



Chromatin-immunoprecipitation reveals the PnPf2 transcriptional network controlling effector-mediated virulence in a fungal pathogen of wheat

Evan John, Karam B Singh, Richard P Oliver, Jessica L Soyer, Jordi Muria-Gonzalez, Daniel Soo, Silke Jacques, Kar-Chun Tan

► To cite this version:

Evan John, Karam B Singh, Richard P Oliver, Jessica L Soyer, Jordi Muria-Gonzalez, et al.. Chromatin-immunoprecipitation reveals the PnPf2 transcriptional network controlling effector-mediated virulence in a fungal pathogen of wheat. 2023. hal-04231656

HAL Id: hal-04231656

<https://hal.science/hal-04231656>

Preprint submitted on 6 Oct 2023

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Title: Chromatin-immunoprecipitation reveals the PnP2 transcriptional network controlling effector-mediated virulence in a fungal pathogen of wheat.

Running title

PnP2 regulation of virulence

Author names and affiliations:

Evan John^{1,#,*}, Karam B. Singh^{1,2}, Richard P. Oliver³, Jessica L. Soyer⁴, Jordi Muria-Gonzalez¹, Daniel Soo¹, Silke Jacques¹ and Kar-Chun Tan^{1,#}

¹Centre for Crop and Disease Management, Curtin University, Perth, Australia

²Agriculture and Food, Commonwealth Scientific and Industrial Research Organisation, Perth, Australia

³School of Biosciences, Nottingham University, Nottingham, United Kingdom

⁴Université Paris-Saclay, INRAE, UR BIOGER, 78850, Thiverval-Grignon, France

*Current address:

Institute of Plant and Microbial Biology, Academia Sinica, Taipei 11529, Taiwan

#Please direct author inquiries to:

Evan John - evan.john@curtin.edu.au

Kar-Chun Tan – Kar-Chun.Tan@curtin.edu.au

Abstract

The regulation of virulence in plant-pathogenic fungi has emerged as a key area of importance underlying host infections. Recent work has highlighted the role of transcription factors (TFs) that mediate the expression of virulence-associated genes. A prominent example is Pf2, a member of the Zn₂Cys₆ family of fungal TFs, where orthologues regulate the expression of genes linked to parasitism in several plant-pathogen lineages. These include PnPf2 which controls effector-gene expression in *Parastagonospora nodorum*, thereby determining the outcome of effector-triggered susceptibility on its host, wheat. PnPf2 is a promising target for disease suppression but the genomic targets, or whether other are regulators involved, remain unknown. This study used chromatin immunoprecipitation (ChIP-seq) and a mutagenesis analysis to investigate these components. Two distinct binding motifs connected to positive gene-regulation were characterised and genes directly targeted by PnPf2 were identified. These included genes encoding major effectors and other components associated with the *P. nodorum* pathogenic lifestyle, such as carbohydrate-active enzymes and nutrient assimilators. This supports a direct involvement of PnPf2 in coordinating virulence on wheat. Other TFs were also prominent PnPf2 targets, suggesting it also operates within a transcriptional network. Several TFs were therefore functionally investigated in connection to fungal virulence. Distinct metabolic and developmental roles were evident for the newly characterised PnPro1, PnAda1, PnEbr1 and the carbon-catabolite repressor PnCreA. Overall, the results uphold PnPf2 as the central transcriptional regulator orchestrating genes that contribute to virulence on wheat and provide mechanistic insight into how this occurs.

Importance

Fungal pathogens cause large crop losses worldwide and consequently much attention has focused on improving host genetic resistance to diseases. These pathogens use effectors, which require coordinated expression at specific stages of the pathogenic lifecycle, to manipulate the host plant metabolism in favour of infection. However, our understanding of the underlying regulatory network in coordination with other genes involved in fungal pathogenicity is lacking. The Pf2 TF orthologues are key players underpinning virulence and effector gene expression in several fungal phytopathogens, including *P. nodorum*. This study provided significant insight into the DNA-binding regulatory mechanisms of *P. nodorum* PnPf2, as well as further evidence that it is central to the coordination of virulence. In the context of crop protection, the Pf2 taxonomic orthologues present opportune targets in major fungal pathogens that can be perturbed to reduce the impact of effector triggered-susceptibility and improve disease resistance.

Keywords

Transcription factor, virulence, regulation, gene expression, orthologue

Abbreviations

Transcription factor (TF), DNA-binding domain (DBD), chromatin immunoprecipitation (ChIP), carbohydrate-active enzyme (CAZyme), amino acid (a.a), gene ontology (GO)

1. Background

The *Parastagonospora nodorum*-wheat interaction has become a model fungal-plant pathosystem to study the molecular virulence factors underpinning infection. The fungus produces small secreted effector proteins that selectively interact with host-receptors encoded by dominant susceptibility-genes (1, 2). These interactions occur in a gene-for-gene manner that causes 'effector-triggered susceptibility' in the host plant, quantitatively affecting the disease which manifests as septoria nodorum blotch. Several effectors acting in this manner have now been identified and characterised for their role in virulence (3–7). These studies have also described a consistent pattern: the expression of these genes is maximal two to four days after infection and then declines. Furthermore, expression levels can vary by the presence or absence of their matching wheat receptors, as well as by epistasis, whereby one effector gene causes suppression of another (8–10). Yet relatively little is known concerning the mechanisms governing the effector gene regulation. In particular, are there common or distinct regulatory pathways involved? Do these components specifically control effector gene expression, or co-regulate other metabolic and developmental pathways? New knowledge in this area could present suitable targets to suppress for disease control.

Many fungi possess a Zn₂Cys₆ transcription factor (TF) Pf2 that has been associated with the regulation of effector gene expression. One example is the AbPf2 orthologue in *Alternaria brassicicola* that is critical for virulence on *Brassica* spp. (11). Gene deletion of *AbPf2* resulted in the down-regulation of a number of effector like genes, as well as putative cell-wall degrading enzymes. In *P. nodorum*, at least two key effector genes, *ToxA* and *Tox3*, require PnPf2 to be expressed (12). An RNA-seq

analysis also revealed PnPf2 regulates many more putative effectors, CAZymes, hydrolases/peptidases and nutrient transporters (13). The PtrPf2 orthologue in *Pyrenophora tritici-repentis* controls *ToxA* expression and virulence on wheat, much like for the homologous *ToxA* gene in *P. nodorum* (12). In *Leptosphaeria maculans*, the causal agent of blackleg disease on *Brassica* spp., the LmPf2 orthologue also regulates several effector genes, including *AvrLm4-7*, *AvrLm6*, *AvrLm10A* and *AvrLm11*, as well as CAZyme expression (14).

The Pf2 orthologues can be traced across several Ascomycota fungal lineages that include the Dothideomycetes, Leotiomyces and Soradiomyces (15). Gene-deletion in the plant pathogens *Botrytis cinerea*, *Fusarium* spp., *Magnaporthe oryzae* and *Zymoseptoria tritici* all suppressed fungal virulence as well as their capacity to utilise alternative carbon sources (16–19). Analogous carbohydrate regulatory roles have been attributed in the saprophytic fungi *Neurospora crassa* and *Trichoderma reesei* (20, 21). In *N. crassa*, the putative orthologue Col-26 is a critical component within a signalling-network that responds to glucose availability and promotes the expression of CAZymes for plant cell-wall degradation (22–24). There is a strong association between CAZyme gene content and plant-pathogenic lifestyles (25).

There are some key factors yet to be established among Pf2 orthologues. Which DNA-regulatory elements are bound? Are Pf2-regulated genes directly targeted or is their expression modulated by indirect factors? Are there other TFs intimately connected with Pf2 regulation of virulence? The research presented here provides such critical insight for PnPf2 in *P. nodorum*, and establishes its direct role in effector expression and CAZyme regulation. A subsequent functional investigation of several

118 connected and directly targeted TFs provides further evidence for the central role held
119 by PnPf2 in the transcriptional network that orchestrates virulence in *P. nodorum*.
120

2. Results

2.1. PnPf2 possesses typical Zn₂Cys₆ TF domains and localises to the nucleus.

Conserved domains or distinguishing features were analysed in the 652 amino acid (a.a) PnPf2 protein. The Zn₂Cys₆ DNA binding domain was located N-terminally at a.a 9 to 54 with an overlapping nuclear localisation signal (NLS) (KKGPKGSR; a.a 51 to 58) (**Fig. 1A**). A 'fungal TF domain' was identified from a.a 223 to 294 within a conserved 'middle homology region' (a.a 104 to 320). These features are frequently observed in Zn₂Cys₆ TFs and have been linked to the modulation of TF activity (26, 27, 15). A structurally disordered domain, typically associated with post-translational modifications and intermolecular interactions (28), was also identified at the C-terminus of PnPf2. Together, these features suggested that PnPf2 possesses the typical functional domains underpinning DNA-binding Zn₂Cys₆ TF activity (26). Nuclear localisation of the C-terminally tagged PnPf2-GFP fusion protein was also observed (**Fig. 1B**). Along with the functional domains identified, this supported DNA-binding regulatory activity by PnPf2.

Fig. 1 HERE

2.2. Two direct PnPf2 target motifs are associated with gene-regulation

A chromatin immunoprecipitation (ChIP) analysis was used to define PnPf2-DNA binding *in situ*. The *Pf2-HA* (native promoter) and *Pf2-HA_OE* (overexpression promoter) strains expressing the 3x haemagglutinin (HA) tagged PnPf2-HA fusion protein retained PnPf2 function, in contrast to a *pf2-HA_KO* deletion control (**Text S1**). A ChIP-seq analysis was therefore undertaken using the *Pf2-HA* and *Pf2-HA_OE* strains to identify 'summits' within enriched 'peak' regions. Summits corresponded to the best estimate of PnPf2-DNA binding loci within the peaks (29). The *Pf2-HA* and *Pf2-HA_OE* samples provided complementary datasets; the overexpression mutant to compensate for lower *PnPf2* expression under culture conditions (13, 30) and broaden the DNA interactions captured. A total of 997 summits were obtained from the *Pf2-HA* dataset and 2196 from *Pf2-HA_OE*, corresponding to 740 and 1588 peak regions respectively. There were 588 shared peaks identified between the samples (**File S1**), indicating strong overlap between the predicted PnPf2-targeted regions. The *Pf2-HA_OE* dataset broadened the scope of putative binding sites. A quantitative PCR (qPCR) analysis was then undertaken on independently prepared ChIP DNA, comparing the *Pf2-HA* and *Pf2-HA_OE* samples to the *pf2-HA_KO* control. Fold-enrichment values relative to *pf2-HA_KO* strongly correlated with ChIP-seq summit - $\text{Log}_{10}(\text{Q-values})$, a proxy measure for PnPf2-DNA binding affinity, in both the *Pf2-HA* ($P < 0.01$ with Pearson's $r = 0.77$) and *Pf2-HA_OE* ($P < 0.01$ with Pearson's $r = 0.74$) datasets (**Text S1**). The high reproducibility across separate methodologies provided confidence in the robustness of ChIP-seq summit calls.

Text S1 HERE

File S1 HERE

Previous RNA-seq differential-expression analyses had identified an enriched consensus motif (5'-WMGGVCCGAA-3') in the promoter regions of both AbPf2 and PnPf2-regulated genes (11, 13). Despite harbouring the typical 'CGG' Zn₂Cys₆ binding triplet (26), an interaction with PnPf2 was not observed in a heterologous system, indicating regulatory cofactors may be required (31, 13). A search for DNA-regulatory elements that interact with PnPf2 from the ChIP-seq dataset identified two enriched motifs in the merged *Pf2-HA* and *Pf2-HA_OE* peak regions (**Fig. 2A**). The first motif designated as M1 (5'-RWMGGVCCGA-3') closely matches the consensus motif from AbPf2 and PnPf2-regulated gene promoters (11, 13). The second motif designated as M2 (5'-CGGCSBBWYYKCGGC-3') is novel for PnPf2, encompassing two copies of the canonical 'CGG' Zn₂Cys₆ binding triplets (26), separated by eight nucleotides. Interestingly, M2 matches the AmyR regulatory response element that was modelled in *A. nidulans* (32). Both M1 and M2 are close to the ChIP-seq summits for the *Pf2-HA* and *Pf2-HA_OE* datasets (**Fig. 2B**), suggesting they accurately reflected DNA-binding loci.

The previous RNA-seq analysis had defined genes positively or negatively-regulated by PnPf2 from their expression changes in the *PnPf2*-deletion mutant *pf2ko* relative to wildtype SN15 (13). These gene sets include the *in vitro* culture conditions that were replicated here for ChIP to maximise TF-DNA yields, as well as an early fungal infection stage *in planta* (72 hrs). Instances of M1 and M2 were then identified across the promoters of the positive (i.e. *pf2ko*-down) and negative (*pf2ko*-up) PnPf2-regulated gene sets (**File S2**). Both M1 and M2 were significantly enriched in the

positively-regulated gene promoter set only (**Fig. 2A**). This indicates both motifs correspond to cis-regulatory elements that induce, rather than repress, gene expression.

A novel approach here sought to detect specifically the PnP2-motif interactions. This utilised a *dTomato* reporter gene fused to the promoter of SNOG_15417. The promoter was chosen to encompass a ChIP-seq peak region with both the M1 and M2 motifs for a gene that is positively-regulated by PnP2 (**Fig. 2C; File S2**). Integration of the construct at a predefined genomic locus in the SN15 background permitted evaluation of the reporter-gene expression in the resultant strain (*p15417_M1M2*) in comparison with strains where the CGG triplets in M1 and/or M2 had been substituted (*p15417_m1M2*, *p15417_M1m2* and *p15417_m1m2*). Significantly reduced expression was observed in the strains where M1 had been mutated, indicating that it is a functionally important and direct PnP2 target in the SNOG_15417 promoter (**Fig. 2C**). No significant expression change was detected where only the M2 motif was mutated.

Fig. 2 HERE

2.3. PnPf2 directly targets genes associated with the pathogenic lifestyle of *P. nodorum*

Genes with a ChIP-seq summit in their promoter region, considered a putative PnPf2 target, were cross-referenced with the *pf2ko* RNA-seq data analysis (**File S2**). There were 1286 targets identified from either ChIP-seq dataset, 484 of which were considered ‘high-confidence’ with a promoter summit in both *Pf2-HA* and *Pf2-HA_OE* (**Fig. 3A**). Of the 484 direct targets, 72 genes were positively-regulated in contrast to 6 negatively-regulated genes under the same *in vitro* conditions used for ChIP-seq. This indicates PnPf2 functions mainly as a positive regulator of gene expression. When expanded to also encompass PnPf2-regulated genes *in planta*, 93 were positively while 27 were negatively-regulated genes (**Fig. 3B**). Differential expression was not detected in the *pf2ko* mutant for 364 genes. This suggests other regulatory factors play a considerable role in their expression.

File S2 HERE

The characterised effector genes present in *P. nodorum* SN15, *ToxA*, *Tox1*, *Tox3* and *Tox267* (7), plus 29 other effector-like genes whose expression was altered in the *pf2ko* mutant, were assessed for evidence of direct regulation by PnPf2. Two distinct ChIP-seq summits were identified in the bi-directional *Tox3* promoter (**Fig. S1**). Both the upstream gene (i.e. SNOG_08982, encoding a protein disulphide-isomerase) and downstream gene (*Tox3*) are positively regulated by PnPf2. A ChIP-seq summit was also identified in the *Tox1* promoter, but only from the *Pf2-HA_OE* dataset (**Fig. S1**). Unlike *Tox3*, *Tox1* necrosis-inducing activity is still detected in the *pf2ko* background (12), indicating the summit may represent a weak enhancer element. The

ToxA gene is only expressed during infection but in a PnPf2-dependent manner. A weak promoter summit was observed despite multiple instances of the M1 motif, suggesting another factor(s) is required to facilitate PnPf2-DNA binding that was absent under the ChIP-seq experimental conditions. No distinct PnPf2 summit was observed in the promoter of *Tox267*, whose expression is not significantly altered in the *pf2ko* mutant, although two instances of M1 were identified >1000 bp upstream (**Fig. S1**). In total, 11 of the 29 PnPf2-regulated effector-like genes showed evidence of direct PnPf2-promoter binding through ChIP-seq summits (**Table S1-A**).

Fig. S1 HERE

A gene-ontology (GO) enrichment and network analysis was then carried out to identify major functional gene classes that are directly regulated by PnPf2. Five distinct groups representing TFs, redox molecules, CAZymes, cell-signalling molecules and nutrient transporters were significantly enriched among the GO networks (**Fig. 3C-D**). The enrichment of CAZymes, redox molecules and nutrient transporters is consistent with enriched functional GO classes that were observed for the *pf2ko* differentially expressed genes (13). In contrast, the TFs and cell-signalling molecules were not enriched, indicating they could be tightly controlled by redundant pathways in addition to PnPf2. Nevertheless, it was striking that TF genes were particularly enriched in the high-confidence set of 484 targets (**Fig. 3C-D**). They made up 9.1% of these genes in contrast to 3.5% of the total genes annotated for SN15. Five TFs were directly targeted and positively regulated, providing a direct connection with PnPf2 in the regulation of virulence (**Table S1-B**).

Fig. 3 HERE

Table S1 HERE

2.4. PnPf2 is the central transcriptional regulator of host-specific virulence

The identification of TFs as major PnPf2 targets prompted a functional exploration of other TFs with a putative intermediate role in regulating virulence. Three directly-regulated TF genes were therefore targeted for deletion where orthologues had virulence-associated roles (**Table 1**). These included SNOG_03490 (*PnPro1*), SNOG_04486 (*PnAda1*) and SNOG_08237. Two additional TF-encoding genes were simultaneously investigated. This included SNOG_08565, identified in the PnPf2 lineage of fungal TFs (15) suggesting a possible common evolutionary origin. It also included SNOG_03067 (*PnEbr1*), which is co-expressed with *PnPf2*, *ToxA*, *Tox1* and *Tox3* high during early infection (**Fig. S2**), suggesting a similarly important role in disease.

Fig. S2 HERE

Gene deletion strains for the five TFs were phenotypically characterised in comparison to wildtype SN15 and *pf2ko*. The *pro1_KO*, *ada1_KO* and *ebr1_KO* deletion mutants presented distinct phenotypic abnormalities (**Fig. 4**). The *pro1_KO* mutants were abolished in their ability to form pycnidia and sporulate both during infection and on nutrient-rich agar. However, vegetative growth was expansive in both conditions (**Fig. 4A-B**), suggesting PnPro1 acts to suppress hyphal development. Although *PnPro1* is positively-regulated by PnPf2, there was no distinct phenotypic overlap with the *pf2ko* mutant. The *ada1_KO* mutant was significantly reduced in virulence on detached leaves (**Fig. 4A-B**). Dark brown discolouration at the site of infection suggested a hypersensitive response had contained the infection. We also

observed an increased susceptibility to oxidative (H₂O₂) stress for *ada1_KO* mutants similar to *pf2ko*. Furthermore, sporulation was reduced in *ada1_KO* relative to SN15 (**Fig. 4C-E**). The *ebr1_KO* mutants exhibited vegetative growth defects with an uneven growth perimeter around the colony edges coinciding with perturbed virulence (**Fig. 4A-B**). Similar hyphal-branching defects were described following deletion of *PnEbr1* orthologues in *Fusarium* spp. (33, 34). Interestingly, the *ebr1_KO* mutants were also susceptible to H₂O₂ stress at a level comparable to *pf2ko* and *ada1_KO*. Furthermore, pycnidia were abnormally developed, although still viable for the production of conidia, but were not detected on infected leaves (**Fig. 4C-D**). We did not observe morphological or virulence defects for the *08237_KO* or *08565_KO* mutants (**Text S2**) which were not investigated in further detail.

Fig. 4 HERE

During the course of this study the carbon-catabolite repressor (CCR) element was modelled as the binding site for the Cre-1 TF that suppresses CAZyme expression in *N. crassa* (22). We noted this was near identical to a motif (5'-RTSYGGGGWA-3') that is also enriched in PnPf2-regulated gene promoters (13) but not identified from the ChIP-seq peaks. Since Cre-1 orthologues are conserved CCR regulators in filamentous fungi (35, 36), and since the CCR element is also enriched in PnPf2 regulated gene promoters, a putative Cre-1 orthologue (PnCreA) was investigated in *P. nodorum* to identify common regulatory pathways with PnPf2. Both *PnCreA* overexpression and gene-deletion mutants (*CreA_OE* and *creA_KO*) were created and then investigated alongside *pf2ko* and a *PnPf2* overexpression mutant (*Pf2_OE*). Despite clear phenotypic-growth abnormalities (**Fig. 5**), neither the *CreA_OE* nor

creA_KO mutants exhibited virulence defects on wheat leaves (**Text S2**). The *creA_KO* strain was enhanced in starch utilisation (**Fig. 5**), an indicator substrate for CCR activity (37). In contrast, there was a moderate reduction of *pf2ko* to utilise starch, similar to observations in other fungal *PnPf2*-orthologue mutants (17, 19, 21). These results support contrasting roles between PnCreA and PnPf2 for the regulation of some CAZyme-related genes.

Fig. 5 HERE

Text S2 HERE

Table 1 HERE

3. Discussion

Prior to this research, PnPf2 had been identified as an important regulator of *P. nodorum* virulence on wheat (12, 13), but important details and mechanistic insights were missing. We sought to take further steps and establish the DNA-binding elements targeted by PnPf2 and identify genes that were directly under its direct regulation. Two distinct regulatory motifs M1 and M2 were identified and linked to positive gene-regulation by PnPf2. M1 was strikingly similar to an enriched sequence in AbPf2 positively-regulated gene promoters (11), possibly representing a conserved Pf2 binding mechanism. It will be pertinent to explore this motif as a regulatory target for other fungal Pf2 orthologues (11–14, 16–21). Interestingly, the M2 motif matches the extensively characterised AmyR regulatory-response element in *A. nidulans* (38, 32). Polysaccharide metabolism has long been established as a regulatory function for AmyR (39, 40). Therefore, some shared regulatory pathways likely exist with Pf2 orthologues given the evidence for at least one conserved binding mechanism. However, there are major a.a polymorphisms between AmyR and Pf2 orthologues at the Zn₂Cys₆ DNA-binding domain (15) and M1 has not been reported as an AmyR target despite extensive motif investigation (38, 32). It is therefore conceivable that M1 is a regulatory binding element unique to Pf2 orthologues and therefore useful to identify putative direct targets such as *ToxA* in *P. nodorum*.

The ChIP-seq PnPf2-DNA binding dataset also facilitated the identification of *P. nodorum* genes under direct PnPf2 regulation. Among these genes is the *Tox3* effector and the adjacent gene, SNOG_08982, encoding a protein disulphide isomerase. This class of protein catalyses cysteine-cysteine bond formation which has been connected to fungal effector protein production (41). Therefore, it would be worth

exploring any involvement of SNOG_08982 in the post-translational modification of Tox3 and other effectors. PnPf2 binding was also detected in the *Tox1* promoter. A partial reduction in *Tox1* expression was reported in the *pf2ko* mutant (13), indicating PnPf2 is not essential but enhances expression under favourable conditions. *ToxA* is only expressed *in planta*, but is PnPf2 dependent (12). Despite multiple instances matching the M1 motif, there was little evidence for PnPf2-*ToxA* promoter binding, suggesting chromatin inaccessibility or the absence of essential binding-cofactors under the ChIP culture conditions. Direct PnPf2 regulation of *Tox267* was not evident. The other recently-cloned effector gene *Tox5* is not present in the SN15 isolate used in this study, but is homologous to *Tox3* may be under PnPf2 control (6). Nevertheless, several other effector-like genes were identified as direct PnPf2 targets (**Table S1-A**). Importantly this analysis provided strong evidence that PnPf2 is a key direct-regulator of effectors, the major *P. nodorum* virulence factors in the lifestyle of this pathogen.

Evidence for regulation of effector expression has been reported for another *P. nodorum* TF PnCon7 (42), yet its apparent requirement for fungal viability renders it difficult to investigate a precise functional role. Here, several novel TFs were functionally investigated based on their connection to PnPf2 (**Table 1**). We did not observe any change in the necrosis-inducing activity on wheat of fungal culture filtrates derived from the respective mutants. However, developmental virulence roles, including oxidative stress tolerance and hyphal development, were identified for *P. nodorum* PnAda1 and PnEbr1. It is possible that the direct regulation of PnAda1 by PnPf2 contributes to the susceptibility to oxidative stress also identified in the *pf2ko* mutant. The PnCreA orthologue of *N. crassa* Cre-1 was also investigated, following the striking observation that the *N. crassa* Cre-1 CCR element (5'-TSYGGGG-3') was

enriched in PnPf2-regulated gene promoters (13). Furthermore, Cre-1 and the PnPf2 orthologue Col-26 are both key components of a transcriptional network controlling CAZyme production in *N. crassa* (22–24, 43). Here, the *creA_KO* strain displayed an enhanced capacity to utilise starch, which was moderately impaired in the *pf2ko* mutant (**Fig. 5**). This indicates PnCreA and PnPf2 shared a similar function to the respective *N. crassa* orthologues (21). Surprisingly however, despite vegetative growth abnormalities on agar, there was no distinct change in the virulence profile of either the *creA_KO* or *CreA_OE* mutants (**Text S2**). We also failed to detect the CCR element in the promoters of *ToxA*, *Tox1*, *Tox3* or *Tox267* (**File S2**). This suggests that the regulation of host-specific virulence factors critical for *P. nodorum* infection are not subject to CCR by PnCreA.

This investigation, along with previous TF studies in *P. nodorum* (44, 45, 12, 42), indicate PnPf2 is central to the transcriptional-regulatory network controlling virulence, for which a tentative model is proposed (**Fig. 6**). Having expanded our understanding, it also raised some key questions. For many genes directly targeted by PnPf2, differential expression in *pf2ko* has not been observed (364 of 484 high-confidence targets). Such discrepancies are also reported in ChIP-seq experiments on filamentous fungi (**Table S2**). One aspect to consider is that functional TF binding requires specific cofactors/coregulators before gene expression is eventually modulated (46, 47). Furthermore, TF-DNA interactions can be redundant or non-functional (48–50). It is therefore plausible that many binding sites are transiently occupied by PnPf2 in this manner, acting as a biological sink. A change in the epigenetic landscape, for example during growth *in planta*, could open up genomic regions for which PnPf2 exhibits a high affinity and then actively binds. Performing

PnPf2 ChIP during early infection will likely prove highly useful in this regard if sufficient fungal material can be obtained. ChIP-seq targeting histone marks specific for euchromatin or heterochromatin under infection conditions, or methylation-sensitive sequencing are alternatives to provide insight into the genome accessibility of PnPf2 (14, 51–53). The identification of both the M1 and M2 motifs carrying alternatively oriented ‘CGG’ triplets, typical of Zn₂Cys₆ monomers (26), was suggestive of PnPf2 dimerisation with other Zn₂Cys₆ TFs. However, deletion of the putative ancestral PnPf2 homologue SNOG_08565 did not provide any phenotypic response that would indicate a connection. Therefore, future investigations will do well to explore these interactions, for example through co-immunoprecipitation/affinity purification analysis or a yeast-2-hybrid screen, to delineate PnPf2-DNA binding mechanisms. Functional investigation of the PnPf2 ‘middle homology region’ and C-terminal disordered region may also provide insight into the upstream signalling pathways that activate or repress PnPf2 activity through these domains.

To conclude, this study presents direct evidence of DNA binding in a Pf2 orthologue, where virulence-regulatory functions are consistently observed in phytopathogenic fungi. In *P. nodorum*, PnPf2 is central to the transcriptional regulation of virulence and directly controls effector expression. The current research on PnPf2 now provides a platform to further investigate its signalling pathways and molecular interactions that could be inhibited for targeted disease control.

Fig. 6 HERE

Table S2 HERE

4. Materials and methods

4.1. Annotations and PnPf2 domain analysis

The *P. nodorum* annotated genome for the reference isolate SN15 (54) was used consistent with annotations in the previous RNA-seq analysis (13). The PnPf2 polypeptide sequence was submitted to Interproscan (Release 82.0) for Interpro and Conserved-Domain-Database domain identification (55). NLStradamus was used to predict the nuclear localisation signal (56). The disordered region was predicted using IUPRED2A (28).

4.2. Generation and assessment of fungal mutants

The molecular cloning stages, the constructs generated and diagrammatic overview of the final transformed *P. nodorum* mutants generated in this study are detailed in **Text S3**. The respective primers designed for fragment amplification and/or screening are outlined in **File S3**. Fungal mutants used in this study are summarised in **Table 2**. Their phenotypic and gene-expression analysis procedures are also described in **Text S3**.

Table 2. HERE

Text S3 HERE

File S3 HERE

4.3. Chromatin immunoprecipitation sample preparation

The *Pf2-HA*, *Pf2-HA_OE* and *pf2-HA_KO* strains were prepared following 3 days standardised growth in 100 mL Fries3 liquid medium (**Text S3**). Prior to harvesting, a 5 mL crosslinking solution (10% w/v formaldehyde, 20 mM EDTA and 2 mM PMSF dissolved in 50 mM NaOH) was added with continuous shaking at 100 rpm for 10 min. To this, 5 mL quenching solution (1.25 M glycine) was added before another 10 min shaking. Whole protein extracts were then obtained as described (**Text S3**) with modifications for ChIP. The 50 mM Tris was replaced with 50 mM HEPES in the lysis buffer while gentle rotation of the resuspended fungal material was replaced by eight rounds of sonication using a Bandelin (Berlin, Germany) UW3100+SH70+MS73 tip sonicator to fragment the fungal DNA (set at 15 sec on/off with 60% amp and 0.8 duty cycle). Samples were held in an ice block during sonication. The supernatant was then retrieved from two rounds of centrifugation (5000 g, 4 °C for 5 min). A 100 µL aliquot of the supernatant was reserved as an 'input control' against which ChIP samples were to be normalised. A 1000 µL aliquot was then precleared for immunoprecipitation by gently rotating with 20 µL Protein A dynabeads (10001D - Thermofisher, Waltham, Massachusetts) for 1 hr at 4 °C. The supernatant was then retrieved and incubated with 2.5 µg anti-HA polyclonal antibody (71-5500 - Thermofisher) for 16 hrs at 4 °C. Another 20 µL Protein A dynabeads were then added and gently rotated for 2 hrs at 4 °C. The dynabeads were then retrieved and washed twice with 1mL ice-cold lysis buffer, once with high-salt buffer (lysis buffer + 500 mM NaCl), once with LiCl buffer (250 mM LiCl, 10 mM Tris-HCl, 1 mM EDTA, 0.5% NP40 and 0.5% NaDOC) and once with TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8). Samples were then incubated in a shaking incubator for 10 min (300 rpm, 65 °C) with 200 µL elution buffer (0.1 M NaHCO₃, 10 mM EDTA and 1% SDS) before

transferring the supernatant to a fresh tube. The input control was also supplemented with 100µL elution buffer at this stage and 8 µL NaCl solution (5 M) was added to both samples before de-crosslinking for 16 hrs at 65 °C. To these samples, 200 µL of H₂O and 100 µg RNase A (QIAGEN, Hilden, Germany) were added before incubating for 1 hr at 65 °C. Ten µg Proteinase K (Sigma-Aldrich, St. Louis, Missouri) was then added before incubating a further 1 hr at 50 °C.

For ChIP-qPCR, DNA (for both the *Pf2-HA*, *Pf2-HA_OE* and *pf2-HA_KO* ChIP and input control samples) was recovered from Proteinase K treated samples using the GenElute PCR purification kit (Sigma-Aldrich).

For ChIP-seq analysis, DNA (for both *Pf2-HA* and *Pf2-HA_OE* ChIP and input control samples) was purified from the Proteinase K treated samples by mixing in 1 volume (400 µL) of phenol:chloroform. This was centrifuged for 5 min at 16000 g and the aqueous phase retrieved. To this, 400µL chloroform was added, mixed and spun (16000 g 5 min) before 350 µL of the aqueous phase was transferred to a fresh tube. 35 µL sodium acetate (3 M, pH 5.2) was added with 1 µL of glycogen (20mg/mL). Samples were mixed by inversion and 1 mL 100% ethanol added before precipitation at -80 °C for 1-2 hrs. Pellets were retrieved by spinning 16000 g for 10 min at 4 °C, then washed in 1 ml of ice-cold 70% ethanol before drying and resuspension in 30 µL Tris-Cl (10 mM).

Two independent DNA preparations for each sample (i.e. the ChIP and input samples for both *Pf2-HA* and *Pf2-HA_OE*), beginning with the fungal growth stage in Fries3 broth, were pooled to ensure sufficient DNA was obtained for generating ChIP-

seq libraries. The pooled DNA was measured using a TapeStation system (Agilent, Santa Clara, California). 10 ng of each sample was processed using the TruSeq ChIP Library Preparation Kit (Illumina, San Diego, California). Libraries were size-selected (100-300 bp) and split across four separate lanes for sequencing in a NextSeq 500 sequencer (Illumina) to obtain 2 x 75 bp paired-end reads (Australian Genome Research Facility, Melbourne, Australia).

4.4. ChIP-seq analysis

An overview of the following data analysis pipeline from QC of raw reads through to genome mapping, ChIP-seq peak/summit calling, target gene prediction, ChIP-qPCR validation, GO enrichment analysis and motif position-weight-matrix (PWM) modelling **Text S1**.

4.4.1. Raw read filtering, mapping and peak/summit calling

Raw reads were checked using FASTQC (Version 0.11.9) (57) and the adapter sequences were trimmed using Cutadapt (Version 1.15) along with nucleotides where the Illumina quality scores were below 30 (58). Optical duplicates were then removed using the 'dedupe' option in Clumpify (version 1.15) from the BBTools package (59). Reads were subsequently mapped to the SN15 genome (54) using BWA-MEM (60). Reads mapping to a single locus as the best match (primary alignments) were retained for downstream analysis and the datasets from sample libraries originally split across the NextSeq lanes were merged using SAMtools (Version 1.10) to produce the final mapped-read datasets (61). MACS (Version 2.2.7.1) was used for calling enriched regions (i.e. peaks) and summits (highest nucleotide point or points within peak regions) from ChIP sample reads relative to the input samples (for *Pf2-HA* and *Pf2-*

HA_OE). A Q-value peak enrichment threshold of 0.01 was used and the BAMPE option utilised to assess read depth from cognate pairs (62, 63). Paired read lengths from the cognate pairs were assessed using Deeptools 'bamPEFragmentSize' (Version 3.3.0) to verify they corresponded to 100-300 bp size selected fragments (64).

4.4.2. Modelling binding-site motifs

The overlapping peak regions identified from the *Pf2-HA* and *Pf2-HA_OE* samples were merged using MAnorm (65) to create a consensus set of enriched peak regions containing the putative PnPf2 binding sites. From this set, overrepresented PWMs up to 20 bp long were modelled with MEME (version 5.1.1) (66, 67). For the resulting PWMs, 500 bp genomic regions centred at ChIP-seq summits were extracted and analysed using CentriMo (Version 5.1.1) to verify that the motif instances were also centred at the respective summits for both the *Pf2-HA* and *Pf2-HA_OE* samples (68). Gene promoters (spanning annotated transcription start sites to the nearest upstream gene feature or 1500 bp) with ≥ 1 occurrence of each motif were determined using FIMO (69). These were cross-referenced with the differentially expressed genes (i.e. expressed significantly up or down in *pf2ko* relative to SN15) defined in a previous RNA-seq analysis (13). Fisher's exact test with Bonferroni corrected P-values (70) was used to identify *pf2ko* differentially expressed gene-promoter sets significantly enriched ($P_{adj} < 0.01$) for the respective motifs vs the background rate in SN15.

4.4.3. PnPf2 target gene-promoter analysis

Genes targeted by PnPf2 were determined based on the proximity of summits to annotated genes, which were identified using ChIPseeker (Version 1.24.0) (71).

Genes with ≥ 1 summit falling within their promoter region from the *Pf2-HA* or *Pf2-HA_OE* datasets were considered PnPf2 targets. High-confidence PnPf2 targets corresponded to genes with a promoter summit in *Pf2-HA* and *Pf2-HA_OE*. ChIP-qPCR was then undertaken to verify that the ChIP-seq peak regions in *Pf2-HA* and *Pf2-HA_OE* would also correlate with quantitative enrichment against the *pf2-HA_KO* control strain. Quantitative PCR primer pairs (**File S3**) were designed to flank ChIP-seq summits in a selection of gene promoters (*ToxA*, *Tox1*, *Tox3*, SNOG_03901, SNOG_04486, SNOG_12958, SNOG_15417, SNOG_15429, SNOG_16438, SNOG_20100 and SNOG_30077) and a selection of non-summit control regions (*Act1* and SNOG_15429 coding sequences and the TrpC terminator). The 'input %' values were calculated for each sample using the method described previously (72) and used to calculate fold-differences (normalised to *Act1*) for *Pf2-HA* and *Pf2-HA_OE* relative to the *pf2-HA_KO* control for comparison with the *Pf2-HA* and *Pf2-HA_OE* $-\text{Log}_{10}(\text{Q-values})$ at the respective ChIP-seq summit loci. Pearson's correlation coefficient was calculated for the ChIP-qPCR fold-difference and ChIP-seq $-\text{Log}_{10}(\text{Q-values})$ at the respective loci and used as the test statistic to assess whether the association was significant (SPSS version 27.0).

The PnPf2 target genes were cross-referenced with the *pf2ko* expression patterns (expressed significantly up or down in *pf2ko*) defined previously (13) to link direct binding with the modulation of gene expression. The SN15 effector-like genes annotated previously (13) were compiled among the PnPf2 targets. Annotated homologues were identified from the corresponding records in UniProt (release 2020_05) (73). Both the high-confidence and total PnPf2 target-gene sets were then used for GO enrichment/network analysis using the SN15 GO annotations defined

previously (13). The ‘enricher’ function in the Clusterprofiler package (Version 3.16.0) (74) was invoked to identify the overrepresented GO classes ($P < 0.01$) in PnPf2 targets.

4.5. Data availability statement

The ChIP-seq reads are available under BioProject ID: PRJNA824526, corresponding to BioSamples SAMN27406642 (*Pf2-HA* strain) and SAMN27406643 (*Pf2-HA_OE* strain).

5. Acknowledgements

This study was supported by the Centre for Crop and Disease Management, a joint initiative of Curtin University (<https://www.curtin.edu.au/>) and the Grains Research and Development Corporation (<https://grdc.com.au/>) under the research grant CUR00023 Project F3 awarded to KCT). EJ was supported by an Australian Government Research Training Program Scholarship (<https://www.dese.gov.au/>) administered through Curtin University (<https://www.curtin.edu.au/>). The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

We would like to acknowledge Dr. Carl Mousley for helpful suggestions relevant to ChIP-seq motif validation, Dr. Darcy Jones for bioinformatics advice and support, as well as Johannes Debblor for assistance with molecular cloning.

6. References

1. McDonald MC, Solomon PS. 2018. Just the surface: advances in the discovery and characterization of necrotrophic wheat effectors. *Current Opinion in Microbiology* 46:14–18.
2. Kanyuka K, Igna AA, Solomon PS, Oliver RP. 2022. The rise of necrotrophic effectors. *New Phytologist* 233:11–14.
3. Friesen TL, Stukenbrock EH, Liu Z, Meinhardt S, Ling H, Faris JD, Rasmussen JB, Solomon PS, McDonald BA, Oliver RP. 2006. Emergence of a new disease as a result of interspecific virulence gene transfer. 8. *Nature Genetics* 38:953–956.
4. Liu Z, Faris JD, Oliver RP, Tan K-C, Solomon PS, McDonald MC, McDonald BA, Nunez A, Lu S, Rasmussen JB, Friesen TL. 2009. SnTox3 acts in effector triggered susceptibility to induce disease on wheat carrying the *Snn3* gene. *PLOS Pathogens* 5:e1000581.
5. Liu Z, Zhang Z, Faris JD, Oliver RP, Syme R, McDonald MC, McDonald BA, Solomon PS, Lu S, Shelver WL, Xu S, Friesen TL. 2012. The cysteine rich necrotrophic effector SnTox1 produced by *Stagonospora nodorum* triggers susceptibility of wheat lines harboring *Snn1*. *PLOS Pathogens* 8:e1002467.
6. Kariyawasam GK, Richards JK, Wyatt NA, Running KLD, Xu SS, Liu Z, Borowicz P, Faris JD, Friesen TL. 2022. The *Parastagonospora nodorum* necrotrophic effector SnTox5 targets the wheat gene *Snn5* and facilitates entry into the leaf mesophyll. *New Phytologist* 233:409–426.
7. Richards JK, Kariyawasam GK, Seneviratne S, Wyatt NA, Xu SS, Liu Z, Faris JD, Friesen TL. 2022. A triple threat: the *Parastagonospora nodorum* SnTox267 effector exploits three distinct host genetic factors to cause disease in wheat. *New Phytologist* 233:427–442.

8. Phan HTT, Rybak K, Furuki E, Breen S, Solomon PS, Oliver RP, Tan K-C. 2016. Differential effector gene expression underpins epistasis in a plant fungal disease. *The Plant J* 87:343–354.
9. Peters-Haugrud AR, Zhang Z, Richards JK, Friesen TL, Faris JD. 2019. Genetics of variable disease expression conferred by inverse gene-for-gene interactions in the wheat-*Parastagonospora nodorum* pathosystem. *Plant Physiology* 180:420–434.
10. John E, Jacques S, Phan HTT, Liu L, Pereira D, Croll D, Singh KB, Oliver RP, Tan K-C. 2022. Variability in an effector gene promoter of a necrotrophic fungal pathogen dictates epistasis and effector-triggered susceptibility in wheat. *PLOS Pathogens* 18:e1010149.
11. Cho Y, Ohm RA, Grigoriev IV, Srivastava A. 2013. Fungal-specific transcription factor *AbPf2* activates pathogenicity in *Alternaria brassicicola*. *The Plant J* 75:498–514.
12. Rybak K, See PT, Phan HTT, Syme RA, Moffat CS, Oliver RP, Tan K-C. 2017. A functionally conserved Zn2Cys6 binuclear cluster transcription factor class regulates necrotrophic effector gene expression and host-specific virulence of two major Pleosporales fungal pathogens of wheat. *Mol Plant Pathol* 18:420–434.
13. Jones DAB, John E, Rybak K, Phan HTT, Singh KB, Lin S-Y, Solomon PS, Oliver RP, Tan K-C. 2019. A specific fungal transcription factor controls effector gene expression and orchestrates the establishment of the necrotrophic pathogen lifestyle on wheat. 1. *Sci Rep* 9:1–13.
14. Clairet C, Gay EJ, Porquier A, Blaise F, Marais CL, Balesdent M-H, Rouxel T, Soyer JL, Fudal I. 2021. Regulation of effector gene expression as concerted waves in *Leptosphaeria maculans*: a two-players game. *BioRxiv* 2021.12.15.472773.

15. John E, Singh KB, Oliver RP, Tan K-C. 2021. Transcription factor lineages in plant-pathogenic fungi, connecting diversity with fungal virulence. *Fungal Genetics and Biology* In press.
16. Chung H, Choi J, Park S-Y, Jeon J, Lee Y-H. 2013. Two conidiation-related Zn(II)2Cys6 transcription factor genes in the rice blast fungus. *Fungal Genet Biol* 61:133–141.
17. Oh M, Son H, Choi GJ, Lee C, Kim J-C, Kim H, Lee Y-W. 2016. Transcription factor ART1 mediates starch hydrolysis and mycotoxin production in *Fusarium graminearum* and *F. verticillioides*. *Mol Plant Pathol* 17:755–768.
18. Habig M, Bahena-Garrido SM, Barkmann F, Haueisen J, Stukenbrock EH. 2020. The transcription factor Zt107320 affects the dimorphic switch, growth and virulence of the fungal wheat pathogen *Zymoseptoria tritici*. *Mol Plant Pathol* 21:124–138.
19. Han JW, Kim DY, Lee YJ, Choi YR, Kim B, Choi GJ, Han S-W, Kim H. 2020. Transcription factor PdeR is involved in fungal development, metabolic change, and pathogenesis of gray mold *Botrytis cinerea*. *J Agric Food Chem* 68:9171–9179.
20. Nitta M, Furukawa T, Shida Y, Mori K, Kuhara S, Morikawa Y, Ogasawara W. 2012. A new Zn(II)2Cys6-type transcription factor BglR regulates β -glucosidase expression in *Trichoderma reesei*. *Fungal Genet Biol* 49:388–397.
21. Xiong Y, Wu VW, Lubbe A, Qin L, Deng S, Kennedy M, Bauer D, Singan VR, Barry K, Northen TR, Grigoriev IV, Glass NL. 2017. A fungal transcription factor essential for starch degradation affects integration of carbon and nitrogen metabolism. *PLOS Genetics* 13:e1006737.
22. Wu VW, Thieme N, Huberman LB, Dietschmann A, Kowbel DJ, Lee J, Calhoun S, Singan VR, Lipzen A, Xiong Y, Monti R, Blow MJ, O'Malley RC, Grigoriev IV, Benz JP,

Glass NL. 2020. The regulatory and transcriptional landscape associated with carbon utilization in a filamentous fungus. PNAS 117:6003–6013.

23. Gabriel R, Thieme N, Liu Q, Li F, Meyer LT, Harth S, Jecmenica M, Ramamurthy M, Gorman J, Simmons BA, McCluskey K, Baker SE, Tian C, Schuerg T, Singer SW, Fleißner A, Benz JP. 2021. The F-box protein gene *exo-1* is a target for reverse engineering enzyme hypersecretion in filamentous fungi. Proc Natl Acad Sci U S A 118:e2025689118.
24. Li J, Liu Q, Li J, Lin L, Li X, Zhang Y, Tian C. 2021. RCO-3 and COL-26 form an external-to-internal module that regulates the dual-affinity glucose transport system in *Neurospora crassa*. Biotechnology for Biofuels 14:33.
25. Hane JK, Paxman J, Jones DAB, Oliver RP, de Wit P. 2020. “CATASrophy,” a genome-informed trophic classification of filamentous plant pathogens – how many different types of filamentous plant pathogens are there? Front Microbiol 10.
26. MacPherson S, Laroche M, Turcotte B. 2006. A fungal family of transcriptional regulators: the zinc cluster proteins. Microbiol Mol Biol Rev 70:583–604.
27. Schubert S, Popp C, Rogers PD, Morschhäuser J. 2011. Functional dissection of a *Candida albicans* zinc cluster transcription factor, the multidrug resistance regulator Mrr1. Eukaryotic Cell 10:1110–1121.
28. Erdős G, Dosztányi Z. 2020. Analyzing Protein Disorder with IUPred2A. Current Protocols in Bioinformatics 70:e99.
29. Zhang Y, Liu T, Meyer CA, Eeckhoute J, Johnson DS, Bernstein BE, Nusbaum C, Myers RM, Brown M, Li W, Liu XS. 2008. Model-based Analysis of ChIP-Seq (MACS). Genome Biology 9:R137.

30. Ipcho SVS, Hane JK, Antoni EA, Ahren D, Henrissat B, Friesen TL, Solomon PS, Oliver RP. 2012. Transcriptome analysis of *Stagonospora nodorum*: gene models, effectors, metabolism and pantothenate dispensability. *Mol Plant Pathol* 13:531–545.
31. Siggers T, Duyzend MH, Reddy J, Khan S, Bulyk ML. 2011. Non-DNA-binding cofactors enhance DNA-binding specificity of a transcriptional regulatory complex. *Mol Syst Biol* 7:555.
32. Kojima T, Kunitake E, Ihara K, Kobayashi T, Nakano H. 2016. A robust analytical pipeline for genome-wide identification of the genes regulated by a transcription factor: combinatorial analysis performed using gSELEX-Seq and RNA-Seq. *PLOS ONE* 11:e0159011.
33. Zhao C, Waalwijk C, de Wit PJGM, van der Lee T, Tang D. 2011. EBR1, a novel Zn(2)Cys(6) transcription factor, affects virulence and apical dominance of the hyphal tip in *Fusarium graminearum*. *Mol Plant Microbe Interact* 24:1407–1418.
34. Jonkers W, Xayamongkhon H, Haas M, Olivain C, van der Does HC, Broz K, Rep M, Alabouvette C, Steinberg C, Kistler HC. 2014. *EBR1* genomic expansion and its role in virulence of *Fusarium* species. *Environ Microbiol* 16:1982–2003.
35. Adnan M, Zheng W, Islam W, Arif M, Abubakar YS, Wang Z, Lu G. 2017. Carbon catabolite repression in filamentous fungi. *Int J Mol Sci* 19:48.
36. John E, Singh KB, Oliver RP, Tan K-C. 2021. Transcription factor control of virulence in phytopathogenic fungi. *Mol Plant Pathol* 22:858–881.
37. Gomi K. 2019. Regulatory mechanisms for amylolytic gene expression in the koji mold *Aspergillus oryzae*. *Bioscience, Biotechnology, and Biochemistry* 83:1385–1401.

38. Wang P, Kojima T, Kobayashi T, Nakano H. 2012. Comprehensive analysis of the DNA-binding specificity of an *Aspergillus nidulans* transcription factor, AmyR, using a bead display system. *Biosci Biotechnol Biochem* 76:1128–1134.
39. Tani S, Katsuyama Y, Hayashi T, Suzuki H, Kato M, Gomi K, Kobayashi T, Tsukagoshi N. 2001. Characterization of the *amyR* gene encoding a transcriptional activator for the amylase genes in *Aspergillus nidulans*. *Curr Genet* 39:10–15.
40. Nakamura T, Maeda Y, Tanque N, Makita T, Kato M, Kobayashi T. 2006. Expression profile of amylolytic genes in *Aspergillus nidulans*. *Bioscience, Biotechnology, and Biochemistry* 70:2363–2370.
41. Marín-Menguiano M, Moreno-Sánchez I, Barrales RR, Fernández-Álvarez A, Ibeas JL. 2019. N-glycosylation of the protein disulfide isomerase Pdi1 ensures full *Ustilago maydis* virulence. *PLOS Pathogens* 15:e1007687.
42. Lin S-Y, Chooi Y-H, Solomon PS. 2018. The global regulator of pathogenesis PnCon7 positively regulates *Tox3* effector gene expression through direct interaction in the wheat pathogen *Parastagonospora nodorum*. *Molecular Microbiology* 109:78–90.
43. Craig JP, Coradetti ST, Starr TL, Glass NL. 2015. Direct target network of the *Neurospora crassa* plant cell wall deconstruction regulators CLR-1, CLR-2, and XLR-1. *mBio* 6.
44. IpCho SVS, Tan K-C, Koh G, Gummer J, Oliver RP, Trengove RD, Solomon PS. 2010. The transcription factor StuA regulates central carbon metabolism, mycotoxin production, and effector gene expression in the wheat pathogen *Stagonospora nodorum*. *Eukaryotic Cell* 9:1100–1108.
45. Chooi Y-H, Zhang G, Hu J, Muria-Gonzalez MJ, Tran PN, Pettitt A, Maier AG, Barrow RA, Solomon PS. 2017. Functional genomics-guided discovery of a light-activated

737 phytotoxin in the wheat pathogen *Parastagonospora nodorum* via pathway activation.
738 Environ Microbiol 19:1975–1986.

739 46. Slattery M, Zhou T, Yang L, Dantas Machado AC, Gordân R, Rohs R. 2014. Absence
740 of a simple code: how transcription factors read the genome. Trends in Biochemical
741 Sciences 39:381–399.

742 47. Reiter F, Wienerroither S, Stark A. 2017. Combinatorial function of transcription factors
743 and cofactors. Current Opinion in Genetics & Development 43:73–81.

744 48. Carey MF, Peterson CL, Smale ST. 2012. Confirming the Functional Importance of a
745 Protein–DNA Interaction. Cold Spring Harb Protoc 2012:pdb.top070060.

746 49. Spivakov M. 2014. Spurious transcription factor binding: non-functional or genetically
747 redundant? Bioessays 36:798–806.

748 50. Osterwalder M, Barozzi I, Tissières V, Fukuda-Yuzawa Y, Mannion BJ, Afzal SY, Lee
749 EA, Zhu Y, Plajzer-Frick I, Pickle CS, Kato M, Garvin TH, Pham QT, Harrington AN,
750 Akiyama JA, Afzal V, Lopez-Rios J, Dickel DE, Visel A, Pennacchio LA. 2018.
751 Enhancer redundancy allows for phenotypic robustness in mammalian development.
752 Nature 554:239–243.

753 51. Soyer JL, Ghalid ME, Glaser N, Ollivier B, Linglin J, Grandaubert J, Balesdent M-H,
754 Connolly LR, Freitag M, Rouxel T, Fudal I. 2014. Epigenetic control of effector gene
755 expression in the plant pathogenic fungus *Leptosphaeria maculans*. PLOS Genetics
756 10:e1004227.

757 52. Soyer JL, Möller M, Schotanus K, Connolly LR, Galazka JM, Freitag M, Stukenbrock
758 EH. 2015. Chromatin analyses of *Zymoseptoria tritici*: methods for chromatin
759 immunoprecipitation followed by high-throughput sequencing (ChIP-seq). Fungal Genet
760 Biol 79:63–70.

53. Bewick AJ, Hofmeister BT, Powers RA, Mondo SJ, Grigoriev IV, James TY, Stajich JE, Schmitz RJ. 2019. Diversity of cytosine methylation across the fungal tree of life. 3. Nature Ecology & Evolution 3:479–490.
54. Syme RA, Tan K-C, Hane JK, Dodhia K, Stoll T, Hastie M, Furuki E, Ellwood SR, Williams AH, Tan Y-F, Testa AC, Gorman JJ, Oliver RP. 2016. Comprehensive annotation of the *Parastagonospora nodorum* reference genome using next-generation genomics, transcriptomics and proteogenomics. PLOS ONE 11:e0147221.
55. Blum M, Chang H-Y, Chuguransky S, Grego T, Kandasaamy S, Mitchell A, Nuka G, Paysan-Lafosse T, Qureshi M, Raj S, Richardson L, Salazar GA, Williams L, Bork P, Bridge A, Gough J, Haft DH, Letunic I, Marchler-Bauer A, Mi H, Natale DA, Necci M, Orengo CA, Pandurangan AP, Rivoire C, Sigrist CJA, Sillitoe I, Thanki N, Thomas PD, Tosatto SCE, Wu CH, Bateman A, Finn RD. 2020. The InterPro protein families and domains database: 20 years on. Nucleic Acids Res <https://doi.org/10.1093/nar/gkaa977>.
56. Nguyen Ba AN, Pogoutse A, Provart N, Moses AM. 2009. NLStradamus: a simple Hidden Markov Model for nuclear localization signal prediction. BMC Bioinformatics 10:202.
57. Andrews S. 2010. Babraham Bioinformatics - FastQC a quality control tool for high throughput sequence data. <https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>. Retrieved 26 April 2021.
58. Martin M. 2011. Cutadapt removes adapter sequences from high-throughput sequencing reads. 1. EMBnet.journal 17:10–12.
59. Bushnell B. 2016. BBMap. SourceForge. <https://sourceforge.net/projects/bbmap/>. Retrieved 26 April 2021.

785 60. Li H. 2013. Aligning sequence reads, clone sequences and assembly contigs with
786 BWA-MEM. arXiv:13033997 [q-bio].

787 61. Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, Marth G, Abecasis G,
788 Durbin R, 1000 Genome Project Data Processing Subgroup. 2009. The sequence
789 alignment/map format and SAMtools. *Bioinformatics* 25:2078–2079.

790 62. Feng J, Liu T, Qin B, Zhang Y, Liu XS. 2012. Identifying ChIP-seq enrichment using
791 MACS. 9. *Nat Protoc* 7:1728–1740.

792 63. Gaspar JM. 2018. Improved peak-calling with MACS2. *bioRxiv* 496521.

793 64. Ramírez F, Ryan DP, Grüning B, Bhardwaj V, Kilpert F, Richter AS, Heyne S, Dündar
794 F, Manke T. 2016. deepTools2: a next generation web server for deep-sequencing data
795 analysis. *Nucleic Acids Res* 44:W160–W165.

796 65. Shao Z, Zhang Y, Yuan G-C, Orkin SH, Waxman DJ. 2012. MAnorm: a robust model
797 for quantitative comparison of ChIP-Seq data sets. *Genome Biology* 13:R16.

798 66. Bailey TL, Boden M, Buske FA, Frith M, Grant CE, Clementi L, Ren J, Li WW, Noble
799 WS. 2009. MEME SUITE: tools for motif discovery and searching. *Nucleic Acids Res*
800 37:W202-208.

801 67. Bailey TL, Elkan C. 1994. Fitting a mixture model by expectation maximization to
802 discover motifs in biopolymers. *Proc Int Conf Intell Syst Mol Biol* 2:28–36.

803 68. Bailey TL, Machanick P. 2012. Inferring direct DNA binding from ChIP-seq. *Nucleic*
804 *Acids Res* 40:e128–e128.

805 69. Grant CE, Bailey TL, Noble WS. 2011. FIMO: scanning for occurrences of a given
806 motif. *Bioinformatics* 27:1017–1018.

70. Armstrong RA. 2014. When to use the Bonferroni correction. *Ophthalmic Physiol Opt* 34:502–508.
71. Yu G, Wang L-G, He Q-Y. 2015. ChIPseeker: an R/Bioconductor package for ChIP peak annotation, comparison and visualization. *Bioinformatics* 31:2382–2383.
72. Lin X, Tirichine L, Bowler C. 2012. Protocol: Chromatin immunoprecipitation (ChIP) methodology to investigate histone modifications in two model diatom species. *Plant Methods* 8:48.
73. Bursteinas B, Britto R, Bely B, Auchincloss A, Rivoire C, Redaschi N, O'Donovan C, Martin MJ. 2016. Minimizing proteome redundancy in the UniProt Knowledgebase. *Database : J Biol Databases Curation* 2016.
74. Yu G, Wang L-G, Han Y, He Q-Y. 2012. clusterProfiler: an R package for comparing biological themes among gene clusters. *OMICS: A Journal of Integrative Biology* 16:284–287.

7. Tables

Table 1. Rationale for the investigation of novel transcription factors (TFs) in this study

TF investigated	Involvement	Virulence-associated orthologues ^A
<i>PnPro1</i> (SNOG_3490)	Directly-positively regulated by PnPf2	<i>AbPro1</i> (<i>Ab</i>), <i>MoPRO1</i> (<i>Mo</i>), <i>GzZC232</i> (<i>Fg</i>), <i>UvPro1</i> (<i>Uv</i>)
<i>PnAda1</i> (SNOG_04486)	Directly-positively regulated by PnPf2	<i>GzbZIP001</i> (<i>Fg</i>), <i>FpAda1</i> (<i>Fp</i>)
SNOG_08237	Directly-positively regulated by PnPf2	<i>CoHox1</i> (<i>Co</i>)
SNOG_08565	Shared ancestral lineage with PnPf2 orthologues	-
<i>PnEbr1</i> (SNOG_03067)	Co-expressed with <i>PnPf2</i> , <i>ToxA</i> , <i>Tox1</i> and <i>Tox3</i> during infection	<i>EBR1</i> (<i>Fg</i>), <i>EBR1</i> (<i>Fo</i>), <i>MoCod2</i> and <i>Cnf2</i> (<i>Mo</i>)
<i>PnCreA</i> (SNOG_13619)	Enriched CreA-binding motif (22) in PnPf2-regulated gene promoters	<i>CreA</i> (<i>Af</i>), <i>Cre1</i> (<i>Fo</i>), <i>CreA</i> (<i>Pe</i>)

^A Putative orthologues were inferred by cross-referencing a previous TF-orthology analysis and literature review (15, 36). Abbreviations: *Ab*; *Alternaria brassicicola*, *Af*; *Aspergillus flavus*, *Co*; *Colletotrichum orbiculare*, *Fg*; *Fusarium graminearum*, *Fo*; *Fusarium oxysporum*, *Fp*; *Fusarium pseudograminearum*, *Mo*; *Magnaporthe oryzae*, *Pe*; *Penicillium expansum*.

Table 2. Overview of the strains referenced in this study ^A

Strain ID	Description ^B
SN15	Wildtype <i>P. nodorum</i> reference isolate
<i>pf2ko</i>	Original <i>PnPf2</i> deletion mutant (12, 13)
<i>pf2_KO</i>	<i>PnPf2</i> deletion mutant to facilitate targeted complementation
<i>Pf2_OE</i>	<i>PnPf2</i> overexpression (<i>pGpdA</i> promoter)
<i>Pf2-GFP</i>	<i>PnPf2</i> with <i>GFP</i> tag, native promoter
<i>Pf2-GFP_OE</i>	<i>PnPf2</i> with <i>GFP</i> tag, overexpression promoter
<i>Pf2-HA</i>	<i>PnPf2</i> with 3xHA tag, native promoter
<i>Pf2-HA_OE</i>	<i>PnPf2</i> with 3xHA tag, overexpression promoter
<i>pf2-HA_KO</i>	<i>PnPf2</i> coding sequence replaced with 3xHA tag
<i>CreA_OE</i>	<i>PnCreA</i> overexpression (<i>pGpdA</i> promoter)
<i>creA_KO</i>	<i>PnCreA</i> deletion mutant
<i>CreA_Ec</i>	<i>PnCreA</i> ectopically-integrated construct
SN15-GFP	SN15 constitutively expressing <i>GFP</i>
<i>pTef1-dTom</i>	SN15 constitutively expressing <i>dTomato</i> (defined locus)
<i>p15417_M1M2</i>	SN15 expressing <i>dTomato</i> (defined locus) SNOG_15417 promoter, no mutations
<i>p15417_m1M2</i>	SN15 expressing <i>dTomato</i> (defined locus) SNOG_15417 promoter, M1 mutated
<i>p15417_M1m2</i>	SN15 expressing <i>dTomato</i> (defined locus) SNOG_15417 promoter, M2 mutated
<i>p15417_m1m2</i>	SN15 expressing <i>dTomato</i> (defined locus) SNOG_15417 promoter, M1+2 mutated
<i>pro1_KO</i>	<i>PnPro1</i> deletion mutant
<i>Pro1_comp</i>	<i>PnPro1</i> complemented in <i>pro1_KO</i> background
<i>ada1_KO</i>	<i>PnAda1</i> deletion mutant
<i>Ada1_comp</i>	<i>PnAda1</i> complemented in <i>ada1_KO</i> background
<i>08237_KO</i>	SNOG_08237 deletion mutant
<i>08237_comp</i>	SNOG_08237 complemented in <i>08237_KO</i> background
<i>08565_KO</i>	SNOG_08565 deletion mutant
<i>08565_comp</i>	SNOG_08565 complemented in <i>08565_KO</i> background
<i>ebr1_KO</i>	<i>PnEbr1</i> deletion mutant
<i>Ebr1_Ec</i>	<i>PnEbr1</i> ectopically-integrated construct

^A Strains are listed in corresponding order to the detailed description of their generation in Text S3.

^B Abbreviations: GFP – Green fluorescent protein, HA – Haemagglutinin, M1 – Motif1, M2 – Motif 2.

8. Figures

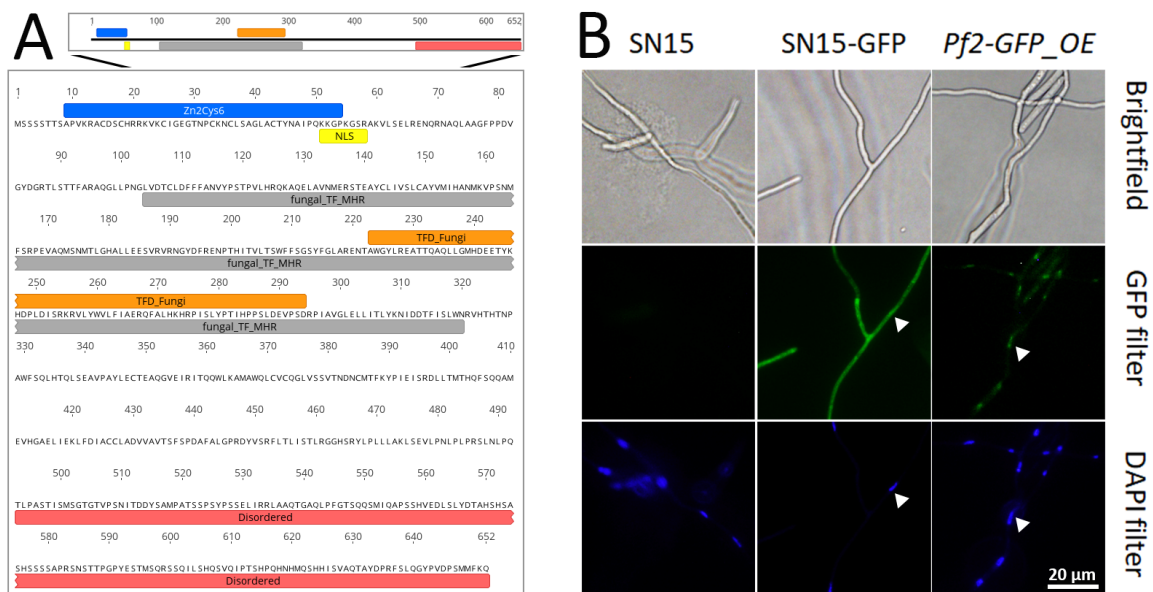


Fig. 1 PnPf2 domain analysis and cellular localisation. A) Predicted domains and features identified in the 652 amino acid PnPf2 protein sequence typical of a Zn₂Cys₆ transcription factor (TF). The region corresponding to the N-terminal Zn₂Cys₆ DNA binding domain (Zn₂Cys₆ - Interpro IPR001138) is depicted in blue and the nuclear localisation signal (NLS) is in yellow. The fungal transcription factor domain (TFD_fungi - Interpro IPR007219) corresponds to orange within the 'middle homology region' (fungal_TF_MHR - Conserved Domain Database CD12148) depicted in grey. A C-terminal disordered protein region lacking secondary structure was identified that corresponds to the red bar. B) Epifluorescence microscopy depicting nuclear localisation of the GFP-tagged PnPf2 translational fusion specific to the *Pf2-GFP_OE* overexpression strain, in contrast to the wildtype (SN15) and the positive control strain expressing cytoplasmic GFP (SN15-GFP). Arrows indicate the corresponding locations of fungal nuclei under the respective filters determined by DAPI staining of a germinated pycnidiospore. A fluorescence signal was not detected in the *Pf2-GFP*

853 strain, where expression was driven by the native *PnPf2* promoter, indicating PnPf2
854 accumulates at relatively low abundance.

855

856

857

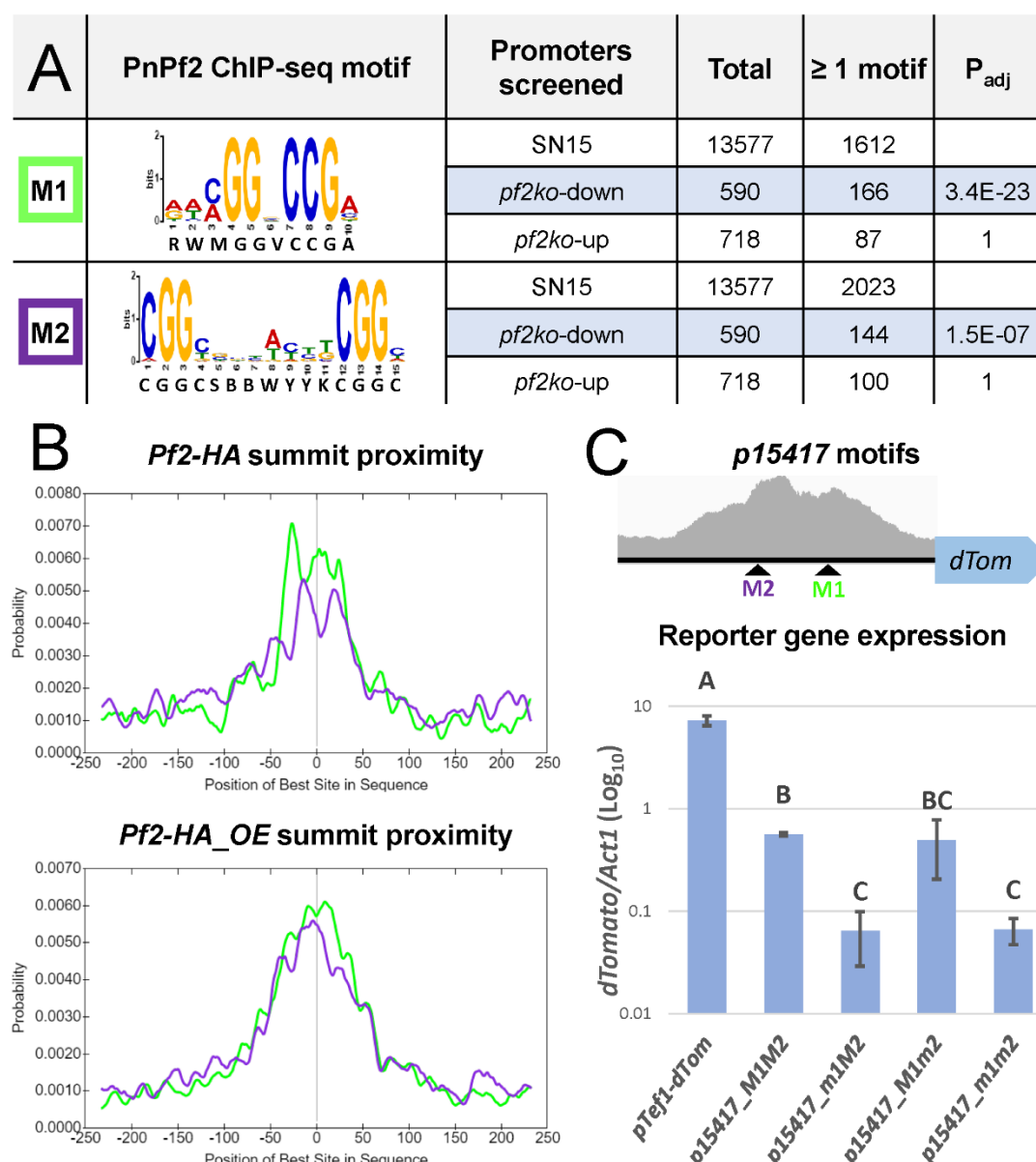


Fig. 2 Identification of PnPf2 regulatory element motifs. **A**) The M1 motif (5'-RWMGGVCCGA-3') and M2 motif (5'-CGGCSBBWYYKCGGC-3') were modelled from the merged set of *Pf2*-HA and *Pf2*-HA_OE sample ChIP-seq peak regions. Their detection (≥ 1 occurrence) in the promoters of PnPf2 positively (*pf2ko*-down) or negatively (*pf2ko*-up) regulated gene promoters (13) are indicated relative to all SN15 promoters. The P_{adj} value reflects the test for significant enrichment (Fisher's test with Bonferroni P_{adj} < 0.01), where both motifs were enriched in the *pf2ko*-down genes relative to SN15. **B**) The position of motif occurrences relative to ChIP-seq summits,

demonstrating their higher likelihood at close proximity to the best estimate of PnPf2-DNA binding loci. **C)** Gene expression analysis assessing the effect of M1 and M2 motif mutation in *P. nodorum in situ*. The motif loci within a ChIP-seq peak in the SNOG_15417 gene promoter region (*p15417*) are depicted. The *dTomato* reporter gene was fused to a constitutive promoter control (*pTef1-dTom*) or the SNOG_15417 gene promoter (*p15417_M1M2*) in the *P. nodorum* background. The motifs were also mutated through substitution of the respective 'CGG' triplets, alone or in combination (*p15417_m1M2*, *p15417_M1m2* and *p15417_m1m2*). The strains *p15417_m1M2* and *p15417_m1m2* where M1 had been mutated exhibited significantly reduced expression relative to the non-mutated promoter in *p15417_M1M2*. This suggested PnPf2 regulatory activity had been impaired following M1 mutation but not M2 mutation in *p15417*. Letters indicate statistically distinct groupings by ANOVA with Tukey's-HSD ($P < 0.05$).

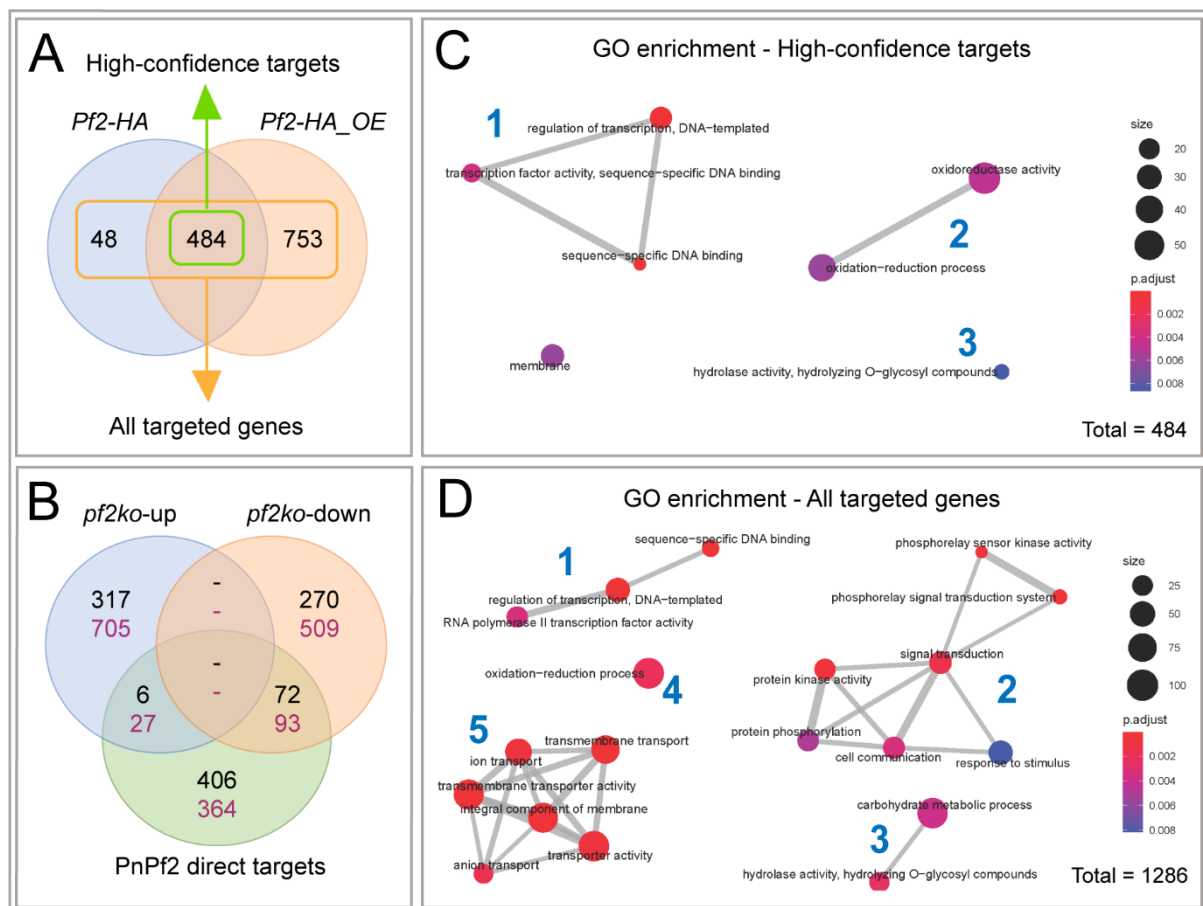
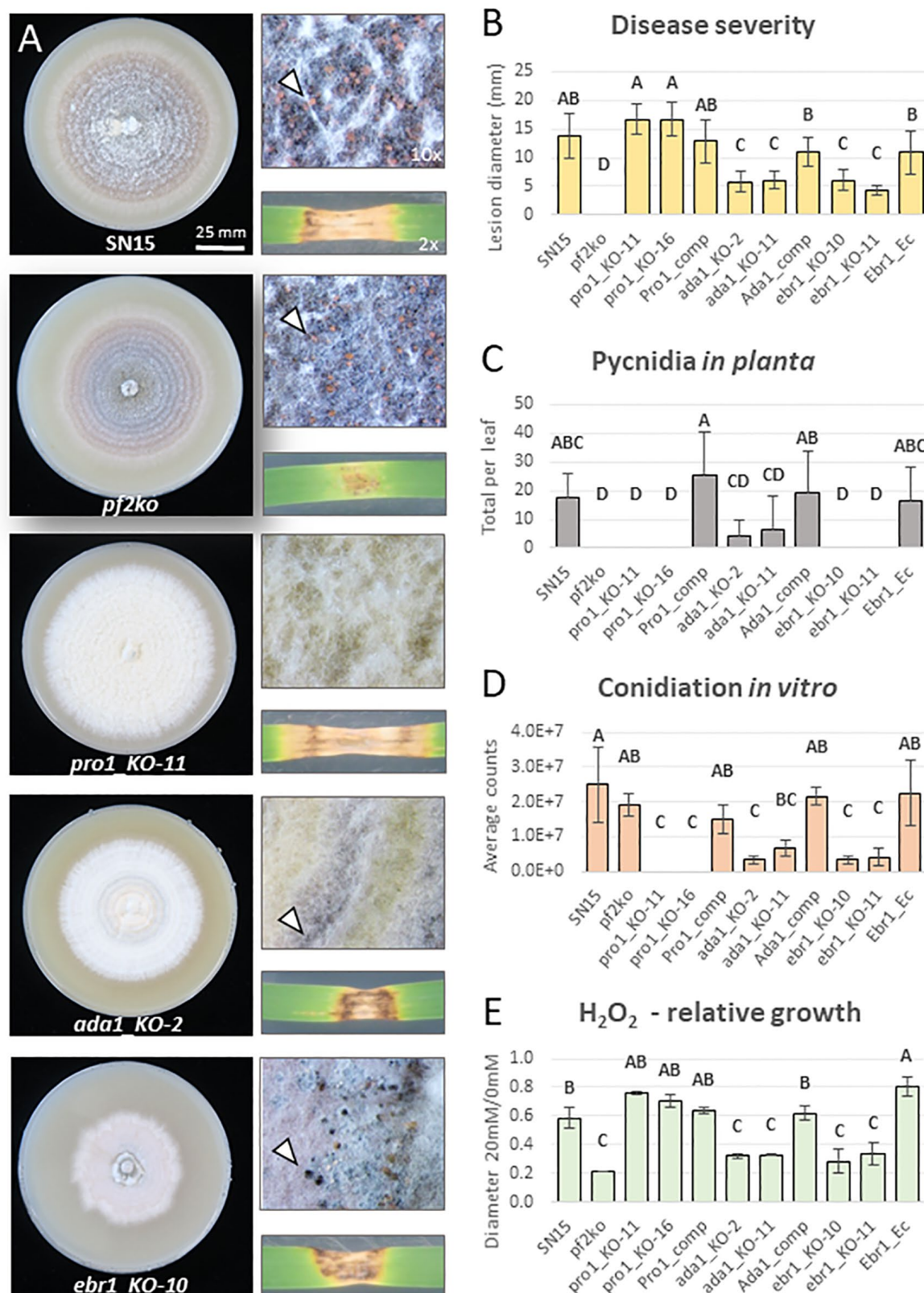


Fig. 3 Gene expression and gene-ontology (GO) analysis of PnPf2 direct targets. **A)** Overview of the genes targeted by PnPf2 in their promoter region based on the respective *Pf2-HA* and *Pf2-HA_OE* ChIP-seq datasets. There were 484 genes considered high-confidence PnPf2 targets among the 1286 putative targets from either construct. **B)** The high-confidence targets in comparison with their expression pattern in the *pf2ko* deletion mutant. Black numbers correspond to the *in vitro* growth conditions replicated for ChIP-seq sample preparation while purple numbers also encompass differentially expressed genes during early infection. The greater overlap with PnPf2 positively-regulated (*pf2ko*-down - 72 genes) than repressed (*pf2ko*-up - 6 genes) suggested PnPf2 predominantly functions as a positive regulator of gene expression. **C-D)** A summary of significantly-enriched GO terms among PnPf2-targeted genes. The high-confidence (*Pf2-HA* and *Pf2-HA_OE*) and total identified

894 targets (*Pf2-HA* or *Pf2-HA_OE*) are both displayed for comparison. Bubble sizes are
 895 proportionate to gene counts, colours to the enrichment test *P* values and the lines
 896 between bubbles to the total shared terms. Numbers in blue indicate connected gene
 897 networks representing transcription factors [number 1], redox molecules [2],
 898 carbohydrate-active enzymes [3], cell-signalling molecules [4], and trans-membrane
 899 transporters [5].



900

901

Fig. 4 Phenotypic assessment of transcription factor (TF) gene deletion mutants. **A)** Representative images after 12 days of growth on nutrient-rich agar (V8PDA) and infection on detached wheat leaves (cv. Halberd). Arrows demonstrate pycnidia if they were detected in the respective mutants. **B)** Average lesion sizes representing disease severity. **C)** Pycnidia counts, a measure of pathogenic fitness following the infection. **D)** Average conidial (pycnidiospore) counts on V8PDA. **E)** Growth inhibition on 20mM H₂O₂ relative to 0mM on minimal medium agar. Letters indicate statistically distinct groupings by ANOVA with Tukey's-HSD (P<0.05).

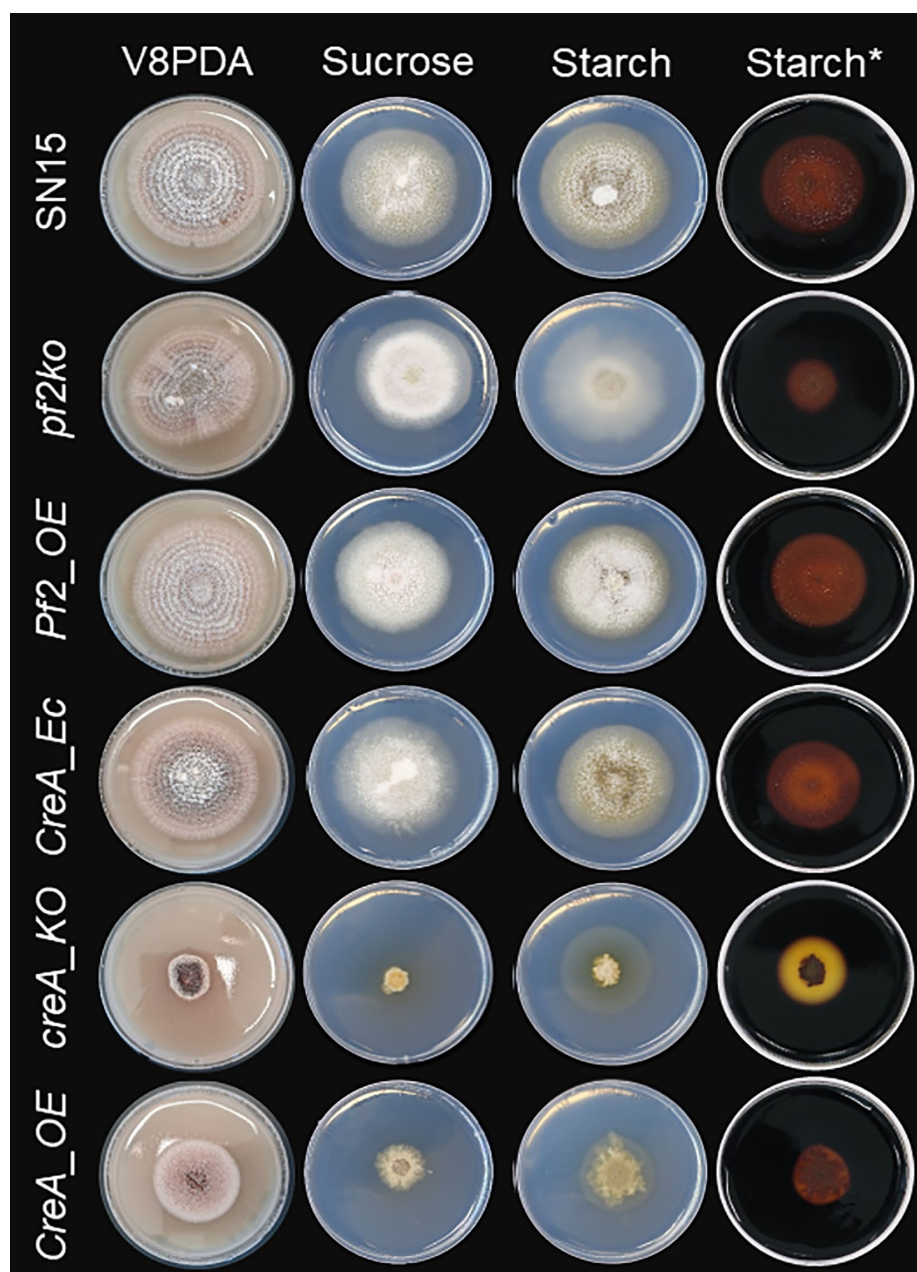


Fig. 5 Assessment of *PnPf2* and *PnCreA* mutant growth on different substrates.

Images following 12 days of growth on nutrient-rich V8PDA and minimal-medium agar with a primary (sucrose) or secondary (starch) carbon source. Wildtype SN15 and the respective mutants are listed on each row. The capacity to fully utilise starch was enhanced in the *creA_KO* mutant despite growth defects, suggesting impaired carbon-catabolite repression. Starch utilisation was moderately reduced in the *pf2ko* mutant.

*Post-stained with Lugol's iodine to assist visualisation of starch hydrolysis.

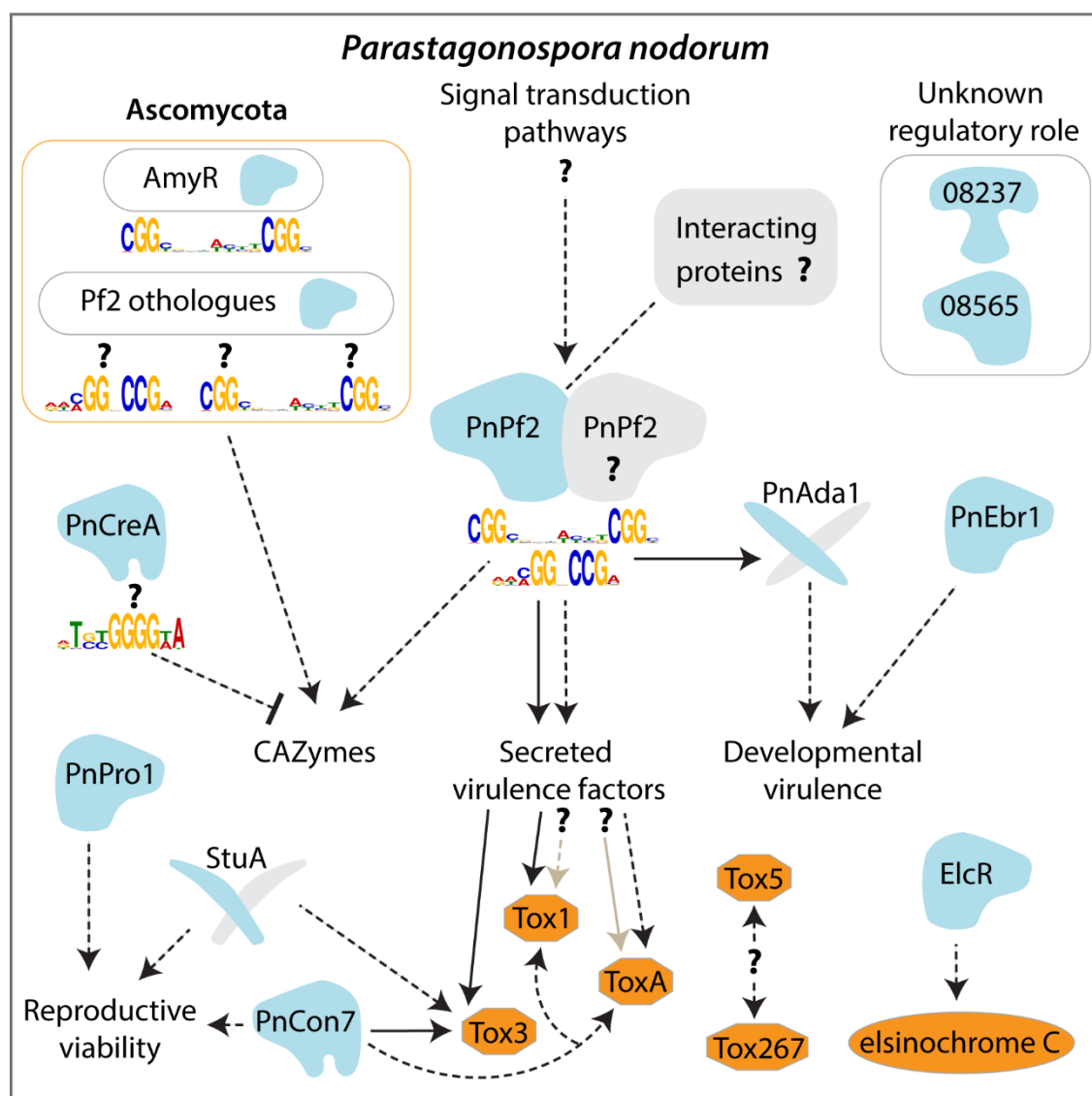


Fig. 6 The proposed model of the PnPf2-centred regulatory network in the virulence of *P. nodorum*. The Pf2 taxonomic orthologues and AmyR regulators in the Ascomycota fungi (discussed in the main text) are presented for context regarding the two PnPf2 regulatory motifs described in this study. Transcription factors (TFs) and virulence factors that are cloned and characterised in *P. nodorum* are depicted (blue and orange shapes respectively; grey shapes indicate putative interacting proteins). Dashed arrows show where gene regulation occurs and solid arrows represent direct regulation. Question marks are presented where evidence is tentative and requires validation. PnPf2 controls the expression of key virulence

factors. The effector *Tox3* is directly regulated and *ToxA*, based on promoter-motif and gene expression data, is likely a direct target during plant infection. PnPf2 also directly targets the *Tox1* promoter as a possible enhancer while regulators of *Tox267* and *Tox5* require investigation. Carbohydrate-active enzymes (CAZymes) are also regulated by PnPf2, with a subset putatively repressed by PnCreA for which no distinct role in virulence has been established. Developmental virulence, such as oxidative stress tolerance and hyphal growth, were processes attributed in this study to the PnPf2 targets PnAda1 and PnEbr1. PnPro1 and StuA (44) are essential for reproduction by sporulation, while no distinct role was identified for the putative TFs encoded by SNOG_08237 and SNOG_08565. Elsewhere, PnCon7 has been reported to regulate effector expression but is an essential viability factor, while production of a phytotoxic metabolite elsinochrome C is controlled by the pathway-specific ElcR gene-cluster TF (45, 42).

9. Supplemental item legends

Text S1 Chromatin immunoprecipitation (ChIP) strain assessment and overview of ChIP-seq/ChIP-qPCR.

Text S2 Supplemental transcription factor mutant phenotype assessment.

Text S3 Supplemental materials and methods.

Fig. S1 A depiction of the PnPf2 targeting of characterised effector genes in *P. nodorum* SN15. The *Pf2-HA* and *Pf2-HA_OE* ChIP-seq read peaks are presented at the *Tox3*, *Tox1*, *ToxA* and *Tox267* promoters. Peak summits were evident in the *Tox3* and *Tox1* promoters. Red dots represent instances of the M1 motif (5'-RWMGGVCCGA-3') and blue dots M2 (5'-CGGCSBBWYYKCGGC-3').

Fig. S2 A heatmap depiction of *Parastagonospora nodorum* SN15 hierarchical cluster analysis. Clustering was based on microarray gene-expression data during infection (*in planta*) or axenic (*in vitro*) growth obtained from a previous study (30). Clusters were cut into the 10 most distant clusters to identify genes co-expressed with *PnPf2*, *ToxA*, *Tox1* and *Tox3*, which included the Zn₂Cys₆ transcription factor *PnEbr1* (SNOG_03037) therefore investigated in this study.

File S1 A spreadsheet detailing the genomic coordinates for ChIP-seq peak regions [columns A-D], the respective summit loci [E], the pileup height of the mapped reads [F] and the summit -Log₁₀(Q-values) representing the difference of ChIP reads relative to the input control sample [G]. The *Pf2-HA* strain encompasses columns A-G and the *Pf2-HA_OE* strain encompasses column H-N. Also included are the genomic coordinates for the peak regions obtained by merging the overlapping regions from the *Pf2-HA* and *Pf2-HA_OE* samples using MAnorm (65) [O-S].

File S2 A spreadsheet of PnPf2 regulation data across the *P. nodorum* SN15 genome for the respective annotated genes [column A]. Listed are whether ChIP-seq

promoter summits were called from the *Pf2-HA* and *Pf2-HA_OE* samples [B-C], whether the enriched PnPf2 target motifs [D-E] or the putative PnCreA motif [G] were present in the gene promoter regions, and whether the gene was also down-regulated in the *pf2ko* mutant [G]. Also listed are the functional annotations [G-M]; whether the gene was classed as effector-like [H], a TF [I], the associated GO IDs/terms [J-K] and Interpro domain information [L-M]. The final columns list the respective gene expression data for *pf2ko* compared with SN15 either *in vitro* (*iv*) or *in planta* (*ip*) [13-22]. *Information indicated was derived from Jones et al. (2019) for comparative purposes.

File S3 A spreadsheet compilation of the primers used in this study organised by their general use [column 1] with the primer ID and sequence [2-3] and descriptions for their use [4-5]. Highlighted in italics are restriction enzyme recognition sites, in bold are overlapping regions used in cloning and in red are sites for incorporating single nucleotide changes during cloning.