A functionally conserved Zn$_2$Cys$_6$ binuclear cluster transcription factor class regulates necrotrophic effector gene expression and host-specific virulence of two major Pleosporales fungal pathogens of wheat

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SUMMARY

The fungus *Parastagonospora nodorum* is the causal agent of Septoria nodorum blotch of wheat (*Triticum aestivum*). The interaction is mediated by multiple fungal necrotrophic effector–dominant host sensitivity gene interactions. The three best-characterized effector–sensitivity gene systems are SnToxA–Tsn1, SnTox1–Snn1 and SnTox3–Snn3. These effector genes are highly expressed during early infection, but expression decreases as the infection progresses to tissue necrosis and sporulation. However, the mechanism of regulation is unknown. We have identified and functionally characterized a gene, referred to as *PtrPf2*, which encodes a putative zinc finger transcription factor. *PtrPf2* deletion resulted in the down-regulation of SnToxA and SnTox3 expression. Virulence on Tsn1 and Snn3 wheat cultivars was strongly reduced. The SnTox1–Snn1 interaction remained unaffected. Furthermore, we have also identified and deleted an orthologous *PtrPf2* from the tan spot fungus *Pyrenophora tritici-repentis* which possesses a near-identical ToxA that was acquired from *P. nodorum* via horizontal gene transfer. *PtrPf2* deletion also resulted in the down-regulation of *PtrToxA* expression and a near-complete loss of virulence on *Tsn1* wheat. We have demonstrated, for the first time, evidence for a functionally conserved signalling component that plays a role in the regulation of a common/horizontally transferred effector found in two major fungal pathogens of wheat.

Keywords: effector regulation, *Parastagonospora nodorum*, *Pyrenophora tritici-repentis*, Septoria nodorum blotch, SnTox3, tan spot, ToxA.

INTRODUCTION

*Parastagonospora* (syn. *Stagonospora*, *Phaeosphaeria*, *Septoria*) *nodorum* (Berk.) Quaedvlieg, Verkle & Crous is the causal agent of Septoria nodorum blotch (SNB) of wheat (Quaedvlieg et al., 2013; Solomon et al., 2006a). The fungus causes significant damage to the leaves and glumes of wheat, and is responsible for substantial yield losses in many wheat-growing areas (Eyal, 1999; Murray and Brennan, 2009; Oliver et al., 2014, 2016). *Parastagonospora nodorum* belongs to the order Pleosporales, which predominantly consists of fungal pathogens that possess a necrotrophic lifestyle. Many have been shown or hypothesized to use host-specific effectors to facilitate disease development (Friesen et al., 2008a). *Parastagonospora nodorum* uses a series of proteinaceous necrotrophic effectors (NEs) to confer virulence on wheat that carries matching dominant sensitivity/susceptibility genes (Tan et al., 2010). Thus far, three proteinaceous NEs have been identified at the gene level. SnToxA encodes a 13.2-kDa mature protein which causes necrosis on wheat varieties that carry *Tsn1* located on wheat chromosome 5BL (Faris et al., 2010; 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 encodes a cysteine-rich, 17.5-kDa mature protein. 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). The role of NEs in *P. nodorum* is well defined. *Parastagonospora nodorum* mutants carrying effector gene deletions are poorly pathogenic on wheat cultivars with matching receptors (Friesen et al., 2006; Liu et al., 2009, 2012). Furthermore, quantitative trait locus (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, SnTox5–Snn5, SnTox6–Snn6 and SnTox7–Snn7 (Friesen et al., 2008a; Gao et al., 2015; Shi et al., 2015; Tan et al., 2015). However, the genes that code for these NEs and the host dominant susceptibility receptors remain unidentified.

The acquisition of NE genes confers virulence and broadens the host range of a plant pathogen (Mehrabi et al., 2011). *Pyrenophora tritici-repentis* is the causal agent of tan spot (syn. yellow spot) of wheat. Like *P. nodorum*, *Py. tritici-repentis* relies on
several NEs to facilitate host infection. Among these is a near-identical ToxA-encoding gene, called \( \text{PtrToxA} \), which may have been acquired from \( P. \text{nodorum} \) through a horizontal gene transfer event (Ciuffetti et al., 1997; Friesen et al., 2006). The acquisition of \( \text{PtrToxA} \) in \( P. \text{tritici-repentis} \) has led to the emergence of a devastating disease of wheat that is the dominant wheat pathogen in Australia and is important in many other parts of the world (Oliver et al., 2016). Similar to the \( P. \text{nodorum} \) \( \text{SnToxA} \) NE, \( \text{PtrToxA} \) causes necrosis on \( Tsn1 \) wheat (Liu et al., 2006). The horizontally transferred region is at least 11 kb and includes DNA upstream of \( \text{PtrToxA} \) which consists of a constitutive promoter (Lorang et al., 2001). It therefore seems likely that \( \text{SnToxA} \) and \( \text{PtrToxA} \) share a common signalling pathway. Evidence for an effector gene regulatory mechanism in \( P. \text{nodorum} \) was first reported by IpCho et al. (2010). Deletion of the APSES class transcription factor gene \( \text{SnStuA} \) in \( P. \text{nodorum} \) impaired vegetative development, abolished the ability to sporulate and caused a reduction in pathogenicity on wheat. Interestingly, the expression of \( \text{SnTox3} \) was down-regulated in the \( \text{snstuA} \) mutants (IpCho et al., 2010).

The SNB pathosystem is dictated by effector–host sensitivity receptor epistatic interactions. Several studies have demonstrated that \( \text{SnToxA–Tsn1} \) (Friesen et al., 2008b), \( \text{SnTox2–Sn2} \) (Friesen et al., 2008b), \( \text{SnTox5–Snn5} \) (Friesen et al., 2012) and \( \text{SnTox6–Snn6} \) (Gao et al., 2015) interactions are epistatic to \( \text{SnTox3–Snn3} \). However, the mechanism of epistasis is unclear. We have demonstrated recently that the \( \text{SnTox1–Sn1} \) interaction is epistically dominant to \( \text{SnTox3–Snn3} \) in the establishment of SNB in popular Australian wheat varieties (Phan et al., 2016). When \( \text{SnTox1} \) was removed from \( P. \text{nodorum} \) via gene deletion, the \( \text{SnTox3–Snn3} \) interaction played an active role in SNB. Gene expression analysis indicated that \( \text{SnTox3} \) expression increased in the absence of \( \text{SnTox1} \). The mechanism associated with the repression of \( \text{SnTox3} \) expression by \( \text{SnTox1} \) is unknown at this stage. Similarly in \( P. \text{tritici-repentis} \), \( \text{PtrToxA} \)-induced symptoms on wheat are epistatic to other effector-induced symptoms (Manning and Ciuffetti, 2015). However, the mechanism underlying the epistatic regulation is unknown. The expression of NE genes in both fungi is complex and a knowledge of how these NE genes are regulated is still lacking.

The Pleosporales fungus \( \text{Alternaria brassicicola} \) is the causal agent of dark leaf spot on many \( \text{Brassica} \) species. A putative GAL4-like transcription factor gene, \( \text{AbPf2} \), of \( A. \text{brassicicola} \) was identified and characterized for its role in vegetative fitness and plant virulence. Mutants deleted in \( \text{AbPf2} \) were able to grow slowly on various \( \text{Brassica} \) hosts, but were unable to cause any disease symptoms (Cho et al., 2013). RNA sequencing (RNAseq) analysis indicated that \( \text{AbPf2} \) functions as a positive regulator of genes encoding secreted proteins, some of which possess hallmarks of effectors. However, evidence on the use of host-specific effectors to mediate virulence on \( \text{Brassicae} \) remains unsubstantiated at this stage (Cho, 2015). In this study, we sought to determine the role of the Pf2 family in two economically important Pleosporales fungi in which proteinaceous NE–host susceptibility/sensitivity gene interactions are well defined (Ciuffetti et al., 2010; Oliver et al., 2012). A simple homology search in the \( P. \text{nodorum} \) genome identified a gene that is orthologous to \( \text{AbPf2} \), which we refer to as \( \text{PnPf2} \). A functional orthologue of \( \text{PnPf2} \) was also identified in the genome of \( P. \text{tritici-repentis} \): \( \text{PtrPf2} \). Functional analyses of \( \text{PnPf2} \) and \( \text{PtrPf2} \) provided a compelling insight into their role in the regulation of major effector genes and in conferring host-specific virulence on wheat.

**RESULTS**

**Identification of the \( \text{AbPf2} \) orthologue in \( P. \text{nodorum} \) and \( P. \text{tritici-repentis} \)**

The putative GAL4-like transcription factor gene \( \text{AbPf2} \) of \( A. \text{brassicicola} \) functions as a virulence factor and positively regulates genes encoding effector-like proteins (Cho et al., 2013). To determine whether \( P. \text{nodorum} \) possess an orthologue, the \( \text{AbPf2} \) polypeptide sequence of 651 amino acids (Genbank Accession AFQ61041) was used to search the ‘\( \text{Phaeosphaeria nodorum} \) SN15’ Genbank non-redundant (nr) protein database, and identified \( \text{SNOG}_00649 \) (Genbank Accession XP_001791330) as the best BLAST hit.

The corrected open reading frame (ORF) and amino acid sequence of \( \text{SNOG}_00649 \) are located at https://github.com/robysyme/Parastagonospora_nodorum_SN15 (Syme et al., 2016). The \( \text{SNOG}_00649 \) ORF consists of four RNAseq-validated exons that encode a 652-amino-acid polypeptide (Syme et al., 2016). CD-BLAST analysis identified two conserved domains: a GAL4-like \( \text{Zn}2\text{Cys}6 \) binuclear cluster domain from amino acid 11 to 46, and a fungal transcription factor regulatory middle homology region from amino acid 104 to 340 (Fig. S1, see Supporting Information). The \( \text{SNOG}_00649 \) polypeptide shares 76% amino acid identity with \( \text{AbPf2} \). Phylogenetic analysis of the \( \text{SNOG}_00649 \) polypeptide indicated that it groups with \( \text{AbPf2} \) and shares closest sequence resemblance to an orthologue from the canola blackleg fungus \( \text{Leptosphaeria maculans} \) (Fig. 1). Hereafter, we designate the \( \text{SNOG}_00649 \) gene as \( \text{PnPf2} \). Interestingly, \( \text{Pf2} \) orthologues are exclusively derived from Pleosporales fungal pathogens.

In \( P. \text{tritici-repentis} \) wild-type isolate M4 (race 1 strain from Australia; \( \text{ToxA}^{+} \), \( \text{ToxB}^{−} \), \( \text{ToxC}^{+} \)), \( \text{BLAST} \)P analysis identified two potential \( \text{Pf2} \) orthologues: \( \text{M4}_04272 \) and \( \text{M4}_04272 \). \( \text{M4}_04272 \) was supported by RNAseq datasets derived from \( \text{in vitro-grown} \) and infected wheat. \( \text{M4}_04272 \) was identified via \( \text{in silico} \) gene prediction as RNAseq data were unavailable (See et al., unpublished data). \( \text{M4}_04272 \) consists of four exons that encode a 650-amino-acid polypeptide. Nucleotide sequence analysis indicates that \( \text{M4}_04272 \) is near-identical to its orthologue \( \text{PTRG}_06982 \) (Genbank ID: XP_001937314) from \( P. \text{tritici-repentis} \) Pt-1C-BFP,
an isolate from the USA with a reported genome sequence (Manning et al., 2013). The former possess two missing nucleotides in the second intron. In addition, M4_04272 and PTRG_06982 possess different annotations at the 5′ region (Fig. S2A, see Supporting Information). M4_11517 consists of three exons that encode a 272-amino-acid polypeptide. The nucleotide gene sequence of M4_11517 shows 100% sequence identity to the gene sequence of PTRG_11777 (XP_001942108) of Py. tritici-repentis Pt-1C-BFP, with the exception of one single nucleotide polymorphism (SNP) located outside the coding regions (Fig. S2B). The annotation of both ORFs differs at the 5′ region (Fig. S2B). CD-BLAST analysis (Marchler-Bauer et al., 2015) of M4_04272 identified two conserved domains that are similar to predictions for AbPf2 and PnPf2 (Fig. S1). The fungal transcription factor regulatory middle homology region was predicted for the M4_11517 polypeptide and its orthologue PTRG_11777. The GAL4 domain was not predicted for either polypeptide using current gene annotations. PnPf2 and M4_04272 shared 76% amino acid identity, whereas similarity between PnPf2 and M4_11517 was much lower at 55% between the aligned amino acid residues. Thus, M4_04272 is the closest orthologue to PnPf2 based on sequence analyses. Hereafter, M4_04272 is referred to as PtrPf2.

PnPf2 and PtrPf2 are expressed during wheat infection

The expression profile of PnPf2 during wheat infection was determined using quantitative real-time polymerase chain reaction (PCR). Infection time points, presented as days post-infection (dpi), were chosen to represent all major stages of infection, as described by Solomon et al. (2006c): early penetration (3 dpi), colonization (6 dpi), and pycnidiation (8 and 10 dpi). The expression...
level of PnPf2 was maximal at 3 and 6 dpi, but decreased during the onset of pycnidiation (Fig. 2A). The expression of SnToxA, SnTox1 and SnTox3 was also examined. All three effector genes demonstrated maximal expression at 3 dpi, followed by a dramatic decrease in expression from 6 dpi which coincided with tissue necrosis (Fig. 2B). SnTox1, SnTox3 and PnPf2, but not SnToxA, transcripts were also readily detected during growth in Fries 3 broth (Fig. 2C).

For Py. tritici-repentis, PtrPf2 was constitutively expressed between 3 and 10 dpi on wheat (Fig. 2D). Similar to SnToxA, the expression of PtrToxA was maximal during early infection (3 dpi), but gradually decreased from 6 dpi onwards, which coincided with the onset of chlorosis and necrosis of the host tissue (Fig. 2E). PtrPf2 and PtrToxA were expressed during axenic growth in Fries 3 broth (Fig. 2F).

**Comparative analysis of the ToxA promoter region in P. nodorum and Py. tritici-repentis reveals a high level of genetic polymorphism**

As SnToxA and PtrToxA demonstrated differential expression during in vitro growth, we hypothesized that genetic polymorphisms were present within the promoter region of the Pf2 orthologues. We compared the 5′ untranslated region (UTR) of two P. nodorum isolates (SN15, Australia and SN4, USA) with that of two Py. tritici-repentis isolates (M4, Australia and Pt-1C-BFP, USA). Sequence alignment of the 5′ ToxA UTR promoter region revealed evidence of genetic polymorphism, including sectional deletions and SNPs between the two species and P. nodorum strains (Fig. 3). This may account for the differences in SnToxA and PtrToxA in vitro expression profiles.

**PnPf2 is deleted in P. nodorum SN15**

As PnPf2 is expressed during infection, we deleted the gene in the P. nodorum SN15 wild-type background using targeted gene deletion to determine its function in virulence on wheat (Fig. S4, see Supporting Information). Mutants that carry the appropriate gene deletion were identified using PCR. A robust quantitative PCR method was used to determine the copy number of PnPf2 deletion constructs in all transformants to identify appropriate mutants that carry single-copy integration (Solomon et al., 2008). Consequently, strains deleted in PnPf2 (pf2-63 and pf2-69) and an ectopic (Ect) mutant were retained for phenotypic characterization (Table 1). The Ect strain contains a selectable marker insertion elsewhere in the genome other than in PnPf2, and thus all assayed phenotypes should be similar to SN15.
A preliminary analysis of pf2-63 and pf2-69 indicated that vegetative growth on an undefined solid medium was indistinguishable from that of SN15 and Ect (Fig. S5A, see Supporting Information). Furthermore, no significant differences in asexual sporulation were observed in the absence of PnPf2 (Fig. S5B). This suggests that PnPf2 plays a dispensable role in vegetative growth.

**PnPf2 deletion reduces P. nodorum virulence on Tsn1 and Snn3 wheat cultivars**

The role of PnPf2 in virulence was examined using a whole-plant spray infection assay. Wheat cultivars BG261 (Tsn1, snn1, snn3), Calingiri (tsn1, Snn1, snn3) and BG220 (tsn1, snn1, Snn3) were used as hosts as they contain differential sensitivities to all three effectors (Liu et al., 2009, 2012; Tan et al., 2014). *Parastagonospora nodorum pf2-63* and pf2-69 were able to cause full infection on Calingiri, but gave no significant necrosis on BG261 and BG220 (Fig. 4A). We tested the disease susceptibility of two other independent Snn1- and Snn3-specific wheat cultivars: Chinese Spring (Snn1, tsn1, snn3) and Wyalkatchem (tsn1, snn1, Snn3) (Tan et al., 2014). PnPf2 deletion impaired virulence on Wyalkatchem, but remained fully pathogenic on Chinese Spring (Fig. S6, see Supporting Information). Apart from BG261, we were unable to source another independent Tsn1 wheat cultivar that was insensitive to SnTox1 and SnTox3.

*Parastagonospora nodorum* produces asexual pycnidiospores in pycnidia during late infection to facilitate secondary local infection. *Parastagonospora nodorum* SN15 and Ect were able to produce pycnidia on BG261, Calingiri and BG220. Not surprisingly, pf2-63 and pf2-69 were able to form pycnidia on Calingiri (Fig. 4B). We then genetically complemented pf2-69 with PnPf2 and its native promoter and terminator to give *P. nodorum pf2::PnPf2*. Genetic complementation restored full virulence on Tsn1 and Snn3 wheat cultivars (Fig. S7, see Supporting Information). This suggests that PnPf2 deletion abolished SnToxA and SnTox3 production, and thus the fungus cannot complete its infection life cycle on hosts that carry Tsn1 or Snn3 only.

**PnPf2 regulates SnToxA and SnTox3 expression**

To test the hypothesis that PnPf2 functions as a positive regulator of SnToxA and SnTox3 expression, we performed a series of effector infiltration experiments using culture filtrates derived from wild-type and gene deletion strains. SnTox1 activity from the culture filtrate of pf2-69 was detected on Calingiri (Snn1). However, infiltration of BG220 (Snn3) with the culture filtrate derived from

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**Table 1** Fungal strains used throughout this study.

| Strain          | Description                                      | Source                      |
|-----------------|--------------------------------------------------|-----------------------------|
| SN15            | *Parastagonospora nodorum* wild-type              | Department of Agriculture, Western Australia |
| Ect             | *Parastagonospora nodorum* SN15 ectopic transformant | This study                  |
| pf2-63          | SN15 deleted in PnPf2                             | This study                  |
| pf2-69          | SN15 deleted in PnPf2                             | This study                  |
| pf2::PnPf2      | pf2-69 complemented with PnPf2                    | This study                  |
| pf2-tox1-2      | pf2-69 deleted in snTox1                          | This study                  |
| pf2-tox1-6      | pf2-69 deleted in SnTox1                          | This study                  |
| pf2-tox1-16     | pf2-69 deleted in SnTox1                          | This study                  |
| pf2-Hph-5       | pf2-69 carrying an ectopic Hph integration        | This study                  |
| tox1-6          | SN15 deleted in SnTox1                            | This study                  |
| M4              | *Pyrenophora tritici-repentis* wild-type          | Meckering, Western Australia |
| E-1             | *Pyrenophora tritici-repentis* M4 ectopic transformant | This study                  |
| pf2-2-a         | M4 deleted in PtrPf2                              | This study                  |
| pf2-2-b         | M4 deleted in PtrPf2                              | This study                  |
| pf2-2-c         | M4 deleted in PtrPf2                              | This study                  |

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pf2-69 did not result in necrosis (Fig. 5A). SnTox3 activity was restored in the genetically complemented strain pf2::PnPf2.

SnToxA activity cannot be assayed in culture filtrates as P. nodorum SN15 does not express SnToxA in vitro (Tan et al., 2015). To determine whether PnPf2 regulates the expression of SnToxA, we used wheat cv. Halberd which is sensitive to SnToxA, SnTox1 and SnTox3 (Tan et al., 2015), and demonstrated strong susceptibility to pf2-69 (Fig. 5B). At 3 dpi, SnToxA and SnTox3 expression was strongly reduced compared with that in SN15, whereas SnTox1 expression was unaffected (Fig. 5C). This suggests that PnPf2 functions as a regulator of SnToxA and SnTox3, but not SnTox1, expression.

Biochemical complementation with SnToxA and SnTox3 restores virulence in a PnPf2 deletion background

To test whether the reduction in virulence caused by the PnPf2 deletion is or is not a pleiotropic effect, we infected BG261 and BG220 which had been pre-infiltrated with SnToxA and SnTox3, respectively, 24 h previously. Pycnidiation was used as a measure of virulence. Pre-infiltration of SnToxA on BG261 restored pf2-69 virulence on Tsn1 wheat (Fig. 6A). Similarly, pre-infiltration of SnTox3 on BG220 restored the ability of pf2-69 to proliferate and produce pycnidia (Fig. 6B). This further indicates that the reduction in virulence of pf2 deletion mutants on BG261 and BG220 can be attributed solely to the inability to produce sufficient levels of SnToxA and SnTox3.

Deletion of SnTox1 in the pnpf2 background eliminates virulence on Snn1 wheat

It was anticipated that SnTox1 deletion in the pf2 background would result in a reduction in the virulence of P. nodorum on Snn1 wheat. Parastagonospora nodorum strains deleted in SnTox1 were created using the pf2-69 background. PCR analysis identified three appropriate SnTox1 knockout strains (pf2-tox1-2, pf2-tox1-6 and pf2-tox1-16) and one ectopic mutant harbouring the Hph selectable marker outside of SnTox1 (Fig. S8, see Supporting Information). As expected, all pf2-tox1 strains were non-pathogenic on the Snn1 wheat cultivar Calingiri (Fig. 7A).

PnPf2 is dominant over SnTox1 epistatic regulation of SnTox3 expression

We have demonstrated previously that the SnTox1–Snn1 interaction is epistatic to SnTox3–Snn3. Hence, no effect of SnTox3–Snn3 is observed during SNB unless infection is carried out with a P. nodorum strain deleted in SnTox1 (tox1-6) (Phan et al., 2016). A significant increase in SnTox3 expression was observed in P. nodorum tox1-6 during wheat infection and growth in vitro. Thus, SnTox1 functions as a negative regulator of SnTox3 expression, unlike PnPf2. We then sought to determine whether PnPf2 or SnTox1 function as the dominant regulator of SnTox3 expression using quantitative real-time PCR analysis of in vitro-grown P. nodorum mutants that lacked PnPf2 and/or SnTox1. SnTox3 expression was observed in SN15 and tox1-6. However, SnTox3 transcripts were barely detectable in all P. nodorum strains that lacked PnPf2, including those that carried the SnTox1 double
deletion (Fig. 7B). This suggests that PnPf2 plays a dominant role over SnTox1 in the regulation of SnTox3 expression. We used QTL mapping of wheat to determine whether the SnTox3–Snn3 interaction is observed during SNB in the absence of SnTox1. Parastagonospora nodorum pf2-tox1-6 was used to infect seedlings of a double haploid (DH) mapping population constructed from a cross between Calingiri (tsn1, Snn1, snn3) and Wyalkatchem (tsn1, snn1, Snn3). The mutant was generally much less pathogenic on the DH population than on SN15, but comparable with tox1-6 (Fig. S9, see Supporting Information). As expected, SnTox3-responsive QTLs on chromosomes 5BS (Snn3) and 4BL were not detected from pf2-tox1-6 infection (Table 2). Previously detected SNB QTLs on 2DS and 3AL were observed from pf2-tox1-6 infection (Table 2). In addition, new SNB QTLs on 2AS and 6A were detected.

**PtrPf2 regulates PtrToxA expression and virulence**

As PtrToxA may have been acquired through a horizontal gene transfer event from *P. nodorum*, we hypothesized that PtrPf2 functions similarly to regulate its expression in *Py. tritici-repentis*. To test this, we deleted PtrPf2 from *Py. tritici-repentis* M4 using homologous recombination (Moffat et al., 2014) and determined the effect on PtrToxA expression and virulence. An ectopic (E-1) and three PtrPf2 deletion strains (pf2-a, pf2-b and pf2-c) were selected for phenotypic analyses (Fig. S11, see Supporting Information; Table 1). As PtrToxA is secreted into the culture filtrate of *Py. tritici-repentis* (unlike *P. nodorum* SnToxA) (Moffat et al., 2014; Tan et al., 2014), we could determine whether PtrPf2 regulates PtrToxA production by infiltration experiments into wheat cv. BG261 (Tsn1). Culture filtrates from M4 and E-1 produced a strong necrotic response after 4 days, but no visible signs of chlorosis or necrosis were seen with pf2-a, pf2-b and pf2-c (Fig. 8A). The role of PtrPf2 in virulence was then examined using a detached leaf assay on BG261. As conidiation was abolished in pf2-a, pf2-b and pf2-c, the infection assay was inoculated with mycelial plugs and lesions were allowed to develop. As expected, *Py. tritici-repentis* pf2-a, pf2-b and pf2-c displayed a near-complete loss of virulence on BG261 compared with M4 and E-1 (Fig. 8B). This strongly indicates that PtrPf2 positively regulates PtrToxA in *Py. tritici-repentis*. The near-complete loss in virulence in Tsn1 wheat was independently confirmed using another wheat cultivar, Yitpi, which is insensitive to ToxA (Fig. S12, see Supporting Information). Quantitative real-time PCR analysis indicated that the expression of PtrToxA is barely detectable in the ptrpf2 mutants, thus indicating that the loss of detectable PtrToxA activity is associated with transcriptional down-regulation (Table 3). *Pyrenophora tritici-repentis* M4 is a race 1 pathogen and thus
produces the ToxC effector that induces chlorosis (Moffat et al., 2014). The wheat cv. 6B365 is sensitive to the chlorosis-inducing effect of ToxC, but is insensitive to ToxA (Moffat et al., 2014). We infiltrated culture filtrates of M4, E1 and all ptrpf2 deletion strains into wheat cv. 6B365 with inconsistent results (data not shown). However, the Australian commercial wheat cv. Machete (insensitive to ToxA) gave distinct chlorosis with M4 and E-1, but not with the ptrpf2 deletion strains (Fig. 8C). At this stage, the identity of the strong chlorosis-inducing factor is unknown. Virulence assay indicated that Py. tritici-repentis mutants deleted in PtrPf2 cause small lesions on wheat cv. Machete which resemble a hypersensitive response (Fig. 8D).

**PtrPf2 is required for conidiation and normal vegetative growth**

Vegetative growth of ptrpf2 strains on solid V8-PDA medium was compared with that of M4 and E-1. All ptrpf2 strains produced fewer aerial hyphae and the colony morphology lacked radial crevassing, relative to M4 and E-1 (Fig. S13, see Supporting Information). Conidiation was abolished in all ptrpf2 strains (Table S1, see Supporting Information). Thus, PtrPf2 plays a substantial regulatory role in vegetative morphogenesis, not seen by PnPf2 in P. nodorum. As a result of the resulting phenotype in the ptrpf2 mutants, genetic complementation cannot be performed using established methods as transformation requires protoplasts derived from germinated conidiospores (Ciuffetti et al., 1997; Moffat et al., 2014). We attempted to generate sufficient quantities of protoplasts using macerated fungal hyphae, but were unsuccessful.

**DISCUSSION**

We have dissected the role of a member of a unique class of GAL4-like Zn$_2$Cys$_6$ transcription factors in two major fungal pathogens of wheat in the regulation of effector expression and host genotype-specific virulence. Deletion of the Pf2 orthologue in both P. nodorum and Py. tritici-repentis abolished ToxA expression and...
virulence on *Tsn1* wheat. In addition, *PnPf2* deletion in *P. nodorum* caused a strong reduction in *SnTox3* expression and subsequently reduced virulence on *Snm3* wheat. To our knowledge, this is the first study to demonstrate that a common regulatory mechanism exists between two closely related fungi that regulate functionally conserved effector(s). This enhances our knowledge of the two pathosystems and opens up a new route to discover further effectors in Pleosporales fungi. At this stage, it is not known whether *PnPf2* and *PtrpF2* function as direct or indirect regulators of effector gene expression in *P. nodorum* and *Py. tritici-repentis*, respectively.

The regulation of effector gene expression in phytopathogenic fungi is a relatively unexplored area of phytopathology. Soyer et al. (2015) used RNA interference to silence *LmStuA*, which encodes a putative helix-loop-helix transcription factor belonging to the APSES protein family, in the canola blackleg pathogen *Leptosphaeria maculans*. Silencing of *LmStuA* expression abolished *L. maculans* virulence on canola. Transcriptome analysis identified the down-regulation of expression of three avirulence genes: *AvrLm1*, *AvrLm4-7* and *AvrLm6*. Perturbation of StuA-mediated signalling also resulted in abnormal vegetative growth, loss of sporulation and virulence in *P. nodorum*. In addition, the expression of *SnTox3* was significantly reduced and could be attributed to pleiotropic effects caused by *StuA* inactivation (IpCho et al., 2010; Soyer et al., 2015). However, it is still not known whether *SnTox3* in *P. nodorum* and *AvrLm4-7*, *AvrLm1* and *AvrLm6* in *L. maculans* are subjected to direct or indirect regulation by *SnStuA* and *LmStuA*, respectively.

A peculiar phenomenon associated with effector gene regulation in *P. nodorum* has been demonstrated recently by Phan et al. (2016). The *SnTox1–Snm1* interaction is epistatic over *SnTox3–Snm3* in the development of SNB. Removal of *SnTox1* in *P. nodorum* unmasked the *SnTox3–Snm3* interaction during SNB. The mechanism of the epistatic interaction can be explained, in part, by an increased expression of *SnTox3*. In this study, we independently validated that *SnTox1* is a negative regulator of *SnTox3* expression, but is recessive to *PnPf2*. This is demonstrated by the lack of expression of *SnTox3* in the *pnpf2* background in *in vitro* and the *SnTox3–Snm3* interaction being absent during SNB. The exact mechanism by which *SnTox1* and *PnPf2* function to co-regulate *SnTox3* expression has yet to be elucidated.

**Wor1** (White-opaque regulator 1) encodes a nuclear protein that was first characterized as a master regulator of morphological switching and virulence in *Candida albicans*, an opportunistic fungal pathogen of humans (Huang et al., 2006). Recently, the role of *Wor1* orthologues has been examined in several plant-pathogenic fungi in terms of the virulence and regulation of secreted proteins, some of which possess effector-like characteristics (Michielse et al., 2009; Mirzadi Gohari et al., 2014; Okmen et al., 2014; Santhanam and Thomma, 2013). In the vascular wilt

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**Table 2** *PnPf2* deletion abolishes the *SnTox3–Snm3* interaction during Septoria nodorum blotch (SNB).

| Chromosome arm | QTL | Locus/QTL flanking markers | LOD | \( R^2 \) | Effect |
|---------------|-----|---------------------------|-----|------|--------|
| 2AS2          | Qn8b.cur-2AS2 | wmc382a – barc124a | 5.7 | 0.90 |
| 2DS           | Qn8b.cur-2DS  | cfl36 – wpt-669517   | 6.4 | 0.90 |
| 3AL           | Qn8b.cur-3AL  | lpt-1143 – wpt-4859  | 6.0 | 0.90 |
| 6A1           | Qn8b.cur-6A1  | gwp4329 – wpt-4370   | 6.0 | 0.90 |

A summary of quantitative trait loci (QTLs) identified in this study from *Parastagonospora nodorum p2-tox1-6* seedling infection. Flanking markers, logarithm of odds (LOD) scores, phenotype contribution (\( R^2 \)) and parental effect of these QTLs are shown. Positive and negative effects indicate that the allele was inherited from Calingiri and Wyalkatchem, respectively. A genetic map is provided in Fig. S10 (see Supporting Information).
fungus *Fusarium oxysporum* f. sp. *lycopersici*, the *Wor1* orthologue *Sge1* is essential for virulence on tomatoes and functions as a positive regulator of four effector genes: *SIX1*, *SIX2*, *SIX3* and *SIX5* (Michielse et al., 2009). BLAST analysis indicates that *P. nodorum* (SNOG_30643) and *Py. tritici-repentis* (PTRG_01056) possess a *Wor1* orthologue. It remains to be determined whether the *Wor1* orthologue plays a concerted role with Pf2 in the regulation of effector gene expression in *P. nodorum* and *Py. tritici-repentis*.

This study has demonstrated that, in both *P. nodorum* and *Py. tritici-repentis*, the Pf2 transcription factor plays a crucial role in the regulation of *ToxA* expression and possibly other effectors. However, under in vitro conditions, *ToxA* is highly expressed in *Py. tritici-repentis*, but is undetectable in *P. nodorum* (unattainable under our laboratory conditions) (Moffat et al., 2014; Tan et al., 2014). We have yet to identify the signal that induces *SnToxA* *expression in vitro*. In addition, evidence of extensive genetic polymorphism at the 5′ *ToxA* UTR between *P. nodorum* and *Py. tritici-repentis* was observed (Fig. 3). At this stage, the link between *ToxA* expression and genetic polymorphism in the 5′ UTR between the two pathogens is unclear, but this observation has opened up opportunities to explore possible links.

*PtrPf2* plays a critical role in vegetative growth and conidiospore production in *Py. tritici-repentis*, but not in *P. nodorum*. The *ptrpf2* mutants retain the ability to infect wheat cultivars that possess *Snn1* (i.e. Halberd, Chinese Spring and Calingiri), but not cultivars that lack *Snn1*. Microscopy analysis revealed that *pf2*-69, like SN15, was able to infect the *Snn1* host through direct entry via the stomata and epidermal penetration using hyphopodia (Solomon et al., 2004) (Fig. 5B). This strongly indicates that *PnPf2* does not play a significant role in the regulation of the basic machinery used by *P. nodorum* to facilitate infection on wheat, other than effector-assisted entry.

**Table 3** *PtrPf2* is a positive regulator of *PtrToxA* expression.

| Strain | *PtrToxA/PtrAct1* | Standard error of the mean |
|--------|------------------|---------------------------|
| M4     | 29474.32         | 28241.12                  |
| E-1    | 9640.70          | 4070.35                   |
| pf2-a  | 0.82             | 0.03                      |
| pf2-b  | 2.13             | 1.19                      |
| pf2-c  | 3.19             | 1.48                      |

Quantitative real-time polymerase chain reaction analysis of *PtrToxA* expression (normalized to *PtrAct1* expression) in *Pyrenophora tritici-repentis* M4, E-1 and *ptrpf2* mutants grown in vitro. The experiment was performed with biological replicates.
The discovery of a functionally conserved transcription factor class in Pleosporales fungi with regard to its role in the regulation of small secreted protein and effector gene expression poses several key research questions. First, do PnPf2 and PtrPf2 regulate the expression of small secreted proteins and effectors other than ToxA and SnTox? A preliminary study using a whole-plant infection assay demonstrated that the virulence of pf2-63 and pf2-69 on wheat cv. BG223 (Smn2, tsn1, smn1, smn3) was greatly reduced (Fig. S14, see Supporting Information). Smn2 is located on chromosome 2DS (Friesen et al., 2007). Hence, it is unlikely that the 2DS QTLs detected in this study are associated with SnTox2 sensitivity. In addition, the removal of SnTox1 in pf2-69 resulted in a complete loss of virulence on wheat cv. Calingiri, which has been previously demonstrated to show susceptibility to a mutant strain of P. nodorum SN15 carrying SnToxA, SnTox1 and SnTox3 deletions (Tan et al., 2015). Therefore, it is reasonable to conclude that PnPf2 regulates SnTox2 and other novel effectors. We have recently commenced extensive functional characterization of PnPf2 and PtrPf2 using chromatin immunoprecipitation sequencing, comparative transcriptomic and proteomic approaches to identify targets such as potential new effectors. Second, do Pf2 orthologues in other Pleosporales regulate the expression of effector genes? Third, what is the function of the second Pf2-like gene M4_11517 in Py. tritici-repentis? Fourth, what is the evolutionary history of the Pf2 transcription factors in the Pleosporales? Finally, what are the signals that activate Pf2 expression? The identification of these signal is paramount in the formulation of strategies to simultaneously shut down the expression of multiple effector genes from two major fungal pathogens of wheat.

EXPERIMENTAL PROCEDURES

Phylogenetic tree construction

Phylogenetic analysis of PnPf2 (SNOG_00649) and PtrPf2 (M4_04272) with orthologues and homologues from other fungal pathogens was performed using MEGA6 software (Tamura et al., 2013). Orthologues and homologues were selected from fungal pathogens based on the top 100 BlastP hits to Apf2 via the nr database. Manual annotations were conducted for incorrectly annotated genes, where stated. All polypeptide sequences were aligned using ClustalW set at the ‘Gonnet’ weight matrix, ‘gap opening penalty’ of 10, ‘gap extension penalty’ of 20 and ‘gap separation distance’ of 5. The phylogenetic tree was constructed using the ‘neighbour-joining’ algorithm, ‘p-distance’ substitution model. Bootstrap analysis set at 1000 repetitions was used to test the statistical significance of the phylogenetic tree.

Fungal culture

All fungal strains were maintained on V8-PDA agar [150 mL/L Campbell’s V8 juice, 3 g/L CaCO₃, 10 g/L Difco potato dextrose agar (PDA) and 10 g/L agar] at 21 °C under a 12-h photoperiod. All fungal strains used in this study are described in Table 1.

The production of NEs and plant infiltration

Parasagamonospora nodorum and Py. tritici-repentis culture filtrates containing NEs were produced from growth in Fries 3 medium broth (Liu et al., 2004). Culture filtrates containing effectors were sequentially filtered using gauze, miracloth, Whatman paper and 0.22-µm sterilizers.

For heterologous effector production, SnTox3 was expressed in Pichia pastoris using the pGAPzA expression vector (Liu et al., 2009). SnToxA was expressed in Escherichia coli BL21E using the pET21a expression vector (Tan et al., 2012). Protein preparations containing the expressed effector were harvested and desalted with 10 mM sodium phosphate buffer, pH 7.0, prior to infiltration into the first leaf of 2-week-old wheat seedlings using a needleless 1-cm³ syringe. Infiltrated leaves were monitored for necrotic development over 7 days post-infiltration.

Gene expression analysis

For P. nodorum, RNA isolation and in planta gene expression analysis were performed as described previously with minor modifications (Solomon et al., 2003). Briefly, detached wheat leaves (cv. Halberd) maintained in 75 mg/L benzimidazole agar were inoculated with 1 × 10⁶ pycnidiospores in 0.02% Tween 20 to facilitate infection. Lesions were excised from infected wheat, freeze dried and subjected to RNA extraction using TRIzol reagent (Invitrogen, La Jolla, CA, USA). Dnae-treated and reverse transcribed as described previously (Tan et al., 2008). Quantitative real-time PCR was performed using a Quantitect SYBR Green RT-PCR kit (Qiagen, Valencia, CA, USA) and a Bio-Rad Hercules, CA, USA) CFX96 system employing SN15 genomic DNA as a quantitative standard. The primer pair Pi2qPCRf and Pi2qPCRr was used to amplify a 130-bp region of PnPf2. The primer pair ToxAqPCRf and ToxAqPCRr was used to amplify a 134-bp region of SnTox1. The primer pair ToxAqPCRf and ToxAqPCRr was used to amplify a 143-bp region of SnTox3. The primer pair 20078-F and 20078-R was used to amplify a 189-bp region of SnToxA. The housekeeping gene actin (Act1) was used to normalize gene expression employing the primer pair Act1qPCRf and Act1qPCRr (Tan et al., 2008).

For Py. tritici-repentis, in planta gene expression analysis was carried out on 2-week-old infected Yitpi seedlings (Moffat et al., 2014). Lesions were excised from the plant and subjected to RNA isolation, CDNA synthesis and quantitative real-time PCR as described above. The primer pair Ptpf2F2 and Ptpf2R2 was used to amplify a 108-bp region of PtrPf2. The primer pair ToxAqPCRf and ToxAqPCRr was used to amplify a 126-bp region of SnToxA. The primer pair ToxAqPCRf and ToxAqPCRr was used to amplify a 134-bp region of SnTox1. The primer pair 20078-F and 20078-R was used to amplify a 189-bp region of SnToxA. The housekeeping gene actin (Act1) was used to normalize gene expression employing the primer pair Act1qPCRf and Act1qPCRr (Tan et al., 2008).

Construction of PnPf2 and PtrPf2 gene knockout vectors

Parasagamonospora nodorum SN15 strains carrying deletion in PnPf2 were created through genetic transformation using a gene knockout vector generated from fusion PCR (Solomon et al., 2006b). The primer pair 5_Pf2F and 5_Pf2R was used to amplify a 653-bp 5’ UTR fragment. This was fused to a phleumycin resistance cassette (Ble) amplified from pAN8-1 using pAN8f and pAN8r. The resulting construct was then fused to an
PCR was used to determine the copy number of the deletion were identified using a series of PCRs (Fig. S11B, C). Quantitative  

M4 (Moffat et al., 2016) was amplified with nested primers and then transformed into mon  

et al, 2003). Mutants that carry single-copy integration (Fig. S11D) (Moffat et al., 2003). Two gene deletion mutants (pf2-63 and pf2-69) and an ectopic strain (Ect) that carry single-copy integration were retained for further studies.

Similarly, for Py. tritic-repentis, PtrPf2 was deleted through genetic transformation using a gene knockout vector generated from fusion PCR. The primer pair PtrPf2_5'r and PtrPf2_5't was used to amplify a 1.3-kbp 5' UTR fragment. The primer pair PtrPf2_3't and PtrPf2_3'f was used to amplify a 1.2-kbp 3' UTR fragment. These two fragments were simultaneously fused to a phleomycin resistance cassette (Ble) amplified from pAN8-1 using pAN8f and pAN8r (Fig. S11A). The PtrPf2 knockout vector was amplified with primers and then transformed into Py. tritirepentis M4 (Moffat et al., 2014). Mutants that carry the appropriate gene deletion were identified using a series of PCRs (Fig. S11B, C). Quantitative PCR was used to determine the copy number of the Pf2 deletion construct in all transformants to identify appropriate mutants that carry single-copy integration (Fig. S11D) (Moffat et al., 2014; Solomon et al., 2008). Three gene deletion mutants (pf2-a, pf2-b and pf2-c) and an ectopic strain (E-1) were retained for further studies.

All PCR amplifications were performed with Phusion Taq DNA polymerase (New England Biolabs, Ipswich, MA, USA).

Genetic complementation

A 4255-bp region, containing Pf2, the 982-bp native promoter and 985-bp terminator regions, was amplified using Pf2compFGib and Pf2compR-Gib. The resulting DNA fragment was fused to a 5424-bp hygromycin resistance cassette (Hph) amplified from pAN7-1 using pAN7f/gib and pAN7r/gib (Fig. S15A, see Supporting Information). The genetic complementation construct Pf2-Hph was transformed into P. nodorum pf2-69. The transformation procedure produced 35 hygromycin-resistant transformants. The insert copy number was determined using the robust quantitative PCR method described by Solomon et al. (2008) with minor modifications. Briefly, P. nodorum MM102 strain containing one predetermined copy of Hph was used as a genomic DNA quantitative standard at 20, 6.7, 2 and 0.67 ng/µL. All reactions were performed with a Quantitect SYBR Green RT-PCR kit (Qiagen) and Bio-Rad CFX96 system. To determine the copy numbers of the Pf2-Hph complementation cassette, 6.7 ng/µL of genomic DNA from two transformants, pf2::Pf2-17 and pf2::Pf2-31, were used in each quantitative PCR (Fig. S15B). Mutants carrying a single copy will amplify at a Cq value similar to that of the MM102 6.7 ng/µL standard. Both transformants contained single-copy integration based on the relative quantification of the template amount (i.e. pf2::Pf2-17, 6.6 ng/µL; pf2::Pf2-31, 6.3 ng/µL) compared with the MM102 (6.7 ng/µL) genomic standard. Parastagonospora nodorum pf2::Pf2-17 was selected for further analysis and is referred to as pf2::Pf2.

PnPf2 and SnTox1 double deletion in P. nodorum

The SnTox1 deletion construct was constructed by Tan et al. (2015) by replacing the effector gene with the Hph cassette (Fig. S8A). This was transformed into P. nodorum pf2-69 to facilitate SnTox1 deletion homologous gene recombination (Solomon et al., 2004). A PCR assay was used to select for transformants deleted in SnTox1 (Fig. S8B). Following this, quantitative PCR was used to determine the insert copy number (Fig. S8C). Mutants that carry single-copy integration were selected for further analysis.

Infection assays

Whole-plant infection assay was performed as described in Solomon et al. (2005). Pycnidiospore inoculum was prepared to a concentration of 1 × 106 spores/mL in 0.5% w/v gelatin and sprayed onto 2-week-old wheat seedlings using a hand-held air brush sprayer. Plants were placed in 100% relative humidity at 21 °C, followed by 5 days at 21 °C under a 12-h photoperiod to facilitate SNB development prior to examination. Disease severity was visually determined. A score of zero indicates no disease symptoms, whereas a score of nine indicates a fully necrotized plant.

A detached leaf assay on benznidizole agar was used to assess the virulence of all Py. tritirepentis strains (Benedikz et al., 1981; Solomon et al., 2004). Small mycelial plug cut-outs were used as inoculum on detached leaves as conidiospore production was abolished in all ptrp2 mutants. Infection was allowed to develop for 7 days prior to examination.

Trypan blue staining of infected wheat leaves was performed as described previously (Solomon et al., 2004).

Genetic mapping and interval QTL analysis

Seedling infection, disease scoring and QTL mapping were performed on a wheat population consisting of 177 DH lines derived from a cross between Calingiri and Wyalkatchem, essentially as described in Phan et al. (2016). The DH population was genotyped with diversity array technology and simple sequence repeat markers (Phan et al., 2016).

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Fig. S2 The homology region is indicated in blue.

Fig. S1 The fungal transcription factor regulatory region is indicated in orange and the fungal transcription factor regulatory region is indicated in purple.

Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher’s website:

Table S1 Quantification of conidiospores from Pyrenophora triticirepentis M4 wild-type, E-1 ectopic and PtrPf2 deletion strains grown on V8-PDA solid medium for 7 days.

Table S2 Primers used throughout this study.

Data S1 Amino acid sequences used to construct a bootstrap consensus phylogenetic tree (Fig. 1).

Fig. S1 Scaled schematics of the CD-BLAST conserved domain structure for SN0G_00649 (PtrPf2), M4_04272 (PtrPf2), M4_11517 and AbPf2. Amino acid numbers are shown. The GAL4-like Zn2Cys6 binuclear cluster domain is indicated in orange and the fungal transcription factor regulatory middle homology region is indicated in blue.

Fig. S2 Nucleotide alignment between M4_04272 and PTRG_06982 (A) and M4_11517 and PTRG_11777 (B). Start (blue) and stop (red) codons are indicated.

Fig. S3 Nucleotide alignment of the putative promoter region of ToxA (yellow) from Parastagonospora nodorum (SN15 and SN4) and Pyrenophora triticirepentis (M4 and Pt-1C-BFP).

Fig. S4 Construction of the PnP2 gene deletion. (A) The 5’ and 3’ untranslated regions (UTRs) of PnP2 were polymerase chain reaction (PCR) amplified (i) and fused to BsuI to give the PnP2 knockout vector (ii). (iii) The vector was transformed into SN15 to facilitate gene knockout via homologous recombination of the 5’ and 3’ flanks. (B) Five knockout mutants (pf2–) and two ectopic strains (PF2–) were selected from PCR screening using 3_00649ScrF/R for insert copy number determination using quantitative PCR. (C) All possess single-copy integration, except for pf2-19. Consequently, pf2-63, pf2-69 and Pf2-18 (Ect) were selected for further studies.

Fig. S5 Assessment of colony morphology (A) and pycnidio-sporation production (B) on 2-week-old Petri dish fungal cultures grown on V8-PDA. Error bars are shown as standard error of the mean. The experiment was performed in biological replicates (n = 3).

Fig. S6 Whole-plant virulence assay of Parastagonospora nodorum strains on wheat cv. Chinese Spring (tsn1, Snn1, Snn3) (A) and Wylatketchem (tsn1, snn1, Snn3) (B).

Fig. S7 Genetic complementation of pf2-69 restores virulence on BG261 and BG220.

Fig. S8 SnTox1 deletion in pf2-69. (A) The SnTox1-Hph gene knockout cassette was polymerase chain reaction (PCR) amplified from Parastagonospora nodorum tox1-6 (i). This was used to delete SnTox1 in pf2-69 (ii), resulting in mutants that lacked PnP2 and SnTox1 (iii). (B) PCR using the primer pair 20078ScreenF/R was used to screen for SnTox1 deletion in all transformants. Mutants deleted in SnTox1 produced a 4.9-kb PCR amplicon. PCR amplification of ectopic transformants resulted in a 2.6-kb band. (C) One ectopic and three toxic mutants were analysed for insert copy number using quantitative PCR. All strains possess single-copy integration.

Fig. S9 The distribution of SN15, pf2-tox1-6 and tox1-6 whole-plant spray disease severity scores on the Calingiri × Wylatketchem DH population at the seedling stage. Data from SN15 and tox1-6 were derived from Phan et al. (2016).

Fig. S10 A genetic map of chromosomes with genetic markers on the right and the centimorgan (cM) distances between loci on the left. Quantitative trait loci (QTLs) associated with pf2-tox1-6 infection are indicated in purple.

Fig. S11 Construction of the PtrPf2 knockout vector. (A) The 5’ and 3’ untranslated region (UTR) of PtrPf2 was polymerase chain reaction (PCR) amplified (i) and fused to BseI to give the PnP2 knockout vector (ii). This was amplified with the nested primer pair PtrPf2-N1/PtrPf2-N2 and transformed into Pyrenophora triticirepentis M4 wild-type to facilitate gene knockout (iii). (B) PtrPf2-specific amplification using the primer pair PtrPf2-SF and PtrPf2-SR identified three knockout (pf2–) and one ectopic (E-1) mutant. (C) Gene disruption of the PtrPf2 locus was confirmed using the primer pair PtrPf2-5’/Phleo5 which amplifies a 1.4-kb fragment in strains that carry the appropriate gene deletion. (D) Transforms were analysed for insert copy...
number using quantitative PCR. All strains possess single-copy integration except for E-1. Pyrenophora tritici-repentis is very difficult to transform. Consequently, we were only able to identify one ectopic mutant. However, double integration did not reduce the level of fitness in the E-1 strain.

**Fig. S12** Virulence of *Pyrenophora tritici-repentis* M4 wild-type, E-1 and *PtrPf2* deletion mutants on wheat cv. Yitpi (*Tsn1*).

**Fig. S13.** Colony morphology of the *P. tritici-repentis* M4 wild-type, E-1 ectopic and *PtrPf2* deletion strains on V8-PDA.

**Fig. S14** Whole-plant virulence assay of *Parastagonospora nodorum* strains on wheat cv. BG223.

**Fig. S15** Genetic complementation of *Parastagonospora nodorum pf2-69*. (a) Fusion polymerase chain reaction (PCR) was used to construct the *PnPf2-Hph* gene complementation vector. (b) *PnPf2-Hph* insert copy number determination using quantitative real-time PCR. Biological triplicates were used in the copy number assay. Error bars are shown as the standard error of the mean.