosciences, Billerica, MA, USA) dissolved in water at a final concentration of 1 mg/mL. Active effector-containing cultures subjected to water alone and Pronase (1 mg/mL) alone were also incubated at room temperature for 4 h and used as controls.

Preliminary size estimation was obtained using Centricon ultrafiltration devices (Millipore, Billerica, MA, USA). Culture filtrate or semi-purified samples containing the newly identified effector were subjected to ultrafiltration using 5-, 10- and 30-kDa MWCO ultrafiltration devices. Concentrates and filtrates were assayed on the differential line LP749-29 for each MWCO filter size. Following infiltration, all plants were placed at 21 °C under a 12-h photoperiod. Plants were evaluated at 3 days post-infiltration. Sensitivity to filtrates indicated that the necrosis-inducing component was smaller than the cut-off of that particular MWCO filter, e.g. sensitivity to the 30-kDa filtrate indicates a size smaller than 30 kDa.

Light dependence for necrosis production was evaluated by infiltrating individual plants of line LP749-29 with active cultures produced from isolate Sn2000KO6-1. Before infiltration, all plants were subjected to a 12-h dark period and then infiltrated with culture filtrates containing the active effector. After infiltration, plants were placed in either a 24-h dark or 12-h light–12-h dark photoperiod, both at 21 °C. Three replicates of three plants each were infiltrated for each treatment.

Heat stability of the SnTox5 protein was evaluated using boiling treatments for 30 and 60 min. A 1-mL volume of culture filtrate of Sn2000KO6-1 was added to a 1.5-mL centrifuge tube and subjected to water that had been brought to a rolling boil. Tubes were removed and allowed to cool to room temperature before infiltrating the treated culture filtrates onto LP749-29 to evaluate the visible level of necrosis development. Two replicates consisting of infiltrations of three leaves each of untreated Sn2000KO6-1 culture filtrates, Sn2000KO6-1 culture filtrates subjected to boiling water for 30 min and Sn2000KO6-1 culture filtrates subjected to boiling water for 60 min were examined.

Disease evaluations

Conidial inoculations were performed as described by Friesen *et al*. (2008b) with slight modifications. The 120 DH lines of the LP749 population, together with parental lines Lebsock and PI94749, were planted in racks consisting of 98 cones (Steuwe & Sons, Corvallis, OR, USA) including a complete border of the susceptible cultivar 'Grandin' to reduce any edge effect. For each of three replicates, each line was planted in a single cone with three seeds in each cone. Conidia were produced and harvested as described in Liu *et al*. (2004b), and plants were inoculated using 1×10^6 spores/mL until runoff. Following inoculations, plants were placed in 100% relative humidity at 21 °C in the light for 24 h, followed by 6 days at 21 °C under a 12-h photoperiod. Plants were evaluated at 7 days post-inoculation using the 0–5 scale described by Liu *et al*. (2004b), where '0' is highly resistant and '5' is highly susceptible. Three replicates were completed for each isolate/strain tested and direct comparisons between wild-type and mutant strains were performed simultaneously.

Molecular mapping and QTL analysis

The SSR primer pair sets CFA2149, CFD22 (Sourdille *et al*., 2004) and WMC349 (Somers *et al*., 2004) were used to amplify DNA of Lebsock and PI94749 employing the conditions described in Chu *et al*. (2010a). All three primer sets revealed polymorphisms and were subsequently used to genotype the entire set of 120 DH lines of the LP749 population. The three resulting SSR markers (*Xcfa2149*, *Xcfd22* and *Xwmc349*) were added to the linkage group for chromosome 4B constructed by Chu *et al*. (2010a) using the 'TRY' command in Mapmaker v2.0 for Macintosh (Lander *et al*., 1987).

Composite interval mapping (CIM) regression analysis was used to evaluate the effects of individual effector–host sensitivity gene interactions using the computer software QGene v4.0 (Joehanes and Nelson, 2008). A permutation test consisting of 1000 iterations indicated that a LOD threshold of about 3.1 showed a significant effect contributed by the effector–host gene interaction.

Statistical analysis

Fisher's protected least-significant differences (LSDs) were used to compare SNB disease severity difference among allelic combinations of lines, and Student's *t*-test was used to test disease difference caused by different isolates within the same allelic combination. LSDs were calcu-

lated using the command PROC GLM and the *t*-test was performed under the command PROC TTEST in SAS (SAS Institute, 2011).

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