Sensitivity to three *Parastagonospora nodorum* necrotrophic effectors in current Australian wheat cultivars and the presence of further fungal effectors

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Abstract

Parastagonospora nodorum is a major fungal pathogen of wheat in Australia causing septoria nodorum blotch (SNB). *P. nodorum* virulence is quantitative and depends to a large extent on multiple effector-host sensitivity gene interactions. The pathogen utilises a series of proteinaceous necrotrophic effectors to facilitate disease development on wheat cultivars that possess appropriate dominant sensitivity loci. Thus far, three necrotrophic effector genes have been cloned. Proteins derived from these genes were used to identify wheat cultivars that confer effector sensitivity. The goal of the study was to determine if effector sensitivity could be used to enhance breeding for SNB resistance.

In this study, we have demonstrated that SnTox1 effector sensitivity is common in current commercial Western Australian wheat cultivars. Thirty-three of 46 cultivars showed evidence of sensitivity to SnTox1. Of these, 19 showed moderate or strong chlorotic/necrotic responses to SnTox1. Thirteen were completely insensitive to SnTox1. Disease susceptibility was most closely associated with SnTox3 sensitivity. In addition, we have identified biochemical evidence of a novel chlorosis-inducing protein or proteins in *P. nodorum* culture filtrates unmasked in strains that lack expression of ToxA, SnTox1 and SnTox3 activities.

Additional keywords: septoria nodorum blotch, necrotrophic effector (NE), SnTox1, ToxA, SnTox3, wheat.

Introduction

Parastagonospora (syn. ana, *Stagonospora*; teleo, *Phaeosphaeria*) *nodorum* (Berk.) Quaedvlieg, Verkley & Crous is the causal agent of septoria nodorum blotch (SNB) on wheat (Quaedvlieg *et al.* 2013; Solomon *et al.* 2006a). The fungus is responsible for yield losses estimated at AUD$108 million per year in Australia (Murray and Brennan 2009; Oliver *et al.* 2012). SNB is quantitatively governed by the interaction of fungal necrotrophic effectors (NEs) that interact with the products of host dominant sensitivity genes (Faris *et al.* 2010; Tan *et al.* 2010). A compatible interaction results in host tissue necrosis that promotes infection by the pathogen. *ToxA* was the first effector gene identified in *P. nodorum*. *ToxA* was originally identified in the tan spot fungus *Pyrenophora tritici-repentis* (*Ptr*), a close relative of *P. nodorum* (Ciuffetti *et al.* 1997; Ellwood *et al.* 2012). Genetic evidence suggests that *PtrToxA* was acquired from *P. nodorum* via horizontal gene transfer (Friesen *et al.* 2006). *ToxA* encodes a small, secreted protein that causes necrosis and promotes infection in wheat carrying the *Tsn1* gene (Tan *et al.* 2012). *Tsn1* is located on chromosome 5B and encodes a protein with distinct NBS-LRR and protein kinase domains (Faris *et al.* 2010). *SnTox3* encodes a small secreted protein (Liu *et al.* 2009). *Snn3-B1* and *Snn3-D1* are key SnTox3 sensitivity genes that mapped to the short arm of chromosome 5B and 5D, respectively (Zhang *et al.* 2011). Both effector genes are ubiquitous in Australian *P. nodorum* isolates. Their roles in virulence were thoroughly demonstrated (Friesen *et al.* 2006; Liu *et al.* 2009; McDonald *et al.* 2013; Oliver *et al.* 2012).

Traditional breeding for SNB resistance is difficult as the trait is quantitative and complex (Czembor *et al.* 2003; Friesen *et al.* 2008). Phenotyping using field-based scoring is laborious and subject to variable weather and uncertainty in the identity of isolates causing infection. Our laboratory has developed and delivered to breeders a simple high-throughput screening procedure to test for effector sensitivity via leaf infiltration of wheat seedlings.

Previously, *ToxA* and *SnTox3* were expressed using microbial expression systems (Liu *et al.* 2009; Tan *et al.* 2012) and used to screen commercial Western Australian (WA) wheat cultivars (Oliver *et al.* 2009; Waters *et al.* 2011). 61% of all tested cultivars were sensitive to ToxA and 90% were sensitive to SnTox3. Due to the prevalence of effector sensitivity, a national cereal breeding program is currently being applied to eliminate ToxA and SnTox3 sensitivity genes in wheat (Oliver and Solomon 2010).

The third characterised effector is the recently discovered *SnTox1* NE gene which encodes a small, cysteine-rich, secreted protein that has no known homologues in other organisms (Liu *et al.* 2012; Liu *et al.* 2004b). The corresponding dominant sensitivity gene *Snn1* has been mapped to the short arm of chromosome 1B (Liu *et al.* 2004a). Functional analysis indicates that *SnTox1* functions as a key virulence factor. Transformation of a non-pathogenic *P. nodorum* strain with *SnTox1* enabled the fungus to infect *Snn1* wheat cultivars. Furthermore, genetic analysis of SNB using the wheat ITMI population indicated that the chromosome region corresponding to *Snn1* accounted for 48% of the disease response (Liu *et al.* 2004b). A recent study on the global distribution of effector genes indicates that *SnTox1* is ubiquitous in Australian *P. nodorum* isolates (McDonald *et al.* 2013).

In this study, we assessed the prevalence of SnTox1 sensitivity in the current Western Australian commercial wheat cultivars using a high-throughput effector infiltration assay on seedlings. We observed that most cultivars were sensitive to SnTox1. Furthermore, we have demonstrated that the SnTox1-Snn1 interaction is important in establishing SNB in a wheat cultivar that has sensitivity to SnTox1. Hence, the elimination of *Snn1* in commercially grown wheat cultivars can potentially reduce the severity of SNB. In addition, mutagenesis studies on *P. nodorum* have revealed evidence of novel effectors that cause chlorosis on several wheat varieties.

Materials and methods

Wheat cultivars

All wheat cultivars used in this study were obtained from the Australian Winter Cereal Collection (Tamworth, Australia). Current SNB disease resistance ratings (DRR) of commercial cultivars were obtained from the Department of Agriculture and Food Western Australia (DAFWA) (Shackley *et al.* 2013). For statistical purposes, a scoring system was assigned to all applicable DRR categories; very susceptible, '1'; susceptible-very susceptible, '2'; susceptible, '3'; moderately susceptible- susceptible, '4'; moderately susceptible, '5'; and moderately resistant-moderately susceptible, '6'.

SnTox1 expression

SnTox1 (SNOG_20078; Genbank acc, XP_001797505) derived from the WA *P. nodorum* SN15 isolate was heterologously expressed in *Pichia pastoris* using pGAPzA (Liu *et al.* 2012). *SnTox1* contains an N-terminal signal peptide that facilitates secretion. Culture filtrates containing the expressed effector were harvested by centrifugation, sterilised through a 0.22 μm filter (Millipore, MA, USA) and desalted via PD-10 size exclusion chromatography (GE Biosciences, Uppsala, Sweden).

Construction of SnTox1 and SnTox3 gene deletion P. nodorum mutants

SnTox1 and *SnTox3* gene deletion constructs were constructed using a fusion PCR approach (Solomon *et al.* 2006b). Firstly, 5' and 3' *SnTox3* untranslated regions (UTRs) were PCR-amplified from genomic DNA using 5'08981KOF, 5'08981KOR, 3'08981KOF and 3'08981KOR (Figure 1; Table 1). Both flanking regions were sequentially fused to a phleomycin resistance cassette PCR-amplified from pAN8 using pAN8f and pAN8r. The *SnTox3* deletion construct was transformed into the Western Australian *P. nodorum* SN15 isolate to facilitate gene knockout (Solomon *et al.* 2004). PCR was using to identify the appropriate mutant deleted in *SnTox3* (SN::*tox3*) for further genetic manipulation. Finally, 5'

and 3' *SnTox1* UTRs were PCR-amplified from genomic DNA using 20078KO5'f, 20078KO5'r, 20078KO3'f and 20078KO3'r (Figure 1; Table 1). Both flanking regions were sequentially fused to a hygromycin resistance cassette derived from pAN7. The *SnTox1* deletion construct was transformed into SN::*tox3* to facilitate double gene knockout. PCR was using to identify the appropriate mutant carrying *SnTox1* deletion. Fungal transformation was performed as described by Solomon *et al.* (2004). Quantitative PCR was used to determine the integration copy number of both gene deletion constructs in all transformants (Solomon *et al.* 2008). Mutants that carried a single integration of each construct were selected for effector studies.

Infection assay

P. nodorum strains were examined their ability to infect using a whole plant spray as described (Solomon *et al.* 2005). Briefly, an inoculum consisting of $1x10^6$ pycnidiospores per ml on 0.02% (v/v) Tween 20 were sprayed onto 2 week old wheat seedlings via an airbrush. Diseases were allowed to develop for 7 days prior to scoring. A score of 0 being uninfected (no disease symptoms observed) and a score of 10 indicates a fully necrotised plant.

Induction of NEs in P. nodorum culture filtrate

P. nodorum culture filtrates were produced as described (Liu *et al.* 2004a). Briefly, $1x10^6$ *P. nodorum* spores were inoculated into a 250 ml conical flask containing 50 ml of Fries broth. The fungus was allowed to grow at 28^oC for 3 days in the dark on an orbital shaker set at 140 rpm. Following this, effector production was achieved by stationary growth for 4 weeks at room temperature. The culture filtrate was collected with cheesecloth filtration and sterilised through a 0.22-μm filter (Millipore, MA, USA). This condition does not induce significant ToxA production in *P. nodorum* (unpublished data).

NE infiltration and scoring

The crude culture filtrate containing SnTox1 was infiltrated into the first leaf of 2 week old wheat seedlings using a 1 cc plastic syringe without a needle. Symptoms were scored at 4 days post-infiltration. Culture filtrate derived from *P. pastoris* transformed with an empty pGAPzA vector was used as a negative infiltration control to ensure that chlorotic/necrotic symptoms from *SnTox1* were caused by the expressed protein. The necrotic reaction was scored at 4 days according to a visual score of 0 to 3 as previously described (Waters *et al.* 2011). A score of '0' indicates insensitivity (no reaction); slight chlorosis, '1'; extensive chlorosis, '2' and necrosis, '3'. Varieties that scored '1' were considered weakly sensitive whereas those that scored '2' or '3' were considered highly sensitive to the effector preparation. Crude culture filtrates derived from *P. nodorum* and other expressed NEs were infiltrated and scored in a similar manner. The experiment was carried out in biological triplicates. Infiltration assays were also performed on the flag leaf of 5 week old adult plants as described above.

Statistical analysis

Statistical analysis was performed using JMP 10.0.0 (SAS Institute, CA, USA) as described in Waters *et al.* (2011) with minor modifications. Briefly, a 2 x 2 Pearson's chi-squared test was used to test effector sensitivity datasets and SNB DRR for evidence of correlation. Effector sensitivity scores of 2 and 3 were pooled together and scores of 0 and 1 were similarly pooled. Cultivars that carry SNB resistance scores of 5 and 6 were pooled separately from scores 1 to 4. Cultivars with mixed effector sensitivity were treated as missing values by JMP 10.0.0.

Results

In this study, a crude SnTox1 preparation from *P. pastoris* was used to screen wheat cultivars for effector sensitivity. Attempts to express tagged SnTox1 for purification failed to produce an active protein probably due to folding interference. To ensure that the crude

SnTox1 preparation possessed *Snn1*-specific activity, we tested the recombinant protein on known effector sensitive (M6 and Chinese Spring) and insensitive wheat lines (BG220 and BR34). As expected, M6 and Chinese Spring were sensitive to the SnTox1 preparation whereas BG220 and BR34 were insensitive (data not shown).

The SnTox1 effector preparation was infiltrated into all 46 wheat cultivars recommended for sowing in 2013 by DAFWA (Table 2). Thirty-three (71.7%) of the cultivars showed evidence of SnTox1 sensitivity (score = 1, 2 and 3) and 13 were completely insensitive (score = 0). Nineteen cultivars (41.3%) were highly sensitive to SnTox1 and developed extensive chlorosis or necrosis (score = 2 and 3). Twenty-seven (58.7%) SnTox1-insensitive or weakly sensitive cultivars were identified (score = 0 and 1). We then compared SnTox1 sensitivity and DRR using frequency counts in mosaic plots and the Pearson's chi-squared test (Figure 2A). No significant correlation was observed between SnTox1 sensitivity and SNB DRR ($p = 0.6553$).

The prevalence of ToxA and SnTox3 sensitivity were previously investigated using DRR data derived from 2005 and 2010, respectively (Oliver *et al.* 2009; Waters *et al.* 2011). We re-examined the prevalence of ToxA and SnTox3 sensitivity in the current 2013 cultivars (Figure 2B and 2C). SnToxA H4 was used in all studies as it is the most potent isoform in causing necrosis on *Tsn1* wheat lines (Tan *et al.* 2012). For ToxA, 29 (63.0%) cultivars showed evidence of sensitivity including 4 that showed mixed sensitivity. Seventeen cultivars (37.0%) were completely insensitive to ToxA. For SnTox3, only 4 cultivars were completely insensitive (8.7%). A strong correlation between SnTox3 sensitivity and SNB DRR was observed ($p = 0.0144$) (Figure 2C). When a Fisher's exact test was applied to the data, there is a significant probability that SnTox3 pooled sensitivity scores of 2 and 3 is greater for SNB DRR 1 to 4 than 5 to 6 ($p = 0.0203$). Correlation between SnTox3 sensitivity and SNB DRR was not observed on the 2010 SNB DRR data (Waters *et al.* 2011) when re-analysed using a 2

x 2 chi-squared test (p = 0.4842). This could be due to variations in the list of new wheat cultivars.

To test the importance of *SnTox1* in disease, we performed a whole plant infection assay of SN::*tox13* on Calingiri (*Snn1, tsn1, snn3*) and observed that the mutant is completely attenuated in virulence when compared to SN15 (Figure 3). This indicates that susceptibility appear to be solely accounted by SnTox1-Snn1 interaction in Calingiri. As expected, SN::*tox13* retained wildtype virulence on *Tsn1* wheat varieties such as BG261 (Figure 3).

We then investigated the relationship between sensitivity of wheat cultivars to SnTox1, ToxA and SnTox3 and to the crude SN15 culture filtrate (Figure 4). No correlation was observed between sensitivity to SnTox1 and SN15 crude culture filtrate (Figure 4A). Analysis of the culture filtrate with *Snn1* wheat lines (M6 and Chinese Spring) demonstrated the activity of SnTox1. This indicates that SnTox1 is not the major component in the SN15 culture filtrate that governed effector sensitivity of wheat cultivars used in this study. No correlation was observed between ToxA and crude SN15 culture filtrate (as expected) as the culturing condition is not conducive for ToxA production (Figure 4B). A strong correlation was observed between the response to SnTox3 and the crude SN15 culture filtrate (p < 0.0001; Figure 4C). When a Fisher's exact test was applied to the data, there is a significant probability that SnTox3 pooled sensitivity scores of 2 and 3 is greater for SN15 culture filtrate 2 to 3 than 0 to 1 (p = 0.0023). This indicates that SnTox3 is the major effector in the SN15 crude preparation.

We then compared scores from SnTox1 with crude culture filtrate of a *P. nodorum* mutant (SN::*toxA3*) that lacked *ToxA* and *SnTox3* (Waters *et al.* 2011). The relationship between sensitivity to SnTox1 and the crude SN::*toxA3* culture filtrate sensitivity was statistically assessed. The correlation between the distribution of sensitivity scores between SnTox1 and SN::*toxA3* was close to statistical significance (p = 0.0808) (Figure 5). The result

suggests that the crude SN::*toxA3* culture filtrate possessed other novel effectors in addition to SnTox1. To test this hypothesis, we then generated a *P. nodorum* mutant that lacked *SnTox1* and *SnTox3* (SN::*tox13*) through homologous gene recombination. When grown under the described condition, the crude SN::*tox13* culture filtrate lacked ToxA, SnTox1 and SnTox3. A triple gene deletion cannot currently be performed on *P. nodorum* due to technical limitations. Infiltration assay of the SN::*tox13* culture filtrate revealed 23 (50%) wheat cultivars showing sensitivity (Table 2). Bullaring, Frame, Envoy and Mace were exceptionally sensitive to the crude culture filtrate (Table 2). Several other commercial eastern Australian wheat varieties such as EGA Gregory and Batavia, were also highly sensitive to the crude SN::*tox13* culture filtrate (data not shown). To ensure that the effector activity was not due to residual ToxA, the culture filtrate was tested on the *Tsn1* wheat cultivar BG261. No effector activity was observed. Furthermore, protease treatment of the crude SN::*tox13* culture filtrate prior to infiltration removed all effector-like activities. This indicates that the chlorosis-inducing factor or factors are proteinaceous.

Analysis of the data thus far did not identify significant correlation between SnTox1 and culture filtrate activity. As demonstrated above, this may be due to the interference of unidentified effectors detected in SN::*tox13*. The importance of SnTox1 as an effector component in the culture filtrate can be assessed by comparing average effector sensitivity scores (Figure 6). It was observed that the SN15 culture filtrate gives the highest average score of 2.67 followed by SnTox3 at 2.43. These are significantly higher than SnTox1 and SN::*toxA3* (*SnTox1* expressing) average scores of 1.22 and 1.43, respectively. However, the SN::*tox13* culture filtrate gives an average score of 0.59 which is significantly less than SN::*toxA3*, a difference of 0.84 that can be attributed to the loss of SnTox1. This suggests that uncharacterised effectors are minor components of the *in vitro* secreted effector arsenal compared to SnTox1 tested under the current culturing condition.

Thus far, all effector assays were performed on the first leaf of 2 week old seedlings. Seedling assays can be performed at a relatively high-throughput while requiring much less space for mass cultivation. However, knowledge of effector sensitivity in adult plants requires more study. To bridge this knowledge gap, we selected 11 commercial wheat cultivars ranging from SNB DRR of 1 to 6 and infiltrated these with SnTox1, ToxA and SnTox3 on the flag leaf of six week old plants (Table 3). Varieties such as Magenta, Envoy and Gladius showed a similar effector sensitivity profile on seedling and adult plants. However, cultivars such as Zippy and Fortune showed a different profile. It is interesting to note that ToxA appear to cause less tissue damage in adult plants when infiltrated with the same amount of effector. We then examined the dataset for evidence of correlation in effector sensitivity (Figure 7). The correlation between the distribution of sensitivity scores between SnTox1-infiltrated seedling and adult plants is close to statistical significance ($p = 0.0868$). However, a strong correlation was observed with ToxA. No correlation between seedling and adult plant SnTox3 sensitivity was observed ($p = 0.1368$).

Discussion

SNB remains an important and damaging disease of wheat in WA despite intense research activity over 30 years. No variety with resistance better than a score moderately resistant-moderately susceptible (6/10) is in current use. This study has demonstrated that SnTox1 sensitivity is common Western Australian wheat cultivars. However, there was no obvious correlation between SnTox1 sensitivity and SNB DRR of Western Australian wheat cultivars. It appears that sensitivity to other effectors explains the disease resistance status in most cultivars. Sensitivity to SnTox3 was strongly associated with cultivars with low DRRs. However only 4 of the 46 cultivars tested were insensitive to SnTox3. One of these is the important noodle variety Calingiri, which was sensitive to SnTox1 alone. Infection of this

variety with a strain genetically modified to lack SnTox1 clearly demonstrated the importance of SnTox1 sensitivity (Figure 3).

It appears that sensitivity to *P. nodorum* effectors operates epistatically rather than additively in wheat; sensitivity to one effector carried by the pathogen leads to significant levels of disease. Elimination of the effector or the sensitivity leads to significant reductions in disease. No current WA wheat cultivars are insensitive to all three known effectors. We therefore expect that the elimination of all the dominant sensitivity genes from wheat germplasm will result in a significant increase in SNB resistance.

A comparison of seedling and adult plant effector sensitivity assay has revealed some evidence of differential sensitivity to known effectors. This is not a surprising observation given that SnTox3 sensitivity is governed by two different QTLs mapped to the short arm of chromosome 5B and 5D (Zhang *et al.* 2011). Therefore, it is possible that effector recognition may be governed by more than one interaction with host components that can only be observed at different growth stages or tissue type. We propose that thorough sampling of plant tissue at different age and should be taken into consideration in identifying effector sensitivity/SNB QTLs,

This study has also demonstrated that effectors other than the three known examples, ToxA, SnTox1 and SnTox3 are present in a WA *P. nodorum* isolate. This is consistent with studies in the US and Australia that have shown that many further effectors remain to be discovered (Abeysekara *et al.* 2012; Crook *et al.* 2012; Francki *et al.* 2011; Friesen *et al.* 2012; Oliver *et al.* 2012; Syme *et al.* 2013). In this study, we show that crude culture filtrates of a strain lacking expression of ToxA, SnTox1 and SnTox3 contain effector activity that induced reactions in 23 of the tested cultivars (Table 2). As before, the effector activity appears to be proteinaceous. Efforts are currently underway to isolate the novel effectors and to determine their role in SNB.

We have continued to work with local wheat breeding companies by supplying effectors for use in their breeding operations. Effector sensitivity assay are simple and rapid and provide information that helps breed for resistance to both SNB and tan spot (Oliver *et al.* 2009; Waters *et al.* 2011). In 2012, some 34,000 doses of the three effectors were used. We have now added the SnTox1 test and shown that sensitivity is common in commercial wheat cultivars. We have also demonstrated that SnTox1-Snn1 interaction is an important factor in establishing SNB in the important cultivar Calingiri. Sensitivity to SnTox1 was found in 72% of cultivars. Sensitivity to SnTox3 sensitivity is more common (91% of cultivars) and is probably a more important factor. The availability of the effectors aids in the stepwise elimination of their sensitivity genes. We have recently shown that no significant yield penalty was observed in wheat cultivars that are insensitive SnTox1, ToxA and SnTox3 (Oliver *et al.* in press). Hence, this strategy is expected to aid in the development of wheat cultivars with increased level of resistance to SNB while maintaining yield.

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Table 1. Primers used to construct *SnTox1* and *SnTox3* deletion vectors. The bold text refers to sequences that are complementary to the pAN8f and pAN8r primers that were used to amplify the phleomycin and hygromycin resistance cassettes for fusion PCR.

Primer	Sequence (5' - 3')
5’08981KOF	GCCAGTGTCAATCTCGGTTAAGTTA
5’08981KOR	**TGTGACTTTTGGTTACGCCGTCT**AAATGCATAGCCTATGTTGTGACCT
3’08981KOF	**TCTCCTATGAGTCGTTTACCCAGAA**GGAATGTTCTACAAGGACATATGGG
3’08981KOR	AGTTAATTGCAGTTACTGCGCTGTT
20078KO5'f	TAGCCGTAGGAGTCCTAATTTAGC
20078KO5'r	**TGTGACTTTTGGTTACGCCGTCT**GAATTCCAGGAGGGAGTTTG
20078KO3'f	**TCTCCTATGAGTCGTTTACCCAGAA**GCAGACAAGAATAGTTCTCTCCAC
20078KO3'r	GCTAGACAACCAATACCTGCTG
pAN8f	AGACGGCGTAACCAAAAGTCACA
pAN8r	TTCTGGGTAAACGACTCATAGGAGA

Table 2. Reactions of 46 current Western Australian commercial wheat cultivars to effector preparations.

Wheat cultivar	SNB disease resistance rating	Effector sensitivity					
		SnTox1[A]	ToxA[AB]	SnTox3[AC]	SN15 culture filtrate[AC]	SN::*toxA3* culture filtrate[AC]	SN::*tox13* culture filtrate[A]
Cobra	6	2	0	2	2	1	1
EGA Eagle Rock	6	2	0	2	3	2	1
Magenta	6	1	0	1	3	0	1
Stiletto	6	1	3	2	3	2	1
Yitpi	6	1	3	3	3	2	0
Camm	6	0	3	3	2	1	1
Clearfield STL	6	0	3	3	3	2	0
Endure	6	0	3	0	1	0	0
Envoy	5	2	3	2	2	2	2
Justica CL Plus	5	1	3	1	2	0	0
Katana	5	2	0/3	2	3	1	1
King Rock	5	1	0	2	3	0	0
Calingiri	5	3	0*	0	3	3	0
Carnamah	5	2	0	3	3	1	1
Fang	5	2	0	0	1	1	1
Mace	5	2	0	3	3	3	2
Frame	5	1	3	3	3	1	2
Annuello	5	0	3	3	3	1	1
Fortune	5	3	0	0	0	1	1
H45	5	0	0	3	3	2	0
Corack	4	0	0	3	3	0	1
Estoc	4	0	3	2	2	0	0
Kunjin	4	2	3	3	3	0	0
Scout	4	2	3	3	3	2	0
Wedin	4	0	3	3	3	0	1
Yandanooka	4	3	0	3	3	3	0
Arrino	4	2	0/3	3	3	3	0
Binnu	4	2	0/3	3	3	3	0
Bullaring	4	2	3	3	3	2	2
Tammarin Rock	4	2	3	3	3	1	0
Cascades	4	1	0	3	2	1	0
Gladius	4	1	3	3	2	1	0
Janz	4	1	3	3	3	3	0
Wyalkatchem	4	1	0	3	3	2	0

Continued next page.

Table 2. Continued.

Wheat cultivar	SNB disease resistance rating	Effector sensitivity					
		SnTox1[A]	ToxA[AB]	SnTox3[AC]	SN15 culture filtrate[AC]	SN::*toxA3* culture filtrate[AC]	SN::*tox13* culture filtrate[A]
EGA Bonnie Rock	4	0	0	3	3	2	0
Espada	4	0	3	3	3	0	1
GBA Sapphire	4	0	3	3	3	2	1
Westonia	3	3	0	3	3	2	0
EGA 2248	3	2	3	3	3	3	1
Correll	3	1	0/3	3	3	2	1
Spear	3	0	3	2	3	1	0
Zippy	3	0	3	3	3	1	0
Halberd	2	2	3	3	3	2	1
Axe	2	1	3	3	3	1	0
Eradu	2	1	3	3	3	3	1
Emu Rock	1	1	0	1	2	0	1

[A]This study.

[B]From Oliver *et al.* (2009) and this study. Individuals with mixed reactions to ToxA were observed (scoring either a '0' or '3' in biological replicates) as the result of seed batches that are heterologous for the *Tsn1*/*tsn1* genotype.

[C]From Waters *et al.* (2011) and this study.

*Previously reported in Oliver *et al.* (2009) as heterologous for ToxA sensitivity. Analysis of recent seed batches indicates complete insensitivity to ToxA.

Table 3. An examination of selected wheat cultivars for effector sensitivity in adult plants using a flag leaf infiltration assay.

		Seedling – first leaf			Adult – flag leaf		
Wheat cultivar	**SNB disease resistance rating**	**SnTox1**	**ToxA**	**SnTox3**	**SnTox1**	**ToxA**	**SnTox3**
Magenta	6	1	0	1	2	0	1
Mace	5	2	0	3	2	0	1
Envoy	5	2	3	2	2	2	2
Fortune	5	3	0	0	2	0	2
Calingiri	5	3	0	0	2	0	1
Bullaring	4	2	3	3	2	2	2
Wyalkatchem	4	1	0	3	1	0	2
Gladius	4	1	3	3	0	2	2
Zippy	3	0	3	3	2	2	2
Halberd	2	2	3	3	2	2	1
Emu Rock	1	1	0	1	2	2	0

Figure 1. Schematic illustrations of **A.** *SnTox3* and **B.** *SnTox1* gene deletion constructs. Primer sequences are listed in Table 1.

Figure 2. Relationship between the distribution of 2013 SNB DRR and reactions to **A.** SnTox1 (p = 0.6553), **B.** ToxA (p = 0.1447) and **C.** SnTox3 (p = 0.0144). The *y* axis represents the proportion of effector sensitivity for each DRR score designated on the *x* axis. The right column demonstrates the distribution of effector sensitivity scores. Statistical analysis was performed using frequency counts in mosaic plots and the Pearson's chi-squared test set at a significance threshold of $p < 0.05$.

Figure 3. Assessing SN::*tox13* virulence on wheat cv. Calingiri and BG261 using a whole plant spray assay. Statistical analysis was performed using a pair-wise student *t*-test set at a significant threshold of $p < 0.05$ against SN15. The experiment was performed in biological triplicates. Standard error bars are shown. Asterisks denote a significant difference to SN15.

Figure 4. Relationship between SN15 culture filtrate sensitivity of 46 wheat cultivars and reactions to **A.** SnTox1 (p = 0.3561), **B.** ToxA (p = 0.3375) and **C.** SnTox3 ($p < 0.0001$). The *y* axis represents the proportion of effector sensitivity for each SN15 culture filtrate sensitivity score designated on the *x* axis. The right column demonstrates the distribution of effector sensitivity scores. Statistical analysis was performed using frequency counts in mosaic plots and the Pearson's chi-squared test set at a significance threshold of $p < 0.05$.

Figure 5. Relationship between SN::*toxA3* culture filtrate sensitivity of 46 wheat cultivars and reactions to SnTox1 (p = 0.0808). The *y* axis represents the proportion of SnTox1 sensitivity for each SN::*toxA3* culture filtrate sensitivity score designated on the *x* axis. The right column demonstrates the distribution of SnTox1 sensitivity scores. Statistical analysis was performed using frequency counts in mosaic plots and the Pearson's chi-squared test set at a significance threshold of $p < 0.05$.

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Figure 6. Average effector sensitivity scores from the 46 commercial wheat cultivars. An analysis of variance using the Tukey-Kramer test set at a significance threshold of $p < 0.05$ was used to compare all means. Levels not connected by the same letter are significantly different.

Figure 7. Relationship between seedling and adult plant sensitivity to **A.** SnTox1 ($p = 0.0868$), **B.** ToxA ($p = 0.0057$) and **C.** SnTox3 ($p = 0.1368$). The *y* axis represents the proportion of seedling effector sensitivity whereas the *x* axis denotes adult plant effector sensitivity. The right column demonstrates the distribution of seedling effector sensitivity scores. Statistical analysis was performed using frequency counts in mosaic plots and the Pearson's chi-squared test set at a significance threshold of $p < 0.05$.

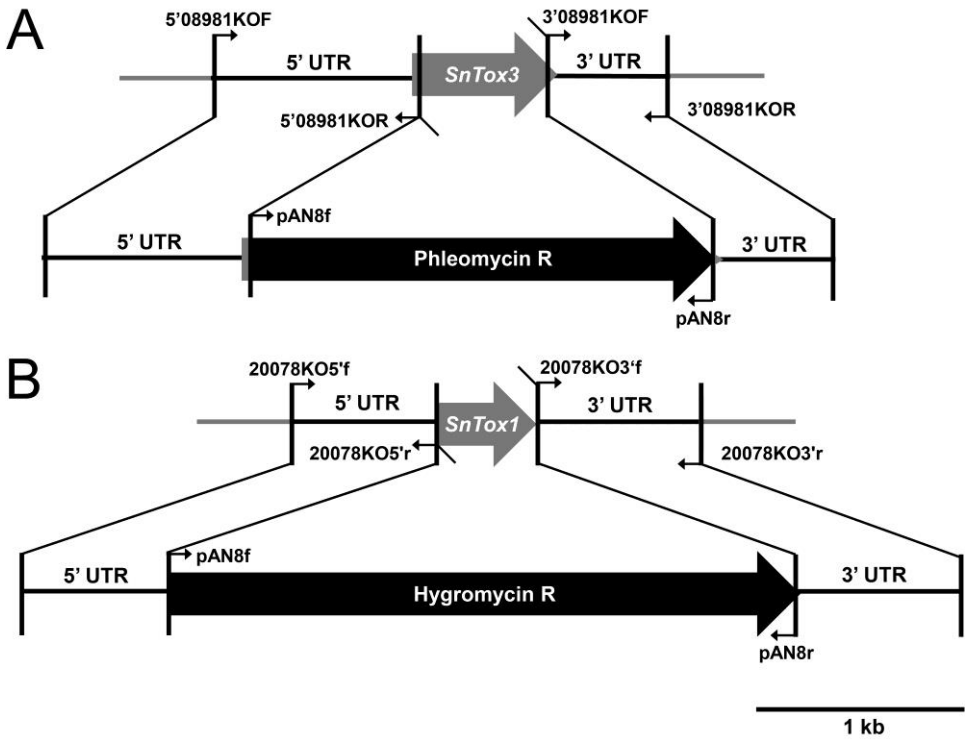

A
5'08981KOF
3'08981KOF
5' UTR
SnTox3
3' UTR
5'08981KOR
3'08981KOR
pAN8f
5' UTR
Phleomycin R
3' UTR
pAN8r
B
20078KO5'f
20078KO3'f
5' UTR
SnTox1
3' UTR
20078KO5'r
20078KO3'r
pAN8f
5' UTR
Hygromycin R
3' UTR
pAN8r
1 kb

Figure 1.

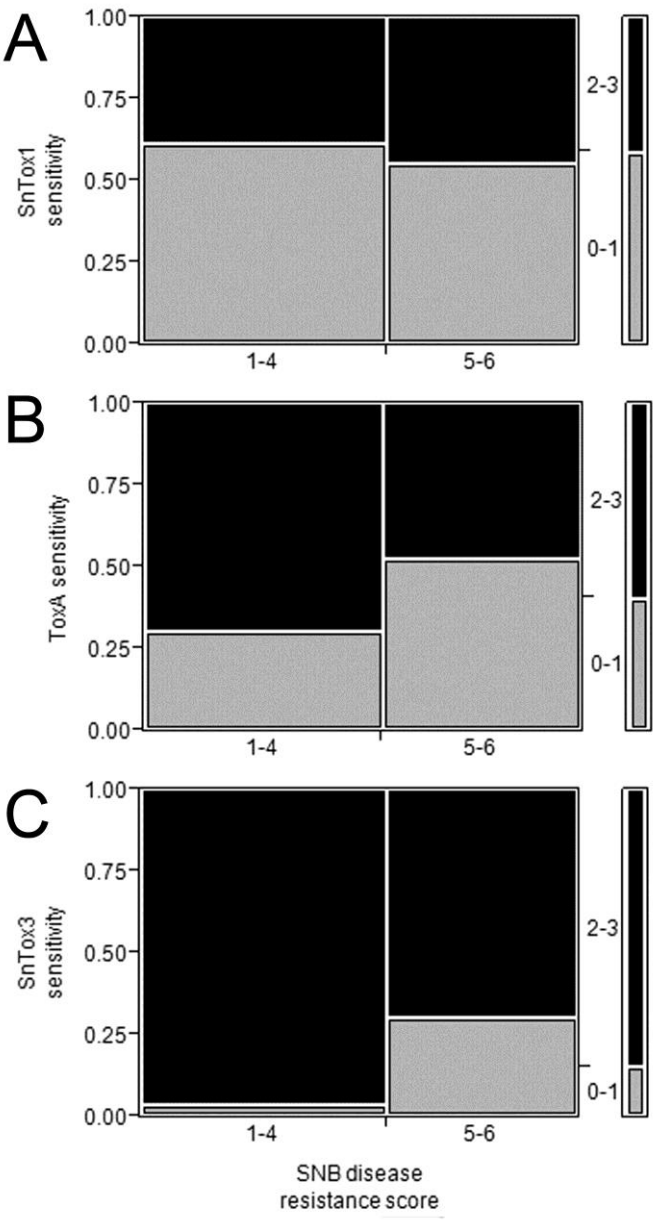

A
1.00
0.75
0.50
0.25
0.00
SnTox1 sensitivity
2-3
0-1
1-4
5-6
B
1.00
0.75
0.50
0.25
0.00
ToxA sensitivity
2-3
0-1
1-4
5-6
C
1.00
0.75
0.50
0.25
0.00
SnTox3 sensitivity
2-3
0-1
1-4
5-6
SNB disease resistance score

Figure 2.

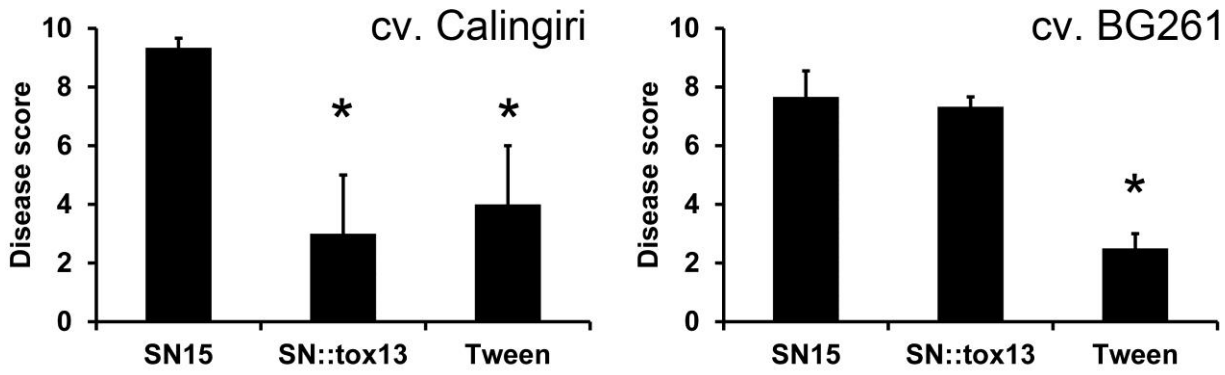

cv. Calingiri
cv. BG261
Disease score
10
8
6
4
2
0
*
*
*
SN15
SN::tox13
Tween
SN15
SN::tox13
Tween

Figure 3.

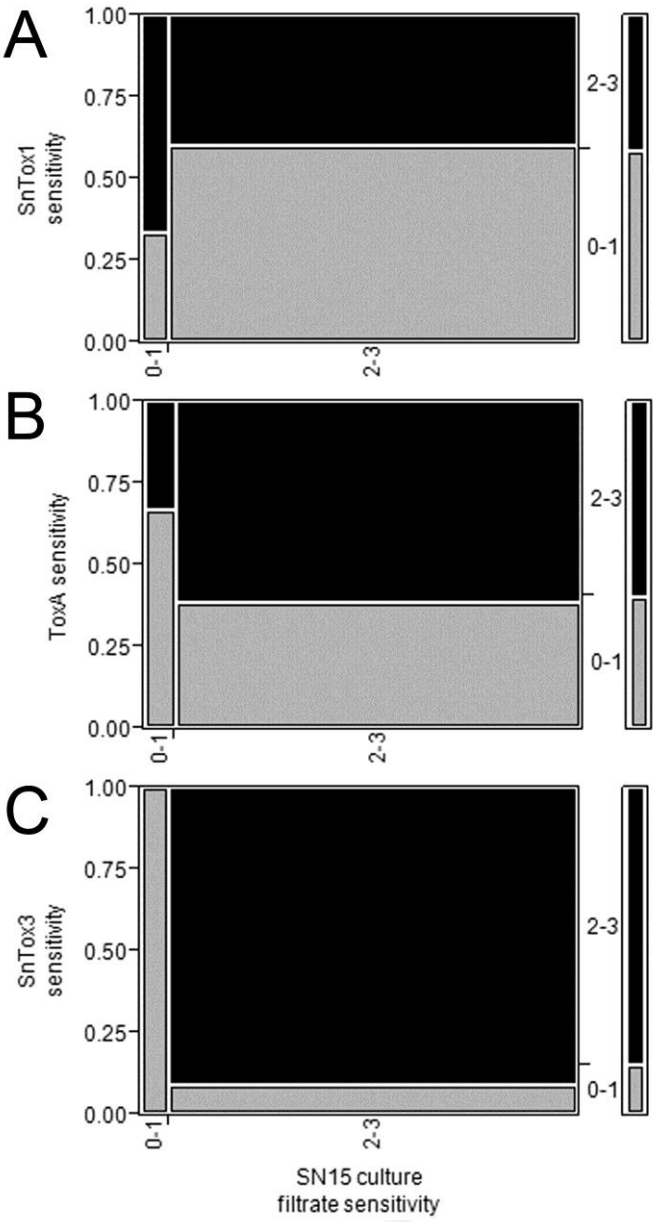

A
1.00
0.75
0.50
0.25
0.00
SnTox1 sensitivity
2-3
0-1
0-1
2-3
B
1.00
0.75
0.50
0.25
0.00
ToxA sensitivity
2-3
0-1
0-1
2-3
C
1.00
0.75
0.50
0.25
0.00
SnTox3 sensitivity
2-3
0-1
0-1
2-3
SN15 culture filtrate sensitivity

Figure 4.

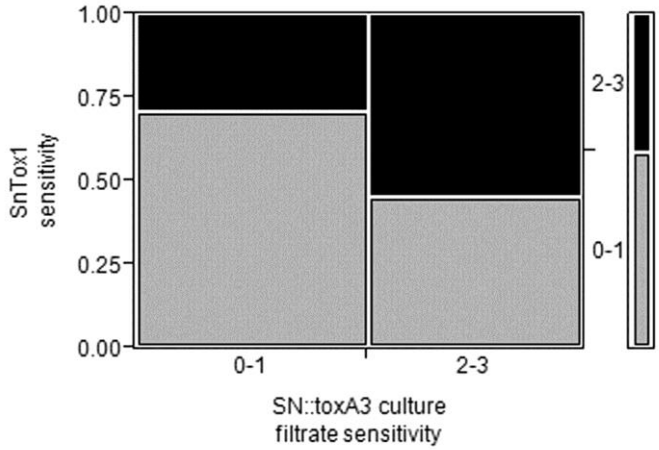

1.00
0.75
0.50
0.25
0.00
SnTox1
sensitivity
2-3
0-1
0-1
2-3
SN::toxA3 culture
filtrate sensitivity

Figure 5.

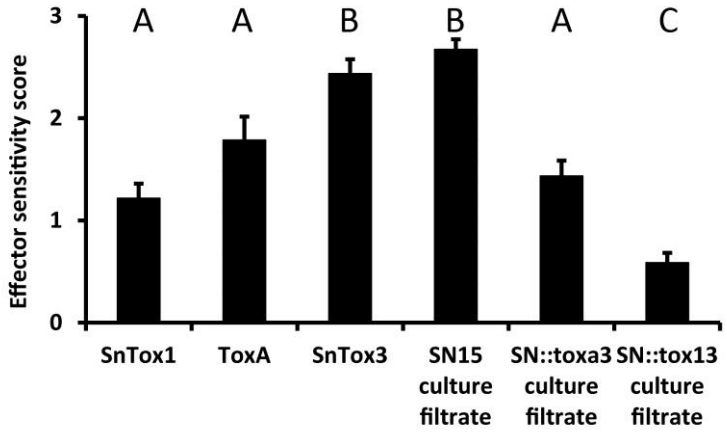
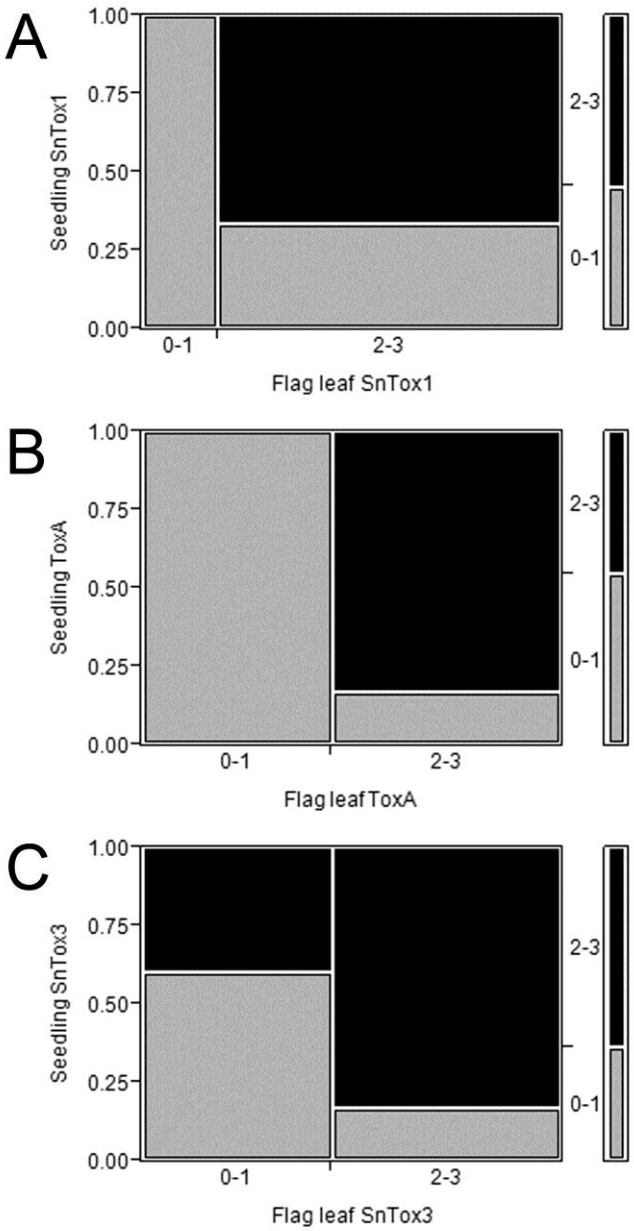

A
A
B
B
A
C
3
2
1
0
Effector sensitivity score
SnTox1
ToxA
SnTox3
SN15 culture filtrate
SN::toxa3 culture filtrate
SN::tox13 culture filtrate

Figure 6.


A
1.00
0.75
0.50
0.25
0.00
Seedling SnTox1
0-1
2-3
Flag leaf SnTox1
2-3
0-1
B
1.00
0.75
0.50
0.25
0.00
Seedling ToxA
0-1
2-3
Flag leaf ToxA
2-3
0-1
C
1.00
0.75
0.50
0.25
0.00
Seedling SnTox3
0-1
2-3
Flag leaf SnTox3
2-3
0-1

1

2 **Figure 7.**