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Evaluating the importance of the tan spot ToxA–*Tsn1* interaction in Australian wheat varieties

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The necrotrophic fungal pathogen *Pyrenophora tritici-repentis* (Ptr) causes the major wheat disease tan spot, and produces multiple necrotrophic effectors that contribute to virulence. The proteinaceous effector ToxA induces necrosis in wheat genotypes possessing the *Tsn1* gene, although the importance of the ToxA–*Tsn1* interaction itself in varietal disease development has not been well studied. Here, 40 Australian spring wheat varieties were assessed for ToxA sensitivity and disease response to a race 1 wildtype Ptr isolate and *ToxA*-deleted strain at both seedling and tillering growth stages. ToxA sensitivity was generally associated with disease susceptibility, but did not always predict spreading necrotic symptoms. Whilst the majority of *Tsn1* varieties exhibited lower disease scores following *toxa* mutant infection, several exhibited no distinct differences between wildtype and *toxa* symptoms. This implies that ToxA is not the major determinant in tan spot disease development in some host backgrounds and indicates the presence of additional effectors. Unexpectedly, several *tsn1* varieties exhibited a reduction in disease severity following *toxa* mutant inoculation, which may suggest an indirect role for ToxA in pathogen fitness. Additionally, increased chlorosis was observed following *toxa* mutant infection in three varieties, and further work is required to determine whether this is likely to be due to ToxA epistasis of ToxC symptoms. Taken together, these observations demonstrate that Ptr interacts with the host in a complex and intricate manner, leading to a variety of disease reactions that are dependent or independent of the ToxA–*Tsn1* interaction.

Keywords: effector, host-selective toxin, *Pyrenophora tritici-repentis*, tan spot, ToxA, yellow spot

Introduction

Tan spot (yellow (leaf) spot) is a major foliar disease of wheat worldwide, and is typified by tan-coloured leaf lesions often surrounded by chlorotic halos. The causal agent is the necrotrophic fungal pathogen, *Pyrenophora tritici-repentis* (Ptr). Globally, tan spot is an economically significant problem, and impacts on yield via a reduction in the leaf area available for photosynthesis. The disease affects the major wheat-growing areas of the world (Lamari & Strelkov, 2010), and is a considerable problem across mainland Australia, where it has been reported to result in the greatest monetary yield losses of all the wheat diseases (Murray & Brennan, 2009). Tan spot was first documented in Australia in the 1950s (Valder & Shaw, 1952), and was initially considered to be a minor disease, but by the 1970s, it had become widespread across the north-eastern wheat belt of Australia and reached a severe epidemic in 1978 causing substantial damage (Rees & Platz, 1979). More than 30 years later, tan spot is still highly prevalent across Australia with losses estimated at A$212 million per annum (Murray & Brennan, 2009; Oliver *et al.*, 2016). In recent years, the Australian breeding industry has focused on improving resistance and has released over a hundred wheat varieties (http://varietycentral.com.au/). Despite this effort, no bread wheat varieties rated better than moderately resistant are currently available for Australian growers. Agronomic practices such as crop rotation, stubble residue management and fungicide application have become the main methods to control tan spot. However, the development and use of resistant varieties remains the most economical and sustainable long-term approach for farmers to manage this disease.

In terms of the mechanisms of pathogenicity that underlie host infection, the manifestation of foliar necrosis and/or chlorosis is the result of effectors secreted by the pathogen. To date, three Ptr effectors have been identified: ToxA, ToxB and ToxC. Of these, ToxA is the most prevalent and the most studied, and is also produced by the septoria nodorum blotch (SNB) wheat fungal pathogen, *Parastagonospora nodorum* (Friesen *et al.*, 2006; Liu *et al.*, 2006) and the spot blotch cereal necrotroph, *Bipolaris sorokiniana* (McDonald *et al.*, 2018). The tan spot disease system is governed primarily by inverse gene-for-gene, race-specific interactions, which involve the recognition of necrotrophic fungal effectors by corresponding wheat sensitivity genes (Tan *et al.*, 2010). Wheat sensitivity to ToxA is governed by the *Tsn1* gene, which encodes an S/TPK and NBS-LRR protein (Faris *et al.*, 2010), and it has been well documented that ToxA causes necrosis on *Tsn1* wheat (Ciuffetti *et al.*, 1997; Faris *et al.*, 2010; Moffat *et al.*, 2014).

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Although the Tsn1 protein itself is not the receptor for ToxA, the *Tsn1*-mediated ToxA interaction is postulated to initiate molecular signalling that leads to the importation of ToxA within mesophyll cell (Faris *et al.*, 2010). Once internalized, ToxA is involved in a multicomplex interaction associated with the chloroplast membrane, whereby reactive oxygen species (ROS) accumulate in the chloroplast leading to cell death by inhibition of photosystem activity (Manning *et al.*, 2009). Therefore, the ToxA–*Tsn1* interaction is a crucial target in breeding efforts towards tan spot resistance.

ToxA is produced by the majority of Ptr isolates worldwide and is the predominant effector in the tan spot–wheat pathosystem (Friesen *et al.*, 2006). The role of ToxA as a virulence factor has been clearly demonstrated *in vivo*, where expression of *ToxA* by an avirulent (non-ToxA producing) isolate rendered that strain pathogenic (Ciuffetti *et al.*, 1997). However, the role of endogenous ToxA in tan spot disease development is not well defined. It is plausible that the relationship between ToxA and disease susceptibility depends not only on the ToxA–*Tsn1* interaction, but also on interactions with other effectors and their corresponding wheat sensitivity genes. The *P. nodorum*–wheat pathosystem has been well studied and multiple effector–host sensitivity gene interactions have been demonstrated to play a role in SNB disease development, acting in an additive manner and via epistasis. For example, the SnToxA–*Tsn1* interaction has been reported to act additively with SnTox2–*Snn2* (Friesen *et al.*, 2007) and SnTox5–*Snn5* (Friesen *et al.*, 2012) in conferring SNB disease susceptibility, with wheat genotypes that harbour multiple susceptibility alleles resulting in significantly higher disease reactions than those containing single alleles. The SnToxA–*Tsn1* interaction has also been shown to interact epistatically to SnTox3–*Snn3* (Friesen *et al.*, 2008), demonstrating the complex gene regulatory interactions of effectors in the host.

Recent work is beginning to demonstrate that such complex effector cross-talk may also exist in the tan spot–wheat pathosystem. Certainly, it is well documented that additional Ptr effectors exist beyond ToxA, ToxB and ToxC. Early studies showed that mutants of the wheat cultivar Kulm that had lost ToxA sensitivity via EMS mutagenesis were still able to develop necrotic lesions surrounded by chlorosis when inoculated with a race 1 (ToxA- and ToxC-producing) isolate (Friesen *et al.*, 2002). In another study, the disease phenotype of a recombinant inbred line (RIL) population, derived from a cross between a ToxA sensitive and an insensitive cultivar, was skewed towards resistance for a race 2 (ToxA-producing) isolate, even though the population segregated 1:1 for ToxA sensitivity (Friesen *et al.*, 2003). Heritability analysis suggested that there were four to five genes that influence the disease response in that population, suggesting that the race 2 isolate possesses pathogenicity factors besides ToxA. More recent studies have further implicated the presence of other effectors in the tan spot–wheat pathosystem. For example, atypical disease phenotypes following infection of differential wheat lines have been noted for some Ptr isolates and postulated to be caused by novel effectors, including an American tan spot isolate that produced typical race 1 necrotic symptoms even though the isolate does not possess the *ToxA* gene (Ali *et al.*, 2010). In previous work, it was demonstrated that a race 1 isolate incapacitated in ToxA production, either by targeted gene deletion (Moffat *et al.*, 2014) or by disruption to the ToxA regulatory pathway via the deletion of a Zn2ys6 binuclear cluster transcription factor (Rybak *et al.*, 2017), was still able to induce chlorosis, suggesting the presence of ToxC or other chlorosis-inducing effectors. Additionally, infection of the ToxA-sensitive wheat line TAM 105 with a ToxA knockout mutant resulted in necrotic lesions comparable to wildtype Ptr (Manning & Ciuffetti, 2015). Moreover, mutant infection induced spreading chlorosis, which was not observed following wildtype inoculation. These observations not only support the presence of additional effectors contributing to the virulence, but also reveal epistasis.

Similarly, a number of wheat genotypes have been identified that are sensitive to ToxA, yet resistant to races 1 and 2 (Noriel *et al.*, 2011; Liu *et al.*, 2015). This lack of association of the ToxA–*Tsn1* interaction with disease demonstrates the complexities underlying the tan spot pathosystem, and may be due to the presence of race-nonspecific resistance. Such a QTL was identified on chromosome 3B in a RIL population derived from a cross between a highly susceptible and a highly resistant wheat line (both sensitive to ToxA) (Kariyawasam *et al.*, 2016). The 3B QTL appeared to prohibit the effector-triggered susceptibility from the ToxA–*Tsn1* interaction, thus it appears that this QTL has an epistatic effect on the ToxA–*Tsn1* interaction in certain host backgrounds. More recently, additive effects have also been observed that contribute collectively to tan spot disease development. A RIL population derived from two susceptible genotypes, one possessing *Tsn1* and the other *Tsc1* (ToxC sensitivity), revealed that lines carrying one susceptibility gene were less susceptible than those carrying both genes (Liu *et al.*, 2017). This demonstrates that Ptr ToxA–*Tsn1* and Ptr ToxC–*Tsc1* interactions have an additive effect on disease, at least in this genetic background. Furthermore, the ToxA–*Tsn1* interaction plays little or no role in the development of tan spot in tetraploid (durum) wheat (Virdi *et al.*, 2016). Altogether, these observations demonstrate the complexity of the wheat–tan spot pathosystem, and point to the involvement of other factors that are dependent on or independent from the ToxA–*Tsn1* interaction.

The relative effects of effector–sensitivity gene interactions in the wheat–tan spot system have yet to be well studied. In Australia, commercial wheat varieties are evaluated against tan spot and other pathogens annually; however, the importance of ToxA itself in varietal disease development has not been evaluated. This study examines tan spot disease reactions of *Tsn1* and *tsn1* host genotypes using a race 1 wildtype isolate and a *ToxA*-deleted mutant strain against 40 Australian spring

wheat varieties. Here it is shown that ToxA is not essential for establishing tan spot disease; however, ToxA influences disease development on particular genotypes. Whether such phenotype data can play a part in unravelling the emerging complexities of the wheat–tan spot pathosystem is discussed.

Materials and methods

Plant and fungal material

A total of 40 commercially available Australian hexaploid spring wheat varieties (*Triticum aestivum*) were evaluated in this study. Their tan spot disease ratings ranged from moderately resistant (MR) to susceptible–very susceptible (S-VS) (https://grdc.com.au/Research-and-Development/National-Variety-Trials/Crop-Variety-Guides; Table S1).

The Ptr isolate M4, collected from Western Australia in 2009, was used in this study, and belongs to race 1 (ToxA- and ToxC-producing) (Moffat *et al.*, 2014). ToxA-knockout strains (*toxa*) have been described previously, whereby the *ToxA* gene was deleted from the genome of the M4 isolate, using a PEG-mediated transformation method (Moffat *et al.*, 2014).

ToxA bioassays

Evaluation of wheat host responses to ToxA was performed on two-leaf-stage seedlings in a controlled environment growth chamber as described earlier (Moffat *et al.*, 2014). Fully extended leaves were infiltrated with the ToxA protein at a concentration of 50 μg mL^{-1}. For each variety, a minimum of three leaves from three independent seedlings were tested. After 4 days, the leaves were evaluated for necrosis and scored as either 'sensitive' (necrosis) or 'insensitive' (no symptoms).

Plant infection assays

For the seedling infection assays, 2-week-old plants were inoculated with spores of wildtype M4, and two independent *toxa* mutants, at a concentration of 2500 conidia mL^{-1} as previously described (Moffat *et al.*, 2015). Disease reactions were evaluated after 7 days using the 1–5 tan spot scoring scale, where 1 = presence of resistant specks; 2 = lesions with little necrosis and chlorosis; 3 = lesions with distinct necrosis and chlorosis; 4 = coalescing type 3 lesions; and 5 = extensive necrosis and chlorosis in the absence of well-defined borders between lesions (Lamari & Bernier, 1989). All infection assays were independently repeated using three biological replicates per variety. Analysis of variance (ANOVA) was performed to compare the means between the two datasets derived from experimental replication (Table S2). No significance difference was found (ANOVA, $P < 0.05$) and therefore the two sets of data were combined to obtain one dataset.

For the adult plant infection assays, wheat seeds were sown in 20 cm pots containing potting mix (Richgro). Three varieties (one seed of each) were planted in each pot, and plants were grown in a controlled environment growth room (Conviron) under a 12 h photoperiod maintained at 22 °C with a relative humidity of 40% and a light intensity of 300 μmol m^{-2} s^{-1}. Plants were fertilized initially with Osmocote Plus Organics all-purpose fertilizer (Scotts), and fertilized fortnightly following seedling emergence with Thrive all-purpose water-soluble fertilizer (Yates). After 6 weeks, plants were inoculated with 2500 conidia mL^{-1} of wildtype or the *toxa* mutant strains. Approximately 50 mL of spore suspension was applied per pot using a hand-held sprayer. Pots were placed in a misting chamber with near 100% relative humidity for 24 h at 22 °C under a 12 h photoperiod. After 7 days, flag–2 leaves were scored for disease based on the scale described by Lamari & Bernier (1989). All infection experiments were independently repeated using three replicates (pots) per treatment and performed as blind experiments arranged in a randomized design. No significant difference was found between the two datasets (ANOVA, $P < 0.05$), and therefore the two sets of data were combined (Table S2).

Statistical analysis

All statistical analyses were performed using JMP v. 11.0.0 software. Student's *t*-tests were used to compare the means of disease scores. The strength of the linear relationship between each pair of disease scores was calculated using the correlation multivariate platform in JMP. A standard least squares fitted linear regression model was used to assess the association between tan spot disease severity and wheat variety sensitivity to ToxA.

Results

Varietal ToxA sensitivity

Forty commercially available Australian wheat varieties were assayed for sensitivity to ToxA (Table 1). Eighteen resulted in necrosis and were determined to be ToxA-sensitive, while the remaining 22 varieties were scored as insensitive. ToxA sensitivity was generally associated with disease susceptibility (Table 2). None of the ToxA-sensitive varieties were rated better than MS in national variety field trials, with the majority (10/18) classified as S-VS/S and the remaining eight as MS-S/MS. In contrast, the majority of ToxA-insensitive varieties exhibited a greater degree of tan spot resistance. Overall, *Tsn1* varieties were rated more poorly for tan spot resistance in field disease trials than *tsn1* varieties.

Wildtype infection assays

Wheat varieties at both seedling and tillering growth stages were assessed for resistance to a wildtype race 1 Ptr isolate under controlled conditions. Correlation analysis was performed to compare results with the West Australian variety disease resistance ratings (which are obtained via field assessments). Significant associations were observed between seedling disease scores and disease resistance field ratings (correlation coefficient $r = 0.70$, $P < 0.0001$) and between tillering disease scores and disease resistance field ratings ($r = 0.78$, $P < 0.001$; Fig. 1). Additionally, correlation analysis between the seedling and tillering disease scores under controlled conditions was also significant ($r = 0.75$, $P < 0.005$).

Response of Tsn1 *varieties to wildtype infection*

The majority of the ToxA-sensitive varieties exhibited tan coloured necrotic lesions following wildtype

Table 1 Tan spot disease reactions of Australian wheat varieties inoculated with wildtype *Pyrenophora tritici-repentis* and *toxa* mutant strains at seedling and tillering growth stages.

	Seedling growth stage			Tillering growth stage		
	Disease score (mean ± SE)			Disease score (mean ± SE)		
	Wildtype	*toxa*	Difference	Wildtype	*toxa*	Difference
Tsn1 genotype (ToxA-sensitive)						
Shield	2.6 ± 0.2	1.8 ± 0.1	0.8*	—	—	—
Spitfire	3.1 ± 0.1	2.2 ± 0.2	0.9*	2.9 ± 0.1	2.3 ± 0.3	0.6
Sunguard	3.2 ± 0.2	3.1 ± 0.1	0.1	4.0 ± 0.2	3.1 ± 0.2	0.9*
Lancer	3.3 ± 0.2	2.4 ± 0.2	0.8*	3.8 ± 0.1	2.9 ± 0.2	0.9*
Harper	3.3 ± 0.3	2.4 ± 0.3	0.9*	—	—	0.6
EGA Gregory	3.3 ± 0.2	3.3 ± 0.2	0.1	—	—	—
Annuello	3.4 ± 0.3	2.7 ± 0.1	0.7	—	—	—
Grenade CL plus	3.4 ± 0.2	2.2 ± 0.5	1.2	—	—	—
Merlin	3.4 ± 0.1	3.2 ± 0.1	0.3	3.2 ± 0.2	2.0 ± 0.2	1.2
Phantom	3.7 ± 0.1	3.3 ± 0.1	0.4*	4.4 ± 0.1	4.0 ± 0.2	0.4*
Estoc	3.8 ± 0.2	3.6 ± 0.2	0.2	—	—	—
Gazelle	3.8 ± 0.2	3.1 ± 0.1	0.7*	3.3 ± 0.3	2.1 ± 0.2	1.2
EGA Wylie	4.2 ± 0.3	3.7 ± 0.2	0.5	—	—	—
Scout	4.3 ± 0.2	4.1 ± 0.4	0.2	—	—	—
Stiletto	4.4 ± 0.3	3.7 ± 0.2	0.7	—	—	—
Axe	4.4 ± 0.3	4.6 ± 0.2	−0.2	—	—	—
Frame	4.5 ± 0.2	4.5 ± 0.2	0.0	—	—	—
Yitpi	4.6 ± 0.2	4.3 ± 0.3	0.2	4.4 ± 0.1	4.5 ± 0.1	−0.1
Average	3.7 ± 0.1	3.2 ± 0.2	0.5	3.7 ± 0.2	3.0 ± 0.4	0.7
tsn1 genotype (ToxA-insensitive)						
Cosmick	2.0 ± 0.0	2.0 ± 0.0	0.0	—	—	—
Hydra	2.3 ± 0.1	2.0 ± 0.0	0.3	—	—	—
Zen	2.3 ± 0.2	1.8 ± 0.1	0.4	2.7 ± 0.2	2.3 ± 0.2	0.4
Gauntlet	2.3 ± 0.1	1.8 ± 0.3	0.5	—	—	—
Impress CL Plus	2.7 ± 0.2	2.5 ± 0.1	0.2	—	—	—
EGA Bonnie Rock	2.9 ± 0.2	2.0 ± 0.2	0.9*	2.8 ± 0.2	2.4 ± 0.1	0.4
Calingiri	2.9 ± 0.2	2.9 ± 0.1	0.0	—	—	—
Magenta	2.9 ± 0.3	2.8 ± 0.3	0.2	3.0 ± 0.2	2.5 ± 0.2	0.6
King Rock	3.0 ± 0.3	3.0 ± 0.1	0.0	—	—	—
Cobra	3.0 ± 0.0	2.5 ± 0.2	0.6*	—	—	—
Wyalkatchem	3.2 ± 0.4	2.9 ± 0.1	0.3	2.3 ± 0.3	1.8 ± 0.2	0.6
Dart	3.2 ± 0.1	3.1 ± 0.2	0.1	—	—	—
Carnamah	3.2 ± 0.3	2.9 ± 0.2	0.3	—	—	—
Mace	3.3 ± 0.2	3.5 ± 0.1	−0.2	2.6 ± 0.2	2.4 ± 0.1	0.3*
Supreme	3.4 ± 0.1	2.5 ± 0.2	0.9*	3.3 ± 0.2	2.7 ± 0.1	0.6
Westonia	3.5 ± 0.3	2.9 ± 0.2	0.6	—	—	—
Suntop	3.5 ± 0.2	3.5 ± 0.2	0.0	—	—	—
Kennedy	3.6 ± 0.2	3.3 ± 0.0	0.3	—	—	—
Wallup	3.6 ± 0.1	3.5 ± 0.0	0.1	—	—	—
Impala	3.7 ± 0.2	3.5 ± 0.0	0.2	—	—	—
EGA Eagle Rock	4.0 ± 0.1	4.0 ± 0.2	0.0	4.3 ± 0.2	4.0 ± 0.2	0.3
Machete	4.2 ± 0.3	4.3 ± 0.2	−0.1	4.7 ± 0.1	4.1 ± 0.1	0.6
Average	3.1 ± 0.1	2.9 ± 0.1	0.3	3.2 ± 0.3	2.8 ± 0.3	0.5

*Significant difference between wildtype and *toxa* mutant scores at $P < 0.05$ (*t*-test).

infection, with average disease scores of 3.7 ± 0.1 and 3.7 ± 0.2 for seedling and tillering stages, respectively (Table 1). Severe necrosis was observed on Frame, Scout, Stiletto and Yitpi at the seedling stage (Fig. 3a). Although infection assays were not performed for all the varieties at the tillering stage, spreading necrosis was evident on Yitpi at both growth stages (Fig. 3). Interestingly, ToxA sensitivity did not always predict extensive spreading necrosis following wildtype spore inoculation. For example, Shield and Spitfire seedlings displayed discrete localized tan necrotic lesions (Fig. 3a) and scored 2.6 ± 0.2 and 3.1 ± 0.1, respectively (Table 1).

Response of tsn1 *varieties to wildtype infection*

In the absence of *Tsn1*, average varietal disease scores were lower following infection with the wildtype isolate (Table 1). This was observed at both the seedling stage

Table 2 Sensitivity of Australian wheat varieties to ToxA.

Variety	ToxA sensitivity[a]	DRR[b]
Frame	+	S-VS
Phantom	+	S-VS
Scout	+	S-VS
Stiletto	+	S-VS
Yitpi	+	S-VS
Axe	+	S
EGA Gregory	+	S
Grenade	+	S
Harper	+	S
Merlin	+	S
Eagle Rock	–	S
Machete	–	S
Annuello	+	MS-S
Estoc	+	MS-S
Gazelle	+	MS-S
Shield	+	MS-S
Spitfire	+	MS-S
Sunguard	+	MS-S
EGA Wylie	+	MS-S
Calingiri	–	MS-S
Carnamah	–	MS-S
Impala	–	MS-S
Kennedy	–	MS-S
Suntop	–	MS-S
Wallup	–	MS-S
Westonia	–	MS-S
Lancer	+	MS
Dart	–	MS
Guantlet	–	MS
Supreme	–	MS
EGA Bonnie Rock	–	MR-MS
Cobra	–	MR-MS
Cosmick	–	MR-MS
Hydra	–	MR-MS
Impress	–	MR-MS
King Rock	–	MR-MS
Mace	–	MR-MS
Zen	–	MR-MS
Magenta	–	MR
Wyalkatchem	–	MR

[a]+, sensitive (extensive necrosis); –, insensitive (absence of necrosis) (Liu *et al.*, 2006).

[b]Tan spot disease resistance ratings (DRR) were adapted from National Variety Trial (NVT) 2016 data where MR = moderately resistant, MS = moderately susceptible, S = susceptible and VS = very susceptible.

(3.1 ± 0.1 for *tsn1* varieties compared to 3.7 ± 0.1 for *Tsn1*) and tillering stage (3.2 ± 0.3 for *tsn1* compared to 3.7 ± 0.2 for *Tsn1*). Disease reactions ranged from small dark brown lesions (e.g. Cosmick) to substantial tan necrosis (e.g. Machete; Fig. 3). Wyalkatchem and Mace displayed resistant reactions to wildtype infection at the tillering stage (Table 1).

Symptom types

A broad range of disease reactions were observed following infection with the wildtype strain, from very little necrosis either with or without chlorosis, through to extensive necrosis. The disease scores ranged from 2.0 to 4.6 for seedlings, and 2.3 to 4.7 for tillering plants, yet the range of symptoms cannot be fully described by a numerical score. Examples of the range of symptoms are shown in Figure 3. These include dark brown flecks only (Cosmick, Hydra), tan necrosis varying from small and distinct lesions (Supreme, Shield) to extensive necrosis (Frame, Yitpi), either with or without chlorosis (Phantom/Carnamah and Spitfire, respectively).

Infection assays with *toxa* mutant

Response of Tsn1 *varieties to* toxa *mutant infection*

The association between the seedling and tillering disease scores for *toxa* mutant inoculation was significant, with a correlation coefficient of 0.73 ($P < 0.005$; Fig. 1).

As expected, the majority of *Tsn1* varieties exhibited lower disease scores following *toxa* mutant infection compared to wildtype, with average mean disease score reductions of 0.5 and 0.7 for seedling and tillering stages, respectively (Table 1; Fig. 2). For example, Spitfire and Shield seedlings exhibited significant reductions in disease scores (*t*-test $P < 0.05$), whereby tan necrotic lesions observed following wildtype inoculation were absent following *toxa* mutant infection (Fig. 3a).

Notable differences between wildtype and *toxa* mutant disease symptoms were also observed that could not be fully described by the numerical score alone. For example, wildtype inoculation of Gazelle resulted in spreading necrosis (Fig. 3); however, the *toxa* mutant strain induced more pronounced chlorosis and markedly reduced necrosis, and resulted in a significant difference in seedling disease scores (*t*-test $P < 0.05$).

No distinct differences between wildtype and *toxa* mutant symptoms were discernible for a number of varieties, including EGA Gregory, Axe and Yitpi (Table 1). Notably, Yitpi displayed severe disease symptoms with spreading tan necrosis and chlorosis when infected with either wildtype or the *toxa* mutant at both growth stages (Fig. 3). These severe symptoms were also observed for Frame and Scout seedlings, which both scored above 4.0 following *toxa* mutant infection (Fig. 3a; Table 1).

Response of tsn1 *varieties to* toxa *mutant infection*

Surprisingly, a number of *tsn1* varieties exhibited a reduction in mean disease scores following *toxa* mutant inoculation, with average reductions of 0.3 and 0.5 for seedlings and tillering stages, respectively (Table 1). The overall mean disease scores between wildtype and *toxa* mutant infection was significantly different at the seedling and tillering stages, albeit at a lower level of significance in comparison to the *Tsn1* varieties (Fig. 2).

Seedlings of variety Supreme showed a significant reduction in tan necrotic lesions following *toxa* mutant infection when compared to wildtype (*t*-test $P < 0.05$) (Table 1; Fig. 3a). However, most *tsn1* varieties displayed small but nonsignificant reductions in disease symptoms, such as Impala seedlings and tillering EGA Bonnie Rock plants, which displayed reduced lesion coalescence (Fig. 3a,b).

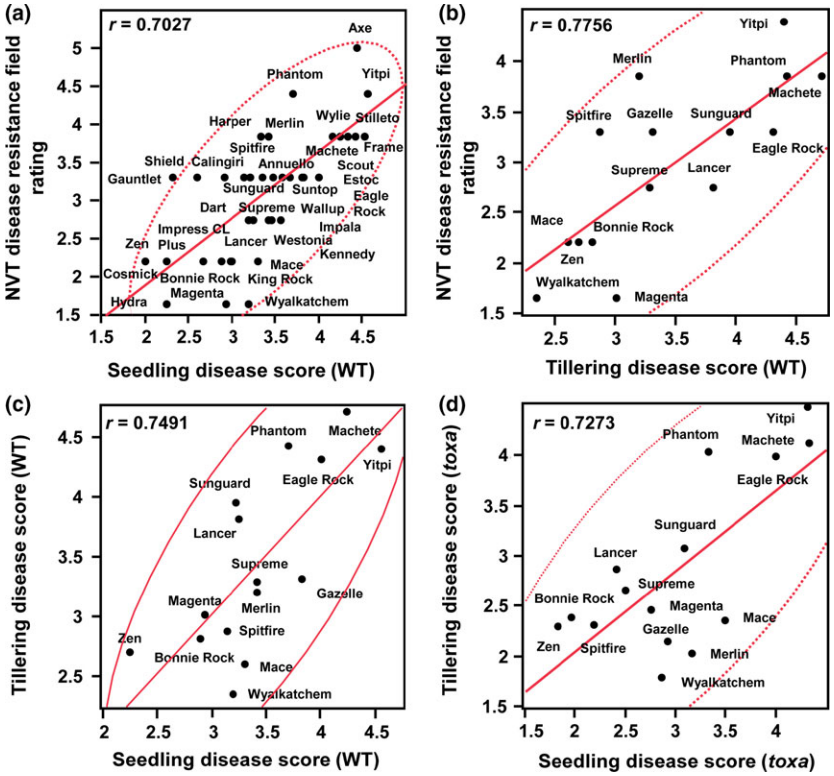

Figure 1 Correlation of wheat variety disease scores following *Pyrenophora tritici-repentis* infection assessed under different growth stages and experimental conditions: (a) national variety trial (NVT; https://www.nvtonline.com.au/crop-guides/wa/) field disease ratings versus wildtype (WT) infection of seedlings under controlled conditions; (b) NVT field disease ratings versus wildtype infection of tillering plants under controlled conditions; (c) wildtype infection of tillering versus seedling plants under controlled conditions; (d) *toxa* mutant infection of tillering and seedling plants under controlled conditions. Scatterplots depict bivariate analysis with designated variables on each axis and density ellipse at 95% confidence. Value of *r* indicates Pearson correlation coefficient ($P < 0.005$). [Colour figure can be viewed at wileyonlinelibrary.com]

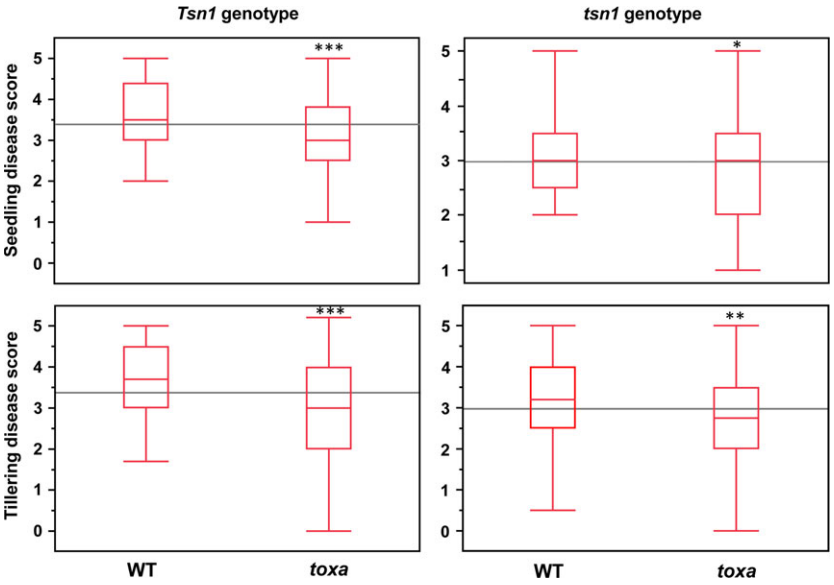

Figure 2 Box plot diagram showing median disease scores of *Tsn1* and *tsn1* wheat varieties infected at either the seedling or tillering stage. Varieties were inoculated with wildtype (WT) or ToxA-deficient mutant strains (*toxa*). The median values are represented by the red horizontal line within each box, while the overall mean is represented by a black horizontal line. Asterisks denote a significant difference (*t*-test, $*P < 0.01$, $**P < 0.001$, $***P < 0.0001$).

Notably, Carnamah seedlings produced a distinct difference in disease symptoms following *toxa* mutant infection, with the marked reduction in tan necrosis and a clear increase in chlorosis (Fig. 3a).

ToxA sensitivity and disease severity

Regression analysis of seedling disease scores between *Tsn1* and *tsn1* varieties showed that for the wildtype infection, ToxA sensitivity had a significant effect on disease severity at both the seedling ($r^2 = 0.11$, $P < 0.0001$) and tillering stage ($r^2 = 0.06$, $P = 0.0001$). This implies that ToxA has a significant role in race 1–host disease development. When seedlings were inoculated with the *toxa* mutants, a less significant and weaker association between *toxa* mutant infection and host ToxA sensitivity was found at both the seedling ($r^2 = 0.02$, $P = 0.0125$) and tillering ($r^2 = 0.02$, $P = 0.0443$) stages.

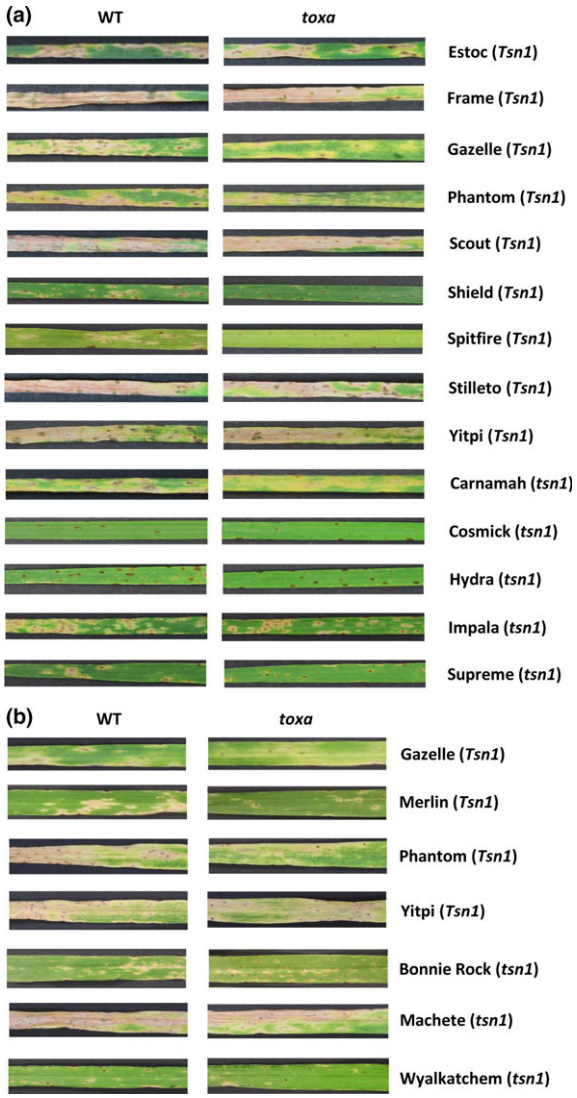

Figure 3 Examples of tan spot disease symptoms of various wheat varieties inoculated with wildtype *Pyrenophora tritici-repentis* (WT) and a ToxA-deficient mutant (*toxa*) at the seedling stage (a) and tillering stage (b). Images are of representative leaves and were taken 7 days post-inoculation. Varieties harbouring *Tsn1* or *tsn1* are indicated.

Discussion

ToxA sensitivity was generally associated with tan spot disease susceptibility, with *Tsn1* varieties rated more susceptible than *tsn1* varieties both in this study and in national variety field trials. Infection with the *toxa* mutants resulted in average reduction of 0.5 to 0.7 in disease scores for *Tsn1* varieties. However, ToxA sensitivity did not always predict extensive necrosis, as evidenced by wildtype infection of MS-S *Tsn1* varieties such as Shield, which displayed minimal tan necrotic lesions. Thus the degree of ToxA-induced necrosis must therefore depend on other factors within specific host genotypes.

Although ToxA-insensitive varieties on the whole were less susceptible to tan spot disease, the majority of varieties still scored 3 and above at both seedling and tillering stages, indicative of necrotic lesions. Moreover, some *tsn1* varieties were highly susceptible to wildtype infection, such as Machete, EGA Eagle Rock and Westonia, presumably due to the involvement of other unidentified effector(s). Furthermore, a number of *Tsn1* varieties showed minimal to no reduction in disease following *toxa* mutant infection, such as Yitpi and Frame which gave high disease scores at the seedling stage and exhibited severe necrosis. Such varying reactions to the *toxa* mutant implies that in some genotypes at least, ToxA is not the major determinant in disease development.

An unexpected reduction in disease scores of *tsn1* wheat varieties was observed at both stages following *toxa* mutant infection. It is possible that the deletion of the *ToxA* gene may have reduced the overall fitness of the pathogen. It is also plausible that ToxA may play an indirect role in fungal growth and development. This finding is in agreement with a previous study that showed that a *ToxA*-expressing race 3 Ptr strain showed a slight increase in mycelial growth in a *tsn1* variety (Manning & Ciuffetti, 2015). This suggests that ToxA may act independently of *Tsn1* to provide additional benefits, such as a growth advantage, in the absence of its recognition partner. A strong correlation has also been reported between *ToxB* transcripts and appressorium abundance (Amaike *et al.*, 2008). This has been further supported by a study that showed the level of *ToxB* RNA silencing had an effect on appressoria formation (Aboukhaddour *et al.*, 2012).

Disease scores from both seedling and tillering stages, assessed herein, positively correlated with variety field trial disease ratings. Therefore controlled disease assessment at both growth stages may be a useful indicator of field trial disease ratings. However, disease assessment in the field can be complicated by a number of factors including complexes of foliar diseases, environmental variations, natural senescence and damage, as well as variation in pathogen isolates and races.

Overall, the correlation between seedling and tillering disease scores was significant. Distinct genetic disease resistance at different growth stages has been reported for other wheat diseases such as leaf rust and powdery mildew, including seedling susceptibility and adult plant resistance (APR) (Li *et al.*, 2014; Milus *et al.*, 2015). For tan spot disease, such phenomena have not been particularly well characterized, although positive correlations between seedling resistance and APR have been reported in American spring wheat varieties (Tadesse *et al.*, 2011). A recent study evaluated 20 spring wheat genotypes, including six Australian varieties also examined in this study, for APR to tan spot (Dinglasan *et al.*, 2016). The level of disease severity at different growth stages varied for each variety, yet both this study and the present study observed a high level of disease severity for EGA Wylie seedlings, which was more severe than the field trial-derived variety rating of MS-S. Thus EGA Wylie, Mace and Wyalkatchem to a lesser extent appear to display increased resistance in more mature plants,

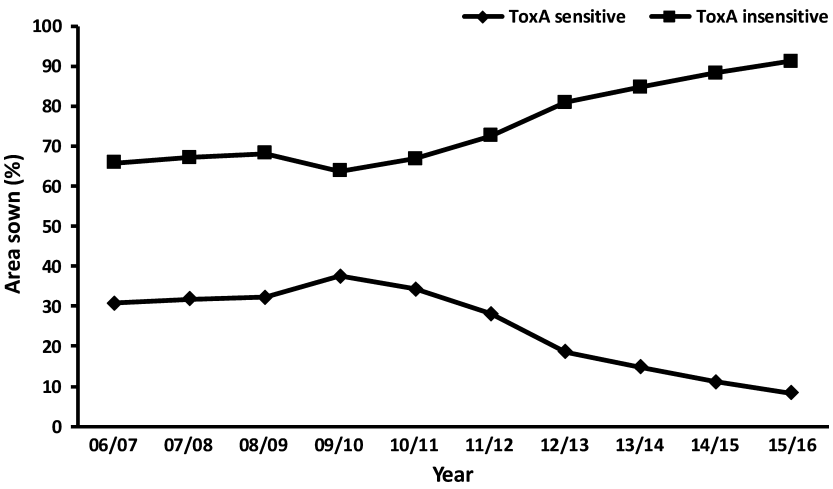

Figure 4 Estimated percentage area sown of ToxA-sensitive (*Tsn1*) and ToxA-insensitive (*tsn1*) wheat varieties in Western Australia from 2006 to 2016. Data from the Wheat Variety Guide for Western Australia 2012–2016 (https://grdc.au/WAWheatVarietyGuide).

and could serve as candidates for further investigation of APR in tan spot disease. Conversely, Dinglasan and colleagues also reported Suntop seedlings to be less susceptible than adult plants, which was also observed here to some extent in varieties such as Phantom, further highlighting the complexities of tan spot resistance. Differences observed between the two studies of the same varieties may be explained by assessment under different environmental conditions as well as use of different fungal isolates. In other wheat diseases, such as septoria tritici blotch (STB) (Brown *et al.*, 2015) and SNB (Shankar *et al.*, 2008), independent QTLs that correspond to disease resistance either at the seedling or adult stages have been identified. Such complexity is now becoming apparent for the tan spot–wheat pathosystem, involving multiple effector–host interaction(s) which may be dependent on plant growth stages.

Wheat genetic studies have identified several race-nonspecific QTLs on chromosomes 1BS, 3BL and 5AL that confer resistance to tan spot in certain host genotypes (Faris & Friesen, 2005; Chu *et al.*, 2008; Kariyawasam *et al.*, 2016). For example, a small but significant reduction in the disease scores of a RIL population using a race 2 *toxa* mutant strain has been reported (Kariyawasam *et al.*, 2016). However, this effect was only observed in lines that did not harbour the race-nonspecific 3B resistance allele and thus the identified 3B QTL appears to have an epistatic effect on the ToxA–*Tsn1* interaction within the host background studied. Further examination of race-nonspecific and race-specific resistance in additional host genetic backgrounds is required if they are to be implemented into commercial wheat breeding programmes.

Epistasis in tan spot was first demonstrated in the ToxA-sensitive cultivar TAM 105, in which more extensive chlorosis was observed upon *toxa* mutant infection compared to the wildtype, while heterologous expression of *ToxA* in a race 3 isolate (ToxC-producing only) was able to induce an antagonistic effect that reduced the spreading chlorosis symptoms (Manning & Ciuffetti, 2015). Of the 40 Australian varieties examined in this study, Carnamah, Gazelle and Phantom exhibited spreading chlorosis following *toxa* mutant infection, which was most evident at the seedling stage. This is likely to be ToxC, as a previous study showed that the *toxa* mutant strain produced spreading chlorosis on the differential line 6B365 (Moffat *et al.*, 2014). Interestingly, Carnamah is a ToxA-insensitive variety, thus the observed increase in chlorosis observed in the absence of ToxA cannot be explained by ToxA epistasis of ToxC symptoms. However, unlike TAM105, a clear reduction in necrosis was also apparent following *toxa* mutant infection compared to wildtype in these varieties. Further work is required to determine whether this is likely to be the result of reduction in necrosis, thus permitting free diffusion of ToxC or some other chlorosis-inducing factor. Another more intriguing possibility is whether ToxA recognition by *Tsn1* leads to the suppression of other host effector sensitivity genes required for additional symptoms. It is certainly evident that the ToxA–*Tsn1* interaction alone is not a prerequisite for pathogenicity of race 1 Ptr isolates, and pathologists have started to recognize that race 1 Ptr isolates harbour additional uncharacterized effectors in addition to ToxA and ToxC (Moffat *et al.*, 2014; Manning & Ciuffetti, 2015; Rybak *et al.*, 2017).

This study used the widely accepted 0–5 scale disease rating based on lesion types (Lamari & Bernier, 1989). However, a broad range of symptoms was observed that could not be fully captured in such a scale. A quantitative approach to evaluating disease response may not always encompass the qualitative aspect of the symptom variations displayed by different host genotypes, as dissimilarities in the symptoms are often subtle and tend to yield statistically insignificant results. Although alternative rating scales have been proposed in the past based on leaf area affected, lesion length and size, a rating scale with a wider range may help to differentiate genotypes with extreme or intermediate reactions, such as a modified 0–9 scale that considers lesion size, type and severity, allowing for good differentiation (Dinglasan *et al.*, 2016).

Although the removal of the *ToxA* gene in Ptr does not severely impede the ability of the pathogen to infect

in all varieties, the absence of the *Tsn1* gene in the wheat germplasm does generally improve resistance to tan spot disease. Because ToxA is found ubiquitously in Australian Ptr isolates and the removal of ToxA sensitivity gene from wheat has been shown to have no effect on yield penalty (Oliver *et al.*, 2014), it is therefore pragmatic that the wheat breeding industry continues their effort to breed ToxA-insensitive varieties. Indeed, there has been a rise in the area sown to ToxA-insensitive wheat varieties in Western Australia since 2009 and a 26% decrease in *Tsn1* varieties sown between 2009 and 2016 (Fig. 4). This is a reflection of rapid and successful adoption by Australian wheat breeders of ToxA-assisted germplasm screening and variety selection (Vleeshouwers & Oliver, 2014). It is likely that ToxA-sensitive varieties will be phased out over the next few years, and the discovery of unknown effectors and their corresponding host sensitivity genes are now the focus of current research. The availability of additional effectors will ultimately improve varietal resistance to tan spot disease through both effector-assisted germplasm screening and the identification of new molecular markers for the corresponding host sensitivity genes.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Table S1. Australian wheat varieties examined in this study.

Table S2. Analysis of variance (ANOVA) comparing the means of the two datasets derived from two independent experimental replications for wildtype (WT) and *toxa* mutant infections at seedling and tillering growth stages. No significant differences were found at $P < 0.05$.