A triple threat: the *Parastagonospora nodorum* SnTox267 effector exploits three distinct host genetic factors to cause disease in wheat

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Summary

- *Parastagonospora nodorum* is a fungal pathogen of wheat. As a necrotrophic specialist, it deploys effector proteins that target dominant host susceptibility genes to elicit programmed cell death (PCD). Here we identify and functionally validate the effector targeting the host susceptibility genes Snn2, Snn6 and Snn7.
- We utilized whole-genome sequencing, association mapping, gene-disrupted mutants, gain-of-function transformants, virulence assays, bioinformatics and quantitative PCR to characterize these interactions.
- A single proteinaceous effector, SnTox267, targeted Snn2, Snn6 and Snn7 to trigger PCD. Snn2 and Snn6 functioned cooperatively to trigger PCD in a light-dependent pathway, whereas Snn7-mediated PCD functioned in a light-independent pathway. Isolates harboring 20 SnTox267 protein isoforms quantitatively varied in virulence. The diversity and distribution of isoforms varied between populations, indicating adaptation to local selection pressures. SnTox267 deletion resulted in the upregulation of effector genes SnToxA, SnTox1 and SnTox3.
- We validated a novel effector operating in an inverse-gene-for-gene manner to target three genetically distinct host susceptibility genes and elicit PCD. The discovery of the complementary gene action of Snn2 and Snn6 indicates their potential function in a guard or decoy model. Additionally, differences in light dependency in the elicited pathways and upregulation of unlinked effectors shed new light onto a complex fungal necrotroph–host interaction.

Introduction

The plant immune system functions predominantly through the recognition of microbe-produced or damage-derived molecules. This recognition of nonself leads to the induction of various defense responses. Perception of conserved microbial motifs known as pathogen-associated molecular patterns (PAMPs) can elicit a basal defense response classically known as PAMP-triggered immunity (PTI) (Boller & Felix, 2009). Pathogen-produced effector molecules may act to suppress or mask pathogen presence to effectively overcome PTI. However, plants have evolved receptor-like proteins to detect the presence of an effector or alternatively, the effector-mediated modification of host cellular machinery (Jones & Dangl, 2006). Although often described as separate immune pathways, evidence suggests that PTI and effector-triggered immunity (ETI) constituents substantially overlap and may function synergistically (Thomma et al., 2011; Ngou et al., 2021). ETI operates in a gene-for-gene manner and is effective at sequestering biotrophic pathogens (Flor, 1956; Jones & Dangl, 2006; Dodds & Rathjen, 2010). Many necrotrophic pathogens, however, have evolved with their respective hosts to target the host defense response to trigger programmed cell death (PCD) and obtain nutrients from the dying tissue. This interaction has been characterized as an inverse gene-for-gene relationship where a dominant host susceptibility gene is targeted by a necrotroph-produced effector molecule (Friesen et al., 2007).

*Parastagonospora nodorum* is a model necrotrophic pathogen that causes the economically important disease septoria nodorum blotch (SNB) of common wheat (*Triticum aestivum*) and durum wheat (*T. durum* ssp. *durum*). In contrast to the barrage of lytic enzymes often used by necrotrophic generalist pathogens, *P. nodorum* deploys an arsenal of specialized necrotrophic effector proteins that target recognition components of the host PCD...
pathways, resulting in necrotrophic effector triggered susceptibility. To date, nine necrotrophic effector–susceptibility gene interactions have been described, including SnToxA–Tsn1 (Friesen et al., 2006), SnTox1–Snn1 (Liu et al., 2004, 2012), SnTox2–Snn2 (Friesen et al., 2007), SnTox3–Snn3–B1/Snn3–D1 (Liu et al., 2009; Zhang et al., 2011), SnTox4–Snn4 (Abeysekara et al., 2009), SnTox5–Snn5 (Friesen et al., 2012), SnTox6–Snn6 (Gao et al., 2015) and SnTox7–Snn7 (Shi et al., 2015). Among these identified effector genes, SnToxA, SnTox1, SnTox3 and SnTox5 have been cloned, and among the host susceptibility genes, Tsn1, Snn1 and Snn3D–1 have been cloned (Friesen et al., 2006; Liu et al., 2009; Faris et al., 2010; Liu et al., 2012; Shi et al., 2016; Zhang et al., 2021; Kariyawasam et al., 2022). The cloned susceptibility genes represent distinctly different gene classes, including an intracellular protein with protein kinase, nucleotide binding and leucine rich-repeat domains (Tsn1), a wall-associated kinase (Snn1), and a protein kinase-major sperm protein (Snn3–D1) (Faris et al., 2010; Shi et al., 2016; Zhang et al., 2021). The identified necrotrophic effector proteins exhibit typical effector properties, including the presence of a secretion signal, relatively small size (<50 kDa), and above average cysteine content. Additionally, natural populations of P. nodorum show varying levels of presence/absence variation of the effector loci, likely reflecting the distribution of corresponding host susceptibility genes in locally planted wheat cultivars or the maintenance of beneficial alternate effector functions (Richards et al., 2019). These alternate functions include the ability of SnTox1 to protect from host-produced chitinases (Liu et al., 2016) or SnTox3 to suppress host defense through a direct interaction with PR1 proteins (Breen et al., 2016; Sung et al., 2021). The characterization of these effectors and corresponding host susceptibility targets has shown how P. nodorum specifically hijacks the host immune response and elicits cell death that ultimately provides nutrients for completion of the pathogenic life cycle.

This study shows that three previously characterized necrotrophic effectors (SnTox2, SnTox6 and SnTox7), which were originally hypothesized to function independently and target three distinct host susceptibility genes (Snn2, Snn6 and Snn7), are actually a single effector that targets two pathways involving three critical genes. One pathway was determined to be light-dependent and requires the cooperative function of both Snn2 and Snn6 to produce necrosis. The second pathway involves the host susceptibility gene Snn7 and is postulated to be separate due to its light-independent nature. Both pathways are targeted by a novel effector, designated as SnTox267, to elicit cell death, facilitating the completion of its pathogenic life cycle. Population-specific SnTox267 protein isoforms were identified and found to vary significantly in their ability to induce necrosis. Sequence analysis also indicated that subpopulation-specific selection pressures may contribute to local SnTox267 diversification. Additionally, the deletion of SnTox267 resulted in the differential upregulation of effector genes SnToxA, SnTox1 and SnTox3, indicating the potential of intertwined effector expression networks or the existence of an underlying trans-acting regulatory mechanism at this genomic locus. Taken together, these results advance our understanding of complex necrotroph–host interactions.

Materials and Methods

Variant identification in a Parastagonospora nodorum natural population

A natural population consisting of 197 P. nodorum isolates was used in this study and was previously described and sequenced by Richards et al. (2019). Isolates were collected from wheat producing regions in the United States, including 105 collected from the Upper Midwest, 84 collected from the southeastern United States and eight from the Pacific Northwest. Genome sequencing, as well as single nucleotide polymorphism (SNP) and insertion/deletion (InDel) identification was conducted in a previous study (Richards et al., 2019) and the derived genotypic data were used in the present research. Briefly, the genome of each isolate was sequenced to approximately 28x coverage. Sequencing reads for each isolate were trimmed using TRIMMOMATIC (Bolger et al., 2014) and mapped to the Sn4 reference genome (Richards et al., 2018) using BWA–MEM (Li & Durbin, 2010). SAMTOOLS ‘mpileup’ was used to identify variants (Li et al., 2009). Genotypic data for the natural population were retrieved from https://github.com/jkzrich/npnordorum_popgen.

Phenotyping of differential wheat lines

Inoculum production and inoculation procedures were conducted as described previously (Friesen & Faris, 2012). Experimental details are provided in Supporting Information Methods S1.

Association mapping and candidate gene identification

A genome-wide association study (GWAS) was conducted using TASSEL (Bradbury et al., 2007). Polymorphic sites with >30% missing data or a minor allele frequency <5% were removed. A general linear model using the first three components of a principal component analysis (PCA) as a fixed effect to correct for population structure was tested. P-values were adjusted using a Bonferroni multiple comparison correction and loci with an adjusted P-value of <0.001 were considered significant. Linkage disequilibrium (LD) was calculated between each marker on chromosome 14 and plotted with LDHEATMAP (Shin et al., 2006). Candidate genes within the LD block were identified from the Sn4 genome annotation (https://github.com/jkzrich/npnordorum_popgen). Secretion signals in candidate genes were predicted with SIGNALP 5.0 (Almagro Armenteros et al., 2019) and EFFECTORP (Siperschneider et al., 2016) was used to predict effectors. The DIANNA 1.1 web server was used to predict disulfide bond formation between cysteine residues (Ferré & Clote, 2006). PHYRE2 was used to predict structural homology. Chimera (Pettersen et al., 2004) was used to superimpose the predicted SnTox267 protein onto the SnTox3 crystal structure and Kex2 cleavage site prediction was conducted as described by Outram et al. (2021). Genomic locations of previously characterized effector genes and SnTox267 were plotted using CHROMPLOT (Oróstica & Verdugo, 2016).
Development of gene-disrupted mutants

The SnTox267 gene was disrupted using a split-marker approach as described previously (Catlett et al., 2003). The detailed protocol used can be found in Methods S2 and Table S1.

Generation of gain-of-function transformants

The SnTox267 genomic region was cloned into the pFPL-Rh vector as described previously with minor modifications (Gong et al., 2014). This construct was used to transform avirulent isolate Sn79-1087. Experimental details can be found in Methods S2 and Table S1.

Phenotyping of host populations

Host populations segregating for Snn2, Snn6 and Snn7 were phenotyped via inoculations and infiltration of culture filtrates. Experiment details are provided in Methods S4.

Development of a Chinese Spring × CS-Timstein-2D F2 population

The phenotyping, genotyping and subsequent quantitative trait loci (QTL) analysis in the CS × CS-Tm-2D population are detailed in Methods S5.

QTL analysis of the BR34 × Grandin recombinant inbred line (BG) population and International Triticeae Mapping Initiative (ITMI) populations

Interval mapping (IM) was conducted using the R/qtl ‘scanone’ function with the Haley–Knott regression method. A logarithm of the odds (LOD) threshold at the significance of α = 0.05 was determined through permutation analysis with 1000 iterations for each phenotype separately. All significant QTL identified from CIM were fitted into a multiple QTL model to determine the percentage of variation explained by each locus using the ‘fitqtl’ function (Broman et al., 2003).

Population genetic and haplotype analysis

Raw sequencing files were obtained from the NCBI SRA (BioProject PRJNA398070) and used for variant confirmation in the SnTox267 coding region. Sequencing reads were trimmed and mapped to the Sn4 reference genome as described previously (Richards et al., 2019). Duplicates were marked using ‘MarkDuplicates’ in Picard (http://broadinstitute.github.io/picard/). The Genome Analysis Toolkit HaplotypeCaller (Poplin et al., 2017) was used to identify SNPs and InDels within the SnTox267 coding region for each sample individually specifying ‘—intervals Chr14:67371-68168 —emitRefConfidence GVCF’. Genotypes were determined using ‘GenotypeGVCFs’ with default settings. De novo genome assembly of each isolate was completed using SPAdes with default settings and automatic k-mer selection (Bankevich et al., 2012). BLAST databases were generated for each assembly and subsequently searched for the SnTox267 coding sequence. Total genomic variants, without filtering for missing data or minor allele frequency, were used to create consensus sequences of the SnTox267 coding region using SAMTOOLS and BCFTOOLS ‘consensus’ (Li et al., 2009). Sequences of the coding region were aligned using CLUSTAL Omega (Sievers & Higgins, 2014) and imported into DNASP v.6 for population genetic analysis (Rozas et al., 2017). Nucleotide diversity was calculated in 20-bp sliding windows in 5-bp steps across the coding region.

Differential effector expression

A time course experiment was designed to test the level of SnToxA, SnTox1 and SnTox3 expression in both the wild-type (WT) Sn4 and Sn4ΔTox267 backgrounds. Experimental details can be found in Methods S6.

Results

Association mapping identifies a single locus associated with virulence on Snn2 and Snn6

A GWAS was conducted to identify the effectors that elicit necrosis via interaction with host susceptibility genes Snn2 and Snn6. A previously described P. nodorum population consisting of 197 isolates from a wide geographical range in the United States was used to phenotype the wheat lines BG223 (Snn2+) and ITMI37 (Snn6+) (Richards et al., 2019). We hypothesized that the use of single susceptibility gene differential lines would enable the detection of the corresponding effector loci by eliminating any major background gene interactions. Phenotyping of BG223 and ITMI37 with the P. nodorum natural population revealed average reactions ranging from 0 to 3.5 (Fig. 1a; Table S2). Additionally, the virulence phenotypes on the two differential lines were highly correlated (r² = 0.59). Previously generated whole-genome sequencing reads for the natural population (NCBI BioProject PRJNA398070) were aligned to the Sn4 reference genome (Richards et al., 2018) for the identification of SNPs and InDels. Following filtering for minor allele frequency (5%) and missing data (30%), 322,613 polymorphic markers were identified and then used to conduct association mapping. Surprisingly, the same genomic locus on P. nodorum chromosome 14 was identified as significantly associated with virulence on both BG223 and ITMI37 (Fig. 1b,c). In both analyses, the marker SNP_66420 was the most significant association (BG223: P = 2.96 × 10⁻12; ITMI37: P = 3.23 × 10⁻14). This marker was located 931 bp upstream of the predicted effector-encoding gene CJF_13380, a 798-bp single exon gene encoding a 265 amino acid protein containing 10 cysteine residues (Fig. S1). SNP_66420 is located within a LD block of c. 20 kb. In addition to CJF_13380, the LD block harbors six genes. One gene encodes a predicted secreted protein, yet it was not considered an SnTox267 candidate because the mature protein had a relatively high estimated molecular weight (74.5 kDa) and was not predicted to be an effector via EffectorP. An additional gene

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encoded a LETM1/MDM38-like protein and the remaining four genes encoded proteins with unknown functional domains (Fig. S1). The cysteine residues in CJJ_13380 were predicted to form disulfide bonds, as is commonly observed in fungal effector proteins. A signal peptide was predicted in the first 16 amino acids and the mature 27.4 kDa protein was predicted to be an effector via E FFECTORP (Sperschneider et al., 2016). B LASTP searches of the NCBI nonredundant protein database failed to identify any homologous proteins, indicating that CJJ_13380 was unique to P. nodorum. Interestingly, although having low homology at the amino acid level (22% in the locally aligned region from residues 71–265), protein structural homology searches identified a high-confidence (93.4%) match to the SnTox3 crystal structure (Fig. S2). However, unlike SnTox3 and SnTox5 (Kariyawasam et al., 2022), SnTox267 was not predicted to contain a Kex2 cleavage site.

A total of 176 isolates had a reliable SNP_66420 genotype and two alleles were observed. Isolates having the ‘A’ allele had average disease reactions on BG223 and ITMI37 of 0.77 and 0.59, respectively (Fig. 1d). These average reaction types represent near-immune responses. Conversely, isolates harboring the ‘G’ allele exhibited average disease reactions of 1.89 and 1.96 on BG223 and ITMI37, respectively. The intermediate reaction types observed in isolates with the virulent allele could be attributed to the differential lines used harboring single susceptibility targets and the typically additive nature of necrotrophic effector–host susceptibility target interactions. These results provided the initial evidence that a single predicted effector protein...
Functional validation indicates CJJ_13380 exploits three genetically distinct host factors

Following the identification of CJJ_13380 as a strong candidate effector, we next attempted to functionally validate its role in virulence through the generation of gene-disrupted mutants. A split-marker approach was used to replace CJJ_13380 with a hygromycin resistance gene cassette. Two independent mutants, as well as an ectopic transformant that contained a nondisrupted CJJ_13380 and the hygromycin resistance gene inserted elsewhere in the genome, were obtained and used to inoculate the BR34 × Grandin recombinant inbred line (RIL) population (hereafter referred to as the BG population) originally used to map and characterize the Snn2 susceptibility gene (Friesen et al., 2007). Phenotyping of the Snn2 differential line BG296 showed a marked reduction in necrosis for the Sn4ΔCJJ_13380 isolates when compared to the WT Sn4 and ectopic transformants (Fig. 2a). Furthermore, inoculation of the Sn4ΔCJJ_13380 deletion mutants onto the BG population, which segregates for Snn2, and subsequent QTL analysis failed to detect the Snn2 QTL (Fig. 2b). Inoculation with the WT and ectopic isolates, however, identified the expected QTL corresponding to Snn2 (Fig. 2b). To further verify the role of CJJ_13380 in Snn2-mediated susceptibility, the avirulent P. nodorum isolate Sn79-1087 was transformed with a 2086-bp genomic region containing the putative promoter and coding sequence of the candidate gene from isolate Sn4. The resulting transformed isolate was named Sn79+CJJ_13380. Inoculation of WT Sn79-1087 exhibited a near-immune response on lines containing Snn2, but inoculation with the Sn79+CJJ_13380 strain showed obvious necrotic symptoms (Fig. 3a). Inoculation of the Sn79+CJJ_13380 transformant onto the BG population followed by QTL analysis identified a highly significant QTL corresponding to the Snn2 locus (Fig. 3c). The QTL explained approximately 92% of the phenotypic variation and was the only significant locus identified. Finally, to confirm that the necrotic symptoms were due to the recognition of a secreted CJJ_13380 protein, cell-free culture filtrates of Sn79+CJJ_13380 were infiltrated into leaves of the BG population and lines were evaluated.

Fig. 2 Disruption of CJJ_13380 results in the loss of virulence on lines harboring Snn2. (a) Wheat line BG296 (Snn2 differential line) inoculated with Sn4 wild-type (WT), Sn4 ectopic transformant, and two independent mutants with a disrupted CJJ_13380 gene. (b) Quantitative trait locus (QTL) analysis of the BR34 × Grandin (BG) population with the Sn4 WT, Sn4 ectopic and two CJJ_13380-disrupted isolates. x-axes, genetic positions along wheat chromosome 2D (in cM); y-axis, logarithm of the odds (LOD). The red dotted line corresponds to a LOD threshold of P = 0.05 as determined by 1000 permutations conducted for each analysis separately.
for necrosis (Fig. 3b). QTL analysis again confirmed \( \text{snn}2 \) as the sole locus involved in this interaction (Fig. 3c). These results indicated that \( \text{CJJ}_{13380} \) was a secreted protein that targeted \( \text{snn}2 \) to cause necrosis and was therefore \( \text{SnTox2} \).

Because the identical SNP in the putative promoter of \( \text{CJJ}_{13380} \) also was associated with virulence on \( \text{ITMI37} \), the \( \text{snn6} \) differential line, we used the same deletion and expression transformants to inoculate the ITMI population, which segregated for \( \text{snn6} \). Inoculation of the \( \text{Sn4ACJJ}_{13380} \) knockout mutants onto the \( \text{snn6} \) differential line \( \text{ITMI37} \) showed a marked reduction in necrotic symptoms compared to inoculations with \( \text{Sn4 WT} \) and the \( \text{Sn4 ectopic transformant} \) (Fig. 4a). Inoculation of the \( \text{Sn4 WT} \) and \( \text{Sn4 ectopic transformant} \) onto the ITMI population detected a QTL corresponding to \( \text{snn6} \). However, inoculation of \( \text{Sn4ACJJ}_{13380} \) mutants onto the ITMI population and subsequent QTL analysis failed to detect significance at the \( \text{snn6} \) locus (Fig. 4b). Additionally, inoculation of the \( \text{Sn79+CIJ}_{13380} \) isolate onto \( \text{ITMI37} \) showed typical disease development, whereas \( \text{ITMI37} \) inoculations with \( \text{Sn79-1087 WT} \) exhibited an immune response (Fig. 5a). Moreover, inoculation of \( \text{Sn79+CIJ}_{13380} \) onto the ITMI wheat population detected a single, highly significant QTL corresponding to \( \text{snn6} \) that explained approximately 42.13% of the phenotypic variation (Fig. 5b). Finally, infiltration of culture filtrates of the \( \text{Sn79+CIJ}_{13380} \) isolate into leaves of the ITMI population also mapped sensitivity to the \( \text{snn6} \) locus and explained 54.67% of the variation (Fig. 5c). These results indicate that in addition to eliciting necrosis via \( \text{snn}2 \), \( \text{CJJ}_{13380} \) also targets \( \text{snn6} \) to cause necrosis.

Previous research mapped the dominant susceptibility gene \( \text{snn7} \), which was targeted by the SnTox7 effector, to chromosome 2D in a Chinese Spring (CS) \( \times \) CS-Timstein 2D (substitution of CS chromosome 2D by Timstein chromosome 2D) population (Shi et al., 2015). The characteristics derived from previous partial purification of SnTox7 including an estimated size of 10–30 kDa, were similar to those observed for the partially purified SnTox2 and SnTox6 (Friesen et al., 2007; Gao et al., 2015), as well as the effector identified in this study. Therefore, we tested the potential for this novel effector to elicit cell death via targeting of \( \text{snn7} \). A CS \( \times \) CS-Tm 2D F2 population was
developed to verify whether the CJJ_13380 candidate gene also was targeting Snn7 on chromosome 2D. Nine markers specific to chromosome 2D were used for genotyping and development of the corresponding linkage group. The F2 population was infiltrated with culture filtrates of Sn79+CJJ_13380 and QTL analysis revealed a highly significant peak corresponding to the Snn7 locus, indicating that Snn7 also is targeted by CJJ_13380 and conditions sensitivity to it (Fig. S3).

Taken together, these results indicated that CJJ_13380 was a single secreted necrotrophic effector protein that elicited cell death from wheat lines harboring Snn2, Snn6 or Snn7. We propose the effector nomenclature SnTox267 to reflect the three known host sensitivity/susceptibility targets.

Snn2 and Snn6 function cooperatively to mediate SnTox267 elicited necrosis

Because SnTox267 induced necrosis on lines harboring Snn2, Snn6 or Snn7, we wanted to determine if the host targets represented distinct pathways or were polymorphic components of a single pathway. Previously, the Snn2–SnTox2 and Snn6–SnTox6 interactions were shown to be light-dependent, whereas the Snn7–SnTox7 interaction was shown to be light-independent and therefore likely functions in a separate pathway (Friesen et al., 2007; Gao et al., 2015; Shi et al., 2015). We also confirmed in this study the light-dependency or -independency of these reactions using side-by-side SnTox267 infiltrations (Fig. S4). Therefore, we specifically examined the potential of Snn2 and Snn6 functioning in the same pathway. An F2 population (n = 159) was developed from a cross between the SnTox267-sensitive lines BG223 (Snn2 differential line) and Opata 85 (sensitive parent of the ITMI population and possesses Snn6) to test whether Snn2 and Snn6 are complementary genes. If Snn2 and Snn6 elicited cell death independently, we expected an F2 segregation ratio of 15 : 1 (sensitive : insensitive) for two dominant genes with redundant functions. However, all 159 F2 progeny were sensitive to SnTox267 infiltrations. This indicated that BG223 and Opata85 each harbored functional copies of both Snn2 and Snn6, therefore, all F2 progeny were fixed at both loci. Based on these results, we hypothesized that both Snn2 and Snn6 are required for SnTox267 sensitivity. If this model was correct, the insensitive parents of the BG and ITMI populations would harbor a

Fig. 4 Disruption of CJJ_13380 results in the loss of virulence on lines harboring Snn6. (a) Wheat line ITMI37 (Snn6 differential line) inoculated with Sn4 wild-type (WT), Sn4 ectopic transformatant and two independent mutants with a disrupted CJJ_13380 gene. (b) Quantitative trait locus (QTL) analysis of the (ITMI, International Triticeae Mapping Initiative) population with the Sn4 WT, Sn4 ectopic and two CJJ_13380-disrupted isolates. x-axes, genetic positions along wheat chromosome 6A (in cM); y-axis, logarithm of the odds (LOD). The red dotted line corresponds to a LOD threshold of P = 0.05 as determined by 1000 permutations conducted for each analysis separately.
functional copy of one susceptibility gene (either Snm2 or Snm6) and a nonfunctional copy of the remaining susceptibility gene, which compromises the pathway and leads to insensitivity. Crossing the two insensitive (resistant) lines would then restore the functional pathway in the progeny and result in SnTox267 sensitivity. To test this hypothesis, we phenotyped F1 and F2 progeny of a cross between BR34 (insensitive parent of the BG population) and W7984 (insensitive parent of the ITMI population) by infiltrating Sn79+SnTox267 culture filtrate. All F1 progeny were sensitive (n = 3; Fig. 6) and sensitivity in the F2 individuals (n = 49) segregated 29 : 20 (sensitive : insensitive), fitting the expected 9 : 7 ratio for two genes with complementary gene

Fig. 5 Inoculation of gain-of-function transformant Sn79+CJJ13380 and infiltration of culture filtrates result in virulence or cell death on lines harboring Snm6. (a) Inoculation of ITMI37 (Snm6 differential line) with gain-of-function transformant Sn79+CJJ13380 and Sn79 wild-type (WT) (ITMI, International Triticeae Mapping Initiative). (b) Infiltration of Sn79+CJJ13380 culture filtrates on wheat lines Opata 85 (sensitive) and W7984 (insensitive). (c) Quantitative trait locus (QTL) analysis of the ITMI population with the Sn79+CJJ13380 isolate (left) and infiltration of Sn79+CJJ13380 culture filtrates (right). x-axes, genetic positions along wheat chromosome 6A (in cM); y-axis, logarithm of the odds (LOD). The red dotted line corresponds to a LOD threshold of P = 0.05 as determined by 1000 permutations conducted for each analysis separately.

Fig. 6 Snm2 and Snm6 are both required to produce SnTox267-mediated cell death. Culture filtrates of the gain-of-function transformant Sn79+CJJ13380 were infiltrated in wheat lines BR34 (snn2/Snm6), F1 plants (n = 3) derived from a cross of BR34 and W7984, W7984 (Snm2/snn6), and F2 (n = 49) plants derived from a cross of BR34 and W7984. ‘I’, insensitive reactions; ‘S’, sensitive reactions.
action ($\chi^2$ goodness-of-fit test: $\chi^2 = 0.17$, $P = 0.68$; Fig. 6). Taken together, these results indicated that Snn2 and Snn6 function as two components of the same molecular pathway where both dominant genes were required for necrosis development induced by SnTox267.

SnTox267 exhibits an uneven distribution of nucleotide diversity

Fungal effector genes, including the previously cloned SnToxA, SnTox1 and SnTox3, have the propensity to exhibit presence/absence variation. BLAST searches of de novo assembled genomes from each isolate in the natural population ($n = 197$) revealed that a total of 95.43% (188 of 197) of isolates had full or partial SnTox267 sequence represented in their respective de novo assemblies (deposited at doi: 10.5281/zenodo.4560540). These results indicated that SnTox267 is absent from only a small proportion of the natural population.

Sequence variants in the SnTox267 coding region were extracted and resulted in the identification of 22 polymorphic nucleotides, corresponding to an overall nucleotide diversity of 0.005. A higher level of nucleotide diversity was observed among isolates from the Upper Midwest population compared to the Southern/Eastern population with population-specific nucleotide diversity values of 0.006 and 0.002, respectively. Among the identified SNPs, six were synonymous changes, 15 were nonsynonymous changes and one introduced a premature stop codon (Fig. 7a). Sliding window analysis of nucleotide diversity determined that variation was not uniformly distributed and was generally concentrated in the 3' half of the gene (Fig. 7b). Taken
together, these results indicated that the presence of *SnTox267* was largely conserved within a diverse *P. nodorum* natural population; however, it possessed considerable nucleotide diversity that was localized to the 3′ portion of the gene.

**SnTox267** protein isoform diversity varies between populations and contributes to virulence

Within the entire population, a total of 32 nucleotide haplotypes, considering SNPs and InDels, were identified in *SnTox267* with a haplotype diversity (*H*<sub>D</sub>) of 0.86. This value of *H*<sub>D</sub> indicated a high probability that two randomly chosen isolates would possess different haplotypes. However, a large proportion of the population (30.32%, *n* = 57) comprised a single nucleotide haplotype. Examination of translated protein sequences of each haplotype resulted in a total of 20 protein isoforms with two predominant isoforms comprising 34.57% (Isoform 1) and 32.45% (Isoform 2) of the population. Only two amino acid substitutions differentiated these two isoforms, an aspartic acid to alanine substitution at residue 137 (D137A) and a threonine to serine substitution at residue 138 (T138S; Fig S5). Interestingly, the prevalence of these two isoforms varied substantially between the previously defined *P. nodorum* populations (Fig S6; Richards et al., 2019). Among isolates harboring the *SnTox267* Isoform 1, 83.08% were from the Upper Midwest (54.5% of all Upper Midwest isolates), 13.85% were from the Southern/Eastern United States (14.1% of all Southern/Eastern isolates) and 3.08% from Oregon (25.0% of all Oregon isolates). By contrast, among isolates with *SnTox267* Isoform 2, 68.85% of isolates were from the Southern/Eastern United States (65.6% of all Southern/Eastern isolates), 18.03% from Oklahoma (68.8% of all Oklahoma isolates), 8.20% from Oregon (62.5% of all Oregon isolates) and only 4.92% from the Upper Midwest (3.0% of all Upper Midwest isolates). Large differences also were observed in the total number of isoforms present in discrete populations. The Upper Midwest population contained 17 protein isoforms, whereas the Southern/Eastern population had only six isoforms present (Fig 7c). This stark contrast in the total number of isoforms detected in each population suggests *SnTox267* has been evolving more rapidly or over a longer evolutionary period in the Upper Midwest compared to the Southern/Eastern United States. The differences in geographical prevalence of the predominant isoforms also indicated that local selection pressures were actively driving the maintenance of specific isoforms in each population, potentially due to allelic differences in corresponding host susceptibility genes.

Virulence phenotypes of isolates harboring specific protein isoforms were examined to determine the potential effects of nonsynonymous substitutions. A protein isoform was considered to confer virulence if disease reactions on BG223 and ITMI37 were > 1.0 and only isoforms with sufficient representation were compared (*n* > 10). Three of the isoforms were considered virulent, with average disease reactions on BG223 and ITMI37 in the ranges 1.66–2.17 and 1.80–2.11, respectively (Fig 7d). Significant differences in virulence between these isoforms were detected, indicating that the subtle mutations differentiating them may have quantitatively contributed to virulence (Dunn’s multiple comparison test, Bonferroni-adjusted *P*-value < 0.10). A premature stop codon was identified in isoform 4 resulting in average disease reactions on BG223 and ITMI37 of 0.85 and 0.63, respectively (Fig 7d). Although the remaining 16 isoforms are represented by < 10 isolates per isoform and were therefore not included in statistical comparisons, it is worth noting that 11 isoforms appeared to confer virulence, with average disease reactions on BG223 and ITMI37 in the ranges 1.33–2.42 and 1.44–2.17, respectively (Table S3). The remaining five isoforms, all represented by single isolates from the Upper Midwest, were considered avirulent with average disease reactions on BG223 and ITMI37 in the ranges 0–0.875 and 0–1.125, respectively. Two of these isoforms contained premature stop codons indicating a truncated protein likely led to the loss of virulence. Two isoforms harbored a six amino acid deletion (discussed further below) that likely disrupted protein function. The remaining avirulent isoform was found to have two nonsynonymous mutations that differentiated it from the rest of the isoforms. One mutation induced a phenyalanine to tyrosine substitution at residue 187 (F187Y) and the other caused a threonine to alanine substitution at residue 244 (T244A). Interestingly, valine and serine substitutions also were identified in three and six isoforms, respectively, at residue 244. The T244S substitution had no apparent effect on virulence, as these isoforms were all classified as virulent. Three isoforms had the T244V substitution and were avirulent; however, two of these isoforms had premature stop codons. The changes in amino acids at this position may have altered the phenotype due to changes in hydrophobicity. As threonine is a hydrophilic amino acid, a substitution to serine, also a hydrophilic amino acid, may have minimal effect. The substitutions to hydrophobic residues such as alanine and valine may have led to altered effector activity. Although represented by a small sample size, these results indicated that the F187Y, T244A or T244V mutations may have led to a loss of virulence, but further experiments are warranted to independently validate these effects.

Alignments of the protein isoform amino acid sequences revealed a variable amino acid repeat stemming from an 18-bp in-frame InDel (Fig S5; Table S3). The 18-bp InDel translated to a VANAPE motif that was predominantly present in two tandem copies in 91.4% of isolates. Three isolates possessed isoforms with three tandem copies, whereas isoforms from 14 isolates harbored only one copy. Among the 14 isolates harboring isoforms with only one copy of the VANAPE motif, 12 also belonged to haplotypes containing a premature stop codon. The two remaining isolates that possessed the single VANAPE motif without a nonsense mutation had markedly lower virulence of 0.375 on both BG223 and ITMI37. However, as a consequence of the small sample size, it is difficult to say with any certainty that this deletion alone leads to compromised effector function.

Disruption of *SnTox267* results in compensatory upregulation of *SnToxA*, *SnTox1* and *SnTox3*

Following confirmation that *SnTox267* functioned in the elicitation of necrosis from lines containing *Snn2*, *Snn6* and *Snn7*, we
wanted to understand the transcriptional regulation during infection time points. A quantitative real-time (qRT-)PCR experiment was conducted using the Sn4 WT isolate inoculated onto the Snn2 line Grandin. Time points assayed included 24, 48, 72, 96 and 120 h postinoculation (hpi). SnTox267 expression was normalized against the housekeeping actin gene. Expression appeared to peak at 24 hpi and slowly declined throughout the time points examined, indicating that SnTox267 is activated and functions in early infection (Fig. S7).

*Parastagonospora nodorum* isolate Sn4 harbors functional copies of several characterized NEs in addition to SnTox267, including SnTox1, SnTox3 and SnToxA. However, QTL analysis following the inoculation of Sn4 WT and the Sn4 ectopic transformant onto the ITMI population, which also segregates for Snn1 and Snn3, did not detect a disease association with either Snn1 or Snn3 (Fig. 8a). Interestingly, inoculations with both Sn4ΔSnTox267 mutants resulted in a significant disease association with Snn1 and Snn3 (Fig. 8a). We hypothesized that the deletion of SnTox267 may alter the expression levels of additional effector genes, including SnToxA, SnTox1 and SnTox3, as a compensatory measure to account for the loss of an important effector.

In order to test the hypothesis that expression of SnTox1, SnTox3 and SnToxA is altered in the Sn4ΔSnTox267 isolate, expression patterns of these effector genes were compared to the Sn4 WT isolate using qPCR at 24, 48, 72, 96 and 120 hpi. Expression of each gene was measured relative to the fungal actin gene. When comparing expression of each effector gene between Sn4 WT and Sn4ΔSnTox267, higher expression of SnToxA, SnTox1 and SnTox3 was observed in Sn4ΔSnTox267 at each time point compared to the Sn4 WT (Fig. 8b). The expression of the three effectors in Sn4ΔSnTox267 was significantly higher compared to that of Sn4 WT during the first 48 h of the infection process. Specifically, expression of SnToxA and SnTox3 was significantly upregulated at 24 and 48 hpi, whereas SnTox1 was significantly upregulated at 48 hpi. Additionally, expression of SnToxA and SnTox3 appeared to be sustained longer, as these genes were significantly upregulated at 120 hpi (Fig. 8b). These results indicated that *P. nodorum* was attempting to compensate for the loss of SnTox267 in Sn4ΔSnTox267 by the upregulation of other genetically unlinked necrotrophic effector genes during the initial stages of the infection process.

**Discussion**

The functional characterization of SnTox267 and genetic dissection of host susceptibility has further clarified our understanding of inverse gene-for-gene interactions. Previously, the Snn2, Snn6 and Snn7 genes were thought to function as susceptibility targets via independent interactions with unique effectors (Friesen et al., 2007; Gao et al., 2015). However, the results from this study indicate that a single effector has co-opted these host targets or pathways to elicit cell death. From a fundamental perspective, this novel adaptive mechanism illustrates the plasticity of effector-producing fungal pathogens and their ability to use multiple evolutionary scenarios to their advantage. A similar, yet

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**Fig. 8** Disruption of SnTox267 resulted in the upregulation of unrelated effectors, leading to detection of corresponding susceptibility loci. (a) Quantitative trait locus (QTL) analysis of the International Triticeae Mapping Initiative (ITMI) population inoculated with Sn4 wild-type (WT), Sn4 ectopic transformant and two Sn4ΔCJ1380 mutants. QTL corresponding to Snn1 on wheat chromosome 1B (left) and Snn3 on chromosome 5B (right) were only detected following inoculation with the gene-disrupted mutants. x-axes, genetic positions (cM); y-axes, logarithm of the odds (LOD). The red dotted line corresponds to a LOD threshold of \( P = 0.05 \) as determined by 1000 permutations conducted for each analysis separately. (b) A quantitative PCR assay to evaluate differences in SnTox1, SnTox3 and SnToxA expression at five *in planta* time points (24, 48, 72, 96 and 120 h postinoculation (hpi)). x-axes, time points; y-axis, relative expression values. Bar colors correspond to either the WT or knockout strain Sn4ΔCJ13380-5 (legend on the right). Error bars, SEM. ‘*’ represents significant upregulation in the knockout compared to the WT at the same time point (Student’s t-test, \( P < 0.05 \)).
distinctly different interaction has been identified in the *Parastagonospora nodorum–wheat* pathosystem with the homoeologous loci *Snn3-B1* and *Snn3-D1*. Previously, the *P. nodorum* effector SnTox3 was observed to interact with two host sensitivity genes, *Snn3-B1* and *Snn3-D1*, which were homoeologous and located on chromosome 5B of the B-genome containing polyploids and 5D of the diploid D-genome progenitor *Aegilops tauschii* (Zhang et al., 2011). *Snn3-B1* and *Snn3-D1* were derived from a common ancestor and evolved independently without selection pressure before the introduction of *SnTox3* (Zhang et al., 2011). Although representative of multiple host targets, the SnTox3-Snn3-B1/D1 interactions are inherently different than the reported SnTox267 interactions. The homoeologous locations of the *Snn3* genes and phenotypic similarities of the induced necrosis point to a single host pathway being exploited. By contrast, the nonhomoeologous positions of the SnTox267 susceptibility loci, as well as the variable light-dependency of SnTox267-induced necrosis, illustrate a different scenario where the pathogen has taken multiple evolutionary steps to hijack host cell-death pathways.

Effector recognition by two host genes resulting in cell death has been reported previously in the hemibiotrophic *Leptosphaeria maculans*–oilseed rape pathosystem. The effector *AvrLm4–7* is recognized by both the *Rlm4* and *Rlm7* resistance genes (Parlange et al., 2009) where a single amino acid change governs recognition specificity by *Rlm4*, whereas *Rlm7* retains the ability to recognize either isoform. Additionally, the *L. maculans* effector *AvrLm1* is recognized by both host resistance genes *Rlm1* and *LepR3*, the latter being cloned and characterized as a receptor-like kinase (Gout et al., 2006; Larkan et al., 2013). By contrast with the inverse gene-for-gene relationship of SnTox267 with its corresponding susceptibility genes, these unique examples of gene-for-gene interactions result in an evolutionary advantage for the host, because the recognition of the pathogen-produced effectors results in a defense response that impedes the pathogen.

As the host targets of SnTox267 presently are unidentified, it was initially unclear if *Snn2*, *Snn6* and *Snn7* represented translocated paralogs, nonhomologous genes with common structural motifs targeted by SnTox267, or genes within the same pathway. The observed complementary gene action of *Snn2* and *Snn6* support our hypothesis that these two genes function cooperatively in the same pathway. It is expected that host gene diversification leading to escape of effector targeting in a necrotrophic system (i.e. resistance) would occur in genes involved in direct or indirect effector interactions rather than downstream signaling components which typically remain conserved as a result of potential roles in other physiological processes (Michelmore et al., 2013). Therefore, the possibility exists that both *Snn2* and *Snn6* are involved in effector interaction, with SnTox267 potentially hijacking a two-component system operating in a guard or decoy model (Van Der Hoorn & Kamoun, 2008). In these scenarios, a nucleotide-binding leucine-rich repeat receptor (NLR) typically guards the effector target or a decoy target. Once modification of, or recognition by the target is sensed by the NLR, a resistance response is triggered, resulting in programmed cell death (PCD). More recently, the requirement of two host NLR genes, one of which possesses an integrated domain, to confer resistance has been observed in several pathosystems (Loutre et al., 2009; Okuyama et al., 2011; Cesari et al., 2013; Wang et al., 2013; Césari et al., 2014). The integrated domains of NLRs are widespread in plant species and are postulated to act as decoy targets or sensors, but also may retain biochemical or functional activity (Cesari et al., 2014; Wu et al., 2015; Kroj et al., 2016). Therefore, a possible hypothesis is that *Snn2* and *Snn6* are two functional susceptibility genes working cooperatively in a guard or decoy model, analogous to the aforementioned resistance genes, with the key difference being the triggering of necrotrophic effector triggered susceptibility rather than effector-triggered immunity (ETI).

The light-independent nature of *Snn7*-mediated necrosis points to the existence of a second pathway exploited by SnTox267. Although the precise mechanisms that govern light-dependency in effector-mediated cell death reactions are not fully understood, previous research has implicated light-dependency as requisite for specific effector-induced cell death responses (Peever & Higgins, 1989; Tang et al., 1999). In the *P. nodorum–wheat* pathosystem, the previously characterized effectors SnToxA, SnTox1 and SnTox3 were demonstrated to induce necrosis only in the presence of light (Friesen et al., 2006; Liu et al., 2009, 2012). It also was shown that ToxA-mediated cell death required reactive oxygen species (ROS) production, which was inhibited under dark conditions (Manning et al., 2009). The observation that *Snn7*-mediated cell death functions in the absence of light indicates that SnTox267 may elicit necrosis from a ROS-independent pathway. Taken together, the identification of SnTox267 has revealed a complex set of host components, some of which may operate cooperatively, involved in the elicitation of PCD.

Analysis of SnTox267 within a natural population of *P. nodorum* revealed the largely conserved presence of the gene. The proportion of isolates harboring a functional copy of SnTox267 is comparable to levels observed for SnTox1, which was found in 95.4% of the same population used in this study, as well as in 84% of isolates in a global collection (McDonald et al., 2013; Richards et al., 2019). The prevalence of both SnTox267 and SnTox1 are substantially higher than that of SnToxA and SnTox3 which were present in 63.4% and 58.9% of isolates in the collection used in this study, respectively (Richards et al., 2019). These differences may be attributed to multiple roles of the specific effector protein. For example, the presence of SnToxA within a *P. nodorum* population was found to be highly correlated with the presence of the host sensitivity gene *Tsn1* in local wheat germplasm (Richards et al., 2019). The strong selection pressure exerted by the SnToxA–Tsn1 interaction likely drives the maintenance of SnToxA within the population; however, a fitness penalty may exist as it is readily removed in the absence of Tsn1. By contrast with the levels of PAV observed for SnToxA, the presence of SnTox267 in nearly all isolates examined suggests that it may possess an advantageous secondary function. Although it remains to be experimentally validated, a chitin-binding or other beneficial effector function would explain the propensity to maintain this gene within the pathogen population at similar...
levels of prevalence observed with SnTox1, which has been shown to bind chitin in the fungal cell wall and protect P. nodorum from wheat chitinases (Liu et al., 2016). Another possible explanation for the conserved presence of SnTox267 is the existence of multiple host targets. Currently, three genetically distinct susceptibility loci functioning in two separate pathways have been identified, which increases the probability of the introduction of at least a single pathway capable of inducing SnTox267-mediated necrosis. Furthermore, the presence of multiple host sensitivity genes suggests that a gene family, or minimally a structural motif, acts as a common target for SnTox267 and additional sensitivity loci may exist in wheat germplasm. This would further broaden the utility of SnTox267, and therefore its maintenance in the natural population.

Kariyawasam et al. (2022) showed that SnTox5 has a high level of structural homology to the recently released crystal structure of SnTox3 (Outram et al., 2021). A structural alignment prediction using SnTox3 and SnTox5 resulted in 98% of the mature SnTox5 being modeled with 100% confidence using SnTox3 as the template. Additionally, a Kex2-processed pro-domain that has been verified in SnTox3 also was predicted in SnTox5. Remarkably, structural homology analysis indicated that the predicted SnTox267 protein also shared some structural similarities with SnTox3. Phyre2 was used to compare SnTox3 and SnTox267 resulting in 40% of the mature SnTox267 being modeled with 93.4% confidence using SnTox3 as the template, all of which aligned with the C-terminal region of the protein. However, bioinformatic prediction based on sequence motifs and intrinsic disorder failed to predict a pro-domain in SnTox267. Further work is underway to evaluate the conserved and unique regions of these proteins and their function in virulence.

Genetic compensation or transcriptional adaptation are phenomena observed in other eukaryotic organisms, including zebrabfish and Arabidopsis, although the exact mechanism that controls the compensatory response has not been elucidated (El-Brolosy & Stainer, 2017). The deletion of SnTox267 led to the serendipitous discovery of altered expression profiles of physically unlinked effector genes (Fig. S8), indicating that genetic compensation or transcriptional adaptation may be active in P. nodorum. Previous research demonstrated similar compensatory effects following effector disruption in P. nodorum. Faris et al. (2011) provided the initial evidence that effector gene expression can directly impact the contribution of a given effector–susceptibility gene interaction to disease. SnToxA was found to be expressed significantly higher in isolate Sn5 compared to isolate Sn4, which led to a greater contribution to disease via interaction with Tsn1. Deletion of an effector gene also has been shown to modify unrelated effector expression and subsequently alter host interaction outcomes. Inoculation of a recombinant inbred line (RIL) population segregating for host susceptibility genes Tsn1, Snn1 and Snn3 with P. nodorum isolate Sn2000 (produces SnToxA and SnTox1) resulted in the detection of three quantitative trait loci (QTL), including the expected Tsn1 and Snn1 loci. However, inoculation with a Sn2000 SnToxA-disrupted mutant led to the detection of Snn1 at a greater magnitude, explaining substantially more phenotypic variation than inoculations with the wild-type (WT) isolate. It subsequently was discovered that SnTox1 was expressed at a significantly higher level in the knockout isolate compared to the WT (Peters et al., 2019). Likewise, inoculation of a wheat mapping population segregating for Snn1 and Snn3 with isolate SN15 only detected the Snn1 QTL (Phan et al., 2016). However, the Snn3 QTL was detected upon inoculation with a SnTox1-disrupted mutant of SN15, which also had significantly higher SnTox3 expression compared to the WT (Phan et al., 2016). In our study, the deletion of SnTox267 appeared to significantly enhance the expression of SnToxA, SnTox1 and SnTox3 through some form of genetic compensation. One explanation is that a common transcription factor may be regulating all four of these effector genes. Previously, Rybak et al. (2016) demonstrated that the zinc-finger transcription factor PnPf2 regulated both SnToxA and SnTox3, but did not appear to regulate SnTox1 expression. If a transcription factor activates SnToxA, SnTox1, SnTox3 and SnTox267 expression and acts in a dose-dependent manner, the loss of a binding site via deletion of the SnTox267 genomic region could result in increased binding to promoters of the three remaining effectors and subsequent higher expression. Alternatively, a trans-acting repressor, such as a small RNA molecule, may be encoded at this genomic locus and when deleted, lifted suppression of the other effector genes. Future RNAseq experiments using effector knockouts in P. nodorum may provide further insight into the observed genetic compensation and potentially identify previously masked effectors.

These results demonstrate that SnTox267 is a proteinaceous effector that exploits two host pathways to elicit cell death and cause disease. This effector is largely conserved throughout a natural population of P. nodorum, suggesting the presence of an advantageous secondary effector function or the presence of multiple host targets beyond Snn2, Snn6 and Snn7. Deletion of SnTox267 resulted in the compensatory upregulation of three unlinked effector genes, adding more evidence for the existence of a complex regulatory mechanism.

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Author contributions

JKR conceptualized and conducted the experiments, analyzed the data, and wrote and revised the manuscript. GK conducted experiments, analyzed data and revised the manuscript. SS conducted experiments. NAW analyzed data and revised the manuscript. JSX developed crosses and provided critical germplasm. ZL revised the manuscript. JDF provided germplasm and revised the manuscript. TLF conceptualized the experiments and analyses, obtained funding, and wrote and revised the manuscript.
Data availability

The Sn4 reference genome assembly, as well as the raw sequencing data for the natural population, are available at the NCBI BioProject PRJNA398070. De novo genome assemblies for each isolate are available at Zenodo (doi: 10.5281/zenodo.4560540). The genotypic data for the natural population and the Sn4 genome annotation are available at the following GitHub repository: https://github.com/jkzrich/pnodorum_popgen.

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Fig. S4 Light-dependency of SnTox267 interactions with Snn2, Snn6, and Snn7.

Fig. S5 Amino acid alignment of SnTox267 protein isoforms.

Fig. S6 Abundant isoform geographical distribution.

Fig. S7 SnTox267 temporal expression in planta.

Fig. S8 Genomic locations of cloned and characterized P. nodorum effectors.

Methods S1 Phenotyping of differential wheat lines.

Methods S2 Development of gene-disrupted mutants.

Methods S3 Generation of gain-of-function transformants.

Methods S4 Phenotyping host populations.

Methods S5 Phenotyping, genotyping and QTL analysis of the CS × CS-Tm-2D F2 population.

Methods S6 Differential effector expression.

Table S1 Primers used in this study.

Table S2 Phenotypic scores for the natural population inoculated on BG223 and ITMI37.

Table S3 Average virulence of SnTox267 isoforms.

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