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# Generation of a *ToxA* knockout strain of the wheat tan spot pathogen *Pyrenophora tritici-repentis*

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## SUMMARY

The necrotrophic fungal pathogen *Pyrenophora tritici-repentis* causes tan spot, a major disease of wheat, throughout the world. The proteinaceous effector ToxA is responsible for foliar necrosis on ToxA-sensitive wheat genotypes. The single copy *ToxA* gene was deleted from a wild-type race 1 *P. tritici-repentis* isolate via homologous recombination of a knockout construct. Expression of the *ToxA* transcript was found to be absent in transformants (*toxa*), as was ToxA protein production in fungal culture filtrates. Plant bioassays were conducted to test transformant pathogenicity. The *toxa* strains were unable to induce necrosis on ToxA-sensitive wheat genotypes. To our knowledge, this is the first demonstration of a targeted gene knockout in *P. tritici-repentis*. The ability to undertake gene deletions will facilitate the characterization of other pathogenicity effectors of this economically significant necrotroph.

**Keywords:** homologous recombination, transformation, yellow leaf spot, yellow spot, necrotrophic effector, toxin, Triticum aestivum.

## INTRODUCTION

The necrotrophic fungus *Pyrenophora tritici-repentis* (Died.) Drechs. [anamorph: *Drechslera tritici-repentis* (Died.) Shoem.] is an economically significant pathogen and is the causal agent of tan (or yellow) spot, a devastating foliar disease of wheat. This leaf spotting disease affects the major wheat (*Triticum aestivum* L.) and durum wheat (*T. turgidum* L.) growing areas of the world, and causes severe yield losses by reducing the leaf photosynthetic area (De Wolf *et al*., 1998). It is the most economically damaging disease of wheat in Australia (Murray and Brennan, 2009).

*Pyrenophora tritici-repentis* produces at least three effectors (host-selective toxins), referred to as ToxA, ToxB and ToxC (Lamari *et al*., 2003). These effectors interact in a highly specific manner with the host plant (Tan *et al*., 2010), leading to the development of two distinct foliar symptoms: tan necrosis and/or extensive chlorosis. The type of symptom depends on both the effectors produced by a particular isolate and the susceptibility genes present in the infected wheat host. So far, eight races of *P. tritici-repentis* have been defined, based on their ability to produce the three effectors alone or in combination (Lamari *et al*., 2003). ToxA causes the most severe damage by inducing necrosis on the leaves of ToxA-sensitive wheat genotypes (possessing the *Tsn1* susceptibility gene) (Faris *et al*., 2010), whereas ToxB and ToxC both induce chlorosis, although on different wheat genotypes harbouring the *Tsc2* and *Tsc1* loci, respectively (Effertz *et al*., 2002; Friesen and Faris, 2004). ToxA is the predominant effector in the tan spot–wheat pathosystem, and is present in the majority of isolates worldwide (Friesen *et al*., 2006). A single copy gene (*ToxA*) encodes the small (13.2 kDa) secreted ToxA protein (Ciuffetti *et al*., 1997; Tuori *et al*., 1995). The *ToxA* gene is sufficient for the pathogenicity of *P. tritici-repentis*, as transformation of a non-pathogenic isolate with *ToxA* was sufficient to render that isolate pathogenic on ToxA-sensitive wheat lines (Ciuffetti *et al*., 1997).

ToxA was first identified over 20 years ago as a necrosis toxin (Ballance *et al*., 1989), and isolation of the *ToxA* gene followed 8 years later (Ciuffetti *et al*., 1997). However, there have been no reports of *ToxA* gene knockout mutants, and moreover, to our knowledge, there have been no reports of any gene deletions of *P. tritici-repentis*. Recent work has been successful in the generation of partial knockdown mutants of *P. tritici-repentis* using a sense- and antisense-mediated RNA-silencing mechanism to reduce the expression of *ToxB* and an exo-1,3-β-glucanase gene (Aboukhaddour *et al*., 2012; Fu *et al*., 2013). Here, we report the successful generation of a *ToxA* knockout strain, and investigate mutant pathogenicity on a set of differential wheat genotypes. To our knowledge, this is the first study in which a gene has been successfully deleted in *P. tritici-repentis*.

## RESULTS

### *ToxA* gene disruption

Using a polymerase chain reaction (PCR) fusion strategy, a phleomycin resistance cassette (*Phleo*$^R$) was integrated into the *ToxA* target site by means of flanking sequences homologous to the targeted *ToxA* locus. Protoplasts of an Australian *P. tritici-repentis* isolate (M4) were transformed with the knockout construct, and putative transformants were screened via PCR for the absence of the *ToxA* gene. Four transformants found to have undergone homologous recombination were selected for further study, and were designated as *toxa-1*, *toxa-2*, *toxa-3* and *toxa-4*.

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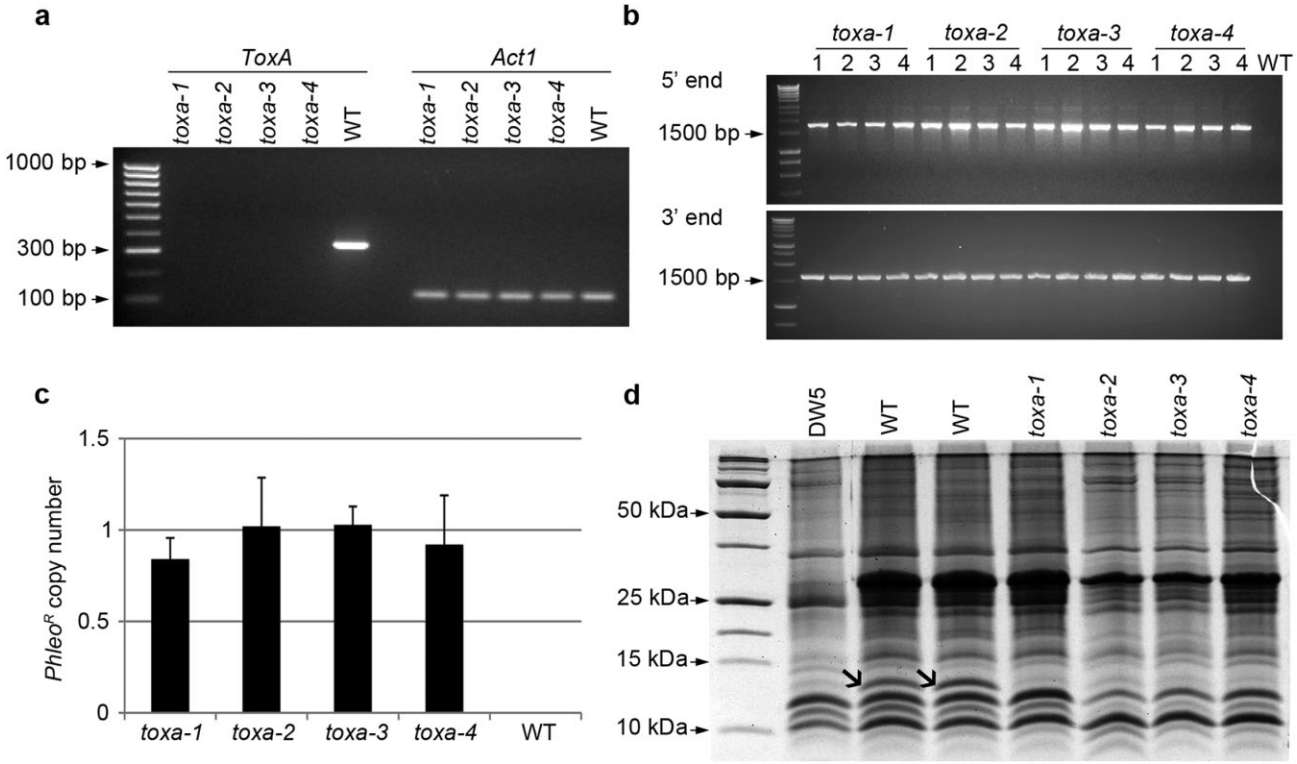


**Fig. 1** Confirmation of four independent *ToxA* knockout strains. (a) Reverse transcription-polymerase chain reaction (RT-PCR) detection of *ToxA* transcript (PCR product size of 393 bp) in the four knockout strains and wild-type (WT), as visualized by agarose gel electrophoresis. *Act1* was included as a positive control (PCR product size of 150 bp). (b) Correct integration of the *ToxA* gene deletion cassette at 5' and 3' ends in four replicates of the knockout strains. PCR product sizes for the 5' and 3' amplicons are 1.7 kb and 1.6 kb, respectively. (c) Phleomycin resistance cassette (*Phleo*$^R$) copy number as detected by quantitative PCR (qPCR). Error bars depict standard deviation. (d) Sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of culture filtrate proteins. Open arrows indicate ToxA (13.2 kDa). Two independent wild-type (WT) culture filtrate samples were analysed.

Absence of *ToxA* gene expression was confirmed in all four transformants based on reverse transcription-polymerase chain reaction (RT-PCR) analysis (Fig. 1a).

To exclude the possibility that the *ToxA* gene deletion construct had integrated ectopically elsewhere in the genome, mutants were screened via PCR to ensure that the construct had integrated at the intended homologous site (Fig. 1b). Correct targeted integration was confirmed for all four transformants.

The copy number of the phleomycin cassette was determined for each of the four mutants via quantitative polymerase chain reaction (qPCR) (Fig. 1c). As expected, the phleomycin resistance gene was not detected in the wild-type isolate. The *Phleo*$^R$ copy numbers revealed for each of the transformants were closely correlated with the known single copy of the *Act1* actin gene in the *P. tritici-repentis* genome sequence, thus confirming single integration of the *ToxA* knockout construct.

ToxA protein production was evaluated by sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of crude culture filtrates of wild-type, mutants and the race 5 isolate DW5, which does not produce ToxA (but instead produces ToxB) (Fig. 1d). The presence of notable bands with a mass of approximately 13.2 kDa, corresponding to the expected size of the ToxA protein (Tuori *et al*., 1995), was detected in the wild-type isolate, but not in the four transformants or DW5, although faint bands of a similar size were visualized in the knockouts and race 5 isolate. Therefore, to confirm the absence of ToxA, these gel bands were excised from wild-type, *toxa-1* and DW5, and subjected to peptide analysis by electrospray ionization mass spectrometry. As expected, ToxA was only identified within the wild-type isolate, and was absent from knockout and DW5 culture filtrates, which both contained other low-molecular-weight proteins (Fig. S1, see Supporting Information).

## Functional analysis of *ToxA* knockout mutants

In order to assess mutants for pathogenicity and to functionally confirm the deletion of *ToxA*, a set of five tan spot differential wheat genotypes, which differ in their effector sensitivities, were inoculated (Table 1) (Ciuffetti *et al*., 1997; Lamari *et al*., 1998, 2003). Also included was the ToxA-sensitive line BG261 (a *Parastagonospora nodorum* ToxA differential line) (Friesen *et al*., 2006). Plants were inoculated with conidial suspensions of the

**Table 1** Differential wheat genotypes and their corresponding effector sensitivities (adapted from Lamari *et al*., 1998). N and C denote necrosis and chlorosis, respectively, and a dash indicates insensitivity.

| | Differential wheat line | | | | |
|---|---|---|---|---|---|
| Effector | Glenlea | 6B662 | Katepwa | 6B365 | Auburn |
| ToxA | N | – | N | – | – |
| ToxB | – | C | C | – | – |
| ToxC | – | – | – | C | – |

wild-type isolate and two of the independent knockout mutants (*toxa-1* and *toxa-2*). As expected, the spreading necrosis typical of ToxA was observed on the ToxA-sensitive lines (cv. Glenlea, BG261 and cv. Katepwa) following infection with the wild-type isolate (Fig. 2). However, this spreading necrosis was absent on these lines following inoculation with the two mutants, thus functionally confirming *toxa-1* and *toxa-2* as lacking *ToxA*. The average disease score of the wild-type isolate was determined to be significantly higher than that of the mutants on these wheat lines (Fig. 3).

All three strains were unable to induce chlorosis on the ToxB-sensitive wheat line 6B662, although the wild-type isolate and both mutants induced chlorosis on the ToxC-sensitive wheat line 6B365 (Figs 2 and 3). No strain was able to successfully infect cv. Auburn, which is resistant to all races and insensitive to all known effectors produced by *P. tritici-repentis* (Ciuffetti *et al*., 1997). Katepwa has been reported to be resistant to race 3 (ToxC-producing) isolates (Lamari *et al*., 1998), and so the observation of mild chlorosis following inoculation with the *ToxA* knockouts is purportedly the result of an as yet unknown effector.

To further functionally validate the *ToxA* deletion, the differential wheat lines were infiltrated with crude culture filtrates of wild-type, *toxa-1* and *toxa-2* mutant strains and purified ToxA protein (Fig. 4). The knockout mutants were unable to cause necrosis on the three ToxA-sensitive lines (cv. Glenlea, BG261 and cv. Katepwa).

The wild-type isolate (M4) used in this study was collected from Australia, where, so far, all isolates tested (including M4) have been found to possess *ToxA* and to lack *ToxB* (Antoni *et al*., 2010). Inoculation with M4 conidia and culture filtrate infiltration induced chlorosis on the ToxC differential wheat line 6B365, thus confirming that this isolate also produces ToxC and hence belongs to race 1 ($ToxA^+$, $ToxB^-$, $ToxC^+$). Both of the *toxa* mutants tested also retained this chlorosis-inducing ability (Figs 2 and 4).

No observable effect on colony morphology, growth rate, sporulation or spore germination was detected in the knockouts compared with the wild-type (data not shown).

## DISCUSSION

Deletion of *ToxA* from *P. tritici-repentis* significantly reduced the extent of disease caused by this pathogen on the ToxA-sensitive

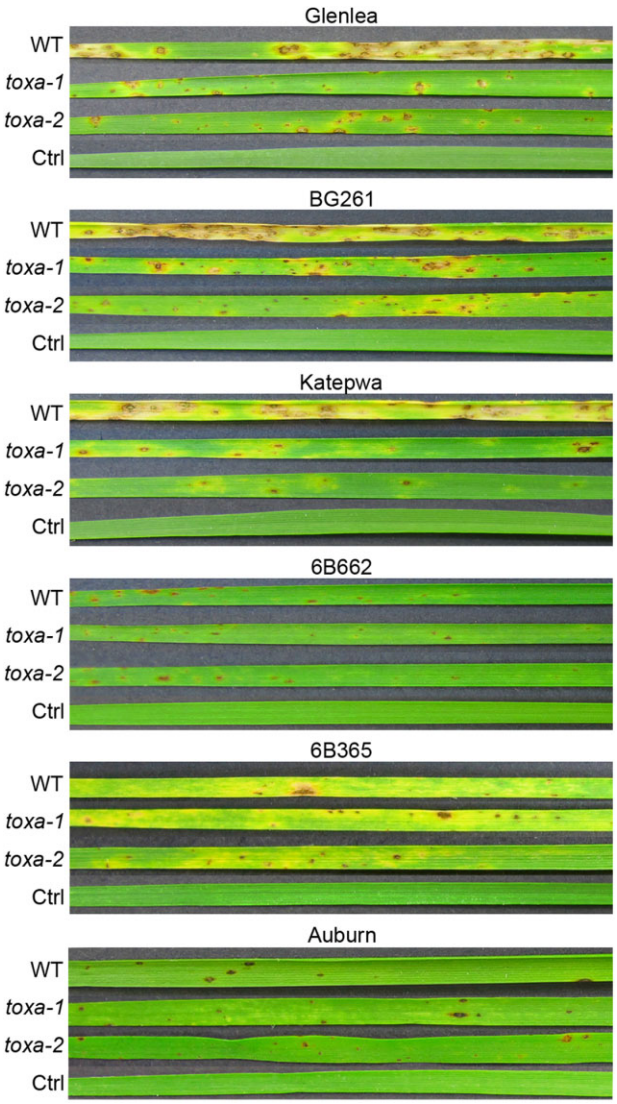


**Fig. 2** Reactions of a differential set of wheat lines to inoculation with wild-type (WT), *toxa* mutants and DW5. Inoculation with 0.25% gelatin (Ctrl) is included as a negative control. Images were taken at 7 days post-inoculation and show representative second leaf symptoms.

(*Tsn1*) wheat genotypes tested, and thus emphasizes the role of ToxA as a major necrotrophic effector. Although non-*ToxA*-containing isolates are found in certain parts of the world (e.g. North America, the Middle East and North Africa) (Friesen *et al*., 2005; Lamari *et al*., 2005; Strelkov *et al*., 2002), the majority of *P. tritici-repentis* isolates worldwide produce ToxA (Friesen *et al*., 2006; Lamari and Strelkov, 2010). In recent years, an increase in the prevalence of tan spot disease, particularly in Europe, has been reported (Crop Monitor, 2008/2009, Jorgensen and Olsen, 2007); however, the proportion of isolates that produce ToxA has yet to be determined, and the distribution of *ToxA* within *P. tritici-repentis* isolates worldwide requires further research.

 

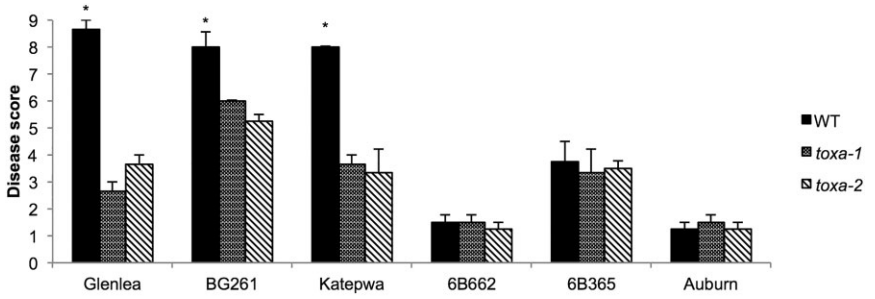


**Fig. 3** Pathogenicity assays of the *ToxA* knockout strains. Average disease scores of individual plants from one round of infection. Leaf symptoms were assessed for disease severity at 7 days post-inoculation using a nine point scale, where 1 represents absence of infection and 9 denotes total necrosis. Analysis of variance (ANOVA) revealed a significant effect of fungal strain on disease score ($P \leq 0.05$). Average disease scores with asterisks are significantly different as determined by Fisher's least-significant difference (LSD) *post-hoc* analysis ($P \leq 0.01$).

The ToxA–Tsn1 interaction is the best characterized host–effector interaction identified in wheat. The dominant *Tsn1* gene was identified on the long arm of chromosome 5B (Faris *et al*., 1996), and has been demonstrated to confer sensitivity to purified ToxA using *Tsn1*-disrupted mutants (Liu *et al*., 2006). Cloning of *Tsn1* revealed disease resistance gene features, such as serine/threonine protein kinase (S/TPK) and nucleotide-binding site-leucine-rich repeat (NBS-LRR) domains. All three domains are required for ToxA sensitivity, as demonstrated via induced mutagenesis (Faris *et al*., 2010). Although *Tsn1* is required to mediate ToxA recognition, it is unlikely to be the actual ToxA receptor. The Tsn1 protein does not contain any apparent transmembrane domains, and thus is probably located within the cell cytoplasm, and yeast two-hybrid assays suggest that the protein does not interact directly with ToxA (Faris *et al*., 2010). However, ToxA has been reported to interact with a chloroplast-localized protein known as ToxABP1 (Manning *et al*., 2007). Viral-induced gene silencing of *ToxABP1* in wheat has been reported to reduce the extent of ToxA-induced necrosis, although it is likely that other ToxA–host protein interactions are required for full necrosis (Manning *et al*., 2010).

In order to fully understand the *P. tritici-repentis*–wheat pathosystem, the role of ToxA in disease needs to be determined for all ToxA-producing races (races 1, 2, 7 and 8). An early report found that ToxA insensitivity was associated with resistance to race 2 ($ToxA^+$ $ToxB^-$ $ToxC^-$) (Lamari and Bernier, 1991). However, in another study, inoculation of this same race 2 isolate on different ToxA-insensitive wheat genotypes resulted in necrotic lesions, although they developed more slowly compared with those on *Tsn1* genotypes (Friesen *et al*., 2003). Therefore, although the production of ToxA is sufficient for pathogenicity (Ciuffetti *et al*., 1997), it does not appear to be necessary for disease on all host genotypes. Previous work to determine whether ToxA insensitivity equates to race 1 ($ToxA^+$ $ToxB^-$ $ToxC^+$) resistance has shown that wheat lines with mutations for ToxA insensitivity are susceptible following inoculation with two race 1 isolates (Friesen *et al*., 2002). This earlier work demonstrated that host sensitivity to ToxA is not necessarily equivalent to resistance to race 1, and one or more other effectors are in play, including ToxC. Indeed, our results agree with this, as we observed symptoms on BG261. The determination of the precise role of ToxA in tan spot disease will require further characterization of the *ToxA* knockout described herein, against a range of wheat genotypes, as well as the generation of *toxa* mutant strains of other races.

The ToxA–Tsn1 interaction has also been evaluated using segregating wheat lines. For example, a population of recombinant inbred lines (derived from Salamouni and Katepwa) has been evaluated for reaction to race 1 and race 2 isolates (Faris *et al*., 2012). Unsurprisingly, the *Tsn1* locus was significantly associated with disease for all isolates tested. However, the amount of variation explained by *Tsn1* varied considerably (ranging between 5% and 30%), which is suggestive of possible *ToxA* gene regulation variation among *P. tritici-repentis* isolates. This has been demonstrated for ToxA-producing isolates of another necrotrophic fungal pathogen of wheat, *Parastagonospora nodorum* (syn. *Stagonospora nodorum*; teleomorph: *Phaeosphaeria nodorum*), which expresses *ToxA* at different levels, such that expression of *ToxA* at higher levels causes more disease on *Tsn1* wheat (Faris *et al*., 2011). It is also conceivable that broad-spectrum or race non-specific resistance mechanisms may impede ToxA–Tsn1 interactions (Faris and Friesen, 2005), or that the consequences of host–effector interactions are reduced or masked as a result of epistatic effects with other host–effector interactions (Friesen *et al*., 2008).

The *ToxA* gene has been successfully deleted in *P. nodorum* and, similar to the approach presented herein, culture filtrates of these mutants were infiltrated on the *P. nodorum* differential line BG261 and were unable to induce necrosis, demonstrating that *ToxA* is necessary for complete virulence on *Tsn1* wheat lines (Friesen *et al*., 2006). The authors also performed infection assays on a wheat population segregating for *Tsn1* (BR34 × Grandin), and found that the *Tsn1* quantitative trait locus (QTL) associated with the disease phenotype was eliminated following inoculation with *ToxA*-disrupted *P. nodorum* mutants. In the same study,

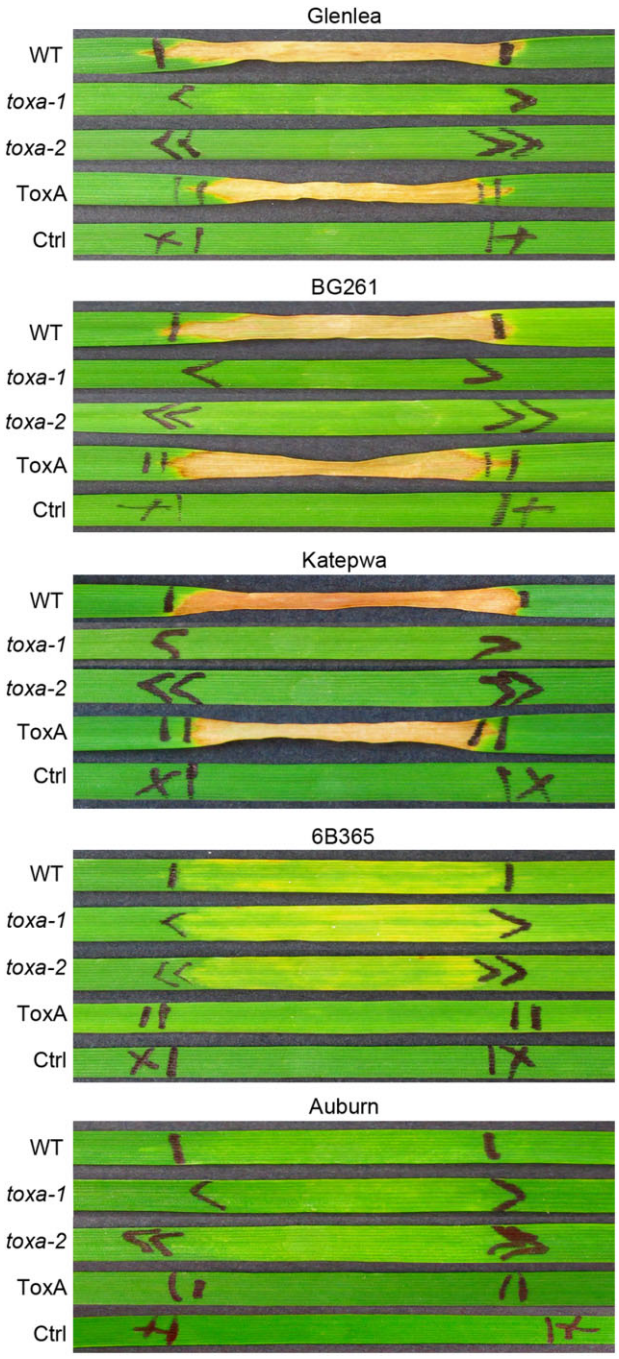


**Fig. 4** Sensitivity of differential wheat lines infiltrated with culture filtrates of wild-type (WT) and *toxa* mutants. Infiltrations with purified ToxA protein (ToxA) and Fries medium (Ctrl) are included as positive and negative controls, respectively. Images were taken at 10 days post-infiltration and show representative second leaf symptoms.

expression of either *ToxA* from *P. tritici-repentis* or from *P. nodorum* in an avirulent (*ToxA*-lacking) *P. nodorum* strain was sufficient to confer virulence that co-segregated with *Tsn1*. Despite predicted amino acid differences, this demonstrates that *P. tritici-repentis ToxA* and *P. nodorum ToxA* are functionally identical in their interaction with *Tsn1*, and there is evidence that the *ToxA* gene was horizontally transferred from *P. nodorum* to *P. tritici-repentis* (Friesen *et al*., 2006). The full suite of native effectors will differ between the two pathogens, and so it is important to dissect separately the role of ToxA within each pathosystem.

The work presented herein opens up several new vistas for future research. First, removal of ToxA, the predominant effector of *P. tritici-repentis*, will facilitate the identification of other, as yet uncharacterized, effectors, whose effects have so far been masked by extensive ToxA-induced necrosis. This is in agreement with previous work, whereby inoculation of *Tsn1*-disrupted mutants still resulted in disease (Friesen *et al*., 2003), and sensitivity to ToxA was a non-significant factor in disease development following inoculation of the BR34 × Grandin population with race 1 and 2 isolates (Faris and Friesen, 2005).

Second, the generation of *toxa* mutants will enable the screening of *Tsn1* wheat cultivars for sensitivity to other novel effectors. In particular, this will permit the screening of ToxA-sensitive wheat mapping populations for novel effector sensitivity and disease resistance QTLs, and thus facilitate the identification of molecular markers for wheat breeders and potential targets for crossing. Thus far, the identification of novel QTLs has been limited to just a handful of mapping populations with ToxA-insensitive parents.

Third, the development of a targeted gene knockout method for such an economically significant and global wheat pathogen is noteworthy. Over the last 2 years, an RNA-silencing method has been successfully developed to reduce gene expression in *P. tritici-repentis* (Aboukhaddour *et al*., 2012; Fu *et al*., 2013). This technique was used to create *ToxB*-silenced transformants, with ToxB production ranging from 15% to 81% of Western blot band intensity relative to the wild-type (Aboukhaddour *et al*., 2012). In the case of ToxB, such an approach was well justified as the *ToxB* gene is found in multiple copies, the number of which varies among isolates (Martinez *et al*., 2001; Strelkov *et al*., 2005). Thus, to knock out all individual copies of *ToxB* would be inherently more challenging than RNA silencing. However, the RNA-silencing strategy is less suitable for single copy effector gene discovery, as it results in partial gene knockdowns with variable expression levels, and thus will not eliminate gene expression entirely. Here, we show, as proof-of-concept, that *P. tritici-repentis* possesses the necessary machinery to bring about the precise integration of exogenous sequences through homologous recombination, and paves the way for the creation of knockouts of other potential genes of interest, and the introduction of specific mutations into a target gene.

The results of this study reiterate the need to increase the area sown to ToxA-insensitive wheat varieties and, ultimately, to phase out *Tsn1* cultivars, particularly in wheat-growing regions with a high proportion of *ToxA*-expressing isolates. A recent wheat

 

cultivar trial found that there was no yield penalty associated with growing ToxA-insensitive varieties and, moreover, in the presence of disease, ToxA-insensitive lines substantially outperformed the sensitive lines (Oliver *et al*., 2014).

In Australia, effector-assisted breeding has been adopted in response to the significant combined losses caused by ToxA-producing pathogens ($212 million caused by tan spot and $108 million caused by *Septoria nodorum* blotch) (Murray and Brennan, 2009). Semi-purified ToxA has been delivered to wheat breeders since 2009 as a selection tool towards the development of disease-resistant germplasm, with current delivery of 30 000 doses per annum (Vleeshouwers and Oliver, 2014). As a result, there has been a considerable decrease in the area sown to ToxA-sensitive wheat varieties, a major step to reduce the huge scale of losses caused by tan spot. However, in accordance with this study and earlier findings, screening germplasm with ToxA should not be used in place of fungal inoculation by breeding programmes, as ToxA sensitivity is not always required for susceptibility to race 1. This is probably because of the presence of ToxC and other effectors not yet identified, and there are currently no Australian commercial wheat varieties rated as resistant to tan spot (DAFWA, 2014). A thorough understanding of the role of ToxA and other effectors in tan spot disease is required, and knockout capability can be expected to expedite strategies targeting the release of resistant lines.

## EXPERIMENTAL PROCEDURES

### Fungal material and growth conditions

The pathogenic *P. tritici-repentis* race 1 isolate M4 was collected from Meckering, Western Australia in 2009. PCR amplification of M4 gDNA confirmed the presence of the *ToxA* gene and the absence of *ToxB* using the primers ToxAscreeningF/R and TB10f/TB12r, respectively (Antoni *et al*., 2010). Fungi were grown on V8-PDA plates (Campbell's V8 juice, 150 mL/L; potato dextrose agar, 10 g/L; $CaCO_3$, 3 g/L; agar, 15 g/L) and incubated at 22 °C under 12-h cycles of light. Sporulation was induced by flooding the plates with ultrapure water and flattening colonies using an L-shaped glass rod. Plates were placed under near-UV and fluorescent lights for 24 h, followed by incubation at 15 °C in darkness for 24 h. Liquid cultures were started with crushed mycelia in Fries medium (Liu *et al*., 2004), and grown at 27 °C and 100 rpm in darkness. For culture filtrates, liquid cultures were shaken for 3 days, followed by 2.5 weeks of stationary growth. The filtrate was harvested by filtration through sterile gauze, MiraCloth (Merck Millipore, Billerica, MA, USA), and passed through a 0.2-μm syringe filter unit (Pall Life Sciences, Port Washington, NY, USA). For spore germination assays, 100 conidia suspended in water were germinated per strain on 1.5% agarose at 4 °C. After 17 h, the number of germ tubes was counted per conidium. Three independent replicates were performed per fungal strain.

### Development of the *ToxA* knockout construct

A fusion PCR approach was undertaken for the inactivation of *ToxA*, whereby two homologous flanking regions and the phleomycin resistance cassette were amplified separately and then fused in a single PCR. Flanking regions of the *ToxA* gene (PTRG_04889) were amplified from genomic M4 DNA. A 1639-bp upstream flanking region was amplified using PtrToxA5'f and PtrToxA5'r primers, whereas PtrToxA3'f and PtrToxA3'r primers were used to amplify a 1561-bp downstream sequence. A phleomycin cassette (*Phleo*$^R$) was amplified from pAN8-1 using the primers pAN8f and pAN8r, as described previously (Solomon *et al*., 2006). Incorporated into the 5' regions of the PtrToxA3'f and PtrToxA5'r primers were 25 bp and 23 bp of sequence homologous to the 3' and 5' ends of the phleomycin fragment. Fragments were gel extracted using a QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany), and equimolar amounts were combined as template for a single fusion PCR using primers PtrToxA5'f and PtrToxA3'r at a final concentration of 50 μM. The fusion PCR was performed using iProof High-Fidelity Master Mix (Bio-Rad, Hercules, CA, USA) with the following cycling conditions: 98 °C/30 s; (98 °C/5 s, 67 °C/30 s, 72 °C/5 min) × 35; 72 °C/5 min. The fusion PCR product (5624 bp) was gel extracted and resuspended in sterile water. A further amplification with nested primers (PtrToxA5'Nf and PtrToxA3'Nr) was performed to generate the final gene deletion construct. All primer sequences are detailed in Table 2.

**Table 2** Primers used throughout this study. Bold text refers to sequence complementary to the phleomycin resistance cassette primers (pAN8f and pAN8r).

| Primer name | Sequence |
|---|---|
| PtrToxA5'f | TCCGCTCTCGATTACCGGCTCA |
| PtrToxA5'r | **TGTGACTTTTGGTTACGCCGTCT**TGTCAATGTCGACTTGGCCGATG |
| PtrToxA3'f | **TCTCCTATGAGTCGTTTACCCAGAA**TCAATGGGAATAAGTCTCCCCACCA |
| PtrToxA3'r | GCGCTCTCGGTACGCTCCTC |
| pAN8f | AGACGGCGTAACCAAAAGTCACA |
| pAN8r | TTCTGGGTAAACGACTCATAGGAGA |
| PtrToxA5'Nf | TGTTCGAGCCTGGTTCAGAT |
| PtrToxA3'Nr | CCTATCTTAAGGGCGGCTTC |
| PtrToxAF2 | ACCGGCAGGACTAATCGCCTCA |
| PtrToxAR2 | CCAACACGTGCCGTTCCGGT |
| Phleo5 | CTCCGTCTTCCGTAGCCGTG |
| Phleo3 | CCAATACGCCGGCCGAAAC |
| Act1F4 | CGAGACCTTCAACGCTCCCGC |
| Act1R4 | GCGTGGGGAAGAGCGAAACCC |
| PhleoF4 | GACCGAGATCGGCGAGCAGC |
| PhleoR4 | TCAAGCTCCTGGGACCCGTGG |

## Fungal transformation

A polyethylene glycol (PEG)-mediated protoplast transformation method was used (Aboukhaddour *et al*., 2012). Transformations were performed using 5 μg of DNA per $1 \times 10^7$ protoplasts. Protoplasts were overlaid with RM agar (Aboukhaddour *et al*., 2012) amended to a final concentration of 10 μg/mL phleomycin. Resistant colonies were transferred to V8-PDA agar containing 15 μg/mL phleomycin for a second round of screening. Putative transformants were screened via PCR in order to verify the absence of the *ToxA* gene (primers PtrToxAF2 and PtrToxAR2) and correct genomic integration of the gene deletion construct (primer combinations PtrToxA5'f/Phleo5 and Phleo3/PtrToxA3'r which amplify the 5' and 3' flanking regions from positive transformants, respectively). The thermal cycling conditions were as follows: 94 °C/3 min; (94 °C/30 s, 58 °C/30 s, 72 °C/2 min) × 35; 72 °C/5 min. Single spore re-isolation was performed for all true transformants to ensure mutant purity.

## RNA extraction and transcript expression

Total RNA was isolated from 1-week-old fungal liquid cultures using TRIzol Reagent, according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). RNA was reversed transcribed using iScript reverse transcriptase (Bio-Rad), according to the manufacturer's protocol. The resulting cDNA was quantified using a NanoDrop 2000 UV-Vis spectrophotometer (ThermoScientific, Waltham, MA, USA). Primers PtrToxAF2 and PtrToxAR2 were used to test for *ToxA* expression in wild-type and transformants. As a control, expression of the housekeeping actin gene (*Act1*) was tested using Act1F4 and Act1R4 primers. PCR products were visualized by agarose gel electrophoresis on a 1.5% agarose gel using SYBR Safe DNA Gel Stain (Life Technologies, Carlsbad, CA, USA).

## Determination of copy number

qPCR was performed to confirm single integration of the *ToxA* knockout construct into the M4 genome, as has been described previously to determine the copy number of introduced gene cassettes in fungal transformants (Solomon *et al*., 2008). Genomic DNA was extracted from wild-type and transformants using the Biosprint 15 DNA kit (Qiagen) according to the manufacturer's instructions. For detection of the *ToxA* knockout construct copy number, a 150-bp region of the phleomycin resistance cassette (*Phleo*$^R$) was amplified using the primers PhleoF4 and PhleoR4. As an endogenous control, primers Act1F4 and Act1R4 were used to amplify a 150-bp fragment from the single copy actin gene (*Act1*) (Ellwood *et al*., 2012). Thermal cycling conditions were 95 °C/15 min, (94 °C/15 s, 63 °C/30 s, 72 °C/30 s) × 35, and were performed in a CFX96 Real-Time PCR Detection System (Bio-Rad). Each 20-μL qPCR consisted of 50 ng of DNA, 10 μL of QuantiTect SYBR Green PCR mix (Qiagen) and 300 nM of the appropriate primers. PCR efficiencies of the target (*Phleo*$^R$) and reference (*Act1*) qPCR amplifications were tested to be approximately equal. The *Phleo*$^R$ copy number was normalized to the *Act1* copy number using the $\Delta\Delta C_t$ method. Samples were analysed in triplicate with two technical replicates.

## ToxA protein production

Fungal culture filtrates were analysed by SDS-PAGE performed on a Mini-PROTEAN 3 vertical gel apparatus (Bio-Rad). Culture filtrates were passed through PD-10 desalting columns to remove salts and low-molecular-weight impurities, according to the manufacturer's instructions (GE Healthcare, Fairfield, CT, USA). Proteins were resolved via a 16.5% polyacrylamide separating gel using the Tris–tricine buffer separation system (Schagger and Vonjagow, 1987). Approximately 40 μg of each total protein sample was loaded per lane and the Precision Plus Protein Standard (Bio-Rad) was used as protein molecular weight standard. Gels were fixed and visualized via Coomassie G250 colloidal staining (Neuhoff *et al*., 1988). Bands of the expected ToxA size were excised individually from the gel for M4, DW5 and *toxa-1*, trypsin digested and peptides were extracted according to standard techniques (Bringans *et al*., 2008). Peptides were analysed by electrospray ionization mass spectrometry using the Shimadzu Prominence nano-high-performance liquid chromatography (HPLC) system (Shimadzu, Kyoto, Japan) coupled to a 5600 triple time-of-flight mass spectrometer (AB Sciex, Framingham, MA, USA). Tryptic peptides were loaded onto an Agilent Zorbax 300SB-C18, 3.5 μm (Agilent Technologies, Santa Clara, USA), and separated with a linear gradient of water–acetonitrile–0.1% formic acid (v/v/v). Spectra were analysed to identify proteins of interest using Mascot sequence matching software (Matrix Science, London, UK) with the Ludwig NR database.

## Plant material and pathogenicity assays

Wheat seeds (*Triticum aestivum* L.) were obtained from the Australian Winter Cereals Collection (AWCC), Tamworth, NSW, Australia. For culture filtrate assays, seeds were sown in Grade 2 vermiculite (The Perlite and Vermiculite Factory, Jandakot, WA, Australia) in seed trays, and grown at 20 °C under a 12-h day/night cycle in a controlled growth chamber. Fully extended leaves of 2-week-old wheat plants were infiltrated with crude culture filtrate from the wild-type or *toxa* mutants. A needleless 1-mL syringe was used to infiltrate the adaxial surface of second leaves, and the infiltration boundaries were marked with a permanent marker pen. Leaves were evaluated at 10 days post-infiltration. All infiltration experiments were repeated twice with consistent results, using a minimum of four plants per line each time. The ToxA protein was purified as described previously (Tan *et al*., 2012) and infiltrated at 50 μg/mL.

For the infections, pots (10 cm in diameter) containing P500 perlite (The Perlite and Vermiculite Factory) and vermiculite were sown with four seeds and grown at 21 °C under a 12-h day/night cycle. Inoculum was prepared consisting of approximately 2000 conidia/mL in 0.25% gelatin. Infection assays were performed by evenly spraying 2-week-old plants (at the two- to three-leaf stage) with inoculum using a spray bottle until run-off. The plants were incubated in a misting chamber for 2 days (relative humidity ≥ 95%) with continuous moisture supplied by a humidifier. At 7 days post-inoculation, the second leaves were harvested and photographed, and plants were visually assessed for disease severity based on the Australian wheat disease resistance ratings scale (DAFWA, 2014), whereby varieties are rated between 1 and 9 (1, absence of infection (resistant); 9, total necrosis (very susceptible)). All infection experiments were independently repeated twice with consistent results, using a minimum of four replicates (pots) per treatment and performed as blind experiments.

Disease scores were analysed by analysis of variance (ANOVA) to determine any significant differences between isolate virulence ($P \leq 0.05$), followed by Fisher's least significant difference (LSD) *post-hoc* analysis to identify which mean values were significantly different ($P \leq 0.01$). Prior to ANOVA, raw data were checked to ensure homogeneity of variance.

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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:

**Fig. S1** *Pyrenophora tritici-repentis* proteins identified from sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) excised bands of culture filtrates. (a) SDS-PAGE of culture filtrate proteins (as shown in Fig. 1a) with arrows indicating excised bands. Two bands (upper and lower) were excised from three strains: wild-type (WT), DW5 and *toxa-1*. (b) The peptide fragmentation data from liquid chromatography-tandem mass spectrometry (LC/MS/MS) were searched against the non-redundant Ludwig NR database using the MASCOT sequence matching software. Only significant hits (score >35) with a significance threshold of $P < 0.01$ and a minimum of two peptide matches are shown.

 