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2 **Sensitivity to three *Parastagonospora nodorum***

3 **necrotrophic effectors in current Australian wheat**

4 **cultivars and the presence of further fungal effectors**

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1 **Abstract**

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

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

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

1 **Introduction**

2 *Parastagonospora* (syn. ana, *Stagonospora*; teleo, *Phaeosphaeria*) *nodorum* (Berk.)
3 Quaedvlieg, Verkley & Crous is the causal agent of septoria nodorum blotch (SNB) on wheat
4 (Quaedvlieg *et al.* 2013; Solomon *et al.* 2006a). The fungus is responsible for yield losses
5 estimated at AUD\$108 million per year in Australia (Murray and Brennan 2009; Oliver *et al.*
6 2012). SNB is quantitatively governed by the interaction of fungal necrotrophic effectors
7 (NEs) that interact with the products of host dominant sensitivity genes (Faris *et al.* 2010; Tan
8 *et al.* 2010). A compatible interaction results in host tissue necrosis that promotes infection by
9 the pathogen. *ToxA* was the first effector gene identified in *P. nodorum*. *ToxA* was originally
10 identified in the tan spot fungus *Pyrenophora tritici-repentis* (*Ptr*), a close relative of *P.*
11 *nodorum* (Ciuffetti *et al.* 1997; Ellwood *et al.* 2012). Genetic evidence suggests that *PtrToxA*
12 was acquired from *P. nodorum* via horizontal gene transfer (Friesen *et al.* 2006). *ToxA*
13 encodes a small, secreted protein that causes necrosis and promotes infection in wheat
14 carrying the *Tsn1* gene (Tan *et al.* 2012). *Tsn1* is located on chromosome 5B and encodes a
15 protein with distinct NBS-LRR and protein kinase domains (Faris *et al.* 2010). *SnTox3*
16 encodes a small secreted protein (Liu *et al.* 2009). *Snn3-B1* and *Snn3-D1* are key *SnTox3*
17 sensitivity genes that mapped to the short arm of chromosome 5B and 5D, respectively
18 (Zhang *et al.* 2011). Both effector genes are ubiquitous in Australian *P. nodorum* isolates.
19 Their roles in virulence were thoroughly demonstrated (Friesen *et al.* 2006; Liu *et al.* 2009;
20 McDonald *et al.* 2013; Oliver *et al.* 2012).

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

1 Previously, *ToxA* and *SnTox3* were expressed using microbial expression systems (Liu *et al.*
2 2009; Tan *et al.* 2012) and used to screen commercial Western Australian (WA) wheat
3 cultivars (Oliver *et al.* 2009; Waters *et al.* 2011). 61% of all tested cultivars were sensitive to
4 *ToxA* and 90% were sensitive to *SnTox3*. Due to the prevalence of effector sensitivity, a
5 national cereal breeding program is currently being applied to eliminate *ToxA* and *SnTox3*
6 sensitivity genes in wheat (Oliver and Solomon 2010).

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

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

25 **Materials and methods**

1 *Wheat cultivars*

2 All wheat cultivars used in this study were obtained from the Australian Winter Cereal
3 Collection (Tamworth, Australia). Current SNB disease resistance ratings (DRR) of
4 commercial cultivars were obtained from the Department of Agriculture and Food Western
5 Australia (DAFWA) (Shackley *et al.* 2013). For statistical purposes, a scoring system was
6 assigned to all applicable DRR categories; very susceptible, ‘1’; susceptible-very susceptible,
7 ‘2’; susceptible, ‘3’; moderately susceptible- susceptible, ‘4’; moderately susceptible, ‘5’; and
8 moderately resistant-moderately susceptible, ‘6’.

9 *SnTox1 expression*

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

16 *Construction of SnTox1 and SnTox3 gene deletion P. nodorum mutants*

17 *SnTox1* and *SnTox3* gene deletion constructs were constructed using a fusion PCR
18 approach (Solomon *et al.* 2006b). Firstly, 5’ and 3’ *SnTox3* untranslated regions (UTRs) were
19 PCR-amplified from genomic DNA using 5’08981KOF, 5’08981KOR, 3’08981KOF and
20 3’08981KOR (Figure 1; Table 1). Both flanking regions were sequentially fused to a
21 phleomycin resistance cassette PCR-amplified from pAN8 using pAN8f and pAN8r. The
22 *SnTox3* deletion construct was transformed into the Western Australian *P. nodorum* SN15
23 isolate to facilitate gene knockout (Solomon *et al.* 2004). PCR was used to identify the
24 appropriate mutant deleted in *SnTox3* (SN::tox3) for further genetic manipulation. Finally, 5’

1 and 3' *SnTox1* UTRs were PCR-amplified from genomic DNA using 20078KO5'f,
2 20078KO5'r, 20078KO3'f and 20078KO3'r (Figure 1; Table 1). Both flanking regions were
3 sequentially fused to a hygromycin resistance cassette derived from pAN7. The *SnTox1*
4 deletion construct was transformed into SN::*tox3* to facilitate double gene knockout. PCR was
5 used to identify the appropriate mutant carrying *SnTox1* deletion. Fungal transformation was
6 performed as described by Solomon *et al.* (2004). Quantitative PCR was used to determine
7 the integration copy number of both gene deletion constructs in all transformants (Solomon *et*
8 *al.* 2008). Mutants that carried a single integration of each construct were selected for effector
9 studies.

10 *Infection assay*

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

16 *Induction of NEs in P. nodorum culture filtrate*

17 *P. nodorum* culture filtrates were produced as described (Liu *et al.* 2004a). Briefly,
18 1×10^6 *P. nodorum* spores were inoculated into a 250 ml conical flask containing 50 ml of
19 Fries broth. The fungus was allowed to grow at 28°C for 3 days in the dark on an orbital
20 shaker set at 140 rpm. Following this, effector production was achieved by stationary growth
21 for 4 weeks at room temperature. The culture filtrate was collected with cheesecloth filtration
22 and sterilised through a 0.22-μm filter (Millipore, MA, USA). This condition does not induce
23 significant ToxA production in *P. nodorum* (unpublished data).

24 *NE infiltration and scoring*

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

14 *Statistical analysis*

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

22 **Results**

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

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

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

14 The prevalence of ToxA and SnTox3 sensitivity were previously investigated using
15 DRR data derived from 2005 and 2010, respectively (Oliver *et al.* 2009; Waters *et al.* 2011).
16 We re-examined the prevalence of ToxA and SnTox3 sensitivity in the current 2013 cultivars
17 (Figure 2B and 2C). SnToxA H4 was used in all studies as it is the most potent isoform in
18 causing necrosis on *Tsn1* wheat lines (Tan *et al.* 2012). For ToxA, 29 (63.0%) cultivars
19 showed evidence of sensitivity including 4 that showed mixed sensitivity. Seventeen cultivars
20 (37.0%) were completely insensitive to ToxA. For SnTox3, only 4 cultivars were completely
21 insensitive (8.7%). A strong correlation between SnTox3 sensitivity and SNB DRR was
22 observed ($p = 0.0144$) (Figure 2C). When a Fisher's exact test was applied to the data, there is
23 a significant probability that SnTox3 pooled sensitivity scores of 2 and 3 is greater for SNB
24 DRR 1 to 4 than 5 to 6 ($p = 0.0203$). Correlation between SnTox3 sensitivity and SNB DRR
25 was not observed on the 2010 SNB DRR data (Waters *et al.* 2011) when re-analysed using a 2

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

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

8 We then investigated the relationship between sensitivity of wheat cultivars to
9 SnTox1, ToxA and SnTox3 and to the crude SN15 culture filtrate (Figure 4). No correlation
10 was observed between sensitivity to SnTox1 and SN15 crude culture filtrate (Figure 4A).
11 Analysis of the culture filtrate with *Snn1* wheat lines (M6 and Chinese Spring) demonstrated
12 the activity of SnTox1. This indicates that SnTox1 is not the major component in the SN15
13 culture filtrate that governed effector sensitivity of wheat cultivars used in this study. No
14 correlation was observed between ToxA and crude SN15 culture filtrate (as expected) as the
15 culturing condition is not conducive for ToxA production (Figure 4B). A strong correlation
16 was observed between the response to SnTox3 and the crude SN15 culture filtrate ($p <$
17 0.0001; Figure 4C). When a Fisher's exact test was applied to the data, there is a significant
18 probability that SnTox3 pooled sensitivity scores of 2 and 3 is greater for SN15 culture filtrate
19 2 to 3 than 0 to 1 ($p = 0.0023$). This indicates that SnTox3 is the major effector in the SN15
20 crude preparation.

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

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

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

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

15 **Discussion**

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

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

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

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

17 This study has also demonstrated that effectors other than the three known examples,
18 ToxA, SnTox1 and SnTox3 are present in a WA *P. nodorum* isolate. This is consistent with
19 studies in the US and Australia that have shown that many further effectors remain to be
20 discovered (Abeysekara *et al.* 2012; Crook *et al.* 2012; Francki *et al.* 2011; Friesen *et al.*
21 2012; Oliver *et al.* 2012; Syme *et al.* 2013). In this study, we show that crude culture filtrates
22 of a strain lacking expression of ToxA, SnTox1 and SnTox3 contain effector activity that
23 induced reactions in 23 of the tested cultivars (Table 2). As before, the effector activity
24 appears to be proteinaceous. Efforts are currently underway to isolate the novel effectors and
25 to determine their role in SNB.

1 We have continued to work with local wheat breeding companies by supplying
2 effectors for use in their breeding operations. Effector sensitivity assay are simple and rapid
3 and provide information that helps breed for resistance to both SNB and tan spot (Oliver *et al.*
4 2009; Waters *et al.* 2011). In 2012, some 34,000 doses of the three effectors were used. We
5 have now added the SnTox1 test and shown that sensitivity is common in commercial wheat
6 cultivars. We have also demonstrated that SnTox1-Snn1 interaction is an important factor in
7 establishing SNB in the important cultivar Calingiri. Sensitivity to SnTox1 was found in 72%
8 of cultivars. Sensitivity to SnTox3 sensitivity is more common (91% of cultivars) and is
9 probably a more important factor. The availability of the effectors aids in the stepwise
10 elimination of their sensitivity genes. We have recently shown that no significant yield
11 penalty was observed in wheat cultivars that are insensitive SnTox1, ToxA and SnTox3
12 (Oliver *et al.* in press). Hence, this strategy is expected to aid in the development of wheat
13 cultivars with increased level of resistance to SNB while maintaining yield.

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18 **References**

19 Abeysekara NS, Faris JD, Chao S, McClean PE, Friesen TL (2012) Whole-genome QTL
20 analysis of Stagonospora nodorum blotch resistance and validation of the SnTox4-Snn4
21 interaction in hexaploid wheat. *Phytopathology* **102**, 94-104.

22

23 Ciuffetti LM, Tuori RP, Gaventa JM (1997) A single gene encodes a selective toxin causal to
24 the development of tan spot of wheat. *Plant Cell* **9**, 135-44.

- 1
- 2 Crook AD, Friesen TL, Liu ZH, Ojiambo PS, Cowger C (2012) Novel necrotrophic effectors
3 from *Stagonospora nodorum* and corresponding host sensitivities in winter wheat germplasm
4 in the southeastern United States. *Phytopathology* **102**, 498-505.
- 5
- 6 Czembor PC, Arseniuk E, Czaplicki A, Song Q, Cregan PB, Ueng PP (2003) QTL mapping
7 of partial resistance in winter wheat to *Stagonospora nodorum* blotch. *Genome* **46**, 546-54.
- 8
- 9 Ellwood SR, Syme RA, Moffat CS, Oliver RP (2012) Evolution of three Pyrenophora cereal
10 pathogens: Recent divergence, speciation and evolution of non-coding DNA. *Fungal Genetics
11 and Biology* **49**, 825-829.
- 12
- 13 Faris JD, Zhang Z, *et al.* (2010) A unique wheat disease resistance-like gene governs effector-
14 triggered susceptibility to necrotrophic pathogens. *Proceedings of the National Academy of
15 Sciences U S A* **107**, 13544-13549.
- 16
- 17 Francki MG, Shankar M, Walker E, Loughman R, Golzar H, Ohm H (2011) New quantitative
18 trait loci in wheat for flag leaf resistance to *Stagonospora nodorum* blotch. *Phytopathology*
19 **101**, 1278-84.
- 20
- 21 Friesen TL, Chu C, Xu SS, Faris JD (2012) SnTox5-Snn5: a novel *Stagonospora nodorum*
22 effector-wheat gene interaction and its relationship with the SnToxA-Tsn1 and SnTox3-Snn3-
23 B1 interactions. *Molecular Plant Pathology* **13**, 1101-9.
- 24

1 Friesen TL, Faris JD, Solomon PS, Oliver RP (2008) Host-specific toxins: effectors of
2 necrotrophic pathogenicity. *Cellular Microbiology* **10**, 1421-8.

3

4 Friesen TL, Stukenbrock EH, *et al.* (2006) Emergence of a new disease as a result of
5 interspecific virulence gene transfer. *Nature Genetics* **38**, 953-956.

6

7 Liu Z, Faris JD, *et al.* (2009) SnTox3 acts in effector triggered susceptibility to induce disease
8 on wheat carrying the Snn3 gene. *PLoS Pathogens* **5**, e1000581.

9

10 Liu Z, Zhang Z, *et al.* (2012) The cysteine rich necrotrophic effector SnTox1 produced by
11 *Stagonospora nodorum* triggers susceptibility of wheat lines harboring Snn1. *PLoS Pathogens*
12 **8**, e1002467.

13

14 Liu ZH, Faris JD, Meinhardt SW, Ali S, Rasmussen JB, Friesen TL (2004a) Genetic and
15 physical mapping of a gene conditioning sensitivity in wheat to a partially purified host-
16 selective toxin produced by *Stagonospora nodorum*. *Phytopathology* **94**, 1056-1060.

17

18 Liu ZH, Friesen TL, Rasmussen JB, Ali S, Meinhardt SW, Faris JD (2004b) Quantitative trait
19 loci analysis and mapping of seedling resistance to *Stagonospora nodorum* leaf blotch in
20 wheat. *Phytopathology* **94**, 1061-1067.

21

22 McDonald MC, Oliver RP, Friesen TL, Brunner PC, McDonald BA (2013) Global diversity
23 and distribution of three necrotrophic effectors in *Phaeosphaeria nodorum* and related
24 species. *New Phytologist* **199**, 241-251.

- 1
- 2 Murray GM, Brennan JP (2009) Estimating disease losses to the Australian wheat industry.
- 3 *Australasian Plant Pathology* **38**, 558-570.
- 4
- 5 Oliver R, Lichtenzveig J, Tan KC, Waters O, Rybak K, Lawrence J, Friesen T, Burgess P
- 6 (2013) Absence of detectable yield penalty associated with insensitivity to Pleosporales
- 7 necrotrophic effectors in wheat grown in the West Australian wheat belt. *Plant Pathology* **In**
- 8 **press**.
- 9
- 10 Oliver RP, Friesen TL, Faris JD, Solomon PS (2012) *Stagonospora nodorum*: from pathology
- 11 to genomics and host resistance. *Annual Review of Phytopathology* **50**, 23-43.
- 12
- 13 Oliver RP, Rybak K, Solomon PS, Ferguson-Hunt M (2009) Prevalence of ToxA-sensitive
- 14 alleles of the wheat gene *Tsn1* in Australian and Chinese wheat cultivars. *Crop & Pasture*
- 15 *Science* **60**, 348-352.
- 16
- 17 Oliver RP, Solomon PS (2010) New developments in pathogenicity and virulence of
- 18 necrotrophs. *Current Opinion in Plant Biology* **13**, 415-9.
- 19
- 20 Quaedvlieg W, Verkley GJ, Shin HD, Barreto RW, Alfenas AC, Swart WJ, Groenewald JZ,
- 21 Crous PW (2013) Sizing up Septoria. *Studies in mycology* **75**, 307-90.
- 22
- 23 Shackley B, Zaicou-Kunusch C, Dhammu H, Shankar M, Amjad M, Young K (2013) 'Wheat
- 24 variety guide for WA 2013.' (Department of Agriculture and Food: Perth)

- 1
- 2 Solomon PS, IpCho SVS, Hane JK, Tan KC, Oliver RP (2008) A quantitative PCR approach
3 to determine gene copy number. *Fungal Genetics Report* **55**, 5-8.
- 4
- 5 Solomon PS, Lowe RGT, Tan K-C, Waters ODC, Oliver RP (2006a) *Stagonospora nodorum*:
6 cause of stagonospora nodorum blotch of wheat. *Molecular Plant Pathology* **7**, 147-156.
- 7
- 8 Solomon PS, Rybak K, Trengove RD, Oliver RP (2006b) Investigating the role of
9 calcium/calmodulin-dependent protein kinases in *Stagonospora nodorum*. *Molecular*
10 *Microbiology* **62**, 367-81.
- 11
- 12 Solomon PS, Tan KC, Oliver RP (2005) Mannitol 1-phosphate metabolism is required for
13 sporulation *in planta* of the wheat pathogen *Stagonospora nodorum*. *Molecular Plant-*
14 *Microbe Interactions* **18**, 110-5.
- 15
- 16 Solomon PS, Tan KC, Sanchez P, Cooper RM, Oliver RP (2004) The disruption of a G α
17 subunit sheds new light on the pathogenicity of *Stagonospora nodorum* on wheat. *Molecular*
18 *Plant-Microbe Interactions* **17**, 456-66.
- 19
- 20 Syme RA, Hane JK, Friesen TL, Oliver RP (2013) Resequencing and comparative genomics
21 of *Stagonospora nodorum*; sectional gene absence and effector discovery. *G3*, In press.
- 22

1 Tan KC, Ferguson-Hunt M, *et al.* (2012) Quantitative variation in effector activity of ToxA
2 isoforms from *Stagonospora nodorum* and *Pyrenophora tritici-repentis*. *Molecular Plant-*
3 *Microbe Interactions* **25**, 515-22.

4

5 Tan KC, Oliver RP, Solomon PS, Moffat CS (2010) Proteinaceous necrotrophic effectors in
6 fungal virulence. *Functional Plant Biology* **37**, 907-912.

7

8 Waters ODC, Lichtenzveig J, Rybak K, Friesen TL, Oliver RP (2011) Prevalence and
9 importance of sensitivity to the *Stagonospora nodorum* necrotrophic effector SnTox3 in
10 current Western Australian wheat cultivars. *Crop & Pasture Science* **62**, 556-562.

11

12 Zhang Z, Friesen TL, Xu SS, Shi G, Liu Z, Rasmussen JB, Faris JD (2011) Two putatively
13 homoeologous wheat genes mediate recognition of SnTox3 to confer effector-triggered
14 susceptibility to *Stagonospora nodorum*. *Plant Journal* **65**, 27-38.

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

Primer	Sequence (5' - 3')
5'08981KOF	GCCAGTGTCAATCTCGGTTAAGTTA
5'08981KOR	TGTGACTTTGGTTACGCCGTCAAATGCATAGCCTATGTTGTGACCT
3'08981KOF	TCTCCTATGAGTCGTTACCCAGAAAGGAATGTTCTACAAGGACATATGGG
3'08981KOR	AGTTAATTGCAGTTACTGCGCTGTT
20078KO5'f	TAGCCGTAGGAGTCCTAATTAGC
20078KO5'r	TGTGACTTTGGTTACGCCGTCTGAATTCCAGGAGGGAGTTG
20078KO3'f	TCTCCTATGAGTCGTTACCCAGAAAGCAGACAAGAACAGTTCTCTCCAC
20078KO3'r	GCTAGACAACCAATACCTGCTG
pAN8f	AGACGGCGTAACCAAAAAGTCACA
pAN8r	TTCTGGGTAAACGACTCATAGGAGA

4

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

3 Continued next page.

1 **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

2 ^AThis study.

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

6 ^CFrom Waters *et al.* (2011) and this study.

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

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

Wheat cultivar	SNB disease resistance rating	Seedling – first leaf			Adult – flag leaf		
		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

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4

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

3 **Figure 2.** Relationship between the distribution of 2013 SNB DRR and reactions to **A.** SnTox1 ($p =$
4 0.6553), **B.** ToxA ($p = 0.1447$) and **C.** SnTox3 ($p = 0.0144$). The y axis represents the proportion of
5 effector sensitivity for each DRR score designated on the x axis. The right column demonstrates the
6 distribution of effector sensitivity scores. Statistical analysis was performed using frequency counts
7 in mosaic plots and the Pearson's chi-squared test set at a significance threshold of $p < 0.05$.

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

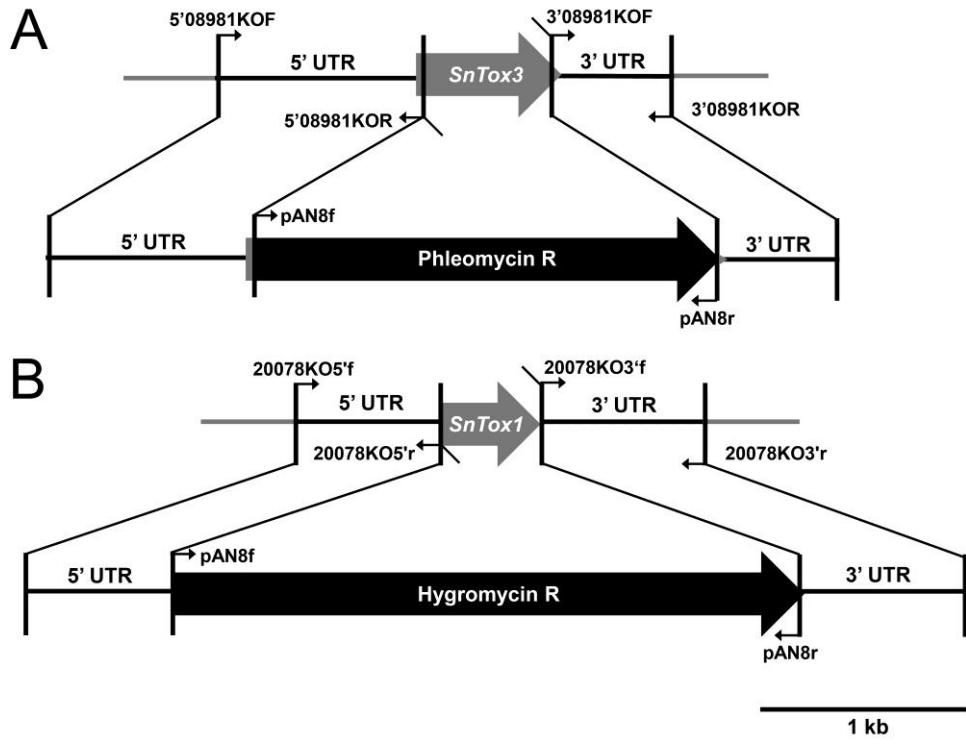
12 **Figure 4.** Relationship between SN15 culture filtrate sensitivity of 46 wheat cultivars and reactions
13 to **A.** SnTox1 ($p = 0.3561$), **B.** ToxA ($p = 0.3375$) and **C.** SnTox3 ($p < 0.0001$). The y axis
14 represents the proportion of effector sensitivity for each SN15 culture filtrate sensitivity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

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

24 \

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

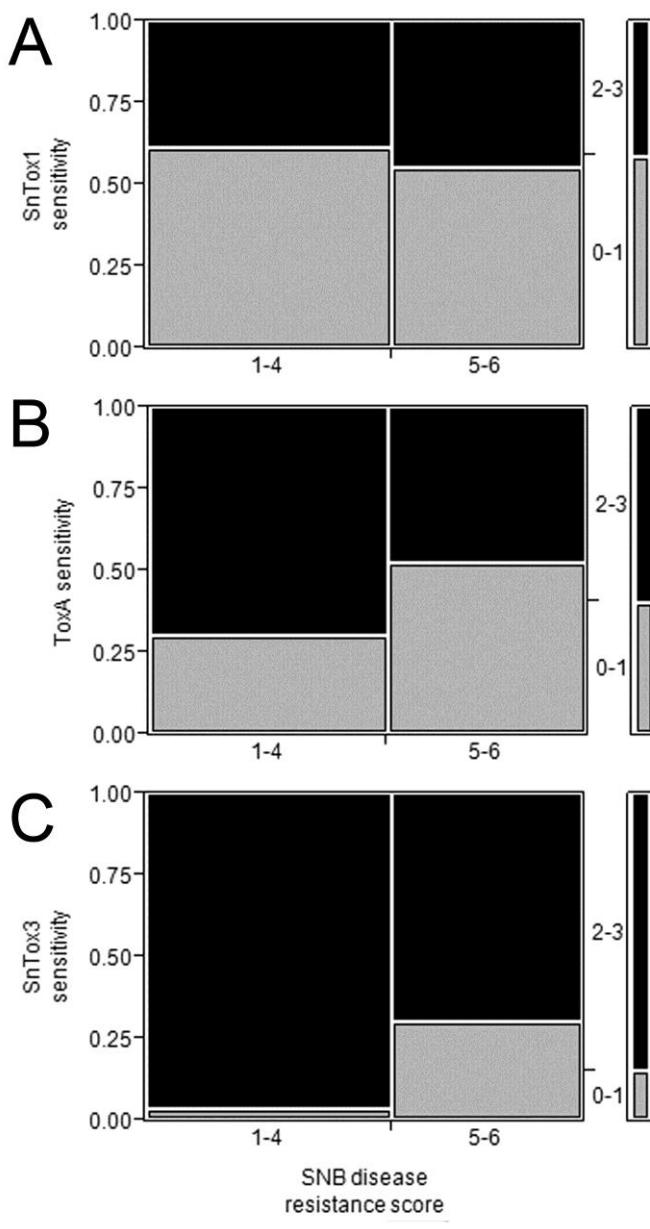
4 **Figure 7.** Relationship between seedling and adult plant sensitivity to **A.** SnTox1 ($p = 0.0868$), **B.**
5 ToxA ($p = 0.0057$) and **C.** SnTox3 ($p = 0.1368$). The y axis represents the proportion of seedling
6 effector sensitivity whereas the x axis denotes adult plant effector sensitivity.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



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2 **Figure 1.**

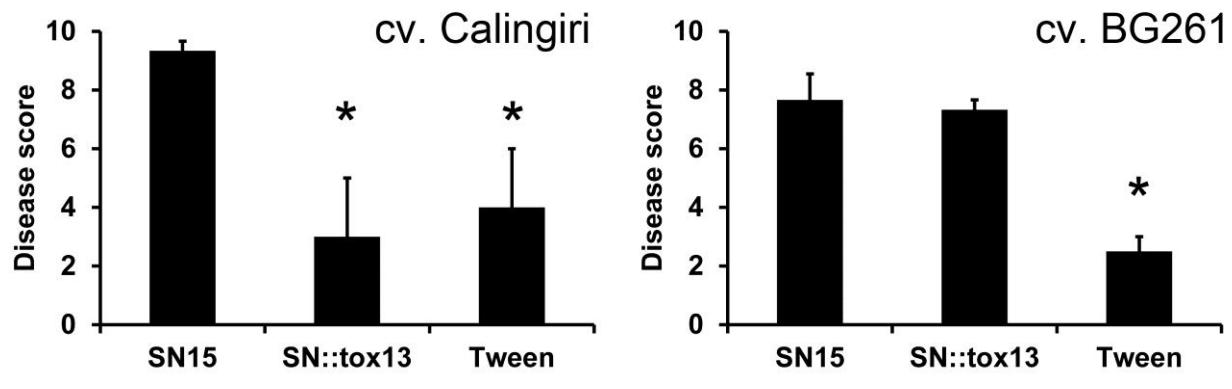
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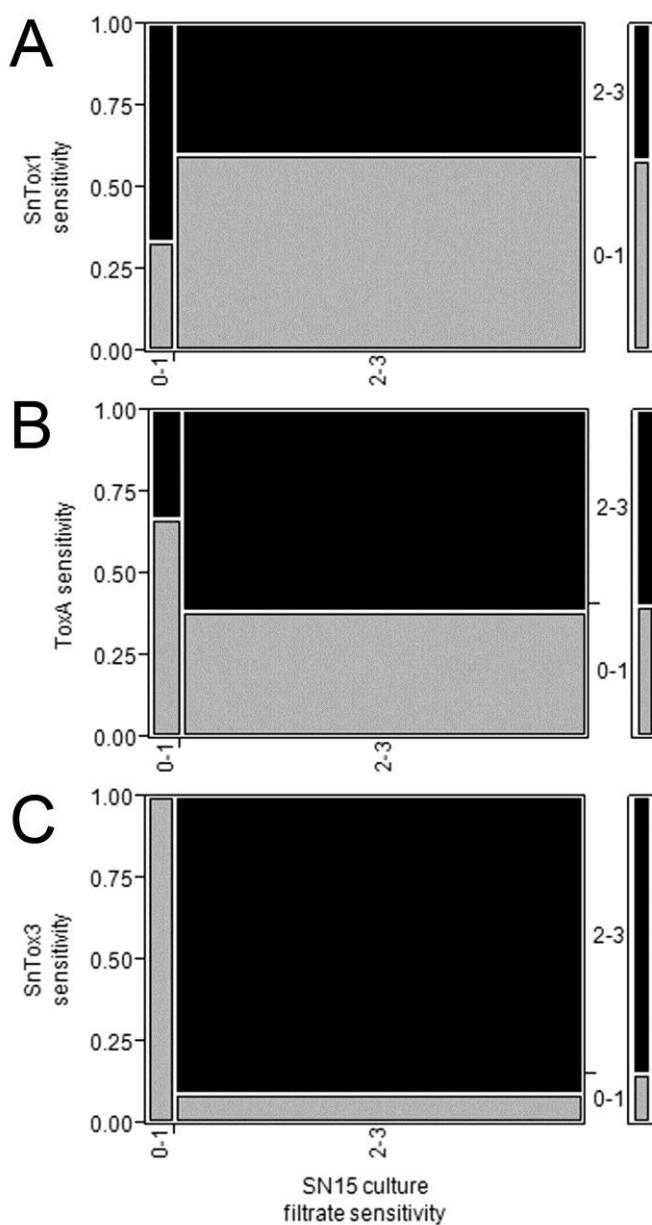


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2 **Figure 2.**

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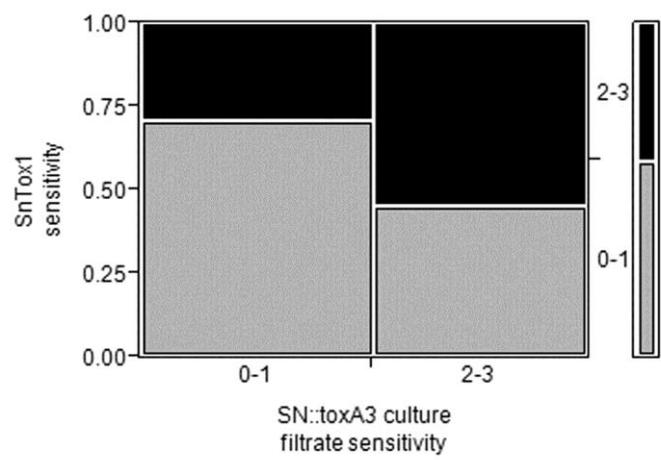


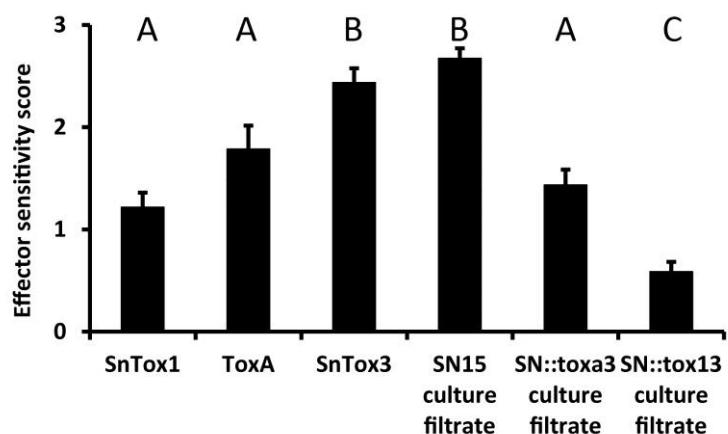


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2 **Figure 4.**

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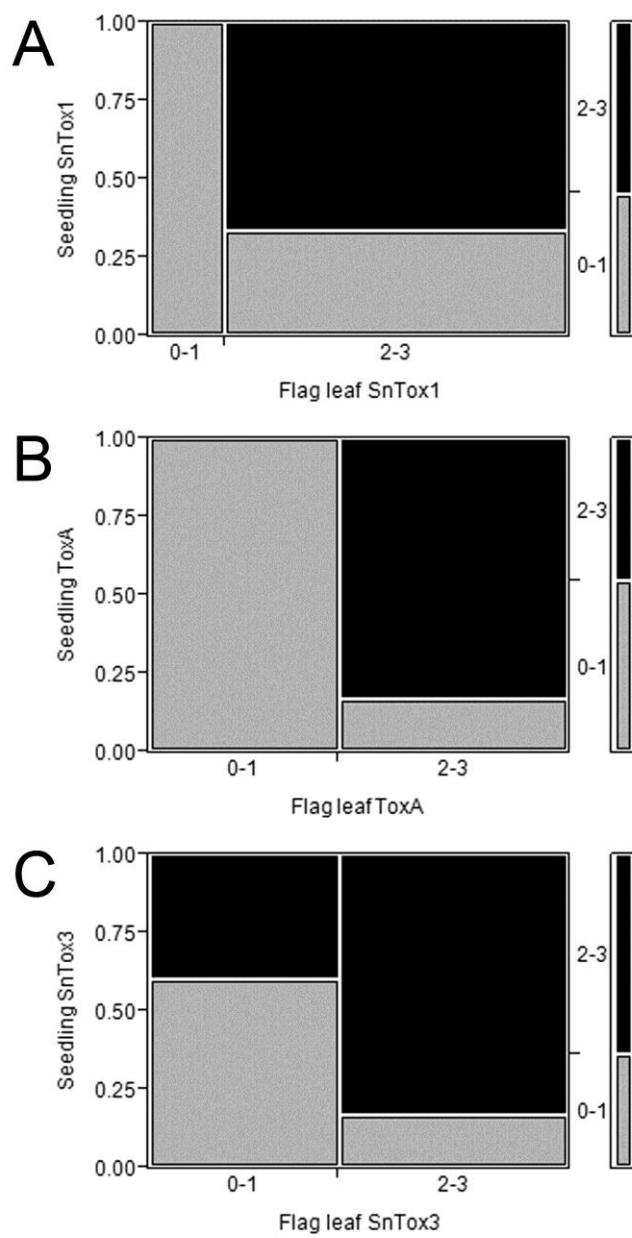




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2 **Figure 6.**

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2 **Figure 7.**