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# Differential effector gene expression underpins epistasis in a plant fungal disease

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## SUMMARY

**Fungal effector–host sensitivity gene interactions play a key role in determining the outcome of septoria nodorum blotch disease (SNB) caused by *Parastagonospora nodorum* on wheat. The pathosystem is complex and mediated by interaction of multiple fungal necrotrophic effector–host sensitivity gene systems. Three effector sensitivity gene systems are well characterized in this pathosystem; SnToxA–*Tsn1*, SnTox1–*Snn1* and SnTox3–*Snn3*. We tested a wheat mapping population that segregated for *Snn1* and *Snn3* with SN15, an aggressive *P. nodorum* isolate that produces SnToxA, SnTox1 and SnTox3, to study the inheritance of sensitivity to SnTox1 and SnTox3 and disease susceptibility. Interval quantitative trait locus (QTL) mapping showed that the SnTox1–*Snn1* interaction was paramount in SNB development on both seedlings and adult plants. No effect of the SnTox3–*Snn3* interaction was observed under SN15 infection. The SnTox3–*Snn3* interaction was however, detected in a strain of SN15 in which *SnTox1* had been deleted (*tox1–6*). Gene expression analysis indicates increased *SnTox3* expression in *tox1–6* compared with SN15. This indicates that the failure to detect the SnTox3–*Snn3* interaction in SN15 is due – at least in part – to suppressed expression of *SnTox3* mediated by SnTox1. Furthermore, infection of the mapping population with a strain deleted in *SnToxA*, *SnTox1* and *SnTox3* (*toxa13*) unmasked a significant SNB QTL on 2DS where the SnTox2 effector sensitivity gene, *Snn2*, is located. This QTL was not observed in SN15 and *tox1–6* infections and thus suggesting that *SnToxA* and/or *SnTox3* were epistatic. Additional QTLs responding to SNB and effectors sensitivity were detected on 2AS1 and 3AL.**

**Keywords: *Parastagonospora nodorum*, necrotrophic effector, NE, epistasis, SnTox1, SnTox3, *Triticum aestivum*.**

## INTRODUCTION

*Parastagonospora* (syn. *Stagonospora*; *Phaeosphaeria, Septoria*) *nodorum* (Berk.) Quaedvlieg, Verkley & Crous is the causal agent of septoria nodorum blotch (SNB) on wheat (Solomon *et al.*, 2006; Quaedvlieg *et al.*, 2013). The fungus causes significant damage to leaves and glumes of wheat (*Triticum aestivum*) and is responsible for substantial yield losses in many wheat growing areas. In Western Australia, losses are estimated to amount to AUD$108 million (~US$97 million) per annum (Eyal *et al.*, 1987; Murray and Brennan, 2009; Oliver *et al.*, 2009).

Resistance to SNB is complex and often reported to be quantitative (Nelson and Gates, 1982; Schnurbusch *et al.*, 2003; Xu *et al.*, 2004; Aguilar *et al.*, 2005; Friesen *et al.*, 2008a). For the past two decades, much effort has been made using genetic markers to breed for SNB resistance but with limited progress due to the complexity of the disease (Ma and Hughes, 1995; Czembor *et al.*, 2003; Schnurbusch *et al.*, 2003). The main difficulty in this process was the lack of understanding of the underlying mechanism of disease resistance/susceptibility and pathogen virulence. Studies since 2006 have shown that the paramount factors controlling the level of SNB are multiple fungal necrotrophic effectors (NEs) (Friesen *et al.*, 2006; Friesen and Faris, 2010; Oliver *et al.*, 2012). These effectors interact either directly or indirectly with the products of dominant sensitivity/susceptibility genes located throughout the wheat genome. When such an interaction is present, host tissue necrosis and/or chlorosis occurs that promotes infection by the pathogen leading to disease (Faris *et al.*, 2010; Tan *et al.*, 2010).

To date, three NE proteins encoded on single copy genes have been identified. *SnToxA* encodes a 13.2 kDa mature protein that causes necrosis on wheat varieties that carry *Tsn1* (Friesen *et al.*, 2006; Faris *et al.*, 2010). A near-identical *PtrToxA* is also found in the tan spot fungus and may have been acquired from *P. nodorum* through horizontal gene transfer (Ciuffetti *et al.*, 1997; Friesen *et al.*, 2006). *SnTox1* encodes a 10.3 kDa mature cysteine-rich protein with a chitin-like binding motif at the C-terminus. Sensitivity to SnTox1 is conferred by the *Snn1* gene located on wheat chromosome 1BS (Liu *et al.*, 2004, 2012). *SnTox3* is an intronless gene that codes for a 17.5 kDa mature protein with six cysteine residues. Sensitivity to SnTox3 is conferred by *Snn3-B1* and *Snn3-D1* located on wheat chromosomes 5BS and 5DS, respectively (Liu *et al.*, 2009; Zhang *et al.*, 2011). In addition, QTL analyses have revealed that the *P. nodorum*–wheat pathosystem is riddled with further effector–host sensitivity gene interactions such as SnTox2–*Snn2,* SnTox4–*Snn4* and SnTox5–*Snn5,* SnTox6-*Snn6* and SnTox7-*Snn7* (Friesen *et al.*, 2008a; Gao *et al.*, 2015; Shi *et al.*, 2015). Genes that code for these fungal effectors and host dominant susceptibility genes remained unidentified.

The NE model for SNB predicts that the severity of disease is a function of the number of effector/sensitivity gene interactions that operate in a given pathosystem (Friesen *et al.*, 2007; Tan *et al.*, 2012). However, investigations into the prevalence of SnToxA, SnTox1 and SnTox3 sensitivity in wheat cultivars revealed a poor correlation between the number of interactions, significance effect of each interaction and SNB severity (Oliver *et al.*, 2009; Waters *et al.*, 2011; Tan *et al.*, 2014). In this study, our aim was to model effector–host sensitivity gene interactions in the establishment of SNB. Infection trials were performed using an established mapping population in which the SnTox1–*Snn1* and SnTox3–*Snn3* interactions are defined and SnToxA sensitivity is absent. Our results have revealed evidence that SnTox1–*Snn1* is epistatic to SnTox3–*Snn3* during the establishment of SNB as well the presence of more disease QTLs. The mechanism of epistasis was investigated and appears to involve differential *SnTox3* expression.

## RESULTS

### Response to SnTox1 and SnTox3 effectors

SnTox1 and SnTox3 were used to screen 177 lines of the Calingiri × Wyalkatchem (C × W) doubled-haploid (DH) population for effector sensitivity. To ensure that SnTox1 possessed *Snn1*-specific activity, we tested the recombinant protein on known effector-sensitive (Chinese Spring) and insensitive (BG261) wheat lines (Liu *et al.*, 2012). As expected, Chinese Spring was sensitive to the SnTox1 whereas BG261 was insensitive (Figure S1). To ensure that SnTox3 possessed *Snn3*-specific activity, we tested the recombinant protein on known effector-sensitive (BG220) and effector-insensitive (BG261) wheat lines (Liu *et al.*, 2009). As expected, BG220 was sensitive to SnTox3 whereas BG261 was insensitive (Figure S1). These effectors were infiltrated into leaves of Calingiri, Wyalkatchem and DH lines of the C × W population. Infiltrated leaves were scored as reported in Tan *et al.* (2012) for chlorosis and/or necrosis in the infiltrated zones. Calingiri was sensitive to SnTox1 and insensitive to SnTox3 whilst Wyalkatchem was insensitive to SnTox1 and sensitive to SnTox3 according to their genotype groups (Figure 1(a)). The DH lines of the C × W population segregated in a ratio of 90:87 and 102:75 sensitive to insensitive to SnTox1 and SnTox3, respectively. A chi-squared test indicated that SnTox1 sensitivity fits a 1:1 ratio ($P = 0.88$) for the segregation of one gene controlling the effector sensitivity while that of SnTox3 was borderline insignificant suggesting there are other genes involved in response to SnTox3 ($P = 0.05$).

Segregation of the SnTox1 sensitivity in the population was aligned with diversity array technology (DArT) and simple sequence repeat (SSR) markers confirmed the co-location with *Snn1* on chromosome 1BS (Figure 2). 1BS explained 99% of the variation. Sensitivity to SnTox3 was mapped to 5BS and 4BL QTLs explaining 95 and 8% of the phenotypic variance observed in the experiment, respectively (Table 1). Both SnTox3 sensitivity QTLs are located in alleles that derived from Wyalkatchem. The 1BS and 5BS QTLs observed in this study are linked with markers that were previously demonstrated to be associated with *Snn1* (psp3000 and cfp618) and *Snn3* (wmc149 and cfd20), respectively (Friesen *et al.*, 2008b; Reddy *et al.*, 2008) (Figure 2). The 4BL QTL was not observed as a SnTox3 sensitivity locus in previous reports (Friesen *et al.*, 2008b; Liu *et al.*, 2009; Zhang *et al.*, 2011) and appears as a unique attribute of the Calingiri × Wyalkatchem population thus far.

### Response to *P. nodorum* SN15 infection on seedlings

Parents and DH lines were evaluated for susceptibility to the wild-type *P. nodorum* strain SN15 at the seedling stage using conidial inoculation (Figure 1(b)). The average disease score (DS) of SN15 on C × W lines was 7.3 (Figure 1(c)). Calingiri was significantly more susceptible (DS, 7.7) than Wyalkatchem (DS, 5.0) (Table 2). When analysed based on genotype groupings, DH lines that were sensitive to SnTox1 were significantly more susceptible to SN15 than DH lines that were SnTox1 insensitive (Table 2). However, we did not observe any significant differences in susceptibility to SN15 between SnTox3 sensitive and insensitive DH lines (Table 2).

QTL analysis was carried out to identify the genomic regions that associated with the reaction to the disease at the seedling stage caused by SN15. Average means for

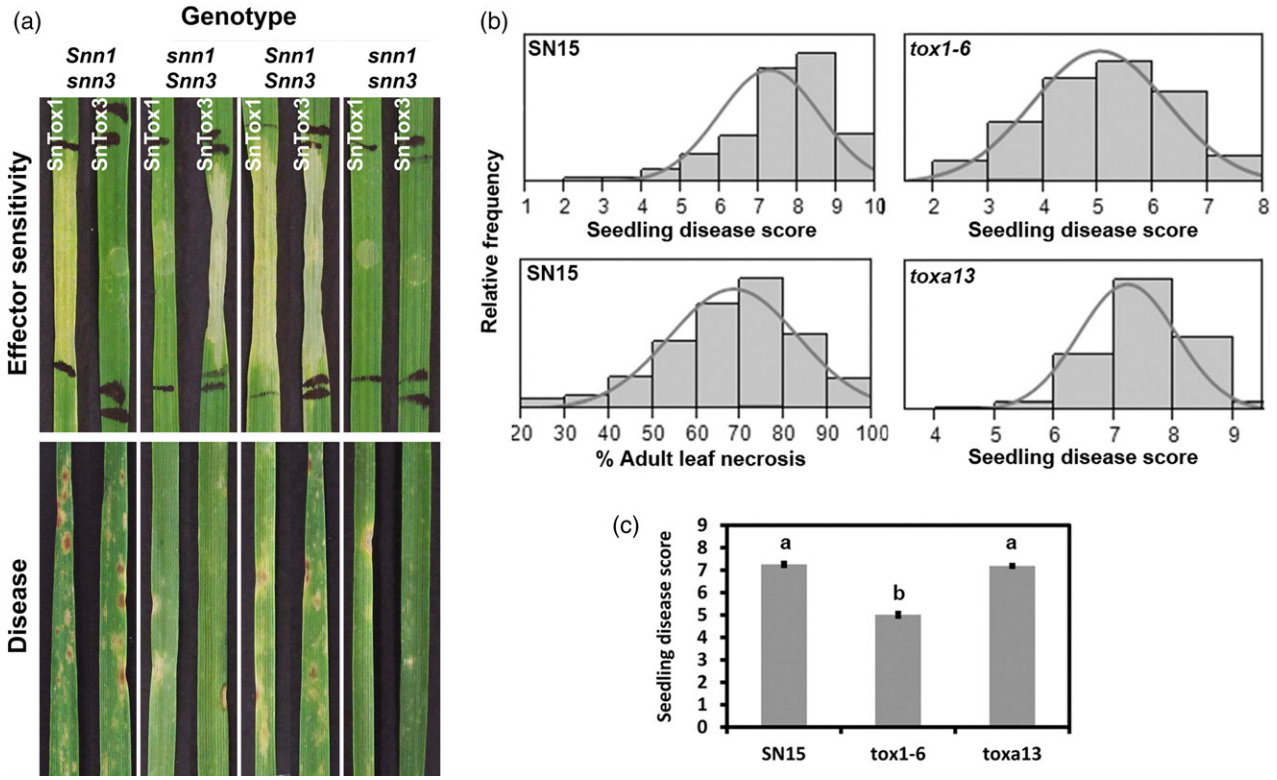


**Figure 1.** The SnTox1–*Snn1* interaction is a major SNB determinant in the C × W population.
(a) Visual assessment of effector sensitivity and disease symptom on C × W DH lines. SnTox1 and SnTox3 infiltrations and disease symptoms from conidial inoculation of *P. nodorum* SN15 on DH lines of four genotype groups: *Snn1*/*Snn3, Snn1*/*snn3, snn1*/*Snn3, snn1*/*snn3*. Representative DH lines for each genotype are shown.
(b) The distribution of SN15, *tox1–6* and *toxa13* disease severity scores on the C × W population at the seedling stage and SN15 at the adult stage.
(c) Statistical analysis was used to compare the average SNB seedling scores between SN15, *tox1–6* and *toxa13*. An analysis of variance (ANOVA) using the Tukey–Kramer test was used to compare all treatments. [a,b]Levels not connected by the same letter are significantly different. SN15–*tox1–6* $P < 0.001$; SN15–*toxa13* $P = 0.7530$; *toxa13*–*tox1–6* $P < 0.001$. Standard error bars are shown.

DSs were used for composite interval mapping (CIM). QTLs with logarithm of the odds (LOD) score of greater than 2.5 were identified in the genomic regions of 1BS, 2AS1 and 3AL (Table 1, Figure 2). The 1BS region corresponding to *Snn1* based on psp3000 and cfp618 markers had the largest contribution to seedling disease with 18% phenotype explained (Table 1 and Figure 2). Two other SNB QTLs were identified on chromosomes 2AS1 and 3AL, for which the susceptibility allele was derived from Calingiri (Table 1). Both QTLs contributed to 14% to the disease. We did not observe the 5BS and 4BL QTLs that were detected in response to SnTox3 infiltration.

### Response to *P. nodorum* SN15 infection on adult plants

To determine if the involvement of the SnTox3–*Snn3* interaction in SNB is age specific, 8-week-old wheat was infected with SN15 in an outdoor field trial. Mean reaction types of the DH lines ranged from 4.2 to 75% necrosis with an overall average of 32.34%. Calingiri and Wyalkatchem DSs were significantly different with average necrotic area of 29.2 and 12.5%, respectively (Table 2). SNB susceptibility was highly associated with sensitivity to SnTox1; DH lines that harboured the *Snn1* allele were more susceptible to SNB than those that harboured the *snn1* allele (Table 2). QTL analysis using CIM revealed a major QTL on chromosome 1BS, which explained 19% of the phenotypic variance. Another QTL on chromosome 6BS was also found to be significantly associated with SNB susceptibility albeit with smaller effect (Table 1 and Figure 2). Again, we did not observe the 5BS and 4BL QTLs that were associated with variation in sensitivity to SnTox3. Thus, SnTox1–*Snn1* is epistatic to the SnTox3–*Snn3* interaction.

### Response to *P. nodorum tox1–6* infection on seedlings

To dissect the mechanism of epistasis, we then created a *P. nodorum* SN15 strain deleted in *SnTox1* (*tox1–6*) and repeated the infection assay on the C × W population at the seedling stage (adult plant assays are not permitted due to genetically-modified organism biosafety regulations). The *P. nodorum tox1–6* isolate was less pathogenic overall than was SN15 (Figure 1C). There were no significant differences in disease susceptibility between the *Snn1* and *snn1* lines (Table 2). However, lines that were sensitive to SnTox3 (DS, 5.4) were significantly more susceptible to

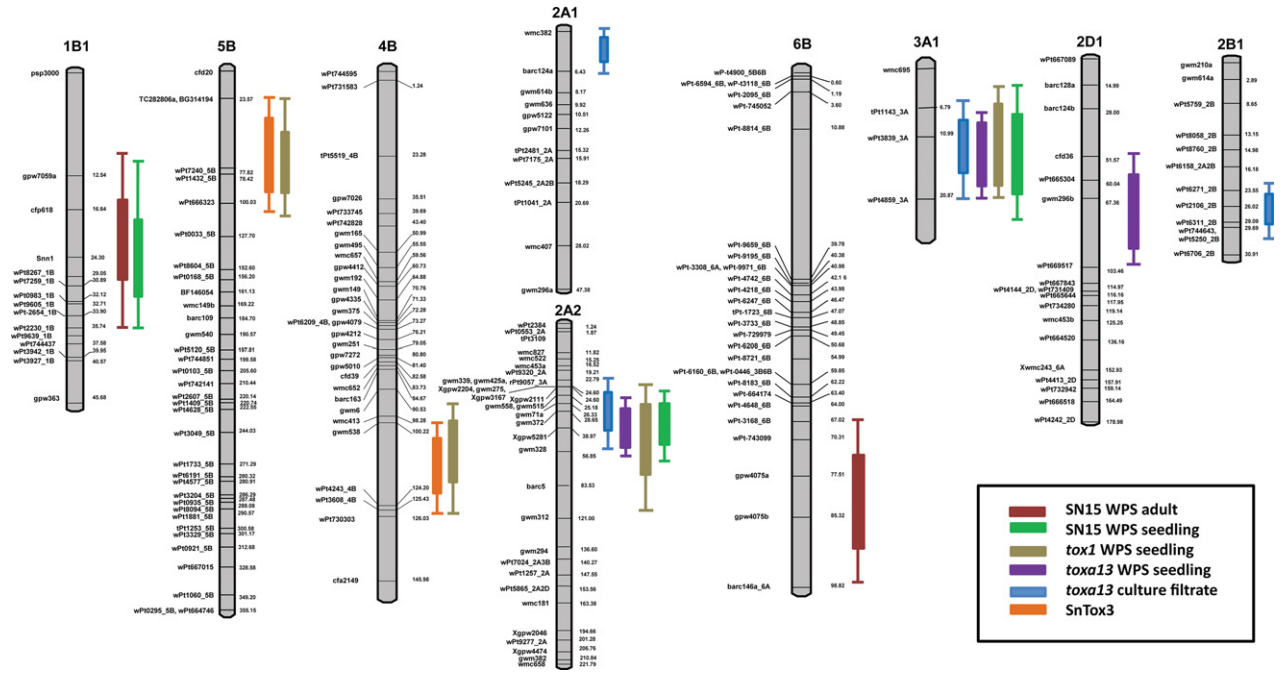


**Figure 2.** Composite interval mapping of quantitative trait loci (QTL) associated with *toxa13* CF sensitivity and SNB caused by SN15, *tox1–6* and *toxa13*. Genetic maps of the chromosomes with genetic markers on the right and the centimorgan (cM) distances between loci are shown on the left. *Snn1* (bold) was mapped as a trait marker in 1B1 in response to the SnTox1 effector.

*P. nodorum tox1–6* than were SnTox3-insensitive lines (DS, 4.7; $P = 0.0004$; Table 2). Using QTL mapping, we were able to detect the *Snn3* 5BS and 4BL QTLs which contributed to 9 and 10% of the disease, respectively (Table 1). This further demonstrated that SnTox1–*Snn1* is epistatic to SnTox3–*Snn3* in the establishment of SNB. Thus the removal of the *SnTox1* gene exposed the SnTox3–*Snn3* interaction that had been suppressed when SN15 was used as the infecting pathogen.

QTL analysis of the mean disease reaction scores conferred by *tox1–6* on the genetic linkage map developed in this study revealed four QTLs (2AS1, 5BS, 4BL and 3AL) that are associated with *tox1–6* seedling SNB (Figure 2 and; Table 1). The 2AS1 and 3AL QTLs were also associated with variation in the disease to conidial inoculation with SN15 while the 5BS and 4BL QTLs were associated with variation in sensitivity to SnTox3 (Figure 2). 3AL QTL was present contributing 8% to the total disease incidence (Table 1). However, the largest contribution to the disease caused by *P. nodorum tox1–6* was from 2AS1 QTL. This contribution was much greater in *tox1–6* suggesting that its effect may have been largely masked by the effect of the SnTox1–*Snn1* interaction (Table 1).

### Response to *P. nodorum toxa13* culture filtrate and infection

*P. nodorum toxa13* is a SN15 mutant that lacks *SnToxA, SnTox1* and *SnTox3* but retains pathogenicity and necrosis-inducing activities in the culture filtrate (CF) (Tan *et al.*, 2015). The strain and its CF were infected onto and infiltrated into the C × W population. A QTL on 2AS (2AS1) was associated with variation in sensitivity to the CF of *toxa13* that contributed to 13% of the phenotype observed (Figure 2 and Table 1). The virulence of *P. nodorum toxa13* on the C × W population at the seedling stage was comparable with that of SN15 (Figure 1(c)). The 2AS1 and 3AL QTLs were also associated with variation in susceptibility in the seedling disease. The contribution of the 2AS1 QTL in SNB was much greater in the *SnTox1* mutants (*toxa13 and tox1–6*) than SN15 thus indicating that SnTox1–*Snn1* was epistatic to the effector–2AS1 interaction. As expected, the 5BS and 4BL QTLs were not detected during *P. nodorum toxa13* infection and CF infiltration as *SnTox3* was deleted.

A significant seedling disease QTL, which is on an allele derived from Wyalkatchem, was detected on 2DS close to the marker cfd36. This marker is also located close to the genomic location of *Snn2* on 2DS (Shi *et al.*, 2015). Intriguingly, the 2DS QTL was not observed with the SN15 and *tox1–6* infections. We have also identified two additional QTLs (2AS2 and 2BS) that were associated with variation in sensitivity to *toxa13* CF. However, these QTLs were not observed to be associated with variation in the seedling disease.

### *SnTox3* expression was elevated in the absence of *SnTox1*

We previously demonstrated that the *SnTox1* and *SnTox3* genes were highly expressed during the early stage of

**Table 1** A summary of SNB and effector sensitivity QTLs identified in this study. Details of the flanking markers, LOD scores, phenotype contribution ($R^2$) and parental effect of these QTLs are indicated. Positive and negative effects indicate the allele was inherited from Calingiri and Wyalkatchem, respectively

| Treatment | Chromosome arm | QTL | Locus/QTL flanking markers | LOD | $R^2$ | Effect |
|---|---|---|---|---|---|---|
| SnTox1 | 1BS | QSnb.fcu–1BS | *Snn1* | 151 | 99 | 1.96 |
| SnTox3 | 5BS | QSnb.fcu–5BS | *Snn3* | 268 | 95 | −3.95 |
| | 4BL | Qsnb.cur–4BL | wmc413–wPt-730303 | 2.5 | 8 | −1.15 |
| Seedling infection with SN15 | 1BS | QSnb.fcu–1BS | *Snn1* | 7 | 18 | 0.92 |
| | 2AS1 | Qsnb.cur–2AS1 | gwm515–gwm328 | 3 | 7 | 0.52 |
| | 3AL | Qsnb.cur–3AL | tPt-1143–wPT-4859 | 3 | 7 | 0.57 |
| Adult plant infection with SN15 | 1BS | QSnb.fcu–1BS | *Snn1* | 7.3 | 19 | 13 |
| | 6BS | Qsnb.cur–6BS | wPt-3168–Xbarc146a | 3.0 | 10 | 9.11 |
| Seedling infection with *tox1-6* | 2AS1 | Qsnb.cur–2AS1 | gwm339–gwm312 | 7.4 | 29 | 1.34 |
| | 5BS | QSnb.fcu–5BS | *Snn3* | 3.5 | 9 | −0.74 |
| | 4BL | Qsnb.cur–4BL | barc163–wPt-4243 | 2.7 | 10 | −0.76 |
| | 3AL | Qsnb.cur–3AL | tPt-1143–wPT-4859 | 2.7 | 8 | 0.67 |
| Seedling infection with *toxa13* | 2AS1 | Qsnb.cur–2AS1 | tPt-8937–gwm312 | 3.2 | 14 | 0.51 |
| | 2DS | Qsnb.cur–2DS | cfd36–wPt-669517 | 5.2 | 15 | −0.53 |
| | 3AL | Qsnb.cur–3AL | tPt-1143–wPT-4859 | 3.2 | 9 | 0.41 |
| Culture filtrate of *toxa13* | 2AS1 | Qsnb.cur–2AS1 | wPt-9320–gwm328 | 4 | 13 | 0.79 |
| | 3AL | Qsnb.cur–3AL | tPt-1143–wPT-4859 | 3.9 | 11 | 0.72 |
| | 2AS2 | Qsnb.cur–2AS2 | wmc382a–barc124a | 2.9 | 8 | −0.64 |
| | 2BS | Qsnb.cur–2BS | wPt-6271–wPt-6311 | 2.6 | 7 | −0.59 |

**Table 2** Least significant difference (LSD) was used to compare the susceptibility of different genotype combination the C × W population to SN15 and *tox1–6* infection. SN15 was used to assess seedling and adult plant infection. *P. nodorum tox1–6* was used to assess seedling plant infection only

| Genotype | No. of lines | Mean | Symptom range | LSD | *P*-value |
|---|---|---|---|---|---|
| SNB adult (SN15) | | | % necrosis | | |
| Calingiri | – | 29.2 | 18.82–39.52 | 2.03 | 0.034[a] |
| Wyalkatchem | – | 12.5 | 2.16–22.85 | | |
| Flag combined–*Snn1* | 86 | 35.5 | 32.38–38.61 | 1.98 | 0.005[a] |
| Flag combined–*snn1* | 83 | 29.1 | 25.90–32.24 | | |
| Flag combined–*Snn3* | 75 | 33.5 | 30.12–36.93 | −2.43 | 0.358 |
| Flag combined–*snn3* | 94 | 31.4 | 28.35–34.43 | | |
| Flag combined–*snn1/Snn3* | 40 | 31.3 | 26.42–36.25 | −2.46 | 0.206 |
| Flag combined–*snn1/snn3* | 43 | 27.0 | 22.21–31.7 | | |
| SNB seedling (SN15) | | | 1 (resistant) to 9 (fully necrotised) | | |
| Calingiri | – | 7.7 | 6.23–9.10 | 0.82 | 0.016[a] |
| Wyalkatchem | – | 5.0 | 2.52–7.48 | | |
| Seedling–*Snn1* | 86 | 7.5 | 7.31–7.74 | 0.21 | 0.001[a] |
| Seedling–*snn1* | 84 | 7.0 | 6.70–7.23 | | |
| Seedling–*Snn3* | 75 | 7.4 | 7.09–7.61 | −0.18 | 0.395 |
| Seedling–*snn3* | 95 | 7.2 | 7.01–7.41 | | |
| Seedling–*snn1/Snn3* | 40 | 7.2 | 6.89–7.55 | −0.06 | 0.088 |
| Seedling–*snn1/snn3* | 44 | 6.8 | 6.50–7.15 | | |
| SNB seedling (*tox1–6*) | | | 1 (resistant) to 9 (fully necrotised) | | |
| Calingiri | – | 5.3 | 3.87–6.80 | −0.4 | 0.089 |
| Wyalkatchem | – | 3.7 | 2.20–5.13 | | |
| Seedling–*Snn1* | 85 | 4.9 | 4.61–5.13 | −0.11 | 0.161 |
| Seedling–*snn1* | 84 | 5.1 | 4.87–5.40 | | |
| Seedling–*Snn3* | 74 | 5.4 | 5.10–5.64 | 0.3 | 0.0004[a] |
| Seedling–*snn3* | 95 | 4.7 | 4.48–4.95 | | |

[a]Denote: significant difference ($P < 0.05$).

infection (Liu *et al.*, 2009; Ipcho *et al.*, 2012). Here, we investigated if these effector genes are expressed in the presence and absence of their corresponding effector sensitivity genes and if their expression levels are associated with the SNB susceptibility using quantitative real-time PCR (qRT-PCR). Twenty DH lines that encompass all four genotypic combinations (*Snn1/Snn3, Snn1/snn3, snn1/Snn3* and *snn1/snn3*) were used for expression analysis of *SnTox1* and *SnTox3* with SN15. No significant differences in *SnTox1* and *SnTox3* expression were observed during infection on all the genotype combinations (Figure 3). This indicates that differences in the wheat genotype do not influence *SnTox1* and *SnTox3* expression in SN15.

We then determined the *SnTox3* expression levels in *P. nodorum tox1–6* infection on *Snn1/Snn3, Snn1/snn3, snn1/Snn3* and *snn1/snn3* genotypes to see if *SnTox1* deletion resulted in differential *SnTox3* expression under this combination of genotypes (Figure 4(a)). No significant differences in *SnTox3* expression in *P. nodorum tox1–6* against all possible genotype combinations were observed.

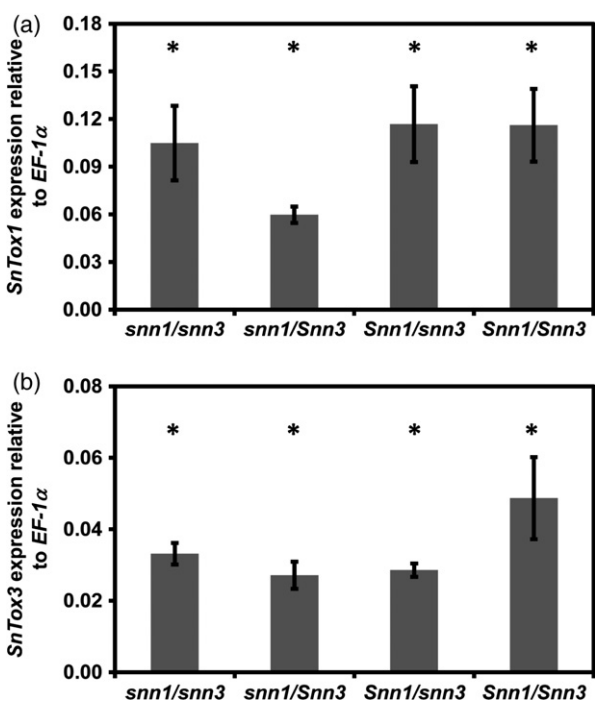


**Figure 3.** The host genotype does not affect the level of *SnTox3* expression in SN15 during infection.
(a) *SnTox1* expression was compared among *snn1/Snn3, snn1/snn3, Snn1/Snn3* and *Snn1/snn3* C × W DH genotype groups. No significant differences in *SnTox1* expression was observed ($P = 0.371$).
(b) No significant differences in *SnTox3* expression was observed in all genotype groups ($P = 0.396$). Average gene expression was calculated from four lines per genotype performed in biological replicates ($n = 4$). Standard error bars are shown. '*'Indicates similar statistical grouping using ANOVA.

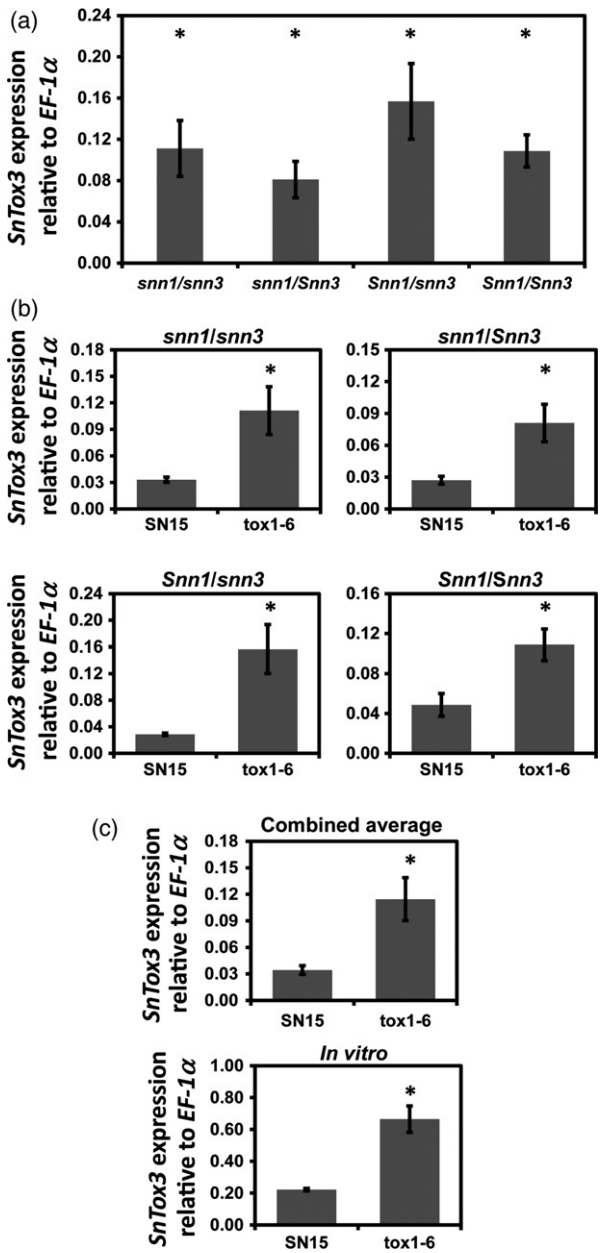


**Figure 4.** *SnTox3* expression is elevated in the *SnTox1* deletion background.
(a) Comparison of *SnTox3* expression in *P. nodorum tox1–6* during infection of *snn1/Snn3, snn1/snn3, Snn1/Snn3* and *Snn1/snn3* C × W DH groups. '*'Indicates similar statistical grouping using ANOVA ($P = 0.808$). Average gene expression was calculated from four lines per genotype performed in biological replicates ($n = 4$).
(b) A comparison of *SnTox3* expression in SN15 and *tox1–6* during infection on different genotype combinations. Average gene expression was calculated from four lines per genotype performed in biological replicates ($n = 4$).
(c) *SnTox3* expression in SN15 and *tox1–6* during growth *in vitro*. '*'Denotes significant difference in a *t*-test. The *SnTox3* expression profile was also tested on two other *tox1* isogenic strains *in vitro* (Figure S4). The experiment was performed in biological triplicates. Standard error bars are shown for all graphs.

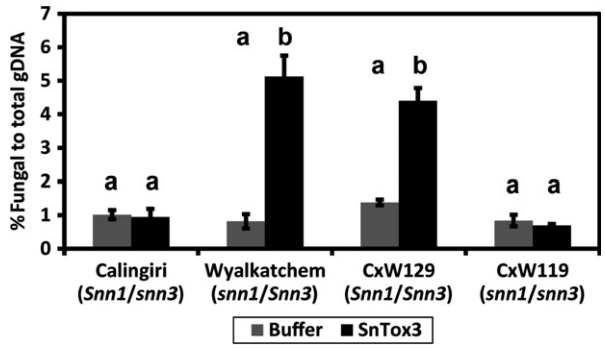


**Figure 5.** SnTox3 infiltration increases the virulence of *P. nodorum* SN15 on *Snn3* wheat cultivars.
Total fungal DNA was measured from the amplification of *Act1* normalised against total DNA. A *t*-test was used to compare the amount of fungal DNA between SnTox3- and buffer-infiltrated treatments infected with SN15. [a,b]Levels not connected by the same letter are significantly different ($P < 0.05$). Standard error bars are shown. The experiment was performed in biological triplicates.

This confirms that the wheat genotype does not influence *SnTox3* expression in *P. nodorum tox1–6.*

The expression of *SnTox3* in SN15 and *tox1–6* on all four possible wheat combinations was compared. Expression data were collected from lines representing all four possible genotype combinations. *SnTox3* expression was consistently greater in *tox1–6* than SN15, ranging from 2.2- to 5.4-fold more (Figure 4(b)). When averaged, *SnTox3* expression in *P. nodorum tox1–6* was 3.3-fold more than in SN15 (Figure 4(b)). The expression of *SnTox3* was also significantly higher in *tox1–6* than SN15 when grown in a broth medium conducive to SnTox3 production (Figure 4(c)). This suggests that the regulation of *SnTox3* expression is linked to *SnTox1* at the pathogen level independent of the host. To ensure that this observation is not a strain-specific artefact, we then examined *SnTox3* expression in two other independent *SnTox1*-deleted strains *tox1–7* and *tox1–8 in vitro.* Like *tox1–6, SnTox3* expression was significantly higher in *tox1–7* and *tox1–8* than SN15 (Figure S4).

To determine if an oversupply of SnTox3 increases the virulence of *P. nodorum,* we pre-infiltrated *Snn3* and *snn3* lines with SnTox3 protein and a buffer control. Following this, we infected infiltrated plants with *P. nodorum* SN15. This approach provides the SN15 wild-type with a surplus of SnTox3 during the infection process. Fungal biomass was determined using qRT-PCR analysis of the genomic DNA content. It was observed that there was 3.2- to 6.2-fold more fungal biomass in *Snn3* wheat lines infiltrated with SnTox3 than those infiltrated with the buffer control (Figure 5). Conversely, SnTox3 infiltration in wheat cultivars that lacked *Snn3* did not result in an increased fungal biomass of SN15 (Figure 5). This suggests that an oversupply of SnTox3 greatly accelerates SN15 growth on *Snn3* but not *snn3* wheat lines.

## DISCUSSION

Effector-assisted breeding has been enthusiastically adopted by Australian plant breeders to combat both SNB and tan (yellow leaf) spot (TS) (Vleeshouwers and Oliver, 2014). In the case of TS, insensitivity to ToxA is a dominating factor and strongly predicts SNB susceptibility at both seedling and adult stages. The use of ToxA to screen germplasm for sensitivity throughout the breeding process has accordingly made a demonstrable impact on disease losses (Vleeshouwers and Oliver, 2014). In the case of SNB, a more complex situation exists and therefore the priorities and strategies for the use of effectors are not obvious. Three SNB effectors have been deployed for effector-assisted breeding and others remain to be discovered. All recommended wheat cultivars for sowing in 2013 by the Department of Agriculture and Food of Western Australia were sensitive to at least one of the known effectors. Seventy per cent and more than 90% of those cultivars were sensitive to SnTox1 and SnTox3 respectively (Tan *et al.*, 2014). Moreover, *SnTox1* and *SnTox3* were present in 96% of Australian *P. nodorum* isolates (McDonald *et al.*, 2013). This current study was designed therefore to investigate how these two defined interactions operate together in causing SNB using a DH population developed from two popular varieties, Calingiri and Wyalkatchem, uniquely sensitive to SnTox1 and SnTox3 respectively.

### Effector sensitivity assay predicts adult plant responses

SNB is a complex disease. Numerous QTLs identified in almost every wheat chromosome have been reported, some of them are specifically identified at seedling stage, some are exclusive to adult plants (Czembor *et al.*, 2003; Schnurbusch *et al.*, 2003; Arseniuk *et al.*, 2004; Aguilar *et al.*, 2005; Friesen *et al.*, 2008a, 2012; Shankar *et al.*, 2008; Abeysekara *et al.*, 2009; Oliver *et al.*, 2012). It is therefore crucial that SNB resistance operates both at seedling and adult plant stages. This study shows that *Snn1* confers susceptibility to SNB and was a contributor to the disease on seedlings and in adult plants. Similar results were also demonstrated by Friesen *et al.* (2009) where SnToxA–*Tsn1* and SnTox2–*Snn2* interactions predicted adult resistance. Thus, SnTox1 sensitivity is a good indicator of the plant response at the seedling and adult growth stages to *P. nodorum* isolates possessing *SnTox1.* It was noted that in all infection and CF infiltration assays, only a portion of the phenotypic variation can be explained by genetic factors. For instance, only 29% of the phenotypic variation was explained by QTLs where the LOD score is >2.5 in the adult plant infection assay. This can be partly explained by environmental factors such as natural infections (Schnurbusch *et al.*, 2003), minor QTLs (LOD score <2.5), the not fully covered genetic linkage map used

in this study and/or parents of the DH population that may possess a common SNB susceptibility allele.

**The role of effector epistasis**

Both epistatic and additive interactions have been observed in previous studies with SNB effectors (Friesen *et al.*, 2007, 2008b, 2009) but the mechanism(s) have not been analysed. Three compatible interactions (SnTox3–*Snn3,* SnTox2–*Snn2* and SnToxA–*Tsn1*) were analysed in both SNB seedling and adult plants. SnTox2–*Snn2* and SnToxA–*Tsn1* interactions were found to be additive and contributed significantly to the SNB susceptibility in both seedling and adult stages whilst the SnTox3–*Snn3* interaction was not detected (Friesen *et al.*, 2009). In addition to SnToxA–*Tsn1* and SnTox2–*Snn2,* the SnTox5–*Snn5* interaction is epistatic to SnTox3–*Snn3* (Friesen *et al.*, 2012). The frequent observation that the SnTox3–*Snn3* interaction can be eliminated by most of the other known NE interactions is intriguing and so far unexplained. We investigated two questions to explain the epistasis of SnTox1–*Snn1* over SnTox3–*Snn3* in order to further dissect this phenomenon. Firstly, is the epistasis occurring at the host or pathogen level? Lastly, was *SnTox3* expression suppressed in the presence of *SnTox1*?

The mechanism of necrotrophic effector epistasis has remained obscure until now. Friesen *et al.* (2008b) proposed that possible mechanisms of epistasis might involve effector gene expression, host gene action, and/or interaction among associated pathways. Another explanation is that several pathways may be involved in effector-mediated disease. Multiple effectors could be using the same pathway and only the most efficient effector will prevail and manifest disease. We examined several possible mechanisms to explain the epistasis of SnTox3–*Snn3* by SnTox1–*Snn1.* Firstly, we evaluated *Snn1* and *Snn3* for evidence of interactions that could explain the mechanism of epistasis but found no evidence that suggests a QTL × QTL effect. Interaction between QTLs involved in disease resistance has been observed in other pathosystems (Wang *et al.*, 1994; Lefebvre and Palloix, 1996; Kump *et al.*, 2011; Liu *et al.*, 2014). Uphaus *et al.* (2007) examined SNB QTLs for glume resistance and found no evidence of QTL interactions. This eliminates the role of epistasis dictated at the host level. We then examined for indication of effector gene expression regulation by the recognition loci in wheat. We found no evidence to indicate that expression of *SnTox1* and *SnTox3* was influenced by the plant genotype differing in the *Snn1/snn1*–1BS and *Snn3/snn3*–5BS loci. Faris *et al.* (2011) observed that the expression of *SnToxA* was upregulated in the *P. nodorum* SN5 American wild-type isolate in response to different wheat genotypes. This phenomenon appears isolate-specific as the expression of *SnToxA* from the *P. nodorum* SN4 American wild-type isolate did not differ significantly between different wheat genotypes (Faris *et al.*, 2011).

We then hypothesised that epistasis between the SnTox1–*Snn1* and SnTox3–*Snn3* is occurring at the pathogen level. We observed a significantly higher expression of *SnTox3* in the absence of *SnTox1* (Figure 4) indicating that the SnTox1–*Snn1* epistasis over SnTox3–*Snn3* observed in this study occurred at the pathogen level through transcriptional suppression. This finding is consistent with the observation of Faris *et al.* (2011) of a positive correlation between the expression level of *SnToxA* and disease contribution of the SnToxA–*Tsn1* interaction.

**Unmasking other SNB and effector sensitivity QTLs**

In addition to *Snn3* on 5BS, a homeologous *Snn3* was identified from the 'D' genome donor *Aegilops tauschii,* (Zhang *et al.*, 2011). In this study, we did not observe the homeolog but identified another minor SnTox3 sensitivity QTL on 4BL that was detected together with *Snn3* from SnTox3 infiltration and reaction to *tox1–6* infection. The 4BL QTL was also identified as a glume blotch resistance QTL in the Arina × Forno winter wheat population through a common genetic marker (gwm538) (Schnurbusch *et al.*, 2003).

Other SNB QTLs were identified on chromosomes 2AS1, 3AL, 2DS and 6BS. Not surprisingly, the phenotypic effects of these SNB QTLs was greater in *P. nodorum* mutant strains that lacked *SnTox1* and all three cloned effectors thus further demonstrating evidence of effector epistasis. The 6BS QTL was observed in adult SNB in this study while previously, Aguilar (2004) identified a 6B QTL that was associated with seedling SNB. The 2AS1 SNB QTL detected in this study was also identified as a SNB QTL in the Arina × Forno population infected with *P. nodorum* through a common genetic marker (gwm372) (Abeysekara *et al.*, 2009). The 2AS1 and 3AL SNB QTLs also co-located with effector sensitivity QTLs that were detected in *P. nodorum toxa13* CF infiltration assays. As such, a biochemical purification approach is currently being used to identify these effectors.

Interestingly, the *toxa13* isolate is as virulent as SN15. The presence of *SnToxA, SnTox1* and *SnTox3* may have an epistatic effect on the activity or expression of undiscovered effectors as in the case of *SnTox3* in the *tox1–6* isolate. In the TS fungus *Pyrenophora tritici-repentis,* ToxA is epistatic to the activity of undiscovered host-selective toxins and disease (Manning and Ciuffetti, 2015). We detected the 2DS QTL in the seedling disease only when *SnToxA, SnTox1* and *SnTox3* were deleted in *P. nodorum.* We hypothesise that the removal of *SnToxA, SnTox1* and *SnTox3* unmasked the SnTox2–*Snn2* interaction which then contributed to the disease using the C × W population. However, the activity of SnTox2 was not detected in the *toxa13* CF. This *in vitro* condition also does not induce

significant SnToxA production in our hands (Tan *et al.*, 2014). This suggests that *SnTox2* is not expressed *in vitro* or it is masked by other undiscovered effectors in the CF.

In conclusion, the epistatic effect was not due to interactions of genes within the host but rather through interference in gene expression levels in the pathogen. Furthermore, we have further demonstrated functional redundancy of the *P. nodorum* effector system with evidence of increased disease contributions in the absence of *SnToxA, SnTox1* and *SnTox3.* More work will certainly be followed to obtain a deeper insight into the mechanism of epistasis and effector gene regulation. As such, we are using several approaches that include RNAseq to identify candidate effector genes in *SnToxA, 1* and *3* knockout mutants and exonuclease protection assays of effector gene promoters to identify DNA-binding regulatory proteins.

## EXPERIMENTAL PROCEDURES

### Biological material

A wheat population consisting of 177 DH lines derived from a cross between Calingiri and Wyalkatchem were used for molecular mapping and genetic analysis of reactions to effectors and SNB caused by an Australian *P. nodorum* wild-type isolate.

All *P. nodorum* strains used in this study were maintained on V8-PDA agar (150 ml $L^{-1}$ Campbell's V8 juice, 3 g $L^{-1}$ $CaCO_3$, 10 g $L^{-1}$ Difco PDA and 10 g $L^{-1}$ agar) at 21°C under a 12-h photoperiod prior to scoring.

### Construction of *SnTox1* gene deletion *P. nodorum* mutants

In this study, *P. nodorum* SN15 strains carrying a *SnTox1* deletion were constructed through homologous recombination using the *SnTox1* gene knockout method described in Tan *et al.* (2014). The *SnTox1* deletion construct harbouring a phleomycin resistance cassette (Tan *et al.*, 2014) was transformed into SN15. PCR was used to identify the appropriate mutants deleted in *SnTox1.* PCR was used to validate transformants that carry *SnTox1* deletion. Three isolates carrying the desired *SnTox1* deletion were identified by PCR. All three strains possessed a single integration of the *SnTox1* knockout cassette (Figure S2). From here, *tox1* mutants were confirmed to carry only a single copy insertion using primers 5_Tox1qPCR-F and 5_Tox1qPCR-R (Figure S2 and Table S1) by a robust quantitative PCR approach (Solomon *et al.*, 2008). *P. nodorum tox1–6* possesses single copy vector integration and was retained for phenotypic analysis.

### Effector expression and infiltration

Proteins derived from *SnTox1* (SNOG_20078; GenBank acc, XP_001797505) and *SnTox3* (SNOG_08981; GenBank acc; XP_001799284) were expressed in *Pichia pastoris* using the pGAPzA expression vector. CFs containing the expressed protein were harvested and desalted with 10 mM sodium phosphate buffer pH 7.0 as previously described (Liu *et al.*, 2009).

CF containing necrosis-inducing factors from *P. nodorum tox-a13* (SN15 deleted in *SnToxA, SnTox1* and *SnTox3*) was produced in Fries 3 broth as previously described (Liu *et al.*, 2004; Tan *et al.*, 2015). The CF was filter sterilised prior to plant infiltration.

Effector assays were performed using a simple leaf infiltration technique (Oliver *et al.*, 2009). A 1-mL plastic syringe was used to infiltrate the expressed proteins into the first leaf of 2-week-old wheat seedlings. Plants were kept in a Conviron growth chamber for 5 days at 21°C under a 12-h photoperiod prior to scoring. Following this, plants were visually evaluated for effector sensitivity on a scale of '0' to '4' as described in Tan *et al.* (2012). A score of 0 indicates insensitivity (no reaction); 1, slight chlorosis; 2, extensive chlorosis; 3, extensive chlorosis with some necrosis; and 4, complete necrosis. CF 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* and *SnTox3* were caused by the expressed protein (Waters *et al.*, 2011; Tan *et al.*, 2014). All infiltrations were carried out in biological triplicates.

### Whole plant infection assay

Seedling infection was performed by whole plant spray as described in Solomon *et al.* (2003). Pycnidiospore inoculum was prepared to a concentration $1 \times 10^6$ spores $ml^{-1}$ in 0.5% (w/v) of gelatin. The 177 DH lines, together with parental lines were planted in a completely randomised design in three replicates. Six seeds of each line were planted in a 12 cm-dimension pot and considered as a repeat. Two-week-old wheat seedlings were sprayed with the inoculum preparation using a hand-held air brush sprayer onto 2-week-old seedlings until run-off. Plants were placed in 100% relative humidity at 21°C in the light for 48 h, followed by 5 days at 21°C under a 12-h photoperiod prior to scoring. An observed score of one indicates no disease symptoms and a score of nine indicates a fully necrotised plant.

For the adult plant assay, the C × W population was evaluated for SNB susceptibility using an outdoor field trial. Here, 177 DH lines and parents were planted in a randomised complete block in hill-plots seeding method of 8–10 seeds each hill/line. The field trial arranged in 10 hills wide with 30 cm spacing between hills in all directions. The field trial was bordered by two rows Yitpi variety. The SN15 pycnidiospore inoculum consisting of $1 \times 10^6$ spores $mL^{-1}$ in 0.5% w/v gelatin was sprayed on 8-week-old plants until run-off. Disease was allowed to develop for 14 days before scoring. Within each hill plot, three individual plants were chosen randomly for disease scoring. Disease severity was scored on flag leaves-2 (Flag-2) and flag leaves-3 (Flag-3) as percentages of necrotic leaf-area (Eyal *et al.*, 1987). The infection assay was conducted in the winter growing season that is conducive to the development of SNB (http://www.herbiguide.com.au/Descriptions/hg_Septoria_Nodorum_Blotch_of_Wheat.htm).

### Molecular mapping and QTL analysis

In total, 365 DArT and 117 wheat genomic SSR and EST-SSR markers were used to construct a genetic map of this C × W population (Figure S3). The DArT marker set was generated from wheat DArT (Diversity Arrays Technology Pty Ltd, Australia; http://www.diversityarrays.com/). The 117 genomic SSR and EST-SSR markers were added only to chromosomes/chromosome arms of interest including 1BS, 5BS, 4B and 2A. The mapped markers were identified to be polymorphic between Calingiri and Wyalkatchem of the total 345 SSR and 50 EST-SSR markers screened. The wheat SSR markers were carefully chosen as being either mapped in the international consensus wheat physical/genetics map in those particular wheat chromosomes or linked to our interested traits published in other studies (Somers *et al.*, 2004; Sourdille *et al.*, 2004; Friesen *et al.*, 2008a; Reddy *et al.*, 2008; Zhang *et al.*, 2011).

Genetic linkage mapping was conducted with MULTIPOINT v. 3.2 software (MultiQTL Ltd, Institute of Evolution, Haifa University, Israel). The Kosambi mapping function was used to convert the recombination frequencies into genetic distance (cM). Chi-squared analysis was applied to test the segregation of the mapped markers against the expected Mendelian segregation ratio for co-dominant inheritance in a DH population. Groups of linked markers that were similarly distorted were accepted for linkage mapping and QTL analysis. Independent markers showing significant segregation distortion and markers with missing data (10%) were rejected for linkage and QTL analysis to avoid bias and false linkages. Association between each linkage group and the putative QTL regions related to effector responses in effector assay, SN15 conidial responses in seedling experiment and percentage of green area in adult field trial were determined by CIM using MULTIQTL software, version 2.5 (MultiQTL Ltd, Institute of Evolution, Haifa University, Israel). Significance levels for each QTL detected were determined with 1000 permutations and the standard deviation for each QTL determined by 1000 Bootstraps. QTLs with a LOD score 2.5 were retained for analysis.

### RNA isolation and gene expression analysis

To investigate the expression of *SnTox1* and *SnTox3* during infection, 2-week-old seedlings of a subset of C × W parents and 20 progeny lines were sprayed with SN15 and *tox1–6* spores in two independent experiments with the same conditions as described above. These 20 lines were carefully chosen so that they represented all four genotype groups (*Snn1*/*Snn3, Snn1*/*snn3, snn1*/*Snn3* and *snn1*/*snn3*; five lines each genotype group). Thereafter, infection was allowed to develop for 48 h prior to sampling. For *SnTox3 in vitro* expression analysis, SN15 and *tox1–6* were grown in Fries 3 broth for 3 days at 22°C. RNA isolation, cDNA synthesis and gene expression analysis were performed as previously described (Tan *et al.*, 2008). Intron-spanning primers ActinF and ActinR designed to amplify the *Act1* (GenBank acc, EAT90788) gene were used to check all cDNA samples via PCR analysis (Tan *et al.*, 2008). All cDNA preparations were shown to be free of genomic DNA. This was repeated with *tox1–7* and *tox1–8*. The expression of *SnTox1* and *SnTox3* were determined via qRT-PCR on a CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA). Elongation factor 1α (*EF-1α* (GenBank acc, XP_001801902) and actin *Act1* housekeeping genes were used as a constitutively expressed control for normalisation using the EF-1alphaF/R and ActinqpcrF/R primer pairs (Tan *et al.*, 2008). All reactions were performed in technical duplicates from three biological triplicates. Primers used for the qRT-PCRs are described in Table S1.

### Fungal biomass analysis

Fungal biomass was determined using qRT-PCR. SnTox3 and a phosphate buffer control were infiltrated into selected wheat cultivars at the 2-week-old seedling stage. Infiltrated leaves were left for 24 h to dry prior to a spray application of SN15 pycnidiospores as described above. Infection was allowed to develop for 4 days prior to sampling and DNA extraction of the infiltrated region using a Biosprint genomic DNA extraction kit (Qiagen, Qiagen, Venlo, Netherlands). Following this, 125 ng of total genomic DNA was used in each qPCR reaction. The amount of *P. nodorum* SN15 genomic DNA was determined using the ActinqpcrF/R primer pair measured against a SN15 only genomic DNA standard. Fungal biomass was expressed as a percentage of SN15 DNA relative to total DNA.

### Statistical analysis

Statistical analysis was performed using JMP 10.0.0 (SAS Institute, CA, USA). Bartlett's test was carried out to determine the homogeneity of variances among different leaves. A *t*-test was used to test disease difference caused by different allelic groups of *Snn1* or *snn1* and *Snn3* or *snn3* groups. LSDs were calculated using the Student's *t*-test. Statistical analysis for LSD was deemed significantly different if the *P*-value is ≤0.05. One-way analysis of variance (ANOVA) and all pairs Tukey honest significant difference (HSD) analysis were used to compare *SnToxA, SnTox1* and *SnTox3* expression in all four genotype groups (*Snn1/snn3, Snn1/Snn3, snn1/Snn3,* and *snn1/snn3*). Statistical analyses for ANOVA and *t*-test were deemed significantly different if the *P*-value was <0.05. Standard error bars are shown when possible.

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## SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

**Figure S1.** SnTox1 and SnTox3 infiltrations on differentially sensitive wheat lines.

**Figure S2.** Copy number of the *SnTox1*–phleomycin resistance gene knockout cassette normalised to a single copy of *Act1.*

**Figure S3.** Genetic map of the C × W DH population. Linkage groups are indicated.

**Figure S4.** *SnTox3* expression was elevated in two other *P. nodorum SnTox1* deletion strains (*tox1–7* and *tox1–8*) compared to SN15.

**Table S1.** Primers used in this study.

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