The Cysteine Rich Necrotrophic Effector SnTox1 Produced by Stagonospora nodorum Triggers Susceptibility of Wheat Lines Harboring Snn1

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Abstract
The wheat pathogen Stagonospora nodorum produces multiple necrotrophic effectors (also called host-selective toxins) that promote disease by interacting with corresponding host sensitivity gene products. SnTox1 was the first necrotrophic effector identified in S. nodorum, and was shown to induce necrosis on wheat lines carrying Snn1. Here, we report the molecular cloning and validation of SnTox1 as well as the preliminary characterization of the mechanism underlying the SnTox1-Snn1 interaction which leads to susceptibility. SnTox1 was identified using bioinformatics tools and verified by heterologous expression in Pichia pastoris. SnTox1 encodes a 117 amino acid protein with the first 17 amino acids predicted as a signal peptide, and strikingly, the mature protein contains 16 cysteine residues, a common feature for some avirulence effectors. The transformation of SnTox1 into an avirulent S. nodorum isolate was sufficient to make the strain pathogenic. Additionally, the deletion of SnTox1 in virulent isolates rendered the SnTox1 mutated strains avirulent on the Snn1 differential wheat line. SnTox1 was present in 85% of a global collection of S. nodorum isolates. We identified a total of 11 protein isoforms and found evidence for strong diversifying selection operating on SnTox1. The SnTox1-Snn1 interaction results in an oxidative burst, DNA laddering, and pathogenesis related (PR) gene expression, all hallmarks of a defense response. In the absence of light, the development of SnTox1-induced necrosis and disease symptoms were completely blocked. By comparing the infection processes of a GFP-tagged avirulent isolate and the same isolate transformed with SnTox1, we conclude that SnTox1 may play a critical role during fungal penetration. This research further demonstrates that necrotrophic fungal pathogens utilize small effector proteins to exploit plant resistance pathways for their colonization, which provides important insights into the molecular basis of the wheat-S. nodorum interaction, an emerging model for necrotrophic pathosystems.

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Introduction
Like other parasites, fungal pathogens secrete a battery of molecules known as effectors during the infection process. These effectors can alter plant biological processes resulting in successful colonization [1,2]. Conversely, recognition of effectors by the plant innate immune system can initiate a defense response resulting in effector-triggered immunity (ETI) [3,4]. ETI is characterized by the accumulation of reactive oxygen species (ROS), transcriptional induction of pathogenesis-related (PR) genes and production of antimicrobial chemical compounds, eventually leading to rapid and localized plant cell death, known as the hypersensitive response (HR) [3]. In ETI, the perception of the fungal effector is mediated by the corresponding plant resistance gene (R) which acts in a gene-for-gene manner [6,7]. Currently, it is believed that this localized suicide of plant cells induced by ETI halts further growth of the biotrophic fungal pathogen, which requires living plant tissue for survival.

Necrotrophic fungal pathogens are known to produce host selective toxins (HSTs), including low molecular weight metabolites and small secreted proteins that function as essential determinants of pathogenicity or virulence [8,9]. HSTs can therefore be viewed as effectors of necrotrophic pathogenicity and hence we prefer the term necrotrophic effector (NE) [10,11]. These effectors play significant roles in determining the outcomes of plant-pathogen interactions by specifically interacting (directly or indirectly) with the products of corresponding host genes [12,13]. However, in contrast to ETI in the classical gene-for-gene model, the necrosis induced by effectors from necrotrophic fungal pathogens results in disease susceptibility; thus, it can be described...
In this manuscript we describe the cloning of SnTox1 from Stagonospora nodorum, the gene encoding the first host selective toxin (SnTox1) identified in this fungus. SnTox1 induces necrosis and promotes disease on wheat lines harboring the Snn1 gene. We verified the function of the SnTox1 gene by expressing it in a yeast culture where the resulting culture filtrate induced necrosis but only on wheat lines that carried a functional Snn1. The SnTox1 gene was also transformed into an avirulent S. nodorum isolate, resulting in an isolate that was virulent on wheat lines harboring Snn1. SnTox1 was also disrupted in virulent S. nodorum isolates resulting in the elimination of disease on Snn1 differential wheat lines. Additionally, we investigated the host response to SnTox1 and S. nodorum strains producing SnTox1 and discovered that several hallmarks of a resistance response were present during the susceptible reaction, showing that the necrotrophic pathogen S. nodorum is likely using SnTox1 to stimulate a host resistance pathway involving Snn1 to induce disease.

Of the S. nodorum effector genes, SnToxA and SnTox3 have been cloned with the aid of the S. nodorum genome sequence information [14,29,33]. The SnToxA gene is essentially identical to the ToxA gene isolated from the wheat tan spot pathogen P. tritic-repentis. Mature ToxA consists of a 13.2 kDa protein containing two cysteine residues as well as an RGD-containing vitronectin-like motif that is present in a solvent-exposed loop in the active protein [34–38]. The RGD motif has been shown to be essential for internalization and internalization has been shown to be critical for the induction of necrosis [37,39,40]. SnTox3 encodes an approximately 17.5 kDa mature protein with six cysteine residues and has no homology to genes in the public databases [14].

Here, we report the molecular cloning and characterization of the SnTox1 gene which encodes the SnTox1 protein, and we show that SnTox1 is specifically recognized by the corresponding wheat sensitivity/susceptibility gene Snn1. The characterization of the SnTox1-Snn1 interaction provides strong evidence that necrotrophic fungal pathogens use necrotrophic effectors to subvert the host resistance mechanism to cause disease.

Results

SNOG_20078 was identified as the SnTox1-encoding gene

Whole genome reference sequences have proven to be powerful for the identification of fungal and oomycete effector genes [1,41]. The annotated S. nodorum genome sequence supports a minimum of 10,762 nuclear genes with 1,782 predicted to encode extracellular proteins [33]. A specific set of criteria was used to prioritize the genes and generate a list of candidates. The criteria (size less than 30 kDa, predicted to be secreted, expressed in planta, etc, see Materials and Methods) were based on the characteristics of the previously cloned SnToxA and SnTox3 genes. We focused on the top 100 genes and as expected, SnToxA and SnTox1 were identified among them. PCR analysis was conducted to confirm the absence of genes in the S. nodorum avirulent isolate Sn79-1087 (data not shown). Genes meeting these criteria were expressed in the Pichia pastoris heterologous expression system [14]. This process and the subsequent screening of a set of differential lines (see Materials and Methods) led us to identify SNOG_20078 as the SnTox1-encoding gene.

Culture filtrates of P. pastoris strain X33 transformed with the coding region of SNOG_20078 cDNA were infiltrated into the leaves of W-7984, Chinese Spring (CS), CS 1BS-18 and CS ems237. W-7984 and CS carry the dominant Snn1 allele that confers sensitivity to SnTox1 [27], CS 1BS-18 and CS ems237 are nearly identical to CS, but harbor mutations at the Snn1 locus, resulting in insensitivity to SnTox1. Necrosis developed in the SnTox1-sensitive lines W-7984 and CS, but not in CS 1BS-18 and CS ems237 (Figure 1) suggesting that SNOG_20078 was the SnTox1-encoding gene. To map the gene conferring sensitivity, the same culture filtrates were subsequently infiltrated into the entire ITMI mapping population, which segregates for Snn1/snn1. All lines sensitive to the partially purified native SnTox1 [27] were also sensitive to the culture filtrates of the SNOG_20078 transformed yeast strain. This strongly indicated that SNOG_20078 was the SnTox1-encoding gene and therefore we designated it SnTox1.

SnTox1 gene structure and genomic location

SnTox1 is located in supercontig 10 of the assembled SN15 genome sequence ([33], Figure 2A). Within a ~7.6 kb region, there are three genes upstream (SNOG_07154-SNOG_7156) and one downstream (SNOG_07153) of SnTox1 (Figure 2A). Similarly, there is a short truncated molly-type retrotransposable element
mature protein (Figure S3). A BlastP search of the NCBI non-redundant database with the SnTox1 protein sequence as a query led to the identification of SNOG_20078 (Snn1), Chinese Spring (CS) (Snn1), CS 1BS-18 (snn1) and CS ems237 (snn1) to culture filtrates of a Pichia pastoris strain transformed with SNOG_20078. doi:10.1371/journal.ppat.1002467.g001

(183 bp) sequence following SnTox1 (http://genome.jgi-psf.org/cgi-bin/browserLoad/?db=Stan1&position=scaffold_10). The sequencing of the 5' and 3' RACE fragments revealed three exons as well as 5' and 3' untranslated regions (UTRs) in the full-length transcript of SnTox1 (Figure 2B). Putative TATA and CAAT boxes were identified 114 bp and 570 bp upstream of the ATG start site, respectively (Figure S1).

The SnTox1 protein consists of 117 amino acids with the first 17 amino acids predicted as a signal peptide. Interestingly, 16 of the remaining 100 amino acids are cysteine residues (Figure 2C). No prosequence was predicted using the web-based program ProP 1.0 (http://www.cbs.dtu.dk/services/ProP/) and after the cleavage of the predicted signal sequence the mature protein was estimated to be 10.33 kDa. To demonstrate that SnTox1 was produced in yeast culture and to verify the size of SnTox1, we applied western blot analysis to the protein samples prepared from SnTox1 yeast culture filtrates. The antibody for SnTox1 was generated from rabbit immunized with a BSA-conjugated 14 amino acid long SnTox1 peptide (see Material and Methods). A single band was only observed in protein samples prepared from SnTox1 yeast culture filtrates, but not from the control culture filtrates (yeast strain transformed with an empty vector). Furthermore, the western band was visualized between the size standard of 10 and 15 kDa, but much closer to 10 kDa (Figure S2). The estimated size of SnTox1 obtained from the western blot agreed with the predicted molecular weight of 10.33 kDa for the mature protein.

A BlastP search of the NCBI non-redundant database with the SnTox1 protein sequence as a query led to the identification of three putative proteins with unknown functions, one from S. nodorum (SNOG_06487) and two from P. tritici-repentis (PTRT_04748 and PTRT_03544) with similarities of 38%, 56%, and 43%, respectively. The conserved amino acids between SnTox1 and these proteins were mostly distributed in the predicted signal sequence and the N-terminal region of the mature protein (Figure S3).

SnTox1 contains a C-terminal chitin-binding-like motif

Amino acid alignment with manual adjustment indicated that SnTox1 contained local similarity with cysteine-rich C. fulvum Avr4-like fungal effectors (Figure 3A) from Cercospora beticola, Mycosphaerella fijiensis [42] and two ascomycete human pathogens, Microsporum gypseum and Geomyces pannorum (this study). These conserved motifs were identified within the chitin-binding domain (ChtBD) including the C-terminal conserved chitin-binding (CB) motif (Figure 3A). Three-dimensional (3D) structure-based sequence alignment suggested that the putative CB motif in SnTox1 was more similar to those of plant-specific ChtBDs (ChtBD1, or CBM18 superfamily, pfam00187) than to Avr4 proteins, which are related to invertebrate ChtBDs (ChtBD2, or the CBM14 superfamily, pfam01607) [43] (Figure 3B). SnTox1 contained all secondary-structure-related residues including the strictly conserved β-strand-forming “CCS” motif found only in plant-specific ChtBD1 proteins [44] (Figure 3B). In contrast, all Avr4-like proteins lacked the “CCS” motif and had a loosely conserved “QWN” motif at the same positions as that found in the antimicrobial protein tachycin, a representative ChtBD2 [44]. There were several insertions found between conserved regions in SnTox1 which also lacked the C-terminal extension after the conserved CB motif, suggesting a significant sequence divergence between SnTox1 and Avr4-like proteins.

SnTox1 is present in most virulent isolates and absent in avirulent isolates

The distribution of SnTox1 in different S. nodorum isolates and related fungal species (Table S1 and S2) was investigated using PCR assays and DNA dot blots. Among the 777 isolates that were sampled from wheat fields around the world, 85% (661) possess the SnTox1 gene (Table S1). Dot blot analysis of a subset of a global collection (Table S2) showed that SnTox1 was absent in all S. nodorum isolates collected from wild grasses which are avirulent on wheat (Figure 4A). Additionally, SnTox1 was absent in related fungal species including P. tritici-repentis, P. teres, P. bromi and M. graminicola.

To investigate sequence variation in SnTox1, the gene was PCR-amplified and sequenced from 159 global S. nodorum isolates. We found 12 different nucleotide haplotypes, 11 of which encode different protein isoforms, consistent with strong diversifying selection. The 11 protein isoforms involve amino acid changes at eight positions within SnTox1; however, all cysteine residues remain unchanged across all isoforms (Figure 2D). The nucleotide sequences of all 12 haplotypes have been submitted to GenBank and the accession number for each haplotype is provided at the end of the text. Four codons exhibit significant positive selection using PAML (Table 1). These findings provide strong evidence that positive diversifying selection, consistent with a co-evolutionary process, has been operating on SnTox1.

To investigate sequence variation of the SnTox1 genomic region in virulent and avirulent isolates, we used PCR to amplify the four genes flanking SnTox1 (SNOG_07153, SNOG_07154, SNOG_07155, and SNOG_07156, see Figure 2A for their locations). Only SNOG_07154 located directly upstream of SnTox1 could not be amplified from the avirulent isolate Sn79-1087 (data not shown), which suggested that a region containing all or part of
as well as the entire SnTox1 sequence may be missing in Sn79-1087. PCR primers were designed within the two genes SNOG_07153 and SNOG_07155 and used to amplify DNA from different virulent isolates as well as Sn79-1087. The amplified fragment in SN15 was 4.1 kb as expected but 4.5 kb in Sn1501 and 2.3 kb in Sn79-1087 (Figure 4B). Sequencing revealed that a 3.1 kb region including SnTox1 and the last 85 bp of the 3'9 end of SNOG_07154 coding region was replaced by a 1.3 kb sequence in Sn79-1087 (Figure 4C). The 1.3 kb insertion in Sn79-1087 does not share homology with any other known sequence in the NCBI database. Sequence analysis also revealed that two indels occur in the SnTox1 genomic region with one indel of 400 bp in the upstream, and the other indel of 167 bp at the end of the 3'UTR region (Figure 4C).

Addition of the SnTox1 gene to an avirulent isolate is sufficient to change the host range

The avirulent isolate Sn79-1087 does not produce any known S. nodorum necrotrophic effectors, nor does it induce a susceptible response on any of the wheat lines that we have tested. Therefore, a 1.1 kb SnTox1 genomic region (Figure S1) containing the native promoter, open reading frame, and the native terminator was cloned into the pDAN vector (Figure S4A) and transformed into Sn79-1087. Southern blot analysis indicated all but one transfor-
mant possessed the 1.1 kb SnTox1 fragment (Figure S4B). Transformsants A1 and A3, designated Sn79+SnTox1A1 and Sn79+SnTox1A3, were selected for further analysis. We confirmed that culture filtrates of Sn79-1087 did not cause necrosis nor did spore inoculations cause disease on CS, which contains Snn1 (Figure 5A). However, infiltration of culture filtrates from Sn79+SnTox1A1 and Sn79+SnTox1A3 produced necrosis on the leaves of CS (Figure 5A) and inoculation of CS with conidia of Sn79+SnTox1A1 and Sn79+SnTox1A3 produced lesions on the leaves of CS (Figure 5B). The two transformants did not cause disease on CS 1BS-18 or CS ems237, which lack a functional Snn1 gene (Figure 5B).

Deletion of SnTox1 in virulent isolates renders them nonpathogenic on Snn1 differential lines

The virulent isolate Sn2000 was used in the original identification of SnTox1 and Snn1 [27]. Therefore, this isolate was used to conduct gene knock outs of SnTox1. We exploited a PCR-based split marker method to replace the majority of the SnTox1 gene with the hygromycin resistance gene (hygR). The transformants were verified using Southern blot analysis with a probe amplified from the SnTox1 region that was replaced by hygR (Figure S4C). In two transformants designated Sn2000ΔSnTox1–9 and Sn2000ΔSnTox1–15, the SnTox1 gene was successfully replaced, and one transformant designated Sn2000ΔSnTox1-ECT was identified as an ectopic insertion due to it being hygromycin resistant but still having an intact and functional SnTox1 gene (Figure S4D).

Spores of the three transformed fungal strains along with wild type Sn2000 were inoculated onto the Snn1 differential wheat line W-7984 [27]. The ectopic strain Sn2000ΔSnTox1-ECT induced similar reaction as the wild type including defined tan necrotic lesions with widespread small white flecking, whereas the two knockout strains induced almost no reaction on the leaves (Figure 6A) indicating SnTox1 is an important virulence factor for Sn2000. Sn2000ΔSnTox1–9 and the Sn2000 wild type were also inoculated onto CS. Compared to the wild type, the virulence of
Sn2000ΔSnTox1–9 on CS was substantially reduced, but not completely eliminated (Figure 6B), which is due to CS having at least one additional necrotrophic effector sensitivity gene that likely interacts with another effector produced by Sn2000 (Faris and Friesen, unpublished).

The wheat ITMI population was used to originally map the QTL associated with disease susceptibility caused by Sn2000, in which two significant QTL were identified, one on chromosome 1BS corresponding to the Snn1 locus and the other on chromosome 4BL, explaining 48% and 9% of the disease, respectively [45]. We inoculated the three fungal strains: Sn2000ΔSnTox1–9, Sn2000ΔSnTox1–15 and Sn2000ΔSnTox1-ECT along with wild type Sn2000 onto the ITMI population. For Sn2000, as expected, we detected two significant QTL with one being at the Snn1 locus and the other being on chromosome 4BL, accounting for 50 and 17% of the disease variation, respectively. A very similar result was obtained for Sn2000ΔSnTox1-ECT where the Snn1 QTL and the QTL on chromosome 4BL were detected explaining 50 and 15% of the variation, respectively (Figure 6C). However, in the inoculation

Figure 4. Distribution of SnTox1 in S. nodorum isolates and variation in its genomic region. A. DNA dot blot analysis of SnTox1 gene distribution. Among 93 fungal isolates or species (Table S1), SnTox1 is absent in related fungal species (dash-lined box), S. nodorum avirulent isolates (box), and 6 virulent isolates not containing SnTox1 (circles). The remaining virulent isolates (72 out of 79) all contained SnTox1. B. PCR amplification of the ~4.1 kb genomic region of SnTox1 in different S. nodorum isolates. Two primers located within SNOG_7153 and SNOG_7155 (see Fig 4C arrows for primer locations) were used to amplify the SnTox1 genomic region in four S. nodorum isolates including the avirulent isolate (Sn79-1087). A difference in fragment size was observed among isolates. C. A diagram of the SnTox1 genomic region in different S. nodorum isolates. Five predicted genes (arrow blocks) and a truncated molly type repeat (gray rectangle) are schematically drawn. The 4.1 Kb region was amplified with two primers in SNOG_7153 and SNOG_7155 (small black arrows) and was investigated by cloning and sequencing. In Sn79-1087, a portion of SNOG_7154 and the entire SnTox1 gene was missing and was replaced by a 1.3 kb region (rectangle). Additionally, two indels (dash-lined rectangle) were identified in the upstream region and the 3’UTR of SnTox1.

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with the two SnTox1 knock out strains, the QTL conferred by Snl1 on chromosome 1BS became undetectable showing no association with disease, but the QTL on chromosome 4B was retained and became more significant overall accounting for 40–50% of the disease variation (Figure 6C). The QTL analysis in the ITMI population clearly demonstrated that SnTox1 codes for the SnTox1 protein which plays a significant role in disease by interacting with the host gene Snl1.

![Image](image-url)

**Figure 5. SnTox1 makes an avirulent isolate pathogenic.** A. Reaction of Chinese Spring to infiltration with culture filtrates from the avirulent isolate Sn79-1087 and its SnTox1 transformants. The culture filtrates from Sn79-1087 did not produce necrosis on CS, but the two SnTox1 transformants Sn79+SnTox1A1 and Sn79+SnTox1A3 did. B. Disease reaction of CS (Snl1) and CS 1BS-18 (Snl1 deleted) or CS ems237 (Snl1 mutated) to the inoculation with the avirulent isolate Sn79-1087 and its SnTox1 transformants. Sn79-1087 did not cause disease on CS, but the two Sn79+SnTox1A1 and Sn79+SnTox1A3 did produce tan necrotic lesions and widespread flecking on CS (Snl1). However, the two transformants were unable to cause disease on CS 1BS-18 and CS ems237 which carry the recessive allele (snn1).

### Table 1. Summary of a likelihood ratio test using PAML for positive selection in the SnTox1 gene.

| Model pairs | dN/dS | Log likelihood (Δ) | P-value | Positively selected codons | Posterior prob. |
|-------------|-------|---------------------|---------|---------------------------|-----------------|
| M1a (nearlyneutral) | 0.52 | −553.98 | | 48 | 0.975 |
| M2a (selection) | 4.49 | −546.04** | | 68 | 0.978 |
| M1a/M2a LRT | | <0.001 | 72 | 0.997 |
| M7 (beta) | 0.50 | −554.07 | | 106 | 0.974 |
| M8 (beta & ω) | 4.49 | −546.127** | | | |
| M7/M8 LRT | | <0.001 | | |

* Asterisks indicate which model resulted in a statistically higher likelihood score for the SnTox1 codon alignment.

** P-value for the likelihood ratio test (LRT) between the log-likelihoods of comparable models. In both cases the null hypothesis is rejected in favor of the selection model.

* Specific codons within the amino acid sequence were tested for positive selection. Positively selected codons with a Bayes Empirical Bayes posterior probability higher than 0.95 are listed on the right half of the table [78].

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Figure 6. *SnTox1* disruption affects virulence only on *Snn1* differential lines. **A.** Reaction of W-7984 to inoculation with Sn2000 and its *SnTox1* disrupted (Sn2000ΔSnTox1-9 and Sn2000ΔSnTox1-15) and ectopic (Sn2000ΔSnTox1-ECT) strains. Compared to the wild type and ectopic strain, the two *SnTox1* disrupted strains completely lost virulence on W-7984 which only contains the *SnTox1* sensitivity gene (*Snn1*). **B.** Reaction of CS to inoculation with Sn2000 and its *SnTox1* disrupted strain (Sn2000ΔSnTox1-9). Compared to wild type, the *SnTox1* disrupted strain (Sn2000ΔSnTox1-9) showed significantly reduced virulence on CS which is not only sensitive to *SnTox1* but to another necrotrophic effector produced by Sn2000. **C.** Interval map of chromosome 1B (left) and 4B (right) from QTL mapping in the ITMI population inoculated with Sn2000, and its *SnTox1* disrupted and ectopic strains. Strains are depicted by different colors as indicated. A centiMorgan scale is on the left of the map and markers are shown in their relative position along the right. The *Snn1* locus on the tip of chromosome 1B is shown in red. An LOD scale is shown along the x axis, and the critical LOD threshold of 3.0 is represented by the dotted lines.

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The transcription of *SnTox1* peaks at 3 days post inoculation correlating with the onset of necrotic lesion development.

*SnTox1* had a very similar expression pattern as *SnToxA* and *SnTox3* during infection in a microarray analysis that examined the expression of all fungal genes at 3, 5, 7, and 10 days post inoculation (DPI) in the wheat cultivar ‘Amery’ inoculated with SN15 (Ip-Cho and Oliver unpublished data). The analysis showed that the expression of all three genes was highest at 3 DPI (Figure S5). In this work, *SnTox1* expression was examined after inoculation of CS with *Sn79+SnTox1A1*, in which no other toxin-sensitivity gene interactions were involved. In the current study, relative expression of *SnTox1* to the fungal actin gene was examined at 10 time points ranging from 1 h to 7 d post inoculation using relative-quantitative PCR. Our analysis confirmed that *SnTox1* expression was maximized at 3 DPI (Figure 7A). The expression of *SnTox1* showed a slow increase between 6 and 12 HPI, increasing to about the same level as the actin gene at 24 HPI and increasing dramatically to 2.5 times higher than the actin gene expression at 48 HPI (Figure 7A). Once gene expression peaked at 3 DPI, the *SnTox1* transcription levels started to drop significantly from 3 to 4 DPI and returned to similar levels as the actin gene between 5 and 6 DPI. The accelerated increase of *SnTox1* expression from 24 HPI to 3 DPI indicates that *SnTox1* plays an important role in the early stage of infection.

The symptom development was examined macroscopically on CS inoculated with *Sn79+SnTox1A1* (Figure 7B). Disease symp-
toms were first visible on leaves at 2 DPI as white flecks and progressed into larger necrotic and chlorotic lesions. Interestingly, tan necrotic lesions start to develop at 3 DPI within the chlorotic areas, which correlates with the maximum expression of SnTox1 (Figure 7A). By 5 DPI, necrotic lesions became evident and the chlorotic areas enlarged (Figure 7B). The overall phenotype of the lesions changed very little from 5 to 7 DPI with only a slight change in size of individual lesions (Figure 7B).

The SnTox1 protein contains 16 cysteine residues that likely form multiple disulfide bonds and are important for SnTox1 stability. The SnTox1 protein contains 16 cysteine residues all of which are predicted to be involved in the formation of disulfide bonds with confidence levels greater than 7 (0 to 9 scale, [46]) (Figure 8A). The prediction software DIANNA [47] was used to identify the most likely connectivity of the cysteine residues as follows: 1–11, 2–5, 3–6, 4–13, 7–9, 8–16, 10–12, and 14–15 (Figure 8B). The stability of SnTox1 was tested by incubation of an SnTox1-containing yeast culture filtrate with different concentrations of dithiothreitol (DTT) and different incubation periods. The complete elimination of SnTox1 activity required 4 h in 40 mM DTT (Figure 8C). Additionally, the stability of SnTox1 was tested by directly heating the SnTox1 yeast culture filtrates on a hot plate. Strikingly, the culture filtrates maintained necrotic activity even after boiling for 30 min and did not completely lose activity until after 1 h (Figure 8D). Together, these results show that SnTox1 is a highly stable protein with the ability to resist physical and chemical degradation.

Figure 8. SnTox1 protein is cysteine-rich and highly stable. A. Disulfide bond prediction. The prediction was conducted using the web-based program DISULFIND (http://disulfind.dsi.unifi.it/). AA: amino acid sequence, DB_state: predicted disulfide bonding state (1 = disulfide bonded, 0 = not disulfide bonded); DB_conf: confidence of disulfide bonding state prediction (0 = low to 9 = high). B. The best predicted connectivity of disulfide bonds was determined using the web-based program DIANNA 1.1 (http://clavelius.bc.edu/closetlab/DIANNA/). C. Dithiothreitol (DTT) treatment of SnTox1. Reaction of CS to the SnTox1 yeast culture filtrates that were treated with DTT at the indicated concentration for 2 h or 4 h at room temperature. D. Heat treatment of SnTox1. Reaction of CS to the SnTox1 yeast culture filtrates that were heated to boiling on a hot plate for 30 min or 1 h.

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**SnTox1 triggers an oxidative burst**

The oxidative burst is one of the best-known biochemical responses of plant cells during a resistance response. The oxidative burst can be visualized by 3'–3' diaminobenzidine (DAB) staining for H$_2$O$_2$ production [48]. Chinese Spring (CS, *Sn1*) wheat leaves were infiltrated with SnTox1 yeast culture filtrate or a control yeast culture filtrate and collected at 48 h post-infiltration. The CS ems237 line (*snn1*) was included for infiltration and DAB staining as a comparison. Leaves were stained with 1 mg/ml DAB solution followed by clearing of chlorophyll. Dense brown DAB staining was observed on the leaves of CS (*Sn1*) infiltrated with SnTox1, but DAB staining did not appear on leaves of CS infiltrated with the control culture filtrates deficient in SnTox1, nor did DAB staining appear when SnTox1 was infiltrated into leaves of CS ems237 lines (*snn1*) (Figure 9A), clearly showing that the production of H$_2$O$_2$ is induced only during the SnTox1-*Sn1* interaction. A control without DAB staining was also conducted on CS leaves infiltrated with SnTox1 yeast culture. After clearing

![Figure 9. SnTox1 triggers H$_2$O$_2$ production.](image)

**Figure 9. SnTox1 triggers H$_2$O$_2$ production.** A. H$_2$O$_2$ production in infiltrated leaves. CS (*Sn1*) or CS ems237(*snn1*) leaves were infiltrated with SnTox1 (+SnTox1) or control culture filtrates (-SnTox1) and stained with 1 mg/ml DAB (+DAB) followed by clearing of chlorophyll. Leaves infiltrated with SnTox1 were cleared without staining as a control (-DAB) to show that the toxin reaction itself does not cause the brown color. B. H$_2$O$_2$ production in the inoculated leaves. CS leaves were inoculated with Sn79+SnToxA1 and collected at each day post inoculation for DAB staining followed by clearing of chlorophyll. Photograph was taken before and after DAB staining and clearing. 2 DPI and 7 DPI are shown. C. Location of H$_2$O$_2$ production in plant cells. The DAB-stained CS leaves were examined under the light microscope for the cellular location of DAB staining. The strong DAB staining was localized to the chloroplasts (white arrows) only in CS infiltrated with SnTox1 (400x magnification).

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the leaf, no browning was observed indicating that, in the absence of DAB, the SnTox1 reaction itself was not able to cause brown staining on the leaf (Figure 9A).

The production of H$_2$O$_2$ was also detected during the fungal infection. The CS leaves inoculated with Sn79+SnTox1A1 were collected daily from 1 to 7 days post inoculation and stained with DAB followed by the same procedure for leaf clearing. The accumulation of brown staining on the leaf was readily visible from 2 DPI (Figure 9B). The generation of reactive oxygen species (ROS) associated with a hypersensitive response in planta often occurs in the chloroplast [49]. Using DAB stained CS leaves from the SnTox1 infiltration, we observed that chloroplasts had the highest intensity of brown color (Figure 9C).

SnTox1 triggers stronger up-regulation of PR-genes

Up-regulation of plant defense or signaling pathway genes including pathogenesis-related (PR) genes are hallmarks of a resistance response. Using RT-PCR, we examined the transcription level of 28 wheat genes (Table S3) in CS (Sn79) and CS ems237 (snn1) leaves that were collected at different time points from 1 h to 72 h after being infiltrated with SnTox1 culture filtrates as well as control culture filtrates. Three genes including PR-1-A1, a thaumatin-like protein gene, and a chitinase were found to be significantly up-regulated in CS leaves infiltrated with SnTox1 compared to the control leaf samples infiltrated with culture filtrates deficient in SnTox1 (Figure 10A). In the CS ems237 line which has a mutated snn1 gene, a transcript was undetectable for the PR-1-A1 gene and was at a significantly lower level for the thaumatin and chitinase genes as detected by RT-PCR (Figure 10A). Quantitative PCR (qPCR) analysis confirmed the higher expression of the three genes in SnTox1 infiltrated CS leaves compared to the control infiltrated CS leaves. Not only did all three genes show maximum expression at 36 HPI, but each had at least two-fold higher expression in SnTox1-infiltrated samples than the control (Figure 10B). qPCR also showed much lower expression of the three genes in the CS ems237 line infiltrated with either SnTox1 or the control yeast culture filtrates in comparison to CS infiltrated with control culture filtrates (Figure 10B). The reason for this is not clear, but it could be explained by the idea that Snn1 may play a role in sensing other environmental stimuli that can trigger PR gene expression.

The SnTox1-Snn1 interaction triggers DNA laddering

Programmed cell death (PCD) triggered by biotrophic effectors is often evidenced by DNA laddering in plants [16,50]. To determine if the necrosis induced by SnTox1 on Snn1 lines was a result of PCD, we isolated DNA from infiltrated CS leaf samples and checked for evidence of DNA laddering. For negative comparisons where no necrosis developed, DNA fragmentation was also examined in CS leaves infiltrated with control culture filtrates (no SnTox1) and CS ems237 (mutated snn1) infiltrated with SnTox1 or control culture filtrates. In the CS leaf samples infiltrated with SnTox1, DNA laddering was detected as early as 10 h after infiltration and was most evident at 36 h after infiltration (Figure 11); however, in the leaf samples from the other three treatments, no DNA laddering was observed at any time point (Figure 11), indicating that SnTox1-induced necrosis on lines harboring Snn1 is a result of host-controlled PCD.

Light is required for the development of necrosis and disease induced by SnTox1

Light has been found to be important in the development of necrosis induced by necrotrophic effectors from *P. triticici-repentis* and *S. nodorum* [12,37]. Therefore, we investigated whether the development of necrosis induced by SnTox1 as well as the disease development caused by the SnTox1-Snn1 interaction was light dependent. After infiltration with SnTox1 yeast culture filtrates, CS plants were incubated in a growth chamber but covered for 2 days. The plants in the dark did not exhibit a necrotic reaction in the infiltrated area on the leaves, while the plants grown in the same growth chamber without covering showed necrosis (Figure 12) indicating the development of necrosis induced by SnTox1 is light dependent. Interestingly, necrosis did develop on the dark treated plants once they were treated with a 12 h light-dark cycle for 2 additional days.

A very similar situation was observed in the inoculation experiment. CS leaves showed no disease symptoms at 3 days post inoculation when plants were kept in the dark and similar to the infiltration experiment, the lesions developed once the dark-treated plants were moved to the light again (Figure 12).

SnTox1 has an important function during the penetration period

To investigate the role of SnTox1 in disease development, we tagged both the avirulent isolate Sn79-1087 and the pathogenic strain Sn79+SnTox1A1 with GFP and compared their infection processes by fluorescence microscopy in wheat lines CS (Sn79) and the Snn1 mutant, CS ems237 (snn1). The inoculation of CS with the SnTox1-producing strain Sn79+SnTox1A1 resulted in an infection (susceptible interaction); however, the other three combinations (CS inoculated with Sn79-1087, CS ems237 inoculated with Sn79-1087, and CS ems237 inoculated with Sn79+SnTox1A1) gave no disease (resistant interaction) (Figure 13).

Within 24 HPI, there was little difference observed between resistant and susceptible interactions. During this period, conidia germinated, grew short hyphae and began the penetration process. The pathogen was able to initiate penetration in both types of reactions visualized by the formation of the indistinct penetration structure called a hyphopodia [26]; Figure 13 A, B) and by autofluorescence of the damaged epidermal cell walls (Figure 13 A, B). We observed mainly direct penetration of the leaf surface over both periclinal and anticlinal epidermal cell walls.

A strong green autofluorescence was observed beneath the epidermis by 2 DPI in the susceptible interaction, suggesting that the pathogen had successfully penetrated through the epidermal cell layer and started the infection of mesophyll cells (Figure 13C). However, in the resistant interaction, the pathogen grew extensively on the leaf surface and no green autofluorescence was visible (Figure 13D). At 4 DPI, the infection area had enlarged in the susceptible interaction as shown by more mesophyll cells producing a fluorescent signal (Figure 13E). On the leaves of the resistant interactions, most of the fungal mycelium was dead, likely due to scarcity of nutrients, and only a few hyphae continued to grow over the leaf surface with repeated unsuccessful attempts to penetrate (Figure 13 F). The susceptible interaction had induced widespread lesion formation on the leaves by 7 DPI, however, no symptoms were found on the leaves of the resistant interaction (Figure 13 G, H). Examination under the fluorescent microscope of the necrotic lesion formed from the susceptible reaction revealed the extensive growth of fungal mycelium within the lesion (Figure S6).

Light is required for penetration during infection

The fungal infection process was also compared microscopically on Snn1-containing plants that were either grown under a normal light/dark cycle or in complete darkness after inoculation. The pathogen was able to germinate and generate hyphopodia within
Figure 10. SnTox1-Snn1 interaction induces increased defense gene expression. A. Expression of three pathogenesis-related (PR) genes using RT-PCR. CS (Snn1) and CS-ems237-1 (snn1) leaf samples were collected at 1, 2, 4, 8, 12, 24, 36, 48, 60, and 72 h after infiltration with SnTox1 or control yeast culture filtrates. RT-PCR was conducted to compare the expression of three PR-genes (PR-1-A1, chitinase, and thaumatin) among four
24 HPI in both conditions (data not shown). However, at 48 HPI, only the plants grown in a normal light/dark cycle showed successful penetration through the epidermal cell layer and the initiation of the infection of mesophyll cells, evidenced by the autofluorescence of the mesophyll cells (Figure 14A). In the plants that were kept in the dark, no autofluorescence was observed in the mesophyll cells and the pathogen still remained on the leaf surface without having successfully penetrated the epidermis (Figure 14B).

Discussion

Molecular cloning of SnTox1

The necrotrophic fungal pathogen S. nodorum produces multiple necrotrophic effectors (host-selective toxins) that function as virulence factors during the infection process. The cloning of these necrotrophic effector genes is an essential step in the characterization and elucidation of the molecular and biochemical mechanism of fungal pathogenesis in the wheat-S. nodorum pathosystem. Besides the traditional biochemical and genetic tools, new genomic strategies have been recently applied for the identification and cloning of effector genes in a number of fungi and oomycetes as more genome and other sequence data becomes available. A typical procedure would include a process of data mining to identify candidate genes that meet a set of specific criteria followed by gene validation through functional analysis. High throughput functional genomics [1] as well as comparative genomics and association genetics [41] have been successfully used for the identification of pathogen effector genes in fungi and oomycetes. In the current study, we used a set of criteria to mine the S. nodorum genome sequence dataset for the identification of necrotrophic effector genes. This strategy led to the successful identification of SnTox1 from S. nodorum. Through heterologous expression, gene transformation, and gene disruption, we have provided convincing evidence that the candidate gene SNOG20078 (Gene ID: 5974395) is the SnTox1-encoding gene. This research further highlights the value of genome sequence data along with efficient bioinformatics tools in identifying effector genes. We are continuing to use this strategy to identify additional S. nodorum necrotrophic effector genes.

The genomic location of SnTox1

SnTox1 was identified using a set of criteria based on the cloned S. nodorum effector genes SnToxA and SnTox3; however, the SnTox1 gene does have some unique features. Unlike many previously identified effector genes including those from Leptosphaeria maculans
cause the frequency of SnTox1 to decrease if there is a fitness cost associated with producing the effector. But the large effective population sizes of S. nodorum [65] make the complete loss of the effector through genetic drift unlikely.

Observed diversity at the SnTox1 locus was found to fit a model of diversifying selection significantly better than a neutral model. Positive selection was found for 4 of the SnTox1 codons, consistent with the growing list of prokaryotic and eukaryotic effector candidates that exhibit positive selection [66]. None of the non-synonymous substitutions were found in the signal peptide, the putative chitin-binding domain, the putative Avr4-like domain or any of the cysteine codons. This suggests that the effector's functional domains were preserved, while more flexible amino acid sites were subject to diversifying selection. Possible differences in activity between different protein variants of SnTox1 are currently being tested.

SnTox1 is a small secreted and cysteine rich necrotrophic effector

Similar to SnTox1 and SnTox2, SnTox1 was shown to play a significant role in disease development. Results presented here on the SnTox1-Snn1 interaction provide further evidence that the necrotrophic wheat-S. nodorum system is largely based on specific host-effector interactions that act in ETS [14,15], which essentially has the opposite outcome of ETI that operates in many biotrophic systems [3,4].

One of the most striking features of the SnTox1 protein as a necrotrophic effector is the high cysteine residue content. This feature is often associated with fungal avirulence gene products such as the Avr and ECP effectors from Cladosporium fulvum [64], SIX (secreted in xylem) effectors from Fusarium oxysporum f.sp. lycopersici [55], and Nip1 from Rhynchosporium secalis [67]. The predicted mature SnTox1 protein has 100 amino acids, 16 of which are cysteine residues, the richest of all effectors that have been identified. The high content of cysteine residues and high stability suggest that SnTox1 may function in the plant apoplastic space which is abundant in plant defense components. We are currently investigating the subcellular location of SnTox1. Most small cysteine-rich secreted effectors from the tomato fungal pathogen C. fulvum such as Avr2, Avr4, Avr9, and ECP2 are associated with the growing list of prokaryotic and eukaryotic effector candidates that exhibit positive selection [66].

The population genetics of SnTox1

Using a worldwide collection of 777 S. nodorum isolates, SnTox1 was found to be present in ~83% of isolates, a markedly higher frequency than found for SnToxA (~36%) and SnTox3 (~60%) [14,63]. Like the other NEs, SnTox1 was shown to have a presence/absence polymorphism within individual wheat fields. This type of polymorphism has been reported in other fungal pathosystems, as reviewed in Stergiopoulos and de Wit [64]. The frequency of SnTox1 varied significantly across regional populations. We hypothesize that regional differences in the frequency of SnTox1 reflect regional differences in the frequency of Snn1. However this correlation was not apparent when tested on a small worldwide collection of wheat. We found that Snn1 is most prevalent in durum wheat lines and much more rare among hexaploid bread wheat lines throughout the world (data not shown). This could indicate that the maintenance of Snn1 in durum wheat is associated with another important trait. Widespread deployment of wheat cultivars lacking Snn1 could cause the frequency of SnTox1 to decrease if there is a fitness cost

Figure 12. The SnTox1-Snn1 interaction is light dependent. The light dependency of the SnTox1-Snn1 interaction was tested in both infiltration (left panel) and inoculation (right panel) experiments. Chinese Spring plants (Snn1) were grown under a normal light-dark cycle (12 h photoperiod) or under complete dark conditions for 48 h after being infiltrated with SnTox1 culture filtrates or inoculated with conidia from Sn79+SnTox1A1. No necrosis or lesions developed in the plants under dark treatment in either experiment, however, once the dark treated plants were moved back to a normal light cycle, necrosis and lesions developed subsequently on the leaves after an additional 48 h.

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Figure 13. Comparison of the fungal infection process with or without an SnTox1-Snn1 interaction. The fungal infection process was examined and compared at 1, 2, 4 and 7 days post inoculation (DPI) between the susceptible interaction (A, C, E, G) and resistant interaction (B, D, F and H) using GFP-tagged fungal strains and confocal fluorescent microscopy. A, B, Infection at 1 DPI. The fungus was able to form penetration structures (hyphopodium, white arrow) in both interactions. C, D, Infection at 2 DPI. The fungus breached the epidermal layer and started infecting the mesophyll layer in the susceptible interaction shown by the autofluorescence (C). No autofluorescence was observed in the resistant interaction (D). E, F, Infection at 4 DPI. The SnTox1+ strain continued infecting mesophyll cells shown by the larger area having autofluorescence in the susceptible interaction (E), but the SnTox1- strain remained on the leaf surface and continued attempting to penetrate in the resistant interaction (F). G, H, Infection at 7 DPI. The disease lesions have fully developed in the susceptible interaction (G), but no disease symptom was observed in the resistant interaction (H). Scale bar = 20 µm.

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interaction with investigation for an added function for SnTox1 in addition to its activity and functional roles of the putative CB domain in SnTox1. However, further tests are needed to determine the binding in the positions of six of the cysteine residues at the C-terminus.

The SnTox1–Snn1 interaction enables direct penetration

Successful penetration is a prerequisite for a pathogen to function exclusively in the apoplast to inhibit and protect against plant hydrolytic enzymes [64]. ECP6, another C. falcum effector containing LysM chitin binding domains was recently found functioning apoplastically as a scavenger of fungal chitin to prevent it from eliciting PAMP-triggered immunity in planta [68]. Interestingly, we observed that SnTox1 has some similarity to C. falcum Avr4 within the chitin-binding domain and in the positions of six of the cysteine residues at the C-terminus. However, further tests are needed to determine the binding activity and functional roles of the putative CB domain in SnTox1. The presence of a potential chitin binding domain provides a point of investigation for an added function for SnTox1 in addition to its interaction with Snn1.

SnTox1 induces a light dependent reaction

It is well known that plant defenses against pests and pathogens are commonly influenced by environmental conditions, including light. Many studies have demonstrated the requirement of illumination for the interaction of plants with a diversity of bacterial and fungal pathogens as well as the isolated pathogenic elicitors [72,73]. The effect of light on the disease development of SNB was first noticed by Baker and Smith [69] who observed that the necrotic reaction and lesion coalescence tended to be suppressed in the absence of light. The necrotrophic effector ToxA, was also shown to induce light-dependent necrosis on Tsn1 lines [37]. Among the S. nodorum necrotrophic effectors published to date, all effectors except SnTox3 have been shown to be light dependent [13,32]. Using heterologously expressed SnTox1 and the avirulent isolate carrying the SnTox1 gene, we showed clearly that the necrosis and disease susceptibility induced by SnTox1 on Ssn1 lines were completely dependent on light. The requirement of light for resistance to biotrophic disease as well as susceptibility to necrotrophic disease suggests a common host mechanism shared by reactions to the two classes of disease interactions.

The molecular mechanism underlying the light dependency of plant pathogen interactions is still poorly understood; however,
SnTox1 functions like a biotrophic effector to induce PCD in Snm1 host lines

Biotic effectors often function as elicitors of programmed cell death (PCD) thereby activating the resistance response in host plants containing the corresponding resistance genes. The host resistance reaction begins with the direct or indirect recognition of the pathogen-produced effector by the resistance gene product, followed by a complicated signaling pathway and a series of biochemical and physiological responses in host plant cells [74]. The host response often includes an oxidative burst, cell wall restructuring, PR-gene expression and antimicrobial compound production culminating in a localized cell death at the point of infection. This PCD is known as a hypersensitive response and is typically aimed at halting further colonization by the pathogen [5]. A set of biochemical tests has shown that SnTox1 is able to induce resistance-like host responses and PCD evidenced by the H2O2 production, stronger expression of PR-genes, and DNA laddering in lines carrying Snm1. It is important to note that SnTox1 physiologically evoked a widespread necrotic flecking on the Snm1 line, which is symptomatically similar to the hypersensitive response in biotrophic disease systems. However, this necrosis spreads into larger lesions resulting in susceptibility (sporulation) rather than resistance (prevention or inhibition of sporulation). Together, this indicates that SnTox1 is likely functioning biochemically and physiologically similar to a biotrophic effector (avirulence factor) in the presence of Snm1 but with a different end result.

A number of other necrotrophic effectors have also been shown to invoke a host resistance response [9,17,40]. It has generally been thought that necrotrophic plant pathogenic fungi possess simplistic infection mechanisms that rely on lytic and degrading enzymes [11]. In contrast, biotrophic fungal pathogen interactions have been considered more sophisticated due to the formation of special penetration and feeding structures, secretion of effectors to overcome plant PAMP triggered immunity and a constantly changing effector complement to avoid recognition by the plant innate immune system. However, three genes conferring sensitivity to necrotrophic effectors as well as susceptibility to the corresponding necrotrophic fungal pathogens have been cloned, and all possess resistance gene-like features [15,22,23]. Therefore, it seems that necrotrophic fungal pathogens may subvert plant resistance mechanisms for their own good. Here, we clearly showed that SnTox1 is an important virulence factor for S. nodorum in the presence of Snm1 and that the host response to SnTox1 shows several similarities to a classical resistance response induced by many biotrophic effectors, however, the outcome of the host recognition was susceptibility rather than resistance.

SnTox1 is the third effector gene that we have cloned and characterized from S. nodorum, which further strengthens the hypothesis that the wheat-S. nodorum pathosystem is based largely on host-effector interactions. The three effector genes cloned have provided molecular tools to study the mechanisms underlying disease in this system, an emerging model for necrotrophic fungal diseases.

Materials and Methods

Bioinformatics for prioritizing candidate genes

A series of experimental and bioinformatic criteria associated with effectors were evaluated to produce a candidate gene ranking of the predicted genes in the S. nodorum genome. These criteria were based on the known and predicted properties of effectors. Genes matching different criteria were given scores from 1 to 6. The sum of scores for each gene was ranked and the top 100 genes were considered. The criteria used data from mass-spectrometry analyses of culture filtrates, a genome sequence scan of the strains Sn4 and Sn79-1087, an in planta microarray experiment and various bioinformatics analyses. The criteria were as follows: predicted to be less than 30 kDa (1 point), cysteine rich (>1 standard deviation more cys residues than expected of a protein of that size) (2 points), detected by MS in culture filtrates (6 points), located within 5 kb of repetitive sequences (2 points), absence of homologues in the NCBI nr database (2 points), presence of RXLR or RGD motifs (2 points), predicted to be secreted (3 points), presence of a modified version of the gene in Sn4 (3 points), absence of the gene in Sn79-1087 (4 points), and a gene expression profile similar to Tox1 and Tox3 (3 points).

Yeast expression of SNOG_20078 for verification that it was SnTox1

The total RNA of 7 day old mycelium of Sn15 grown in Fries media [27] was prepared using the RNeasy plant mini kit (Qiagen) and treated with RNase-free DNase I (Promega). First-strand cDNA was synthesized from 2 μg of total RNA using TaqMan Reverse Transcription Reagents (Applied Biosystems). The coding region of SNOG_20078 was amplified from the above cDNA sample using primers 20078CF_EcoRI and 20078CR_Apal containing the indicated restriction site (Table S4). The cloning of SNOG_20078 into the corresponding sequencing and expression vectors, yeast transformation, and preparation of culture filtrates from yeast cultures all followed the procedure described by Liu et al. [14]. The pGAPZ A vector containing the SNOG_20078 gene was linearized with AscI before transformation. Culture filtrates of the yeast culture transformed with the SNOG_20078 coding region were infiltrated into wheat lines including BR34 (snm1), Grandin (snm1), BG220 (snm1), BG223 (snm1), BG261 (snm1), W-7984 (snm1), Chinese Spring (Snn1), Opataaa5 (snm1), and ND495 (snm1). Because the culture filtrates caused necrosis on W-7984 and CS, which both possess Snm1 [27], it was infiltrated onto CS 1BS-18, CS ems237, and the ITMI population [27] for verification of SnTox1 based on its interaction with Snm1. CS 1BS-18 carries a deletion in
the distal end of chromosome 1B that harbors the Sn1 locus [27]. CS ems237 is an SnTox1 insensitive mutant derived from CS by EMS (ethane methyl sulfonate) mutagenesis (Faris et al., unpublished data).

Generation of an SnTox1 antibody and western blot analysis of SnTox1

A 14 amino acid long peptide (sequence: CKNGKQA-AHEAQPQ), designated SnTox1:50–63, was synthesized by GenScript (Piscataway, NJ). The peptide SnTox1:50–63 (4.7 mg, 0.003 mM) was first conjugated to bovine serum albumin (BSA, 20 mg, 0.0003 mM), Sigma-Aldrich, St Louis, MO) in the presence of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC, 20 mg, Pierce Biotechnology, Rockford, IL) in 2 mL of 100 mM 2-(N-morpholino)ethanesulfonic acid buffer, pH 6 overnight at 4°C. The protein was separated from EDC through a size-based column (D-Salt Excellulose, Thermo Scientific, Rockford, IL) and concentrations were determined by the method of Bradford (Bio-Rad Laboratories, Inc. Hercules, CA) using BSA as the calibration standard. Success of the conjugation reaction was assessed on a 13% SDS-PAGE gel. One hundred milligrams of the immunogen were immunized into New Zealand White Rabbits at 3 week intervals for a total of six immunization cycles. The final sera were collected eight days after immunization and were used for western blot analysis.

To prepare the SnTox1 protein sample for western blot analysis, 5 mL of culture filtrate from an SnTox1 yeast culture and control yeast culture (yeast strain transformed with an empty vector) was precipitated from 20 mL of methanol and incubating in a -20 freezer overnight. After centrifuging for 10 min at 13,000 rpm on a HERMLE Z 523K centrifuge with a 220,80 V20 rotor (Labnet), the pellet was retained, air dried and resuspended in 500 μL of a 1X sample loading buffer. Protein gels were loaded with 50 μL of the resulting sample solution. SDS-PAGE, protein transferring, and color development followed a routine protocol described in Meinhardt et al. [36]. To ensure the quality of protein sample preps, the same amount of sample solution was also run on a gel and visualized by coomassie blue staining.

Amplification of full-length transcript of SnTox1

The same RNA extracted from SN15 was used to amplify the 5’ and 3’ ends of the cDNA of SnTox1. The 5’ and 3’ RACE were performed using the Smart RACE cDNA amplification kit (Stratagene, LaJolla, CA) according to the instructions in the user manual with gene-specific primers 20078CF and 20078CR (Table S1). The procedure described by Liu et al. [14] was followed for the cloning and sequencing of the amplified 5’ and 3’ RACE fragments. The obtained sequences from 5’ and 3’ RACE fragments were used to assemble the full length cDNA and determine the 5’ and 3’ UTRs based on the SN15 genome sequence.

Searches for SnTox1 homologs and protein alignments

SnTox1 and Avr4 homologs were identified from the NCBI non-redundant (nr) protein database [http://www.ncbi.nlm.nih.gov/BLAST/] using BLAST searches. The chitin-binding domains of Avr4 and its homologues were identified using Reverse Position-Specific (RPS)-BLAST searches (www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Amino acid alignments were performed using the MegAlign programs from Lasergene 8.1 software (DNASTAR Inc. Madison, WI). Three-dimensional (3D) structure-based sequence alignment of the putative chitin-binding motifs identified in SnTox1 with those of ChbBD1 and ChbBD2 proteins were performed following the previously published data on related structures [43,44,75].

Analyses of SnTox1 diversity

SnTox1 presence and absence was screened in 777 S. nodorum isolates from seven geographical regions: Australia, Central Asia, East Asia, Europe, Middle East, North America, South America and South Africa (Table S1) using PCR with primer pair Tox1F_Coding and Tox1R_Coding (Table S2). A secondary PCR screen using the conserved primer pair Tox1_XF and Tox1_XR (Table S4) was conducted to confirm questionable PCR amplifications. PCR amplification was performed in 20 μl reactions containing 0.05 μM of each primer (supplied by Microsynth), 1X Dream Taq Buffer (Fermentas), 0.4 μM dNTPs (Fermentas) and 0.5 units of Dream Taq™ DNA polymerase (Fermentas). The PCR cycle parameters were: 2 min initial denaturation at 96°C followed by 35 cycles of 96°C for 30 s, 58°C for 45 s and 72°C for 1 min. A final 5 min extension was made at 72°C. To demonstrate the wide distribution of SnTox1, a subset of the global collection (79 isolates), along with 10 avirulent isolates and several related fungal species including Pti2 (P. tritici-repentis), NDI98-19 (P. teres f. teres), Sm15A (P. bromii) and S. tr. 9715 (M. graminicola) (Table S2) were evaluated in a dot blot analysis. For dot blot analysis, the DNA of fungal samples was isolated using a BioSprint 15 instrument (QIAGEN) with the corresponding kit (QIAGEN). The DNA samples were blotted onto a nylon membrane using a Bio-Dot microfiltration apparatus (BIO-RAD) following the instructions in the user manual. The entire SnTox1 coding region was PCR amplified from the genomic DNA of SN15 and used as a probe for Southern blot analysis. Probe preparation, DNA hybridization, membrane washing and image acquisition followed the protocol described by Faris et al. [76]. The membrane was stripped and hybridized to the S. nodorum actin gene probe to ensure the quality for all the DNA samples.

Sequences for the entire coding region were obtained using the primer pair Tox1UTR_F and Tox1UTR_R and the primer pair Tox1Fout and Tox1Rout (Table S4). In cases of poor amplification primer Tox1F_Coding and a new conserved reverse primer, Tox1R_Conserved (Table S4), were used to confirm observed sequence variation. Sequencing reactions were conducted in 10 μl volume using the BigDye Terminator v3.1 Sequencing Standard Kit (Applied Biosystems) with both the forward and the reverse primer. The cycling parameters were 96°C for 2 min followed by 55 or 99 cycles of 96°C for 10 s, 50°C for 5 s and 60°C for 4 min. The products were cleaned with the illustra Sephadex G-50 fine DNA Grade column (GE healthcare) according to the manufacturer’s recommendations and then sequenced with a 3730x/Genetic Analyzer (Applied Biosystems). Alignment of forward and reverse sequences for each isolate was performed in SeqScape software V2.5 (Applied Biosystems, Foster City, CA). Translation and identification of protein haplotypes was also performed using this software.

Tests for positive selection in SnTox1

Codeml implemented in the software PAML [http://abacus. gene.ucl.ac.uk/software/paml.html] was used to test for positive diversifying selection [77]. The program uses four different codon substitution models implemented in a maximum-likelihood framework to test which model, neutral or selection, best fits the data. Each model assumes a different range of values for the estimated value ω (the ratio of non-synonymous to synonymous nucleotide substitutions). Under purifying selection, non-synonymous substitutions are expected to be rare, thus ω will remain low. Under positive selection, non-synonymous substitutions are expected to be frequent, thus ω will be greater than 1. Under neutral evolution, the value of ω is 1. The models implemented in the current analysis are the general time reversible model (GTR) and the Kimura 2 parameter model (K2P) and both models are used with a proportion of invariable sites (I) and a gamma distribution of substitution rates (Γ).
below 1. If non-synonymous mutations offer a selective advantage, they will be fixed at a higher rate than synonymous mutations and $\omega$ will be greater than one. We compared the null model M1a (neutral), which assumes two site classes, purifying ($0<\omega_0<1$) or neutral ($\omega_0 = 1$) to the alternative model M2a (selection), which adds another class of diversifying sites ($\omega_2>1$). We also compared the more complex null model M7 (neutral) that assumes a beta distribution and adds an additional site resistance gene) cassette. The resulting plasmid, designated pDAN-

Investigation of the genomic region of SnTox1 in different S. nodorum isolates

Based on the annotated SN15 genome sequence, four genes SNOG_07153-SNOG_07156 were predicted within a ~7.6 kb region containing SnTox1. Primers were designed (Table S4) to amplify the gene region from start to stop codon for the four genes identified in the avirulent isolate Sn79-1087. Since all the genes except SNOG_07154 were present in Sn79-1087, one new primer designed in SNOG_07153 (20078g3R, Table S4) was used with the SNOG07155 forward primer to amplify the whole region in several virulent isolates as well as Sn79-1087. The amplified fragments with different sizes were cloned into the pCR-4 TOPO cloning vector (Invitrogen) for sequencing. The sequences from different isolates were analyzed manually to identify the variations in the SnTox1 genomic region with the aid of the genome sequence (http://genome.jgi-psf.org/Stano1/Stano1.home.html).

SnTox1 gene transformation into the avirulent isolate Sn79-1087

A 1.1 kb sequence of the SnTox1 genomic region including a putative promoter and terminator was amplified from the Sn2000 isolate using primers 20078gF_XbaI and 20078gR_XbaI, each containing an XbaI restriction site sequence (Table S4). The amplified fragment was cloned into the pCR-4 TOPO vector (Invitrogen) for sequencing to verify the identity and XbaI restriction sites. The SnTox1 gene fragment was then released from pCR-4 TOPO plasmid and cloned into the pDAN vector that carries the HY phosphotransferase cassette (HY-G). The cloned fragment was then released from the pDAN vector using a split marker and 9 flanking region with the HY fragment and the other to fuse and amplify the SnTox1’ flanking region with the HY fragment by adding the corresponding first round templates and primers. At least 100 µl of PCR reaction was set up for each reaction in the second round. Standard PCR conditions and Taq polymerase (NEB BioLabs) were used for both rounds of amplification except that round 2 used a longer extension time due to the longer template. A small amount of product from the second round of PCR was evaluated on a 1.0% agarose gel to ensure a successful fusion and amplification for each fragment. The remaining product was combined and concentrated by routine ethanol precipitation [79]. The pellet was finally resuspended in 20 µl of TE (10 mM Tris and 1 mM EDTA) for transformation of Sn2000 protoplasts.

Fungal protoplasting and transformation followed the procedure described by Liu et al. [14]. The regenerated clones were screened using the PCR primers 20078KOF and 20078KOR (Table S4) which amplifies the partial coding region of SnTox1 that was replaced by the hygromycin-resistance gene cassette. The ectopic transformant and two knock out transformants were verified by Southern blot analysis using the SnTox1 coding region as a probe. Spores of the knock out and ectopic strains as well as wild type Sn2000 were inoculated onto wheat lines W-7984, and CS for testing the effect of the SnTox1 knock out.

QTL analysis of SnTox1 knock out strains

The International Triticeae Mapping Initiative (ITMI) mapping population was originally used to map the Snml1 gene, which confers sensitivity to SnTox1, and quantitative trait loci conferring resistance/susceptibility to Sn2000 [27,45]. The same 106 recombinant inbred (RI) lines of this population were used to evaluate the genetically modified fungal strains including two Sn2000 SnTox1 knock out transformants (Sn2000SnTox1−9 and Sn2000SnTox1−15), one Sn2000 ectopic transformant (Sn2000SnTox1-ECT), and the wild type Sn2000 as a control. All strains were evaluated with three biological replications by inoculating their conidia onto the ITMI population as previously described [45]. The disease rating was conducted 7 days post-inoculation using a 0–5 rating scale as described by Liu et al. [45]. Composite interval mapping with the average of three disease readings was performed as previously described [30].

Disulfide bond prediction

The web-based program DISULFIND (http://disulfind.dsi.unifi.it/) was used to predict if a particular cysteine residue was involved in the formation of a disulfide bond (DB_state) as well as the confidence level of the prediction. The site of each cysteine residue was predicted as either involved (1) or not involved (0) in a DB. The scale of confidence of disulfide bonding state prediction ranges from 0 (low) to 9 (high) [46]. The web-based program DISULFIND 1.1 (http://clavius.bc.edu/~clotelab/DiANNA/) [47] was used to determine the best connectivity prediction of cysteine residues in SnTox1.
qPCR analysis of SnTox1 transcription during infection

The secondary leaves of CS (≈2 week old plants) were inoculated with a fungal strain modified from the avirulent isolate Sn79-1087 by addition of the SnTox1 gene. The leaf tissues were collected from the inoculated leaves at 1 h, 3 h, 6 h, 12 h, 24 h, 2 d (day), 3 d, 4 d, 5 d, 6 d, and 7 d post inoculation. The RNA was extracted from leaf samples using the RNase-free DNase I (Promega). RNA sample quantification, cDNA synthesis, and gene transcript abundance analysis were performed as previously described [15]. The gene specific primers SnTox1qPCRF and SnTox1qPCRR (Table S4) designed within the two exons were used for the SnTox1 gene in qPCR. The previously reported primers ActinF and ActinR [14] were used for the S. nodorum actin gene as internal control.

Dithiothreitol (DTT) and heat treatment of the SnTox1 protein

Because all cysteine residues were predicted to form disulfide bonds, the protein stability of SnTox1 was investigated by DTT and heat treatment. For DTT treatment, the SnTox1 P. pastoris culture filtrate were treated with DTT (Fisher Scientific, Pittsburgh, PA) at final concentrations of 0, 20, or 40 mM and incubated at room temperature for 2 h or 4 h. For heat treatment, the P. pastoris culture filtrate was scaled in a 2 ml centrifuge tube and heated for 30 min or 1 h on a hot plate setting at 100°C. All treated culture filtrates were then infiltrated into CS leaves.

DAB staining of hydrogen peroxide

The fully expanded secondary leaves of CS and CS ems237 were infiltrated with the culture filtrates from yeast transformed with SnTox1 or culture filtrates from yeast transformed with an empty vector (as control). At 24, 48 and 72 hours post infiltration, leaf samples were collected and leaf segments with an infiltrated area were cut and stained in a freshly made 1 mg/ml 3′-3′ diaminobenzidine (DAB) (Sigma) solution. The preparation of a DAB staining solution and the staining process followed a procedure described by Thordal-Christensen et al. [48]. The stained leaf tissue was cleared for chlorophyll by placing them on a paper pre-soaked with ethanol/acetic acid solution (3:1, V/V) in a component (Carl Zeiss Light Microscopy, Germany) equipped with a GFP filter and UV light (Nikon, Japan).

The two GFP-tagged fungal strains were inoculated onto both genotypes of Sn1 (CS) and sn1l (CS ems237) as described in Liu et al. [45]. The inoculated leaves were collected at 1 h, 3 h, 6 h, 12 h, 24 h, 2 d, 4 d, and 7 d post inoculation. The leaves were cut into 5 cm long segments and directly mounted onto glass slides. The specimens were examined immediately using a Zeiss Axioplan 2 Imaging Research Microscope with ApoTome confocal component (Carl Zeiss Light Microscopy, Germany) equipped with filter blocks with spectral properties matching those of GFP.

DNA laddering analysis

The fully expanded secondary leaves of CS and CS ems237 were infiltrated with SnTox1 yeast culture filtrates and control culture filtrates. Leaf samples were taken at 1, 2, 4, 8, 10, 24, 36, 48, 60, and 72 hour post infiltration. The DNA was extracted from the collected leaf samples using the CTAB method [17]. The 5 μl of DNA from each sample were separated on a 2% agarose gel. The gel was stained in ethidium bromide solution for 1 hour, destained in water for 1 h and photographed using a Gel LOGIC 100 image system (Kodak).

Wheat defense gene expression using RT-PCR and qPCR

The fully expanded secondary leaves of CS and CS ems237 were infiltrated with SnTox1 yeast culture filtrates and control culture filtrates. Five centimeter segments of infiltrated leaf tissue was collected at 1, 2, 4, 8, 10, 24, 36, 48, 60, and 72 hour post infiltration. Three leaves from different plants were collected as three replications for each time point. Total RNA was extracted from all leaf samples using the RNeasy plant kit (QIAGEN) and treated with RNase-free DNaseI (Promega). The RNA quantification and first strand cDNA synthesis were conducted as previously described [15]. Using the cDNA samples, we examined the expression of a total of 28 wheat genes that have been reported or predicted to be involved in the defense response [Liu et al. unpublished, 21] (Table S5). The RT-PCR and agarose gel electrophoresis were performed using a standard procedure. The same cDNA samples from the three replications were used to conduct the qPCR analysis for three genes: PR-1-A1, chitinase (PR-3), and thaumatin-like protein (PR-5) following the description by Faris et al. [15].

Generation of GFP tagged strains and microscopy

The gGFP vector [91] was used to transform the green fluorescence protein gene into two fungal strains that were only different in the production of SnTox1. One was the avirulent Sn79-1087 that did not produce SnTox1 nor did it cause disease, and the other was an Sn79-1087 SnTox1 transformant (Sn79+SnTox1A1) that expressed SnTox1 and caused disease on Sn1 lines. Since Sn79+SnTox1A1 already carried the hygromycin resistance resulting from the SnTox1 transformation, the plasmid pH99 [82] containing genetic resistance, was used with gGFP for co-transformation of this fungus. The plasmid DNA preparation, fungal protoplasting, and fungal transformation followed the same methods described above. For all transformations, at least 20 μg of each plasmid DNA linearized with the corresponding restriction enzyme (gGFP with BglII and pH99 with EcoRV) was used. The transformants with the strongest GFP signal were selected for both strains under the Nikon Eclipse TE-2000U microscope equipped with a GFP filter and UV light (Nikon, Japan).

The two GFP-tagged fungal strains were inoculated onto both genotypes of Sn1 (CS) and sn1l (CS ems237) as described in Liu et al. [45]. The inoculated leaves were collected at 1 h, 3 h, 6 h, 12 h, 24 h, 2 d, 4 d, and 7 d post inoculation. The leaves were cut into 5 cm long segments and directly mounted onto glass slides. The specimens were examined immediately using a Zeiss Axioplan 2 Imaging Research Microscope with ApoTome confocal component (Carl Zeiss Light Microscopy, Germany) equipped with filter blocks with spectral properties matching those of GFP.

GenBank accession/ID numbers

The S. nodorum gene SnOG_20078 has been deposited in Genbank with identity numbers of 5974395 for gene ID and XP_001797505.1 for protein ID. The nucleotide sequence of 12 different haplotypes of SnTox1, designated Tox1-H1–H13, was submitted to GenBank with accession numbers from JN791682 to JN791693. The other genes and proteins referred to in this paper included Cladosporium fulvum Avr4 protein (CAA69643.1), Mycosphaerella fijiensis Avr4-like protein [Protein ID: Myc61:37167], Ceratospora beticola Avr4-like protein (GU574324), Microsorum gypseum Avr4-like protein (GeneID:10030079) and Geomyces pannorum Avr4-like protein (DY991214).

Supporting Information

Figure S1 SnTox1 nucleotide and deduced amino acid sequences. The sequence of the 1.2 Kb SnTox1 genomic region from Sn15 is shown. The putative TATA box is highlighted in red, the 5′ and 3′ UTRs are highlighted in yellow and the start and stop codons are indicated in bold. The introns are highlighted in gray. The underlined sequences are the primers that were used to amplify the genomic region (≈1.1 kb) for transformation into the avirulent isolate Sn79-1087. The translated protein sequence is shown under the DNA sequence of the coding region with the first
Amino acids highlighted in blue as the predicted signal peptide. The 16 cysteine residues are highlighted in green.

**Figure S2** Analysis of SnTox1 protein expressed in *Pichia pastoris*. A. Western blot analysis of the SnTox1 protein expressed in *Pichia pastoris*. M: protein size marker (BIO-RAD, Cat #161-0377). 1 and 2: Independently prepared protein samples from culture filtrates of *P. pastoris* transformed with SnNOG_20078; 3 and 4: Independently prepared protein samples from culture filtrates of *P. pastoris* transformed with an empty vector. B. SDS-PAGE with Coomassie blue 250 staining to visualize the total protein in each sample. The same amount of each protein sample shown in panel A were loaded onto the gel.

**Figure S3** Amino acid sequence alignment of SnTox1 with its homologs obtained from BlastP searches. The partial protein sequence of SnTox1 was aligned with three homologs, SNOG06478 from *Stagonospora nodorum*, partial protein sequence of SnTox1 was aligned with three homologs obtained from BlastP searches.

**Figure S4** Molecular manipulation and characterization in *SnTox1* gene transformation and disruption. A. Map of the pDAN vector containing the *SnTox1* genomic region that was used to transform the avirulent isolate Sn79-1087. The plasmid contained the Hygromycin resistance gene (*Hys*) as a selectable marker. To facilitate cloning, the *XbaI* restriction site was incorporated into the primers that were used to amplify the *SnTox1* genomic region. The plasmid was linearized with *EcoRV* before transformation. B. Southern blot analysis of fungal transformants for *SnTox1* integration. The *SnTox1* genomic region (1.1 kb) was amplified from the virulent isolate Sn2000 (V) and transformed into the avirulent isolate Sn79-1087 (A). All transformants (No. 1–7) contained *SnTox1* insertion except No. 2 based on the Southern analysis using the *SnTox1* coding region as a probe. C. A PCR-based split marker strategy for replacement of the *SnTox1* gene in isolate Sn2000. Overlapping PCR was used to fuse the 5’ flanking region and 3’ flanking region with the HY and YG fragments which were amplified from the *Hys*. The two fused PCR fragments were used to transform Sn2000 to replace the *SnTox1* coding region through homologous recombination. The green boxed region was amplified and used as a probe in Southern analysis of the *SnTox1* disruption. D. Southern blot analysis of fungal transformants for *SnTox1* knockout out. Genomic DNA from the wild type, knock out, and ectopic fungal strain was digested with *XbaI* and blotted to a nylon membrane, which was then hybridized with a probe from the deleted region of *SnTox1* (green box in C). The wild type (lane 1) and ectopic type, *Sn2000SnTox1-ECT* (lane 4) contained a 5.0 kb fragment, but the fragment was absent in the two disrupted strains, *Sn2000SnTox1-9* and 15 (lanes 2 and 3).

**Figure S5** Gene expression patterns for *SnTox4*, *SnTox3* and *SnTox1* during infection revealed by microarray analysis. The expression level of the three effector genes, *SnTox4*, *SnTox3* and *SnTox1*, was examined and compared to that of the *Act1* gene at 3, 5, 7 and 10 days after inoculation. The x axis shows the number of days post-infection. The y axis represents relative gene expression levels normalized to *Act1*.

**Figure S6** A close-up of a necrotic lesion induced by a GFP-tagged *SnTox1* transformed fungal strain. A. Microscopic examination under normal light; B. Microscopic examination under UV light with GFP filter indicating the extensive growth of the fungus within the lesion of dead cells (200 x magnification).

**Table S1** *SnTox1* distribution and haplotypes in a global collection of *S. nodorum* isolates.

**Table S2** *S. nodorum* isolates and related fungal species used in dot blot analysis of *SnTox1* presence.

**Table S3** Defense response genes investigated in *SnTox1-Snn1* interaction.

**Table S4** Primers used in this research.

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**Author Contributions**

Conceived and designed the experiments: ZL RPO BAM TLF. Performed the experiments: ZL ZZ RS MCM. Analyzed the data: ZL ZZ JDF BAM SL. Contributed reagents/materials/analysis tools: PSS WLS SX. Wrote the paper: ZL TLF.
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