School of Molecular & Life Sciences

Characterisation of transcriptional elements regulating virulence during *Parastagonospora nodorum* infection of wheat

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I. DECLARATION

I declare that this thesis is my own account of research undertaken by me. This work has not been submitted to any other tertiary education institution. Any material that has been published in scientific journals is made explicit in the respective chapters with co-author attribution statements attached as appendices.

Evan John

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IV. THESIS ABSTRACT

The following thesis details an investigation into the transcriptional elements that regulate virulence in *Parastagonospora nodorum*, the cause of septoria nodorum blotch (SNB) disease on wheat. Transcription factors (TFs) are a major focus of this research, which are first systematically reviewed and analysed in plant-pathogenic fungi to provide context for the research conducted here in *P*. *nodorum*. Hence the contents of this thesis is of interest not only to researchers, breeders and cereal growers looking to find solutions to SNB and related diseases, but also to plant pathologists in general, who will gain useful insights into TF evolution and their control of fungal virulence.

In **Chapter 1**, I first introduce the problem of plant-pathogenic fungi and a key challenge that remains; to better characterise the regulatory factors that control their pathogenic lifestyles. This precedes an overview of the current state of research into the lifecycle and molecular mechanisms of virulence in *P*. *nodorum*, necrotrophic pathogen that is a significant problem in wheat-growing regions worldwide. I finish the chapter by detailing specific aims for the research presented, where the general goal was to establish novel insights into the transcriptional regulation of virulence in *P. nodorum*, for improving the management and control of SNB.

The focus of **Chapter 2 and 3** was to systematically review (**Chapter 2**) and then identify evolutionary trends (**Chapter 3**) in TFs that regulate virulence in plant-pathogenic fungi. The comprehensive literature review in **Chapter 2** highlighted the diverse TF regulatory roles that underpin fungal development and virulence. It also highlighted the need to identify and characterise TFs that are specifically associated

with phytopathogenicity. Therefore, a TF orthology analysis detailed in **Chapter 3** sought to better identify trends in the specific TF content associated with individual fungal lineages and plant-pathogenic lifestyles. By compiling and analysing the TF repertoires from a taxonomically diverse range of fungi, this provided a novel resource that was used to identify specific cases of TF conservation, expansion and loss associated with fungal virulence.

The remainder of the thesis concerns functional research into virulence regulation in *P. nodorum*. **Chapter 4** details general materials and methods applicable to subsequent chapters. The research aim in **Chapter 5** was to define the regulatory origins and mechanisms of PnPf2, a TF that controls the expression of important *P. nodorum* virulence factors that include necrotrophic effectors (NEs). A detailed phylogenetic analysis suggested PnPf2 evolved from fungal regulators of polysaccharide metabolism. A chromatin-immunoprecipitation analysis also demonstrated that PnPf2 directly controls NE expression, binds two distinct promoter-regulatory motifs, and coordinates virulence through a transcriptional regulatory network that incorporates other TFs. These insights not only contribute to the identification of novel virulence factors directly regulated by PnPf2, but also demonstrate a potentially-conserved mechanism by which PnPf2 orthologues control gene expression in other necrotrophic fungi.

The research aim in **Chapter 6** was to identify and functionally characterise the promoter elements regulating the expression of the NE *Tox1*, and to define their role SNB. A novel 401 bp promoter element (PE401) was investigated in detail. A functional assessment through promoter substitution demonstrated that PE401 repressed *Tox1* expression and thereby reduced *P. nodorum* virulence on susceptible

wheat cultivars that carry *Snn1*. However, the presence of PE401 allowed another major SNB disease trait to manifest. The prevalence of PE401 was highly variable in regional *P*. *nodorum* populations, insights which have significant consequences for SNB disease monitoring and breeding for resistance in wheat. Importantly, the analysis of PE401 also sheds light on the transcriptional suppression of NEs mediated through epistasis, which is discussed in detail.

In **Chapter 7** the significant outcomes for the study of TF regulation in fungal virulence and then PnPf2 as well as NE regulation in *P*. *nodorum* are summarised. The implications and future scientific directions for plant-pathologists and SNB disease management are discussed.

V. LIST OF ABBREVIATIONS

Abbreviation	Definition
SNB	septoria nodorum blotch
SM	secondary metabolite
STB	septoria tritici blotch
TS	Tan/yellow spot
QTL	quantitative trait loci
MAPK	mitogen-activated protein kinase
CWDE	cell wall-degrading enzymes
TF	transcription factor
PDA	potato dextrose agar
DLA	detached leaf assay
PEG	polyethylene glycol
GG	Golden gate
Avr	avirulence (effector)
R	resistance (gene)
DBD	DNA binding domain
Zn2Cys6	zinc cluster (domain)
C2H2	Cys2His2 domain (domain)
GATA	Cys4 domain (domain)
bZIP	basic leucine-zipper domain (domain)
bHLH	basic helix-loop-helix domain (domain)
HD/Hoc	homeobox domain (domain)
MOR	morphogenesis-related (domain)
SIX	secreted in xylem
ROS	reactive-oxygen species
AA	amino acid
ORGID	organism identification
ANOVA	analysis of variance

ANCOVA	analysis of covariance
R	correlation coefficient
NCBI	National centre for biotechnology information
qPCR	quantitative PCR
GFP	green fluorescent protein
SQ	starting quantity
HA	haemagglutinin
PhleoR	Phleomycin resistance
HygR	Hygromycin resistance
KO	knockout
CAZyme	Carbohydrate-active enzyme
DE	differentially expressed
NJ	neighbour joining (tree)
ML	maximum likelihood (tree)
PWM	position weight matrix
HR	homologous recombination
Ec	ectopic control
ChIP	chromatin immunoprecipitation
GO	gene ontology
NLS	nuclear localisation signal
PE401	401 bp promoter element
ETS	effector-triggered susceptibility
DH	double haploid (population)
RI	recombinant inbred (population)
ITMI	International Triticeae mapping initiative (population)
CxW	Calingiri Wyalkatchem (population)
LOD	logarithm of the odds (score)
UTR	untranslated region

VI. LIST OF FIGURES, TABLES & SUPPLEMENTARY ITEMS

Supplementary items link https://figshare.com/s/b0bd50b709e823cbf43e

Figures link https://figshare.com/s/7bd77b9de6e422d48173

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Chapter 1

A general introduction

Section 1.1 of this chapter has been published prior to submission of this thesis, forming part of the following review:

John, E., Singh, K.B., Oliver, R.P. & Tan, K-C (2021) Transcription factor control of virulence in phytopathogenic fungi. *Molecular Plant Pathology*, 22, 858-881. https://doi.org/10.1111/mpp.13056.

Formatting has been undertaken to suit this thesis, but in all other aspects the content remains the same. A signed co-author contribution statement demonstrating that the work contained herein is my own is provided in **Appendix 1**.

1.1. Plant-pathogenic fungi

The symptoms of plant diseases such as rusts, blasts, smuts, blotches, blights and mildews, along with various prescriptions for their mitigation have been recorded since antiquity (Dark and Gent, 2001; Dugan, 2008; Wu et al., 2019). Until the establishment of the germ theory of disease in the 19th century, the nature of the causal agents remained obscure (Kelman and Peterson, 2002). Subsequently, numerous plant-pathogenic microorganisms have been identified (Crous et al., 2015; Lucas, 2020). Consequences of disease outbreaks range from economic to humanitarian and environmental, the threat of which is heightened by increased anthropogenic migration and transport, allowing diseases to spread (Fones et al., 2017; Santini et al., 2018; Kamoun et al., 2019; Savary et al., 2019).

Crop pathogens pose a distinct challenge, as they exploit the lack of diversity in agroecosystems to proliferate rapidly (Brown, 2015; McDonald and Stukenbrock, 2016; Möller and Stukenbrock, 2017). While modern agricultural practices and technologies have enabled substantial yield increases, continued population growth tied with limited arable land are placing pressure on necessary production (Cole et al., 2018; World Food Program, 2019; Fones et al., 2020). Therefore, disease management forms an integral component of food and resource security and the economic security of producers (Avery et al., 2019; Islam et al., 2019; Kettles and Luna, 2019). A lack of durable resistance in many crops and evolved pathogen resistance to available chemical controls provide further challenges, meaning novel solutions must be developed (Burdon et al., 2016; Nelson et al., 2018; Fisher et al., 2018). Hence, an important goal in fungal plant pathology is to characterise the molecular mechanisms of disease that can be exploited for plant protection (Sacristan and Garcia-Arenal, 2008; Oliver, 2012; Fones et al., 2020).

Fungi represent the majority of pathogens posing a severe threat to plant health, with bacteria, oomycetes and viruses largely making up the rest (de Wit, 2015; Hawksworth and Lücking, 2017; Doehlemann et al., 2017). Nevertheless, most fungi are non-pathogenic and most plants are resistant to all but a few species. This begs the question, what is it that allows a disease to develop? Recent advances have shed light on various aspects of fungal virulence by highlighting the role of microbial effector-host receptor interactions (de Wit et al., 2017; Han and Kahmann, 2019; Kanja and Hammond-Kosack, 2020), secondary metabolite (SM) biosynthesis (Chooi et al., 2014; Macheleidt et al., 2016; Collemare et al., 2019), signal transduction/cellular metabolism (Bielska et al., 2014; Ikeda et al., 2019), cellular trafficking/secretion systems (Rascle et al., 2018; Park et al., 2018; Le Marquer et al., 2019) and the channelling of non-coding RNAs (Sesma, 2016; Hua et al., 2018; Cai et al., 2019). However, a universal strategy does not appear to exist. While the detection of positive selection and the development of machine learning approaches such as EffectorP have assisted the identification of pathogenicity-related genes, such genes have been difficult to functionally annotate (Aylward et al., 2017; Sperschneider et al., 2018; Haridas et al., 2020; Feurtey et al., 2020). Moreover, an organism may be mutualistic or symbiotic until a change in conditions renders it pathogenic (Lo Presti et al., 2015; van der Does and Rep, 2017). Therefore understanding what regulates those aspects pertaining to fungal virulence is critical to understanding the nature of plant diseases.

The protection of plants from fungal pathogens is significantly enhanced by incorporating durable resistance through the host immune system (Brown, 2015). This has been a key goal since the discovery that interactions between a microbial pathogen and the host-immune system occur in a ‘gene-for-gene’ manner (Flor, 1956;

Hammond-Kosack and Jones, 1996; Dangl and Jones, 2001). This describes the manner by which a pathogen produces small molecules known as ‘effectors’ that function to facilitate infection either by subverting a plant hosts physiology/metabolism, acting directly as toxins or (conversely) by offering fungal protection from host-secreted toxins or evading recognition by components of the host immune system. To counter these effector molecules, plants have evolved immune-receptors that specifically recognise a cognate-effector that actives ‘effector-triggered immunity’, which typically takes the form of a hypersensitive response (HR) whereby highly-oxidative molecules are released and a localised cell death contains the pathogen at the site of infection (Balint-Kurti, 2019; Cui et al., 2015). Effectors include small-secreted proteins/peptides, secondary metabolites or short-interfering RNAs that are targeted at the host (Lo Presti et al., 2015). Host receptor (*R*) genes commonly encode proteins with nucleotide-binding site (NBS) and leucine-rich repeat (LRR) domains known as NBS-LRR proteins or NLR proteins (Wang and Chai, 2020). The continued evolution of fungal effector-coding genes to evade recognition and plant *R* genes to detect these changes is directly connected in the gene-for-gene manner. Hence, durable genetic resistance from a plant-protection standpoint is achieved by characterising these interactions at the molecular level and incorporating the appropriate combination of *R* genes into plant germplasm that are less-susceptible to be overcome.

1.2. *Parastagonospora nodorum*, a necrotrophic fungal pathogen of wheat

1.2.1. The lifecycle and historical context

This thesis focuses on the causal agent of septoria nodorum blotch (SNB), *Parastagonospora nodorum* (syn. *Stagonospora nodorum*, *Septoria nodorum*, *Phaeosphaeria nodorum*, *Leptosphaeria nodorum*). SNB is a fungal disease that has been identified on several grass species but is primarily associated with wheat. It is characterised by chlorotic and necrotic lesions which can form on both leaves and glumes (**Figure 1.1**). This inhibits the photosynthetic capacity of the host plant causing a reduction in yield, and in the case of severe infections, grain quality (Baker, 1978; Karjalainen et al., 1983; Eyal, 1999; Solomon et al., 2006b; Oliver et al., 2011; Zhang and Nan, 2018; Mehra et al., 2019). The lifecycle of *P*. *nodorum* involves both asexual and sexual (heterothallic) reproduction prior to spore dissemination through rain splash and wind respectively (**Figure 1.1**).

SNB was first reported in 1845 in the United Kingdom, where archives indicate it was the major disease of wheat from the early 1900s until the mid-1980s (Berkeley, 1845; Bearchell et al., 2005; Shaw et al., 2008). The pathogen has since been identified in the majority of wheat-growing regions worldwide (Leath, 1993; Stukenbrock et al., 2006). Recent reports indicate it is widespread in northern Europe, northern America, south-western Australia, northern India and Iran (Cowger et al., 2020; Ghaderi et al., 2020; Hafez et al., 2020; Lin et al., 2020b; Phan et al., 2020; Rana et al., 2020; Willocquet et al., 2020). Direct losses are difficult to estimate in the field as it is often mistaken for septoria tritici blotch (STB caused by *Zymoseptoria tritici*) and tan (syn. yellow leaf) spot (TS caused by *Pyrenophora tritici-repentis*), wheat

diseases which co-occur with SNB (Loughman et al., 1993; Bhathal et al., 2003; Oliver et al., 2016; Ficke et al., 2017; Figueroa et al., 2018; Abdullah et al., 2020). The most comprehensive analysis conducted in Australia suggested costs around AUD$108 million are directly attributable to SNB even in the presence of control measures (Murray and Brennan, 2009).

Strobilurins and azole fungicides are widely used to manage the disease (along with STB and TS) both through seed treatment and foliar sprays (Salam et al., 2013; Oliver et al., 2016; Ficke et al., 2017). Although not reported in some SNB endemic regions such as Western Australia, fungicide resistance has been reported in European populations (Blixt et al., 2009; Pereira et al., 2017). Therefore, genetic resistance to SNB is desirable to reduce the application costs and reliance on chemical controls. Co-evolving gene-for-gene interactions with the host-immune system were not identified, such as those typical of biotrophic/hemibiotrophic pathogens (Flor, 1956; Hammond-Kosack and Jones, 1996; Dangl and Jones, 2001; Friesen and Faris, 2010). Hence, the deployment of major*R* genes which could trigger host immunity to contain the pathogen was not viable. Instead, a number of minor wheat quantitative trait loci (QTLs) have been described which are associated with SNB severity (Schnurbusch et al., 2003; Xu et al., 2004; Singh et al., 2019; Downie et al., 2020; Lin et al., 2020a; Lin et al., 2021). Therefore, QTLs for SNB resistance must be stacked which is difficult to achieve through traditional breeding methods that make use of mixed pathogen complements for disease screening. Recent advances in our molecular understanding of the disease have begun to alleviate this issue (Oliver et al., 2014; Oliver et al., 2016; Keller et al., 2018).

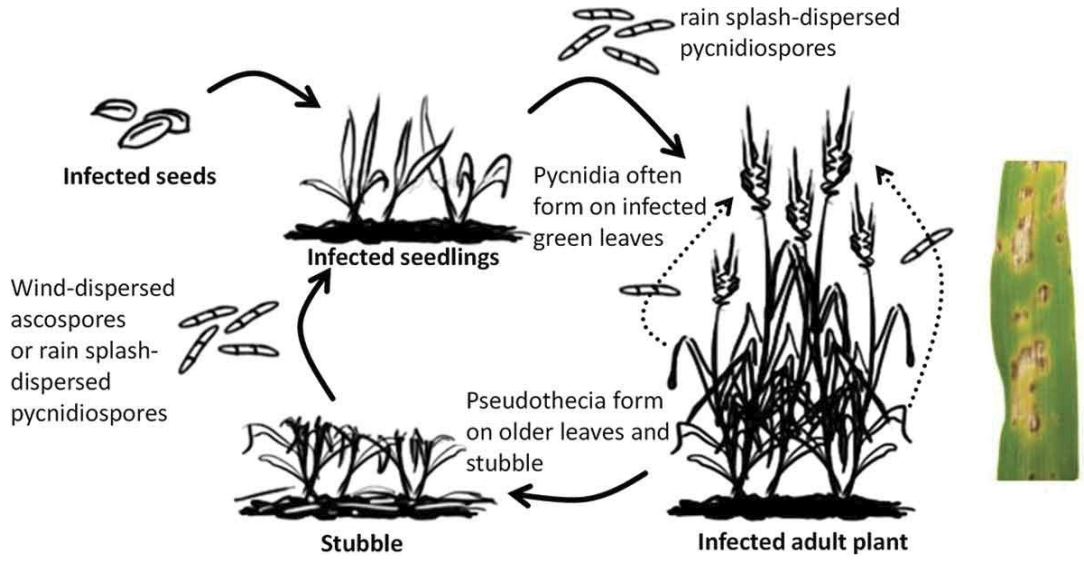


Figure 1.1 - The *P. nodorum* infection cycle

P. nodorum inoculum may be stubble or seed-borne in the form of rain splash-disseminated asexual pycnidiospores or wind-dispersed ascospores (the sexual reproductive propagule). Under moist conditions favourable to growth, hyphae stemming from germinated spores penetrate susceptible plant tissue directly through the cuticle, as well as through stomatal openings. The fungus proceeds in a typical necrotrophic fashion, killing the host and feeding off the dead tissue, whereupon proliferation leads to the formation of pycnidia for repeated dissemination of pycnidiospores throughout the growing season by rain splash. Under favourable conditions, this cycle takes 10-12 days. If compatible (heterothallic) mating types meet, pseudothecia form (sexual reproductive bodies, taking 20-24 days to develop) to complete the sexual reproductive cycle. Figure adapted from Chooi *et al.* (2014) - Creative Commons CC BY.

1.2.2. On the molecular mechanisms of virulence

Several lines of investigation have been pursued in the effort to define the molecular mechanisms by which *P. nodorum* causes disease on wheat (a summary of research efforts is provided in **Table 1.1**). Microscopic analysis has revealed that the fungus can infect both via the direct penetration of host-epidermal cells or through stomatal openings. This precedes both intra- and intercellular growth throughout the epidermis and mesophyll tissue (Baker, 1978; Bird and Ride, 1981; Karjalainen and Lounatmaa, 1986; Zinkernagel et al., 1988; Solomon et al., 2006e). It was originally hypothesised that the infection was primarily facilitated by the secretion of a broad set of phytotoxic metabolites and hydrolytic cell wall-degrading enzymes (CWDEs) (Kent and Strobel, 1976; Devys et al., 1980; Magro, 1984; Lehtinen, 1993; Lalaoui et al., 2000; Carlile et al., 2000; Friesen and Faris, 2010; Oliver et al., 2011). Through biochemical approaches, some non-selective phytotoxins secreted by the fungus were isolated such as the melleins and septorins (Devys et al., 1978; Devys et al., 1992). However, these could not explain the specificity or severity of SNB of wheat.

New avenues for research were opened when targeted genetic manipulation in *P. nodorum* was first demonstrated in 1988 (Cooley et al., 1988). The focus of molecular research then shifted towards the characterisation of the metabolic and signal transduction components that are required for pathogenic development (Solomon et al., 2006b). Through gene disruption of nitrate assimilatory genes, this pathway was found to be dispensable during infection, indicating nitrogen scarcity was not a major factor of SNB (Cutler and Caten, 1999; Howard et al., 1999). Lipids were established as the primary energy source during *P. nodorum* germination and host penetration following gene deletion of the malate synthase gene *Mls1* (Solomon et al., 2004a). Meanwhile, mannitol and trehalose were shown to be critical primary

metabolites for sporulation and therefore pathogen dissemination (Solomon et al., 2005a; Solomon et al., 2006d; Lowe et al., 2009). Core eukaryotic signal transduction mechanisms such as the heterotrimeric G-protein, calcium/calmodulin and mitogen-activated protein kinase (MAPK) pathways were also shown to control sporulation, stress tolerance and/or host penetration (Solomon et al., 2004b; Solomon et al., 2005b; Solomon et al., 2006c; Gummer et al., 2012; John et al., 2016). Recently, functional genomics guided approaches have identified novel SMs that are secreted by *P. nodorum* during infection. Some of these (such as phomacins and elsinichrome C) elicit broad phytotoxic activities, suggesting they contribute to the virulence of *P. nodorum* (Chooi et al., 2014; Muria-Gonzalez et al., 2015; Chooi et al., 2017; Li et al., 2018; Li et al., 2020).

The aforementioned studies have established some of the fundamental developmental and general-virulence pathways in *P. nodorum*. However, it was evidence from wheat QTL-mapping studies utilising semi-purified culture filtrates that indicated host-specific toxins were key factors in the severity of SNB (Keller et al., 1994; Wicki et al., 1999; Liu et al., 2004a; Liu et al., 2004b). Their characterisation represented a promising avenue for identifying robust molecular markers which could assist disease resistance breeding efforts. This prompted a pioneering effort to assemble the *P. nodorum* genome, which was achieved through Sanger sequencing and released in 2004, the first reported for a Dothideomycete fungus (Hane et al., 2007). A new understanding of the major determinants underpinning SNB was then established, as it was confirmed that *P. nodorum* produces proteinaceous necrotrophic effectors (NEs) (Liu et al., 2006; Friesen et al., 2007; Oliver and Solomon, 2010). NEs are small (<30Kda), cysteine-rich proteins that are secreted to subvert plant immunity through an ‘inverse’ gene-for-gene interaction that results in ‘effector-

triggered susceptibility' (Friesen et al., 2007; Tan et al., 2010; Friesen and Faris, 2010; McDonald and Solomon, 2018). NE interactions occur on wheat lines carrying a receptor encoded by a dominant-susceptibility gene and elicits a necrotic response in the host tissue that facilitates an infection (**Figure 1.2**). This contrasts with the traditional avirulence (Avr) effector model that typifies biotrophic and hemibiotrophic pathogens, where Avr effector recognition by host receptors triggers an immune response that contains the infection (effector-triggered immunity). For these pathogens, the incorporation of immune receptors (i.e. 'R genes') into plant hosts provides strong genetic resistance, until effectors evolve that can evade recognition (McDonald and Linde, 2002; Rouxel and Balesdent, 2017; Sánchez-Vallet et al., 2018). On the other hand, the removal of host receptors that recognise NEs improves the genetic potential for resistance to NE-producing pathogens such as *P*. *nodorum* (Tan et al., 2010; Vleeshouwers and Oliver, 2014).

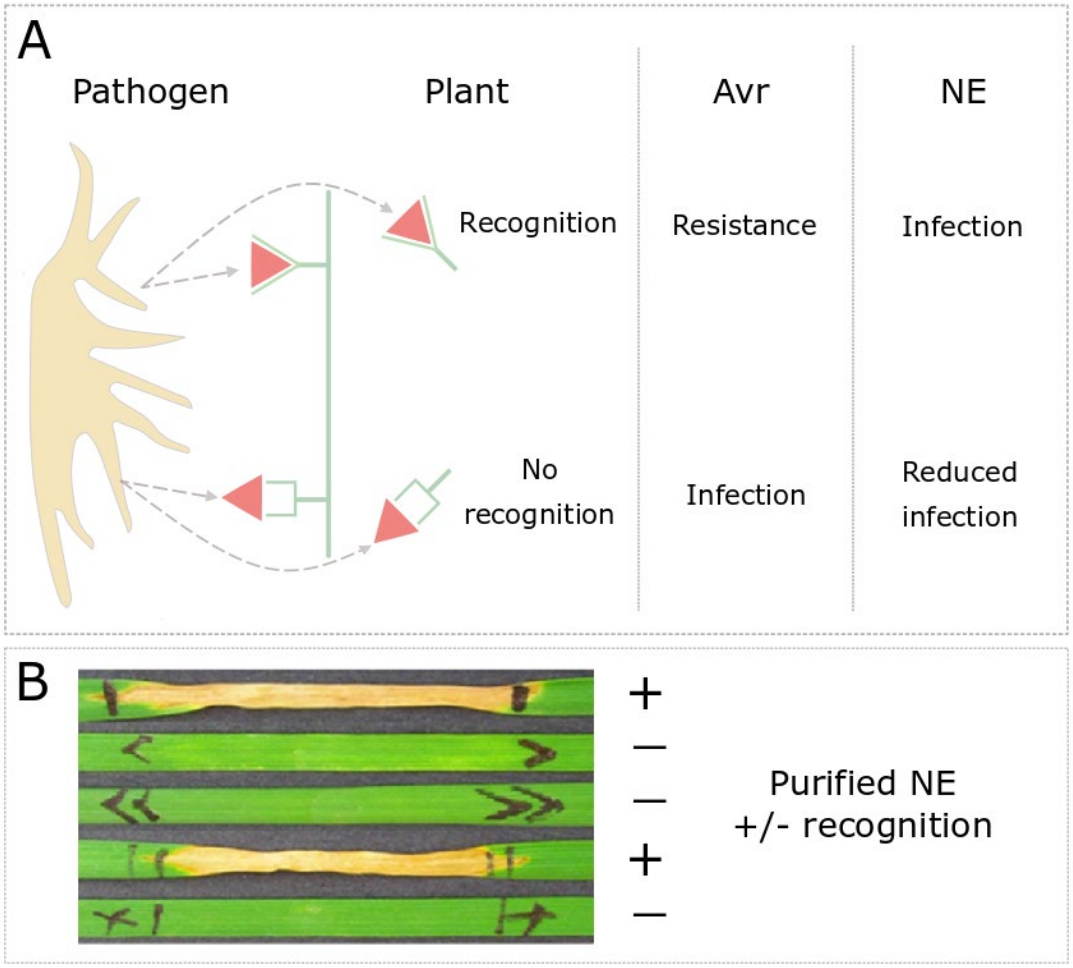


Figure 1.2 – Avirulence (Avr) vs necrotrophic effectors (NEs) in fungal virulence

A) In the traditional gene-for-gene model of plant-pathogen interactions, Avr effectors (encoded by *Avr* genes) are secreted to manipulate plant development/metabolism. Plant hosts have developed a network of intra/extracellular receptors (encoded by *R* genes) to activate effector-triggered immunity. A NE instead targets plant receptors (encoded by '*S*' genes) to elicit effector-triggered susceptibility, where the absence of either component reduces the potential for a necrotrophic pathogen to infect. Effectors are depicted as red triangles and plant receptors by the proximal green shape outline. B) Necrotic symptoms of the purified-NE 'ToxA' following leaf infiltration into wheat lines with (+) or without (-) the corresponding receptor gene *Tsn1*. Infiltration image courtesy of the Centre for Crop and Disease Management (http://ccdm.com.au).

There have been significant efforts to uncover both the pathogen NE genes and their corresponding host-susceptibility receptors in the *P. nodorum*-wheat pathosystem (Friesen et al., 2007; Tan et al., 2010; Friesen and Faris, 2010; McDonald and Solomon, 2018). Genetic evidence has revealed at least 10 interactions so far, three of which (ToxA-*Tsn1*, Tox1-*Snn1* and Tox3-*Snn3D1*) are cloned and characterised in both the pathogen and host (Friesen et al., 2006; Faris et al., 2010; Liu et al., 2006; Liu et al., 2009; Liu et al., 2012; Shi et al., 2016; Phan et al., 2016; Ruud et al., 2017; Downie et al., 2020; Zhang et al., 2021). Two additional NE genes have been cloned in *P. nodorum*, *Tox5* and *Tox267*, which will assist efforts to fine map and identify the corresponding receptor gene targets in wheat (Kariyawasam et al., 2021; Richards et al., 2021). With its genetic tractability and completed genomes now available for multiple isolates, *P. nodorum* has become a model system for studying necrotrophic fungal-plant interactions (Syme et al., 2016; Richards et al., 2017; Richards et al., 2019). A direct outcome has been the discovery that *ToxA* was horizontally transferred to the wheat pathogens *P. tritici-repentis* and *Bipolaris sorokiniana*, which significantly enhanced their virulence on *Tsn1* wheat (Friesen et al., 2006; Friesen et al., 2018; McDonald et al., 2018; McDonald et al., 2019). This opens the possibility that the exchange of other *P. nodorum* NEs also occurs between necrotrophic pathogens, with implications for the evolution and management of these diseases.

The symptoms induced by purified NEs in susceptible wheat lines are stark (**Figure 1.2**), a phenotypic response exploited to fast-track the removal of the susceptibility genes in wheat-breeding programs (Oliver et al., 2014). Hence, it is somewhat paradoxical that the absence of one or more of these interactions does not

reliably confer increased SNB resistance (Phan et al., 2018; McDonald and Solomon, 2018; Cowger et al., 2020). Some interactions are also not detected in field trials despite the use of wheat mapping populations with known susceptibility genes (Ruud et al., 2019; Lin et al., 2021). An explanation lies in recent evidence that suggests many NE-receptor SNB interactions are either redundant or epistatic, whereby they mask the disease contribution of others (Tan et al., 2015; Phan et al., 2016; Peters-Haugrud et al., 2019; Downie et al., 2020; Richards et al., 2021). Additional NE properties also exist. For example, ToxA and Tox3 interact with PR-1 pathogenesis-related proteins while Tox1 suppresses wheat chitinase activity to further manipulate host defenses (Breen et al., 2016; Liu et al., 2016; Breen et al., 2017; Sung et al., 2021). On the other hand, a fitness cost is likely associated with NEs, given the extreme presence/absence variation in *P. nodorum* isolates around the world (McDonald et al., 2013; Richards et al., 2019; Cowger et al., 2020; Lin et al., 2020b). These issues complicate SNB-resistance breeding through receptor removal and demand a better understanding of the individual and connected roles played by NEs in the *P. nodorum*-wheat pathosystem, where the host-isolate genetic composition is subject to change. Hence, understanding the components regulating the expression of both NEs and the other virulence factors such as SMs or CWDEs is of fundamental importance if we are to delineate the context and the extent to which they contribute to SNB.

Table 1.1 – Scientific investigation into the virulence mechanisms of *P. nodorum*

A summary of the published research on the diverse aspects pertaining to virulence in *P. nodorum* [column 1], the components studied [2], the virulence functions reported with any associated functionally-defined genes [3-4] and the associated publications [5]. 'ND' indicates the virulence/gene function were not determined in the corresponding study references.

Aspect	Component	Virulence function	Protein functionally studied	Reference
Developmental stage of infection	Infection-related morphogenesis	Plant penetration/expansion	ND	(Baker, 1978; Bird and Ride, 1981; Karjalainen and Lounatmaa, 1986; Zinkernagel et al., 1988; Solomon et al., 2006e)
Enzymatic activity	Cell wall-degrading enzymes	ND	ND	(Magro, 1984; Lehtinen, 1993; Lalaoui et al., 2000)
	Protease	ND	Snp1 (Trypsin protease)	(Carlile et al., 2000; Bindschedler et al., 2003)
	Lipase	ND	Lip1 (Carboxylic ester hydrolase)	(Feng et al., 2012)
Secondary metabolite production	Cationic acids (unstable)	Phytotoxic activity	ND	(Kent and Strobel, 1976)
	Melleins	Germination inhibitor	SNOG_00477 (Polyketide synthase)	(Devys et al., 1980; Devys et al., 1992; Keller et al., 1994; Bousquet and Kollmann, 1998; Wicki et al., 1999; Yang et al., 2013; Chooi et al., 2015a)
	Mycophenolic acid	ND	ND	(Devys et al., 1980; Bousquet and Kollmann, 1998)

	Septorines	Phytotoxic activity	ND	(Devys et al., 1978; Devys et al., 1982; Bousquet and Kollmann, 1998; Yang et al., 2013)
	Butyrophenones	ND	ND	(Yang et al., 2013)
	Alternariol	ND (mycotoxin)	SnPKS19 (Polyketide synthase) + Sch1 (Short chain dehydrogenase)	(Tan et al., 2009; Chooi et al., 2015b)
	Elsinichrome C	Phytotoxic activity	EclR (Zn2Cys6 transcription factor)	(Chooi et al., 2017)
	a-pyrones	Germination inhibitor	AlpA (Polyketide synthase)	(Li et al., 2018)
	Stemphyloxin II	Phytotoxic activity	SthR (Zn2Cys6 transcription factor)	(Li et al., 2019)
	Phomacins	Phytotoxic activity	PhmR (Zn2Cys6 transcription factor) + PhmA (Polyketide synthase/non ribosomal peptide synthase)	(Li et al., 2020)
	Volatile organic compounds (sesquiterpenes)	ND (general toxicity)	Sts1 + Sts2 (Sesquiterpene synthases)	(Muria-Gonzalez et al., 2020)
Primary metabolism	Nitrate reduction	ND	Nia1 + Nii1 (Nitrate/nitrite reductase)	(Cutler and Caten, 1999; Howard et al., 1999)
	Leucine biosynthesis	Auxotroph	LeuA (3-isopropylmalate dehydrogenase)	(Cooley et al., 1999)
	Polyamine biosynthesis	Auxotroph	Odc1 (Ornithine decarboxylase)	(Bailey et al., 2000)
	Di/tripeptide assimilation	ND	Ptr2 (Di/tripeptide transporter)	(Solomon et al., 2003)
	γ-aminobutyric acid metabolism	Stress tolerance + sporulation	Sdh1 (Succinic semialdehyde dehydrogenase)	(Mead et al., 2013)

	Haem biosynthesis	Auxotroph	Als1 (δ-Aminolaevulinic acid synthase)	(Solomon et al., 2006a)
	Mannitol metabolism	ND (sporulation)	Mdp1 (Mannitol-1-phosphate dehydrogenase) + Mdh1 (Mannitol 2-dehydrogenase)	(Solomon et al., 2005a; Solomon et al., 2006d)
	Lipid oxidation	Germination + penetration	Mls1 (Malate synthase)	(Solomon et al., 2004a)
	Methylglyoxal detoxification	ND (methylglyoxal sensitivity)	Gox1 (Glyoxylase I)	(Solomon and Oliver, 2004)
	Arabitol + xylitol metabolism	ND (osmotic stress tolerance)	Abd1 (L-arabitol dehydrogenase) + Xdh1 (Xylitol dehydrogenase)	(Lowe et al., 2008)
	Trehalose biosynthesis	Germination + sporulation + stress tolerance	Tps1 (Trehalose 6-phosphate synthase)	(Lowe et al., 2009)
	Undetermined metabolic pathways	ND (sporulation)	Sch1 + Sch3 (Short chain dehydrogenases)	(Tan et al., 2008; Casey et al., 2010)
	Coenzyme A biosynthesis	ND	Pbl1 (Pantothenate-β-alanine ligase)	(Ipcho et al., 2012)
Signal transduction	G protein signalling	Sporulation + penetration + protease secretion + stress tolerance + vegetative growth	Gna1 + Gba1 + Gga1 (Heterotrimeric G proteins)	(Solomon et al., 2004b; Tan et al., 2008; Gummer et al., 2012; Casey et al., 2010; Gummer et al., 2013)
	Fus3/Kss1 MAPK signalling	Sporulation + vegetative growth + penetration	Mak2 (Mitogen activated protein kinase)	(Solomon et al., 2005b)
	Hog1 MAPK signalling	ND (sporulation + stress tolerance)	Hog1 (Mitogen activated protein kinase) + Nik1 (Histidine kinase)	(John et al., 2016)

	Calcium/calmodulin signalling	ND (sporulation)	CpkA + CpkB + CpkC (Ca/Cm-dependent protein kinases)	(Solomon et al., 2006c)
Necrotrophic effectors	ToxA	Necrosis (*Tsn1* wheat) + TaPR1-5 binding	ToxA	(Sarma et al., 2005; Liu et al., 2006; Faris et al., 2010; Vincent et al., 2011; Lu et al., 2014)
	Tox3	Necrosis (*Snn3* wheat) + TaPR1 binding	Tox3	(Liu et al., 2009; Winterberg et al., 2014; Breen et al., 2016; Ruud et al., 2017; Zhang et al., 2021)
	Tox1	Necrosis (*Snn1* wheat) + Chitin binding	Tox1	(Liu et al., 2012; Liu et al., 2016; Shi et al., 2016)
	Tox5	Necrosis (*Snn5* wheat)	Tox5	(Friesen et al., 2012; Kariyawasam et al., 2021)
	Tox267	Necrosis (*Snn2/6/7* wheat)	Tox267	(Friesen et al., 2008; Gao et al., 2015; Shi et al., 2015; Richards et al., 2021)
Gene regulation	Transcription factor (Zn2Cys6)	ND (malayamycin response + sporulation)	Mrg1	(Li et al., 2008)
	Transcription factor (APSES)	Sporulation + carbon metabolism + *Tox3* expression	StuA	(IpCho et al., 2010)
	Transcription factor (C2H2)	Essential gene + *Tox3* + *ToxA* expression	PnCon7	(Lin et al., 2018)
	Transcription factor (Zn2Cys6)	*ToxA* + *Tox3* expression	PnPf2	(Rybak et al., 2017; Jones et al., 2019)

1.2.3. On the regulation of virulence

While novel *P. nodorum* virulence factors continue to be uncovered, relatively little has been established regarding their specific regulatory components. One characteristic of the NE genes is that they display maximal gene expression at the onset of disease symptoms ~2-3 days post-inoculation (Rybak et al., 2017; Richards et al., 2021; Kariyawasam et al., 2021). However, attempts to isolate the unique nutritional or chemical signals that trigger or repress gene expression have proven unsuccessful (McDonald and Solomon, 2018).

Recent work characterising transcription factors (TFs) began to shed light on the specifics of individual NE gene expression (TFs are defined and their functional roles in plant-pathogenic fungi are reviewed in depth in **Chapter 2**). One of these was PnCon7, identified through a yeast-2-hybrid screen as a direct positive regulator of *Tox3* (Lin et al., 2018). In this study, *pncon7* knockout mutants could not be obtained which indicated the TF was also critical for fungal viability. In a separate study, another TF PnPf2 was found to be an essential regulator of both *ToxA* and *Tox3* gene expression (Rybak et al., 2017). Unlike *PnCon7*, *PnPf2* gene deletion had minimal effect on saprophytic development but abolished *P. nodorum* virulence on *Tsn1* and *Snn3* selective wheat lines. Interestingly, the *P. tritici-repentis* orthologue PtrPf2 is also essential for *ToxA* expression, while in another necrotroph of the Pleosporales order, *A. brassicicola*, AbPf2 is a critical regulator of virulence (Cho et al., 2013; Rybak et al., 2017). RNA-seq analyses in both *A. brassicicola* and *P. nodorum* utilising *pf2* knockout mutants suggested a wide range of uncharacterised effector-like genes and CWDEs are (either directly or indirectly) also targeted (Cho et al., 2013; Jones et al., 2019). These attributes suggest that further exploration into both the evolution and mechanisms of PnPf2 regulation in *P. nodorum* will better define the regulation of NEs

and other components of virulence that are integral to host infection. An independent regulatory pathway remains to be characterised for the NE *Tox1*, since *PnPf2* deletion caused only a moderate reduction in gene expression (Jones et al., 2019). Expression variation between isolates, and polymorphisms in the promoter of *Tox1* have also been noted (Gao et al., 2015; Peters-Haugrud et al., 2019). Therefore, a functional investigation into the DNA regulatory elements in the *Tox1* promoter region represents a promising avenue to define its regulatory role in SNB.

1.3. Thesis aims

An overarching goal for the work presented in this thesis was to establish novel insights into the transcriptional regulation of virulence in *P. nodorum* and advance scientific knowledge of the disease. The TF PnPf2 was prioritised as a fundamental component to further characterise and explore its regulatory role. This was due to the previously established regulatory role in both *ToxA* and *Tox3* expression and the identification of a broad array of necrotrophic-lifestyle genes under PnPf2 control (Rybak et al., 2017; Jones et al., 2019). The significance of PnPf2 in regulating *P. nodorum* virulence, coupled with the available RNA-seq dataset provided a sound basis on which to apply a novel chromatin immunoprecipitation (ChIP) analysis to establish the genetic targets and binding mechanisms. The common virulence-regulatory role for PnPf2 orthologues in other fungi of the Pleosporales order (Cho et al., 2013; Rybak et al., 2017) raised further questions regarding the evolutionary origins and significance of this TF. Therefore, as a major component of this research, TFs in plant-pathogenic fungi at large were first analysed to provide both the context for their regulatory role in *P. nodorum* and a novel resource for the field of molecular plant pathology. A TF-centered analysis was not applicable to studying another major *P. nodorum* NE *Tox1*. Hence, a promoter-centred approach to elucidate the regulation of this major virulence factor was considered a suitable avenue to pursue. In light of these opportunities to explore the transcriptional regulation of virulence, three specific aims for the major components of the thesis are defined as follows:

1) To systematically review and identify evolutionary trends in TFs that regulate virulence in plant-pathogenic fungi (**Chapters 2 and 3**).

2) To define the origins and mechanisms of PnPf2-mediated virulence in *P. nodorum* through a functional exploration of direct genetic targets and the associated transcriptional network (**Chapter 5**).
3) To identify and functionally characterise the promoter elements regulating *Tox1*-mediated virulence in *P. nodorum* populations and determine their significance in SNB (**Chapter 6**).

These aims are detailed further and then addressed in the context of the respective chapters with the outcomes discussed in detail. In **Chapter 7** the significant outcomes and future directions for plant-pathologists, cereal breeders and growers are then summarised.

1.4. References

Abdullah, A.S., Gibberd, M.R. & Hamblin, J. (2020) Co-infection of wheat by *Pyrenophora tritici-repentis* and *Parastagonospora nodorum* in the wheatbelt of Western Australia. *Crop and Pasture Science*, 71, 119–127. https://doi.org/10.1071/CP19412.

Avery, S.V., Singleton, I., Magan, N. & Goldman, G.H. (2019) The fungal threat to global food security. *Fungal Biology*, 123, 555–557. https://doi.org/10.1016/j.funbio.2019.03.006.

Aylward, J., Steenkamp, E.T., Dreyer, L.L., Roets, F., Wingfield, B.D. & Wingfield, M.J. (2017) A plant pathology perspective of fungal genome sequencing. *IMA Fungus*, 8, 1–15. https://doi.org/10.5598/imafungus.2017.08.01.01.

Bailey, A., Mueller, E. & Bowyer, P. (2000) Ornithine decarboxylase of *Stagonospora (Septoria) nodorum* is required for virulence toward wheat. *Journal of Biological Chemistry*, 275, 14242–14247. https://doi.org/10.1074/jbc.275.19.14242.

Baker, E.A. (1978) *Septoria* - the lurking threat to wheat yields. *EPPO Bulletin*, 8, 9–20. https://doi.org/10.1111/j.1365-2338.1978.tb02143.x.

Balint-Kurti P. (2019) The plant hypersensitive response: concepts, control and consequences. *Molecular Plant Pathology*, 20(8), 1163–1178. https://doi.org/10.1111/mpp.12821

Bearchell, S.J., Fraaije, B.A., Shaw, M.W. & Fitt, B.D.L. (2005) Wheat archive links long-term fungal pathogen population dynamics to air pollution. *Proceedings of the National Academy of Sciences*, 102, 5438–5442. https://doi.org/10.1073/pnas.0501596102.

Berkeley, M.J. (1845) Disease in the wheat crop. *Gardeners' Chronicle*, 35.

Bhathal, J.S., Loughman, R. & Speijers, J. (2003) Yield reduction in wheat in relation to leaf disease from yellow (tan) spot and septoria nodorum blotch. *European Journal of Plant Pathology*, 109, 435–443. https://doi.org/10.1023/A:1024277420773.

Bielska, E., Higuchi, Y., Schuster, M., Steinberg, N., Kilaru, S., Talbot, N.J., et al. (2014) Long-distance endosome trafficking drives fungal effector production during plant infection. *Nature Communications*, 5, 1–14. https://doi.org/10.1038/ncomms6097.

Bindschedler, L.V., Sanchez, P., Dunn, S., Mikan, J., Thangavelu, M., Clarkson, J.M., et al. (2003) Deletion of the SNP1 trypsin protease from *Stagonospora nodorum* reveals another major protease expressed during infection. *Fungal Genetics and Biology*, 38, 43–53. https://doi.org/10.1016/S1087-1845(02)00517-0.

Bird, P.M. & Ride, J.P. (1981) The resistance of wheat to *Septoria nodorum*: fungal development in relation to host lignification. *Physiological Plant Pathology*, 19, 289-IN3. https://doi.org/10.1016/S0048-4059(81)80063-X.

Blixt, E., Djurle, A., Yuen, J. & Olson, Å. (2009) Fungicide sensitivity in Swedish isolates of *Phaeosphaeria nodorum*. *Plant Pathology*, 58, 655–664. https://doi.org/10.1111/j.1365-3059.2009.02041.x.

Bousquet, J.F. & Kollmann, A. (1998) Variation in metabolite production by *Septoria nodorum* isolates adapted to wheat or to barley. *Journal of Phytopathology*, 146, 273–277. https://doi.org/10.1111/j.1439-0434.1998.tb04690.x.

Breen, S., Williams, S.J., Outram, M., Kobe, B. & Solomon, P.S. (2017) Emerging insights into the functions of pathogenesis-related protein 1. *Trends in Plant Science*, 22, 871–879. https://doi.org/10.1016/j.tplants.2017.06.013.

Breen, S., Williams, S.J., Winterberg, B., Kobe, B. & Solomon, P.S. (2016) Wheat PR-1 proteins are targeted by necrotrophic pathogen effector proteins. *The Plant*

Journal, 88, 13–25. https://doi.org/10.1111/tpj.13228.
Brown, J.K.M. (2015) Durable resistance of crops to disease: a Darwinian perspective. *Annual Review of Phytopathology*, 53, 513–539. https://doi.org/10.1146/annurev-phyto-102313-045914.
Burdon, J.J., Zhan, J., Barrett, L.G., Papaïx, J. & Thrall, P.H. (2016) Addressing the challenges of pathogen evolution on the world's arable crops. *Phytopathology*, 106, 1117–1127. https://doi.org/10.1094/PHYTO-01-16-0036-FI.
Cai, Q., He, B., Weiberg, A., Buck, A.H. & Jin, H. (2019) Small RNAs and extracellular vesicles: New mechanisms of cross-species communication and innovative tools for disease control. *PLOS Pathogens*, 15, e1008090. https://doi.org/10.1371/journal.ppat.1008090.
Carlile, A.J., Bindschedler, L.V., Bailey, A.M., Bowyer, P., Clarkson, J.M. & Cooper, R.M. (2000) Characterization of SNP1, a cell wall-degrading trypsin, produced during infection by *Stagonospora nodorum*. *Molecular Plant-Microbe Interactions*, 13, 538–550. https://doi.org/10.1094/MPMI.2000.13.5.538.
Casey, T., Solomon, P.S., Bringans, S., Tan, K.-C., Oliver, R.P. & Lipscombe, R. (2010) Quantitative proteomic analysis of G-protein signalling in *Stagonospora nodorum* using isobaric tags for relative and absolute quantification. *PROTEOMICS*, 10, 38–47. https://doi.org/10.1002/pmic.200900474.
Cho, Y., Ohm, R.A., Grigoriev, I.V. & Srivastava, A. (2013) Fungal-specific transcription factor *AbPf2* activates pathogenicity in *Alternaria brassicicola*. *The Plant Journal*, 75, 498–514. https://doi.org/10.1111/tpj.12217.
Chooi, Y.-H., Krill, C., Barrow, R.A., Chen, S., Trengove, R., Oliver, R.P., et al. (2015a) An in planta-expressed polyketide synthase produces (R)-mellein in the wheat pathogen *Parastagonospora nodorum*. *Applied and Environmental Microbiology*, 81, 177–186. https://doi.org/10.1128/AEM.02745-14.
Chooi, Y.-H., Muria-Gonzalez, M.J., Mead, O.L. & Solomon, P.S. (2015b) *SnPKS19* encodes the polyketide synthase for alternariol mycotoxin biosynthesis in the wheat pathogen *Parastagonospora nodorum*. *Applied and Environmental Microbiology*, 81, 5309–5317. https://doi.org/10.1128/AEM.00278-15.
Chooi, Y.-H., Muria-Gonzalez, M.J. & Solomon, P.S. (2014) A genome-wide survey of the secondary metabolite biosynthesis genes in the wheat pathogen *Parastagonospora nodorum*. *Mycology*, 5, 192–206. https://doi.org/10.1080/21501203.2014.928386.
Chooi, Y.-H., Zhang, G., Hu, J., Muria-Gonzalez, M.J., Tran, P.N., Pettitt, A., et al. (2017) Functional genomics-guided discovery of a light-activated phytotoxin in the wheat pathogen *Parastagonospora nodorum* via pathway activation. *Environmental Microbiology*, 19, 1975–1986. https://doi.org/10.1111/1462-2920.13711.
Cole, M.B., Augustin, M.A., Robertson, M.J. & Manners, J.M. (2018) The science of food security. *NPJ science of food*, 2, 14. https://doi.org/10.1038/s41538-018-0021-9.
Collemare, J., O'Connell, R. & Lebrun, M.-H. (2019) Nonproteinaceous effectors: the terra incognita of plant–fungal interactions. *New Phytologist*, 223, 590–596. https://doi.org/10.1111/nph.15785.
Cooley, R.N., Monk, T.P., McLoughlin, S.B., Foster, S.G. & Dancer, J.E. (1999) Gene disruption and biochemical characterisation of 3-isopropylmalate dehydrogenase from *Stagonospora nodorum*. *Pesticide Science*, 55, 364–367. https://doi.org/10.1002/(SICI)1096-9063(199903)55:3<364::AID-PS910>3.0.CO;2-K.
Cooley, R.N., Shaw, R.K., Franklin, F.C.H. & Caten, C.E. (1988) Transformation of the

phytopathogenic fungus *Septoria nodorum* to hygromycin B resistance. *Current Genetics*, 13, 383–389. https://doi.org/10.1007/BF00365659.
Cowger, C., Ward, B., Brown-Guedira, G. & Brown, J.K.M. (2020) Role of effector-sensitivity gene interactions and durability of quantitative resistance to Septoria nodorum blotch in eastern U.S. wheat. *Frontiers in Plant Science*, 11. https://doi.org/10.3389/fpls.2020.00155.
Crous, P.W., Hawksworth, D.L. & Wingfield, M.J. (2015) Identifying and naming plant-pathogenic fungi: past, present, and future. *Annual Review of Phytopathology*, 53, 247–267. https://doi.org/10.1146/annurev-phyto-080614-120245.
Cui, H., Tsuda, K., & Parker, J. E. (2015) Effector-triggered immunity: from pathogen perception to robust defense*. Annual Review of Plant Biology*, 66, 487–511. https://doi.org/10.1146/annurev-arplant-050213-040012
Cutler, S.B. & Caten, C.E. (1999) Characterisation of the nitrite reductase gene *(NII1)* and the nitrate-assimilation gene cluster of *Stagonospora (Septoria) nodorum*. *Current Genetics*, 36, 282–289. https://doi.org/10.1007/s002940050502.
Dangl, J.L. & Jones, J.D. (2001) Plant pathogens and integrated defence responses to infection. *Nature*, 411, 826–833. https://doi.org/10.1038/35081161.
Dark, P. & Gent, H. (2001) Pests and diseases of prehistoric crops: a yield 'honeymoon' for early grain crops in europe? *Oxford Journal of Archaeology*, 20, 59–78. https://doi.org/10.1111/1468-0092.00123.
Devys, M., Barbier, M., Bousquet, J.-F. & Kollmann, A. (1992) Isolation of the new (-)-(3 R,4S)-4-hydroxymellein from the fungus *Septoria nodorum* Berk. *Zeitschrift für Naturforschung C*, 47, 779–781. https://doi.org/10.1515/znc-1992-9-1024.
Devys, M., Barbier, M., Kollmann, A. & Bousquet, J.-F. (1982) Septorine and N-methoxy septorine, substituted pyrazines from the fungus *Septoria nodorum* Berk. *Tetrahedron Letters*, 23, 5409–5412. https://doi.org/10.1016/0040-4039(82)80143-3.
Devys, M., Bousquet, J.-F., Kollmann, A. & Barbier, M. (1980) Dihydroisocoumarines et acide mycophenolique du milieu de culture du champignon phytopathogene *Septoria nodorum*. *Phytochemistry*, 19, 2221–2222. https://doi.org/10.1016/S0031-9422(00)82234-7.
Devys, M., Bousquet, J.-F., Kollmann, A. & Barbier, M. (1978) Isolation of a new pyrazine, septorine, from the culture medium of the phytopathogenic fungus *Septoria nodorum* Berk. *Chemischer Informationsdienst*, 9. https://doi.org/10.1002/chin.197835266.
Doehlemann, G., Ökmen, B., Zhu, W. & Sharon, A. (2017) Plant pathogenic fungi. *Microbiology Spectrum*, 5. https://doi.org/10.1128/microbiolspec.FUNK-0023-2016.
Does, H.C. van der & Rep, M. (2017) Adaptation to the host environment by plant-pathogenic fungi. *Annual Review of Phytopathology*, 55, 427–450. https://doi.org/10.1146/annurev-phyto-080516-035551.
Downie, R.C., Lin, M., Corsi, B., Ficke, A., Lillemo, M., Oliver, R.P., et al. (2020) Septoria nodorum blotch of wheat: disease management and resistance breeding in the face of shifting disease dynamics and a changing environment. *Phytopathology*. https://doi.org/10.1094/PHYTO-07-20-0280-RVW.
Dugan, F.M. (2008) Fungi in the ancient world: how mushrooms, mildews, molds, and yeast shaped the early civilizations of Europe, the Mediterranean, and the Near East. American Phytopathological Society (APS Press).
Eyal, Z. (1999) The *Septoria tritici* and *Stagonospora nodorum* blotch diseases of wheat. *European Journal of Plant Pathology*, 105, 629–641. https://doi.org/10.1023/A:1008716812259.

Faris, J.D., Zhang, Z., Lu, H., Lu, S., Reddy, L., Cloutier, S., et al. (2010) A unique wheat disease resistance-like gene governs effector-triggered susceptibility to necrotrophic pathogens. *Proceedings of the National Academy of Sciences*, 107, 13544–13549. https://doi.org/10.1073/pnas.1004090107.

Feng, J., Liu, T., Qin, B., Zhang, Y. & Liu, X.S. (2012) Identifying ChIP-seq enrichment using MACS. *Nature Protocols*, 7, 1728–1740. https://doi.org/10.1038/nprot.2012.101.

Feurtey, A., Lorrain, C., Croll, D., Eschenbrenner, C., Freitag, M., Habig, M., et al. (2020) Genome compartmentalization predates species divergence in the plant pathogen genus *Zymoseptoria*. *BMC Genomics*, 21, 588. https://doi.org/10.1186/s12864-020-06871-w.

Ficke, A., Cowger, C., Bergstrom, G. & Brodal, G. (2017) Understanding yield loss and pathogen biology to improve disease management: Septoria nodorum blotch - a case study in wheat. *Plant Disease*, 102, 696–707. https://doi.org/10.1094/PDIS-09-17-1375-FE.

Figueroa, M., Hammond-Kosack, K.E. & Solomon, P.S. (2018) A review of wheat diseases—a field perspective. *Molecular Plant Pathology*, 19, 1523–1536. https://doi.org/10.1111/mpp.12618.

Fisher, M.C., Hawkins, N.J., Sanglard, D. & Gurr, S.J. (2018) Worldwide emergence of resistance to antifungal drugs challenges human health and food security. *Science*, 360, 739–742. https://doi.org/10.1126/science.aap7999.

Flor, H.H. (1956) The complementary genic systems in flax and flax rust joint contribution from the field crops research branch, agricultural research service, united states department of agriculture and the North Dakota Agricultural Experiment Station. In: Demerec, M. (Ed.) Advances in Genetics. Academic Press, pp. 29–54.

Fones, H.N., Bebber, D.P., Chaloner, T.M., Kay, W.T., Steinberg, G. & Gurr, S.J. (2020) Threats to global food security from emerging fungal and oomycete crop pathogens. *Nature Food*, 1, 332–342. https://doi.org/10.1038/s43016-020-0075-0.

Fones, H.N., Fisher, M.C. & Gurr, S.J. (2017) Emerging fungal threats to plants and animals challenge agriculture and ecosystem resilience. *Microbiology Spectrum*, 5. https://doi.org/10.1128/microbiolspec.FUNK-0027-2016.

Friesen, T.L., Chu, C., Xu, S.S. & Faris, J.D. (2012) SnTox5-*Snn5*: a novel *Stagonospora nodorum* effector-wheat gene interaction and its relationship with the SnToxA-*Tsn1* and SnTox3-*Snn3-B1* interactions. *Molecular Plant Pathology*, 13, 1101–1109. https://doi.org/10.1111/j.1364-3703.2012.00819.x.

Friesen, T.L. & Faris, J.D. (2010) Characterization of the wheat-*Stagonospora nodorum* disease system: what is the molecular basis of this quantitative necrotrophic disease interaction? *Canadian Journal of Plant Pathology*, 32, 20–28. https://doi.org/10.1080/07060661003620896.

Friesen, T.L., Holmes, D.J., Bowden, R.L. & Faris, J.D. (2018) *ToxA* is present in the U.S. *Bipolaris sorokiniana* population and is a significant virulence factor on wheat harboring *Tsn1*. *Plant Disease*, 102, 2446–2452. https://doi.org/10.1094/PDIS-03-18-0521-RE.

Friesen, T.L., Meinhardt, S.W. & Faris, J.D. (2007) The *Stagonospora nodorum*-wheat pathosystem involves multiple proteinaceous host-selective toxins and corresponding host sensitivity genes that interact in an inverse gene-for-gene manner. *The Plant Journal*, 51, 681–692. https://doi.org/10.1111/j.1365-313X.2007.03166.x.

Friesen, T.L., Stukenbrock, E.H., Liu, Z., Meinhardt, S., Ling, H., Faris, J.D., et al.

(2006) Emergence of a new disease as a result of interspecific virulence gene transfer. *Nature Genetics*, 38, 953–956. https://doi.org/10.1038/ng1839.
Friesen, T.L., Zhang, Z., Solomon, P.S., Oliver, R.P. & Faris, J.D. (2008) Characterization of the interaction of a novel *Stagonospora nodorum* host-selective toxin with a wheat susceptibility gene. *Plant Physiology*, 146, 682–693. https://doi.org/10.1104/pp.107.108761.
Gao, Y., Faris, J.D., Liu, Z., Kim, Y.M., Syme, R.A., Oliver, R.P., et al. (2015) Identification and characterization of the SnTox6-*Snn6* interaction in the *Parastagonospora nodorum*–wheat pathosystem. *Molecular Plant-Microbe Interactions*, 28, 615–625. https://doi.org/10.1094/MPMI-12-14-0396-R.
Ghaderi, F., Sharifnabi, B., Javan-Nikkhah, M., Brunner, P.C. & McDonald, B.A. (2020) SnToxA, SnTox1, and SnTox3 originated in *Parastagonospora nodorum* in the Fertile Crescent. *Plant Pathology*, 69, 1482–1491. https://doi.org/10.1111/ppa.13233.
Gummer, J.P.A., Trengove, R.D., Oliver, R.P. & Solomon, P.S. (2012) A comparative analysis of the heterotrimeric G-protein Gα, Gβ and Gγ subunits in the wheat pathogen *Stagonospora nodorum*. *BMC Microbiology*, 12, 131. https://doi.org/10.1186/1471-2180-12-131.
Gummer, J.P.A., Trengove, R.D., Oliver, R.P. & Solomon, P.S. (2013) Dissecting the role of G-protein signalling in primary metabolism in the wheat pathogen *Stagonospora nodorum*. *Microbiology,* 159, 1972–1985. https://doi.org/10.1099/mic.0.067009-0.
Hafez, M., Gourlie, R., Despins, T., Turkington, K., Friesen, T.L. & Aboukhaddour, R. (2020) *Parastagonospora nodorum* and related species in western Canada: genetic variability and effector genes. *Phytopathology*. https://doi.org/10.1094/PHYTO-05-20-0207-R.
Hammond-Kosack, K.E. & Jones, J.D. (1996) Resistance gene-dependent plant defense responses. *The Plant Cell*, 8, 1773–1791. https://doi.org/10.1105/tpc.8.10.1773.
Han, X. & Kahmann, R. (2019) Manipulation of phytohormone pathways by effectors of filamentous plant pathogens. *Frontiers in Plant Science*, 10. https://doi.org/10.3389/fpls.2019.00822.
Hane, J.K., Lowe, R.G.T., Solomon, P.S., Tan, K.-C., Schoch, C.L., Spatafora, J.W., et al. (2007) Dothideomycete–plant interactions illuminated by genome sequencing and EST analysis of the wheat pathogen *Stagonospora nodorum*. *The Plant Cell*, 19, 3347–3368. https://doi.org/10.1105/tpc.107.052829.
Haridas, S., Albert, R., Binder, M., Bloem, J., LaButti, K., Salamov, A., et al. (2020) 101 Dothideomycetes genomes: a test case for predicting lifestyles and emergence of pathogens. *Studies in Mycology*, 96, 141–153. https://doi.org/10.1016/j.simyco.2020.01.003.
Hawksworth, D.L. & Lücking, R. (2017) Fungal diversity revisited: 2.2 to 3.8 million species. *Microbiology Spectrum*, 5. https://doi.org/10.1128/microbiolspec.FUNK-0052-2016.
Howard, K., Foster, S.G., Cooley, R.N. & Caten, C.E. (1999) Disruption, replacement, and cosuppression of nitrate assimilation genes in *Stagonospora nodorum*. *Fungal Genetics and Biology*, 26, 152–162. https://doi.org/10.1006/fgbi.1998.1113.
Hua, C., Zhao, J.-H. & Guo, H.-S. (2018) Trans-kingdom RNA silencing in plant-fungal pathogen interactions. *Molecular Plant*, 11, 235–244. https://doi.org/10.1016/j.molp.2017.12.001.
Ikeda, K., Park, P. & Nakayashiki, H. (2019) Cell biology in phytopathogenic fungi

during host infection: commonalities and differences. *Journal of General Plant Pathology*, 85, 163–173. https://doi.org/10.1007/s10327-019-00846-w.
Ipcho, S.V.S., Hane, J.K., Antoni, E.A., Ahren, D., Henrissat, B., Friesen, T.L., et al. (2012) Transcriptome analysis of *Stagonospora nodorum*: gene models, effectors, metabolism and pantothenate dispensability. *Molecular Plant Pathology*, 13, 531–545. https://doi.org/10.1111/j.1364-3703.2011.00770.x.
IpCho, S.V.S., Tan, K.-C., Koh, G., Gummer, J., Oliver, R.P., Trengove, R.D., et al. (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. https://doi.org/10.1128/EC.00064-10.
Islam, M.T., Kim, K.-H. & Choi, J. (2019) Wheat blast in Bangladesh: the current situation and future impacts. *The Plant Pathology Journal*, 35, 1–10. https://doi.org/10.5423/PPJ.RW.08.2018.0168.
John, E., Lopez-Ruiz, F., Rybak, K., Mousley, C.J., Oliver, R.P. & Tan, K.-C. (2016) Dissecting the role of histidine kinase and HOG1 mitogen-activated protein kinase signalling in stress tolerance and pathogenicity of *Parastagonospora nodorum* on wheat. *Microbiology*, 162, 1023–1036. https://doi.org/10.1099/mic.0.000280.
Jones, D.A.B., John, E., Rybak, K., Phan, H.T.T., Singh, K.B., Lin, S.-Y., et al. (2019) A specific fungal transcription factor controls effector gene expression and orchestrates the establishment of the necrotrophic pathogen lifestyle on wheat. *Scientific Reports*, 9, 1–13. https://doi.org/10.1038/s41598-019-52444-7.
Kamoun, S., Talbot, N.J. & Islam, M.T. (2019) Plant health emergencies demand open science: tackling a cereal killer on the run. *PLOS Biology*, 17. https://doi.org/10.1371/journal.pbio.3000302.
Kanja, C. & Hammond-Kosack, K.E. (2020) Proteinaceous effector discovery and characterization in filamentous plant pathogens. *Molecular Plant Pathology*, 21, 1353-1376. https://doi.org/10.1111/mpp.12980.
Kariyawasam, G.K., Richards, J.K., Wyatt, N.A., Running, K., Xu, S.S., Liu, Z., et al. (2021) The *Parastagonospora nodorum* necrotrophic effector SnTox5 targets the wheat gene *Snn5* and facilitates entry into the leaf mesophyll. *bioRxiv*, 2021.02.26.433117. https://doi.org/10.1101/2021.02.26.433117.
Karjalainen, R., Laitinen, A. & Juuti, T. (1983) Effects of *Septoria nodorum* Berk. on yield and yield components of spring wheat. *Agricultural and Food Science*, 55, 333–344. https://doi.org/10.23986/afsci.72133.
Karjalainen, R. & Lounatmaa, K. (1986) Ultrastructure of penetration and colonization of wheat leaves by *Septoria nodorum*. *Physiological and Molecular Plant Pathology*, 29, 263–270. https://doi.org/10.1016/S0048-4059(86)80026-1.
Keller, B., Wicker, T. & Krattinger, S.G. (2018) Advances in wheat and pathogen genomics: implications for disease control. *Annual Review of Phytopathology*, 56, 67–87. https://doi.org/10.1146/annurev-phyto-080516-035419.
Keller, B., Winzeler, H., Winzeler, M. & Fried, P.M. (1994) Differential sensitivity of wheat embryos against extracts containing toxins of *Septoria nodorum*: first steps towards in vitro selection. *Journal of Phytopathology*, 141, 233–240. https://doi.org/10.1111/j.1439-0434.1994.tb01466.x.
Kelman, A. & Peterson, P.D. (2002) Contributions of plant scientists to the development of the germ theory of disease. *Microbes and Infection*, 4, 257–260. https://doi.org/10.1016/S1286-4579(01)01532-5.
Kent, S.S. & Strobel, G.A. (1976) Phytotoxin from *Septoria nodorum*. *Transactions of the British Mycological Society*, 67, 354–358. https://doi.org/10.1016/S0007-

1536(76)80149-0.
Kettles, G.J. & Luna, E. (2019) Food security in 2044: how do we control the fungal threat? *Fungal Biology*, 123, 558–564. https://doi.org/10.1016/j.funbio.2019.04.006.
Lalaoui, F., Halama, P., Dumortier, V. & Paul, B. (2000) Cell wall-degrading enzymes produced in vitro by isolates of *Phaeosphaeria nodorum* differing in aggressiveness. *Plant Pathology*, 49, 727–733. https://doi.org/10.1046/j.1365-3059.2000.00491.x.
Le Marquer, M., San Clemente, H., Roux, C., Savelli, B. & Frei dit Frey, N. (2019) Identification of new signalling peptides through a genome-wide survey of 250 fungal secretomes. *BMC Genomics*, 20. https://doi.org/10.1186/s12864-018-5414-2.
Leath, S. (1993) Factors associated with global occurrences of *Septoria nodorum* blotch and *Septoria tritici* blotch of wheat. *Plant Disease*, 77, 1266. https://doi.org/10.1094/PD-77-1266.
Lehtinen, U. (1993) Plant cell wall degrading enzymes of *Septoria nodorum*. *Physiological and Molecular Plant Pathology*, 43, 121–134. https://doi.org/10.1006/pmpp.1993.1045.
Li, H., Hu, J., Wei, H., Solomon, P.S., Stubbs, K.A. & Chooi, Y.-H. (2019) Biosynthesis of a tricyclo[6.2.2.02,7]dodecane system by a berberine bridge enzyme-like aldolase. *Chemistry – A European Journal*, 25, 15062–15066. https://doi.org/10.1002/chem.201904360.
Li, H., Hu, J., Wei, H., Solomon, P.S., Vuong, D., Lacey, E., et al. (2018) Chemical ecogenomics-guided discovery of phytotoxic α-pyrones from the fungal wheat pathogen *Parastagonospora nodorum*. *Organic Letters*, 20, 6148–6152. https://doi.org/10.1021/acs.orglett.8b02617.
Li, H., Wei, H., Hu, J., Lacey, E., Sobolev, A.N., Stubbs, K.A., et al. (2020) Genomics-driven discovery of phytotoxic cytochalasans involved in the virulence of the wheat pathogen *Parastagonospora nodorum*. *ACS Chemical Biology*, 15, 226–233. https://doi.org/10.1021/acschembio.9b00791.
Li, W., Csukai, M., Corran, A., Crowley, P., Solomon, P.S. & Oliver, R.P. (2008) Malayamycin, a new streptomycete antifungal compound, specifically inhibits sporulation of *Stagonospora nodorum* (Berk) Castell and Germano, the cause of wheat glume blotch disease. *Pest Management Science*, 64, 1294–1302. https://doi.org/10.1002/ps.1632.
Lin, M., Corsi, B., Ficke, A., Tan, K.-C., Cockram, J. & Lillemo, M. (2020a) Genetic mapping using a wheat multi-founder population reveals a locus on chromosome 2A controlling resistance to both leaf and glume blotch caused by the necrotrophic fungal pathogen *Parastagonospora nodorum*. *TAG. Theoretical and Applied Genetics. Theoretische Und Angewandte Genetik*, 133, 785–808. https://doi.org/10.1007/s00122-019-03507-w.
Lin, M., Ficke, A., Cockram, J. & Lillemo, M. (2020b) Genetic structure of the Norwegian *Parastagonospora nodorum* population. *Frontiers in Microbiology*, 11. https://doi.org/10.3389/fmicb.2020.01280.
Lin, M., Stadlmeier, M., Mohler, V., Tan, K.-C., Ficke, A., Cockram, J., et al. (2021) Identification and cross-validation of genetic loci conferring resistance to Septoria nodorum blotch using a German multi-founder winter wheat population. *TAG. Theoretical and applied genetics. Theoretische und Angewandte Genetik*, 134, 125–142. https://doi.org/10.1007/s00122-020-03686-x.
Lin, S.-Y., Chooi, Y.-H. & Solomon, P.S. (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. https://doi.org/10.1111/mmi.13968.
Liu, Z., Faris, J.D., Meinhardt, S.W., Ali, S., Rasmussen, J.B. & Friesen, T.L. (2004a) Genetic and physical mapping of a gene conditioning sensitivity in wheat to a partially purified host-selective toxin produced by *Stagonospora nodorum*. *Phytopathology*, 94, 1056–1060. https://doi.org/10.1094/PHYTO.2004.94.10.1056.
Liu, Z., Faris, J.D., Oliver, R.P., Tan, K.-C., Solomon, P.S., McDonald, M.C., et al. (2009) SnTox3 acts in effector triggered susceptibility to induce disease on wheat carrying the *Snn3* gene. *PLOS Pathogens*, 5, e1000581. https://doi.org/10.1371/journal.ppat.1000581.
Liu, Z., Friesen, T.L., Ling, H., Meinhardt, S.W., Oliver, R.P., Rasmussen, J.B., et al. (2006) The *Tsn1*-ToxA interaction in the wheat-*Stagonospora nodorum* pathosystem parallels that of the wheat-tan spot system. *Genome*, 49, 1265–1273. https://doi.org/10.1139/g06-088.
Liu, Z., Friesen, T.L., Rasmussen, J.B., Ali, S., Meinhardt, S.W. & Faris, J.D. (2004b) Quantitative trait loci analysis and mapping of seedling resistance to *Stagonospora nodorum* leaf blotch in wheat. *Phytopathology*, 94, 1061–1067. https://doi.org/10.1094/PHYTO.2004.94.10.1061.
Liu, Z., Gao, Y., Kim, Y.M., Faris, J.D., Shelver, W.L., Wit, P.J.G.M. de, et al. (2016) SnTox1, a *Parastagonospora nodorum* necrotrophic effector, is a dual-function protein that facilitates infection while protecting from wheat-produced chitinases. *New Phytologist*, 211, 1052–1064. https://doi.org/10.1111/nph.13959.
Liu, Z., Zhang, Z., Faris, J.D., Oliver, R.P., Syme, R., McDonald, M.C., et al. (2012) The cysteine rich necrotrophic effector SnTox1 produced by *Stagonospora nodorum* triggers susceptibility of wheat lines harboring *Snn1*. *PLOS Pathogens*, 8, e1002467. https://doi.org/10.1371/journal.ppat.1002467.
Lo Presti, L., Lanver, D., Schweizer, G., Tanaka, S., Liang, L., Tollot, M., et al. (2015) Fungal effectors and plant susceptibility. *Annual Review of Plant Biology*, 66, 513–545. https://doi.org/10.1146/annurev-arplant-043014-114623.
Loughman, R., Wilson, R.E. & Thomas, G.J. (1993) The influence of disease complexes involving *Leptosphaeria* (Septoria) *nodorum* on detection of resistance to three leaf spot diseases in wheat. *Euphytica*, 72, 31–42. https://doi.org/10.1007/BF00023770.
Lowe, R.G.T., Lord, M., Rybak, K., Trengove, R.D., Oliver, R.P. & Solomon, P.S. (2008) A metabolomic approach to dissecting osmotic stress in the wheat pathogen *Stagonospora nodorum*. *Fungal Genetics and Biology*, 45, 1479–1486. https://doi.org/10.1016/j.fgb.2008.08.006.
Lowe, R.G.T., Lord, M., Rybak, K., Trengove, R.D., Oliver, R.P. & Solomon, P.S. (2009) Trehalose biosynthesis is involved in sporulation of *Stagonospora nodorum*. *Fungal Genetics and Biology*, 46, 381–389. https://doi.org/10.1016/j.fgb.2009.02.002.
Lu, S., Faris, J.D., Sherwood, R., Friesen, T.L. & Edwards, M.C. (2014) A dimeric PR-1-type pathogenesis-related protein interacts with ToxA and potentially mediates ToxA-induced necrosis in sensitive wheat. *Molecular Plant Pathology*, 15, 650–663. https://doi.org/10.1111/mpp.12122.
Lucas, J.A. (2020) Plant pathology and plant pathogens. John Wiley & Sons.
Macheleidt, J., Mattern, D.J., Fischer, J., Netzker, T., Weber, J., Schroeckh, V., et al. (2016) Regulation and role of fungal secondary metabolites. *Annual Review of Genetics*, 50, 371–392. https://doi.org/10.1146/annurev-genet-120215-035203.
Magro, P. (1984) Production of polysaccharide-degrading enzymes by *Septoria*

nodorum in culture and during pathogenesis. *Plant Science Letters*, 37, 63–68. https://doi.org/10.1016/0304-4211(84)90204-9.
McDonald, B.A. & Linde, C. (2002) Pathogen population genetics, evolutionary potential, and durable resistance. *Annual Review of Phytopathology*, 40, 349–379. https://doi.org/10.1146/annurev.phyto.40.120501.101443.
McDonald, B.A. & Stukenbrock, E.H. (2016) Rapid emergence of pathogens in agro-ecosystems: global threats to agricultural sustainability and food security. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 371, 20160026. https://doi.org/10.1098/rstb.2016.0026.
McDonald, M.C., Ahren, D., Simpfendorfer, S., Milgate, A. & Solomon, P.S. (2018) The discovery of the virulence gene ToxA in the wheat and barley pathogen *Bipolaris sorokiniana*. *Molecular Plant Pathology*, 19, 432–439. https://doi.org/10.1111/mpp.12535.
McDonald, M.C., Oliver, R.P., Friesen, T.L., Brunner, P.C. & McDonald, B.A. (2013) Global diversity and distribution of three necrotrophic effectors in *Phaeosphaeria nodorum* and related species. *New Phytologist*, 199, 241–251. https://doi.org/10.1111/nph.12257.
McDonald, M.C. & Solomon, P.S. (2018) Just the surface: advances in the discovery and characterization of necrotrophic wheat effectors. *Current Opinion in Microbiology*, 46, 14–18. https://doi.org/10.1016/j.mib.2018.01.019.
McDonald, M.C., Taranto, A.P., Hill, E., Schwessinger, B., Liu, Z., Simpfendorfer, S., et al. (2019) Transposon-mediated horizontal fransfer of the host-specific virulence protein ToxA between three fungal wheat pathogens. *mBio*, 10. https://doi.org/10.1128/mBio.01515-19.
Mead, O., Thynne, E., Winterberg, B. & Solomon, P.S. (2013) Characterising the role of GABA and its metabolism in the wheat pathogen *Stagonospora nodorum*. *PLOS ONE*, 8, e78368. https://doi.org/10.1371/journal.pone.0078368.
Mehra, L., Adhikari, K., Ojiambo, P.S. & Cowger, C. (2019) Septoria nodorum blotch of wheat. *The Plant Health Instructor*.
Möller, M. & Stukenbrock, E.H. (2017) Evolution and genome architecture in fungal plant pathogens. *Nature Reviews. Microbiology*, 15, 756–771. https://doi.org/10.1038/nrmicro.2017.76.
Muria-Gonzalez, M.J., Chooi, Y.-H., Breen, S. & Solomon, P.S. (2015) The past, present and future of secondary metabolite research in the Dothideomycetes. *Molecular Plant Pathology*, 16, 92–107. https://doi.org/10.1111/mpp.12162.
Muria-Gonzalez, M.J., Yeng, Y., Breen, S., Mead, O., Wang, C., Chooi, Y.-H., et al. (2020) Volatile molecules secreted by the wheat pathogen *Parastagonospora nodorum* are involved in development and phytotoxicity. *Frontiers in Microbiology*, 11. https://doi.org/10.3389/fmicb.2020.00466.
Murray, G.M. & Brennan, J.P. (2009) Estimating disease losses to the Australian wheat industry. *Australasian Plant Pathology*, 38, 558–570. https://doi.org/10.1071/AP09053.
Nelson, R., Wiesner-Hanks, T., Wisser, R. & Balint-Kurti, P. (2018) Navigating complexity to breed disease-resistant crops. *Nature Reviews Genetics*, 19, 21–33. https://doi.org/10.1038/nrg.2017.82.
Oliver, R. (2012) Genomic tillage and the harvest of fungal phytopathogens. *New Phytologist*, 196, 1015–1023. https://doi.org/10.1111/j.1469-8137.2012.04330.x.
Oliver, R., Lichtenzveig, J., Tan, K.-C., Waters, O., Rybak, K., Lawrence, J., et al. (2014) Absence of detectable yield penalty associated with insensitivity to Pleosporales necrotrophic effectors in wheat grown in the West Australian wheat belt. *Plant Pathology*, 63, 1027–1032. https://doi.org/10.1111/ppa.12191.

Oliver, R.P., Friesen, T.L., Faris, J.D. & Solomon, P.S. (2011) *Stagonospora nodorum*: from pathology to genomics and host resistance. *Annual Review of Phytopathology*, 50, 23–43. https://doi.org/10.1146/annurev-phyto-081211-173019.

Oliver, R.P. & Solomon, P.S. (2010) New developments in pathogenicity and virulence of necrotrophs. *Current Opinion in Plant Biology*, 13, 415–419. https://doi.org/10.1016/j.pbi.2010.05.003.

Oliver, R.P., Tan, K.-C. & Moffat, C.S. (2016) Necrotrophic pathogens of wheat. In: Wrigley, C., Corke, H., Seetharaman, K., and Faubion, J. (Eds.) Encyclopedia of food grains (second edition). Oxford: Academic Press, pp. 273–278.

Park, E., Nedo, A., Caplan, J.L. & Dinesh-Kumar, S.P. (2018) Plant–microbe interactions: organelles and the cytoskeleton in action. *New Phytologist*, 217, 1012–1028. https://doi.org/10.1111/nph.14959.

Pereira, D.A., McDonald, B.A. & Brunner, P.C. (2017) Mutations in the *CYP51* gene reduce DMI sensitivity in *Parastagonospora nodorum* populations in Europe and China. *Pest Management Science*, 73, 1503–1510. https://doi.org/10.1002/ps.4486.

Peters-Haugrud, A.R., Zhang, Z., Richards, J.K., Friesen, T.L. & Faris, J.D. (2019) Genetics of variable disease expression conferred by inverse gene-for-gene interactions in the wheat-*Parastagonospora nodorum* pathosystem. *Plant Physiology*, 180, 420–434. https://doi.org/10.1104/pp.19.00149.

Phan, H.T.T., Jones, D.A.B., Rybak, K., Dodhia, K.N., Lopez-Ruiz, F.J., Valade, R., et al. (2020) Low amplitude boom-and-bust cycles define the Septoria nodorum blotch interaction. *Frontiers in Plant Science*, 10. https://doi.org/10.3389/fpls.2019.01785.

Phan, H.T.T., Rybak, K., Bertazzoni, S., Furuki, E., Dinglasan, E., Hickey, L.T., et al. (2018) Novel sources of resistance to Septoria nodorum blotch in the Vavilov wheat collection identified by genome-wide association studies. *Theoretical and Applied Genetics*, 131, 1223–1238. https://doi.org/10.1007/s00122-018-3073-y.

Phan, H.T.T., Rybak, K., Furuki, E., Breen, S., Solomon, P.S., Oliver, R.P., et al. (2016) Differential effector gene expression underpins epistasis in a plant fungal disease. *The Plant Journal*, 87, 343–354. https://doi.org/10.1111/tpj.13203.

Rana, S.K., Katoch, S. & Sharma, P.N. (2020) Assessment of yield losses caused by *Stagonospora* leaf and glume blotch (SLGB) of wheat; its status and distribution in Himachal Pradesh. *Indian Phytopathology*, 73, 267–272. https://doi.org/10.1007/s42360-019-00185-y.

Rascle, C., Dieryckx, C., Dupuy, J.W., Muszkieta, L., Souibgui, E., Droux, M., et al. (2018) The pH regulator PacC: a host-dependent virulence factor in *Botrytis cinerea*. *Environmental Microbiology Reports*, 10, 555–568. https://doi.org/10.1111/1758-2229.12663.

Richards, J.K., Kariyawasam, G., Seneviratne, S., Wyatt, N.A., Xu, S.S., Liu, Z., et al. (2021) A triple threat: the *Parastagonospora nodorum* SnTox267 effector exploits three distinct host genetic factors to cause disease in wheat. *bioRxiv*, 2021.02.25.432871. https://doi.org/10.1101/2021.02.25.432871.

Richards, J.K., Stukenbrock, E.H., Carpenter, J., Liu, Z., Cowger, C., Faris, J.D., et al. (2019) Local adaptation drives the diversification of effectors in the fungal wheat pathogen *Parastagonospora nodorum* in the United States. *PLOS Genetics*, 15, e1008223. https://doi.org/10.1371/journal.pgen.1008223.

Richards, J.K., Wyatt, N.A., Liu, Z., Faris, J.D. & Friesen, T.L. (2017) Reference quality genome assemblies of three *Parastagonospora nodorum* isolates differing in virulence on wheat. *G3: Genes, Genomes, Genetics*, 8, 393–399.

https://doi.org/10.1534/g3.117.300462.
Rouxel, T. & Balesdent, M.-H. (2017) Life, death and rebirth of avirulence effectors in a fungal pathogen of Brassica crops, *Leptosphaeria maculans*. *New Phytologist*, 214, 526–532. https://doi.org/10.1111/nph.14411.
Ruud, A.K., Dieseth, J.A., Ficke, A., Furuki, E., Phan, H.T.T., Oliver, R.P., et al. (2019) Genome-wide association mapping of resistance to Septoria nodorum leaf blotch in a Nordic spring wheat collection. *The Plant Genome*, 12, 180105. https://doi.org/10.3835/plantgenome2018.12.0105.
Ruud, A.K., Windju, S., Belova, T., Friesen, T.L. & Lillemo, M. (2017) Mapping of SnTox3–*Snn3* as a major determinant of field susceptibility to Septoria nodorum leaf blotch in the SHA3/CBRD × Naxos population. *Theoretical and Applied Genetics*, 130, 1361–1374. https://doi.org/10.1007/s00122-017-2893-5.
Rybak, K., See, P.T., Phan, H.T.T., Syme, R.A., Moffat, C.S., Oliver, R.P., et al. (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. *Molecular Plant Pathology*, 18, 420–434. https://doi.org/10.1111/mpp.12511.
Sacristan, S. & Garcia-Arenal, F. (2008) The evolution of virulence and pathogenicity in plant pathogen populations. *Molecular Plant Pathology*, 9, 369–384. https://doi.org/10.1111/j.1364-3703.2007.00460.x.
Salam, K.P., Thomas, G.J., Beard, C., Loughman, R., MacLeod, W.J. & Salam, M.U. (2013) Application of meta-analysis in plant pathology: a case study examining the impact of fungicides on wheat yield loss from the yellow spot—septoria nodorum blotch disease complex in Western Australia. *Food Security*, 5, 319–325. https://doi.org/10.1007/s12571-013-0255-y.
Sánchez-Vallet, A., Fouché, S., Fudal, I., Hartmann, F.E., Soyer, J.L., Tellier, A., et al. (2018) The genome biology of effector gene evolution in filamentous plant pathogens. *Annual Review of Phytopathology*, 56, 21–40. https://doi.org/10.1146/annurev-phyto-080516-035303.
Santini, A., Liebhold, A., Migliorini, D. & Woodward, S. (2018) Tracing the role of human civilization in the globalization of plant pathogens. *The ISME Journal*, 12, 647–652. https://doi.org/10.1038/s41396-017-0013-9.
Sarma, G.N., Manning, V.A., Ciuffetti, L.M. & Karplus, P.A. (2005) Structure of Ptr ToxA: an RGD-containing host-selective toxin from *Pyrenophora tritici-repentis*. *The Plant Cell*, 17, 3190–3202. https://doi.org/10.1105/tpc.105.034918.
Savary, S., Willocquet, L., Pethybridge, S.J., Esker, P., McRoberts, N. & Nelson, A. (2019) The global burden of pathogens and pests on major food crops. *Nature Ecology & Evolution*, 3, 430–439. https://doi.org/10.1038/s41559-018-0793-y.
Schnurbusch, T., Paillard, S., Fossati, D., Messmer, M., Schachermayr, G., Winzeler, M., et al. (2003) Detection of QTLs for Stagonospora glume blotch resistance in Swiss winter wheat. *Theoretical and Applied Genetics*, 107, 1226–1234. https://doi.org/10.1007/s00122-003-1372-3.
Sesma, A. (2016) RNA metabolism and regulation of virulence programs in fungi. *Seminars in Cell & Developmental Biology*, 57, 120–127. https://doi.org/10.1016/j.semcdb.2016.03.019.
Shaw, M.W., Bearchell, S.J., Fitt, B.D.L. & Fraaije, B.A. (2008) Long-term relationships between environment and abundance in wheat of *Phaeosphaeria nodorum* and *Mycosphaerella graminicola*. *New Phytologist*, 177, 229–238. https://doi.org/10.1111/j.1469-8137.2007.02236.x.
Shi, G., Friesen, T.L., Saini, J., Xu, S.S., Rasmussen, J.B. & Faris, J.D. (2015) The wheat *Snn7* gene confers susceptibility on recognition of the *Parastagonospora*

nodorum necrotrophic effector SnTox7. *The Plant Genome*, 8, plantgenome2015.02.0007. https://doi.org/10.3835/plantgenome2015.02.0007.
Shi, G., Zhang, Z., Friesen, T.L., Raats, D., Fahima, T., Brueggeman, R.S., et al. (2016) The hijacking of a receptor kinase–driven pathway by a wheat fungal pathogen leads to disease. *Science Advances*, 2. https://doi.org/10.1126/sciadv.1600822.
Singh, P.K., Singh, S., Deng, Z., He, X., Kehel, Z. & Singh, R.P. (2019) Characterization of QTLs for seedling resistance to tan spot and Septoria nodorum blotch in the PBW343/Kenya Nyangumi wheat recombinant inbred lines population. *International Journal of Molecular Sciences*, 20, 5432. https://doi.org/10.3390/ijms20215432.
Solomon, P.S., Jörgens, C.I. & Oliver, R.P. (2006a) δ-Aminolaevulinic acid synthesis is required for virulence of the wheat pathogen *Stagonospora nodorum*. *Microbiology,* 152, 1533–1538. https://doi.org/10.1099/mic.0.28556-0.
Solomon, P.S., Lee, R.C., Wilson, T.J.G. & Oliver, R.P. (2004a) Pathogenicity of *Stagonospora nodorum* requires malate synthase. *Molecular Microbiology*, 53, 1065–1073. https://doi.org/10.1111/j.1365-2958.2004.04178.x.
Solomon, P.S., Lowe, R.G.T., Tan, K.-C., Waters, O.D.C. & Oliver, R.P. (2006b) *Stagonospora nodorum*: cause of stagonospora nodorum blotch of wheat. *Molecular Plant Pathology*, 7, 147–156. https://doi.org/10.1111/j.1364-3703.2006.00326.x.
Solomon, P.S. & Oliver, R.P. (2004) Functional characterisation of glyoxalase I from the fungal wheat pathogen *Stagonospora nodorum*. *Current Genetics*, 46, 115–121. https://doi.org/10.1007/s00294-004-0514-8.
Solomon, P.S., Rybak, K., Trengove, R.D. & Oliver, R.P. (2006c) Investigating the role of calcium/calmodulin-dependent protein kinases in *Stagonospora nodorum*. *Molecular Microbiology*, 62, 367–381. https://doi.org/10.1111/j.1365-2958.2006.05380.x.
Solomon, P.S., Tan, K.-C. & Oliver, R.P. (2005a) Mannitol 1-phosphate metabolism is required for sporulation in planta of the wheat pathogen *Stagonospora nodorum*. *Molecular Plant-Microbe Interactions*, 18, 110–115. https://doi.org/10.1094/MPMI-18-0110.
Solomon, P.S., Tan, K.-C., Sanchez, P., Cooper, R.M. & Oliver, R.P. (2004b) The disruption of a Gα subunit sheds new light on the pathogenicity of *Stagonospora nodorum* on wheat. *Molecular Plant-Microbe Interactions*, 17, 456–466. https://doi.org/10.1094/MPMI.2004.17.5.456.
Solomon, P.S., Thomas, S.W., Spanu, P. & Oliver, R.P. (2003) The utilisation of di/tripeptides by *Stagonospora nodorum* is dispensable for wheat infection. *Physiological and Molecular Plant Pathology*, 63, 191–199. https://doi.org/10.1016/j.pmpp.2003.12.003.
Solomon, P.S., Waters, O.D.C., Jörgens, C.I., Lowe, R.G.T., Rechberger, J., Trengove, R.D., et al. (2006d) Mannitol is required for asexual sporulation in the wheat pathogen *Stagonospora nodorum* (glume blotch). *Biochemical Journal*, 399, 231–239. https://doi.org/10.1042/BJ20060891.
Solomon, P.S., Waters, O.D.C., Simmonds, J., Cooper, R.M. & Oliver, R.P. (2005b) The Mak2 MAP kinase signal transduction pathway is required for pathogenicity in *Stagonospora nodorum*. *Current Genetics*, 48, 60–68. https://doi.org/10.1007/s00294-005-0588-y.
Solomon, P.S., Wilson, T.J.G., Rybak, K., Parker, K., Lowe, R.G.T. & Oliver, R.P. (2006e) Structural characterisation of the interaction between *Triticum aestivum* and the Dothideomycete pathogen *Stagonospora nodorum*. *European Journal of Plant Pathology*, 114, 275–282. https://doi.org/10.1007/s10658-005-5768-6.

Sperschneider, J., Dodds, P.N., Gardiner, D.M., Singh, K.B. & Taylor, J.M. (2018) Improved prediction of fungal effector proteins from secretomes with EffectorP 2.0. *Molecular Plant Pathology*, 19, 2094–2110. https://doi.org/10.1111/mpp.12682.

Stukenbrock, E.H., Banke, S. & McDONALD, B.A. (2006) Global migration patterns in the fungal wheat pathogen *Phaeosphaeria nodorum*. *Molecular Ecology*, 15, 2895–2904. https://doi.org/10.1111/j.1365-294X.2006.02986.x.

Sung, Y.-C., Outram, M.A., Breen, S., Wang, C., Dagvadorj, B., Winterberg, B., et al. (2021) PR1-mediated defence via C-terminal peptide release is targeted by a fungal pathogen effector. *New Phytologist*, 229, 3467–3480. https://doi.org/10.1111/nph.17128.

Syme, R.A., Tan, K.-C., Hane, J.K., Dodhia, K., Stoll, T., Hastie, M., et al. (2016) Comprehensive annotation of the *Parastagonospora nodorum* reference genome using next-generation genomics, transcriptomics and proteogenomics. *PLOS ONE*, 11, e0147221. https://doi.org/10.1371/journal.pone.0147221.

Tan, K.-C., Heazlewood, J.L., Millar, A.H., Thomson, G., Oliver, R.P. & Solomon, P.S. (2008) A signaling-regulated, short-chain dehydrogenase of *Stagonospora nodorum* regulates asexual development. *Eukaryotic Cell*, 7, 1916–1929. https://doi.org/10.1128/EC.00237-08.

Tan, K.-C., Oliver, R.P., Peter S., S. & Moffat, C.S. (2010) Proteinaceous necrotrophic effectors in fungal virulence. *Functional Plant Biology : FPB*, 37, 907–912. https://doi.org/10.1071/fp10067.

Tan, K.-C., Phan, H.T.T., Rybak, K., John, E., Chooi, Y.H., Solomon, P.S., et al. (2015) Functional redundancy of necrotrophic effectors – consequences for exploitation for breeding. *Frontiers in Plant Science*, 6. https://doi.org/10.3389/fpls.2015.00501.

Tan, K.-C., Trengove, R.D., Maker, G.L., Oliver, R.P. & Solomon, P.S. (2009) Metabolite profiling identifies the mycotoxin alternariol in the pathogen *Stagonospora nodorum*. *Metabolomics*, 5, 330–335. https://doi.org/10.1007/s11306-009-0158-2.

Vincent, D., Fall, L.A. du, Livk, A., Mathesius, U., Lipscombe, R.J., Oliver, R.P., et al. (2011) A functional genomics approach to dissect the mode of action of the *Stagonospora nodorum* effector protein SnToxA in wheat. *Molecular Plant Pathology*, 13, 467–482. https://doi.org/10.1111/j.1364-3703.2011.00763.x.

Vleeshouwers, V.G.A.A. & Oliver, R.P. (2014) Effectors as tools in disease resistance breeding against biotrophic, hemibiotrophic, and necrotrophic plant pathogens. *Molecular Plant-Microbe Interactions*, 27, 196–206. https://doi.org/10.1094/MPMI-10-13-0313-IA.

Wang, J., & Chai, J. (2020) Structural Insights into the Plant Immune Receptors PRRs and NLRs. *Plant physiology*, 182(4), 1566–1581. https://doi.org/10.1104/pp.19.01252

Wicki, W., Messmer, M., Winzeler, M., Stamp, P. & Schmid, J.E. (1999) In vitro screening for resistance against Septoria nodorum blotch in wheat. *Theoretical and Applied Genetics*, 99, 1273–1280. https://doi.org/10.1007/s001220051333.

Willocquet, L., Meza, W.R., Dumont, B., Klocke, B., Feike, T., Kersebaum, K.C., et al. (2020) An outlook on wheat health in Europe from a network of field experiments. *Crop Protection*, 105335. https://doi.org/10.1016/j.cropro.2020.105335.

Winterberg, B., Du Fall, L.A., Song, X., Pascovici, D., Care, N., Molloy, M., et al. (2014) The necrotrophic effector protein SnTox3 re-programs metabolism and elicits a strong defence response in susceptible wheat leaves. *BMC plant biology*, 14,

215. https://doi.org/10.1186/s12870-014-0215-5.
Wit, P.J.G.M. de de (2015) Plant pathogenic fungi and oomycetes. Principles of Plant-Microbe Interactions; Microbes for Sustainable Agriculture. Springer International Publishing Switzerland, pp. 79–90.
Wit, P.J.G.M. de de, Testa, A.C. & Oliver, R.P. (2017) Fungal plant pathogenesis mediated by effectors. *The Fungal Kingdom*, 767–785. https://doi.org/10.1128/microbiolspec.FUNK-0021-2016.
World Food Program (2019) 2019 - The state of food security and nutrition in the world (SOFI): safeguarding against economic slowdowns and downturns.
Wu, S., Wei, Y., Head, B., Zhao, Y. & Hanna, S. (2019) The development of ancient Chinese agricultural and water technology from 8000 BC to 1911 AD. *Palgrave Communications*, 5, 1–16. https://doi.org/10.1057/s41599-019-0282-1.
Xu, S., Friesen, T.L. & Cai, X. (2004) Sources and genetic control of resistance to *Stagonospora nodorum* blotch in wheat. Research signpost/recent research development in gentics and plant breeding.
Yang, X.-L., Awakawa, T., Wakimoto, T. & Abe, I. (2013) Induced biosyntheses of a novel butyrophenone and two aromatic polyketides in the plant pathogen *Stagonospora nodorum*. *Natural Products and Bioprospecting*, 3, 141–144. https://doi.org/10.1007/s13659-013-0055-2.
Zhang, Y.W. & Nan, Z.B. (2018) First report of leaf blotch caused by *Parastagonospora nodorum* on *Leymus chinensis* (Chinese rye grass) in China. *Plant disease.*, 102, 2661–2661. https://doi.org/10.1094/pdis-06-18-0926-pdn.
Zhang, Z., Running, K.L.D., Seneviratne, S., Haugrud, A.R.P., Szabo-Hever, A., Shi, G., et al. (2021) A protein kinase-major sperm protein gene hijacked by a necrotrophic fungal pathogen triggers disease susceptibility in wheat. *The Plant Journal*, https://doi.org/10.1111/tpj.15194.
Zinkernagel, V., Rless, F., Wendland, M. & Bartscherer, H.-Chr. (1988) Infektionsstrukturen von *Septoria nodorum* in Blättern anfälliger Weizensorten / Infection structures of *Septoria nodorum* in leaves of susceptible wheat cultivars. *Zeitschrift für Pflanzenkrankheiten und Pflanzenschutz / Journal of Plant Diseases and Protection*, 95, 169–175.

Chapter 2

Transcription factor control of virulence in phytopathogenic fungi

The content of this chapter has been published prior to submission of this thesis as part of the following review:

John, E., Singh, K.B., Oliver, R.P. & Tan, K-C (2021) Transcription factor control of virulence in phytopathogenic fungi. *Molecular Plant Pathology*, 22, 858-881. https://doi.org/10.1111/mpp.13056.

Formatting has been undertaken to suit this thesis, but in all other aspects the content remains the same. A signed co-author contribution statement demonstrating that the work contained herein is my own is provided in **Appendix 1**.

2.1. General Introduction

Transcription factors (TFs) are sequence-specific DNA-binding proteins required to modulate gene expression (Charoensawan et al., 2010; Hughes, 2011; Caramori et al., 2019). Consequently, pathogenic fungi rely on a set of suitably operating TFs to orchestrate the expression of genes involved in phytopathogenicity. Characterisation of such regulators provides an avenue to identify virulence factors, informing research strategies aimed at building durable resistance into plants (Nejat et al., 2017; Zhang et al., 2018; Jones et al., 2019a; Keller, 2019). In addition, their direct inhibition is considered an effective method for targeted disease control (Tietjen and Schreier, 2013; Bahn, 2015; Cho, 2015; Sang and Kim, 2019). In recent years, molecular characterisation of TFs has proceeded at a rapid rate and it has become difficult to navigate the wealth of published material concerning aspects of phytopathogenicity. Therefore, the purpose of this review is to provide a systematic overview of what has been established through functional investigation in plant-pathogenic fungi (**Supplementary Item 2.1** catalogues published studies, indicating TFs involved in virulence). The classification of TFs into families is first detailed to provide insight into some of the distinct mechanisms of gene regulation. This precedes an analysis of TF orthologues belonging to several extensively characterised families from the perspective of fungal virulence and pathogenicity (summarised in **Table 2.1**). After establishing where investigations have focused, recommendations for future research efforts are proposed to better characterise disease regulatory pathways for the ultimate goal of better plant protection.

2.2. Transcription factor classification

TFs are classed into families based on their DNA-binding domains (DBDs) which bind through distinct mechanisms (Charoensawan et al., 2010; Hughes, 2011; Wong, 2018). While TFs of a family tend to share affinities for particular core DNA sequences, active binding depends on additional variables including the presence of cofactors/coregulators, post-translational modifications, epigenetic states of the DNA and target site synergists/antagonists (Zabet et al., 2013; Levo et al., 2017; Vandel et al., 2019; Sri Theivakadadcham et al., 2019). With this considered, some conserved DNA-binding features have been elucidated for each family.

The largest class of TFs in fungi are the zinc coordinated 'zinc fingers' which comprises several families. These include TFs harbouring the fungal-specific zinc cluster (Zn2Cys6), Cys2His2 (C2H2) and Cys4 (GATA) DBDs (Iuchi, 2005). The Zn2Cys6 DBD is characterised by two zinc ions, each interacting with three cysteine residues. This structure stabilises a pair of alpha helices within the protein that interacts with DNA via the major groove and exhibits an affinity for CGG triplets. Zn2Cys6 TFs predominantly bind as homodimers or homotypic dimers (same TF family members) (Todd and Andrianopoulos, 1997; MacPherson et al., 2006; Joshua and Höfken, 2017). C2H2 zinc finger TFs generally harbour multiple C2H2 DBDs which can flexibly bind DNA of diverse nucleotide composition and length outside the major groove (Iuchi, 2005; Klug, 2010; Fedotova et al., 2017). The GATA Zinc finger domain is named for the canonical DNA target sequence and comprises four zinc coordinated cysteines. In fungi, GATA TFs often contain additional zinc finger domains, which actively bind other regulatory molecules leading to more precise DNA-binding activity (Scazzocchio, 2000; Chen et al., 2012; Hasegawa and Shimizu, 2017).

The basic leucine-zipper (bZIP) and basic helix-loop-helix (bHLH) TFs are obligate homo(typic) dimer forming proteins that constitute two of the major TF families in fungi. bZIP TFs dimerise through a leucine-rich 'zipper' region and bind to DNA via the major groove with adjacent basic residues (Fujii et al., 2000; Deppmann et al., 2006; Reinke et al., 2013; Llorca et al., 2014; Rodríguez-Martínez et al., 2017). The helix-loop-helix region performs a similar function as the leucine-zipper in the bHLH family (includes the fungal-specific 'APSES' TFs) (Carretero-Paulet et al., 2010; Sailsbery et al., 2012; Sailsbery and Dean, 2012; Shively et al., 2019). For both families, palindromic half-sites are a feature of target DNA sequences.

The homeodomain or homoeobox (HD/Hox) family TFs contain a DBD harbouring a conserved helix-turn-helix structure with affinity for a short AT-rich sequence. The HD/Hox family TFs, which encompasses the fungal-specific Ste12 TFs, actively target more specific sequences by forming heteromeric regulatory complexes (Mann et al., 2009; Bürglin and Affolter, 2016; Bobola and Merabet, 2017).

Less numerous, but well-characterised fungal-specific TFs belong to the Velvet and Gti1/Pac2 DBD families. The Velvet TFs are known to interact with other Velvet TFs and cofactors, recognising an 11 bp DNA sequence through two positively charged residue loops (Ahmed et al., 2013; Calvo et al., 2016). In contrast, the Gti1/Pac2 family TFs bind DNA as monomers, through two separate but essential globular regions targeting a conserved nine bp sequence (Lohse et al., 2010; Cain et al., 2012; Tollot et al., 2016). General binding properties for the families that have been presented, on the basis of extensive characterisation in plant-pathogenic fungi, are presented in **Figure 2.1**.

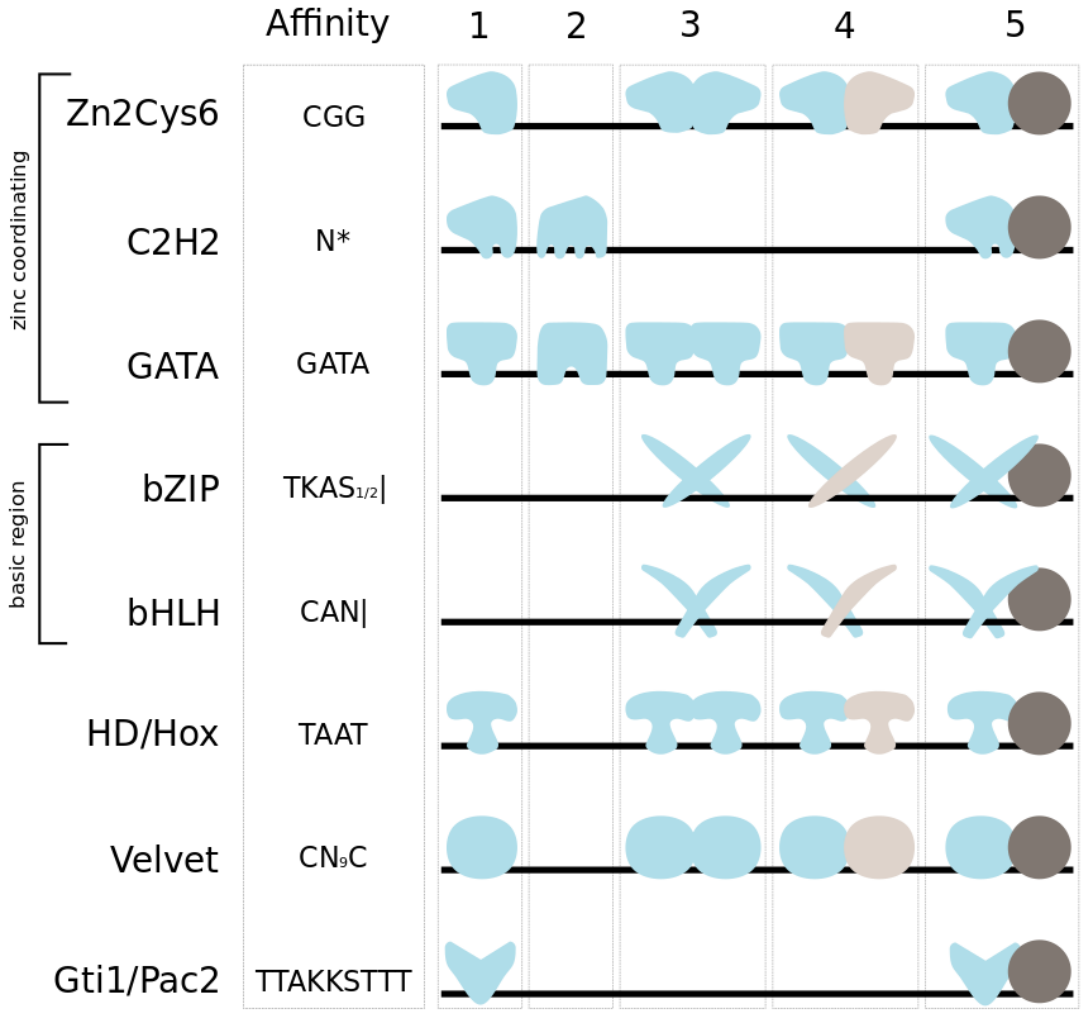


Figure 2.1 - Mechanistic overview of transcription factor (TF) families detailed in this review

A canonical core DNA target sequence with a strong affinity to the domain is indicated. Numbers represent where members of the family bind as a: 1) monomer, 2) multiple DNA-binding domain monomer, 3) homodimer/multimer, 4) homotypic dimer/multimer (TF of same family), 5) coordinated DNA-binding complex (either as a heterodimer/multimer, heterochimeric TF or by synergistic binding of separate TFs).

2.3. TFs and their role in virulence in plant-pathogenic fungi

2.3.1. The Zn2Cys6 family

Zn2Cys6 TFs are fungal-specific and the largest family among plant-pathogenic fungi, particularly in ascomycetes (Todd et al., 2014; Shelest, 2017). A significant number of Zn2Cys6 TF-encoding genes reside within secondary metabolite (SM) gene clusters, whose activation or functional products have not been resolved (Deepika et al., 2016; Romsdahl and Wang, 2019; Keller, 2019; Graham-Taylor et al., 2020). Nevertheless, diverse and interesting roles for Zn2Cys6 TFs have been reported, some as specific regulators of fungal virulence along with more general developmental regulators.

2.3.1.1. Mtf4 + VdFtf1 - Host sensors & secretion of virulence factors

Mtf4 and VdFtf1 are distinct Zn2Cys6 TFs specifically regulating fungal pathogenicity in their respective species. Mtf4 belongs to the cucurbit pathogen *Colletotrichum orbiculare* where it was shown to control the development of the appressorium, a mechanical host-penetration structure (Kodama et al., 2019). Further analysis revealed Mtf4 is activated via the morphogenesis-related (MOR) kinase signalling pathway in response to cutin monomers derived from the host (Kodama et al., 2019). In the vascular wilt pathogen *Verticillium dahliae,* VdFtf1 was identified through *Agrobacterium* T-DNA mediated random mutagenesis as a TF required for full virulence on cotton (Zhang et al., 2018). A comparative RNA-seq analysis identified a number of putative plant cell wall-degrading enzymes (CWDEs) down-regulated in the *vdftf1* mutant. Subsequent deletion of one of the encoding genes (*VEDA_09651*) revealed that it played a significant role in host infection (Zhang et al., 2018), demonstrating the utility of this approach for characterising novel virulence factors.

2.3.1.2. Ftf1/2 - Gene expansion & effector regulation

Gene duplication and neofunctionalisation have been recognised as important processes in the evolution of fungal virulence (Skamnioti et al., 2008; Haridas et al., 2020). Ftf1 from the *Fusarium oxysporum* species complex represents an interesting case study for a Zn2Cys6 TF (despite the nomenclature, this TF is not orthologous to VdFtf1). Several accessory chromosomes exist in *F. oxysporum formae speciales* causing vascular wilt on distinct hosts, the acquisition of which can be sufficient to render non-pathogenic strains virulent (Ma et al., 2010). Up to 10 paralogues of *Ftf1* can be found on these chromosomes, the number of which varies depending on the isolate (de Vega-Bartol et al., 2010; Taylor et al., 2019). Ftf1 TF paralogues have been shown to positively regulate a number of key virulence factors such as the SIX (Secreted-In-Xylem) effectors, and increased *Ftf1* gene expression or copy number is positively correlated with virulence (de Vega-Bartol et al., 2010; Niño-Sánchez et al., 2016). It is presumed that *Ftf1* arose from a duplication of *Ftf2*, a paralogue located on the core chromosome 9 (Armitage et al., 2018). Ftf2 shares some conserved regulatory targets with Ftf1 paralogues, many of which are located on the accessory chromosomes with *Ftf1* (van der Does et al., 2016). Interestingly, deletion of the putative *Ftf1*/*Ftf2* orthologue in *Fusarium graminearum*, the cause of fusarium head blight on wheat, did not affect fungal virulence (Son et al., 2011). This was also observed in the rice blast pathogen *Magnaporthe oryzae*, where *fzc76* knockout mutants were fully pathogenic (Lu et al., 2014). Therefore Ftf1 in *F. oxysporum* demonstrates how TF acquisition (through horizontal gene transfer or duplication followed by neofunctionalisation) can enable sufficient expression of host-specific virulence factors important during infection.

2.3.1.3. EBR1 - Hyphal branching

A virulence function for the enhanced branching TF EBR1 was first reported in *F. graminearum* (Zhao et al., 2011). Detailed phenotypic characterisation attributed severe pathogenicity defects in *ebr1* mutants to impaired host penetration as a result of defective growth at the hyphal tip. The orthologue in *F. oxysporum* f. sp. *lycopersici* represents another case of TF gene expansion in this pathogen. Deletion of the core chromosomal *EBR1* orthologue had a moderate effect on hyphal growth and virulence, although this gene fully restored wheat pathogenicity when used to complement the *F. graminearum ebr1* mutant (Zhao et al., 2011). It was proposed that paralogues on *F. oxysporum* accessory chromosomes partially mitigated the effect of *EBR1* gene deletion (Jonkers et al., 2014; Zhao et al., 2011). Further analysis revealed one paralogue, *EBR2* could fully complement the *ebr1* mutant, but only under the control of an *EBR1* promoter (Jonkers et al., 2014). Compared with *EBR1*, the expression of the respective paralogues during infection was relatively low. Hence the exact significance of these extra functional copies during infection remains to be determined (Jonkers et al., 2014; Yang et al., 2020). In *M. oryzae*, the orthologous gene *MoCod2* was required for proliferation beyond the initial sites of plant infection (Chung et al., 2013). This suggests a conserved EBR1 functional role controlling invasive hyphal growth may also exist in plant-pathogenic fungi.

2.3.1.4. Pro1 - Sporulation & development

The Zn2Cys6 TF Pro1 lacks the canonical dimerisation domain, indicating it binds DNA as a monomer, an unusual property for this family (Masloff et al., 2002). Originally Pro1 was reported to orchestrate the formation of sexual reproductive bodies in the ascomycete *Sordaria macrospora* (Masloff et al., 1999). This developmental role is conserved for Pro1 orthologues in *F. graminearum* and the chestnut blight fungus *Cryphonectria parasitica* (Son et al., 2011; Sun et al., 2009). In *C. parasitica*, Pro1 was

also involved in conidiation, a function which can be extended to the rice pathogens *Ustilaginoidea virens* and *M. oryzae* (Lu et al., 2014; Lv et al., 2016). In several plant pathogens, *pro1* mutants also exhibited perturbed hyphal development. This correlated with impaired virulence on the respective hosts; an exception being *C. parasitica* where infections were comparable to the wildtype (Cho et al., 2009; Sun et al., 2009; Son et al., 2011; Lu et al., 2014; Lv et al., 2016).

2.3.1.5. Pf2 - CWDEs and effector/effector-like genes

Pf2 has been identified as an important regulator controlling the necrotrophic lifestyle in fungal phytopathogens of the Pleosporales order. In the black spot fungus *Alternaria brassicicola,* AbPf2 was dispensable for normal growth but crucial for virulence on various Brassicaceae species (Cho et al., 2013). Gene deletion of *Pf2* orthologues in *Parastagonospora nodorum* and *Pyrenophora tritici-repentis* resulted in down-regulation of key necrotrophic effector genes including *ToxA* and *Tox3*, leading to the loss of host-specific virulence on wheat (Rybak et al., 2017). A detailed investigation was undertaken in both *A. brassicicola* and *P. nodorum* through RNA-seq analyses of *abpf2* and *pnpf2* mutants, respectively. This revealed that Pf2 orchestrates the expression of a range of additional targets encoding putative effector-like proteins (small in size, possessing secretion signals and a high cysteine content) and plant CWDEs during early infection in both pathogens (Cho et al., 2013; Jones et al., 2019a). A similar motif resembling a Zn2Cys6-binding site was characterised in both mutants suggesting a conserved nucleotide target exists for Pf2 (MacPherson et al., 2006; Cho et al., 2013; Jones et al., 2019a). In *Zymoseptoria tritici*, a devastating wheat pathogen of the Capnodiales, the putative Pf2 orthologue Zt107320 was reported to regulate carbon sensing/utilisation pathways, mediating virulence and sporulation during infection (Habig et al., 2020). This was similar to what was reported in *M. oryzae*, where MoCod1 was shown to be critical

for invasive growth on rice (Chung et al., 2013). In both *F. graminearum* and *Fusarium verticillioides*, gene deletion of the respective orthologues *FgArt1* and *FvArt1* impaired pathogenicity, in part through changes to starch hydrolysis and SM biosynthesis (Oh et al. 2016). These reports indicate Pf2 taxonomic orthologues regulate pathways involved in carbohydrate acquisition, which can be tightly linked to the secretion of host-specific virulence factors during invasive growth.

2.3.2. The C2H2 family

C2H2 TFs represent a second extensive family of fungal zinc finger regulators. In contrast to the fungal Zn2Cys6 family, C2H2 TFs exist in all eukaryotes (Fedotova et al., 2017). Well-characterised C2H2 TFs are primarily linked to the control of fungal development, stress tolerance and metabolic activities in plant-pathogenic fungi.

2.3.2.1. CreA - Carbon metabolism

The catabolite repressor CreA was originally studied for its regulatory role in central carbon metabolism in the model saprophytic fungus *Aspergillus nidulans* (Dowzer and Kelly, 1989; Dowzer and Kelly, 1991). In the presence of primary carbon sources such as glucose, CreA blocks the expression of enzymes that break down complex carbohydrates (David et al., 2005; Adnan et al., 2017; Assis et al., 2018). Since then, studies in a number of fungal phytopathogens have demonstrated a conserved role for CreA in carbon catabolite repression (Vautard et al., 1999; Tudzynski et al., 2000; Jonkers and Rep, 2009; Cao et al., 2016; Tannous et al., 2018; Fasoyin et al., 2018). A role for CreA in pathogenicity was reported during *Aspergillus flavus* infection of peanuts/maize kernels and *Penicillium expansum* colonisation of apples. In these species *creA* mutants also displayed severely perturbed vegetative development and capacity to synthesise phytotoxic SMs such as aflatoxin and patulin which likely contributed to a

reduction in virulence (Fasoyin et al., 2018; Tannous et al., 2018). Meanwhile, contrasting reports suggest a significant role for FgCreA in the virulence of *F. graminearum* may or may not exist (Son et al., 2011; Hou and Wang, 2018).

2.3.2.2. Crz1 - Stress tolerance

The Ca^{2+}/calcineurin responsive zinc finger Crz1 is a key target downstream of Ca^{2+} ion signalling pathways, originally characterised in the yeast model *Saccharomyces cerevisiae* (Stathopoulos-Gerontides et al., 1999; Thewes, 2014). Orthologues in a number of fungal phytopathogens including *A. flavus*, *Botrytis cinerea*, *Colletotrichum gloeosporioides*, *F. graminearum*, *M. oryzae*, *Penicillium digitatum* and *V. dahliae* have been functionally characterised through gene deletion (Choi et al., 2009; Dubey et al., 2016; Lim et al., 2019; Schumacher et al., 2008; Son et al., 2011; T., Zhang et al., 2013b; Xiong et al., 2015). In each case, severe defects in development and both ionic/cell wall stress tolerances in *crz1* mutants are accompanied by reduced virulence. These studies demonstrate Crz1 is a fundamental regulator of genes essential for fungal development and stress tolerance in plant-pathogenic fungi of diverse hosts.

2.3.2.3. PacC - pH stress tolerance, CWDEs and SMs

In *A. nidulans* and *S. cerevisiae,* the C2H2 TF PacC/Rim101p is activated following proteolytic cleavage under alkaline conditions, leading to activation of pH-responsive genes (Tilburn et al., 1995; Li and Mitchell, 1997; Díez et al., 2002; Lamb and Mitchell, 2003). In plant-pathogenic fungi, gene deletion of *PacC* orthologues mirrors the phenotypes observed in *A. nidulans* and *S. cerevisiae* where *pacC* mutants exhibit growth defects at high pH (Rollins, 2003; Caracuel et al., 2003; Flaherty et al., 2003; Aréchiga-Carvajal and Ruiz-Herrera, 2005; Miyara et al., 2008; Wiemann et al., 2009; Merhej et al., 2011; Cho et al., 2012; Landraud et al., 2013; T., Zhang et al., 2013a; Rascle et al.,

2018; Chen et al., 2018; Wu et al., 2018c). In *B. cinerea, Sclerotinia sclerotiorum* and *Valsa mali*, virulence defects in *pacC* mutants were attributed to an inability to actively acidify the site of host infection (Rollins, 2003; Rascle et al., 2018; Wu et al., 2018c). For *M. oryzae*, *C. gloeosporioides*, *P. digitatum* and *P. expansum*, a virulence function for PacC was instead linked to the regulation of CWDEs (Miyara et al., 2008; Landraud et al., 2013; T., Zhang et al., 2013a; Chen et al., 2018). In contrast, PacC was shown to suppress the expression of genes involved in the biosynthesis of toxic and protective SMs. These included patulin, bikaverin, fumonisins and trichothecene biosynthetic genes in *P. expansum*, *Fusarium fujikori*, *F. verticillioides* and *F. graminearum* respectively (Flaherty et al., 2003; Wiemann et al., 2009; Merhej et al., 2011; Chen et al., 2018). Moreover, PacC suppresses virulence in *F. oxysporum* and was dispensable during infection in both *F. graminearum* and the obligate biotroph *Ustilago maydis* (Caracuel et al., 2003; Aréchiga-Carvajal and Ruiz-Herrera, 2005; Son et al., 2011). Hence in plant pathogens, PacC primarily coordinates pH tolerance along with additional pathways distinct to each species which are often but not always implicated in fungal virulence.

2.3.2.4. Msn2/Vf19 - Development & virulence

The Msn2 C2H2 TF was first characterised in *S. cerevisiae* as an important factor coordinating adaptation to environmental stressors including heat, hyperosmotic and oxidative conditions (Martínez-Pastor et al., 1996; Schmitt and McEntee, 1996). Host virulence was consistently reduced or abolished following the deletion of *Msn2* orthologues in a number of plant-pathogenic fungi. However, rather than perturbed abiotic stress tolerances, abnormality in vegetative growth and the development of asexual spores or sclerotia were common phenotypic defects associated with *msn2* mutants (Chang et al., 2011; Son et al., 2011; Zhang et al., 2014; Tian et al., 2017; Mohammadi et al., 2017; Wu et al., 2018b). An interesting contrast was for the *A. brassicicola*

orthologue Vf19 which was dispensable for normal growth and development, although required for virulence. Following RNA-seq analysis and growth tests on complex carbon sources, this function was attributed to Vf19 regulation of hydrolytic enzymes (Srivastava et al., 2011).

2.3.2.5. Con7 - Development & virulence

The C2H2 TF Con7 was originally identified through a random mutagenesis screen for conidiation defects in *M. oryzae*. Since then, orthologues in other plant pathogens have been characterised through gene deletion (Shi et al., 1998; Odenbach et al., 2007; Son et al., 2011; Tran et al., 2014; Ruiz-Roldán et al., 2015). In addition to conidiation, these studies demonstrated Con7 is an important factor controlling hyphal growth and host invasion. In *M. oryzae* and *F. oxysporum* this was largely attributed to the regulation of cell wall synthesis/modulating enzymes (Odenbach et al., 2007; Ruiz-Roldán et al., 2015). *Verticillium* spp. that lacked *Con7* were abolished in their ability to penetrate host tissue. A gene expression analysis and cross-species functional complementation in *S. cerevisiae* revealed cellular adhesins and secreted enzymes were controlled by Con7 in these species (Tran et al., 2014). Although knockout mutants were not obtainable, suggesting a role in viability, gene knockdown of the *P. nodorum* orthologue *PnCon7* revealed it regulates the expression of necrotrophic effector genes and was correlated with reduced virulence on susceptible wheat (Lin et al., 2018). Furthermore, a yeast-1-hybrid analysis indicated PnCon7 directly targeted a promoter element of the necrotrophic effector *SnTox3*. Interestingly, not all differentially spliced isoforms (a common feature of Con7 orthologues) could bind this element (Ruiz-Roldán et al., 2015; Lin et al., 2018). Hence, through alternative splicing Con7 may flexibly regulate both core developmental processes and specific virulence factors in plant-pathogenic fungi.

2.3.2.6. Tri6 - Toxic SM biosynthesis

Trichothecene SMs are major virulence factors during *F. graminearum* infection of wheat. Deoxynivalenol (DON) is one of the best-studied trichothecenes in *F. graminearum* where it is synthesised through the action of at least 16 genes situated on four chromosomal locations (Alexander et al., 2009; Amarasinghe and Fernando, 2016). One of these is *Tri6*, encoding a C2H2 TF located within the chromosome 1 core gene cluster and is crucial for biosynthesis of DON (Proctor et al., 1995; Hohn et al., 1999; Scherm et al., 2011). Interestingly ChIP-seq and RNA-seq analyses demonstrate Tri6 possesses broader regulatory roles in *F. graminearum*, by controlling the expression directly or indirectly of ~200 genes (Seong et al., 2009; Nasmith et al., 2011). It has since been shown that Tri6 also controls the production of phytotoxic fusaoctaxins and gramillins, demonstrating the TF is a general regulator of SMs involved in the virulence of this pathogen (Shostak et al., 2020).

2.3.2.7. Cmr1 - Melanisation

Melanins refer to a class of dark pigmented, insoluble compounds. They are produced by fungi for cellular protection against physical and chemical environmental stresses or as factors involved in plant penetration and virulence (Howard and Valent, 1996; Eisenman and Casadevall, 2012; Nosanchuk et al., 2015). Melanin regulator Cmr1 is a chimeric TF with both C2H2 and Zn2Cys6 DBDs. It was originally shown to be involved in the biosynthesis of melanins in both *C. orbiculare* (Cmr1) and *M. oryzae* (Pig1) (Tsuji et al., 2000). These pathogens form melanised appressoria to initiate infection, however, Cmr1 and Pig1 were dispensable for melanisation at this stage (Howard and Valent, 1996; Tsuji et al., 2000). Instead, Cmr1 orthologues have been reported to induce melanin biosynthetic genes during normal mycelial growth and the formation of sclerotia and conidia, corresponding to later stages of the infection cycle for a number of ascomycetous

plant pathogens (Tsuji et al., 2000; Eliahu et al., 2007; Kihara et al., 2008; Cho et al., 2012; Fetzner et al., 2014; Zhou et al., 2017; Krishnan et al., 2018; Yonglin Wang et al., 2018b). In an interesting case study, mutations in the *Zmr1* promoter from various wildtype *Z. tritici* isolates and laboratory-induced mutants resulted in changes to melanin production (Krishnan et al., 2018). This had direct implications in a fitness tradeoff. Isolates with reduced melanin grew more vigorously under optimal conditions but were more susceptible to stress induced by succinate dehydrogenase inhibitor fungicides, which are widely used to control this pathogen (Krishnan et al., 2018).

2.3.3. The GATA family

GATA zinc finger TFs are named after the core nucleotide sequence they were originally shown to target and are featured across the eukaryotic taxa (Lowry and Atchley, 2000; Scazzocchio, 2000). In plant-pathogenic fungi, the best studied are conserved GATA TFs governing core metabolic pathways involved in nutrient acquisition and responses to light.

2.3.3.1. AreA + AreB - Nitrogen assimilation

AreA (syn. NIT2) is a well-characterised TF controlling nitrogen assimilation in filamentous fungi (Marzluf, 1997; Tao and Marzluf, 1999). Other TFs and signal transduction molecules which modulate AreA activity have also been identified. Briefly, AreB (a GATA TF that interacts with AreA), NmrA (involved in post-translational modification of AreA) and MeaB (a bZIP TF suppressor of nitrate assimilation) are suppressors of AreA while the Zn2Cys6 TF NirA acts synergistically with AreA on a subset of nitrate assimilation pathways (Bernreiter et al., 2007; Bolton and Thomma, 2008; Michielse et al., 2014; Pfannmüller et al., 2017; Wilson et al., 2010; Wong et al., 2007; Wong et al., 2008; Wong et al., 2009).

Nutritional studies on the tomato pathogen *Cladosporium fulvum* revealed nitrogen starvation resulted in the production and secretion of the avirulence effector Avr9 (Van den Ackerveken et al., 1994). It was therefore hypothesised that nitrogen starvation would act as a general trigger for AreA mediated expression of pathogenicity genes. However, subsequent investigations revealed the connection was limited to Avr9 and nitrogen scarcity was not a significant factor in this pathosystem (Solomon and Oliver, 2001; Thomma et al., 2006). Interestingly, *AreA* gene deletion consistently resulted in perturbed virulence in a wide range of plant-pathogenic fungi (Froeliger et al., 1996; Pellier et al., 2003; Divon et al., 2006; Kim and Woloshuk, 2008; Wilson et al., 2010; Min et al., 2012; Horst et al., 2012; Bi et al., 2017; Fasoyin et al., 2019). These results suggest AreA is still an important general factor for fungal growth and development during infection. It has also been shown that specific nitrogen sources can induce SM biosynthesis through the AreA regulatory network (Keller, 2015; Tudzynski, 2014). In *F. fujikori*, this includes gibberellins, a class of phytohormones employed by the fungus to manipulate host physiology (Michielse et al., 2014). In other pathogens AreA also controls the production of a range of phytotoxic SMs (Fasoyin et al., 2019; Kim and Woloshuk, 2008; López-Berges et al., 2014; Min et al., 2012). These factors highlight additional mechanisms through which AreA may be regulating fungal virulence in these pathosystems.

2.3.3.2. SreA + HapX (bZIP) - Iron homeostasis

Iron is an essential component in fungal metabolism and is strongly linked to the modulation of reactive-oxygen species (ROS). Hence, the maintenance of appropriate iron levels ensures optimal cellular function while avoiding iron toxicity (Haas, 2012; Johnson, 2008). In filamentous fungi, iron homeostasis is mediated by the GATA family TF SreA (siderophore regulator). During iron-replete conditions, SreA negatively regulates the production of iron-chelating siderophores (Gerwien et al., 2018; Johnson,

2008). A similar function has been described in the plant-pathogenic fungi *Bipolaris maydis*, *F. oxysporum*, *F. graminearum*, *V. dahliae* and *U. maydis* (Voisard et al., 1993; López-Berges et al., 2012; N., Zhang et al., 2013; Yonglin Wang et al., 2018a; Wang et al., 2019). *SreA* expression is suppressed by the bZIP TF HapX, a positive regulator of genes important for iron uptake. Deletion of *HapX* resulted in a loss of virulence in *M. oryzae*, *F. oxysporum* and *V. dahliae* and suggests HapX mediated iron acquisition is critical during infection for these pathogens (López-Berges et al., 2012; Kong et al., 2015; Yonglin Wang et al., 2018a).

2.3.3.3. WC-1 + WC-2 - Light response

The GATA TFs WC-1 and WC-2 interact to form the White-Collar Complex (WCC), one of the best-studied light response regulators in filamentous fungi. In the model *Neurospora crassa*, this complex is directly activated through light-mediated stimulus of a conserved sensory domain which induces transcriptional regulation of downstream response elements (Chen et al., 2010; Fuller et al., 2015; Schumacher et al., 2014). Interestingly, WC-1 orthologues in basidiomycetes lack the GATA DBD but can still form the WCC with WC-2, as demonstrated through a yeast-2-hybrid assay in *U. maydis*, where *wco2* (*wc-2*) mutants are highly susceptible to ultra-violet (UV) light induced stress (Fuller et al., 2015; Brych et al., 2016). UV stress tolerance is also mediated by the WCC in ascomycete plant pathogens, in addition to conidiation or vegetative growth (Kihara et al., 2007; Estrada and Avalos, 2007; S., Kim et al., 2011; Canessa et al., 2013; Pruß et al., 2014; Kim et al., 2015; Ruiz-Roldán et al., 2015). While the majority of these pathogens did not rely on the WCC for infection, an interesting case was reported for *Cercospora zeae-maydis*, where WC-1 regulated light-dependent infection via stomatal openings (H., Kim et al., 2011). On the other hand, MGWC-1 from *M. oryzae* acted to suppress infection of rice under light conditions (S., Kim et al., 2011).

2.3.4. The bZIP family

The basic region-leucine zipper or 'bZIP' family of TFs are prevalent across eukaryotes (Amoutzias et al., 2007; Llorca et al., 2014; Reinke et al., 2013). An ability to readily form homo(typic) dimers with other bZIP TFs renders them flexible regulators of diverse cellular processes (Amoutzias et al., 2007; Deppmann et al., 2006; Miller, 2009). Studies in plant-pathogenic fungi have identified key metabolic and stress response pathways regulated by bZIP orthologues.

2.3.4.1. Yap1/Ap1 + Atf1 & Skn7 (Heat shock factor/HSF) - Oxidative stress & SM biosynthesis

Yap1 is the representative member of the fungal-specific activator protein lineage of bZIP TFs, which have been extensively characterised in the yeast model *S. cerevisiae* (Moye-Rowley et al., 1989; Simaan et al., 2019). This subfamily targets both distinct and overlapping chemical stress responses (Rodrigues-Pousada et al., 2010). Highly conserved in filamentous fungi, Yap1 is activated by oxidative stressors which induce cysteine-cysteine intramolecular bond formation, leading to rapid nuclear import and transcriptional activity (Fernandes et al., 1997; Rodrigues-Pousada et al., 2019). Given that production of ROSs form a major component of plant immune responses, a role for Yap1 during infection has been explored in a number of phytopathogens (Camejo et al., 2016; Mendoza-Martínez et al., 2019; Segal and Wilson, 2018). Consistent with this connection, Yap1 orthologues were shown to be important for virulence in *Alternaria alternata*, *C. gloeosporioides*, *M. oryzae* and *U. maydis* (Molina and Kahmann, 2007; Lin et al., 2009; Guo et al., 2011; Li et al., 2017). However, Yap1 was dispensable for pathogenicity in *B. cinerea*, *B. maydis*, *F. graminearum* and *Z. tritici* despite contributing to ROS detoxification (Temme and Tudzynski, 2009; Montibus et al., 2013; Shalaby et

al., 2014; Yang et al., 2015). Some insight comes from the observation that Yap1 can heterodimerise with Skn7 (a TF of the HSF family, see **Table 2.2**), a general stress responder downstream of the histidine kinase-based phosphorelay system (Mulford and Fassler, 2011). In *B*. *maydis*, only the double deletion of both *ChAp1* and *Skn7* genes resulted in reduced virulence on maize (Shalaby et al., 2014). This suggests Yap1 and Skn7 may redundantly target ROS detoxification pathways during plant infection.

Atf1 (also of the bZIP family) is another TF controlling ROS tolerance. Originally characterised in the fission yeast *Schizosaccharomyces pombe*, Atf1, like the human orthologue Atf2, is activated through phosphorylation by the p38/Hog1-mitogen activated protein kinase (MAPK) signalling cascade (Breitwieser et al., 2007; Shiozaki and Russell, 1996). While Atf1 shares some redundancy with Yap1 targets for oxidative stress tolerance, the two act independently (Montibus et al., 2015; Simaan et al., 2019). In plant-pathogenic fungi, Atf1 orthologues were shown to regulate stress response pathways to varying degrees (Nathues et al., 2004; Guo et al., 2010; Temme et al., 2012; Qi et al., 2013; Van Nguyen et al., 2013; Fang et al., 2017). Recently, nitric oxide detoxification and inorganic nitrogen assimilation were novel functions attributed to the Atf1 orthologue in *V*. *dahliae* which was also extended to *F*. *graminearum* (Tang et al., 2020). These additional roles may help explain why Atf1 remains an integral component regulating virulence in plant pathogens even while Yap1 is still functional. Induction or suppression of SM biosynthesis has also been linked to both Yap1 and Atf1 (Hong et al., 2013; Guan et al., 2019; Mendoza-Martínez et al., 2019). SMs can be metabolically taxing to synthesise under stressful conditions, providing an explanation for their suppression, while some exhibit protective properties and are valuable under such circumstances (Keller, 2015).

2.3.4.2. Cpc1/Gcn4 - Amino acid biosynthesis

A general mediator of amino-acid (AA) biosynthetic pathways is the cross-pathway control bZIP TF Cpc1, which binds the TGACTCA sequence (Paluh et al., 1988; Hoffmann et al., 2001; Tian et al., 2007). The DBD is remarkably conserved in eukaryotes, with the yeast orthologue Gcn4 able to functionally complement the human orthologue JUN (Struhl, 1987; Struhl, 1988). In fungal models including *N*. *crassa* and *A. nidulans*, AA starvation leads to Cpc1 mediated activation of AA biosynthetic genes (Paluh et al., 1988; Hoffmann et al., 2001; Tian et al., 2011). Reports to date in plant-pathogenic fungi suggest this function is also a conserved feature of Cpc1, which is dispensable for growth under nutrient-rich conditions (Wang et al., 1998; Schönig et al., 2009; Elliott et al., 2011; Son et al., 2011; Timpner et al., 2013). Accordingly, perturbed development on host tissue by *C*. *parasitica* following *CpCpc1* mutation was attributed to AA starvation (Wang et al., 1998). The vascular wilt pathogens *Verticillium longisporum* and *V*. *dahliae* were similarly perturbed following *Cpc1* gene deletion (Timpner et al., 2013). In the blackleg pathogen of canola *Leptosphaeria maculans*, gene silencing of the *Cpc1* orthologue led to overproduction of the phytotoxin sirodesmin-PL under AA starvation (Elliott et al., 2011). There it was concluded Cpc1 diverts metabolism towards AA biosynthesis, thereby reducing the available precursors necessary for sirodesmin-PL biosynthesis (Elliott et al., 2007; Elliott et al., 2011). However, a strong link between Cpc1 regulation and SM biosynthesis has not yet been reported in other plant-pathogenic fungi. As such, the overall connection between Cpc1 and virulence probably correlates with nutrient availability rather than the induction of host-specific virulence factors.

2.3.5. The bHLH family

Like the bZIP TFs, bHLH regulators readily dimerise with other members of the family in order to bind DNA through the basic residues at the N-termini (Sailsbery et al., 2012;

Shively et al., 2019). Thus far, the ASPES subfamily is the best-characterised group of bHLH TFs in plant-pathogenic fungi (others are outlined in **Table 2.2** and **Supplementary item 2.1**).

2.3.5.1. APSES - Developmental regulators

The Asm1/Phd1/Sok2/Efg1/StuA (APSES) subfamily is unique to fungi and has been divided into four groups, orthologues of StuA, Mbp1/Swi4 & Swi6, Afp1 and Xbp1. Each group is largely conserved across the fungal kingdom (Aramayo et al., 1996; Zhao et al., 2015). Functional investigation in plant-pathogenic fungi has focussed on the StuA orthologues. The *A*. *nidulans* StuA target motif (A/TCGCGT/ANA/C) is enriched in gene promoters regulated by StuA in both *F*. *graminearum* and *U*. *maydis*. Consequently, it has been inferred that a functional StuA binding system is conserved, while a diverse set of pathways are known to be controlled by StuA in plant-pathogenic fungi (Dutton et al., 1997; García-Pedrajas et al., 2010; Koch et al., 1993; Lysøe et al., 2011).

In the ascomycete pathogens, gene deletion or silencing revealed *StuA* is broadly required for asexual reproduction (Ohara and Tsuge, 2004; Nishimura et al., 2009; IpCho et al., 2010; Lysøe et al., 2011; Pasquali et al., 2013; Soyer et al., 2015; Sarmiento-Villamil et al., 2018; Yao et al., 2017; Tiley et al., 2018). Conversely, the *U*. *maydis* orthologue Ust1 suppresses haploid spore formation (García-Pedrajas et al., 2010). In addition to asexual reproductive pathways, StuA is also important for sexual development in several species (Nishimura et al., 2009; García-Pedrajas et al., 2010; Lysøe et al., 2011; Soyer et al., 2015). Biosynthesis of melanins and phytotoxic SMs, as well as glycolysis are examples of the diverse metabolic pathways regulated in some fungi (García-Pedrajas et al., 2010; IpCho et al., 2010; Lysøe et al., 2011; Pasquali et al., 2013; Tiley et al., 2018; Sarmiento-Villamil et al., 2018). StuA has also been shown to play a role in effector gene expression in *L*. *maculans* and *P*. *nodorum* (IpCho et al., 2010; Soyer

et al., 2015). Moreover, deletion or silencing of *StuA* orthologues directly impaired or abolished pathogenicity in all of the fungi studied, with the interesting exceptions of *F. oxysporum* and *V. dahliae*; both pathogens which invade vascular tissue suggesting this infection route may be independent of StuA regulation.

Aside from StuA, regulatory functions have been attributed to APSES orthologues of Mbp1/Swi4 and Swi6. Early studies in *S. cerevisiae*, replicated in the closely related pathogen of cotton *Ashbya gossypii*, demonstrated the two members of the Mbp1/Swi4 and Swi6 group interact to form a homotypic dimer, regulating cellular division and cell wall integrity (Nasmyth and Dirick, 1991; Leem et al., 1998; Nair et al., 2010; Lengeler et al., 2013). Similar roles may explain the defects in both vegetative and invasive hyphal growth, sporulation and susceptibility to several chemical stresses observed in *F. graminearum* and *M. oryzae* deletion mutants (Liu et al., 2013; Park et al., 2013; Qi et al., 2013; Son et al., 2011). In *A. flavus* the Mbp1 orthologue AfRafA lacks the conserved APSES DBD but was still crucial for fungal development, pathogenicity and production of aflatoxin (Yao et al., 2017). These orthologues and the other APSES TFs remain to be explored further in plant-pathogenic fungi.

2.3.6. The HD/Hox family

The HD/Hox TF family is the third largest in fungi after the Zn2Cys6 and C2H2 zinc fingers (Shelest, 2017). Aside from the Ste12 regulator, relatively few reports regarding phytopathogenic fungi are available that describe a role for HD/Hox TFs beyond mating type regulation (see **Table 2.2** and **Supplementary item 2.1**).

2.3.6.1. Ste12 - Invasive growth

In *S*. *cerevisiae* mating and morphological transitions in response to nutritional scarcity are controlled by Ste12, a target of the Fus3/Kss1-MAPK signal transduction cascades (Song et al., 1991; Cook et al., 1996; Tedford et al., 1997). Ste12 is characterised by a conserved N-terminal HD/Hox domain, but in filamentous fungi orthologues contain additional C2H2 domains at the C-terminus (Rispail and Di Pietro, 2010; Wong Sak Hoi and Dumas, 2010). Despite this difference, largely conserved regulatory functions are described regarding the morphological transition and sexual development. Ste12 is critical for pathogenicity in *A*. *alternata*, *C*. *orbiculare*, *F*. *graminearum*, *M*. *oryzae*, *Setosphaeria turcica* and *V*. *dahliae* (Park et al., 2002; Tsuji et al., 2003; Gu et al., 2014; Gu et al., 2015; Sarmiento-Villamil et al., 2018; Ma et al., 2019), and plays some role in the virulence of several other plant pathogens (see **Table 2.1** and **Supplementary Item 2.1**). A common underlying factor is that Ste12 promotes the development of invasive hyphae, which allows the pathogen to colonise the host tissue and acquire nutrients. On nutrient-rich media, vegetative growth of *ste12* mutants is mostly unaffected in plant-pathogenic fungi aside from *B*. *cinerea*, *S*. *sclerotiorum* and *S*. *turcica* (Schamber et al., 2010; Gu et al., 2014; Xu et al., 2018). Beyond morphological transitions, Ste12-mediated virulence has been attributed to reduced CWDE (*A*. *alternata* and *A*. *brassicicola*) and protease (*F*. *graminearum* and *V. dahliae*) secretion (Cho et al., 2009; Gu et al., 2015; Sarmiento-Villamil et al., 2018; Ma et al., 2019). Spliced isoforms and distinct cofactors have also been identified in several *Ste12* orthologues, revealing mechanisms for differential activity (Tsuji et al., 2003; Schamber et al., 2010; Sarmiento-Villamil et al., 2018). Interestingly, host-induced gene silencing of *PstSTE12* in the obligate rust fungus *Puccinia striiformis* f. sp. *tritici* inhibits the growth of the pathogen in wheat and demonstrates Ste12 is a useful target for disease control (Zhu et al., 2018).

2.3.7. The Velvet family

The Velvet TF family consists of four conserved members (VeA, VelB, VelC and VosA) which are unique in filamentous fungi (Bayram and Braus, 2012). The nature of the velvet domain was only recently elucidated after structural analysis in *A*. *nidulans* (Ahmed et al., 2013). This confirmed the domain binds DNA and can interact with other Velvet TFs and an associated methyltransferase LaeA (Ahmed et al., 2013; Bayram et al., 2008). The Velvet family is predominantly associated with fungal metabolism and sporulation, functions which have been summarised previously (Bayram and Braus, 2012; Calvo et al., 2016).

Recent reports have extended this understanding in several plant-pathogenic fungi. In the apple canker pathogen *V*. *mali*, deletion of *VmVeA* and *VmVelB* revealed that they function as suppressors of melanin production and conidiation (Wu et al., 2018a). The two TFs, which interacted in a yeast-2-hybrid assay, were also shown to regulate virulence through pectinase production and the response to several abiotic stresses. In the cereal spot blotch pathogen *Bipolaris sorokiniana*, CsVeA, CsVelB, and CsVelC are all required for full virulence on barley (Wang et al., 2016). Specific functions for CsVeA and CsVelB were reported to include oxidative stress tolerance, conidiation linked to trehalose biosynthesis, hyphal development, pigmentation and biosynthesis of the host-specific virulence factor ND90Pr (Wang et al., 2016). In a separate study, it was shown that CsVosA shares overlapping functions with CsVeA and CsVelB, as well as a role in regulating ionic/heat stress responses (Wang et al., 2015). These results largely mirror those reported for orthologues in *B*. *oryzae* (Wang et al., 2014; Wu et al., 2012). In *M*. *oryzae* MoVeA, MoVelB and to a lesser extent MoVelC are important for hyphal development and conidiation (Kim et al., 2014). MoVeA and MoVelC were both involved in virulence on rice, controlling appressorium formation, cell wall porosity and the

deployment of ROSs. However, MoVosA and MoVelB orthologues were dispensable for virulence, in contrast to what is observed for most plant pathogens (Calvo et al., 2016; Kim et al., 2014). Lastly, an analysis in *P. expansum* found *veA* mutants display perturbed conidiation, invasive growth and biosynthesis of toxic SMs including patulin and citrinin (Assaf et al., 2018). Together, these reports reveal the extensive involvement of Velvet regulators in biosynthetic pathways, which is linked to the production of both specific virulence factors and the core developmental components of plant-pathogenic fungi.

2.3.8. The Gti1/Pac2 family

The last TFs discussed in detail in this review belong to the Gti1/Pac2 family: orthologues of the two respective yeast genes *Gti1* and *Pac2* (Kunitomo et al., 1995; Caspari, 1997). Unlike other TF families, significant duplication/loss events have not been reported for the *Gti1* or *Pac2* genes, which are present ubiquitously as single copies in fungi (Cain et al., 2012). A role in fungal virulence was originally explored in Gti1 orthologs of the human pathogens *Candida albicans* (Wor1) and *Histoplasma capsulatum* (Ryp1), where they function as master regulators of the morphological transition between saprophytic and invasive growth (Huang et al., 2006; Zordan et al., 2006; Nguyen and Sil, 2008). An analogous function in *S. cerevisiae* exists as the switch to pseudohyphal growth exhibited during nutrient starvation (Cain et al., 2012).

This conserved role can be extended to plant pathogens where several studies have found the Gti1 orthologue positively regulates a suite of genes required for host infection. The first report was in *F. oxysporum* f. sp. *lycopersici* (Michielse et al., 2009). Despite the fungus lacking a distinct morphological transition, the phase transition into parasitism was found to be highly dependent on the Gti1 orthologue. Expression of several key effectors encoded by *SIX* genes was a distinguishing feature which suggested the name Sge1

(*SIX* gene expression) (Michielse et al., 2009). In addition, conidiation was reduced and subsequent studies in the banana pathovar *F. oxysporum* f. sp. *cubense* through gene knockout (Hou et al., 2018) and RNAi (Fernandes et al., 2016) identified similar regulatory roles for Sge1 in these contexts. For other members of the *Fusarium* genus, Gti1 orthologues are global regulators of effector gene expression or SM biosynthesis (Jonkers et al., 2012; Brown et al., 2014; Michielse et al., 2015).

Functional analyses of the respective orthologues have also been undertaken in *V. dahliae, Z. tritici, B. cinerea, C. fulvum* and *M. oryzae.* These studies consistently describe a significant role in fungal development, conidiation and virulence, in line with a shift in the expression profile of effector-like genes and/or SMs (Michielse et al., 2011; Brown et al., 2014; Chen et al., 2014; Mirzadi Gohari et al., 2014; Ökmen et al., 2014). In the basidiomycete *U. maydis*, the orthologue Ros1 orchestrates a massive transcriptional reprogramming during the late stage of infection (Tollot et al., 2016). This was found to be crucial for karyogamy and teliospore development, both required for completion of the infection cycle. The genes regulated included 80 TFs and 198 effectors, many of which were identified as direct targets through ChIP-seq analysis (Tollot et al., 2016). The N-terminal DBD of Gti1 orthologues is highly conserved across the fungal taxa along with its cognate DNA target sequence (Cain et al., 2012; Tollot et al., 2016). This is not observed for the C-terminal, evidenced by partial or complete loss of TF function when the region was exchanged between *Fusarium* and *Cladosporium* spp. (Jonkers et al., 2012; Ökmen et al., 2014).

Fewer studies have been conducted on the other member of the Gti1/Pac2 family (i.e. Pac2 orthologues). Originally described as a suppressor of sexual development in *S. pombe* this function was also reported in *U. maydis* (Elías-Villalobos et al., 2011;

Kunitomo et al., 1995). In this pathogen, sexual development is crucial to the infection process and *Pac2* overexpression mutants were abolished in pathogenicity, in contrast to the *pac2* knockout mutants (Elías-Villalobos et al., 2011). Targeted gene knockout in *Fusarium* species indicates that *Pac2* plays only a minor role if any in fungal pathogenicity and development (Michielse et al., 2009; Jonkers et al., 2012). *M. oryzae pac2* mutants were impaired in hyphal growth and displayed some reduced virulence but, unlike *gti1* mutants, were not involved in sexual development (Chen et al., 2014). As these studies did not test *Pac2* overexpression, a potential role as a suppressor of sexual development in plant-pathogenic ascomycetes remains unknown.

Table 2.1 - The transcription factors (TFs) and their families detailed in this review

The respective TF orthologues are grouped by rows corresponding to their respective families. Pathogen abbreviations = *Aa; Alternaria alternata, Ab; Alternaria brassicicola, Af; Aspergillus flavus, Ag; Ashbya gossypii, Bc; Botrytis cinerea, Bs; Bipolaris sorokiniana, Bm; Bipolaris maydis, Bz; Bipolaris zeicola, Ca; Colletotrichum acutatum, Cf; Cladosporium fulvum, Cg; Colletotrichum gloeosporioides, Cl; Colletotrichum lindemuthianum, Co; Colletotrichum orbiculare, Cr; Cryphonectria parasitica, Cz; Cercospora zeae-maydis, Ds; Dothistroma septosporum, Ff; Fusarium fujikori, Fg; Fusarium graminearum, Fo; Fusarium oxysporum, Fp; Fusarium pseudograminearum, Fv; Fusarium verticillioides, Mo; Magnaporthe oryzae, Pd; Penicillium digitatum, Pe; Penicillium expansum, Pn; Parastagonospora nodorum, Ps; Puccinia striiformis Ptr; Pyrenophora tritici-repentis, Ss; Sclerotinia sclerotiorum, Um; Ustilago maydis, Uv; Ustilaginoidea virens, Vd; Verticillium dahliae, Vl; Verticillium longisporum, Vm; Valsa mali, Zt; Zymoseptoria tritici*. *Regulatory roles and a direct virulence function is indicated and may not be applicable to all orthologues in the listed organisms. Refer to the corresponding record in **Supplementary Item 2.1** for species-specific TF details and links to the corresponding publications.

TF family	Orthologue (synonyms)	Pathogens	Reported regulatory functions*
Zn2Cys6	Mtf4	*Co*	Infection-related morphogenesis
	VdFtf1	*Vd*	Carbohydrate metabolism, enzyme secretion
	Ftf1 & Ftf2 (GzZC215, MGG_06243)	*Fg, Fo, Mo*	Effector regulation, infection-related morphogenesis, sporulation
	Ebr1 (MoCod2)	*Fg, Fo, Mo*	SM biosynthesis, hyphal growth, infection-related morphogenesis, sporulation
	Pro1 (AbPro1, GzZC232, MoPro1, UvPro1)	*Ab, Cr, Fg, Mo, Uv*	Sexual development, sporulation, hyphal growth

	Pf2 (AbPf2 & AbEf1, FgArt1, FvArt1, MoCon1, PnPf2, PtrPf2, Zt107320)	*Ab, Fg, Fv, Mo, Pn, Ptr, Zt*	Carbohydrate metabolism, effector production, abiotic stress tolerance, infection-related morphogenesis, sporulation
	NirA (Nir1)	*Ca, Ff, Mo*	Nitrogen metabolism
C2H2	CreA (Cre1, GzC2H079)	*Ab, Af, Bc, Ff, Fg, Mo, Pe, Ss*	Carbon catabolite repression, SM biosynthesis, sporulation
	Crz1 (CrzA, BcCrz1, FgCrz1 & GzC2H013, PdCrz1, VdCrz1)	*Af, Bc, Cg, Fg, Mo, Pd, Vd*	Ca^{2+}/calcineurin signalling, ionic/cell wall stress response, sporulation, hyphal growth, SM biosynthesis
	PacC (Pac1, BcPacC, Rim1, VmPacC)	Ab, Bc, Cg, Fg, Fo, Mo, Pd, Pe, Ss, Um, Vm	pH response regulator, SM biosynthesis, carbohydrate metabolism
	Msn2 (Vf19, Msn1, GzC2H045, MoMsn2/Tdg1, VmSeb1, VdMsn2, ZtVf1)	*Ab, Af, Fg, Mo, Vd, Vm, Zt*	Hyphal growth, sporulation, enzyme secretion, abiotic stress tolerance
	Con7 (Con7p, GzCon7, Con7-1, PnCon7, Vta2)	*Fg, Fo, Mo, Pn, Vd, Vl*	Hyphal growth, cell wall biosynthesis, sporulation, effector regulation, enzyme secretion
	Tri6	*Fg*	SM biosynthesis
	Cmr1 (CmrA, Amr1, Bmr1, Bcsmr1, Pig1, VdCmr1, Zmr1)	*Aa, Ab, Bm, Bo, Bc, Co, Mo, Vd, Zt,*	Melanisation, infection-related morphogenesis, abiotic stress tolerance, sporulation
GATA	AreA (Nrf1, ClnR1, Fnr1, Nut1, Nit2) & AreB (Asd4)	*Af, Cf, Cg, Cl, Ff, Fg, Fo, Fv, Mo, Um*	Nitrogen metabolism, SM biosynthesis, abiotic stress tolerance, effector regulation
	SreA (Sre1, GzGATA007, Urbs1)	*Bm, Fg, Fo, Um*	Iron metabolism, abiotic stress tolerance
	Wc-1 (LreA, BcWcl-1, Blr1, Crp1, WcoA, FgWc-1, FoWc1, MgWc1) & Wc-2 (BcWcl-2, FgWc-2, Wco2)	*Aa, Bc, Bo, Cz, Ff, Fg, Fo, Mo, Um*	Light response/tropism, hyphal growth, sporulation, SM biosynthesis
bZIP	Yap1 (Ap1, AgAp1, ChAp1, Bap1, CgApr, FgAp1, MoAp1, ZtAp1)	*Aa, Af, Ag, Bc, Bm, Cg, Fg, Mo, Um, Zt*	Oxidative stress tolerance, metal toxicity, SM biosynthesis, hyphal development
	Atf1(BcAtf1, CpTf1, FgAtf1, Foatf1, MoAtf1, VDAG_08676)	*Bc, Cp, Fg, Fo, Mo, Vd*	Oxidative stress tolerance, nitrogen metabolism, sporulation
	Cpc1/Gcn4 (CpcA, CpCpc1)	*Cr, Ff, Lm, Vd, Vl*	Amino acid biosynthesis, SM biosynthesis

	MeaB (MobZIP12)	*Af, Ff, Fo, Mo*	Nitrogen metabolism, SM biosynthesis
	HapX (FgHapX, MobZIP13, VdHapX)	*Bm, Fg, Fo, Mo, Vd*	Iron metabolism, abiotic stress tolerance, hyphal growth, sporulation
APSES (bHLH)	StuA (Stu1, FcStuA, FgStuA, FoStuA, LmStuA, Ust1, Vst1, ZtStuA)	*Af, Fc, Fg, Fo, Lm, Mo, Pn, Um, Vd, Zt*	Sporulation, sclerotia formation, melanisation, sexual reproduction, SM biosynthesis, effector regulation
	Swi4 (Mbp1, AfRafA, GzAPSES004, MoAps2) & Swi6 (AFLA_076560, FgSwi6, MOAps1)	*Af, Ag, Fg, Mo*	Hyphal growth, infection-related morphogenesis, stress response, sporulation, SM biosynthesis
Ste12 (HD/Hox)	Ste12 (AbSte12, Cst1, Fost, Mst12, Ztf1, Vph1, MgSte12, PstSte12)	*Aa, Ab, Bc, Co, Cr, Fg, Fo, Mo, Pd, Pe, Ps, Ss, St, Um, Vd, Zt*	Infection-related morphogenesis, sexual development, nutritional response, hyphal growth, sporulation
Velvet	VeA (Vel1, BcVel1, CsVeA, Fgve1, FoVeA, FvVe1, MoVEA, Umv1, VmVeA, Mve1)	*Aa, Af, Bc, Bm, Bs, Ds, Ff, Fg, Fo, Fv, Mo, Pe, Um, Vm, Zt*	SM biosynthesis, hyphal growth, sporulation, sexual development, abiotic stress tolerance, pigmentation, cell wall integrity
	VelB (Vel2, BcVel2, CsVelB, FgVelB, FoVelB, FvVelB, MoVELB, Umv2, VmVelB, ZtVelB)	*Af, Bc, Bm, Bs, Ff, Fg, Fo, Fv, Mo, Um, Vm, Zt*	SM biosynthesis, hyphal growth, sporulation, sexual development, abiotic stress tolerance, pigmentation
	VelC (BcVel3, CsVelC FoVelC, FvVelC, MoVELC, Umv3) & VelD	*Af, Bc, Bs, Fo, Fv, Mo, Um,*	Hyphal growth, sporulation
	VosA (CsVosA, MoVOSA)	*Af, Bm, Bs, Mo,*	SM biosynthesis, hyphal growth, sporulation, sexual development, abiotic stress tolerance, pigmentation
Gti1/Pac2	Wor1 (Gti1, Sge1, BcReg1, CfWor1, FfSge1, Fgp1, MoGti1, Ros1, VdSge1, ZtWor1)	*Bc, Cf, Ff, Fg, Fo, Fv, Mo, Um, Zt*	Infection-related morphogenesis, hyphal growth, effector regulation, SM biosynthesis, sporulation, sexual development
	Pac2 (Fgp2, MoPac2)	*Fg, Fo, Mo, Um*	Hyphal growth, sexual development

2.3.9. Additional TF families of plant-pathogenic fungi

In this review, several of the major fungal TF families have been covered. Priority was given to those encompassing TFs that have been the subject of extensive functional research efforts. However, the breadth of the topic means that it is not possible to cover each family in detail. Hence, a summary of research efforts until the time of writing into several other conserved classes is provided in **Table 2.2**. Along with those already detailed, this highlights the extent to which TFs that bind DNA through a wide range of mechanisms regulate diverse pathways implicated in fungal virulence. However, many are yet to be thoroughly investigated in plant-pathogenic fungi. Previous reviews annotating TF families across the fungal taxon provide further scope on the extent to which this remains the case (Park et al., 2008; Shelest, 2008; Shelest, 2017; Todd et al., 2014).

Table 2.2 - Research on the transcription factors (TFs) of conserved families not covered in this review

The respective TF orthologues are grouped by rows corresponding to their respective families. Pathogen abbreviations = *Aa; Alternaria alternata, Af; Aspergillus flavus, Ag; Ashbya gossypii, Bc; Botrytis cinerea, Bm; Bipolaris maydis, Bz; Bipolaris zeicola, Co; Colletotrichum orbiculare, Fg; Fusarium graminearum, Fp; Fusarium pseudograminearum, Fv; Fusarium verticillioides, Mo; Magnaporthe oryzae, Pd; Penicillium digitatum, Ps; Puccinia striiformis, Sp; Sporisorium scitamineum, Ss; Sclerotinia sclerotiorum, Um; Ustilago maydis, Uv; Ustilaginoidea virens, Vd; Verticillium dahliae, Zt; Zymoseptoria tritici*. *Regulatory roles and a direct virulence function is indicated and may not be applicable to all orthologues in the listed organisms. Refer to the corresponding record in **Supplementary item 2.1** for species-specific TF details and links to the corresponding publications.

TF family	Orthologue (synonyms)	Pathogens	Reported regulatory functions*
bHLH	SreA (GzbHLH013, MoSre1, PdSreA)	*Fg, Mo, Pd*	Fungicide sensitivity (sterol biosynthesis), oxidative stress tolerance, iron metabolism
	SreB (PdSreB, GzbHLH009, FpbHLH9)	*Fg, Fp, Pd*	Fungicide sensitivity (sterol biosynthesis), oxidative stress tolerance
	Crf1 (GzbHLH005)	*Fg, Mo*	Carbon/lipid metabolism, osmotic stress tolerance, hyphal growth, sporulation
HD/Hox	Hbx1 (GzHOME005, Htf1, UvHox2, Vhb1)	*Af, Fg, Mo, Uv, Vd*	Sporulation, SM biosynthesis, abiotic stress tolerance, sclerotia formation
	Hdp1 & Hdp2 (GzHOME009, MoHox1)	*Fg, Mo, Um*	Infection-related morphogenesis, hyphal growth, SM biosynthesis, effector regulation
	MatbE1 & MatbW1	*Sp, Um, Uh*	Sexual development, infection-related morphogenesis
	MoHOX7 (CoHox3, GzHOME002)	*Fg, Mo, Co*	Infection-related morphogenesis, melanisation, sporulation, SM biosynthesis
HSF	Skn7 (MoSKN7	*Aa, Af, Bc, Bm, Fg,Mo*	Abiotic stress tolerance, hyphal growth, sporulation, fungicide sensitivity (Hog1 pathway), melanisation

	Sfl1 (MoSfl1, GzHSF003, VdSfl1)	*Fg, Mo, Vd*	Hyphal growth, infection-related morphogenesis, sclerotia formation, abiotic stress tolerance
FH/WH	MoFkh1 (GzWing010, SsFkh1)	*Fg, Mo, Ss*	Hyphal growth, sporulation, sclerotia formation, melanisation, abiotic stress tolerance
	MoHcm1 (GzWing015, Fox1)	*Fg, Mo, Um*	Hyphal growth, sexual development, effector regulation
	MoFox1 (GzWing027, FoxE2)	*Fg, Mo, Ss*	Sexual development
MADS	Mcm1 (Fgmcm1, Fmt, MoMcm1, PctMCM1-1, SsMads, VdMcm1)	*Fg, Fv, Mo, Ps, Ss, Vd*	Hyphal growth, infection-related morphogenesis, sexual development, SM biosynthesis, sporulation
	Rlm1 (BcMads1, GzMADS003, Fmt2, Mig1)	*Ag, Bc, Fg, Fv, Mo*	Hyphal growth, sexual development, SM biosynthesis, sclerotia formation, protein secretion, light response
HMG box	Mat1 loci genes (Mat-1-1-x, Mat-2-1-x, Prf1)	*Fg, Sp, Um*	Sexual development
SANT/Myb	Flb4 (BzCon1, GzFlbD, MoMyb1)	*Ag, Bz, Fg, Mo*	Hyphal growth, sporulation, sexual development, SM regulation

2.4. Future perspectives

A question was raised at the beginning of this review: what is it that allows a disease to develop in plant-pathogenic fungi? This review outlined the extent to which the functional characterisation of TFs has provided insight into the regulation of pathogenicity. The aim was to systematically summarise these studies, in order to highlight what is known and which areas remain to be explored for the ultimate goal of plant protection.

So far, functionally conserved TFs have been shown to control fundamental pathways such as iron, nitrogen and carbohydrate metabolism, oxidative, pH, osmotic and UV light stress tolerance, as well as vegetative growth, differentiation and both asexual and sexual development. Many of these TFs were first identified based on homology to well-characterised saprophytic fungal models and functional investigation provided useful insight into the extent these pathways operate in the respective pathosystems. Some of these well-characterised regulators such as Ste12, StuA, Gti1 and the Velvet TF orthologues are fungal-specific and broadly required for pathogenicity. As such they represent promising targets for the control of fungal diseases. Indeed this was already effectively demonstrated through host-induced gene silencing of *PstSTE12* in *P. striiformis* f. sp. *tritici* as well as *V. dahliae Sge1* (Song and Thomma, 2018; Zhu et al., 2018). TFs continue to be explored as effective targets through gene silencing measures (Guo et al., 2019; Sang and Kim, 2019). TFs would also seem to be good targets for chemical intervention, which must be safe for non-target organisms including the host plants, animals, humans and beneficial microbes such as mycorrhizal fungi to be commercially viable (Tietjen and Schreier, 2013; Bahn, 2015). The design of screens for inhibitors of such fungal-specific TFs could be optimised using engineered strains, where activity could be measured by TF controlled expression of a reporter gene.

While the core fungal TFs present robust targets for broad-spectrum disease control, regulators specifically controlling the expression of virulence factors would allow an even more targeted approach. Candidates such as VdFtf1, Ftf1/2, Pf2 and Tri6 could be targeted with conceivably few off-target effects on beneficial fungi. It is the novel identification and characterisation of these TFs which warrants further effort, not only for direct pathogen control, but to better elucidate specific disease pathways. This would assist the discovery of novel effectors and damaging SMs, useful tools for improving plant protection (Vleeshouwers and Oliver, 2014). Genome sequencing revealed the huge number of TFs from a range of families which have yet to be characterised (Aylward et al., 2017; Shelest, 2017). Hence, the scope for exploring the TF control of virulence in plant pathogens remains large. Moving forward two broad approaches seem pertinent:

a) Continue to identify novel TFs which are regulators of specific virulence and pathogenicity-related functions. Random mutagenesis, or the large-scale TF knockout studies conducted on *F*. *graminearum*, *A*. *brassicicola* and *M*. *oryzae* are examples of such approaches (Cao et al., 2016; Cho et al., 2012; Lu et al., 2014; Son et al., 2011). Phylogenetic approaches may also identify cases evolutionarily linked to pathogenicity, such as the Ftf1/2 TFs in *F*. *oxysporum* (Niño-Sánchez et al., 2016).

b) Harness molecular techniques to determine TF-DNA binding in order to characterise the precise targets for known regulators of virulence. Some use cases in plant-pathogenic fungi are provided (**Table 2.3**). Further adoption of these techniques will allow us to distinguish direct target genes specific to fungal pathogenicity from pleiotropic effects resulting from gene deletion. It is also of interest to determine the interactions with other regulatory molecules and cofactors. This has already been achieved through yeast-2-hybrid screens, protein arrays, co-immunoprecipitation and bimolecular fluorescence

complementation, identifying interactions with signal transducers and other regulatory molecules (Li et al., 2011; Schumacher et al., 2015; Brych et al., 2016; Liu et al., 2018; Zhu et al., 2019; Liu et al., 2019). When these methods are used in concert with RNA-seq, proteomics and epigenetic tools, the fundamental mechanisms orchestrating the disease regulatory networks for specific pathogens can be established.

Considering the current technological and genomic resources, both avenues are sound approaches which will extend our understanding of the mechanisms, and thereby enhance, both monitoring and management of the diseases caused by plant-pathogenic fungi.

Table 2.3 - Molecular methods to analyse transcription factor (TF)-DNA binding applied to plant-pathogenic fungi

Detailed reviews on these and other methods not yet widely utilised in plant-pathogenic fungi can be found (Slattery et al., 2014; Levati et al., 2016; Viola and Gonzalez, 2016; Orenstein and Shamir, 2017).

Molecular method	Description	Examples
Chromatin immunoprecipitation (ChIP)	*In vivo* system, antibody-mediated pull down of TF followed by enrichment analysis of bound DNA by sequencing or qPCR	MoCrz1 (Kim et al., 2010), Tri6 (Nasmith et al., 2011), FgSR (Liu et al., 2019), Ros1 (Tollot et al., 2016), FgAreA (Wang et al., 2019)
Protein-binding microarray (PBM)	Binding affinity for purified TF measured against an array of DNA sequences *in vitro*	Ftf1 + EBR (van der Does et al., 2016), MAT-1-2-1 (Kim et al., 2015), Fct1 + Fct2 (Kim et al., 2020)
Electrophoretic mobility shift assay (EMSA/Gel shift)	Binding affinity for purified TF against a predetermined DNA sequence measured as shift in migration along a gel	BcabaR1 (Yonglin Wang et al., 2018a), VdPf (Luo et al., 2016), FgSR (Liu et al., 2019), Tri6 (Nasmith et al., 2011), ZEB2 (Park et al., 2015), MAT-1-2-1 (Kim et al., 2015), SsFdh1 (Zhu et al., 2019), Ust1 (Baeza-Montañez et al., 2015), ClSte12 (Hoi et al., 2007), AreA (Mihlan et al., 2003), FgHapX (Wang et al., 2019)
Yeast-1-Hybrid (Y1H)	TF expressed in yeast. TF target determined by mating a compatible strain containing candidate sequence upstream of selectable marker gene system.	PnCon7 (Lin et al., 2018), PnPf2 (Jones et al., 2019b), BcYOH1 etc.(Simon et al., 2013), Rua1 (Teichmann et al., 2010), BcabaR1 (Yingming Wang et al., 2018)

2.5. Supplementary items

Accessible via: https://figshare.com/s/b0bd50b709e823cbf43e

Supplementary item 2.1 - TFs characterised in plant-pathogenic fungi

A catalogue of TFs functionally characterised in plant-pathogenic fungi. This includes summary information on the organism [columns 1-2], the TF name [3], its TF family [4], observable phenotype [5-6], molecular method used to determine its function [7], a link to the relevant citation/publication [8-9] and a description any virulence related role [10].

2.6. References

Adnan, M., Zheng, W., Islam, W., Arif, M., Abubakar, Y.S., Wang, Z., et al. (2017) Carbon catabolite repression in filamentous fungi. *International Journal of Molecular Sciences*, 19. https://doi.org/10.3390/ijms19010048.

Ahmed, Y.L., Gerke, J., Park, H.-S., Bayram, Ö., Neumann, P., Ni, M., et al. (2013) The velvet family of fungal regulators contains a DNA-binding domain structurally similar to NF-κB. *PLOS Biology*, 11, e1001750. https://doi.org/10.1371/journal.pbio.1001750.

Alexander, N.J., Proctor, R.H. & McCormick, S.P. (2009) Genes, gene clusters, and biosynthesis of trichothecenes and fumonisins in *Fusarium*. *Toxin Reviews*, 28, 198–215. https://doi.org/10.1080/15569540903092142.

Amarasinghe, C.C. & Fernando, W.G.D. (2016) Comparative analysis of deoxynivalenol biosynthesis related gene expression among different chemotypes of *Fusarium graminearum* in spring wheat. *Frontiers in Microbiology*, 7. https://doi.org/10.3389/fmicb.2016.01229.

Amoutzias, G.D., Veron, A.S., Weiner, J., Robinson-Rechavi, M., Bornberg-Bauer, E., Oliver, S.G., et al. (2007) One billion years of bZIP transcription factor evolution: conservation and change in dimerization and DNA-binding site specificity. *Molecular Biology and Evolution*, 24, 827–835. https://doi.org/10.1093/molbev/msl211.

Aramayo, R., Peleg, Y., Addison, R. & Metzenberg, R. (1996) *Asm-1*(+), a *Neurospora crassa* gene related to transcriptional regulators of fungal development. *Genetics*, 144, 991–1003.

Aréchiga-Carvajal, E.T. & Ruiz-Herrera, J. (2005) The RIM101/pacC homologue from the Basidiomycete *Ustilago maydis* is functional in multiple pH-sensitive phenomena. *Eukaryotic Cell*, 4, 999–1008. https://doi.org/10.1128/EC.4.6.999-1008.2005.

Armitage, A.D., Taylor, A., Sobczyk, M.K., Baxter, L., Greenfield, B.P.J., Bates, H.J., et al. (2018) Characterisation of pathogen-specific regions and novel effector candidates in *Fusarium oxysporum* f. sp. *cepae*. *Scientific Reports*, 8, 1–15. https://doi.org/10.1038/s41598-018-30335-7.

Assaf, C.E.H., Snini, S.P., Tadrist, S., Bailly, S., Naylies, C., Oswald, I.P., et al. (2018) Impact of *veA* on the development, aggressiveness, dissemination and secondary metabolism of *Penicillium expansum*. *Molecular Plant Pathology*, 19, 1971–1983. https://doi.org/10.1111/mpp.12673.

Assis, L.J. de, Ulas, M., Ries, L.N.A., Ramli, N.A.M.E., Sarikaya-Bayram, O., Braus, G.H., et al. (2018) Regulation of *Aspergillus nidulans* CreA-mediated catabolite repression by the F-Box proteins Fbx23 and Fbx47. *mBio*, 9. https://doi.org/10.1128/mBio.00840-18.

Aylward, J., Steenkamp, E.T., Dreyer, L.L., Roets, F., Wingfield, B.D. & Wingfield, M.J. (2017) A plant pathology perspective of fungal genome sequencing. *IMA Fungus*, 8, 1–15. https://doi.org/10.5598/imafungus.2017.08.01.01.

Baeza-Montañez, L., Gold, S.E., Espeso, E.A. & García-Pedrajas, M.D. (2015) Conserved and distinct functions of the “stunted” (StuA)-homolog Ust1 during cell differentiation in the corn smut fungus *Ustilago maydis*. *Molecular Plant-Microbe Interactions*, 28, 86–102. https://doi.org/10.1094/MPMI-07-14-0215-R.

Bahn, Y.-S. (2015) Exploiting fungal virulence-regulating transcription factors as novel antifungal drug targets. *PLOS Pathogens*, 11, e1004936. https://doi.org/10.1371/journal.ppat.1004936.

Bayram, Ö. & Braus, G.H. (2012) Coordination of secondarymetabolism and

development in fungi: the velvet family of regulatory proteins. *FEMS Microbiology Reviews*, 36, 1–24. https://doi.org/10.1111/j.1574-6976.2011.00285.x.
Bayram, O., Krappmann, S., Ni, M., Bok, J.W., Helmstaedt, K., Valerius, O., et al. (2008) VelB/VeA/LaeA complex coordinates light signal with fungal development and secondary metabolism. *Science*, 320, 1504–1506. https://doi.org/10.1126/science.1155888.
Bernreiter, A., Ramon, A., Fernández-Martínez, J., Berger, H., Araújo-Bazan, L., Espeso, E.A., et al. (2007) Nuclear export of the transcription factor NirA is a regulatory checkpoint for nitrate induction in *Aspergillus nidulans*. *Molecular and Cellular Biology*, 27, 791–802. https://doi.org/10.1128/MCB.00761-06.
Bi, F., Ment, D., Luria, N., Meng, X. & Prusky, D. (2017) Mutation of AREA affects growth, sporulation, nitrogen regulation, and pathogenicity in *Colletotrichum gloeosporioides*. *Fungal Genetics and Biology*, 99, 29–39. https://doi.org/10.1016/j.fgb.2016.12.006.
Bobola, N. & Merabet, S. (2017) Homeodomain proteins in action: similar DNA-binding preferences, highly variable connectivity. *Current Opinion in Genetics & Development*, 43, 1–8. https://doi.org/10.1016/j.gde.2016.09.008.
Bolton, M.D. & Thomma, B.P.H.J. (2008) The complexity of nitrogen metabolism and nitrogen-regulated gene expression in plant pathogenic fungi. *Physiological and Molecular Plant Pathology*, 72, 104–110. https://doi.org/10.1016/j.pmpp.2008.07.001.
Breitwieser, W., Lyons, S., Flenniken, A.M., Ashton, G., Bruder, G., Willington, M., et al. (2007) Feedback regulation of p38 activity via ATF2 is essential for survival of embryonic liver cells. *Genes & Development*, 21, 2069–2082. https://doi.org/10.1101/gad.430207.
Brown, D.W., Busman, M. & Proctor, R.H. (2014) *Fusarium verticillioides SGE1* is required for full virulence and regulates expression of protein effector and secondary metabolite biosynthetic genes. *Molecular Plant-Microbe Interactions*, 27, 809–823. https://doi.org/10.1094/MPMI-09-13-0281-R.
Brych, A., Mascarenhas, J., Jaeger, E., Charkiewicz, E., Pokorny, R., Bölker, M., et al. (2016) White collar 1-induced photolyase expression contributes to UV-tolerance of *Ustilago maydis*. *MicrobiologyOpen*, 5, 224–243. https://doi.org/10.1002/mbo3.322.
Bürglin, T.R. & Affolter, M. (2016) Homeodomain proteins: an update. *Chromosoma*, 125, 497–521. https://doi.org/10.1007/s00412-015-0543-8.
Cain, C.W., Lohse, M.B., Homann, O.R., Sil, A. & Johnson, A.D. (2012) A conserved transcriptional regulator governs fungal morphology in widely diverged species. *Genetics*, 190, 511–521. https://doi.org/10.1534/genetics.111.134080.
Calvo, A.M., Lohmar, J.M., Ibarra, B. & Satterlee, T. (2016) Velvet regulation of fungal development. In: Wendland, J. (Ed.) Growth, Differentiation and Sexuality The Mycota. Cham: Springer International Publishing, pp. 475–497.
Camejo, D., Guzmán-Cedeño, Á. & Moreno, A. (2016) Reactive oxygen species, essential molecules, during plant–pathogen interactions. *Plant Physiology and Biochemistry*, 103, 10–23. https://doi.org/10.1016/j.plaphy.2016.02.035.
Canessa, P., Schumacher, J., Hevia, M.A., Tudzynski, P. & Larrondo, L.F. (2013) Assessing the effects of light on differentiation and virulence of the plant pathogen *Botrytis cinerea*: characterization of the white collar complex. *PLOS ONE*, 8, e84223. https://doi.org/10.1371/journal.pone.0084223.
Cao, H., Huang, P., Zhang, L., Shi, Y., Sun, D., Yan, Y., et al. (2016) Characterization of 47 Cys2-His2 zinc finger proteins required for the development and

pathogenicity of the rice blast fungus *Magnaporthe oryzae*. *New Phytologist*, 211, 1035–1051. https://doi.org/10.1111/nph.13948.
Caracuel, Z., Roncero, M.I.G., Espeso, E.A., González-Verdejo, C.I., García-Maceira, F.I. & Pietro, A.D. (2003) The pH signalling transcription factor PacC controls virulence in the plant pathogen *Fusarium oxysporum*. *Molecular Microbiology*, 48, 765–779. https://doi.org/10.1046/j.1365-2958.2003.03465.x.
Caramori, G., Ruggeri, P., Mumby, S., Atzeni, F. & Adcock, I.M. (2019) Transcription factors. *eLS*, 1–8. https://doi.org/10.1002/9780470015902.a0005278.pub3.
Carretero-Paulet, L., Galstyan, A., Roig-Villanova, I., Martínez-García, J.F., Bilbao-Castro, J.R. & Robertson, D.L. (2010) Genome-wide classification and evolutionary analysis of the bHLH family of transcription factors in Arabidopsis, poplar, rice, moss, and algae. *Plant Physiology*, 153, 1398–1412. https://doi.org/10.1104/pp.110.153593.
Caspari, T. (1997) Onset of gluconate-H+ symport in *Schizosaccharomyces pombe* is regulated by the kinases Wis1 and Pka1, and requires the *gti1+* gene product. *Journal of Cell Science*, 110 (Pt 20), 2599–2608.
Chang, P.-K., Scharfenstein, L.L., Luo, M., Mahoney, N., Molyneux, R.J., Yu, J., et al. (2011) Loss of *msnA*, a putative stress regulatory gene, in *Aspergillus parasiticus* and *Aspergillus flavus* increased production of conidia, aflatoxins and kojic acid. *Toxins*, 3, 82–104. https://doi.org/10.3390/toxins3010082.
Charoensawan, V., Wilson, D. & Teichmann, S.A. (2010) Genomic repertoires of DNA-binding transcription factors across the tree of life. *Nucleic Acids Research*, 38, 7364–7377. https://doi.org/10.1093/nar/gkq617.
Chen, C.-H., Dunlap, J.C. & Loros, J.J. (2010) Neurospora illuminates fungal photoreception. *Fungal Genetics and Biology*, 47, 922–929. https://doi.org/10.1016/j.fgb.2010.07.005.
Chen, Y., Bates, D.L., Dey, R., Chen, P.-H., Machado, A.C.D., Laird-Offringa, I.A., et al. (2012) DNA-binding by GATA transcription factor suggests mechanisms of DNA looping and long-range gene regulation. *Cell reports*, 2, 1197–1206. https://doi.org/10.1016/j.celrep.2012.10.012.
Chen, Y., Li, B., Xu, X., Zhang, Z. & Tian, S. (2018) The pH-responsive PacC transcription factor plays pivotal roles in virulence and patulin biosynthesis in *Penicillium expansum*. *Environmental Microbiology*, 20, 4063–4078. https://doi.org/10.1111/1462-2920.14453.
Chen, Y., Zhai, S., Zhang, H., Zuo, R., Wang, J., Guo, M., et al. (2014) Shared and distinct functions of two Gti1/Pac2 family proteins in growth, morphogenesis and pathogenicity of *Magnaporthe oryzae*. *Environmental Microbiology*, 16, 788–801. https://doi.org/10.1111/1462-2920.12204.
Cho, Y. (2015) How the necrotrophic fungus *Alternaria brassicicola* kills plant cells remains an enigma. *Eukaryotic Cell*, 14, 335–344. https://doi.org/10.1128/EC.00226-14.
Cho, Y., Kim, K.-H., La Rota, M., Scott, D., Santopietro, G., Callihan, M., et al. (2009) Identification of novel virulence factors associated with signal transduction pathways in *Alternaria brassicicola*. *Molecular Microbiology*, 72, 1316–1333. https://doi.org/10.1111/j.1365-2958.2009.06689.x.
Cho, Y., Ohm, R.A., Grigoriev, I.V. & Srivastava, A. (2013) Fungal-specific transcription factor *AbPf2* activates pathogenicity in *Alternaria brassicicola*. *The Plant Journal*, 75, 498–514. https://doi.org/10.1111/tpj.12217.
Cho, Y., Srivastava, A., Ohm, R.A., Lawrence, C.B., Wang, K.-H., Grigoriev, I.V., et al. (2012) Transcription factor *Amr1* induces melanin biosynthesis and suppresses virulence in *Alternaria brassicicola*. *PLOS Pathogens*, 8, e1002974.

https://doi.org/10.1371/journal.ppat.1002974.
Choi, J., Kim, Y., Kim, S., Park, J. & Lee, Y.-H. (2009) *MoCRZ1*, a gene encoding a calcineurin-responsive transcription factor, regulates fungal growth and pathogenicity of *Magnaporthe oryzae*. *Fungal Genetics and Biology*, 46, 243–254. https://doi.org/10.1016/j.fgb.2008.11.010.
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 Genetics and Biology*, 61, 133–141. https://doi.org/10.1016/j.fgb.2013.10.004.
Cook, J.G., Bardwell, L., Kron, S.J. & Thorner, J. (1996) Two novel targets of the MAP kinase Kss1 are negative regulators of invasive growth in the yeast *Saccharomyces cerevisiae*. *Genes & Development*, 10, 2831–2848. https://doi.org/10.1101/gad.10.22.2831.
David, H., Krogh, A.M., Roca, C., Åkesson, M. & Nielsen, J. (2005) CreA influences the metabolic fluxes of *Aspergillus nidulans* during growth on glucose and xylose. *Microbiology*, 151, 2209–2221. https://doi.org/10.1099/mic.0.27787-0.
Deepika, V.B., Murali, T.S. & Satyamoorthy, K. (2016) Modulation of genetic clusters for synthesis of bioactive molecules in fungal endophytes: A review. *Microbiological Research*, 182, 125–140. https://doi.org/10.1016/j.micres.2015.10.009.
Deppmann, C.D., Alvania, R.S. & Taparowsky, E.J. (2006) Cross-species annotation of basic leucine zipper factor interactions: Insight into the evolution of closed interaction networks. *Molecular Biology and Evolution*, 23, 1480–1492. https://doi.org/10.1093/molbev/msl022.
Díez, E., Álvaro, J., Espeso, E.A., Rainbow, L., Suárez, T., Tilburn, J., et al. (2002) Activation of the *Aspergillus* PacC zinc finger transcription factor requires two proteolytic steps. *The EMBO Journal*, 21, 1350–1359. https://doi.org/10.1093/emboj/21.6.1350.
Divon, H.H., Ziv, C., Davydov, O., Yarden, O. & Fluhr, R. (2006) The global nitrogen regulator, FNR1, regulates fungal nutrition-genes and fitness during *Fusarium oxysporum* pathogenesis. *Molecular Plant Pathology*, 7, 485–497. https://doi.org/10.1111/j.1364-3703.2006.00354.x.
Does, H.C. van der, Fokkens, L., Yang, A., Schmidt, S.M., Langereis, L., Lukasiewicz, J.M., et al. (2016) Transcription factors encoded on core and accessory chromosomes of *Fusarium oxysporum* induce expression of effector genes. *PLOS Genetics*, 12, e1006401. https://doi.org/10.1371/journal.pgen.1006401.
Dowzer, C.E. & Kelly, J.M. (1991) Analysis of the *creA* gene, a regulator of carbon catabolite repression in *Aspergillus nidulans*. *Molecular and Cellular Biology*, 11, 5701–5709. https://doi.org/10.1128/MCB.11.11.5701.
Dowzer, C.E. & Kelly, J.M. (1989) Cloning of the *creA* gene from *Aspergillus nidulans*: a gene involved in carbon catabolite repression. *Current Genetics*, 15, 457–459. https://doi.org/10.1007/bf00376804.
Dubey, A.K., Barad, S., Luria, N., Kumar, D., Espeso, E.A. & Prusky, D.B. (2016) Cation-stress-responsive transcription factors SltA and CrzA regulate morphogenetic processes and pathogenicity of *Colletotrichum gloeosporioides*. *PLOS One*, 11, e0168561. https://doi.org/10.1371/journal.pone.0168561.
Dutton, J.R., Johns, S. & Miller, B.L. (1997) StuAp is a sequence-specific transcription factor that regulates developmental complexity in *Aspergillus nidulans*. *The EMBO Journal*, 16, 5710–5721. https://doi.org/10.1093/emboj/16.18.5710.
Eisenman, H.C. & Casadevall, A. (2012) Synthesis and assembly of fungal melanin. *Applied Microbiology and Biotechnology*, 93, 931–940. https://doi.org/10.1007/s00253-011-3777-2.
Eliahu, N., Igbaria, A., Rose, M.S., Horwitz, B.A. & Lev, S. (2007) Melanin biosynthesis

in the maize pathogen *Cochliobolus heterostrophus* depends on two mitogen-activated protein kinases, Chk1 and Mps1, and the transcription factor Cmr1. *Eukaryotic Cell*, 6, 421–429. https://doi.org/10.1128/EC.00264-06.

Elías-Villalobos, A., Fernández-Álvarez, A. & Ibeas, J.I. (2011) The general transcriptional repressor Tup1 is required for dimorphism and virulence in a fungal plant pathogen. *PLOS Pathogens*, 7, e1002235. https://doi.org/10.1371/journal.ppat.1002235.

Elliott, C.E., Fox, E.M., Jarvis, R.S. & Howlett, B.J. (2011) The cross-pathway control system regulates production of the secondary metabolite toxin, sirodesmin PL, in the ascomycete, *Leptosphaeria maculans*. *BMC Microbiology*, 11, 169. https://doi.org/10.1186/1471-2180-11-169.

Elliott, C.E., Gardiner, D.M., Thomas, G., Cozijnsen, A., VAN DE Wouw, A. & Howlett, B.J. (2007) Production of the toxin sirodesmin PL by *Leptosphaeria maculans* during infection of *Brassica napus*. *Molecular Plant Pathology*, 8, 791–802. https://doi.org/10.1111/j.1364-3703.2007.00433.x.

Estrada, A.F. & Avalos, J. (2007) The white collar protein WcoA of *Fusarium fujikuroi* is not essential for photocarotenogenesis, but is involved in the regulation of secondary metabolism and conidiation. *Fungal Genetics and Biology* , 45, 705–718. https://doi.org/10.1016/j.fgb.2007.12.003.

Fang, Y., Xiong, D., Tian, L., Tang, C., Wang, Y. & Tian, C. (2017) Functional characterization of two bZIP transcription factors in *Verticillium dahliae*. *Gene*, 626, 386–394. https://doi.org/10.1016/j.gene.2017.05.061.

Fasoyin, O.E., Wang, B., Qiu, M., Han, X., Chung, K.-R. & Wang, S. (2018) Carbon catabolite repression gene *CreA* regulates morphology, aflatoxin biosynthesis and virulence in *Aspergillus flavus*. *Fungal Genetics and Biology*, 115, 41–51. https://doi.org/10.1016/j.fgb.2018.04.008.

Fasoyin, O.E., Yang, K., Qiu, M., Wang, B., Wang, Sen & Wang, Shihua (2019) Regulation of morphology, aflatoxin production, and virulence of *Aspergillus flavus* by the major nitrogen regulatory gene *areA*. *Toxins*, 11, 718. https://doi.org/10.3390/toxins11120718.

Fedotova, A.A., Bonchuk, A.N., Mogila, V.A. & Georgiev, P.G. (2017) C2H2 zinc finger proteins: the largest but poorly explored family of higher eukaryotic transcription factors. *Acta Naturae*, 9, 47–58.

Fernandes, J.S., Angelo, P.C.S., Cruz, J.C., Santos, J.M.M., Sousa, N.R. & Silva, G.F. (2016) Post-transcriptional silencing of the SGE1 gene induced by a dsRNA hairpin in *Fusarium oxysporum* f. sp *cubense*, the causal agent of Panama disease. *Genetics and molecular research*, 15. https://doi.org/10.4238/gmr.15027941.

Fernandes, L., Rodrigues-Pousada, C. & Struhl, K. (1997) Yap, a novel family of eight bZIP proteins in *Saccharomyces cerevisiae* with distinct biological functions. *Molecular and Cellular Biology*, 17, 6982–6993. https://doi.org/10.1128/mcb.17.12.6982.

Fetzner, R., Seither, K., Wenderoth, M., Herr, A. & Fischer, R. (2014) *Alternaria alternata* transcription factor CmrA controls melanization and spore development. *Microbiology,* 160, 1845–1854. https://doi.org/10.1099/mic.0.079046-0.

Flaherty, J.E., Pirttilä, A.M., Bluhm, B.H. & Woloshuk, C.P. (2003) *PAC1*, a pH-regulatory gene from *Fusarium verticillioides*. *Applied and Environmental Microbiology*, 69, 5222–5227. https://doi.org/10.1128/AEM.69.9.5222-5227.2003.

Froeliger, E.H., Carpenter, B.E. & Froeliger, E. (1996) *NUT1*, a major nitrogen

regulatory gene in *Magnaporthe grisea*, is dispensable for pathogenicity. *Molecular and General Genetics*, 251, 647–656. https://doi.org/10.1007/BF02174113.
Fujii, Y., Shimizu, T., Toda, T., Yanagida, M. & Hakoshima, T. (2000) Structural basis for the diversity of DNA recognition by bZIP transcription factors. *Nature Structural Biology*, 7, 889–893. https://doi.org/10.1038/82822.
Fuller, K.K., Loros, J.J. & Dunlap, J.C. (2015) Fungal photobiology: visible light as a signal for stress, space and time. *Current Genetics*, 61, 275–288. https://doi.org/10.1007/s00294-014-0451-0.
García-Pedrajas, M.D., Baeza-Montañez, L. & Gold, S.E. (2010) Regulation of *Ustilago maydis* dimorphism, sporulation, and pathogenic development by a transcription factor with a highly conserved APSES domain. *Molecular Plant-Microbe Interactions*, 23, 211–222. https://doi.org/10.1094/MPMI-23-2-0211.
Gerwien, F., Skrahina, V., Kasper, L., Hube, B. & Brunke, S. (2018) Metals in fungal virulence. *FEMS Microbiology Reviews*, 42. https://doi.org/10.1093/femsre/fux050.
Graham-Taylor, C., Kamphuis, L.G. & Derbyshire, M.C. (2020) A detailed in silico analysis of secondary metabolite biosynthesis clusters in the genome of the broad host range plant pathogenic fungus *Sclerotinia sclerotiorum*. *BMC Genomics*, 21, 7. https://doi.org/10.1186/s12864-019-6424-4.
Gu, Q., Zhang, C., Liu, X. & Ma, Z. (2015) A transcription factor FgSte12 is required for pathogenicity in *Fusarium graminearum*. *Molecular Plant Pathology*, 16, 1–13. https://doi.org/10.1111/mpp.12155.
Gu, S., Li, P., Wu, M., Hao, Z., Gong, X., Zhang, X., et al. (2014) *StSTE12* is required for the pathogenicity of *Setosphaeria turcica* by regulating appressorium development and penetration. *Microbiological Research*, 169, 817–823. https://doi.org/10.1016/j.micres.2014.04.001.
Guan, X., Zhao, Y., Liu, X., Shang, B., Xing, F., Zhou, L., et al. (2019) The bZIP transcription factor Afap1 mediates the oxidative stress response and aflatoxin biosynthesis in *Aspergillus flavus*. *Revista Argentina de Microbiología*, 51, 292–301. https://doi.org/10.1016/j.ram.2018.07.003.
Guo, M., Chen, Y., Du, Y., Dong, Y., Guo, W., Zhai, S., et al. (2011) The bZIP transcription factor MoAP1 mediates the oxidative stress response and is critical for pathogenicity of the rice blast fungus *Magnaporthe oryzae*. *PLOS Pathogens*, 7, e1001302. https://doi.org/10.1371/journal.ppat.1001302.
Guo, M., Guo, W., Chen, Y., Dong, S., Zhang, X., Zhang, H., et al. (2010) The basic leucine zipper transcription factor Moatf1 mediates oxidative stress responses and is necessary for full virulence of the rice blast fungus *Magnaporthe oryzae*. *Molecular Plant-Microbe Interactions*, 23, 1053–1068. https://doi.org/10.1094/MPMI-23-8-1053.
Guo, X.-Y., Li, Y., Fan, J., Xiong, H., Xu, F.-X., Shi, J., et al. (2019) Host-induced gene silencing of *MoAP1* confers broad-spectrum resistance to *Magnaporthe oryzae*. *Frontiers in Plant Science*, 10. https://doi.org/10.3389/fpls.2019.00433.
Haas, H. (2012) Iron – A key nexus in the virulence of *Aspergillus fumigatus*. *Frontiers in Microbiology*, 3. https://doi.org/10.3389/fmicb.2012.00028.
Habig, M., Bahena-Garrido, S.M., Barkmann, F., Haueisen, J. & Stukenbrock, E.H. (2020) The transcription factor Zt107320 affects the dimorphic switch, growth and virulence of the fungal wheat pathogen *Zymoseptoria tritici*. *Molecular Plant Pathology*, 21, 124–138. https://doi.org/10.1111/mpp.12886.
Haridas, S., Albert, R., Binder, M., Bloem, J., LaButti, K., Salamov, A., et al. (2020) 101 Dothideomycetes genomes: a test case for predicting lifestyles and emergence

of pathogens. *Studies in Mycology*, 96, 141–153. https://doi.org/10.1016/j.simyco.2020.01.003.
Hasegawa, A. & Shimizu, R. (2017) GATA1 Activity governed by configurations of cis-acting elements. *Frontiers in Oncology*, 6. https://doi.org/10.3389/fonc.2016.00269.
Hoffmann, B., Valerius, O., Andermann, M. & Braus, G.H. (2001) Transcriptional autoregulation and inhibition of mRNA translation of amino acid regulator gene *CpcA* of filamentous fungus *Aspergillus nidulans*. *Molecular Biology of the Cell*, 12, 2846–2857.
Hohn, T.M., Krishna, R. & Proctor, R.H. (1999) Characterization of a transcriptional activator controlling trichothecene toxin biosynthesis. *Fungal Genetics and Biology*, 26, 224–235. https://doi.org/10.1006/fgbi.1999.1122.
Hoi, J.W.S., Herbert, C., Bacha, N., O'Connell, R., Lafitte, C., Borderies, G., et al. (2007) Regulation and role of a STE12-like transcription factor from the plant pathogen *Colletotrichum lindemuthianum*. *Molecular Microbiology*, 64, 68–82. https://doi.org/10.1111/j.1365-2958.2007.05639.x.
Hong, S.-Y., Roze, L.V. & Linz, J.E. (2013) Oxidative stress-related transcription factors in the regulation of secondary metabolism. *Toxins*, 5, 683–702. https://doi.org/10.3390/toxins5040683.
Horst, R.J., Zeh, C., Saur, A., Sonnewald, S., Sonnewald, U. & Voll, L.M. (2012) The *Ustilago maydis* Nit2 homolog regulates nitrogen utilization and is required for efficient induction of filamentous growth. *Eukaryotic Cell*, 11, 368–380. https://doi.org/10.1128/EC.05191-11.
Hou, R. & Wang, C. (2018) The function of the carbon metabolism regulator FgCreA in *Fusarium graminearum*. *Scientia Agricultura Sinica*, 51, 257–267.
Hou, X., An, B., Wang, Q., Guo, Y., Luo, H. & He, C. (2018) SGE1 is involved in conidiation and pathogenicity of *Fusarium oxysporum* f.sp. *cubense*. *Canadian Journal of Microbiology*, 64, 349–357. https://doi.org/10.1139/cjm-2017-0638.
Howard, R.J. & Valent, B. (1996) Breaking and entering: host penetration by the fungal rice blast pathogen *Magnaporthe grisea*. *Annual Review of Microbiology*, 50, 491–512. https://doi.org/10.1146/annurev.micro.50.1.491.
Huang, G., Wang, H., Chou, S., Nie, X., Chen, J. & Liu, H. (2006) Bistable expression of *WOR1*, a master regulator of white–opaque switching in *Candida albicans*. *Proceedings of the National Academy of Sciences*, 103, 12813–12818. https://doi.org/10.1073/pnas.0605270103.
Hughes, T.R. (2011) Introduction to "A handbook of transcription factors." In: Hughes, Timothy R. (Ed.) A Handbook of Transcription Factors Subcellular Biochemistry. Dordrecht: Springer Netherlands, pp. 1–6.
IpCho, S.V.S., Tan, K.-C., Koh, G., Gummer, J., Oliver, R.P., Trengove, R.D., et al. (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. https://doi.org/10.1128/EC.00064-10.
Iuchi, S. (2005) C2H2 zinc fingers as DNA-binding domains. In: Iuchi, S. and Kuldell, N. (Eds.) Zinc Finger Proteins: From Atomic Contact to Cellular Function Molecular Biology Intelligence Unit. Boston, MA: Springer US, pp. 7–13.
Johnson, L. (2008) Iron and siderophores in fungal-host interactions. *Mycological Research*, 112, 170–183. https://doi.org/10.1016/j.mycres.2007.11.012.
Jones, D.A.B., John, E., Rybak, K., Phan, H.T.T., Singh, K.B., Lin, S.-Y., et al. (2019a) A specific fungal transcription factor controls effector gene expression and orchestrates the establishment of the necrotrophic pathogen lifestyle on wheat.

Scientific Reports, 9, 1–13. https://doi.org/10.1038/s41598-019-52444-7.
Jones, D.A.B., John, E., Rybak, K., Phan, H.T.T., Singh, K.B., Lin, S.-Y., et al. (2019b) A specific fungal transcription factor controls effector gene expression and orchestrates the establishment of the necrotrophic pathogen lifestyle on wheat. *Scientific Reports*, 9, 1–13. https://doi.org/10.1038/s41598-019-52444-7.
Jonkers, W., Dong, Y., Broz, K. & Kistler, H.C. (2012) The Wor1-like protein Fgp1 regulates pathogenicity, toxin synthesis and reproduction in the phytopathogenic fungus *Fusarium graminearum*. *PLOS Pathogens*, 8, e1002724. https://doi.org/10.1371/journal.ppat.1002724.
Jonkers, W. & Rep, M. (2009) Mutation of *CRE1* in *Fusarium oxysporum* reverts the pathogenicity defects of the *FRP1* deletion mutant. *Molecular Microbiology*, 74, 1100–1113. https://doi.org/10.1111/j.1365-2958.2009.06922.x.
Jonkers, W., Xayamongkhon, H., Haas, M., Olivain, C., Does, H.C. van der, Broz, K., et al. (2014) *EBR1* genomic expansion and its role in virulence of *Fusarium* species. *Environmental Microbiology*, 16, 1982–2003. https://doi.org/10.1111/1462-2920.12331.
Joshua, I.M. & Höfken, T. (2017) From lipid homeostasis to differentiation: old and new functions of the zinc cluster proteins Ecm22, Upc2, Sut1 and Sut2. *International Journal of Molecular Sciences*, 18. https://doi.org/10.3390/ijms18040772.
Keller, N.P. (2019) Fungal secondary metabolism: regulation, function and drug discovery. *Nature Reviews Microbiology*, 17, 167–180. https://doi.org/10.1038/s41579-018-0121-1.
Keller, N.P. (2015) Translating biosynthetic gene clusters into fungal armor and weaponry. *Nature Chemical Biology*, 11, 671–677. https://doi.org/10.1038/nchembio.1897.
Kihara, J., Moriwaki, A., Tanaka, N., Tanaka, C., Ueno, M. & Arase, S. (2008) Characterization of the *BMR1* gene encoding a transcription factor for melanin biosynthesis genes in the phytopathogenic fungus *Bipolaris oryzae*. *FEMS Microbiology Letters*, 281, 221–227. https://doi.org/10.1111/j.1574-6968.2008.01101.x.
Kihara, J., Moriwaki, A., Tanaka, N., Ueno, M. & Arase, S. (2007) Characterization of the *BLR1* gene encoding a putative blue-light regulator in the phytopathogenic fungus *Bipolaris oryzae*. *FEMS Microbiology Letters*, 266, 110–118. https://doi.org/10.1111/j.1574-6968.2006.00514.x.
Kim, H., Ridenour, J.B., Dunkle, L.D. & Bluhm, B.H. (2011) Regulation of stomatal tropism and infection by light in *Cercospora zeae-maydis*: evidence for coordinated host/pathogen responses to photoperiod? *PLOS Pathogens*, 7, e1002113. https://doi.org/10.1371/journal.ppat.1002113.
Kim, H. & Woloshuk, C.P. (2008) Role of AREA, a regulator of nitrogen metabolism, during colonization of maize kernels and fumonisin biosynthesis in *Fusarium verticillioides*. *Fungal Genetics and Biology*, 45, 947–953. https://doi.org/10.1016/j.fgb.2008.03.007.
Kim, H.-J., Han, J.-H., Kim, K.S. & Lee, Y.-H. (2014) Comparative functional analysis of the velvet gene family reveals unique roles in fungal development and pathogenicity in *Magnaporthe oryzae*. *Fungal Genetics and Biology*, 66, 33–43. https://doi.org/10.1016/j.fgb.2014.02.011.
Kim, H.-K., Jo, S.-M., Kim, G.-Y., Kim, D.-W., Kim, Y.-K. & Yun, S.-H. (2015) A large-scale functional analysis of putative target genes of mating-type loci provides insight into the regulation of sexual development of the cereal pathogen *Fusarium graminearum*. *PLOS Genetics*, 11, e1005486. https://doi.org/10.1371/journal.pgen.1005486.

Kim, J.-E., Nam, H., Park, J., Choi, G.J., Lee, Y.-W. & Son, H. (2020) Characterization of the CCAAT-binding transcription factor complex in the plant pathogenic fungus *Fusarium graminearum*. *Scientific Reports*, 10, 4898. https://doi.org/10.1038/s41598-020-61885-4.

Kim, S., Hu, J., Oh, Y., Park, J., Choi, J., Lee, Y.-H., et al. (2010) Combining ChIP-chip and expression profiling to model the MoCRZ1 mediated circuit for Ca2+/calcineurin signaling in the rice blast fungus. *PLOS Pathogens*, 6, e1000909. https://doi.org/10.1371/journal.ppat.1000909.

Kim, S., Singh, P., Park, J., Park, S., Friedman, A., Zheng, T., et al. (2011) Genetic and molecular characterization of a blue light photoreceptor MGWC-1 in *Magnaporthe oryzae*. *Fungal Genetics and Biology*, 48, 400–407. https://doi.org/10.1016/j.fgb.2011.01.004.

Klug, A. (2010) The discovery of zinc fingers and their applications in gene regulation and genome manipulation. *Annual Review of Biochemistry*, 79, 213–231. https://doi.org/10.1146/annurev-biochem-010909-095056.

Koch, C., Moll, T., Neuberg, M., Ahorn, H. & Nasmyth, K. (1993) A role for the transcription factors Mbp1 and Swi4 in progression from G1 to S phase. *Science*, 261, 1551–1557. https://doi.org/10.1126/science.8372350.

Kodama, S., Nishiuchi, T. & Kubo, Y. (2019) *Colletotrichum orbiculare* MTF4 is a key transcription factor downstream of MOR essential for plant signal-dependent appressorium development and pathogenesis. *Molecular Plant-Microbe Interactions*, 32, 313–324. https://doi.org/10.1094/MPMI-05-18-0118-R.

Kong, S., Park, S.-Y. & Lee, Y.-H. (2015) Systematic characterization of the bZIP transcription factor gene family in the rice blast fungus, *Magnaporthe oryzae*. *Environmental Microbiology*, 17, 1425–1443. https://doi.org/10.1111/1462-2920.12633.

Krishnan, P., Meile, L., Plissonneau, C., Ma, X., Hartmann, F.E., Croll, D., et al. (2018) Transposable element insertions shape gene regulation and melanin production in a fungal pathogen of wheat. *BMC Biology*, 16, 78. https://doi.org/10.1186/s12915-018-0543-2.

Kunitomo, H., Sugimoto, A., Wilkinson, C.R. & Yamamoto, M. (1995) *Schizosaccharomyces pombe* pac2+ controls the onset of sexual development via a pathway independent of the cAMP cascade. *Current Genetics*, 28, 32–38. https://doi.org/10.1007/bf00311879.

Lamb, T.M. & Mitchell, A.P. (2003) The transcription factor Rim101p governs ion tolerance and cell differentiation by direct repression of the regulatory genes *NRG1* and *SMP1* in *Saccharomyces cerevisiae*. *Molecular and Cellular Biology*, 23, 677–686. https://doi.org/10.1128/MCB.23.2.677-686.2003.

Landraud, P., Chuzeville, S., Billon-Grande, G., Poussereau, N. & Bruel, C. (2013) Adaptation to pH and role of PacC in the rice blast fungus *Magnaporthe oryzae*. *PLOS ONE*, 8, e69236. https://doi.org/10.1371/journal.pone.0069236.

Leem, S.H., Chung, C.N., Sunwoo, Y. & Araki, H. (1998) Meiotic role of SWI6 in *Saccharomyces cerevisiae*. *Nucleic Acids Research*, 26, 3154–3158.

Lengeler, K.B., Wasserstrom, L., Walther, A. & Wendland, J. (2013) Analysis of the cell wall integrity pathway of *Ashbya gossypii*. *Microbiological Research*, 168, 607–614. https://doi.org/10.1016/j.micres.2013.06.008.

Levati, E., Sartini, S., Ottonello, S. & Montanini, B. (2016) Dry and wet approaches for genome-wide functional annotation of conventional and unconventional transcriptional activators. *Computational and Structural Biotechnology Journal*, 14, 262–270. https://doi.org/10.1016/j.csbj.2016.06.004.

Levo, M., Avnit-Sagi, T., Lotan-Pompan, M., Kalma, Y., Weinberger, A., Yakhini, Z., et

al. (2017) Systematic investigation of transcription factor activity in the context of chromatin using massively parallel binding and expression assays. *Molecular Cell*, 65, 604-617.e6. https://doi.org/10.1016/j.molcel.2017.01.007.
Li, G., Zhou, X., Kong, L., Wang, Y., Zhang, H., Zhu, H., et al. (2011) *MoSfl1* is important for virulence and heat tolerance in *Magnaporthe oryzae*. *PLOS ONE*, 6, e19951. https://doi.org/10.1371/journal.pone.0019951.
Li, W. & Mitchell, A.P. (1997) Proteolytic activation of Rim1p, a positive regulator of yeast sporulation and invasive growth. *Genetics*, 145, 63–73.
Li, X., Wu, Y., Liu, Z. & Zhang, C. (2017) The function and transcriptome analysis of a bZIP transcription factor CgAP1 in *Colletotrichum gloeosporioides*. *Microbiological Research*, 197, 39–48. https://doi.org/10.1016/j.micres.2017.01.006.
Lim, S.-Y., Son, Y.-E., Lee, D.-H., Eom, T.-J., Kim, M.-J. & Park, H.-S. (2019) Function of *crzA* in fungal development and aflatoxin production in *Aspergillus flavus*. *Toxins*, 11, 567. https://doi.org/10.3390/toxins11100567.
Lin, C.-H., Yang, S.L. & Chung, K.-R. (2009) The YAP1 homolog-mediated oxidative stress tolerance is crucial for pathogenicity of the necrotrophic fungus *Alternaria alternata* in citrus. *Molecular Plant-Microbe Interactions*, 22, 942–952. https://doi.org/10.1094/MPMI-22-8-0942.
Lin, S.-Y., Chooi, Y.-H. & Solomon, P.S. (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. https://doi.org/10.1111/mmi.13968.
Liu, L., Wang, Q., Sun, Y., Zhang, Y., Zhang, X., Liu, J., et al. (2018) *Sssfh1*, a gene encoding a putative component of the RSC chromatin remodeling complex, is involved in hyphal growth, reactive oxygen species accumulation, and pathogenicity in *Sclerotinia sclerotiorum*. *Frontiers in Microbiology*, 9. https://doi.org/10.3389/fmicb.2018.01828.
Liu, N., Fan, F., Qiu, D. & Jiang, L. (2013) The transcription cofactor FgSwi6 plays a role in growth and development, carbendazim sensitivity, cellulose utilization, lithium tolerance, deoxynivalenol production and virulence in the filamentous fungus *Fusarium graminearum*. *Fungal Genetics and Biology*, 58–59, 42–52. https://doi.org/10.1016/j.fgb.2013.08.010.
Liu, Z., Jian, Y., Chen, Y., Kistler, H.C., He, P., Ma, Z., et al. (2019) A phosphorylated transcription factor regulates sterol biosynthesis in *Fusarium graminearum*. *Nature Communications*, 10, 1–17. https://doi.org/10.1038/s41467-019-09145-6.
Llorca, C.M., Potschin, M. & Zentgraf, U. (2014) bZIPs and WRKYs: two large transcription factor families executing two different functional strategies. *Frontiers in Plant Science*, 5. https://doi.org/10.3389/fpls.2014.00169.
Lohse, M.B., Zordan, R.E., Cain, C.W. & Johnson, A.D. (2010) Distinct class of DNA-binding domains is exemplified by a master regulator of phenotypic switching in *Candida albicans*. *Proceedings of the National Academy of Sciences*, 107, 14105–14110. https://doi.org/10.1073/pnas.1005911107.
López-Berges, M.S., Capilla, J., Turrà, D., Schafferer, L., Matthijs, S., Jöchl, C., et al. (2012) HapX-mediated iron homeostasis is essential for rhizosphere competence and virulence of the soilborne pathogen *Fusarium oxysporum*. *The Plant Cell*, 24, 3805–3822. https://doi.org/10.1105/tpc.112.098624.
López-Berges, M.S., Schäfer, K., Hera, C. & Di Pietro, A. (2014) Combinatorial function of velvet and AreA in transcriptional regulation of nitrate utilization and secondary metabolism. *Fungal Genetics and Biology*, 62, 78–84. https://doi.org/10.1016/j.fgb.2013.11.002.

Lowry, J.A. & Atchley, W.R. (2000) Molecular evolution of the GATA family of transcription factors: conservation within the DNA-binding domain. *Journal of Molecular Evolution*, 50, 103–115. https://doi.org/10.1007/s002399910012.
Lu, J., Cao, H., Zhang, L., Huang, P. & Lin, F. (2014) Systematic analysis of Zn2Cys6 transcription factors required for development and pathogenicity by high-throughput gene knockout in the rice blast fungus. *PLOS Pathogens*, 10. https://doi.org/10.1371/journal.ppat.1004432.
Luo, X., Mao, H., Wei, Y., Cai, J., Xie, C., Sui, A., et al. (2016) The fungal-specific transcription factor Vdpf influences conidia production, melanized microsclerotia formation and pathogenicity in *Verticillium dahliae*. *Molecular Plant Pathology*, 17, 1364–1381. https://doi.org/10.1111/mpp.12367.
Lv, B., Zheng, L., Liu, H., Tang, J., Hsiang, T. & Huang, J. (2016) Use of random T-DNA mutagenesis in identification of gene *UvPRO1*, a regulator of conidiation, stress response, and virulence in *Ustilaginoidea virens*. *Frontiers in Microbiology*, 7. https://doi.org/10.3389/fmicb.2016.02086.
Lysøe, E., Pasquali, M., Breakspear, A. & Kistler, H.C. (2011) The transcription factor FgStuAp influences spore development, pathogenicity, and secondary metabolism in *Fusarium graminearum*. *Molecular Plant-Microbe Interactions*, 24, 54–67. https://doi.org/10.1094/MPMI-03-10-0075.
Ma, H., Zhang, B., Gai, Y., Sun, X., Chung, K.-R. & Li, H. (2019) Cell-wall-degrading enzymes required for virulence in the host selective toxin-producing necrotroph *Alternaria alternata* of citrus. *Frontiers in Microbiology*, 10. https://doi.org/10.3389/fmicb.2019.02514.
Ma, L.-J., Does, H.C. van der, Borkovich, K.A., Coleman, J.J., Daboussi, M.-J., Pietro, A.D., et al. (2010) Comparative genomics reveals mobile pathogenicity chromosomes in *Fusarium*. *Nature*, 464, 367–373. https://doi.org/10.1038/nature08850.
MacPherson, S., Larochelle, M. & Turcotte, B. (2006) A fungal family of transcriptional regulators: the zinc cluster proteins. *Microbiology and Molecular Biology Reviews*, 70, 583–604. https://doi.org/10.1128/MMBR.00015-06.
Mann, R.S., Lelli, K.M. & Joshi, R. (2009) Hox specificity unique roles for cofactors and collaborators. *Current Topics in Developmental Biology*, 88, 63–101. https://doi.org/10.1016/S0070-2153(09)88003-4.
Martínez-Pastor, M.T., Marchler, G., Schüller, C., Marchler-Bauer, A., Ruis, H. & Estruch, F. (1996) The *Saccharomyces cerevisiae* zinc finger proteins Msn2p and Msn4p are required for transcriptional induction through the stress response element (STRE). *The EMBO Journal*, 15, 2227–2235.
Marzluf, G.A. (1997) Genetic regulation of nitrogen metabolism in the fungi. *Microbiology and Molecular Biology Reviews*, 61, 17–32.
Masloff, S., Jacobsen, S., Pöggeler, S. & Kück, U. (2002) Functional analysis of the C6 zinc finger gene *pro1* involved in fungal sexual development. *Fungal Genetics and Biology*, 36, 107–116. https://doi.org/10.1016/S1087-1845(02)00010-5.
Masloff, S., Pöggeler, S. & Kück, U. (1999) The *pro1(+)* gene from *Sordaria macrospora* encodes a C6 zinc finger transcription factor required for fruiting body development. *Genetics*, 152, 191–199.
Mendoza-Martínez, A.E., Cano-Domínguez, N. & Aguirre, J. (2019) Yap1 homologs mediate more than the redox regulation of the antioxidant response in filamentous fungi. *Fungal Biology*. https://doi.org/10.1016/j.funbio.2019.04.001.
Merhej, J., Richard-Forget, F. & Barreau, C. (2011) The pH regulatory factor Pac1 regulates *Tri* gene expression and trichothecene production in *Fusarium graminearum*. *Fungal Genetics and Biology*, 48, 275–284.

https://doi.org/10.1016/j.fgb.2010.11.008.
Michielse, C.B., Becker, M., Heller, J., Moraga, J., Collado, I.G. & Tudzynski, P. (2011) The *Botrytis cinerea* Reg1 protein, a putative transcriptional regulator, is required for pathogenicity, conidiogenesis, and the production of secondary metabolites. *Molecular Plant-Microbe Interactions*, 24, 1074–1085. https://doi.org/10.1094/MPMI-01-11-0007.
Michielse, C.B., Pfannmüller, A., Macios, M., Rengers, P., Dzikowska, A. & Tudzynski, B. (2014) The interplay between the GATA transcription factors AreA, the global nitrogen regulator and AreB in *Fusarium fujikuroi*. *Molecular Microbiology*, 91, 472–493. https://doi.org/10.1111/mmi.12472.
Michielse, C.B., Studt, L., Janevska, S., Sieber, C.M.K., Arndt, B., Espino, J.J., et al. (2015) The global regulator FfSge1 is required for expression of secondary metabolite gene clusters but not for pathogenicity in *Fusarium fujikuroi*. *Environmental Microbiology*, 17, 2690–2708. https://doi.org/10.1111/1462-2920.12592.
Michielse, C.B., Wijk, R. van, Reijnen, L., Manders, E.M.M., Boas, S., Olivain, C., et al. (2009) The nuclear protein Sge1 of *Fusarium oxysporum* is required for parasitic growth. *PLOS Pathogens*, 5. https://doi.org/10.1371/journal.ppat.1000637.
Mihlan, M., Homann, V., Liu, T.-W.D. & Tudzynski, B. (2003) AREA directly mediates nitrogen regulation of gibberellin biosynthesis in *Gibberella fujikuroi*, but its activity is not affected by NMR. *Molecular Microbiology*, 47, 975–991. https://doi.org/10.1046/j.1365-2958.2003.03326.x.
Miller, M. (2009) The importance of being flexible: the case of basic region leucine zipper transcriptional regulators. *Current protein & peptide science*, 10, 244–269.
Min, K., Shin, Y., Son, H., Lee, J., Kim, J.-C., Choi, G.J., et al. (2012) Functional analyses of the nitrogen regulatory gene *areA* in *Gibberella zeae*. *FEMS Microbiology Letters*, 334, 66–73. https://doi.org/10.1111/j.1574-6968.2012.02620.x.
Mirzadi Gohari, A., Mehrabi, R., Robert, O., Ince, I.A., Boeren, S., Schuster, M., et al. (2014) Molecular characterization and functional analyses of ZtWor1, a transcriptional regulator of the fungal wheat pathogen *Zymoseptoria tritici*. *Molecular Plant Pathology*, 15, 394–405. https://doi.org/10.1111/mpp.12102.
Miyara, I., Shafran, H., Haimovich, H.K., Rollins, J., Sherman, A. & Prusky, D. (2008) Multi-factor regulation of pectate lyase secretion by *Colletotrichum gloeosporioides* pathogenic on avocado fruits. *Molecular Plant Pathology*, 9, 281–291. https://doi.org/10.1111/j.1364-3703.2007.00462.x.
Mohammadi, N., Mehrabi, R., Mirzadi Gohari, A., Mohammadi Goltapeh, E., Safaie, N. & Kema, G.H.J. (2017) The ZtVf1 transcription factor regulates development and virulence in the foliar wheat pathogen *Zymoseptoria tritici*. *Fungal Genetics and Biology*, 109, 26–35. https://doi.org/10.1016/j.fgb.2017.10.003.
Molina, L. & Kahmann, R. (2007) An *Ustilago maydis* gene involved in H2O2 detoxification is required for virulence. *The Plant Cell*, 19, 2293–2309. https://doi.org/10.1105/tpc.107.052332.
Montibus, M., Ducos, C., Bonnin-Verdal, M.-N., Bormann, J., Ponts, N., Richard-Forget, F., et al. (2013) The bZIP transcription factor Fgap1 mediates oxidative stress response and trichothecene biosynthesis but not virulence in *Fusarium graminearum*. *PLOS One*, 8, e83377. https://doi.org/10.1371/journal.pone.0083377.
Montibus, M., Pinson-Gadais, L., Richard-Forget, F., Barreau, C. & Ponts, N. (2015) Coupling of transcriptional response to oxidative stress and secondary

metabolism regulation in filamentous fungi. *Critical Reviews in Microbiology*, 41, 295–308. https://doi.org/10.3109/1040841X.2013.829416.
Moye-Rowley, W.S., Harshman, K.D. & Parker, C.S. (1989) Yeast YAP1 encodes a novel form of the jun family of transcriptional activator proteins. *Genes & Development*, 3, 283–292. https://doi.org/10.1101/gad.3.3.283.
Mulford, K.E. & Fassler, J.S. (2011) Association of the Skn7 and Yap1 transcription factors in the *Saccharomyces cerevisiae* oxidative stress response. *Eukaryotic Cell*, 10, 761–769. https://doi.org/10.1128/EC.00328-10.
Nair, D.R., D'Ausilio, C.A., Occhipinti, P., Borsuk, M.E. & Gladfelter, A.S. (2010) A conserved G1 regulatory circuit promotes asynchronous behavior of nuclei sharing a common cytoplasm. *Cell Cycle*, 9, 3771–3779. https://doi.org/10.4161/cc.9.18.12999.
Nasmith, C.G., Walkowiak, S., Wang, L., Leung, W.W.Y., Gong, Y., Johnston, A., et al. (2011) Tri6 is a global transcription regulator in the phytopathogen *Fusarium graminearum*. *PLOS Pathogens*, 7, e1002266. https://doi.org/10.1371/journal.ppat.1002266.
Nasmyth, K. & Dirick, L. (1991) The role of SWI4 and SWI6 in the activity of G1 cyclins in yeast. *Cell*, 66, 995–1013. https://doi.org/10.1016/0092-8674(91)90444-4.
Nathues, E., Joshi, S., Tenberge, K.B., Driesch, M. von den, Oeser, B., Bäumer, N., et al. (2004) CPTF1, a CREB-like transcription factor, is involved in the oxidative stress response in the phytopathogen *Claviceps purpurea* and modulates ROS level in its host *Secale cereale*. *Molecular Plant-Microbe Interactions*, 17, 383–393. https://doi.org/10.1094/MPMI.2004.17.4.383.
Nejat, N., Rookes, J., Mantri, N.L. & Cahill, D.M. (2017) Plant–pathogen interactions: toward development of next-generation disease-resistant plants. *Critical Reviews in Biotechnology*, 37, 229–237. https://doi.org/10.3109/07388551.2015.1134437.
Nguyen, V.Q. & Sil, A. (2008) Temperature-induced switch to the pathogenic yeast form of *Histoplasma capsulatum* requires Ryp1, a conserved transcriptional regulator. *Proceedings of the National Academy of Sciences*, 105, 4880–4885. https://doi.org/10.1073/pnas.0710448105.
Niño-Sánchez, J., Casado-Del Castillo, V., Tello, V., De Vega-Bartol, J.J., Ramos, B., Sukno, S.A., et al. (2016) The *FTF* gene family regulates virulence and expression of SIX effectors in *Fusarium oxysporum*. *Molecular Plant Pathology*, 17, 1124–1139. https://doi.org/10.1111/mpp.12373.
Nishimura, M., FUKADA, J., MORIWAKI, A., FUJIKAWA, T., OHASHI, M., HIBI, T., et al. (2009) Mstu1, an APSES transcription factor, is required for appressorium-mediated infection in *Magnaporthe grisea*. *Bioscience, Biotechnology, and Biochemistry*, 73, 1779–1786. https://doi.org/10.1271/bbb.90146.
Nosanchuk, J.D., Stark, R.E. & Casadevall, A. (2015) Fungal melanin: what do we know about structure? *Frontiers in Microbiology*, 6. https://doi.org/10.3389/fmicb.2015.01463.
Odenbach, D., Breth, B., Thines, E., Weber, R.W.S., Anke, H. & Foster, A.J. (2007) The transcription factor Con7p is a central regulator of infection-related morphogenesis in the rice blast fungus *Magnaporthe grisea*. *Molecular Microbiology*, 64, 293–307. https://doi.org/10.1111/j.1365-2958.2007.05643.x.
Ohara, T. & Tsuge, T. (2004) *FoSTUA*, encoding a basic helix-loop-helix protein, differentially regulates development of three kinds of asexual spores, macroconidia, microconidia, and chlamydospores, in the fungal plant pathogen *Fusarium oxysporum*. *Eukaryotic Cell*, 3, 1412–1422. https://doi.org/10.1128/EC.3.6.1412-1422.2004.

Ökmen, B., Collemare, J., Griffiths, S., Burgt, A. van der, Cox, R. & Wit, P.J.G.M. de (2014) Functional analysis of the conserved transcriptional regulator CfWor1 in *Cladosporium fulvum* reveals diverse roles in the virulence of plant pathogenic fungi. *Molecular Microbiology*, 92, 10–27. https://doi.org/10.1111/mmi.12535.

Orenstein, Y. & Shamir, R. (2017) Modeling protein-DNA binding via high-throughput in vitro technologies. *Briefings in Functional Genomics*, 16, 171–180. https://doi.org/10.1093/bfgp/elw030.

Paluh, J.L., Orbach, M.J., Legerton, T.L. & Yanofsky, C. (1988) The cross-pathway control gene of *Neurospora crassa*, *cpc-1*, encodes a protein similar to GCN4 of yeast and the DNA-binding domain of the oncogene v-jun-encoded protein. *Proceedings of the National Academy of Sciences*, 85, 3728–3732.

Park, A.R., Son, H., Min, K., Park, J., Goo, J.H., Rhee, S., et al. (2015) Autoregulation of *ZEB2* expression for zearalenone production in *Fusarium graminearum*. *Molecular Microbiology*, 97, 942–956. https://doi.org/10.1111/mmi.13078.

Park, G., Xue, C., Zheng, L., Lam, S. & Xu, J.-R. (2002) MST12 regulates infectious growth but not appressorium formation in the rice blast fungus *Magnaporthe grisea*. *Molecular Plant-Microbe Interactions*, 15, 183–192. https://doi.org/10.1094/MPMI.2002.15.3.183.

Park, Jongsun, Park, Jaejin, Jang, S., Kim, Seryun, Kong, S., Choi, J., et al. (2008) FTFD: an informatics pipeline supporting phylogenomic analysis of fungal transcription factors. *Bioinformatics (Oxford, England)*, 24, 1024–1025. https://doi.org/10.1093/bioinformatics/btn058.

Park, S.-Y., Choi, J., Lim, S.-E., Lee, G.-W., Park, J., Kim, Y., et al. (2013) Global expression profiling of transcription factor genes provides new insights into pathogenicity and stress responses in the rice blast fungus. *PLOS Pathogens*, 9, e1003350. https://doi.org/10.1371/journal.ppat.1003350.

Pasquali, M., Spanu, F., Scherm, B., Balmas, V., Hoffmann, L., Hammond-Kosack, K.E., et al. (2013) *FcStuA* from *Fusarium culmorum* controls wheat foot and root rot in a toxin dispensable manner. *PLOS ONE*, 8, e57429. https://doi.org/10.1371/journal.pone.0057429.

Pellier, A.-L., Laugé, R., Veneault-Fourrey, C. & Langin, T. (2003) CLNR1, the AREA/NIT2-like global nitrogen regulator of the plant fungal pathogen *Colletotrichum lindemuthianum* is required for the infection cycle. *Molecular Microbiology*, 48, 639–655. https://doi.org/10.1046/j.1365-2958.2003.03451.x.

Pfannmüller, A., Boysen, J.M. & Tudzynski, B. (2017) Nitrate assimilation in *Fusarium fujikuroi* is controlled by multiple levels of regulation. *Frontiers in Microbiology*, 8. https://doi.org/10.3389/fmicb.2017.00381.

Proctor, R.H., Hohn, T.M., McCormick, S.P. & Desjardins, A.E. (1995) *Tri6* encodes an unusual zinc finger protein involved in regulation of trichothecene biosynthesis in *Fusarium sporotrichioides*. *Applied and Environmental Microbiology*, 61, 1923–1930.

Pruß, S., Fetzner, R., Seither, K., Herr, A., Pfeiffer, E., Metzler, M., et al. (2014) Role of the *Alternaria alternata* blue-light receptor LreA (white-collar 1) in spore formation and secondary metabolism. *Applied and Environmental Microbiology*, 80, 2582–2591. https://doi.org/10.1128/AEM.00327-14.

Qi, X., Guo, L., Yang, L. & Huang, J. (2013) Foatf1, a bZIP transcription factor of *Fusarium oxysporum* f. sp. *cubense*, is involved in pathogenesis by regulating the oxidative stress responses of Cavendish banana (*Musa* spp.). *Physiological and Molecular Plant Pathology*, 84, 76–85. https://doi.org/10.1016/j.pmpp.2013.07.007.

Rascle, C., Dieryckx, C., Dupuy, J.W., Muszkieta, L., Souibgui, E., Droux, M., et al.

(2018) The pH regulator PacC: a host-dependent virulence factor in *Botrytis cinerea*. *Environmental Microbiology Reports*, 10, 555–568. https://doi.org/10.1111/1758-2229.12663.
Reinke, A.W., Baek, J., Ashenberg, O. & Keating, A.E. (2013) Networks of bZIP protein-protein interactions diversified over a billion years of evolution. *Science*, 340, 730–734. https://doi.org/10.1126/science.1233465.
Rispail, N. & Di Pietro, A. (2010) The homeodomain transcription factor Ste12. *Communicative & Integrative Biology*, 3, 327–332.
Rodrigues-Pousada, C., Devaux, F., Caetano, S.M., Pimentel, C., Silva, S. da, Cordeiro, A.C., et al. (2019) Yeast AP-1 like transcription factors (Yap) and stress response: a current overview. *Microbial Cell*, 6, 267–285. https://doi.org/10.15698/mic2019.06.679.
Rodrigues-Pousada, C., Menezes, R.A. & Pimentel, C. (2010) The Yap family and its role in stress response. *Yeast*, 27, 245–258. https://doi.org/10.1002/yea.1752.
Rodríguez-Martínez, J.A., Reinke, A.W., Bhimsaria, D., Keating, A.E. & Ansari, A.Z. (2017) Combinatorial bZIP dimers display complex DNA-binding specificity landscapes Green, M.R. (Ed.). *eLife*, 6, e19272. https://doi.org/10.7554/eLife.19272.
Rollins, J.A. (2003) The *Sclerotinia sclerotiorum pac1* gene is required for sclerotial development and virulence. *Molecular Plant-Microbe Interactions*, 16, 785–795. https://doi.org/10.1094/MPMI.2003.16.9.785.
Romsdahl, J. & Wang, C.C.C. (2019) Recent advances in the genome mining of *Aspergillus* secondary metabolites (covering 2012–2018). *MedChemComm*, 10, 840–866. https://doi.org/10.1039/C9MD00054B.
Ruiz-Roldán, C., Pareja-Jaime, Y., González-Reyes, J.A. & Roncero, M.I.G. (2015) The transcription factor Con7-1 is a master regulator of morphogenesis and virulence in *Fusarium oxysporum*. *Molecular Plant-Microbe Interactions*, 28, 55–68. https://doi.org/10.1094/MPMI-07-14-0205-R.
Rybak, K., See, P.T., Phan, H.T.T., Syme, R.A., Moffat, C.S., Oliver, R.P., et al. (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. *Molecular Plant Pathology*, 18, 420–434. https://doi.org/10.1111/mpp.12511.
Sailsbery, J.K., Atchley, W.R. & Dean, R.A. (2012) Phylogenetic analysis and classification of the fungal bHLH domain. *Molecular Biology and Evolution*, 29, 1301–1318. https://doi.org/10.1093/molbev/msr288.
Sailsbery, J.K. & Dean, R.A. (2012) Accurate discrimination of bHLH domains in plants, animals, and fungi using biologically meaningful sites. *BMC evolutionary biology*, 12, 154. https://doi.org/10.1186/1471-2148-12-154.
Sang, H. & Kim, J.-I. (2019) Advanced strategies to control plant pathogenic fungi by host-induced gene silencing (HIGS) and spray-induced gene silencing (SIGS). *Plant Biotechnology Reports*. https://doi.org/10.1007/s11816-019-00588-3.
Sarmiento-Villamil, J.L., García-Pedrajas, N.E., Baeza-Montañez, L. & García-Pedrajas, M.D. (2018) The APSES transcription factor Vst1 is a key regulator of development in microsclerotium- and resting mycelium-producing *Verticillium* species. *Molecular Plant Pathology*, 19, 59–76. https://doi.org/10.1111/mpp.12496.
Sarmiento-Villamil, J.L., Prieto, P., Klosterman, S.J. & García-Pedrajas, M.D. (2018) Characterization of two homeodomain transcription factors with critical but distinct roles in virulence in the vascular pathogen *Verticillium dahliae*. *Molecular Plant Pathology*, 19, 986–1004. https://doi.org/10.1111/mpp.12584.

Scazzocchio, C. (2000) The fungal GATA factors. *Current Opinion in Microbiology*, 3, 126–131. https://doi.org/10.1016/S1369-5274(00)00063-1.
Schamber, A., Leroch, M., Diwo, J., Mendgen, K. & Hahn, M. (2010) The role of mitogen-activated protein (MAP) kinase signalling components and the Ste12 transcription factor in germination and pathogenicity of *Botrytis cinerea*. *Molecular Plant Pathology*, 11, 105–119. https://doi.org/10.1111/j.1364-3703.2009.00579.x.
Scherm, B., Orrù, M., Balmas, V., Spanu, F., Azara, E., Delogu, G., et al. (2011) Altered trichothecene biosynthesis in *TRI6*-silenced transformants of *Fusarium culmorum* influences the severity of crown and foot rot on durum wheat seedlings. *Molecular Plant Pathology*, 12, 759–771. https://doi.org/10.1111/j.1364-3703.2011.00709.x.
Schmitt, A.P. & McEntee, K. (1996) Msn2p, a zinc finger DNA-binding protein, is the transcriptional activator of the multistress response in *Saccharomyces cerevisiae*. *Proceedings of the National Academy of Sciences*, 93, 5777–5782. https://doi.org/10.1073/pnas.93.12.5777.
Schönig, B., Vogel, S. & Tudzynski, B. (2009) Cpc1 mediates cross-pathway control independently of Mbf1 in *Fusarium fujikuroi*. *Fungal Genetics and Biology*, 46, 898–908. https://doi.org/10.1016/j.fgb.2009.08.003.
Schumacher, J., Larrinoa, I.F. de & Tudzynski, B. (2008) Calcineurin-responsive zinc finger transcription factor CRZ1 of *Botrytis cinerea* is required for growth, development, and full virulence on bean plants. *Eukaryotic Cell*, 7, 584–601. https://doi.org/10.1128/EC.00426-07.
Schumacher, J., Simon, A., Cohrs, K.C., Traeger, S., Porquier, A., Dalmais, B., et al. (2015) The VELVET complex in the gray mold fungus *Botrytis cinerea*: impact of BcLAE1 on differentiation, secondary metabolism, and virulence. *Molecular Plant-Microbe Interactions*, 28, 659–674. https://doi.org/10.1094/MPMI-12-14-0411-R.
Schumacher, J., Simon, A., Cohrs, K.C., Viaud, M. & Tudzynski, P. (2014) The transcription factor BcLTF1 regulates virulence and light responses in the necrotrophic plant pathogen *Botrytis cinerea*. *PLOS Genetics*, 10. https://doi.org/10.1371/journal.pgen.1004040.
Segal, L.M. & Wilson, R.A. (2018) Reactive oxygen species metabolism and plant-fungal interactions. *Fungal Genetics and Biology*, 110, 1–9. https://doi.org/10.1016/j.fgb.2017.12.003.
Seong, K.-Y., Pasquali, M., Zhou, X., Song, J., Hilburn, K., McCormick, S., et al. (2009) Global gene regulation by *Fusarium* transcription factors Tri6 and Tri10 reveals adaptations for toxin biosynthesis. *Molecular Microbiology*, 72, 354–367. https://doi.org/10.1111/j.1365-2958.2009.06649.x.
Shalaby, S., Larkov, O., Lamdan, N.L. & Horwitz, B.A. (2014) Genetic interaction of the stress response factors ChAP1 and Skn7 in the maize pathogen *Cochliobolus heterostrophus*. *FEMS Microbiology Letters*, 350, 83–89. https://doi.org/10.1111/1574-6968.12314.
Shelest, E. (2008) Transcription factors in fungi. *FEMS microbiology letters*, 286, 145–151. https://doi.org/10.1111/j.1574-6968.2008.01293.x.
Shelest, E. (2017) Transcription factors in fungi: TFome dynamics, three major families, and dual-specificity TFs. *Frontiers in Genetics*, 8. https://doi.org/10.3389/fgene.2017.00053.
Shi, Z., Christian, D. & Leung, H. (1998) Interactions between spore morphogenetic mutations affect cell types, sporulation, and pathogenesis in *Magnaporthe grisea*. *Molecular Plant-Microbe Interactions*, 11, 199–207.

https://doi.org/10.1094/MPMI.1998.11.3.199.
Shiozaki, K. & Russell, P. (1996) Conjugation, meiosis, and the osmotic stress response are regulated by Spc1 kinase through Atf1 transcription factor in fission yeast. *Genes & Development*, 10, 2276–2288. https://doi.org/10.1101/gad.10.18.2276.
Shively, C.A., Liu, J., Chen, X., Loell, K. & Mitra, R.D. (2019) Homotypic cooperativity and collective binding are determinants of bHLH specificity and function. *Proceedings of the National Academy of Sciences*, 116, 16143–16152. https://doi.org/10.1073/pnas.1818015116.
Simaan, H., Lev, S. & Horwitz, B.A. (2019) Oxidant-sensing pathways in the responses of fungal pathogens to chemical stress signals. *Frontiers in Microbiology*, 10. https://doi.org/10.3389/fmicb.2019.00567.
Simon, A., Dalmais, B., Morgant, G. & Viaud, M. (2013) Screening of a *Botrytis cinerea* one-hybrid library reveals a Cys2His2 transcription factor involved in the regulation of secondary metabolism gene clusters. *Fungal Genetics and Biology*, 52, 9–19. https://doi.org/10.1016/j.fgb.2013.01.006.
Skamnioti, P., Furlong, R.F. & Gurr, S.J. (2008) The fate of gene duplicates in the genomes of fungal pathogens. *Communicative & Integrative Biology*, 1, 196–198.
Slattery, M., Zhou, T., Yang, L., Dantas Machado, A.C., Gordân, R. & Rohs, R. (2014) Absence of a simple code: how transcription factors read the genome. *Trends in Biochemical Sciences*, 39, 381–399. https://doi.org/10.1016/j.tibs.2014.07.002.
Solomon, P.S. & Oliver, R.P. (2001) The nitrogen content of the tomato leaf apoplast increases during infection by *Cladosporium fulvum*. *Planta*, 213, 241–249. https://doi.org/10.1007/s004250000500.
Son, H., Seo, Y.-S., Min, K., Park, A.R., Lee, J., Jin, J.-M., et al. (2011) A phenome-based functional analysis of transcription factors in the cereal head blight fungus, *Fusarium graminearum*. *PLOS Pathogens*, 7, e1002310. https://doi.org/10.1371/journal.ppat.1002310.
Song, D., Dolan, J.W., Yuan, Y.L. & Fields, S. (1991) Pheromone-dependent phosphorylation of the yeast STE12 protein correlates with transcriptional activation. *Genes & Development*, 5, 741–750. https://doi.org/10.1101/gad.5.5.741.
Song, Y. & Thomma, B.P.H.J. (2018) Host-induced gene silencing compromises Verticillium wilt in tomato and *Arabidopsis*. *Molecular Plant Pathology*, 19, 77–89. https://doi.org/10.1111/mpp.12500.
Soyer, J.L., Hamiot, A., Ollivier, B., Balesdent, M.-H., Rouxel, T. & Fudal, I. (2015) The APSES transcription factor LmStuA is required for sporulation, pathogenic development and effector gene expression in *Leptosphaeria maculans*. *Molecular Plant Pathology*, 16, 1000–1005. https://doi.org/10.1111/mpp.12249.
Sri Theivakadadcham, V.S., Bergey, B.G. & Rosonina, E. (2019) Sumoylation of DNA-bound transcription factor Sko1 prevents its association with nontarget promoters. *PLOS Genetics*, 15, e1007991. https://doi.org/10.1371/journal.pgen.1007991.
Srivastava, A., Ohm, R.A., Oxiles, L., Brooks, F., Lawrence, C.B., Grigoriev, I.V., et al. (2011) A zinc-finger-family transcription factor, *AbVf19*, is required for the induction of a gene subset important for virulence in *Alternaria brassicicola*. *Molecular Plant-Microbe Interactions*, 25, 443–452. https://doi.org/10.1094/MPMI-10-11-0275.
Stathopoulos-Gerontides, A., Guo, J.J. & Cyert, M.S. (1999) Yeast calcineurin regulates nuclear localization of the Crz1p transcription factor through dephosphorylation.

Genes & Development, 13, 798–803.
Struhl, K. (1987) The DNA-binding domains of the jun oncoprotein and the yeast GCN4 transcriptional activator protein are functionally homologous. *Cell*, 50, 841–846. https://doi.org/10.1016/0092-8674(87)90511-3.
Struhl, K. (1988) The JUN oncoprotein, a vertebrate transcription factor, activates transcription in yeast. *Nature*, 332, 649–650. https://doi.org/10.1038/332649a0.
Sun, Q., Choi, G.H. & Nuss, D.L. (2009) Hypovirus-responsive transcription factor gene *Pro1* of the chestnut blight fungus *Cryphonectria parasitica* is required for female fertility, asexual spore development, and stable maintenance of hypovirus infection. *Eukaryotic Cell*, 8, 262–270. https://doi.org/10.1128/EC.00338-08.
Tang, C., Li, T., Klosterman, S.J., Tian, C. & Wang, Y. (2020) The bZIP transcription factor VdAtf1 regulates virulence by mediating nitrogen metabolism in *Verticillium dahliae*. *New Phytologist*, 226, 1461–1479. https://doi.org/10.1111/nph.16481.
Tannous, J., Kumar, D., Sela, N., Sionov, E., Prusky, D. & Keller, N.P. (2018) Fungal attack and host defence pathways unveiled in near-avirulent interactions of *Penicillium expansum creA* mutants on apples. *Molecular Plant Pathology*, 19, 2635–2650. https://doi.org/10.1111/mpp.12734.
Tao, Y. & Marzluf, G.A. (1999) The NIT2 nitrogen regulatory protein of *Neurospora*: expression and stability of *nit-2* mRNA and protein. *Current Genetics*, 36, 153–158. https://doi.org/10.1007/s002940050485.
Taylor, A., Armitage, A.D., Handy, C., Jackson, A.C., Hulin, M.T., Harrison, R.J., et al. (2019) Basal rot of *Narcissus*: understanding pathogenicity in *Fusarium oxysporum* f. sp. *narcissi*. *Frontiers in Microbiology*, 10. https://doi.org/10.3389/fmicb.2019.02905.
Tedford, K., Kim, S., Sa, D., Stevens, K. & Tyers, M. (1997) Regulation of the mating pheromone and invasive growth responses in yeast by two MAP kinase substrates. *Current Biology*, 7, 228–238. https://doi.org/10.1016/S0960-9822(06)00118-7.
Teichmann, B., Liu, L., Schink, K.O. & Bölker, M. (2010) Activation of the ustilagic acid biosynthesis gene cluster in *Ustilago maydis* by the C2H2 zinc finger transcription factor Rua1. *Applied and Environmental Microbiology*, 76, 2633–2640. https://doi.org/10.1128/AEM.02211-09.
Temme, N., Oeser, B., Massaroli, M., Heller, J., Simon, A., González Collado, I., et al. (2012) BcAtf1, a global regulator, controls various differentiation processes and phytotoxin production in *Botrytis cinerea*. *Molecular Plant Pathology*, 13, 704–718. https://doi.org/10.1111/j.1364-3703.2011.00778.x.
Temme, N. & Tudzynski, P. (2009) Does *Botrytis cinerea* ignore H2O2-induced oxidative stress during infection? Characterization of *Botrytis* activator protein 1. *Molecular Plant-Microbe Interactions*, 22, 987–998. https://doi.org/10.1094/MPMI-22-8-0987.
Thewes, S. (2014) Calcineurin-Crz1 signaling in lower eukaryotes. *Eukaryotic Cell*, 13, 694–705. https://doi.org/10.1128/EC.00038-14.
Thomma, B.P.H.J., Bolton, M.D., Clergeot, P.-H. & DE Wit, P.J.G.M. (2006) Nitrogen controls in planta expression of *Cladosporium fulvum Avr9* but no other effector genes. *Molecular Plant Pathology*, 7, 125–130. https://doi.org/10.1111/j.1364-3703.2006.00320.x.
Tian, C., Kasuga, T., Sachs, M.S. & Glass, N.L. (2007) Transcriptional profiling of cross pathway control in *Neurospora crassa* and comparative analysis of the Gcn4 and CPC1 regulons. *Eukaryotic Cell*, 6, 1018–1029.

https://doi.org/10.1128/EC.00078-07.
Tian, C., Li, J. & Glass, N.L. (2011) Exploring the bZIP transcription factor regulatory network in *Neurospora crassa*. *Microbiology*, 157, 747–759. https://doi.org/10.1099/mic.0.045468-0.
Tian, L., Yu, J., Wang, Y. & Tian, C. (2017) The C2H2 transcription factor VdMsn2 controls hyphal growth, microsclerotia formation, and virulence of *Verticillium dahliae*. *Fungal Biology*, 121, 1001–1010. https://doi.org/10.1016/j.funbio.2017.08.005.
Tietjen, K. & Schreier, P.H. (2013) New targets for fungicides. Modern methods in crop protection research. John Wiley & Sons, Ltd, pp. 197–216.
Tilburn, J., Sarkar, S., Widdick, D.A., Espeso, E.A., Orejas, M., Mungroo, J., et al. (1995) The *Aspergillus* PacC zinc finger transcription factor mediates regulation of both acid- and alkaline-expressed genes by ambient pH. *The EMBO Journal*, 14, 779–790.
Tiley, A.M.M., Foster, G.D. & Bailey, A.M. (2018) Exploring the genetic regulation of asexual sporulation in *Zymoseptoria tritici*. *Frontiers in Microbiology*, 9. https://doi.org/10.3389/fmicb.2018.01859.
Timpner, C., Braus-Stromeyer, S.A., Tran, V.T. & Braus, G.H. (2013) The Cpc1 regulator of the cross-pathway control of amino acid biosynthesis is required for pathogenicity of the vascular pathogen *Verticillium longisporum*. *Molecular Plant-Microbe Interactions*, 26, 1312–1324. https://doi.org/10.1094/MPMI-06-13-0181-R.
Todd, R.B. & Andrianopoulos, A. (1997) Evolution of a fungal regulatory gene family: the Zn(II)2Cys6 binuclear cluster DNA-binding motif. *Fungal Genetics and Biology*, 21, 388–405. https://doi.org/10.1006/fgbi.1997.0993.
Todd, R.B., Zhou, M., Ohm, R.A., Leeggangers, H.A., Visser, L. & Vries, R.P. de (2014) Prevalence of transcription factors in ascomycete and basidiomycete fungi. *BMC Genomics*, 15, 214. https://doi.org/10.1186/1471-2164-15-214.
Tollot, M., Assmann, D., Becker, C., Altmüller, J., Dutheil, J.Y., Wegner, C.-E., et al. (2016) The WOPR protein Ros1 is a master regulator of sporogenesis and late effector gene expression in the maize pathogen *Ustilago maydis*. *PLOS pathogens*, 12, e1005697. https://doi.org/10.1371/journal.ppat.1005697.
Tran, V.-T., Braus-Stromeyer, S.A., Kusch, H., Reusche, M., Kaever, A., Kühn, A., et al. (2014) *Verticillium* transcription activator of adhesion Vta2 suppresses microsclerotia formation and is required for systemic infection of plant roots. *New Phytologist*, 202, 565–581. https://doi.org/10.1111/nph.12671.
Tsuji, G., Fujii, S., Tsuge, S., Shiraishi, T. & Kubo, Y. (2003) The *Colletotrichum lagenarium Ste12*-like gene *CST1* is essential for appressorium penetration. *Molecular Plant-Microbe Interactions*, 16, 315–325. https://doi.org/10.1094/MPMI.2003.16.4.315.
Tsuji, G., Kenmochi, Y., Takano, Y., Sweigard, J., Farrall, L., Furusawa, I., et al. (2000) Novel fungal transcriptional activators, Cmr1p of *Colletotrichum lagenarium* and Pig1p of *Magnaporthe grisea*, contain Cys2His2 zinc finger and Zn(II)2Cys6 binuclear cluster DNA-binding motifs and regulate transcription of melanin biosynthesis genes in a developmentally specific manner. *Molecular Microbiology*, 38, 940–954. https://doi.org/10.1046/j.1365-2958.2000.02181.x.
Tudzynski, B. (2014) Nitrogen regulation of fungal secondary metabolism in fungi. *Frontiers in Microbiology*, 5. https://doi.org/10.3389/fmicb.2014.00656.
Tudzynski, B., Liu, S. & Kelly, J.M. (2000) Carbon catabolite repression in plant pathogenic fungi: isolation and characterization of the *Gibberella fujikuroi* and *Botrytis cinerea creA* genes. *FEMS microbiology letters*, 184, 9–15.

https://doi.org/10.1111/j.1574-6968.2000.tb08982.x.
Van den Ackerveken, G.F.J.M., Dunn, R.M., Cozijnsen, A.J., Vossen, J.P.M.J., Van den Broek, H.W.J. & De Wit, P.J.G.M. (1994) Nitrogen limitation induces expression of the avirulence gene *avr9* in the tomato pathogen *Cladosporium fulvum*. *Molecular and General Genetics*, 243, 277–285. https://doi.org/10.1007/BF00301063.
Van Nguyen, T., Kröger, C., Bönnighausen, J., Schäfer, W. & Bormann, J. (2013) The ATF/CREB transcription factor Atf1 Is essential for full virulence, deoxynivalenol production, and stress tolerance in the cereal pathogen *Fusarium graminearum*. *Molecular Plant-Microbe Interactions*, 26, 1378–1394. https://doi.org/10.1094/MPMI-04-13-0125-R.
Vandel, J., Cassan, O., Lèbre, S., Lecellier, C.-H. & Bréhélin, L. (2019) Probing transcription factor combinatorics in different promoter classes and in enhancers. *BMC genomics*, 20, 103. https://doi.org/10.1186/s12864-018-5408-0.
Vautard, G., Cotton, P. & Fèvre, M. (1999) The glucose repressor CRE1 from *Sclerotinia sclerotiorum* is functionally related to CREA from *Aspergillus nidulans* but not to the Mig proteins from *Saccharomyces cerevisiae*. *FEBS Letters*, 453, 54–58. https://doi.org/10.1016/S0014-5793(99)00691-2.
Vega-Bartol, J.J. de, Martín-Dominguez, R., Ramos, B., García-Sánchez, M.-A. & Díaz-Mínguez, J.M. (2010) New virulence groups in *Fusarium oxysporum* f. sp. *phaseoli*: the expression of the gene coding for the transcription factor Ftf1 correlates with virulence. *Phytopathology*, 101, 470–479. https://doi.org/10.1094/PHYTO-09-10-0252.
Viola, I.L. & Gonzalez, D.H. (2016) Methods to study transcription factor structure and function. In: Gonzalez, D.H. (Ed.) Plant transcription factors. Boston: Academic Press, pp. 13–33.
Vleeshouwers, V.G.A.A. & Oliver, R.P. (2014) Effectors as tools in disease resistance breeding against biotrophic, hemibiotrophic, and necrotrophic plant pathogens. *Molecular Plant-Microbe Interactions*, 27, 196–206. https://doi.org/10.1094/MPMI-10-13-0313-IA.
Voisard, C., Wang, J., McEvoy, J.L., Xu, P. & Leong, S.A. (1993) *Urbs1*, a gene regulating siderophore biosynthesis in *Ustilago maydis*, encodes a protein similar to the erythroid transcription factor GATA-1. *Molecular and Cellular Biology*, 13, 7091–7100. https://doi.org/10.1128/MCB.13.11.7091.
Wang, P., Larson, T.G., Chen, C.-H., Pawlyk, D.M., Clark, J.A. & Nuss, D.L. (1998) Cloning and characterization of a general amino acid control transcriptional activator from the chestnut blight fungus *Cryphonectria parasitica*. *Fungal Genetics and Biology*, 23, 81–94. https://doi.org/10.1006/fgbi.1997.1023.
Wang, R., Leng, Y., Shrestha, S. & Zhong, S. (2016) Coordinated and independent functions of velvet-complex genes in fungal development and virulence of the fungal cereal pathogen *Cochliobolus sativus*. *Fungal Biology*, 120, 948–960. https://doi.org/10.1016/j.funbio.2016.05.004.
Wang, R., Leng, Y. & Zhong, S. (2015) The regulatory gene *VosA* affects conidiogenesis and is involved in virulence of the fungal cereal pathogen *Cochliobolus sativus*. *Fungal Biology*, 119, 884–900. https://doi.org/10.1016/j.funbio.2015.06.009.
Wang, W., Wu, D., Pan, H. & Turgeon, B.G. (2014) Vel2 and Vos1 hold essential roles in ascospore and asexual spore development of the heterothallic maize pathogen *Cochliobolus heterostrophus*. *Fungal Genetics and Biology*, 70, 113–124. https://doi.org/10.1016/j.fgb.2014.07.010.

Wang, Yonglin, Deng, C., Tian, L., Xiong, D., Tian, C. & Klosterman, S.J. (2018a) The transcription factor VdHapX controls iron homeostasis and is crucial for virulence in the vascular pathogen *Verticillium dahliae*. *mSphere*, 3. https://doi.org/10.1128/mSphere.00400-18.

Wang, Yonglin, Hu, X., Fang, Y., Anchieta, A., Goldman, P.H., Hernandez, G., et al. (2018b) Transcription factor VdCmr1 is required for pigment production, protection from UV irradiation, and regulates expression of melanin biosynthetic genes in *Verticillium dahliae*. *Microbiology,* 164, 685–696. https://doi.org/10.1099/mic.0.000633.

Wang, Yingming, Zhou, J., Zhong, J., Luo, D., Li, Z., Yang, J., et al. (2018) Cys2His2 zinc finger transcription factor BcabaR1 positively regulates abscisic acid production in *Botrytis cinerea*. *Applied and Environmental Microbiology*, 84. https://doi.org/10.1128/AEM.00920-18.

Wang, Z., Ma, T., Huang, Y., Wang, J., Chen, Y., Kistler, H.C., et al. (2019) A fungal ABC transporter FgAtm1 regulates iron homeostasis via the transcription factor cascade FgAreA-HapX. *PLOS Pathogens*, 15, e1007791. https://doi.org/10.1371/journal.ppat.1007791.

Wiemann, P., Willmann, A., Straeten, M., Kleigrewe, K., Beyer, M., Humpf, H.-U., et al. (2009) Biosynthesis of the red pigment bikaverin in *Fusarium fujikuroi*: genes, their function and regulation. *Molecular Microbiology*, 72, 931–946. https://doi.org/10.1111/j.1365-2958.2009.06695.x.

Wilson, R.A., Gibson, R.P., Quispe, C.F., Littlechild, J.A. & Talbot, N.J. (2010) An NADPH-dependent genetic switch regulates plant infection by the rice blast fungus. *Proceedings of the National Academy of Sciences*, 107, 21902–21907. https://doi.org/10.1073/pnas.1006839107.

Wong, K.-C. (2018) DNA motif recognition modeling from protein sequences. *iScience*, 7, 198–211. https://doi.org/10.1016/j.isci.2018.09.003.

Wong, K.H., Hynes, M.J. & Davis, M.A. (2008) Recent advances in nitrogen regulation: a comparison between *Saccharomyces cerevisiae* and filamentous fungi. *Eukaryotic Cell*, 7, 917–925. https://doi.org/10.1128/EC.00076-08.

Wong, K.H., Hynes, M.J., Todd, R.B. & Davis, M.A. (2009) Deletion and overexpression of the *Aspergillus nidulans* GATA factor AreB reveals unexpected pleiotropy. *Microbiology*, 155, 3868–3880. https://doi.org/10.1099/mic.0.031252-0.

Wong, K.H., Hynes, M.J., Todd, R.B. & Davis, M.A. (2007) Transcriptional control of *nmrA* by the bZIP transcription factor MeaB reveals a new level of nitrogen regulation in *Aspergillus nidulans*. *Molecular Microbiology*, 66, 534–551. https://doi.org/10.1111/j.1365-2958.2007.05940.x.

Wong Sak Hoi, J. & Dumas, B. (2010) Ste12 and Ste12-like proteins, fungal transcription factors regulating development and pathogenicity. *Eukaryotic Cell*, 9, 480–485. https://doi.org/10.1128/EC.00333-09.

Wu, D., Oide, S., Zhang, N., Choi, M.Y. & Turgeon, B.G. (2012) ChLae1 and ChVel1 regulate T-toxin production, virulence, oxidative stress response, and development of the maize pathogen *Cochliobolus heterostrophus*. *PLOS Pathogens*, 8, e1002542. https://doi.org/10.1371/journal.ppat.1002542.

Wu, Y., Xu, L., Yin, Z., Dai, Q., Gao, X., Feng, H., et al. (2018a) Two members of the velvet family, VmVeA and VmVelB, affect conidiation, virulence and pectinase expression in *Valsa mali*. *Molecular Plant Pathology*, 19, 1639–1651. https://doi.org/10.1111/mpp.12645.

Wu, Y., Xu, L., Yin, Z., Feng, H. & Huang, L. (2018b) Transcription factor VmSeb1 is required for the growth, development, and virulence in *Valsa mali*. *Microbial Pathogenesis*, 123, 132–138. https://doi.org/10.1016/j.micpath.2018.06.043.

Wu, Y., Yin, Z., Xu, L., Feng, H. & Huang, L. (2018c) VmPacC is required for acidification and virulence in *Valsa mali*. *Frontiers in Microbiology*, 9. https://doi.org/10.3389/fmicb.2018.01981.

Xiong, D., Wang, Y., Tang, C., Fang, Y., Zou, J. & Tian, C. (2015) VdCrz1 is involved in microsclerotia formation and required for full virulence in *Verticillium dahliae*. *Fungal genetics and biology*, 82, 201–212. https://doi.org/10.1016/j.fgb.2015.07.011.

Xu, T., Li, J., Yu, B., Liu, L., Zhang, X., Liu, J., et al. (2018) Transcription factor SsSte12 was involved in mycelium growth and development in *Sclerotinia sclerotiorum*. *Frontiers in Microbiology*, 9. https://doi.org/10.3389/fmicb.2018.02476.

Yang, F., Li, W., Derbyshire, M., Larsen, M.R., Rudd, J.J. & Palmisano, G. (2015) Unraveling incompatibility between wheat and the fungal pathogen *Zymoseptoria tritici* through apoplastic proteomics. *BMC Genomics*, 16, 362. https://doi.org/10.1186/s12864-015-1549-6.

Yang, H., Yu, H. & Ma, L.-J. (2020) Accessory chromosomes in *Fusarium oxysporum*. *Phytopathology*, 110, 1488–1496. https://doi.org/10.1094/PHYTO-03-20-0069-IA.

Yao, G., Zhang, F., Nie, X., Wang, X., Yuan, J., Zhuang, Z., et al. (2017) Essential APSES transcription factors for mycotoxin synthesis, fungal development, and pathogenicity in *Aspergillus flavus*. *Frontiers in Microbiology*, 8. https://doi.org/10.3389/fmicb.2017.02277.

Zabet, N.R., Foy, R. & Adryan, B. (2013) The influence of transcription factor competition on the relationship between occupancy and affinity. *PLOS One*, 8, e73714. https://doi.org/10.1371/journal.pone.0073714.

Zhang, H., Zhao, Q., Guo, X., Guo, M., Qi, Z., Tang, W., et al. (2014) Pleiotropic Function of the putative zinc-finger protein MoMsn2 in *Magnaporthe oryzae*. *Molecular Plant-Microbe Interactions*, 27, 446–460. https://doi.org/10.1094/MPMI-09-13-0271-R.

Zhang, N., NurAinIzzati, M.Z., Scher, K., Condon, B.J., Horwitz, B.A. & Turgeon, B.G. (2013) Iron, oxidative stress, and virulence: roles of iron-sensitive transcription factor Sre1 and the redox sensor ChAp1 in the maize pathogen *Cochliobolus heterostrophus*. *Molecular Plant-Microbe Interactions*, 26, 1473–1485. https://doi.org/10.1094/MPMI-02-13-0055-R.

Zhang, T., Sun, X., Xu, Q., Candelas, L.G. & Li, H. (2013a) The pH signaling transcription factor PacC is required for full virulence in *Penicillium digitatum*. *Applied Microbiology and Biotechnology*, 97, 9087–9098. https://doi.org/10.1007/s00253-013-5129-x.

Zhang, T., Xu, Q., Sun, X. & Li, H. (2013b) The calcineurin-responsive transcription factor Crz1 is required for conidation, full virulence and DMI resistance in *Penicillium digitatum*. *Microbiological Research*, 168, 211–222. https://doi.org/10.1016/j.micres.2012.11.006.

Zhang, W.-Q., Gui, Y.-J., Short, D.P.G., Li, T.-G., Zhang, D.-D., Zhou, L., et al. (2018) *Verticillium dahliae* transcription factor VdFTF1 regulates the expression of multiple secreted virulence factors and is required for full virulence in cotton. *Molecular Plant Pathology*, 19, 841–857. https://doi.org/10.1111/mpp.12569.

Zhao, C., Waalwijk, C., Wit, P.J.G.M. de, Lee, T. van der & Tang, D. (2011) EBR1, a novel Zn(2)Cys(6) transcription factor, affects virulence and apical dominance of the hyphal tip in *Fusarium graminearum*. *Molecular Plant-Microbe Interactions*, 24, 1407–1418. https://doi.org/10.1094/MPMI-06-11-0158.

Zhao, Y., Su, H., Zhou, J., Feng, H., Zhang, K.-Q. & Yang, J. (2015) The APSES family proteins in fungi: Characterizations, evolution and functions. *Fungal Genetics*

and Biology, 81, 271–280. https://doi.org/10.1016/j.fgb.2014.12.003.
Zhou, Y., Yang, L., Wu, M., Chen, W., Li, G. & Zhang, J. (2017) A single-nucleotide deletion in the transcription factor gene *Bcsmr1* causes sclerotial-melanogenesis deficiency in *Botrytis cinerea*. *Frontiers in Microbiology*, 8. https://doi.org/10.3389/fmicb.2017.02492.
Zhu, G., Yu, G., Zhang, X., Liu, J., Zhang, Y., Rollins, J.A., et al. (2019) The formaldehyde dehydrogenase SsFdh1 Is regulated by and functionally cooperates with the GATA transcription factor SsNsd1 in *Sclerotinia sclerotiorum*. *mSystems*, 4. https://doi.org/10.1128/mSystems.00397-19.
Zhu, X., Liu, W., Chu, X., Sun, Q., Tan, C., Yang, Q., et al. (2018) The transcription factor PstSTE12 is required for virulence of *Puccinia striiformis* f. sp. *tritici*. *Molecular Plant Pathology*, 19, 961–974. https://doi.org/10.1111/mpp.12582.
Zordan, R.E., Galgoczy, D.J. & Johnson, A.D. (2006) Epigenetic properties of white–opaque switching in *Candida albicans* are based on a self-sustaining transcriptional feedback loop. *Proceedings of the National Academy of Sciences*, 103, 12807–12812. https://doi.org/10.1073/pnas.0605138103.

Chapter 3

Transcription factor orthologues in plant-pathogenic fungi; connecting lineage diversity with fungal virulence

3.1. Background

Plant-pathogenic fungi have adapted to a wide range of ecological niches. It is now clear that pathogenic lifestyles, broadly defined as biotrophic, necrotrophic or hemibiotrophic, have evolved independently and across distant fungal lineages (Aylward et al., 2017; Möller and Stukenbrock, 2017; Ikeda et al., 2019). As such, the molecular mechanisms underpinning host infections are often specific to a pathogen or its close relatives. In recent years, large-scale comparative genomics and machine learning approaches have demonstrated that the enrichment of particular gene classes can be predictive of a pathogenic lifestyle. This includes carbohydrate-active enzymes, putative effectors, genes related to secondary metabolite (SM) biosynthesis and novel classes that currently lack functional annotations (Collemare and Lebrun, 2011; Pusztahelyi et al., 2016; Plissonneau et al., 2017; Hane et al., 2020; Haridas et al., 2020). For these genes to promote the virulence of a pathogen, their expression requires a coordinated regulation to maximise activity at the appropriate stage of infection (van der Does and Rep, 2017). Hence, the evolution of virulence encoding genes simultaneously requires the pathogen to evolve an appropriate regulatory system.

Transcription factors (TFs) regulate diverse aspects of fungal development and virulence, targeting either a small number of genes or by exerting broad control as ‘master’ regulators. Systematic reviews on the matter demonstrate that conserved TFs originally characterised in model saprophytes are now well-studied in plant-pathogenic fungi (Tan and Oliver, 2017; van der Does and Rep, 2017; John et al., 2021). However, there is extensive variation in the number of TFs belonging to different structural families across the fungal taxa (Todd et al., 2014; Shelest, 2017) and the role of most remains undefined. It is therefore anticipated that a functional exploration of the TFs unique to a fungal lineage or lifestyle will reveal novel regulators that distinguish a pathogen from a

non-pathogen. Already, TF gene duplications that are directly implicated in fungal virulence have been described in several lineages of *Fusarium oxysporum*, the causal agent of vascular wilt on a wide range of plants (de Vega-Bartol et al., 2010; Niño-Sánchez et al., 2016; van der Does et al., 2016). On the other hand, broadly conserved regulators of virulence-associated pathways in plant-pathogenic fungi, such as the Velvet TFs, are absent in saprophytic yeasts (Bayram and Braus, 2012; Calvo et al., 2016). Hence, in both their presence or absence, TFs are implicated in the distinct regulatory systems that have evolved to control plant-pathogenic lifestyles.

Random mutagenesis or high-throughput gene knockout studies are traditional approaches that have been used to identify novel virulence-regulating TFs. For example, VdFTF1 in *Verticillium dahliae*, which regulates the expression of secreted virulence factors on cotton, was identified by screening a random mutagenesis library (Zhang et al., 2018). On the other hand, a genome-wide TF knockout screen was undertaken for *Fusarium graminearum*, which identified several virulence regulators otherwise dispensable for saprophytic growth (Son et al., 2011). However, the generation and screening of fungal mutant libraries in this fashion can be labour intensive. A targeted approach that makes use of the annotated fungal genomes now available to disentangle regulators unique to a pathogenic lineage or lifestyle presents a powerful alternative to direct functional TF investigations.

A novel analysis is presented here that simultaneously assesses the TF repertoires (“TFomes”) from 100 phytopathogenic fungi alongside 20 saprophytes or symbionts for comparison. The TFs were refined into orthogroups; the smallest possible set of proteins where all their orthologues are included (Emms and Kelly, 2019). In doing

so, the relationships between TFs across plant-pathogenic fungi were systematically defined. The distinct aims were to:

- Identify evidence for TF conservation, expansion and loss that are associated with plant-pathogenic lifestyles.
- Provide a novel resource for tracing the evolutionary trajectory of TFs that are correlated with virulence in distinct fungal lineages.

Several orthogroups that harbour established fungal-virulence regulators are explored in detail, providing examples of TF conservation, expansion and loss connected to pathogenic lineages, but which also provided novel insights upon which future analyses can build.

3.2. Methods

3.2.1. Compiling fungal TFomes

Non-redundant fungal proteomes were sourced for 100 plant pathogens, 10 saprophytes and 10 symbionts from UniProt (release 2020_05) (Bursteinas et al., 2016) and also MycoCosm (Nordberg et al., 2014) or independent platforms where the proteomes were available with higher coverage assemblies (listed in **Supplementary item 3.1**). Pathogens were selected from diverse taxa of significant research interest (Pedro et al., 2016; Urban et al., 2020). The saprophytes and symbionts included both close and distant pathogen relatives across the taxa. Organisms were assigned a five letter organism ID (ORGID) for brevity (defined in **Supplementary item 3.1**), adapted from their NCBI Taxonomy database naming convention (Schoch et al., 2020). General lifestyles (biotroph, hemibiotroph, necrotroph, saprophyte or symbiont) and plant host-associations/dependency (i.e. obligate, facultative or not associated) were assigned based on descriptions from the published proteome sources and/or literature covering the matter (Möller and Stukenbrock, 2017; Hane et al., 2020; Haridas et al., 2020).

Interpro protein-domain annotations were also downloaded with the respective fungal proteomes. Interproscan (release 82.0) was used to annotate proteomes from the independent servers where these were not available (Blum et al., 2020). TFs were selected from the annotated proteomes by cross referencing a list of Interpro domain accessions that represent sequence-specific DNA-binding domains (DBDs). This list was adapted from a previous study (Shelest, 2017) to include additional known fungal TF DBDs (detailed in **Supplementary item 3.2**). Unique TF protein identifiers were assigned following the format ‘ProteinID | ORGID’ where the ‘ProteinID’ was derived from the downloaded proteome source. The total occurrences of each TF domain across fungal

proteomes, which included cases where multiple exist within a single protein, were then counted using a custom bash script.

3.2.2. TF orthology analysis and species tree inference

An orthology analysis for the entire TF set compiled from the TFomes was undertaken using OrthoFinder (release 2.4.0) (Emms and Kelly, 2019), with MMseqs2 (Steinegger and Söding, 2017) invoked for the sequence search stage. The TF distance-based species tree (which included here several sub-species taxonomic rankings), produced as part of the OrthoFinder analysis by STAG (Emms and Kelly, 2018), was manually rooted at the most distantly related pathogen *Synchytrium endobioticum* (SYNEN), inferred from the phylogeny documented in a previous study (Choi and Kim, 2017). OrthoFinder was subsequently invoked using the '-s' option and supplied the rooted-species tree to resolve the orthogroup 'protein trees' and infer the species-pair taxonomic orthologues. The protein trees were built using the DendroBLAST distance measure (Kelly and Maini, 2013) as part of the default OrthoFinder analysis. Tree visualisations and formatting were subsequently undertaken using iTOL (Letunic and Bork, 2019). NCBI taxonomic rankings for each respective phylum, class, order, family and genus were then sourced using Taxize (version 0.9.99) to demonstrate the clades in the TF distance-based species tree corresponded with designated fungal taxonomies (Chamberlain et al., 2020).

3.2.3. Data analysis and visualisation

Linear regressions were first produced to model the association between proteome size and TFome size for the entire set of 120 fungi, as well as for the lifestyle

and host-association sub-groups. The data analysis, formatting and plotting was undertaken in R (version 4) using the ggplot2, ggpubr, rstats, rstatix and emmeans packages (R Core Team, 2020). Pearson's correlation coefficients were calculated for each linear regression and used as the test statistic to assess whether the association was significant ($P < 0.05$). A covariance analysis (ANCOVA) was also undertaken to test whether the regression-slopes (i.e. the rate of TFome size increase relative to the proteome size) were different between the lifestyle or host-association groups (Bonferroni $Padj < 0.05$).

For visualisation and analysis, fungal TF orthogroup datasets were ordered as per the leaf-node order derived from the species tree, beginning with the root at *S. endobioticum*. Data formatting and the production of heatmaps was undertaken in R (version 4) using the phylogram, pheatmap, ggplot2, ggpubr and the rstats packages (R Core Team, 2020). The species-pair orthologue counts were plotted as a heatmap. To account for differences in individual TFome sizes, the counts were first divided by the sum TFs for each species-pair before plotting. A hierarchical clustering analysis was then conducted to group the TF orthogroups based on their counts per fungus. The Z-scores for each count were first calculated similarly to a previous TF analysis (Charoensawan et al., 2010b), which represented a clustering measure independent of the differences in orthogroup sizes. Clustering distances were calculated based on Pearson’s correlation and the orthogroups were clustered using the Average linkage function.

3.3. Results & Discussion

3.3.1. Correlations between TFome and proteome sizes in plant-pathogenic fungi

Fungal TFomes were defined as the set of proteins harbouring at least one sequence-specific DBD as previously (Park et al., 2008; Shelest, 2008; Todd et al., 2014; Shelest, 2017). From the most recent study (Shelest, 2017), the list of Interpro domains corresponding to fungal TFs was refined (**Supplementary item 3.2**) by incorporating 16 additional sequence-specific DBDs such as the Velvet domain (IPR037525), the high-mobility-group box domain (IPR009071), the APSES/Swi6 domain (IPR001606) and the Sant/Myb domain (IPR001005). Conversely, two domains were excluded (IPR012340 and IPR001878) that did not represent sequence-specific DBDs. Hence, this represented a further refinement of the TF-annotation criteria from the previous studies.

The 120 fungal TFomes were first assessed as a proportion of the proteome sizes, to gauge any broader trends in the TF-regulatory capacity among plant pathogens. These ranged in size from 189 (for *Blumeria graminis* f. sp. *tritici*; BLUGT) to 1,493 (for the *F. oxysporum* biocontrol strain Fo47; FUSO4). The average TFome size was 473 from an average proteome size of 12,355. This corresponded to a median of 437.5 and 12,296.5 respectively. The TF fraction of the proteomes ranged between 1.2% (for *Puccinia triticina*; PUCTR) to 6.9% (for FUSO4) with an average of 3.9%. The highest proportion of TFs were generally observed among the *Fusarium* lineages, while rust pathogens such as *Melampsora* and *Puccinia* spp. shared a relatively low TF content. Overall, a moderate-linear correlation (R = 0.58) was observed between TFome size and proteome size (**Figure 3.1**) (Mukaka, 2012). A previous study that assessed a broad range of eukaryotes reported a stronger correlation (R = 0.78) with proteome size (Charoensawan

et al., 2010a). This was enough to suggest additional lifestyle factors contributed to higher variation among the predominantly plant-pathogenic fungi that were assessed here.

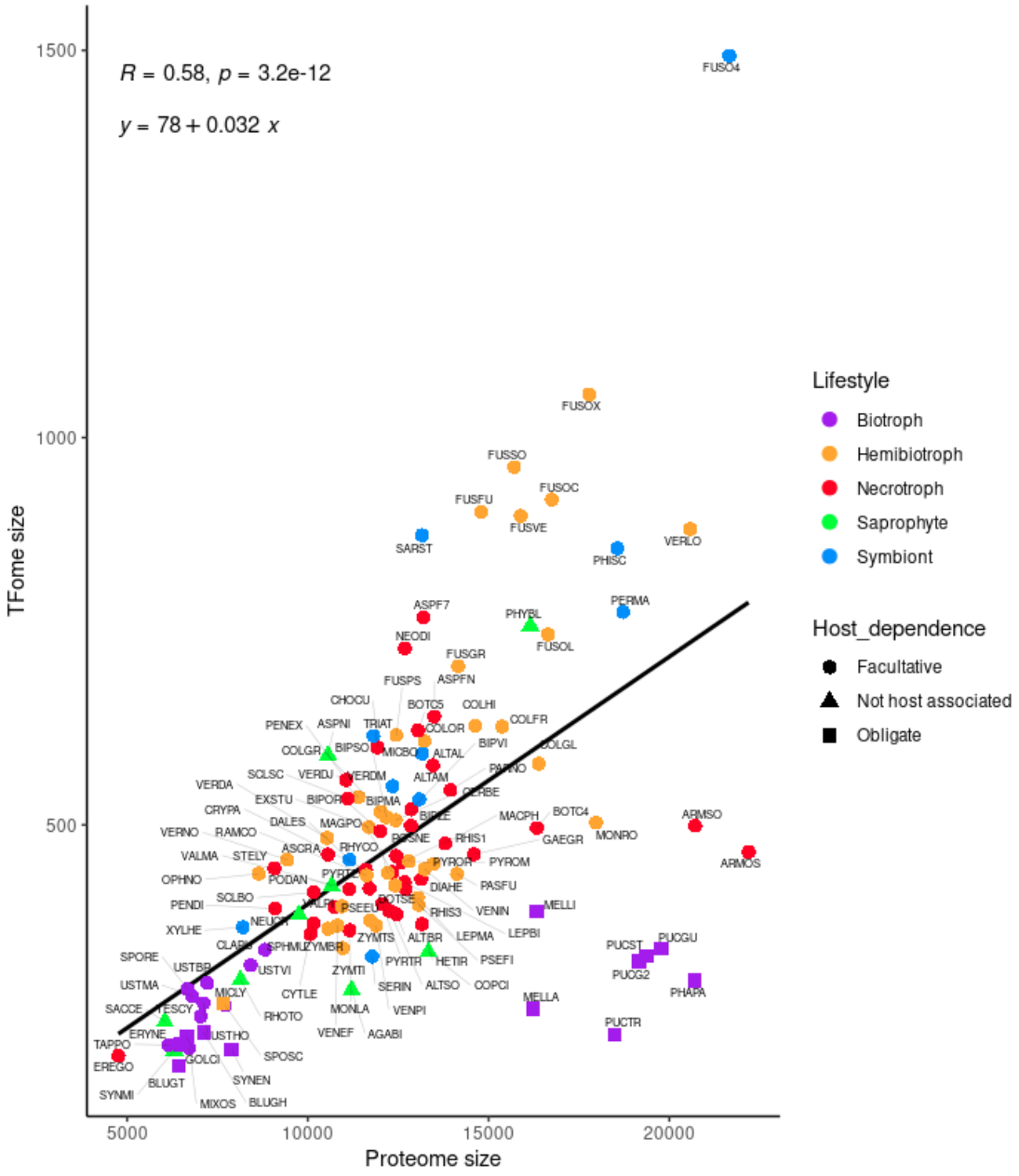
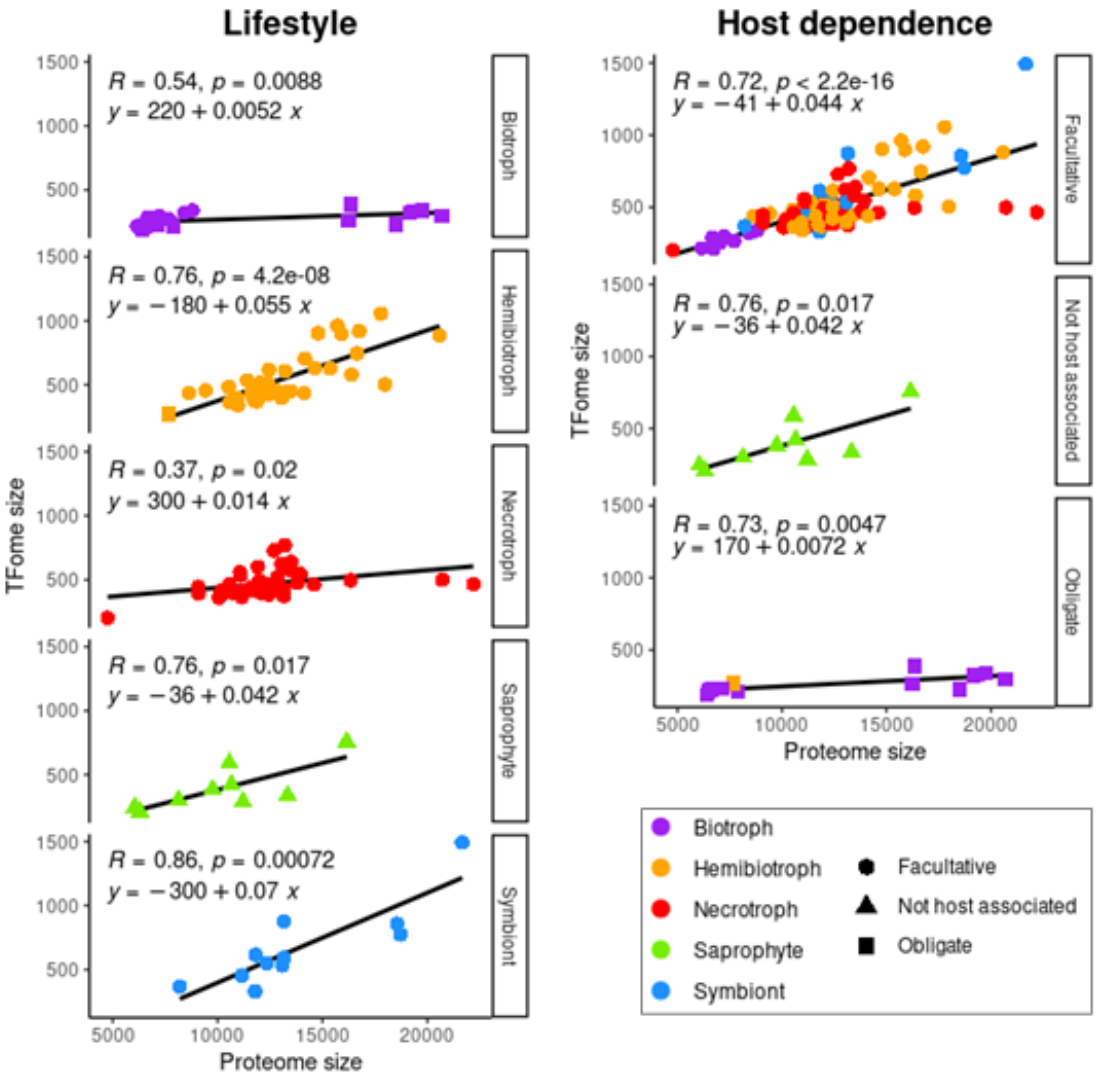


Figure 3.1 - Fungal transcription factor content (TFome) relative to proteome size

TFome vs proteome sizes for the 120 fungi used in this analysis. The five-letter fungal ORGIDs (**Supplemental item 3.1**) are used to label the respective points which provide an indication of the respective lifestyle and host-association/dependency. The correlation coefficient (*R*) and P-value (*p*) used to test for any association ($P < 0.05$) and linear regression (*y*) for the relationship are presented at the upper-left of the plot.

The TF content of fungal sub-groups, defined by their lifestyle or association with/dependence upon a plant host, were then compared to try and identify any distinct size trends relevant to phytopathogenicity. For each pathogenic (i.e. biotrophic, hemibiotrophic or necrotrophic) and non-pathogenic (i.e. saprohphytic or symbiotic) lifestyle explored, TF content was still found to correlate linearly with proteome size (**Figure 3.2**), with no significant differences observed in the rate between these groups. In contrast, for fungi grouped based on their host-associations (i.e. facultative, obligate or not host-associated), the TF content of obligate-host fungi relative to the proteome size increases at a significantly lower rate than the facultative pathogens and non-host saprophytes (**Figure 3.2**). This was typified by the rust and mildew pathogens from the Pucciales (1.7% TF fraction) and Erysiphales (3.2% TF fraction) fungal orders. This provided evidence that a restricted regulatory capacity exists where fungal pathogens rely on a host plant for their survival. Based on this, it was hypothesised that fungi adapt to a broader environmental range, in large-part, by harnessing a broader set of distinct TFs that modulate gene expression in response to diverse stimuli.

While the evidence suggested that a capacity to inhabit diverse ecological niches represents a general selective pressure to maintain TF diversity, the goal remained to define specific TFs that evolve with pathogenicity. A cursory look at TFs classified by their families paralleled previous analyses (Todd et al., 2014; Shelest, 2017), which revealed that the Zn2Cys6 and C2H2 zinc finger DBDs were the most prevalent among fungi. A total of 32,023 Zn2Cys6, 11,436 C2H2, 4,777 homeodomain, 3,168 bZIP and 2,932 CCCH-type TF domains represented the top five observed (**Supplementary item 3.2**). The sizes of such families make it difficult to classify any specific regulators, which then motivated a more refined analysis to identify the TFs that define specific pathogenic lifestyles in distinct fungal lineages.



Fungal division	ANCOVA	Regression line groupings
Lifestyle	Pval = 0.561	Biotroph[A], Hemibiotroph[A], Necrotroph[A], Saprophyte[A], Symbiont[A]
Host dependence	Pval = 9.13E-5*	Facultative[A], Not host associated[A], Obligate[B]

Figure 3.2 - A comparison of the transcription factor proportion of fungal proteomes grouped by lifestyle or host-association

TFome vs proteome sizes are depicted along with the correlation coefficients (*R*) and *P*-values (*p*) for the respective linear regressions (*y*) in each plot. The panel on the left depicts the relationships by lifestyle and the right panel by host-association for the 120

fungal proteomes assessed in this study. The bottom table summarises where a statistically significant difference (Pval < 0.05)* exists across the regression rates determined by ANCOVA. Superscript letters ^{A} or ^{B} indicate where individual differences occur within the groups.

3.3.2. Lineage-specific TF orthogroup expansions among plant-pathogenic fungi

The 56,754 fungal TFs were first resolved into 855 orthogroups (**Supplementary item 3.3**). This refined the DBD classification system where only 70 distinct TF families with ≥10 TFs existed overall (**Supplementary item 3.2**). An assessment was then undertaken to ensure the evolutionary distances used to define the TF orthogroups had been accurate. This first involved a comparison of the TF orthogroup-derived species tree with the clade architecture of the fungal taxonomy tree (**Supplementary item 3.4 & 3.5**). Inspection of the tree leaves and nodes revealed that the topology of both trees were overwhelmingly consistent **Figure 3.3**. This suggested the TF-orthologue distances had correlated well overall with the pattern of species divergence. Building on the taxonomic comparison, a species vs species assessment of the TF fraction that were inferred as taxonomic-orthologues was conducted. This controlled for gene duplications post-speciation and was normalised to the total TFs from the species pair. A distinctly larger proportion of TF orthologues were identified among closely related fungi compared to the more distant lineages (**Figure 3.4**). Taken together, both assessments indicated the orthogroup analysis using fungal TFomes was reliable and could be used to identify novel TF relationships and trends.

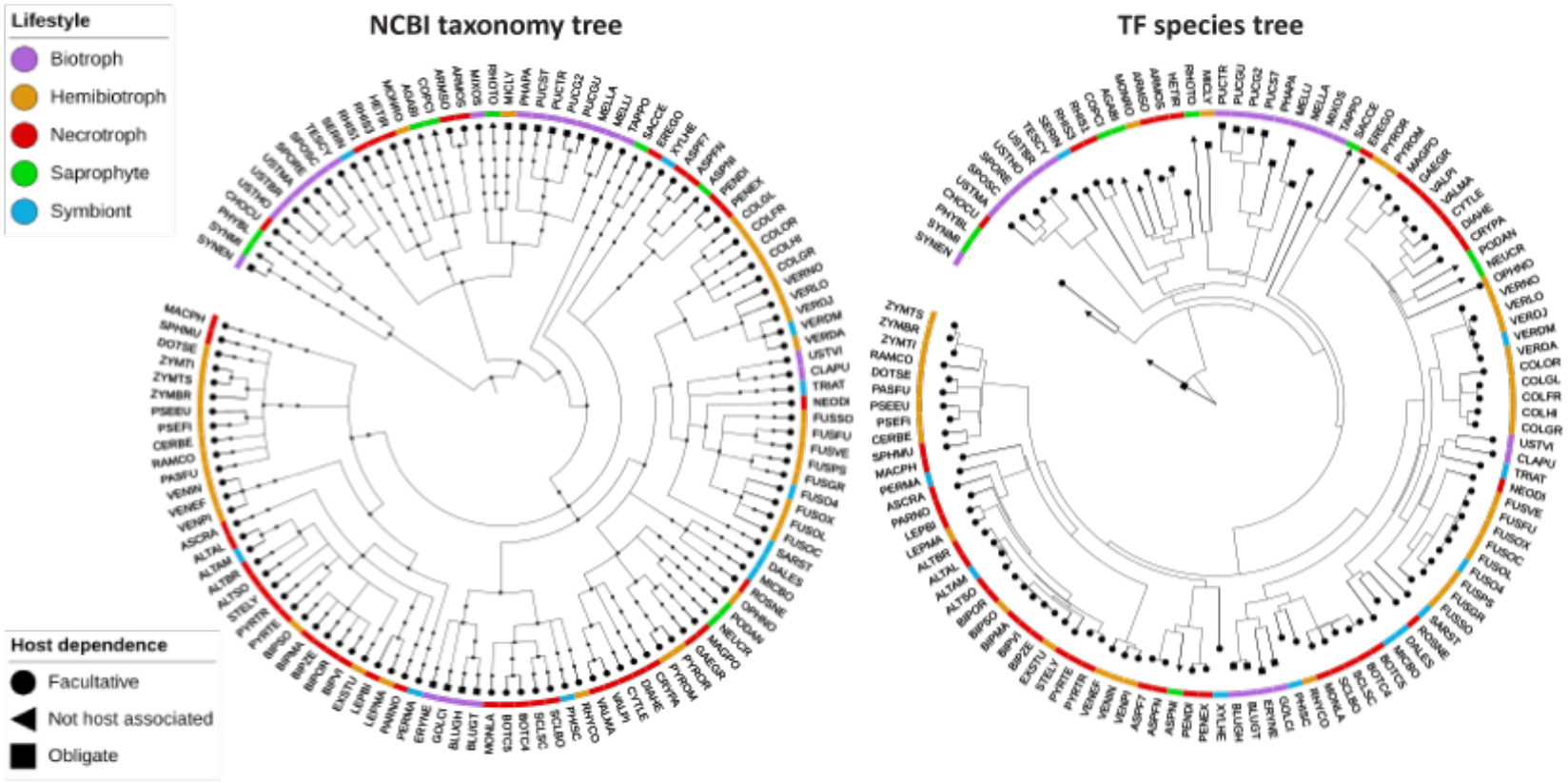


Figure 3.3 – NCBI taxonomy relative to the transcription factor (TF) distance-based species tree

Clade architectures derived from NCBI taxonomic divisions (node dots) are presented in the left tree, demonstrating shared topologies with the TF distance-based species tree. Fungal ORGIDs are used at branch tips due to space constraints. The corresponding trees using the full species names are available for higher resolution viewing as **Supplementary item 3.4 & 3.5**.

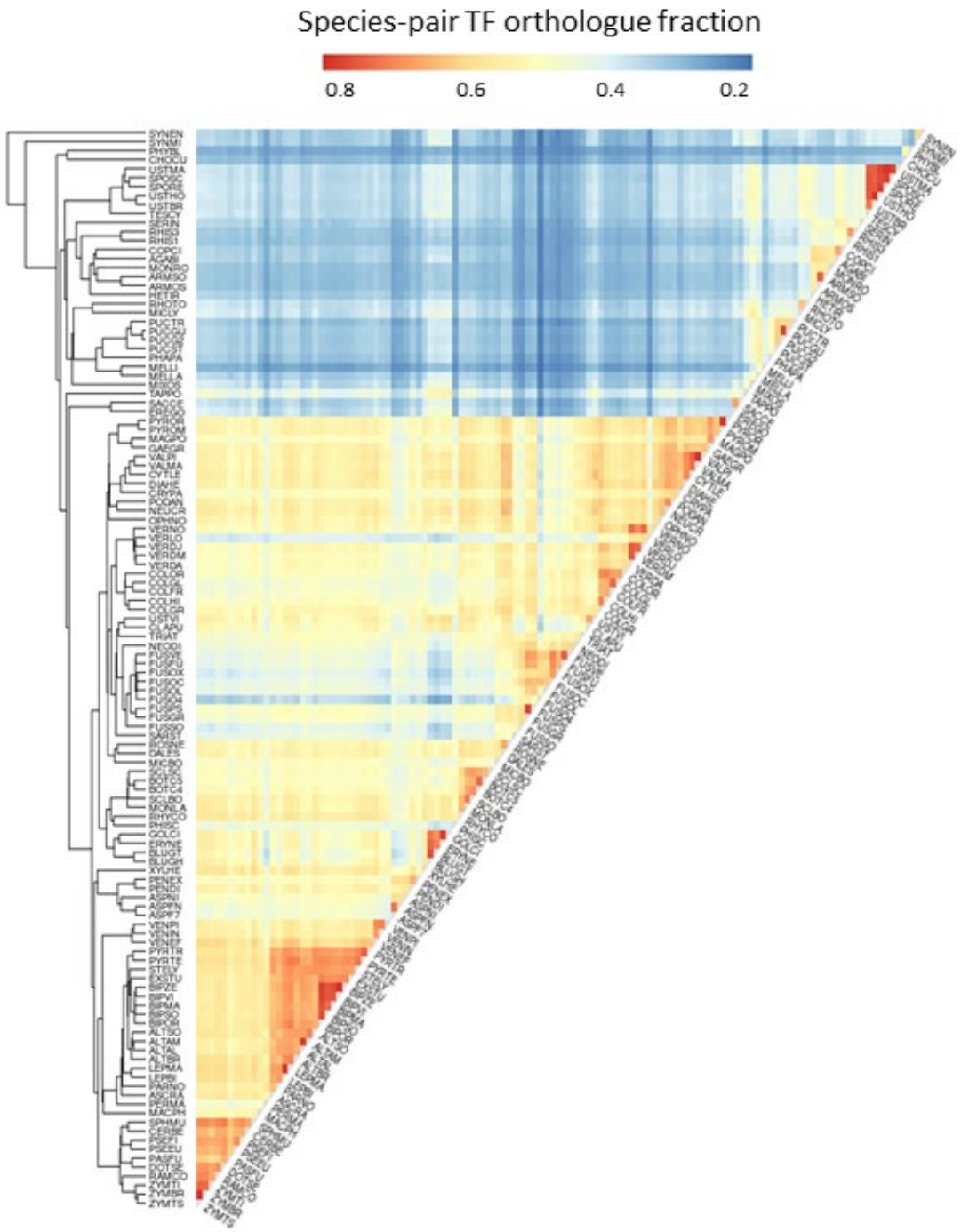


Figure 3.4 – A heatmap of fungal species-pair transcription factor (TF) orthologues

A heatmap depicting the relative fraction of shared TFs (TF orthologues) between each fungal species-pair (total predicted orthologues divided by the mean species-pair TFome size). Fungal ORGIDs are listed for pairwise-comparison on the Y-axis and the diagonal based on the TF-species tree order depicted in **Figure 3.3**.

A key goal of the analysis was to better classify TFs of relevance to fungal virulence by assessing the extent to which TF orthologues are specific to, or expanded in distinct pathogen lineages, a potential indicator for virulence-associated selection. The TF orthogroups were therefore clustered together based on their normalised counts across the 120 fungi to group those highly represented in distinct lineages (**Supplementary item 3.6**). Inspection of the corresponding heatmap (**Figure 3.5**) highlighted that for most fungi, TFs overrepresented at either their genus, species or sub-species level could be easily identified. While the relevant consequences in the capacity for individual fungi to regulate virulence will need to be functionally assessed case-by-case, the analysis demonstrates that evolutionarily recent TF orthologue expansion or loss events are widespread, providing a foundation for future investigations. Some examples are discussed in the next section.

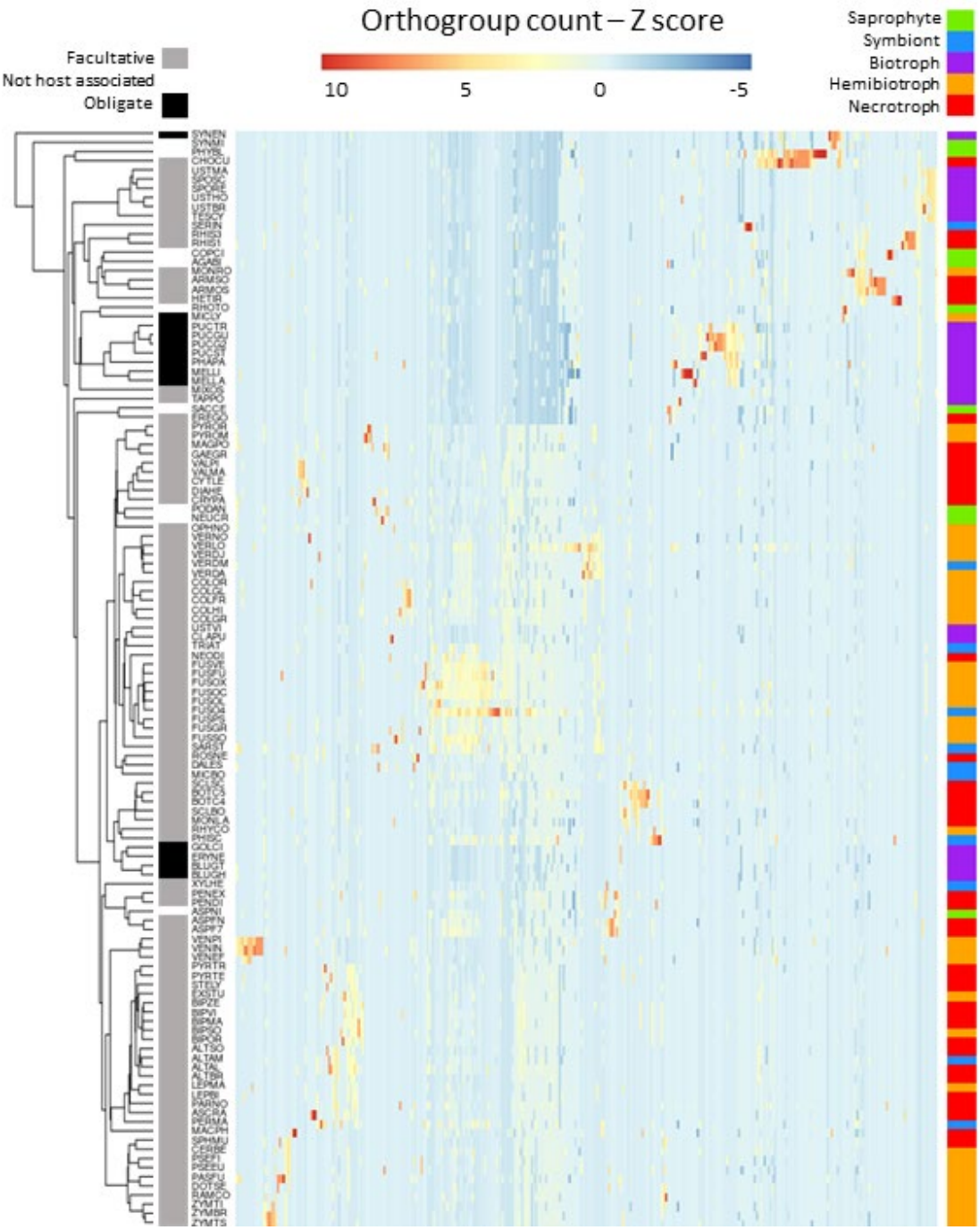


Figure 3.5 – Relative proportion of transcription factors (TFs) within orthogroups across the 120 fungi assessed in this study

A heatmap depicting TF expansion based on relative counts (Z scores) for each of the 855 orthogroups defined in this analysis (arrayed on the X axis and clustered using the Euclidean distance measure). Fungal ORGIDs are listed on the Y-axis based on the TF-

species tree order depicted in **Figure 3.3**. Coloured markers indicate host-association (left) or lifestyle (right).

3.3.3. Regulators of virulence in conserved and recently evolved TFs

The breadth of the 855 orthogroups defined in the analysis constrained the extent to which individual fungal lineages could be assessed in detail. Instead, the evolutionary trajectories for several established virulence regulators are now explored in the context of their respective orthogroups to provide examples where novel insights can be gained into fungal plant pathogenicity. This makes use of the orthogroup 'protein trees' produced from the analysis which allow the origins of TF duplication and/or loss to be traced across fungal lineages. The respective protein trees for each orthogroup (OG0000000 > OG0000854) are provided as supplementary material (**Supplementary item 3.7**). The virulence regulators covered include TFs regulating either a broad or restricted set of genetic pathways, as well as those that are conserved across fungal taxa and those where orthologues are difficult to trace.

3.3.3.1. Pf2 TFs are phylogenetically grouped with regulators of carbon metabolism

Orthogroup OG0000017 comprises 370 putative TFs that belong to the Zn2Cys6 DBD family. These are represented across the Ascomycota, in most cases with multiple TFs identified for each fungus. This includes *Saccharomyces cerevisiae* MAL13, ZNF1, MAL33 and YGR288W that have all been shown to target either the aerobic and/or anaerobic pathways regulating carbon metabolism (Akache et al., 2001; Tangsombatvichit et al., 2015). Analysis of the OG0000017 phylogenetic tree suggests these are all paralogues relative to the other TFs (**Figure 3.6.A**). OG0000017 also includes AmyR (ANIA_02016; **Figure 3.6.B**), a TF well-established as a regulator of polysaccharide hydrolysis in the filamentous saprophyte *Aspergillus nidulans* (Tani et al.,

2001; Nakamura et al., 2006). Their common link to carbon metabolism is also conserved in a clade distal to both (**Figure 3.6.C**), which encompasses Pf2 and several other related TFs that were detailed previously for their fundamental role in phytopathogenicity (**Chapter 2**) (Cho et al., 2013; Chung et al., 2013; Oh et al., 2016; Jones et al., 2019; Habig et al., 2020; Han et al., 2020).

This analysis demonstrates each of the the aforementioned pathogenicity regulators are traceable as direct taxonomic orthologues of *Neurospora crassa* Col-26 (**Figure 3.6.C**), a TF extensively characterised in this saprophytic fungus for its role in polysaccharide metabolism linked to plant cell walls (Xiong et al., 2017; Li et al., 2021). It is interesting to speculate that the ancestral Pf2 TF would have been amenable to the adaptation of a co-regulatory capacity for virulence factors that enable the metabolism of host tissue such as polysaccharide-rich cell walls. It was also intriguing that the Pf2/Col-26 monophyletic clade within OG0000017 did not include TFs from the Eurotiomycetes or Saccharomycetes such as AmyR or MAL13, but is conserved among the other Ascomycete classes. Phenotypic screening of Zn2Cys6 gene-deletion mutant libraries in *Magnaporthe* (*Pyicularia*) *oryzae* and *Fusarium graminearum* covers three and nine of the TFs in OG0000017 respectively (Son et al., 2011; Lu et al., 2014). These mutants did exhibit obvious pathogenicity defects, suggesting the Pf2 orthologues are uniquely adapted for this role among homologous TFs with a shared evolutionary origin. Deeper functional investigation into these TFs would be required to demonstrate this is the case or identify any shared or dissimilar roles with Pf2 in plant-pathogenic fungi.

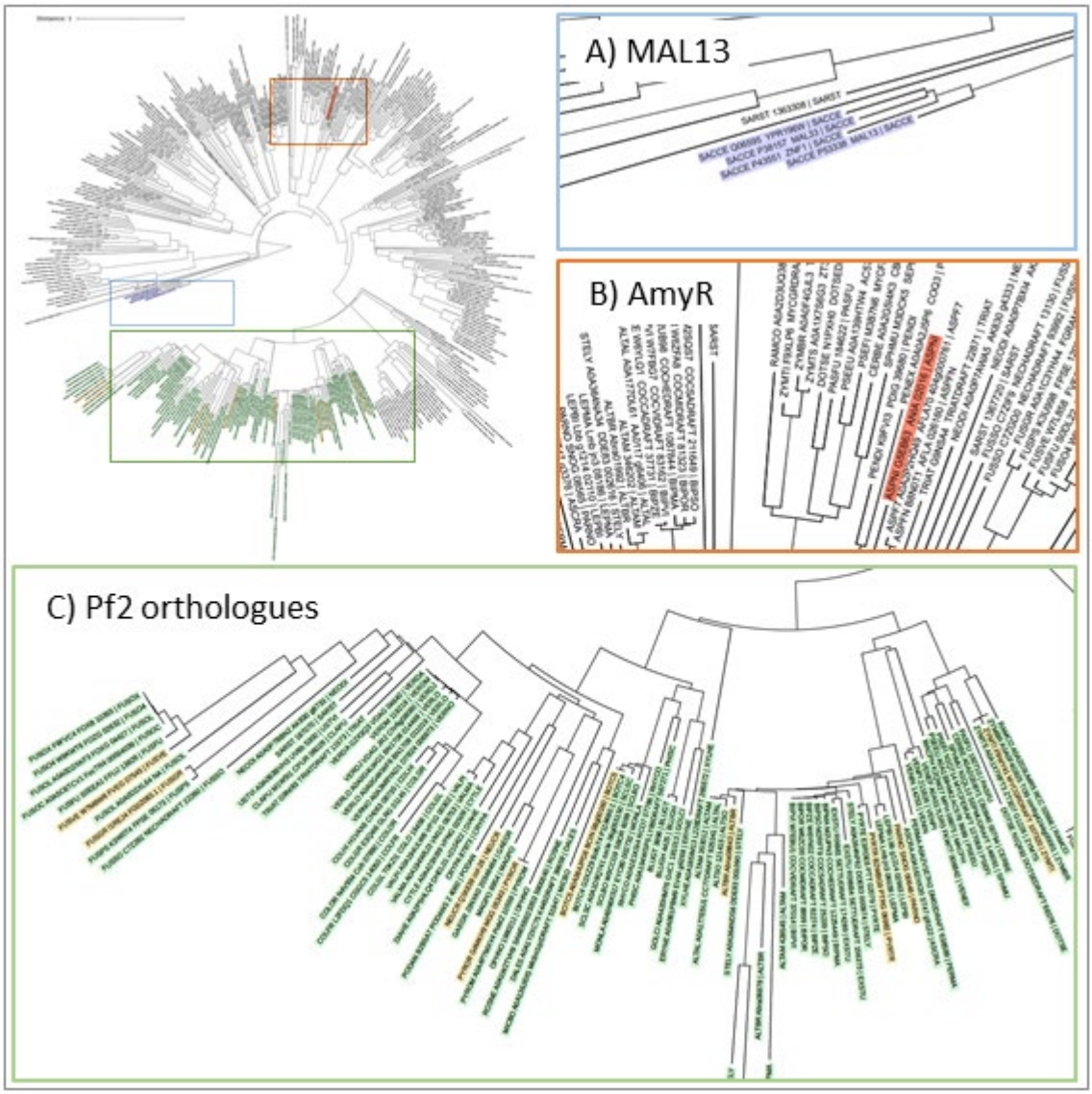


Figure 3.6 – Orthogroup OG0000017 protein tree containing Pf2 orthologues

The *S*. *cerevisiae* MAL13 outgroup (**A**) is highlighted for reference in blue, the *A*. *nidulans* AmyR (**B**) in orange and the Pf2 taxonomic clade (**C**) in green. The functionally defined Pf2 orthologues in the scientific literature are highlighted in yellow (represented by the fungal ORGIDs for FUSVE, FUSGR, NEUCR, PYROR, BOTC5, ALTBR, PYRTR, PARNO and ZYMTI). The branch length is defined by the DendroBLAST distance measure (Kelly and Maini, 2013). A full resolution version of this tree is supplied as **Supplemental item 3.8**.

3.3.3.2. Domain architecture variation in Ste12 TFs

Ste12 TFs are downstream targets of mitogen-activated protein kinase (MAPK) signalling that control expansive hyphal growth (as opposed to yeast-like budding growth) and the pheromone response (sexual development) in *S. cerevisiae* (Rispail and Di Pietro, 2010; Zhou et al., 2020). An analogous role has been attributed in fungal phytopathogens, where Ste12 TFs play a significant role in plant virulence that is manifested by its regulation of invasive hyphal growth (**Chapter 2**). An analysis of the corresponding OG0000022 phylogenetic tree reveals Ste12 TFs are conserved even among the early-diverging fungal lineages such as the Chytridiomycota and Mucoromycota (**Figure 3.7.A**). However, Ste12 orthologues were not detected among plant-pathogenic fungi of the Ustilaginomycetes class. This contrasted with the other Basidiomycetes such as *Puccinia striiformis*, where it is essential for pathogenicity on wheat (Zhu et al., 2018).

Interestingly, Ste12 orthologues in filamentous fungi also harbour C2H2 DBDs located at the C-terminus (Wong Sak Hoi and Dumas, 2010; Gu et al., 2015; Sarmiento-Villamil et al., 2018). In *Ustilago maydis*, a prominent maize pathogen of the Ustilaginomycetes, all three TFs in OG0000022 (Biz1, Mzr1 and Ztf1) play important, interconnected roles promoting infectious growth *in planta* (Flor-Parra et al., 2006; Zheng et al., 2008; Velez-Haro et al., 2020). However, none of these were traceable as Ste12 orthologues (**Figure 3.7.B**). This is likely because they only possess the C2H2 DBD, suggesting the Ste12 domain was lost in this lineage. Paradoxically, the sexual cycle is intimately connected with host invasion in *U. maydis* (Vollmeister et al., 2012), both roles driven by Ste12 regulation in other fungi. This indicates a regulatory shift has occurred in the Ustilaginomycetes, possibly involving Biz1, Mrz1 and Ztf1, following the loss of the Ste12 domain in this lineage. It would be worth exploring whether any conserved

functional link to MAPK signalling or DNA-binding exists in these C2H2-only TFs relative to the Ste12+C2H2 domain TFs in other filamentous fungi.

Virulence-associated roles were not yet reported for the other functionally-characterised fungal TFs in the C2H2-only clade. These included the BrlA conidiation regulators from the Aspergillaceae family (**Figure 3.7.B**) and several that were phenotypically assessed by screening C2H2 gene-deletion mutant libraries in *M. oryzae* and *F. graminearum* (Kwon et al., 2010; Son et al., 2011; Wang et al., 2015; Cao et al., 2016). Taken together, the analysis of the OG0000022 orthogroup presents an interesting case of TF domain loss and adaptation.

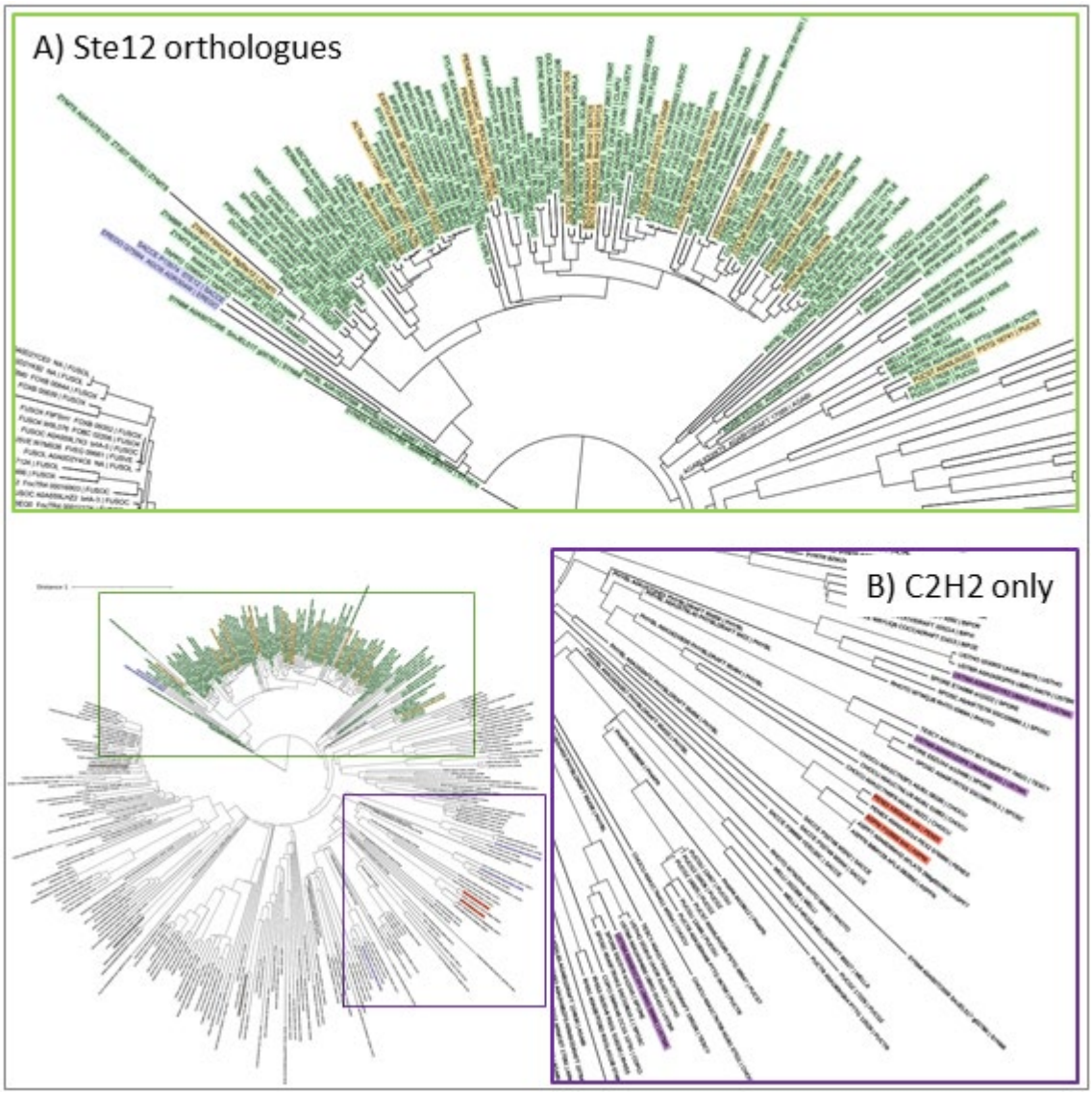


Figure 3.7 – Orthogroup OG0000022 protein tree containing Ste12 orthologues

The Ste12 orthologues (**A**) are highlighted for reference in green with those that have been functionally defined in the literature depicted in yellow (represented by the fungal ORGIDs for ZYMTI, ALTBR, ALTAL, EXSTU, PENDI, PENEX, SCLSC, BOTC5, FUSGR, FUSOX, PYROR, CRYPA, VERDA, COLOR and PUCST). The Ste12 orthologues that lack C-terminal C2H2 domains (for SACCE and EREGO) are highlighted in blue. The ancestral lineage lacking the Ste12 domain (i.e. the C2H2-only proteins) includes the functionally defined *U. maydis* (USTMA) transcription factors Biz1, Mrz1 and Ztf1, which

are highlighted in purple (**B**). The BrlA orthologues within this lineage (represented by PENDI and ASPNI) are highlighted in orange. The branch length is defined by the DendroBLAST distance measure (Kelly and Maini, 2013). A full resolution version of this tree is supplied as **Supplemental item 3.9**.

3.3.3.3. Evidence for further expansion of the EBR1 TFs

The Zn2Cys6 TF EBR1 was first reported and characterised in *F. graminearum* through mutagenesis screening, with the large protein size and positioning of the DBD at the C-terminus unusual features for the Zn2Cys6 family (Dufresne et al., 2008; Zhao et al., 2011). The TF suppresses hyphal branching and promotes radial growth that facilitates host invasion in *Fusarium* spp. (**Chapter 2**). In *F. oxysporum*, gene expansions are a characteristic of lineage-specific chromosomes (Coleman et al., 2009; Niño-Sánchez et al., 2016). This phenomenon is also well-documented for *EBR1*, whereas many as nine paralogues have been reported (Jonkers et al., 2014; van der Does et al., 2016). An analysis here of the corresponding OG0000121 protein tree highlights such cases (**Figure 3.8.A**), but also reveals EBR1 expansion in Ascomycete plant pathogens.

In the *M. oryzae* reference isolate causing rice blast (PYROR) both MoCod2 (MGG_09263) and Cnf2 (MGG_15023) are TFs required for pathogenic development (Chung et al., 2013; Lu et al., 2014). The OG0000121 phylogenetic analysis indicates both are orthologous to *Fusarium* EBR1 (**Figure 3.8.B**). In the *M. oryzae* millet pathovar included in this study (PYROM), this analysis also indicates three additional MoCod2 paralogues are present. The functional connection between MoCod2 and Cnf2 has not yet been explored in *M. oryzae* and may reveal novel insights into the evolution and regulation of virulence.

Duplication events are also evident for EBR1 orthologues in the Mycosphaerellaceae family, which encompasses several damaging pathogens (**Figure 3.8.C**). Although functional analyses are limited to the overexpression of *AlmA* (Mycgr3g111569) in *Zymoseptoria tritici*, this suggested an involvement in hyphal development during axenic growth (Cairns et al., 2015).

It was striking that the majority of pathogens discussed where EBR1 expansion events are observed are considered hemibiotrophs. That is, they exhibit a latent period during infection where they expand throughout the host before the onset of symptoms. It is plausible that a consequence of EBR1 duplication is the up-regulation of genes underpinning radial growth, thereby promoting asymptomatic colonisation of the host. Whilst speculative, the novel evidence for gene expansions in key pathogen lineages, in particular the Mycosphaerellaceae, warrants further exploration to establish their individual and/or connected roles during host infection. Furthermore, the OG0000121 example highlights the utility of the orthology analysis conducted here for identifying TF relationships that traditional homology-based searches can overlook.

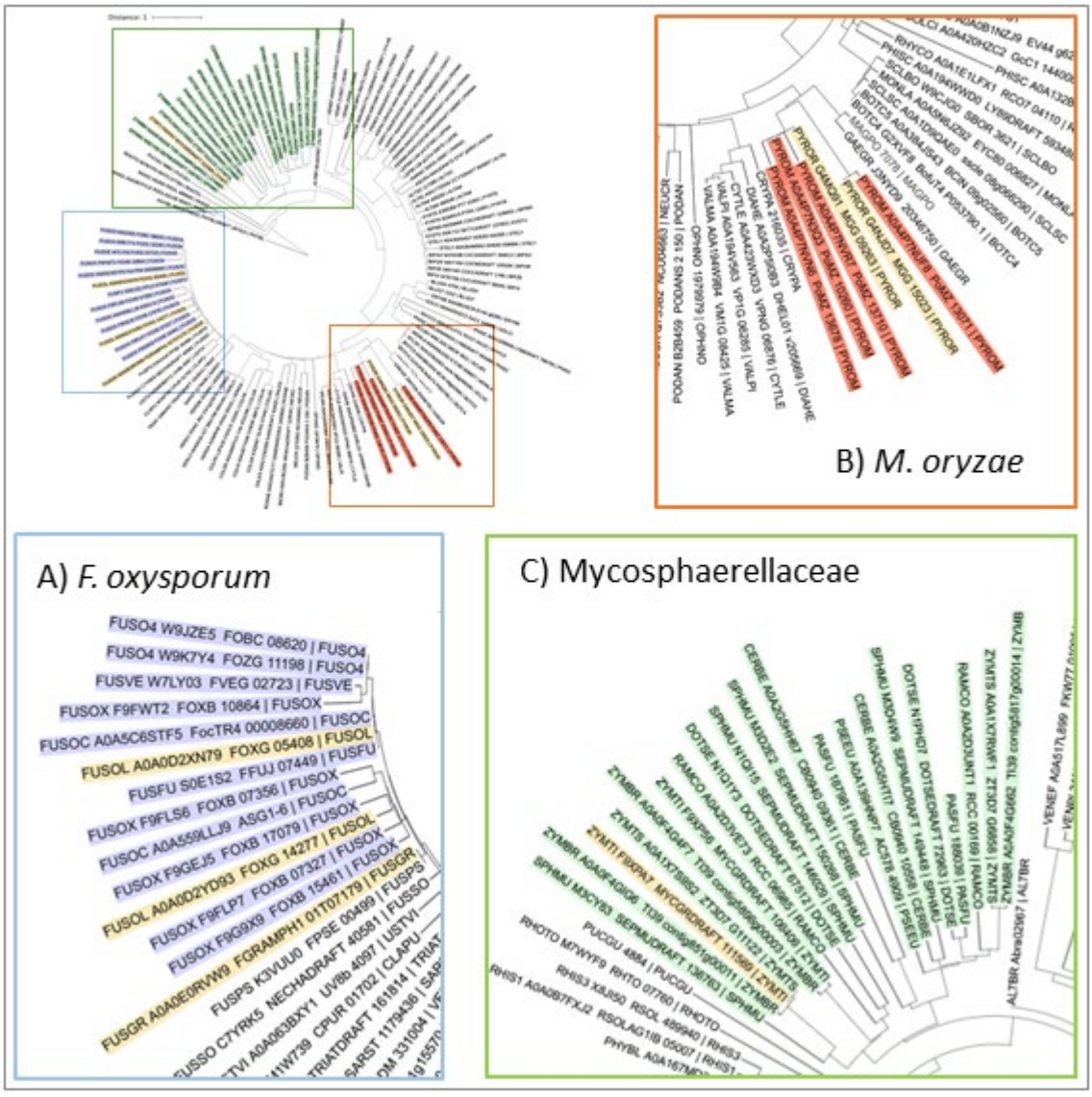


Figure 3.8 - Orthogroup OG0000121 protein tree representing EBR1 orthologues

The functionally-defined EBR1 orthologues are highlighted in yellow (represented by the fungal ORGIDs for FUSOL, FUSGR, ZYMTI and PYROR). Putative copy-number expansions in the (**A**) *Fusarium*, (**B**) *M. oryzae* and (**C**) the Mycosphaerellaceae lineages are depicted in the respective boxes by multiple instances of the same ORGID in the protein identifier at the branch tip. The branch length is defined by the DendroBLAST distance measure (Kelly and Maini, 2013). A full resolution version of this tree is supplied as **Supplemental item 3.10**.

3.3.3.4. Tracing phytotoxic SM cluster regulators

For many of the pathway-specific TFs that regulate SM gene clusters, common lineages are difficult to trace. This may stem from a propensity for both diversifying selection, the frequent loss of SM gene clusters or their horizontal transfer across fungal genomes (Lind et al., 2017; de Jonge et al., 2018; Rokas et al., 2018). Consequently, a close inspection of adjacent branches on the respective TF protein trees produced in this analysis can reveal some surprising relationships among the regulators of phytotoxic SM biosynthesis.

The Zn2Cys6 TF BcBot6 from *Botrytis cinerea*, which regulates the biosynthesis of the phytotoxin botrydial, was grouped within the OG0000292 orthogroup (Porquier et al., 2016). The ortholog to BcBot6 from the respective T4 and B05.10 reference isolates, was an uncharacterised putative TF (DHEL01_v212770) from the sunflower pathogen *Diaporthe helianthi* (**Figure 3.9.A**). *D. helianthi* is reported to secrete a several SMs that are phytotoxic (Ruocco et al., 2018). However, it is not known how these SMs are regulated, or if any are structurally analogous to botrydial. Therefore, it would be appropriate to explore the role of DHEL01_v212770 in *D. helianthi* SM regulation as well as cross complement *bcbot6* deletion mutants of *B. cinerea* to determine if DHEL01_v212770 is functionally conserved.

Another *B. cinerea* phytotoxic SM regulator is BcBOA13, which controls botcinic acid biosynthesis and is present in the B05.10 isolate but missing in the T4 isolate (Dalmais et al., 2011; Porquier et al., 2019). Interestingly, a corresponding putative botcinic acid biosynthetic cluster was previously identified in the genome of *Sclerotinia sclerotiorum*, a broad-host range pathogen that is closely related to *B. cinerea* (Porquier

et al., 2019). However, inspection of the BcBOA13 clade in the OG0000000 protein tree revealed the homologous protein sscle_15g106440 was absent (**Figure 3.9.B**). Closer inspection revealed the DBD for sscle_15g106440 is missing (Uniprot accession A0A1D9QLR3) and was therefore not included as a TF in the current study. It will be important to functionally assess whether this is a mis-annotation, or whether the SM cluster in *S. sclerotiorum* is inactive due to mutation of sscle_15g106440.

The nearest branches in the BcBOA13 clade correspond to TFs regulating the biosynthesis of aspirochlorine, a mycotoxin produced in *Aspergillus* spp. with no structural relationship to botcinic acid having been described (Chankhamjon et al., 2014; Uka et al., 2020). Hence, it is unlikely that BcBOA13 is horizontally transferred among the fungi assessed here.

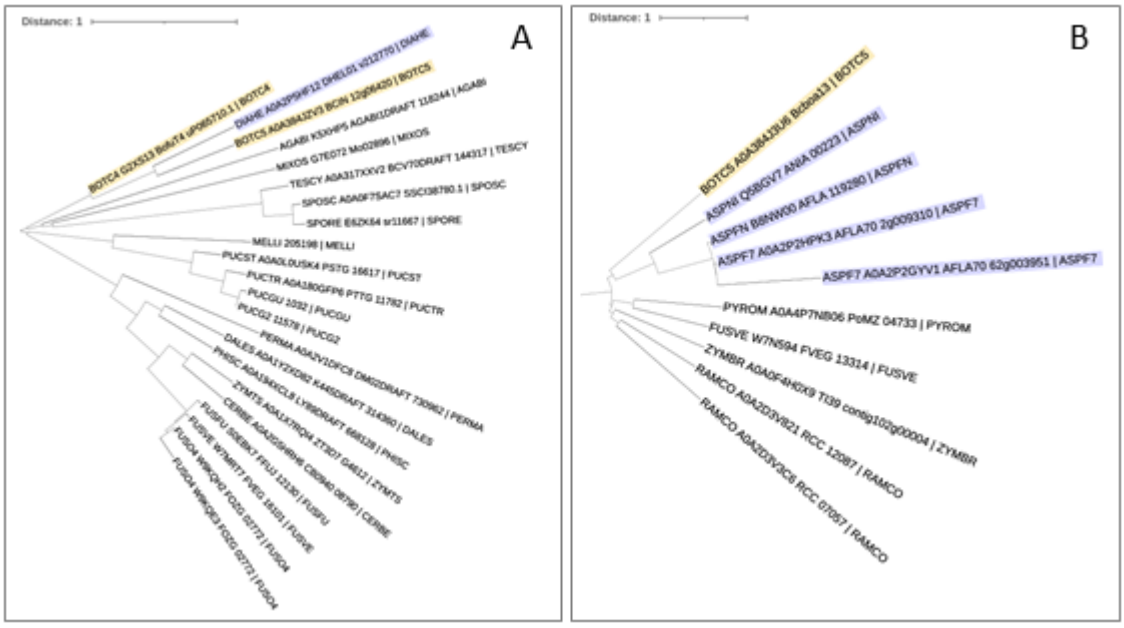


Figure 3.9 – Orthogroup OG0000292 (*BcBot6*) and OG0000000 (node 6240 - *BcBOA13*) protein trees

Panel **A** highlights the BcBot6 transcription factor (TF) for *B*. *cinerea* T4 (BOTC4) and B05.10 (BOTC5) isolates in yellow alongside the putative *D*. *helianthi* (DIAHE) homologue in blue, indicating a shared ancestral origin despite relatively large species distances. Panel **B** highlights the BcBOA13 TF from BOTC5 in yellow and the aspirochlorine TF regulators from *Aspergillus* spp. (corresponding to ORGIDs in blue: ASPNI, ASPFN and ASPF7) a that also suggest a shared ancestral origin despite overall species distances. The branch length is defined by the DendroBLAST distance measure (Kelly and Maini, 2013).

Cercosporin and its structural homologues are potent light-activated phytotoxic SMs produced by *Cercospora* spp. (Daub et al., 2005). Evidence of horizontal transfer, lineage diversification and/or gene loss has recently been detailed for the cercosporin biosynthetic gene cluster in several Ascomycete lineages (de Jonge et al., 2018). Within this set of genes in *Cercospora beticola*, *CbCTB8* encodes a Zn2Cys6 cluster regulator. Despite evidence for positive-diversifying selection relative to other cluster elements (de Jonge et al., 2018), the CbCTB8 clade in the OG0000000 protein tree corresponded well to the evolutionary pathway for the SM cluster. The closest CbCTB8 orthologue in this analysis was *Parastagonospora nodorum* SNOG_08609 (**Figure 3.10**), which has been investigated through gene overexpression. This resulted in the production of elsinichrome C, a phytotoxin that is a structural homologue of cercosporin (Chooi et al., 2017). Other putative orthologues are also positioned in the CbCTB8 TF clade, which correspond to the SM cluster regulators in both the *M. oryzae* rice and millet pathovars (**Figure 3.10**). Therefore, the evolutionary pathway traced here for the ancestral CbCTB8 demonstrates how the TF-orthology analysis serves as a useful proxy for identifying related SM clusters in plant-pathogenic fungi. While the TF based analysis is not a replacement for full SM cluster phylogenies, it provides a useful avenue to explore the regulators of potential phytotoxic compounds, particularly if combined with gene expression data during a host-plant infection.

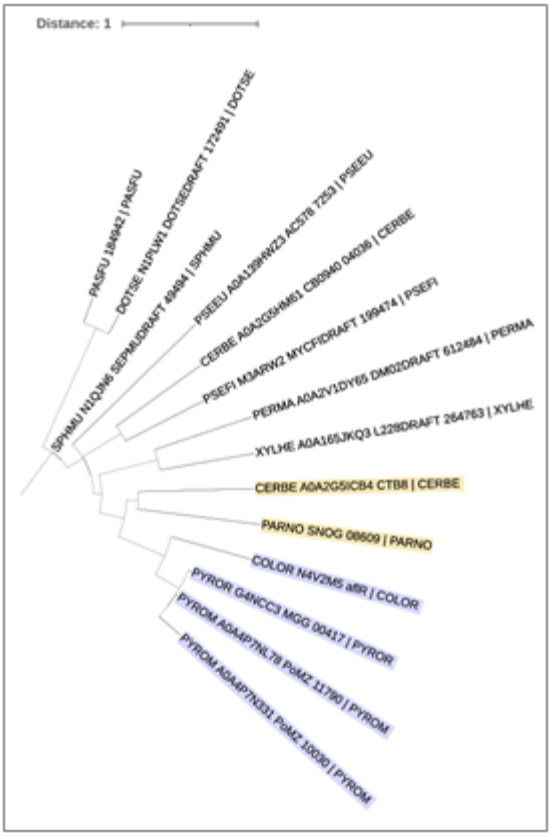


Figure 3.10 – Orthogroup OG0000000 protein tree (node 6486 - CbCTB8)

A tree representing the relationship between transcription factors regulating the biosynthesis of Cercosporin (i.e. CbCTB8) or its structural homologues. The functionally defined CbCTB8 orthologues in the literature (represented by the fungal ORGIDs for CERBE and PARNO) are highlighted in yellow. Other putative homologous regulators reported for the cercosporin secondary metabolite gene clusters (de Jonge et al., 2018) were also grouped in a neighbouring orthogroup-clade and are highlighted in blue (represented by the fungal ORGIDs for PYROM, PYROR and COLOR). The branch length is defined by the DendroBLAST distance measure (Kelly and Maini, 2013).

3.4. Conclusion

The aims of the analysis presented were to identify evidence of conservation, expansion and loss associated with pathogenic lifestyles, and to provide a novel resource for tracing the evolutionary trajectory of TFs that are correlated with virulence. By assessing a taxonomically diverse range of fungal pathogens alongside non-host fungi, a general trend was observed for obligate host-associated pathogens, where the TF content was comparatively low and did not significantly expand with increasing proteome sizes. This suggested that the limited ecological-niche of these fungi lends itself to a reduced regulatory capacity. Furthermore, the TF DBD-based classification system was refined to an extensive set of 855 orthogroups. For TFs that have been under strong diversifying selection, defining true orthologues precisely will require more detailed datasets that can incorporate a greater number of closely related species. Nevertheless, as demonstrated in the virulence regulators discussed, this broad analysis provided novel insights into their evolutionary origins and trajectories.

Importantly, the orthogroups defined in this analysis represent a useful resource for the future exploration of virulence regulating TFs. It is envisaged this will occur using different and complementary approaches:

- The orthogroup lineages (protein trees) of functionally characterised TFs can be explored to define taxonomic orthologues and search for cases of gene duplication or loss in a pathogen of interest.
- Investigate orthogroups that exhibit expansion in a pathogen lineage. These can be identified from the heatmap in **Figure 3.5** which corresponds to columnar layout of the orthogroup counts in **Supplementary item 3.6.** The protein trees for the expanded orthogroups can then be explored in detail to guide further

phylogenetic and functional analyses that investigate any lifestyle-associated selection pressures.

- Search for cases of horizontal-gene transfer events for TFs implicated in the acquisition of fungal virulence. The orthogroup clade of a known virulence regulator is examined in the corresponding protein tree to identify anomalous TF neighbours (i.e. those from taxonomically distant fungi). This is particularly relevant for regulators of phytotoxic SM-gene clusters where genetic mobility is common (Lind et al., 2017; de Jonge et al., 2018; Rokas et al., 2018).

Combined, these represent a powerful means to uncover the fundamental virulence regulators in plant-pathogenic fungi. From an evolutionary standpoint, a detailed analysis of the TFs that have evolved with virulence is also warranted. Recent approaches have used large genomic datasets to demonstrate correlations between pathogenic lifestyles and the prevalence of carbohydrate-active enzymes or other gene families (Hane et al., 2020; Haridas et al., 2020). The evidence from this analysis suggested correlations among pathogen lineages also exist for specific TF orthologues (**Figure 3.5**) which could be incorporated as a useful predictor of fungal virulence.

3.5. Supplementary items

Accessible via: https://figshare.com/s/b0bd50b709e823cbf43e

Supplementary item 3.1 - Organism and TF annotation metadata

A table providing the fungal species/strains used in the analysis [columns 1-2], their assigned five letter ORGIDs [3], alternative names used for each organism in the scientific literature [4], their lifestyle (either necrotroph, biotroph, hemibiotroph, symbiont or saprophyte) [5], host dependence (either facultative or obligate and not applicable for saprophytes) [6], TF count relative to the proteome size [7-9], details on the proteome sources [10-11], references for the associated genome publication [12-13] and the NCBI taxonomic identifiers with phylogenetic rankings for each organism [14-20].

Supplementary item 3.2 – Assessment of total TF DBDs compiled

A table listing the respective Interpro IDs [column 1], Pfam/Superfam IDs [2], DBD/TF family description [3], the total count across species analysed in this study [4] and an indication whether the DBD was in the list adapted for this study [5] (Shelest, 2017).

Supplementary item 3.3 – Orthogroups defined for fungal TFs

Spreadsheets detailing individual TFs assigned to orthogroups (spreadsheet 1) or the unassigned TFs (spreadsheet 2). Orthogroups are listed by size in descending order. TF identifiers follow the format: ‘ProteinID | ORGID’ where the ProteinID was derived from the downloaded proteome source.

Supplementary item 3.4 – TF distance-based species tree

The species tree built from orthogroup TF distances (Emms and Kelly, 2018) and rooted at *Synchytrium endobioticum* (SYNEN) which was used for orthologue inferences. The

full fungal species/strain IDs are included alongside the respective ORGIDs at the tree leaves.

Supplementary item 3.5 - NCBI taxonomy tree

The NCBI taxonomy tree for the fungal species/strains used in the analysis. Node points indicate the respective taxonomic divisions listed in **Supplementary item 3.6**. The full fungal species/strain IDs are included alongside the respective ORGIDs at the tree leaves.

Supplementary item 3.6 – TF orthogroup counts

A spreadsheet detailing the orthogroup counts for each organism [column 1], which are listed using their ORGIDs in the order of the leaf nodes in the TF-species tree. The respective NCBI taxonomic rankings for species, genus, family, order, class and phylum are listed [2-7]. The listed TFs for 855 orthogroups [8-862] are ordered by the hierarchical clustering depicted in **Figure 3.5**.

Supplementary item 3.7 – TF orthogroup protein trees

A folder containing the 855 TF orthogroup protein trees (OG0000000 > OG0000854) produced in the analysis. These are in newick format and can be visualised in the reader's chosen tree-viewing platform such as iTOL (Letunic and Bork, 2019). The branch lengths are defined by the DendroBLAST distance measure (Kelly and Maini, 2013).

Supplementary item 3.8 - Edited OG0000017 protein tree

The full OG0000017 (Pf2) protein tree for high-resolution viewing from **Figure 3.6**. Highlighted are the *S. cerevisiae* MAL13 outgroup in blue, *A. nidulans* AmyR in orange and the Pf2 taxonomic clade (green) with the functionally defined orthologues in yellow

(for the fungi FUSVE, FUSGR, NEUCR, PYROR, BOTC5, ALTBR, PYRTR, PARNO and ZYMTI respectively). The branch length is defined by the DendroBLAST distance measure (Kelly and Maini, 2013).

Supplementary item 3.9 - Edited OG0000017 protein tree

The full OG0000022 (Ste12) protein tree for high-resolution viewing from **Figure 3.7**. Highlighted are the Ste12 orthologues in green with the functionally defined TFs in yellow (for the fungi ZYMTI, ALTBR, ALTAL, EXSTU, PENDI, PENEX, SCLSC, BOTC5, FUSGR, FUSOX, PYROR, CRYPA, VERDA, COLOR and PUCST respectively) and the Ste12 TFs that lack C-terminal C2H2 domains in blue (for SACCE and EREGO). The *U. maydis* C2H2-only TFs Biz1, Mrz1 and Ztf1 are highlighted in purple and the BrlA orthologues (PENDI and ASPNI) in orange. The branch length is defined by the DendroBLAST distance measure (Kelly and Maini, 2013).

Supplementary item 3.10 - Edited OG0000017 protein tree

The full OG0000121 (EBR1) protein tree for high-resolution viewing **Figure 3.8**. Highlighted are the functionally defined EBR1 orthologues in yellow (for the fungi FUSOL, FUSGR, ZYMTI and PYROR respectively) and the putative expansions in the *Fusarium*, *M. oryzae* and the Mycosphaerellaceae lineages. The branch length is defined by the DendroBLAST distance measure (Kelly and Maini, 2013).

3.6. References

Akache, B., Wu, K. & Turcotte, B. (2001) Phenotypic analysis of genes encoding yeast zinc cluster proteins. *Nucleic Acids Research*, 29, 2181–2190. https://doi.org/10.1093/nar/29.10.2181.

Aylward, J., Steenkamp, E.T., Dreyer, L.L., Roets, F., Wingfield, B.D. & Wingfield, M.J. (2017) A plant pathology perspective of fungal genome sequencing. *IMA Fungus*, 8, 1–15. https://doi.org/10.5598/imafungus.2017.08.01.01.

Bayram, Ö. & Braus, G.H. (2012) Coordination of secondary metabolism and development in fungi: the velvet family of regulatory proteins. *FEMS Microbiology Reviews*, 36, 1–24. https://doi.org/10.1111/j.1574-6976.2011.00285.x.

Blum, M., Chang, H.-Y., Chuguransky, S., Grego, T., Kandasaamy, S., Mitchell, A., et al. (2020) The InterPro protein families and domains database: 20 years on. *Nucleic Acids Research*. https://doi.org/10.1093/nar/gkaa977.

Bursteinas, B., Britto, R., Bely, B., Auchincloss, A., Rivoire, C., Redaschi, N., et al. (2016) Minimizing proteome redundancy in the UniProt Knowledgebase. *Database: The Journal of Biological Databases and Curation*, 2016. https://doi.org/10.1093/database/baw139.

Cairns, T.C., Sidhu, Y.S., Chaudhari, Y.K., Talbot, N.J., Studholme, D.J. & Haynes, K. (2015) Construction and high-throughput phenotypic screening of *Zymoseptoria tritici* over-expression strains. *Fungal Genetics and Biology*, 79, 110–117. https://doi.org/10.1016/j.fgb.2015.04.013.

Calvo, A.M., Lohmar, J.M., Ibarra, B. & Satterlee, T. (2016) Velvet regulation of fungal development. In: Wendland, J. (Ed.) Growth, Differentiation and Sexuality The Mycota. Cham: Springer International Publishing, pp. 475–497.

Cao, H., Huang, P., Zhang, L., Shi, Y., Sun, D., Yan, Y., et al. (2016) Characterization of 47 Cys2-His2 zinc finger proteins required for the development and pathogenicity of the rice blast fungus *Magnaporthe oryzae*. *New Phytologist*, 211, 1035–1051. https://doi.org/10.1111/nph.13948.

Chamberlain, S., Szoecs, E., Foster, Z., Boettiger, C., Ram, K., Bartomeus, I., et al. (2020) taxize: Taxonomic information from around the web.

Chankhamjon, P., Boettger-Schmidt, D., Scherlach, K., Urbansky, B., Lackner, G., Kalb, D., et al. (2014) Biosynthesis of the halogenated mycotoxin aspirochlorine in Koji old involves a cryptic amino acid conversion. *Angewandte Chemie International Edition*, 53, 13409–13413. https://doi.org/10.1002/anie.201407624.

Charoensawan, V., Wilson, D. & Teichmann, S.A. (2010a) Genomic repertoires of DNA-binding transcription factors across the tree of life. *Nucleic Acids Research*, 38, 7364–7377. https://doi.org/10.1093/nar/gkq617.

Charoensawan, V., Wilson, D. & Teichmann, S.A. (2010b) Lineage-specific expansion of DNA-binding transcription factor families. *Trends in genetics: TIG*, 26, 388–393. https://doi.org/10.1016/j.tig.2010.06.004.

Cho, Y., Ohm, R.A., Grigoriev, I.V. & Srivastava, A. (2013) Fungal-specific transcription factor *AbPf2* activates pathogenicity in *Alternaria brassicicola*. *The Plant Journal*, 75, 498–514. https://doi.org/10.1111/tpj.12217.

Choi, J. & Kim, S.-H. (2017) A genome tree of life for the fungi kingdom. *Proceedings of the National Academy of Sciences*, 114, 9391–9396. https://doi.org/10.1073/pnas.1711939114.

Chooi, Y.-H., Zhang, G., Hu, J., Muria-Gonzalez, M.J., Tran, P.N., Pettitt, A., et al. (2017) Functional genomics-guided discovery of a light-activated phytotoxin in the wheat pathogen *Parastagonospora nodorum* via pathway activation.

Environmental Microbiology, 19, 1975–1986. https://doi.org/10.1111/1462-2920.13711.
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 Genetics and Biology*, 61, 133–141. https://doi.org/10.1016/j.fgb.2013.10.004.
Coleman, J.J., Rounsley, S.D., Rodriguez-Carres, M., Kuo, A., Wasmann, C.C., Grimwood, J., et al. (2009) The Genome of Nectria haematococca: Contribution of Supernumerary Chromosomes to Gene Expansion. *PLOS Genetics*, 5, e1000618. https://doi.org/10.1371/journal.pgen.1000618.
Collemare, J. & Lebrun, M.-H. (2011) Fungal secondary metabolites: ancient toxins and novel effectors in plant–microbe interactions. Effectors in Plant–Microbe Interactions. John Wiley & Sons, Ltd, pp. 377–400.
Dalmais, B., Schumacher, J., Moraga, J., Pêcheur, P.L., Tudzynski, B., Collado, I.G., et al. (2011) The *Botrytis cinerea* phytotoxin botcinic acid requires two polyketide synthases for production and has a redundant role in virulence with botrydial. *Molecular Plant Pathology*, 12, 564–579. https://doi.org/10.1111/j.1364-3703.2010.00692.x.
Daub, M.E., Herrero, S. & Chung, K.-R. (2005) Photoactivated perylenequinone toxins in fungal pathogenesis of plants. *FEMS Microbiology Letters*, 252, 197–206. https://doi.org/10.1016/j.femsle.2005.08.033.
Does, H.C. van der, Fokkens, L., Yang, A., Schmidt, S.M., Langereis, L., Lukasiewicz, J.M., et al. (2016) Transcription factors encoded on core and accessory chromosomes of *Fusarium oxysporum* induce expression of effector genes. *PLOS Genetics*, 12, e1006401. https://doi.org/10.1371/journal.pgen.1006401.
Does, H.C. van der & Rep, M. (2017) Adaptation to the host environment by plant-pathogenic fungi. *Annual Review of Phytopathology*, 55, 427–450. https://doi.org/10.1146/annurev-phyto-080516-035551.
Dufresne, M., Lee, T. van der, Ben M'barek, S., Xu, X., Zhang, X., Liu, T., et al. (2008) Transposon-tagging identifies novel pathogenicity genes in *Fusarium graminearum*. *Fungal Genetics and Biology*, 45, 1552–1561. https://doi.org/10.1016/j.fgb.2008.09.004.
Emms, D.M. & Kelly, S. (2019) OrthoFinder: phylogenetic orthology inference for comparative genomics. *bioRxiv*, 466201. https://doi.org/10.1101/466201.
Emms, D.M. & Kelly, S. (2018) STAG: species tree inference from all genes. *bioRxiv*, 267914. https://doi.org/10.1101/267914.
Flor-Parra, I., Vranes, M., Kämper, J. & Pérez-Martín, J. (2006) Biz1, a zinc finger protein required for plant invasion by *Ustilago maydis*, regulates the levels of a mitotic cyclin. *The Plant Cell*, 18, 2369–2387. https://doi.org/10.1105/tpc.106.042754.
Gu, Q., Zhang, C., Liu, X. & Ma, Z. (2015) A transcription factor FgSte12 is required for pathogenicity in *Fusarium graminearum*. *Molecular Plant Pathology*, 16, 1–13. https://doi.org/10.1111/mpp.12155.
Habig, M., Bahena-Garrido, S.M., Barkmann, F., Haueisen, J. & Stukenbrock, E.H. (2020) The transcription factor Zt107320 affects the dimorphic switch, growth and virulence of the fungal wheat pathogen *Zymoseptoria tritici*. *Molecular Plant Pathology*, 21, 124–138. https://doi.org/10.1111/mpp.12886.
Han, J.W., Kim, D.Y., Lee, Y.J., Choi, Y.R., Kim, B., Choi, G.J., et al. (2020) Transcription factor PdeR is involved in fungal development, metabolic change, and pathogenesis of gray mold <i>Botrytis cinerea<i>. *Journal of Agricultural and Food Chemistry*, 68, 9171–9179. https://doi.org/10.1021/acs.jafc.0c02420.
Hane, J.K., Paxman, J., Jones, D.A.B., Oliver, R.P. & Wit, P. de (2020) "CATAStrophy,"

a genome-informed trophic classification of filamentous plant pathogens – how many different types of filamentous plant pathogens are there? *Frontiers in Microbiology*, 10. https://doi.org/10.3389/fmicb.2019.03088.
Haridas, S., Albert, R., Binder, M., Bloem, J., LaButti, K., Salamov, A., et al. (2020) 101 Dothideomycetes genomes: a test case for predicting lifestyles and emergence of pathogens. *Studies in Mycology*, 96, 141–153. https://doi.org/10.1016/j.simyco.2020.01.003.
Ikeda, K., Park, P. & Nakayashiki, H. (2019) Cell biology in phytopathogenic fungi during host infection: commonalities and differences. *Journal of General Plant Pathology*, 85, 163–173. https://doi.org/10.1007/s10327-019-00846-w.
John, E., Singh, K.B., Oliver, R.P. & Tan, K.-C. (2021) Transcription factor control of virulence in phytopathogenic fungi. *Molecular Plant Pathology*, 22, 858–881. https://doi.org/10.1111/mpp.13056.
Jones, D.A.B., John, E., Rybak, K., Phan, H.T.T., Singh, K.B., Lin, S.-Y., et al. (2019) A specific fungal transcription factor controls effector gene expression and orchestrates the establishment of the necrotrophic pathogen lifestyle on wheat. *Scientific Reports*, 9, 1–13. https://doi.org/10.1038/s41598-019-52444-7.
Jonge, R. de, Ebert, M.K., Huitt-Roehl, C.R., Pal, P., Suttle, J.C., Spanner, R.E., et al. (2018) Gene cluster conservation provides insight into cercosporin biosynthesis and extends production to the genus *Colletotrichum*. *Proceedings of the National Academy of Sciences,* 115, E5459–E5466. https://doi.org/10.1073/pnas.1712798115.
Jonkers, W., Xayamongkhon, H., Haas, M., Olivain, C., Does, H.C. van der, Broz, K., et al. (2014) *EBR1* genomic expansion and its role in virulence of *Fusarium* species. *Environmental Microbiology*, 16, 1982–2003. https://doi.org/10.1111/1462-2920.12331.
Kelly, S. & Maini, P.K. (2013) DendroBLAST: approximate phylogenetic trees in the absence of multiple sequence alignments. *PLOS ONE*, 8. https://doi.org/10.1371/journal.pone.0058537.
Kwon, N.-J., Garzia, A., Espeso, E.A., Ugalde, U. & Yu, J.-H. (2010) FlbC is a putative nuclear C2H2 transcription factor regulating development in Aspergillus nidulans. *Molecular Microbiology*, 77, 1203–1219. https://doi.org/10.1111/j.1365-2958.2010.07282.x.
Letunic, I. & Bork, P. (2019) Interactive Tree Of Life (iTOL) v4: recent updates and new developments. *Nucleic Acids Research*, 47, W256–W259. https://doi.org/10.1093/nar/gkz239.
Li, Jinyang, Liu, Q., Li, Jingen, Lin, L., Li, X., Zhang, Y., et al. (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. https://doi.org/10.1186/s13068-021-01877-2.
Lind, A.L., Wisecaver, J.H., Lameiras, C., Wiemann, P., Palmer, J.M., Keller, N.P., et al. (2017) Drivers of genetic diversity in secondary metabolic gene clusters within a fungal species. *PLOS Biology*, 15. https://doi.org/10.1371/journal.pbio.2003583.
Lu, J., Cao, H., Zhang, L., Huang, P. & Lin, F. (2014) Systematic analysis of Zn2Cys6 transcription factors required for development and pathogenicity by high-throughput gene knockout in the rice blast fungus. *PLOS Pathogens*, 10. https://doi.org/10.1371/journal.ppat.1004432.
Möller, M. & Stukenbrock, E.H. (2017) Evolution and genome architecture in fungal plant pathogens. *Nature Reviews. Microbiology*, 15, 756–771. https://doi.org/10.1038/nrmicro.2017.76.
Mukaka, M. (2012) A guide to appropriate use of Correlation coefficient in medical

research. *Malawi Medical Journal : The Journal of Medical Association of Malawi*, 24, 69–71.
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. https://doi.org/10.1271/bbb.50694.
Niño-Sánchez, J., Casado-Del Castillo, V., Tello, V., De Vega-Bartol, J.J., Ramos, B., Sukno, S.A., et al. (2016) The *FTF* gene family regulates virulence and expression of SIX effectors in *Fusarium oxysporum*. *Molecular Plant Pathology*, 17, 1124–1139. https://doi.org/10.1111/mpp.12373.
Nordberg, H., Cantor, M., Dusheyko, S., Hua, S., Poliakov, A., Shabalov, I., et al. (2014) The genome portal of the Department of Energy Joint Genome Institute: 2014 updates. *Nucleic Acids Research*, 42, D26-31. https://doi.org/10.1093/nar/gkt1069.
Oh, M., Son, H., Choi, G.J., Lee, C., Kim, J.-C., Kim, H., et al. (2016) Transcription factor ART1 mediates starch hydrolysis and mycotoxin production in *Fusarium graminearum* and *F. verticillioides*. *Molecular Plant Pathology*, 17, 755–768. https://doi.org/10.1111/mpp.12328.
Park, Jongsun, Park, Jaejin, Jang, S., Kim, Seryun, Kong, S., Choi, J., et al. (2008) FTFD: an informatics pipeline supporting phylogenomic analysis of fungal transcription factors. *Bioinformatics (Oxford, England)*, 24, 1024–1025. https://doi.org/10.1093/bioinformatics/btn058.
Pedro, H., Maheswari, U., Urban, M., Irvine, A.G., Cuzick, A., McDowall, M.D., et al. (2016) PhytoPath: an integrative resource for plant pathogen genomics. *Nucleic Acids Research*, 44, D688–D693. https://doi.org/10.1093/nar/gkv1052.
Plissonneau, C., Benevenuto, J., Mohd-Assaad, N., Fouché, S., Hartmann, F.E. & Croll, D. (2017) Using population and comparative genomics to understand the genetic basis of effector-driven fungal pathogen evolution. *Frontiers in Plant Science*, 8. https://doi.org/10.3389/fpls.2017.00119.
Porquier, A., Moraga, J., Morgant, G., Dalmais, B., Simon, A., Sghyer, H., et al. (2019) Botcinic acid biosynthesis in *Botrytis cinerea* relies on a subtelomeric gene cluster surrounded by relics of transposons and is regulated by the Zn2Cys6 transcription factor BcBoa13. *Current Genetics*, 65, 965–980. https://doi.org/10.1007/s00294-019-00952-4.
Porquier, A., Morgant, G., Moraga, J., Dalmais, B., Luyten, I., Simon, A., et al. (2016) The botrydial biosynthetic gene cluster of *Botrytis cinerea* displays a bipartite genomic structure and is positively regulated by the putative Zn(II)2Cys6 transcription factor BcBot6. *Fungal Genetics and Biology*, 96, 33–46. https://doi.org/10.1016/j.fgb.2016.10.003.
Pusztahelyi, T., Holb, I.J. & Pócsi, I. (2016) Plant-Fungal Interactions: Special Secondary Metabolites of the Biotrophic, Necrotrophic, and Other Specific Interactions. In: Mérillon, J.-M. and Ramawat, K.G. (Eds.) Fungal Metabolites Reference Series in Phytochemistry. Cham: Springer International Publishing, pp. 1–58.
R Core Team (2020) R: A language and environment for statistical computing. *R Foundation for Statistical Computing*.
Rispail, N. & Di Pietro, A. (2010) The homeodomain transcription factor Ste12. *Communicative & Integrative Biology*, 3, 327–332.
Rokas, A., Wisecaver, J.H. & Lind, A.L. (2018) The birth, evolution and death of metabolic gene clusters in fungi. *Nature Reviews Microbiology*, 16, 731–744. https://doi.org/10.1038/s41579-018-0075-3.

Ruocco, M., Baroncelli, R., Cacciola, S.O., Pane, C., Monti, M.M., Firrao, G., et al. (2018) Polyketide synthases of *Diaporthe helianthi* and involvement of DhPKS1 in virulence on sunflower. *BMC Genomics*, 19, 27. https://doi.org/10.1186/s12864-017-4405-z.

Sarmiento-Villamil, J.L., Prieto, P., Klosterman, S.J. & García-Pedrajas, M.D. (2018) Characterization of two homeodomain transcription factors with critical but distinct roles in virulence in the vascular pathogen *Verticillium dahliae*. *Molecular Plant Pathology*, 19, 986–1004. https://doi.org/10.1111/mpp.12584.

Schoch, C.L., Ciufo, S., Domrachev, M., Hotton, C.L., Kannan, S., Khovanskaya, R., et al. (2020) NCBI Taxonomy: a comprehensive update on curation, resources and tools. *Database: The Journal of Biological Databases and Curation*, 2020. https://doi.org/10.1093/database/baaa062.

Shelest, E. (2008) Transcription factors in fungi. *FEMS microbiology letters*, 286, 145–151. https://doi.org/10.1111/j.1574-6968.2008.01293.x.

Shelest, E. (2017) Transcription factors in fungi: TFome dynamics, three major families, and dual-specificity TFs. *Frontiers in Genetics*, 8. https://doi.org/10.3389/fgene.2017.00053.

Son, H., Seo, Y.-S., Min, K., Park, A.R., Lee, J., Jin, J.-M., et al. (2011) A phenome-based functional analysis of transcription factors in the cereal head blight fungus, *Fusarium graminearum*. *PLOS Pathogens*, 7, e1002310. https://doi.org/10.1371/journal.ppat.1002310.

Steinegger, M. & Söding, J. (2017) MMseqs2 enables sensitive protein sequence searching for the analysis of massive data sets. *Nature Biotechnology*, 35, 1026–1028. https://doi.org/10.1038/nbt.3988.

Tan, K.-C. & Oliver, R.P. (2017) Regulation of proteinaceous effector expression in phytopathogenic fungi. *PLOS Pathogens*, 13, e1006241. https://doi.org/10.1371/journal.ppat.1006241.

Tangsombatvichit, P., Semkiv, M.V., Sibirny, A.A., Jensen, L.T., Ratanakhanokchai, K. & Soontorngun, N. (2015) Zinc cluster protein Znf1, a novel transcription factor of non-fermentative metabolism in *Saccharomyces cerevisiae*. *FEMS Yeast Research*, 15. https://doi.org/10.1093/femsyr/fou002.

Tani, S., Katsuyama, Y., Hayashi, T., Suzuki, H., Kato, M., Gomi, K., et al. (2001) Characterization of the amyR gene encoding a transcriptional activator for the amylase genes in *Aspergillus nidulans*. *Current Genetics*, 39, 10–15. https://doi.org/10.1007/s002940000175.

Todd, R.B., Zhou, M., Ohm, R.A., Leeggangers, H.A., Visser, L. & Vries, R.P. de (2014) Prevalence of transcription factors in ascomycete and basidiomycete fungi. *BMC Genomics*, 15, 214. https://doi.org/10.1186/1471-2164-15-214.

Uka, V., Cary, J.W., Lebar, M.D., Puel, O., Saeger, S.D. & Mavungu, J.D.D. (2020) Chemical repertoire and biosynthetic machinery of the *Aspergillus flavus* secondary metabolome: A review. *Comprehensive Reviews in Food Science and Food Safety*, 19, 2797–2842. https://doi.org/10.1111/1541-4337.12638.

Urban, M., Cuzick, A., Seager, J., Wood, V., Rutherford, K., Venkatesh, S.Y., et al. (2020) PHI-base: the pathogen–host interactions database. *Nucleic Acids Research*, 48, D613–D620. https://doi.org/10.1093/nar/gkz904.

Vega-Bartol, J.J. de, Martín-Dominguez, R., Ramos, B., García-Sánchez, M.-A. & Díaz-Mínguez, J.M. (2010) New virulence groups in *Fusarium oxysporum* f. sp. *phaseoli*: the expression of the gene coding for the transcription factor Ftf1 correlates with virulence. *Phytopathology*, 101, 470–479. https://doi.org/10.1094/PHYTO-09-10-0252.

Velez-Haro, J.M., Martínez-Soto, D., Guevara-Olvera, L. & Ruiz-Herrera, J. (2020) Ztf1,

an *Ustilago maydis* transcription factor involved in virulence. *European Journal of Plant Pathology*, 156, 189–200. https://doi.org/10.1007/s10658-019-01877-x.
Vollmeister, E., Schipper, K., Baumann, S., Haag, C., Pohlmann, T., Stock, J., et al. (2012) Fungal development of the plant pathogen *Ustilago maydis*. *FEMS Microbiology Reviews*, 36, 59–77. https://doi.org/10.1111/j.1574-6976.2011.00296.x.
Wang, M., Sun, X., Zhu, C., Xu, Q., Ruan, R., Yu, D., et al. (2015) PdbrlA, PdabaA and PdwetA control distinct stages of conidiogenesis in *Penicillium digitatum*. *Research in Microbiology*, 166, 56–65. https://doi.org/10.1016/j.resmic.2014.12.003.
Wong Sak Hoi, J. & Dumas, B. (2010) Ste12 and Ste12-like proteins, fungal transcription factors regulating development and pathogenicity. *Eukaryotic Cell*, 9, 480–485. https://doi.org/10.1128/EC.00333-09.
Xiong, Y., Wu, V.W., Lubbe, A., Qin, L., Deng, S., Kennedy, M., et al. (2017) A fungal transcription factor essential for starch degradation affects integration of carbon and nitrogen metabolism. *PLOS Genetics*, 13, e1006737. https://doi.org/10.1371/journal.pgen.1006737.
Zhang, W.-Q., Gui, Y.-J., Short, D.P.G., Li, T.-G., Zhang, D.-D., Zhou, L., et al. (2018) *Verticillium dahliae* transcription factor VdFTF1 regulates the expression of multiple secreted virulence factors and is required for full virulence in cotton. *Molecular Plant Pathology*, 19, 841–857. https://doi.org/10.1111/mpp.12569.
Zhao, C., Waalwijk, C., Wit, P.J.G.M. de, Lee, T. van der & Tang, D. (2011) EBR1, a novel Zn(2)Cys(6) transcription factor, affects virulence and apical dominance of the hyphal tip in *Fusarium graminearum*. *Molecular Plant-Microbe Interactions*, 24, 1407–1418. https://doi.org/10.1094/MPMI-06-11-0158.
Zheng, Y., Kief, J., Auffarth, K., Farfsing, J.W., Mahlert, M., Nieto, F., et al. (2008) The Ustilago maydis Cys2His2-type zinc finger transcription factor Mzr1 regulates fungal gene expression during the biotrophic growth stage. *Molecular Microbiology*, 68, 1450–1470. https://doi.org/10.1111/j.1365-2958.2008.06244.x.
Zhou, W., Dorrity, M.W., Bubb, K.L., Queitsch, C. & Fields, S. (2020) Binding and regulation of transcription by yeast Ste12 variants to drive mating and invasion phenotypes. *Genetics*, 214, 397–407. https://doi.org/10.1534/genetics.119.302929.
Zhu, X., Liu, W., Chu, X., Sun, Q., Tan, C., Yang, Q., et al. (2018) The transcription factor PstSTE12 is required for virulence of *Puccinia striiformis* f. sp. *tritici*. *Molecular Plant Pathology*, 19, 961–974. https://doi.org/10.1111/mpp.12582.

Chapter 4

General materials and methods

4.1. Background

This chapter details materials and methods that are applicable to both of the subsequent experimental chapters that concern research into the regulation of virulence in *Parastagonospora nodorum*. It serves to avoid repetition and is referred to throughout the respective chapters accordingly.

4.2. Fungal culture maintenance

4.2.1. Routine maintenance

Stock cultures of *P. nodorum* isolates were kept in 20% glycerol at -80ºC and periodically inoculated to sterile V8PDA plates (10 g/L potato dextrose agar, 3 g/L $CaCl_2$, 150 mL/L V8 juice and 15 g/L w/v agar) for 12-14 days under a 12 hr fluorescent light/dark cycle for general use.

4.2.1.1. Spore collection

Pycnidiospores were collected for inoculation in subsequent assays by flooding plates with 10 mL sterile H_2O and transferring suspended material through sterilised glass wool to filter out mycelia in a similar manner to that previously described (Mead et al., 2013).

4.2.1.2. DNA extractions

DNA extractions were performed on fungal material scraped from growth plates using a scalpel which was transferred to 2 mL safelock tubes (Sarstedt, Nümbrecht, Germany) containing a tungsten bead and snap-frozen. Using a TissueLyser II (QIAGEN, Hilden, Germany), material was crushed and then processed using the QIAGEN DNeasy Plant Mini Kit following the manufacturer's protocol.

4.2.2. Growth in modified Fries3 liquid medium

Sterilised Fries3 medium (Liu et al., 2004) consisting 5 g/L Ammonium tartrate ($(NH_4)_2C_4H_4O_6$), 1 g/L NH_4NO_3, 50 mg/L $MgSO_4.7H_2O$, 130 mg/L K_2HPO_4, 260 mg/L KH_2PO_4, 30 g/L sucrose, 1 g/L yeast extract and 2 mL/L trace stock (167 mg/L LiCl, 227 mg/L $CuSO_4.5H_2O$, 34 mg/L H_2MoO_4, 72 mg/L $MnCl_2.4H_2O$, 80 mg/L $CoCl_2.4H_2O$ in H_2O) in H_2O was used for liquid culturing of fungal mycelia.

4.2.2.1. Gene expression *in vitro*

20 µL spores (10^6/mL) were added to 2 mL Fries3 in a sterile 12 well microtiter plate (Corning, Somerville, USA) and incubated in the dark on an orbital shaker for 72 hrs and 100 rpm at 22°C. Mycelia were transferred into 2 mL safelock tubes (Sarstedt) for collection via centrifugation (12000g, 1 min) before washing 1x with Milli-Q purified H_2O and snap-freezing/lyophilisation for 12 hrs in a freeze dryer (Zirbus). Mycelia was then crushed using tungsten beads with a TissueLyser II (QIAGEN) before RNA extraction using a PureLink RNA Mini Kit (Thermofisher, Waltham, USA), DNAse treatment (DNA-free DNA Removal Kit, Thermofisher) and cDNA synthesis (iScript cDNA Synthesis Kit, Biorad, Hercules, USA) for downstream analysis by qPCR.

4.2.2.2. Protein isolation

One mL spores (10^6/mL) were added to 100 mL Fries3 in a sterile 250mL conical flask and incubated in the dark for 72 hrs shaken at 100rpm and 22°C.

4.2.2.2.1. Culture filtrate production

For the production of culture filtrate, flasks were left without shaking for a further 14-21 days and liquid was then filtered through a sterile 0.22 µm sterile filter before use in leaf infiltrations or long-term storage by snap freezing.

4.2.2.2.2. Whole protein extraction

For protein extractions, the mycelia were instead transferred to 50 mL Falcon tubes for collection via centrifugation (5 min, 3000 g) before washing 1x with Milli-Q purified H_2O and snap-freezing/lyophilisation in a freeze-dryer (Zirbus, Harz, Germany) for at least 24 hrs. Mycelia were then weighed and crushed in liquid nitrogen using a mortar/pestle before the addition of 10x (v/w) ice-cold lysis buffer (50 mM Tris, 150 mM NaCl, 1 mM EDTA and 10 mL/L TritonX100 with pH = 8. Additionally, 0.1% v/v NaDOC, 1 mM PMSF and 1% v/v protease inhibitor cocktail (P8215, Sigma-Aldrich, St Louis, USA) were freshly added before use). A 1.6 mL aliquot of resuspended material was then transferred to a sterile 2 mL tube before gentle rotation at 4°C for 20 min. Protein fractions were then separated by taking 1mL supernatant from two rounds of centrifugation (5000 g, 4°C for 5 min). All protein preparations were quantified using a Direct Detect infrared spectrometer (Merck, Kenilworth, USA) prior to downstream analysis.

4.3. Wheat maintenance for infection assays

Wheat seedlings were grown for 12 days in vermiculite supplemented with all minimal amounts of all-purpose fertiliser (Yates, Auckland, New Zealand) under a 12 hr light/dark photoperiod at 22°C in a controlled growth chamber (Conviron, Winnipeg, Canada) before subsequent use.

4.3.1. Whole plant seedling spray

Seedling sprays were carried out according to a method previously described (Phan et al., 2016; Phan et al., 2018). Briefly, seedlings grown in 12cm diameter pots were individually sprayed with 1×10^6 spores ml^{-1} in 0.5% gelatin until saturation using a fine-misting spray bottle. Treatments were performed in triplicates prior to randomisation in the growth chamber (Conviron). Seedlings were kept at 100% humidity for 48 hrs through fine misting before returning to 60% humidity for the remainder of the infection period. For gene expression analyses at the respective time points, 5-6 cm lesions from infected first leaves were removed, snap frozen and lyophilised before RNA extraction and cDNA synthesis as described for samples grown in Fries3 medium. For the assessment of SNB severity, disease symptoms were scored by an independent assessor on a standardised scale of 1-9, where 1 indicates no visible symptoms and 9 indicates a fully-necrotised plant (Phan et al., 2016; Phan et al., 2018).

4.3.2. Detached leaf assay

For the assessment of fungal virulence where sporulation was not viable, the detached leaf assay was used (Solomon et al., 2005). Five cm excisions from the first leaves of wheat seedlings were embedded in 75 mg/L benzimidazole agar containing plates. Five µL spot inoculations of 1×10^6 spores/mL in 0.02% Tween (or a 3 mm mycelial plug from the growth perimeter of a culture plate) were applied to the centre of the leaves before incubation up to 12 days for the assessment of fungal virulence by lesion diameter at respective time points.

4.3.3. Leaf infiltrations using culture filtrate

First leaves on growing seedlings were infiltrated with culture filtrate derived from growth in Fries3 as performed previously (Tan et al., 2015). Seedlings were returned to

the growth chamber (Conviron) and lesions were visually assessed after four days for comparison of necrosis-inducing potential of the secretome for the respective fungal mutants.

4.4. PCRs & Sanger sequencing

The primers used throughout this thesis and a description of their usage are described in **Supplementary item 4.1**.

4.4.1. PCRs for cloning and screening purposes

For amplification of fragments used in cloning and sequencing, Phusion High-Fidelity PCR Master Mix (Thermofisher) was used. Fragments were purified using a GenElute™ PCR Clean-Up Kit (Merck) or a GenElute™ Gel Extraction Kit (Merck) where non-specific amplicons were obvious. For size-screening purposes, MyTaq™ DNA Polymerase (Bioline, London, UK) was used for targets up to 5000 bp.

4.4.2. Sanger sequencing

Sanger sequencing was performed for sequence-screening purposes on purified plasmids or fungal derived PCR products by Macrogen, Inc. (Seoul, South Korea) following the recommended guidelines.

4.4.3. Quantitative PCR

Quantitative PCR (qPCR) was undertaken on a Biorad CFX96™ thermocycler using the RT-PCR SYBR Green master mix (QIAGEN). Reactions were performed using 600 nM primers and 2 ng/µL cDNA in a 20 µL solution. A 35 cycle procedure was undertaken (95^{o}C 15 sec, 58^{o}C 30 sec, 72^{o}C 30 sec) followed by a melt curve analysis step using the Precision Melt Analysis™ software (Biorad) following the manufacturer's

guidelines to ensure primer specificity. For gene expression analysis, the relative abundance of target gene cDNA was calculated with the 2^{dCt} method (Livak and Schmittgen, 2001) using *Act1* amplified with Actin_qPCR_F/R primers which is regularly used as a *P*. *nodorum* internal reference control (Solomon et al., 2006; Rybak et al., 2017; Peters-Haugrud et al., 2019). Fold differences between samples were calculated as 2^{ddCt} values. For calculation of target gDNA copy numbers, a method previously developed for use in *P. nodorum* (Solomon et al., 2008) was employed. Briefly, a standard curve of Starting Quantity (SQ) values was generated using serially diluted reference gDNA (from 5 ng/µL to 0.16 ng/µL) of a predetermined single copy target/control in technical triplicates. Sample reactions were performed at 1.6 ng/µL gDNA and an average ratio of SQ values (target/control) between 0.8 and 1.2 was considered a single copy.

4.5. DNA cloning

4.5.1. Bacterial culturing & transformation

One Shot TOP10 Chemically Competent *E. coli* cells (Invitrogen, Carlsbad, USA) were used for plasmid transformations following the manufacturers protocol and routinely cultured in SOC media (20 g/L tryptone, 5 g/L yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, and 20 mM glucose). Plasmid containing colonies were selected on LB agar (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, 15 g/L agar) with ampicillin (100 mg/L) or spectinomycin (200 mg/L) and 0.04% v/v X-Gal (Promega, Madison, USA) + 50 µM IPTG (Invitrogen) for blue/white screening when required. Plasmids were extracted using the GenElute Plasmid MiniPrep Kit (Sigma-Aldrich).

4.5.2. Type IIS/Golden gate cloning

An in-house Golden Gate (Engler et al., 2008) style cloning system was utilised (originally developed by Dr Jordi Muria-Gonzales). To summarise, BbsI or BsaI restriction

sites flank a LacZ marker gene in a pUC19 vector containing either spectinomycin/ampicillin resistance, termed pGGS-/pGGA- respectively. Four bp overhangs generated by restriction digest with BbsI or BsaI allow LacZ marker replacement with the desired fragment sequence(s) in a predetermined orientation tailored to the production of targeted gene replacement constructs. An overview of the respective fragment (Left flank, Promoter, Basic/CDS, Terminator, Right Flank) overhangs used in the directional multi-fragment assembly is provided in **Figure 4.1**. The one-step digestion/ligation reaction mix consisted 0.08 pmol destination vector, 0.16 pmol fragments (either purified DNA or preassembled in a compatible donor vector), 1.5 µL T4 ligase buffer (Promega), 1.5 µL 10% v/v BSA, 1 µL ATP, 0.5 µL T4 ligase and 0.5 µL either BbsI or BsaI (New England Biolabs, Ipswich, USA) to a total of 20 µL. This was incubated on a 20x cycle (3 min 37°C then 4 min 16°C) before enzyme denaturation for 5 min at 80°C. Where target fragment sequences contained an internal BbsI or BsaI site, 'domestication' was employed (Andreou and Nakayama, 2018) to facilitate introduction of synonymous mutations as required. Where required constructs didn't fit the tailored orientation of this Golden Gate system, two-step BbsI (type IIS restriction enzyme) digestion/ligation with custom-designed fragment overhangs was used following the enzyme manufacturers protocol. Annealed oligonucleotides (produced by mixing 10 µM fragments, heating to 95°C for 5 min, then cooling at a rate of 1°C/min until 10°C) resulting in sequence fragments with appropriate 4 bp overhangs were also incorporated into this system where PCR products were not viable (length too short for PCR or sequence template locally unavailable).

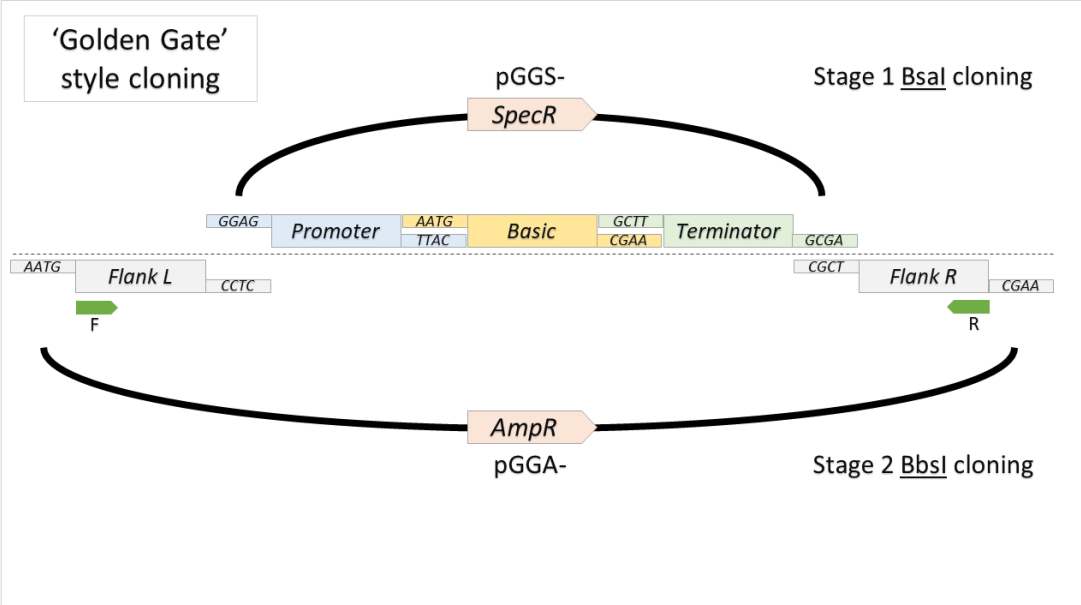


Figure 4.1 - Overview of the 'Golden Gate' style cloning strategy described in this thesis

The universal 4 bp overhangs are indicated for the respective fragments. *SpecR* and *AmpR* refer to the bacterial antibiotic resistance markers for spectinomycin and ampicillin resistance respectively. The first reaction stage incorporating the cloning of fragments into pGGS- is depicted above the dotted line with the second stage assembly incorporating the flanking regions into the destination vector pGGA- underneath. Linear constructs used in subsequent fungal transformations were amplified using primers depicted by the green arrows F/R.

4.5.3. Gibson assembly

Where the required fragments were not amenable to TypeIIS restriction enzyme manipulation (such as the presence of multiple internal recognition sequences making 'domestication' unfeasible), Gibson Assembly (New England Biolabs) was used to clone fragments following the manufacturer's protocol.

4.5.4. Fungal markers & tags

The *A. nidulans pGpdA*/*pTef1* promoters were used for constitutive expression coupled to the *tTrpC*/*tTef1* terminators. Hygromycin (*HygR*) and Phleomycin (*PhleoR*) resistance marker genes were amplified from plasmids Pan7 and Pan8 respectively (Mattern et al., 1988; Punt et al., 1987). The *GFP* gene and *tTrpC-HygR-tTrpC* marker cassette were derived from the plasmid pGpdGFP (Sexton and Howlett, 2001). Other linker, leader and tag sequences (<100 bp) were sourced from synthesised/annealed oligonucleotides (**Supplementary item 4.1**).

4.6. Fungal transformation

P. nodorum transformation was carried out using the polyethylene glycol (PEG) protocol described previously (Solomon et al., 2004). Briefly 10^8 spores were cultured for 20 hrs in 100ml CzV8 media (45.4 g/L Czapek Dox liquid media (Oxoid, Bakingstone, UK), 150 ml/L Campbell's V8 juice, 20.0 g/L casamino acids, 20 g/L peptone, 20 g/L yeast extract, 3 g/L adenine, 0.02 g/L biotin, 0.02 g/L nicotinic acid, 0.02 g/L p-aminobenzoic acid, 0.02 g/L pyridoxine and 0.02 g/L thiamine) at 100 rpm and 22°C in the dark on an orbital shaker. Mycelia were treated with 15mg/mL Extralyse (Laffort, Floirac, France) + 1.2 M $MgSO_4$ for 2 hrs at 28°C to form protoplasts. Five µg of linear DNA construct suspended in STC buffer (1 M sorbitol, 10 mM Tris-HCl, 10 mM $CaCl_2$) was added to 100 µL protoplasts (washed in 2 M sorbitol, resuspended in 1 mL STC buffer) and incubated

for 20 min in 60% PEG. Successful transformants were selected for in Hygromycin (200mg/L) or Phleomycin (50 mg/L) supplemented CzV8 agar (CzV8 + 10 g/L agar and 18 g/L sorbitol) before subculturing, screening (PCR, copy number detection and Sanger sequencing as required) and long term storage in 20% glycerol at -80^{o}C.

4.7. Supplementary items

Accessible via: https://figshare.com/s/b0bd50b709e823cbf43e

Supplementary item 4.1 - Primers and their uses

A 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 GG cloning.

4.8. References

Andreou, A.I. & Nakayama, N. (2018) Mobius assembly: a versatile Golden-Gate framework towards universal DNA assembly. *PLOS ONE*, 13, e0189892. https://doi.org/10.1371/journal.pone.0189892.

Engler, C., Kandzia, R. & Marillonnet, S. (2008) A one pot, one step, precision cloning method with high throughput capability. *PLOS ONE*, 3. https://doi.org/10.1371/journal.pone.0003647.

Liu, Z., Faris, J.D., Meinhardt, S.W., Ali, S., Rasmussen, J.B. & Friesen, T.L. (2004) Genetic and physical mapping of a gene conditioning sensitivity in wheat to a partially purified host-selective toxin produced by *Stagonospora nodorum*. *Phytopathology*, 94, 1056–1060. https://doi.org/10.1094/PHYTO.2004.94.10.1056.

Livak, K.J. & Schmittgen, T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods (San Diego, Calif.)*, 25, 402–408. https://doi.org/10.1006/meth.2001.1262.

Mattern, I., Punt, P. & Hondel, C. (1988) A vector for Aspergillus transformation conferring phleomycin resistance. *Fungal Genetics Reports*, 35, 25. https://doi.org/10.4148/1941-4765.1533.

Mead, O., Thynne, E., Winterberg, B. & Solomon, P.S. (2013) Characterising the role of GABA and its metabolism in the wheat pathogen *Stagonospora nodorum*. *PLOS ONE*, 8, e78368. https://doi.org/10.1371/journal.pone.0078368.

Peters-Haugrud, A.R., Zhang, Z., Richards, J.K., Friesen, T.L. & Faris, J.D. (2019) Genetics of variable disease expression conferred by inverse gene-for-gene interactions in the wheat-*Parastagonospora nodorum* pathosystem. *Plant Physiology*, 180, 420–434. https://doi.org/10.1104/pp.19.00149.

Phan, H.T.T., Rybak, K., Bertazzoni, S., Furuki, E., Dinglasan, E., Hickey, L.T., et al. (2018) Novel sources of resistance to Septoria nodorum blotch in the Vavilov wheat collection identified by genome-wide association studies. *Theoretical and Applied Genetics*, 131, 1223–1238. https://doi.org/10.1007/s00122-018-3073-y.

Phan, H.T.T., Rybak, K., Furuki, E., Breen, S., Solomon, P.S., Oliver, R.P., et al. (2016) Differential effector gene expression underpins epistasis in a plant fungal disease. *The Plant Journal*, 87, 343–354. https://doi.org/10.1111/tpj.13203.

Punt, P.J., Oliver, R.P., Dingemanse, M.A., Pouwels, P.H. & Hondel, C.A. van den (1987) Transformation of *Aspergillus* based on the hygromycin B resistance marker from Escherichia coli. *Gene*, 56, 117–124. https://doi.org/10.1016/0378-1119(87)90164-8.

Rybak, K., See, P.T., Phan, H.T.T., Syme, R.A., Moffat, C.S., Oliver, R.P., et al. (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. *Molecular Plant Pathology*, 18, 420–434. https://doi.org/10.1111/mpp.12511.

Sexton, A.C. & Howlett, B.J. (2001) Green fluorescent protein as a reporter in the *Brassica–Leptosphaeria maculans* interaction. *Physiological and Molecular Plant Pathology*, 58, 13–21. https://doi.org/10.1006/pmpp.2000.0307.

Solomon, P., Ipcho, S., Hane, J., Tan, K.-C. & Oliver, R. (2008) A quantitative PCR approach to determine gene copy number. *Fungal Genetics Reports*, 55, 5–8. https://doi.org/10.4148/1941-4765.1082.

Solomon, P.S., Tan, K.-C. & Oliver, R.P. (2005) Mannitol 1-phosphate metabolism is

required for sporulation in planta of the wheat pathogen *Stagonospora nodorum*. *Molecular Plant-Microbe Interactions*, 18, 110–115. https://doi.org/10.1094/MPMI-18-0110.

Solomon, P.S., Tan, K.-C., Sanchez, P., Cooper, R.M. & Oliver, R.P. (2004) The disruption of a Gα subunit sheds new light on the pathogenicity of *Stagonospora nodorum* on wheat. *Molecular Plant-Microbe Interactions*, 17, 456–466. https://doi.org/10.1094/MPMI.2004.17.5.456.

Solomon, P.S., Waters, O.D.C., Jörgens, C.I., Lowe, R.G.T., Rechberger, J., Trengove, R.D., et al. (2006) Mannitol is required for asexual sporulation in the wheat pathogen *Stagonospora nodorum* (glume blotch). *Biochemical Journal*, 399, 231–239. https://doi.org/10.1042/BJ20060891.

Tan, K.-C., Phan, H.T.T., Rybak, K., John, E., Chooi, Y.H., Solomon, P.S., et al. (2015) Functional redundancy of necrotrophic effectors – consequences for exploitation for breeding. *Frontiers in Plant Science*, 6. https://doi.org/10.3389/fpls.2015.00501.

Chapter 5

Mechanistic insights into PnPf2 regulation of the necrotrophic lifestyle in *Parastagonospora nodorum*.

Content contained within sections 5.2.2 and 5.3.2 of this chapter has been published prior to submission of this thesis, forming part of the following:

Jones, D.A.B., John, E., Rybak, K., Phan, H.T.T., Singh, K.B., Lin, S.-Y., Solomon, P.S., Oliver, R.P., Tan, K.-C (2019) A specific fungal transcription factor controls effector gene expression and orchestrates the establishment of the necrotrophic pathogen lifestyle on wheat. *Sci Rep*, 9, 1–13. https://doi.org/10.1038/s41598-019-52444-7.

A signed co-author contribution statement demonstrating that the work contained herein is my own is provided in **Appendix 2**.

5.1. Background

In *Parastagonospora nodorum*, the Zn2Cys6 transcription factor (TF) PnPf2 is a key regulator of pathogenicity on wheat. The TF controls the expression of proteinaceous necrotrophic effectors (NEs) which underpin the virulence of this pathogen (Rybak et al., 2017; McDonald and Solomon, 2018). Gene deletion of *PnPf2* leads to a loss of pathogenicity on wheat lines carrying the dominant-susceptibility genes, which encode receptor proteins targeted by the NEs ToxA and Tox3. It has also been demonstrated that the PtrPf2 of *Pyrenophora tritici-repentis* positively regulates *ToxA*, a NE gene horizontally transferred between these species and another wheat pathogen *Bipolaris sorokiniana* (Friesen et al., 2006; Rybak et al., 2017; McDonald et al., 2019). Furthermore, the AbPf2 orthologue in *Alternaria brassicicola* is critical for virulence on *Brassica* spp. (Cho et al., 2013). Gene deletion of *AbPf2* resulted in the down-regulation of a number of effector-like genes (encoding small, secreted, cysteine-rich proteins) and plant cell wall-degrading enzymes (CWDEs). Together, these studies depict a key role in the regulation of secreted virulence factors in plant pathogens belonging to the Pleosporales order of fungi.

The predicted PnPf2 orthologues (**Chapter 3**) in several other fungal ascomycete lineages also govern plant pathogenicity. Studies have now detailed this in *Botrytis cinerea*, *Fusarium* spp., *Magnaporthe oryzae* and *Zymoseptoria tritici* (Chung et al., 2013; Oh et al., 2016; Habig et al., 2020; Han et al., 2020), where the regulatory activities were also associated with carbon metabolism. The respective TFs were required for normal growth on several simple or complex carbohydrates in defined media, with some variation on the species-specific profiles. However, chemical complementation of the gene deletion mutants with appropriate carbon sources restored fungal growth (Chung et al., 2013; Oh et al., 2016; Habig et al., 2020;

Han et al., 2020). Similar regulatory roles were also attributed to the inferred PnPf2 orthologues from the saprophytic fungi *Neurospora crassa* and *Trichoderma reesei* (Nitta et al., 2012; Xiong et al., 2017). Collectively, these studies suggest a shared evolutionary origin exists in the regulation of carbon acquisition that has been adapted to fungal virulence. It is now known that there is a strong association between the fungal carbohydrate-active enzyme (CAZyme) repertoire and plant-pathogenic lifestyles (Hane et al., 2020). Accordingly, PnPf2 orthologues are of significant interest as TF regulators that coordinate virulence in fungal pathogens.

In *P. nodorum*, while it had been shown that PnPf2 was a key regulator of *ToxA* and *Tox3* expression, the extent of the regulatory network had not been defined (Rybak et al., 2017). Another well-characterised NE (Tox1) could still be produced in the *PnPf2* knockout (KO) mutant studied (*pf2-69*; referred to in this study as *pf2ko*). However, *Tox1* deletion in the *pf2ko* mutant background rendered it unable to produce the three NEs and avirulent. This contrasted with a *toxA*/*tox1*/*tox3* KO mutant (*toxa13*) that was still highly virulent, suggesting other important virulence factors still expressed in *toxa13* are regulated by PnPf2 in *P. nodorum* (Tan et al., 2015; Rybak et al., 2017).

A concurrent RNA-seq analysis was recently conducted to explore the regulatory landscape of PnPf2 by comparing the expression profile of *pf2ko* with the *P*. *nodorum* wildtype SN15 (Jones et al., 2019). Several effector-like genes were differentially expressed (DE) in *pf2ko*, indicating they are regulated by PnPf2. A larger set of PnPf2-regulated genes were also revealed under both *in vitro* conditions and during early infection *in planta*, highlighting a broader regulatory role. Genes positively-regulated by PnPf2 (i.e. reduced expression in *pf2ko* vs SN15) were enriched for

CAZymes, peptidases and hydrolases, while genes negatively regulated by PnPf2 were enriched for oxidoreductase/respiration activity. Several predicted effectors were also identified among the DE genes. A picture emerged that *P. nodorum* PnPf2 orchestrates the expression of genes conducive to the necrotrophic lifestyle on wheat, such as NEs and plant CWDEs, while repressing general metabolic activity, perhaps to conserve energy (Jones et al., 2019). However, some important questions still remain. Are these genes directly regulated by PnPf2, or is their expression controlled indirectly by another virulence regulator? Does PnPf2 favour a unique DNA target or is there a conserved sequence shared by other fungal Zn2Cys6 TFs?

Building on from the previous insights, the aim of the research presented here was to determine the molecular mechanisms by which PnPf2 regulates virulence in *P. nodorum*. Multiple avenues were pursued, centred on a chromatin immunoprecipitation analysis in order to first, verify TF-binding and define the genes under direct regulation. Second, to model the enrichment of DNA sequences bound by PnPf2, which has not been established for PnPf2 orthologues in other fungi. Lastly, to identify and then functionally characterise other gene regulators that act as PnPf2 intermediates, synergists or antagonists, in the orchestration of virulence in *P. nodorum*.

5.2. Materials and methods

5.2.1. PnPf2 annotation and phylogeny

The *P. nodorum* PnPf2 protein sequence was derived from the gene annotation SNOG_00649 from the genome for the reference isolate SN15 (Syme et al., 2016). PnPf2 taxonomic orthologues were inferred by phylogenetic analysis from the set of 370 fungal Zn2Cys6 TFs previously compiled (i.e. orthogroup OG0000017; **Chapter 3**). First, a neighbour-joining (NJ) consensus tree (branch support as 100 bootstraps) was constructed from MUSCLE aligned protein sequences using Geneious (Geneious Prime 20.2.5). A maximum-likelihood (ML) tree was then constructed from the aligned sequences using PhyML 3.0 (default parameters with smart model selection and LRT/SH branch support) (Guindon et al., 2010; Lefort et al., 2017) and trees were visualised using iTOL (Letunic and Bork, 2019). The PnPf2 orthologues were verified by identifying the corresponding clade (with minimum branch support values of 0.8) in both trees where TF divergence could be traced to a speciation event rather than gene duplication. The functionally defined PnPf2 orthologues in the scientific literature within this clade (detailed in **Chapters 2 and 3**) were re-aligned with AmyR from *Aspergillus nidulans* and MAL13 from *Saccharomyces cerevisiae*; Zn2Cys6 TFs belonging to outgroups. From this, a NJ tree with Jukes-Cantor distances was derived to estimate divergence among PnPf2 functional orthologues (Geneious Prime 20.2.5). The PnPf2 polypeptide sequence was also submitted to Interproscan (Release 82.0) for protein domain analysis (Blum et al., 2020) and NLStradamus for the prediction of nuclear localisation signals (Nguyen Ba et al., 2009). The disordered/binding region was predicted using IUPRED2A (Erdős and Dosztányi, 2020).

5.2.2. Differentially expressed gene promoter motif analysis

The promoter regions, spanning gene transcription start sites to the nearest upstream gene feature (or a maximum length of 1500 bp), were derived from the SN15 genome (Syme et al., 2016). Enriched motifs were identified in promoter sets that were classed by their differentially expressed (DE) gene pattern previously determined by Jones et al., 2019 (genes expressed significantly up or down in *pf2ko* vs SN15 under *in planta* and/or *in vitro* conditions after three days growth). Weeder 2.0 (Zambelli et al., 2014) was used to search for enriched motifs vs the background frequencies in SN15 promoters with the redundancy filter set at 0.5. Utilising the consensus option in MEME (version 5.0.1) (Bailey and Elkan, 1994; Bailey et al., 2009), position weight matrices (PWMs) for the non-redundant motifs from each subset were modelled. Promoters with ≥ 1 occurrence were determined using FIMO (Grant et al., 2011). Fisher's exact test with Bonferroni corrected P-values (Armstrong, 2014) was used to identify *pf2ko* DE gene promoter sets significantly enriched (Padj < 0.01) for the respective motifs vs the background rate in SN15. TOMTOM was used to search the JASPAR-NR database with the significantly enriched motif PWMs for matches (E < 1) to published fungal TF-binding sites (Gupta et al., 2007).

5.2.3. Generation of fungal mutants

The molecular cloning stages, the constructs generated and an overview of the final transformed *P. nodorum* mutants described herein are described in **Figure 5.1**. The primers designed for fragment amplification and/or screening, plus descriptions of their use pertaining to these steps are outlined in **Supplementary item 4.1**. Fungal transformation constructs were produced by the Golden Gate (GG), Type IIS or Gibson Assembly cloning methods detailed in **Chapter 4** and verified through PCR screening and Sanger sequencing. The fungal mutants were produced by

polyethylene-glycol (PEG) mediated transformation and were screened by PCR and qPCR to confirm both targeted and single-copy construct integration following the procedures detailed in **Chapter 4**.

A *PnPf2* KO construct was first produced through GG cloning by attaching ~700 bp flanking fragments located 5' and 3' of *PnPf2* (amplified from SN15 gDNA with Pf2_HR_FL_BsaI_F/R and Pf2_HR_FR_BsaI_F/R respectively) to the *pTef1-PhleoR-tTef1* marker (**Figure 5.1.A**). The resulting construct was amplified with Pf2_HR_FL_F/Pf2_HR_FR_R which was then used to generate the new *pnpf2* mutant (*pf2_KO*) from the wildtype (SN15) by PEG transformation. The *pf2_KO* mutant was amenable to homologous recombination (HR) and marker retrieval at the native locus, which is where *PnPf2* tagged constructs were subsequently introduced (using the *pTrpC-HygR-tTrpC* selectable marker).

The *Pf2-GFP* construct was produced by first amplifying the *PnPf2* region with pPf2_P_BbsI_985_F/Pf2_link_B_BbsI_R from SN15 (encompassing the promoter, coding sequence and incorporating a GGSG peptide linker for protein/GFP tag spatial separation) followed by Type IIS cloning into the linear vector amplified from 'pGpdGFP' (Sexton and Howlett, 2001) using eGFP_BbsI_F/pBack_FL_BbsI_R. The resulting construct was PCR amplified using tTrpc_T_BbsI_F/eGFP_B_BbsI_R to receive the *PnPf2* terminator sequence amplified from SN15 using tPf2_T_BbsI_F/R by Type IIS cloning to yield the plasmid 'Pf2-GFP_HygR' (**Figure 5.1.B**). Two separate linear vectors were amplified from 'Pf2-GFP_HygR' using either Pf2_link_B_BbsI_R/tPf2_T_BbsI_F or pPf2_P_BbsI_R/tPf2_T_BbsI_F. These vectors were used for incorporating the oligo-annealed HA_Oligo_sense/anti fragment (encoding a 3x haemagglutinin tag) by TypeIIS cloning to produce the plasmids 'Pf2-

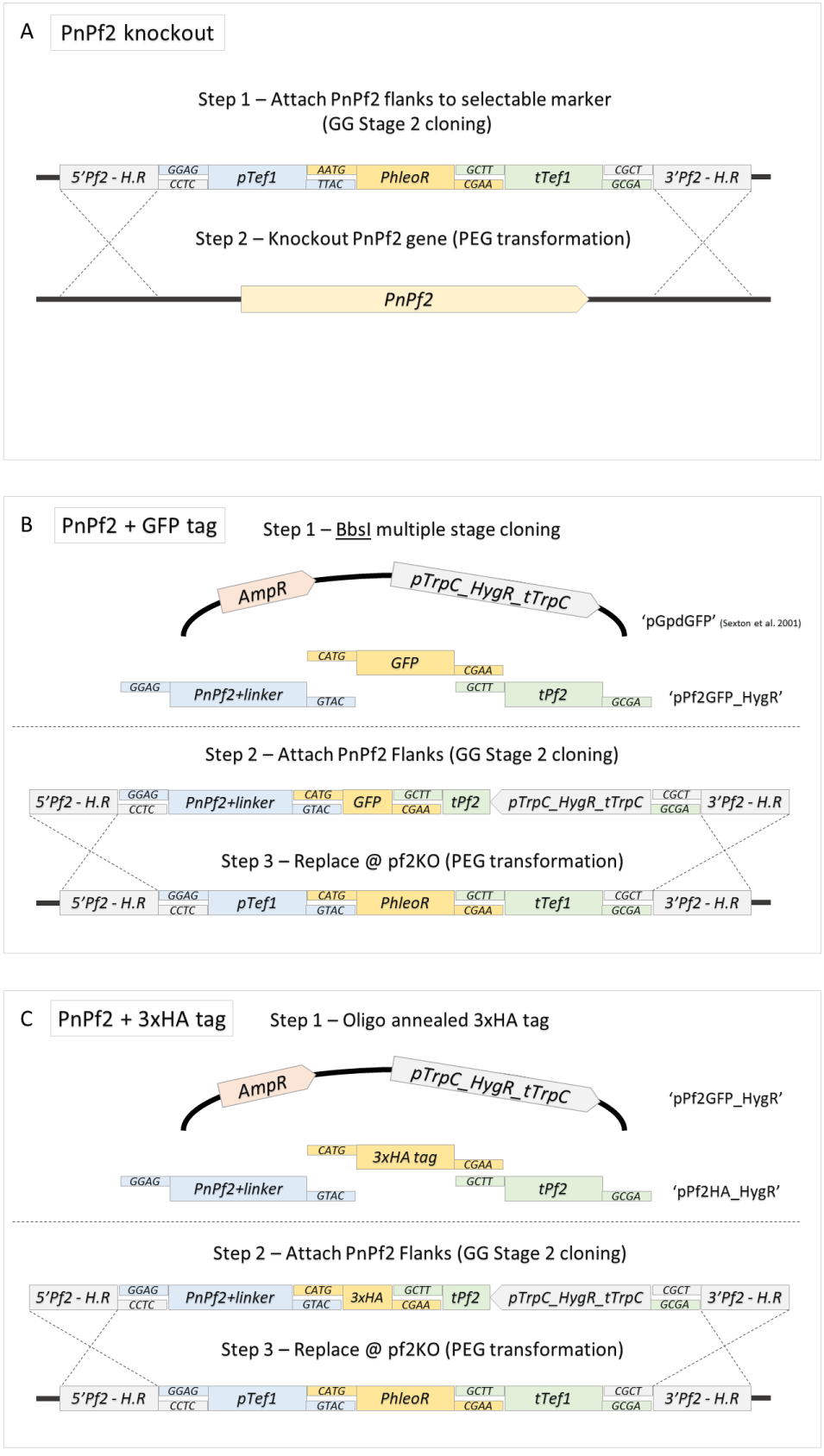
HA_HygR' and 'pf2-HA_KO_HygR' respectively (**Figure 5.1.C**). Linear constructs were amplified from 'Pf2-GFP_HygR', 'Pf2-HA_HygR and 'pf2-HA_KO_HygR' using pPf2_P_BbsI_985_F/pTrpC_T_BbsI_R to attach the same ~700 bp 5' and 3' *PnPf2* flanking fragments (used for *pf2_KO*) by GG cloning. The resulting plasmids were then amplified with Pf2_HR_FL_F/Pf2_HR_FR_R to obtain the linear constructs used for PEG transformation in the *pf2_KO* background to generate the fungal mutants *Pf2-GFP*, *Pf2-HA* and *pf2-HA_KO* respectively (**Figure 5.1.G**).

PnPf2 overexpression mutants were derived using a promoter replacement strategy where a *pGpdA* promoter was used to replace the native *PnPf2* promoter. The replacement construct was produced through GG cloning, where the same ~700 bp 5' *PnPf2* flanking fragment (used for *pf2_KO*) as well as a flanking fragment derived from two PCR products (amplified using pGpd_FR_BbsI_F/R from the Pan8 plasmid (Mattern et al., 1988) and Pf2_start_FR_BbsI_F/Pf2_mid_FR_BbsI_R from SN15 gDNA) were fused to the recycled *pTef1-PhleoR-tTef1* marker (**Figure 5.1.D**). The resulting construct was amplified using Pf2_HR_FL_F/Pf2_mid_FR_R to produce the linear constructs used for PEG transformation in the *Pf2-GFP* and *Pf2-HA* backgrounds, producing the strains *Pf2-GFP_OE* and *Pf2-HA_OE* respectively (**Figure 5.1.G**).

A positive control strain for epifluorescence microscopy (SN15-*GFP*) was produced by random construct integration in SN15. A *pTef1-GFP-tTef1* expression cassette was first derived by GG cloning which produced the plasmid 'pGGS_pTef1_GFP' (**Figure 5.1.E**). A linear plasmid was amplified from this using pTef1_F/pGG_Gibson_R which was used to incorporate the *pGpdA-PhleoR-tTrpC* cassette (amplified from Pan8 with PhleoR_Gibson_F/R) by Gibson assembly. A linear

construct was amplified from the resulting plasmid using pGG_screen_F/R and integrated into SN15 by PEG transformation.

The SNOG_03067 (*PnEbr1*), SNOG_03490 (*PnPro1*), SNOG_04486 (*PnAda1*), SNOG_07185, SNOG_08237 and SNOG_08565 TF KO mutants (outlined in **Figure 5.1.H**) were generated in the SN15 background. HR constructs were derived by attaching 5' and 3' flanks (amplified from SN15 using *TF*_HR_FL_BsaI_F/R and *TF*_HR_FR_BsaI_F/R; *TF* corresponds to the numerical SNOG_ID for the respective gene annotations) to *pGpdA-PhleoR-tTrpC* by GG cloning (**Figure 5.1.F**). The linear constructs used for PEG transformation were amplified from the resulting plasmids using *TF*_HR_FL_F/*TF*_HR_FR_R. Two single copy KO mutants (verified by PCR screening using *TF*_screen_F/R and the qPCR method detailed in **Chapter 4**) were selected for functional and phenotypic characterisation (**Figure 5.1.H**). For gene complementation, the original TF genes were amplified from SN15 gDNA using *TF*_Gibson_F/R and fused to the *pTef1-HygR-tTef1* marker cassette by Gibson assembly. The constructs were amplified from the resulting plasmids using pGG_screen_F/R and integrated into one of the respective KO mutant backgrounds by PEG transformation to produce the complemented mutants (outlined in **Figure 5.1.H**).

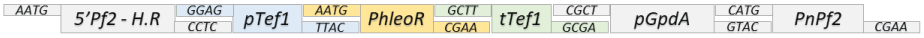

A
PnPf2 knockout
Step 1 – Attach PnPf2 flanks to selectable marker
(GG Stage 2 cloning)
5'Pf2 - H.R
GGAG
CCTC
pTef1
AATG
TTAC
PhleoR
GCTT
CGAA
tTef1
CGCT
GCGA
3'Pf2 - H.R
Step 2 – Knockout PnPf2 gene (PEG transformation)
PnPf2
B
PnPf2 + GFP tag
Step 1 – BbsI multiple stage cloning
AmpR
pTrpC_HygR_tTrpC
'pGpdGFP' (Sexton et al. 2001)
CATG
GFP
CGAA
GGAG
PnPf2+linker
GTAC
GCTT
tPf2
GCGA
'pPf2GFP_HygR'
Step 2 – Attach PnPf2 Flanks (GG Stage 2 cloning)
5'Pf2 - H.R
GGAG
CCTC
PnPf2+linker
CATG
GTAC
GFP
GCTT
CGAA
tPf2
pTrpC_HygR_tTrpC
CGCT
GCGA
3'Pf2 - H.R
Step 3 – Replace @ pf2KO (PEG transformation)
5'Pf2 - H.R
GGAG
CCTC
pTef1
CATG
GTAC
PhleoR
GCTT
CGAA
tTef1
CGCT
GCGA
3'Pf2 - H.R
C
PnPf2 + 3xHA tag
Step 1 – Oligo annealed 3xHA tag
AmpR
pTrpC_HygR_tTrpC
'pPf2GFP_HygR'
CATG
3xHA tag
CGAA
GGAG
PnPf2+linker
GTAC
GCTT
tPf2
GCGA
'pPf2HA_HygR'
Step 2 – Attach PnPf2 Flanks (GG Stage 2 cloning)
5'Pf2 - H.R
GGAG
CCTC
PnPf2+linker
CATG
GTAC
3xHA
GCTT
CGAA
tPf2
pTrpC_HygR_tTrpC
CGCT
GCGA
3'Pf2 - H.R
Step 3 – Replace @ pf2KO (PEG transformation)
5'Pf2 - H.R
GGAG
CCTC
pTef1
CATG
GTAC
PhleoR
GCTT
CGAA
tTef1
CGCT
GCGA
3'Pf2 - H.R

D

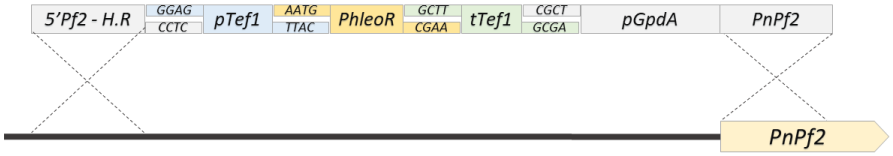
PnPf2 overexpression

- pGpdA+PnPf2

Step 1 – Stage 1/2 GG cloning, modified right flank



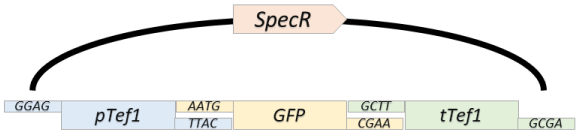
Step 2 – Replace @ PnPf2 promoter (PEG transformation)



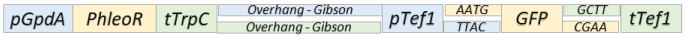
E

GFP positive control

Step 1 – GG assembly of GFP cassette



Step 2 – Attach GFP cassette to Pan8 *PhleoR* cassette (Gibson assembly)



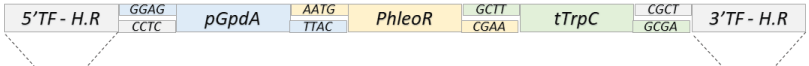
Step 3 – Non-targeted integration into SN15 (PEG transformation)

F

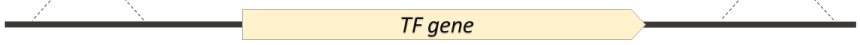
Novel TFs

- Knockout
- Complementation

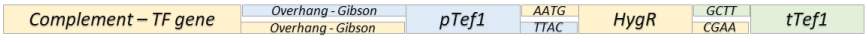
Step 1 – Attach TF flanks to selectable marker (GG Stage 2 cloning)



Step 2 – Knockout TF gene (PEG transformation)



Step 3 – Attach TF gene to new marker (Gibson assembly)



Step 4 – Non-targeted integration into TF KO mutant (PEG transformation)

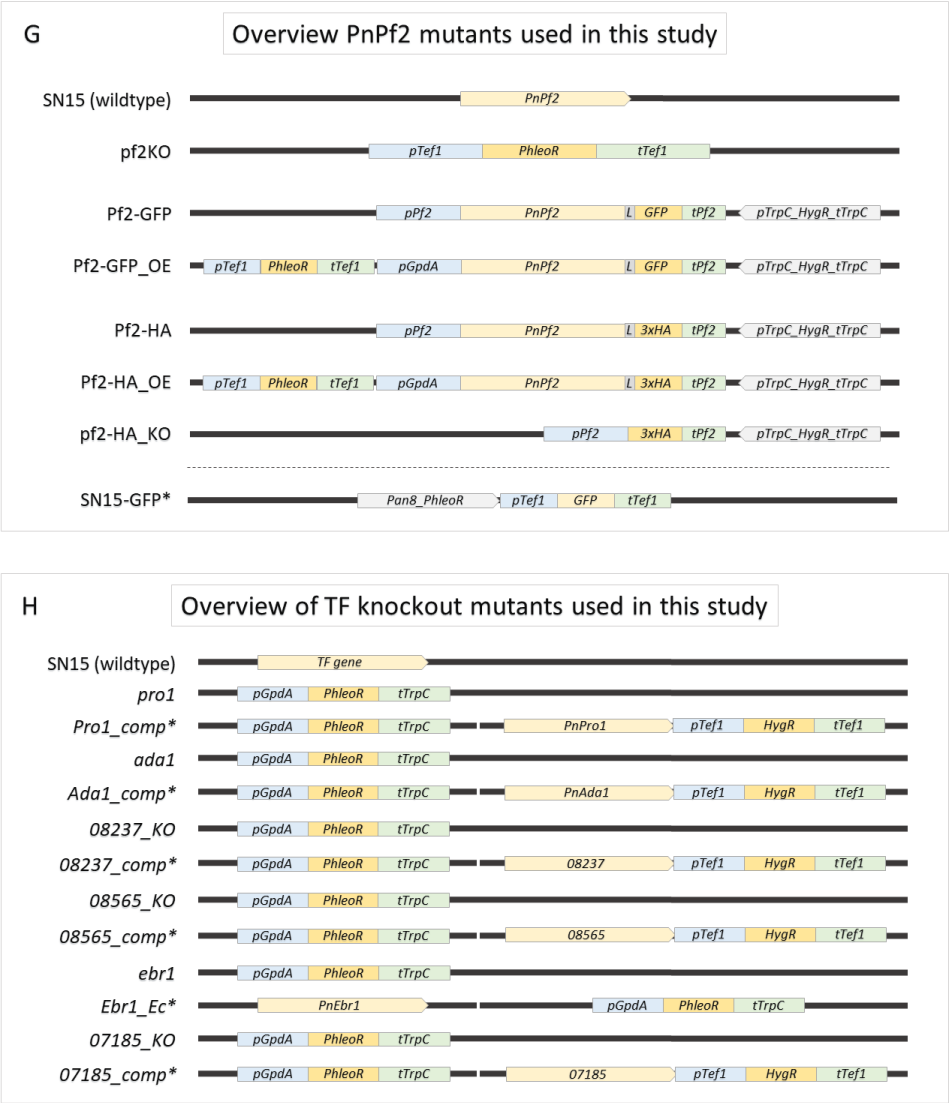


Figure 5.1 - Cloning outline and fungal mutants generated in this chapter

A-F) An overview of the cloning and transformation stages for the replacement constructs used in this study. The respective constructs are indicated in the top left corner for each panel and captions within the panel describe the individual steps undertaken in order. **G**) A summary of the final SN15 mutants at the target loci that were generated and assessed in the study of PnPf2. **H**) A summary of the transcription factor (TF) knockout mutants and their respective complemented (comp) or ectopic (Ec) controls that were generated and phenotypically assessed. *Indicates non-targeted integration of the respective constructs.

5.2.4. Nuclei staining and microscopy

Pycnidiospores of *Pf2-GFP_OE*, SN15 and SN15-GFP were inoculated onto 1% potato dextrose agar (PDA) set on microscope slides and left for 24 hrs in the dark in a humid petri-dish chamber to germinate. Samples were fixed and stained as follows. Fungal material was flooded with a fixing solution (4% w/v formaldehyde and 0.1% Triton in 1x PBS; phosphate buffered saline at pH 7.4) for 10 min, then continuously washed with a rinsing solution (0.1% Triton/PBS) for 10 sec, stained for 10 min (0.1% Triton/PBS with 0.5µg/mL DAPI; 4′,6-diamidino-2-phenylindole) and washed again with the rinsing solution followed by a 1x PBS wash before a coverslip was applied. Samples were viewed on an Olympus BX-51 microscope with a 40x objective lens and a DAPI (Ex 350/50, FT 400, BP 460/50) or FITC (Ex 480/30, FT 505, BP 535/40) filter for visualisation of stained nuclei and GFP fluorescence, respectively.

5.2.5. Fungal mutant gene expression and immunodetection

Gene expression analysis for the *SN15*, *Pf2-HA*, *Pf2-HA_OE* and *pf2-HA_KO* mutants was measured in Fries3 media (Jones et al., 2019) following the method detailed in **Chapter 4**. The *PnPf2* and *Tox3* genes were targeted in the qPCR using Pf2_qPCR_F/R and Tox3_qPCR_F/R respectively. Western blotting was undertaken on whole protein extracts (derived using the method outlined in **Chapter 4**), probed using an anti-HA polyclonal antibody (71-5500, Thermofisher, Waltham, Massachusetts) and detected using an anti-IgG/Pierce ECL chemiluminescence detection system (A16096/32209, Thermofisher).

5.2.6. Chromatin immunoprecipitation (ChIP) sample preparation

Pf2-HA, *Pf2-HA_OE* and *pf2-HA_KO* were grown in 100 mL Fries3 for protein isolation (as detailed in **Chapter 4**). Prior to harvesting, a 5 mL crosslinking solution (10% w/v formaldehyde, 20 mM EDTA and 2mM PMSF dissolved in 50 mM NaOH) was added with continuous shaking at 100rpm for 10 min. To this, 5mL quenching solution (1.25M glycine) was added before another 10 min shaking. Whole protein extracts were then obtained as described previously (**Chapter 4**) with modifications for ChIP (Soyer et al., 2015). The 50 mM Tris was replaced with 50 mM HEPES in the lysis buffer and 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 retrieved from two rounds of centrifugation (5000 g, 4^{o}C for 5 min). A 100 µL aliquot of the supernatant was reserved as an input control used to produce the background level of DNA against which ChIP samples were to be normalised. A 1000 µL aliquot was precleared for immunoprecipitation by gently rotating with 20 uL Protein A dynabeads (10001D, Thermofisher) for 1hr at 4^{o}C. The supernatant was then retrieved and incubated with 2.5 µg anti-HA polyclonal antibody (71-5500, Thermofisher) for 16 hrs at 4^{o}C. Another 20 µL Protein A dynabeads were then added and gently rotated for 2 hrs at 4^{o}C. Dynabeads were then retrieved and washed twice with 1mL 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^{o}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 65ºC. To these samples, 200 µL of H_2O and 100 µg RNAse A (QIAGEN, Hilden, Germany) were added before incubating for 1 hr at 65ºC. Subsequently, 10 µg of Proteinase K (Sigma-Aldrich, St. Louis, Missouri) was added before incubating for a further 1 hr at 50ºC.

For ChIP-qPCR, DNA (for 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. A 35 µL sodium acetate solution (3 M, pH 5.2) was then added with 1 µL of glycogen (20mg/mL). Samples were mixed by inversion and 1 mL 100% ethanol added before precipitation at -80ºC for 1-2 hrs. Pellets were retrieved by spinning 16000 g for 10 min at 4ºC, 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) and 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).

5.2.7. 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, gene ontology (GO) enrichment analysis and PWM motif modelling **Figure 5.2**.

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

Raw reads were checked using FASTQC (Version 0.11.9) (Andrews, 2010) and the adapter sequences were trimmed using Cutadapt (Version 1.15) along with nucleotides where the Illumina quality scores were below 30 (Martin, 2011). Optical duplicates were then removed using the 'dedupe' option in Clumpify (version 1.15) from the BBTools package (Bushnell, 2016). Reads were subsequently mapped to the SN15 genome (Syme et al., 2016) using BWA-MEM (Li, 2013). 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 (Li et al., 2009). 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 (Feng et al., 2012; Gaspar, 2018). 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 (Ramírez et al., 2016).

5.2.7.2. PnPf2 target gene identification

Genes targeted by PnPf2 were determined based on the proximity of summits to annotated genes, which were identified using ChIPseeker (Version 1.24.0) (Yu et al., 2015). Genes with ≥1 summit falling within their ≤1500 bp promoter region (as defined in **Section 5.2.2**) 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 (**Supplementary item 4.1**) 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 (Lin et al., 2012) 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* $-\text{Log}_{10}$(Q-values) at the respective ChIP-seq summit loci. Pearson's correlation coefficient was calculated for the ChIP-qPCR fold-difference and ChIP-seq $-\text{Log}_{10}$(Q-values) at the respective loci and used as the test statistic to assess whether the association was significant (SPSS version 27.0).

5.2.7.3. Modelling PnPf2 binding site motifs

The overlapping peak regions identified from the *Pf2-HA* and *Pf2-HA_OE* samples were merged using MAnorm (Shao et al., 2012) to create a consensus set of enriched peak regions containing the putative PnPf2 binding sites. From this set, overrepresented motif PWMs (up to 20 bp long) were modelled directly with MEME (version 5.1.1) (Bailey et al., 2009; Bailey and Elkan, 1994). 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 (Bailey and Machanick, 2012). Gene promoters with ≥ 1 occurrence of each motif were determined using FIMO (Grant et al., 2011). Fisher's exact test with Bonferroni corrected P-values (Armstrong, 2014) was used to identify *pf2ko* DE gene promoter sets significantly enriched (Padj < 0.01) for the respective motifs vs the background rate in SN15.

5.2.7.4. PnPf2 target gene analysis

The PnPf2 target genes were cross-referenced with the *pf2ko* DE gene patterns (expressed significantly up or down in *pf2ko*) from Jones et al., 2019 to identify cases where direct binding was linked to the modulation of gene expression. The SN15 effector-like genes annotated by Jones et al., 2019 (possessing a secretory signal peptide with no other transmembrane domains and predicted to be effector-like by EffectorP) were compiled among the PnPf2 targets. Annotated homologues were identified from the corresponding records in UniProt (release 2020_05) (Bursteinas et al., 2016). Both the high-confidence and total PnPf2 target gene sets were then used for GO enrichment/network analysis using the SN15 genome-wide GO annotations also used by Jones et al., 2019. The 'enricher' function in the Clusterprofiler package

(Version 3.16.0) (Yu et al., 2012) was invoked to identify the overrepresented GO classes ($P < 0.01$) in PnPf2 targets. The *P. nodorum* TFs compiled among the PnPf2 targets were sourced from the annotations in **Chapter 3**. The TFs that were also down-regulated in *pf2ko* were assessed for functionally-defined species orthologues by cross-referencing the respective orthogroups against the literature reviewed in **Chapter 2** (**Supplementary item 2.1**).

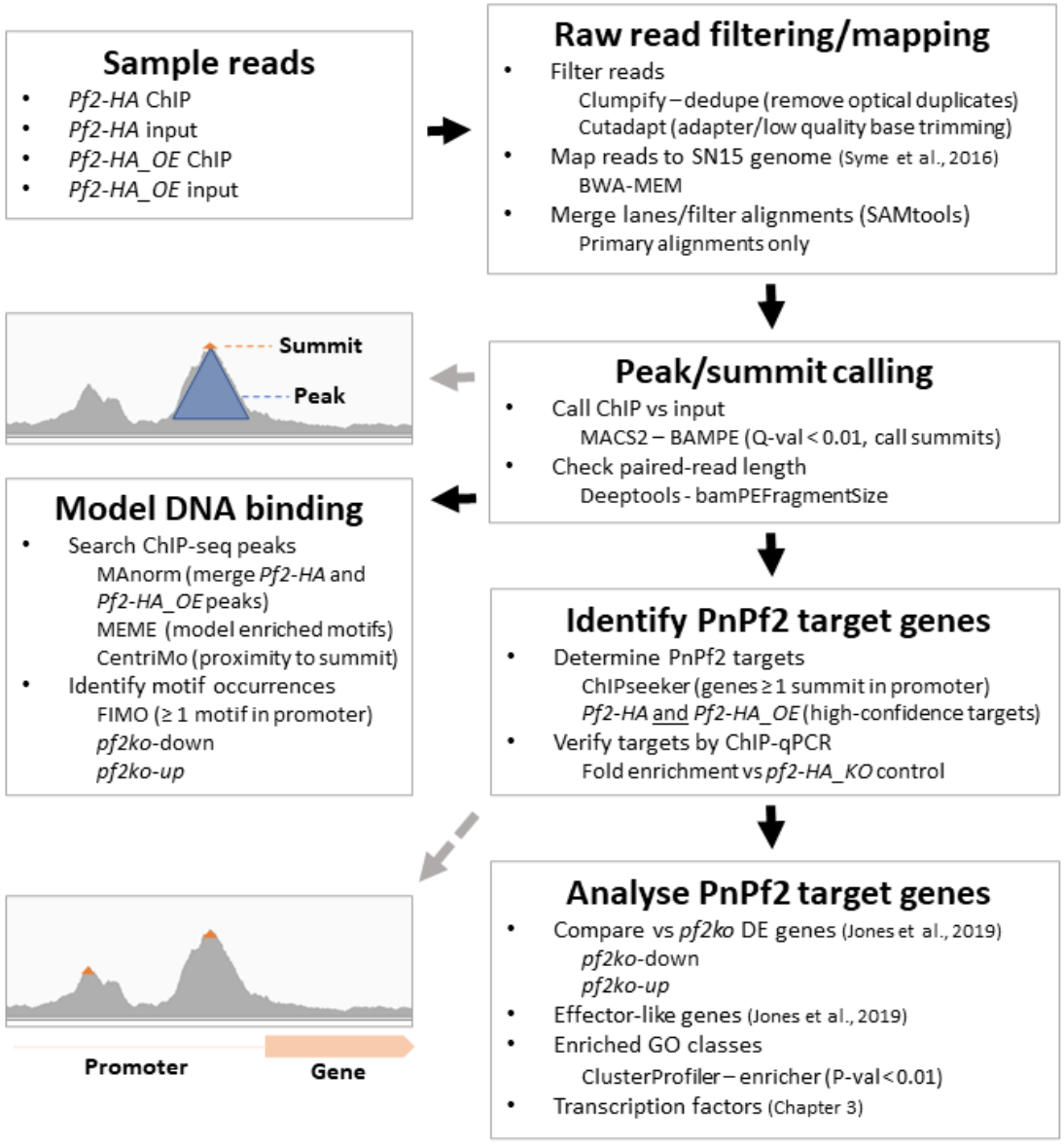


Figure 5.2 - ChIP-seq data analysis overview

A diagrammatic overview of the data processing and analysis pipeline is described in **Section 5.2.7**. Grey arrows indicate visual examples of the output from the corresponding stages while black arrows indicate subsequent procedures.

5.2.8. Novel transcription factor analysis

5.2.8.1. Selection of transcription factors for gene knockout

Three candidate PnPf2 targeted TFs were selected for functional analysis that met all of the following criteria: a ChIP-seq summit in the promoter; down-regulation in *pf2ko* (Jones et al., 2019) and a pathogen orthologue with a defined virulence role. Three genes encoding Zn2Cys6 TFs classed as candidate PnPf2 co-regulators were also selected for functional analysis. One was selected as the other *P*. *nodorum* TF in orthogroup OG0000017 (**Chapter 3**). A hierarchical-cluster analysis was then used to identify Zn2Cys6 TFs co-expressed with *PnPf2*, which made use of genome-wide SN15 microarray expression data from a previous study that spanned multiple infection timepoints and *in vitro* conditions (Ipcho et al., 2012). SN15 genes were clustered together with pheatmap using the normalised microarray gene expression values (Z-scores) (Kolde, 2015). Clustering distances were derived from Pearson's correlation coefficients using the Average linkage function. The candidate PnPf2 coregulators were selected from the Zn2Cys6 TFs clustering within the *PnPf2*/*ToxA*/*Tox1*/*Tox3* co-expression clade.

5.2.8.2. Knockout mutant phenotypic analyses

Two KO mutants and a complemented control mutant (or ectopic control where the original KO mutant was not amenable to PEG transformation) for the six respective TFs (**Figure 5.1.H**) were subject to a comparative phenotypic analysis to identify differences relative to the wildtype SN15 and the *pf2ko* mutant. Colony morphologies were compared following 12 days of routine growth on V8PDA (**Chapter 4**). Lesions on the wheat cv. Halberd and Calingiri were monitored for 12 days post-inoculation (with 3 mm mycelial plugs from the growing tip) using detached leaf assays (DLAs) (**Chapter 4**). Fungal culture filtrates (prepared as described in **Chapter 4**) were

assessed for necrosis inducing activity compared to SN15 after four days following infiltration into five wheat lines that are differentially sensitive to ToxA, Tox1 and Tox3 [Halberd (*Tsn1*, *Snn1*, *Snn3*), Calingiri (*tsn1*, *Snn1*, *snn3*), Estoc (*Tsn1*, *snn1*, *Snn3*), BG220 (*tsn1*, *snn1*, *Snn3*) and BG261 (*Tsn1*, *snn1*, *snn3*)]. Differences in minimum inhibitory concentrations vs SN15 were established after seven days growth on MM agar (**Chapter 4**) by testing a range of concentrations for NaCl, H_20_2, Calcofluor white and Congo Red.

PnEbr1, *PnPro1* and *PnAda1* derived mutants (**Figure 5.1.H**) were then characterised in conjunction with SN15 and *pf2ko* as follows. Virulence was quantified using DLAs after 12 days with 10 replicates (5x Halberd and 5x Calingiri) using two metrics; lesion diameter (mm) and weighted pycnidia counts (scored as immature/black = 1, mature/pink = 2, fully mature/burst = 3). The conidiation rate was assessed *in vitro* by counting pycnidiospores following 12 days growth on V8PDA with three replicates. Osmotic stress inhibition was measured by comparing the radial growth of strains on MM agar plates with and without 20 mM H_2O_2. A relative measure of fitness was obtained by dividing the diameter with/without H_20_2 across three replicates. For each of the aforementioned assays, a one-way ANOVA with Tukeys-HSD post-hoc test was used to test for differences ($P < 0.05$) between SN15, *pf2ko* and the TF mutants generated in this study (SPSS version 27.0).

5.3. Results

5.3.1. Functional domains are conserved in *P. nodorum* PnPf2

PnPf2 was first examined *in silico* to verify the evolutionary trajectory and identify any conserved structural domains linked to TF activity. From the 370 annotated Zn2Cys6 TFs analysed, 92 were PnPf2 species orthologues, supported in a clade comprising 83 fungi from the Sordariomycete, Leotiomycete, Dothideomycete and Xylonomycete lineages (**Figure 5.3**). The remaining 278 TFs analysed included *S. cerevisiae* MAL13 and *A. nidulans* AmyR; carbon metabolic regulators (Charron et al., 1986; Tani et al., 2001; Nakamura et al., 2006) whose evolutionary relationship to PnPf2 was not confidently traced to a distinct speciation event.

The 652 residue PnPf2 protein was found to harbour the Zn2Cys6 DNA-binding domain (IPR001138) located at residues 9 to 54 with an overlapping nuclear localisation signal (NLS) (KKGPKGSR; located from residues 51 to 58). The 'fungal transcription factor domain' (IPR007219) was identified from residues 223 to 294. This domain, also referred to as the 'middle homology region', is presumed to be involved in the modulation of TF activity (MacPherson et al., 2006). A refined phylogenetic analysis revealed these domains are highly conserved among the PnPf2 orthologues that have been functionally defined (**Figure 5.4**). In contrast, a structurally disordered domain associated with post-translational modifications and intermolecular interactions (Erdős and Dosztányi, 2020) was identified at the N-terminus of PnPf2 that was a highly variable region. Together, these results suggested PnPf2 orthologues possess the typical functional domains underpinning Zn2Cys6 TF activity (MacPherson et al., 2006). This indicated the gene expression changes previously observed in the *P. nodorum pf2ko* mutant (Jones et al., 2019) resulted from the loss of activity at gene regulatory elements.

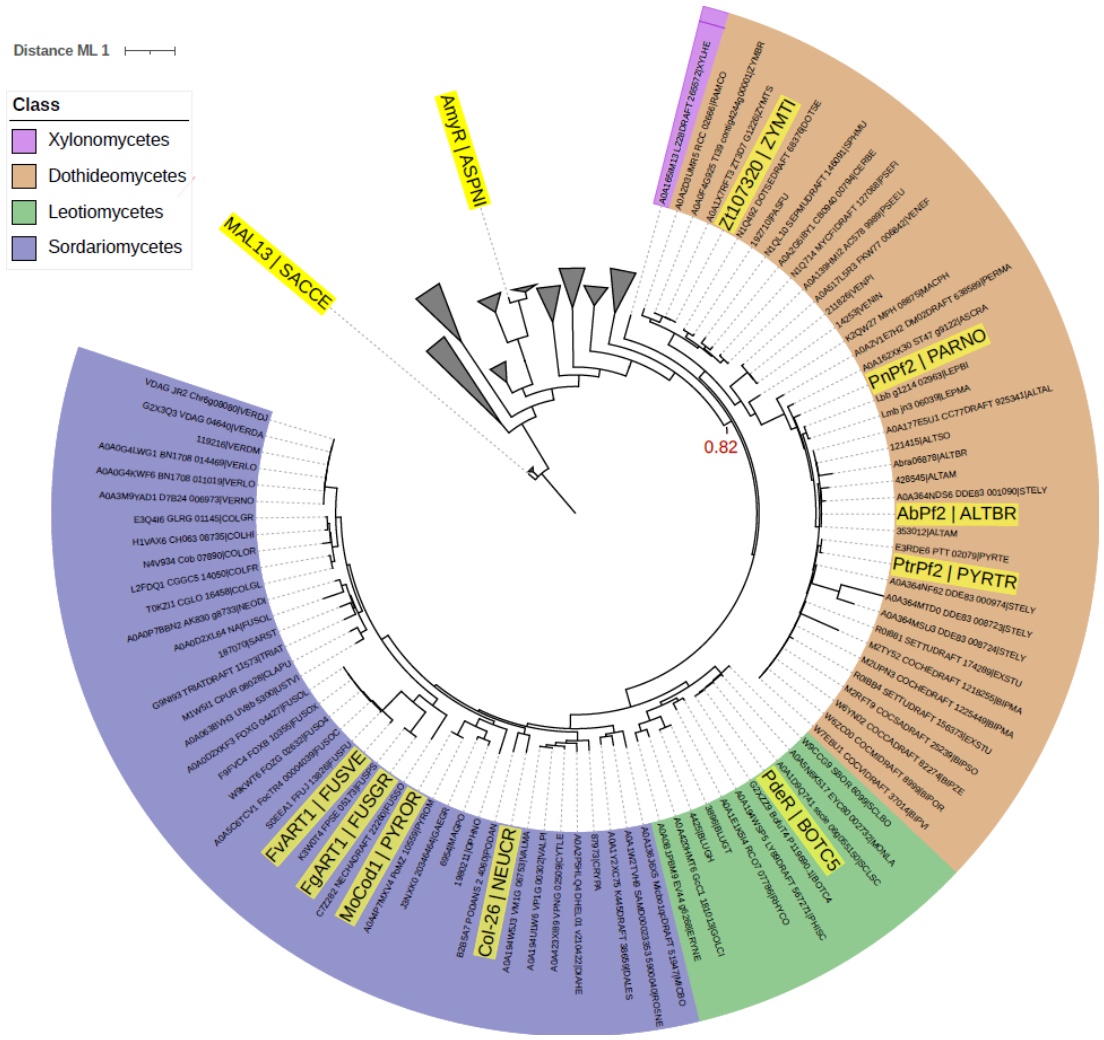


Figure 5.3 - Phylogenetic analysis of PnPf2 transcription factor (TF) lineage highlighting orthologues defined in this study

A phylogenetic analysis of 370 fungal Zn2Cys6 TFs. The 92 annotated PnPf2 orthologues are coloured by fungal taxonomic classes. The PhyML tree support value (0.82) for the PnPf2 clade is indicated in red. Functionally-defined TFs are highlighted in yellow. Non-PnPf2 orthologues (total TFs = 278) were collapsed to assist visualisation with the exception of *S. cerevisiae* MAL13 and *A. nidulans* AmyR, included as functionally-defined outgroups for reference.

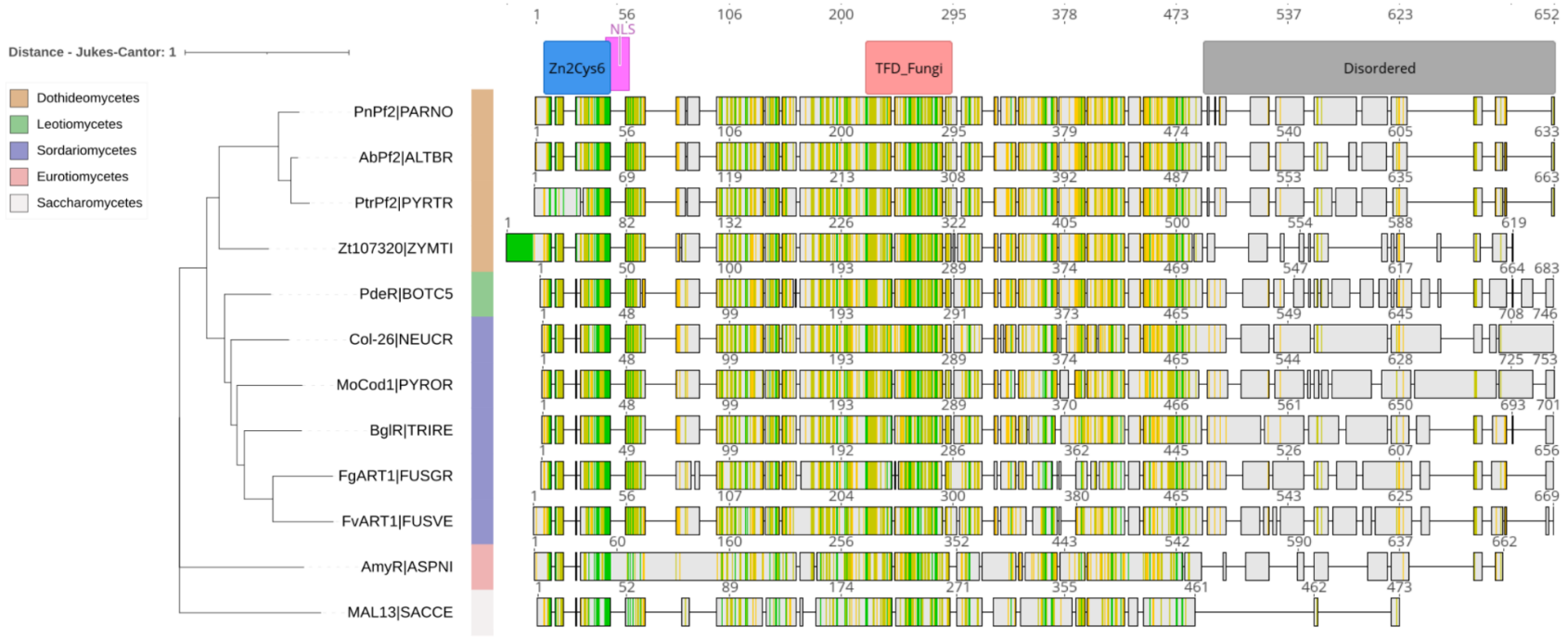


Figure 5.4 - Functional domain conservation of PnPf2 orthologues

The PnPf2 protein sequence with annotates structural domains is presented (top) in an alignment of functionally validated TF orthologues (named with the accompanying ORGIDs defined in **Supplementary item 3.1**). Sequence agreement is indicated on a decreasing scale of green>yellow>grey. A neighbour-joining tree derived from the alignment is presented to depict the genetic distances across each TF. The *S. cerevisiae* MAL13 and *A. nidulans* AmyR sequences were included for reference as outgroups.

5.3.2. Promoter elements are enriched in PnPf2-regulated genes

A broad set of genes were previously reported that were positively (i.e. *pf2ko*-down vs SN15) or negatively (i.e. *pf2ko*-up vs SN15) regulated by PnPf2 during either *in planta* or *in vitro* growth (Jones et al., 2019). A motif enrichment analysis was therefore undertaken to identify putative PnPf2 regulatory binding sites. A consensus motif 5‘-WMGGVCCGAA-3’ (where W=A/T, M=A/C and V=A/C/G) was significantly enriched among the positively regulated gene promoters (**Figure 5.5**) where it was identified at least once in 171 of the 590 genes. This motif contained the ‘CGG’ triplet characteristic of Zn2Cys6-binding sites and strongly resembled an enriched motif associated with AbPf2 regulated genes in *A*. *brassicicola* (MacPherson et al., 2006; Cho et al., 2013), indicative of a conserved regulatory element targeted by PnPf2.

Additional enriched motifs were also detected. The 5‘-RTSYGGGGWA-3’ consensus motif (where R=A/G, S=G/C, Y=C/T and W=A/T) was significantly enriched in the *pf2ko*-down genes under the *in planta* conditions (**Figure 5.5**). This was similar to fungal C2H2 TF-binding sites in the JASPAR database and thus unlikely to be a direct PnPf2 target. The motif is near-identical to the Cre-1 carbon-catabolite repressor element modelled in *N*. *crassa* (Wu et al., 2020). This suggested either 5‘-RTSYGGGGWA-3’ is genetically linked to PnPf2 targets or that a candidate Cre-1 *P*. *nodorum* orthologue (SNOG_13619 in OG0000002; **Chapter 3**) is an intermediate factor controlling a subset of PnPf2-regulated genes *in planta*. Another enriched motif 5‘-CTGYGCCGCA-3’ (where Y=C/T) was identified in PnPf2 repressed genes under the *in vitro* conditions. This was also similar to C2H2 TF-binding sites in the JASPAR database (**Figure 5.5**), suggesting it was not a direct PnPf2 target. The presence of these additional enriched motifs indicated synergistic and/or antagonistic TFs acted on a significant proportion of PnPf2-regulated genes.

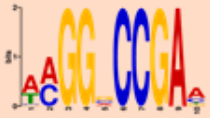
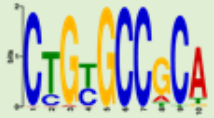
PnPf2 regulation	Promoter motif	JASPAR identity	Promoters screened	≥ 1 match	Total	Padj
Positive (*pf2ko*-down)	5'-WMGGVCCGAA-3' 	MA0429.1 (YLL054C)	SN15	1579	13577	
			pf2ko-down	171	590	4.1E-30
			pf2ko-up	65	718	1.0E+00
			pf2ko-down *in planta*	98	302	9.5E-21
			pf2ko-up *in planta*	40	445	1.0E+00
			pf2ko-down *in vitro*	99	342	5.6E-17
			pf2ko-up *in vitro*	29	324	1.0E+00
Positive (*pf2ko*-down) *in planta*	5'-RTSYGGGGWA-3' 	MA0339.1 (MIG3) MA0441.1 (ZMS1) MA0338.1 (MIG2) MA0431.1 (TDA9)	SN15	2499	13577	
			pf2ko-down	181	590	5.2E-12
			pf2ko-up	123	718	1.0E+00
			pf2ko-down *in planta*	121	302	2.6E-17
			pf2ko-up *in planta*	84	445	1.0E+00
			pf2ko-down *in vitro*	72	342	1.0E+00
			pf2ko-up *in vitro*	48	324	1.0E+00
Negative (*pf2ko*-up) *in vitro*	5'-CTGYGCCGCA-3' 	MA0394.1 (STP1) MA0395.1 (STP2)	SN15	2699	13577	
			pf2ko-down	152	590	1.0E-02
			pf2ko-up	253	718	1.5E-21
			pf2ko-down *in planta*	81	302	8.7E-02
			pf2ko-up *in planta*	115	445	5.0E-02
			pf2ko-down *in vitro*	83	342	1.0E+00
			pf2ko-up *in vitro*	157	324	6.1E-30

Figure 5.5 (previous page) - Promoter elements enriched in PnPf2-regulated genes from comparison of SN15 and *pf2ko* expression

Motifs modelled from PnPf2 positively or negatively regulated gene promoters [columns 1-2]. Motif letters represent nucleotides as follows: M=A/C, V=A/C/G, R=A/G, S=G/C, Y=C/T and W=A/T. Also indicated in this figure are the fungal TF-binding sites with shared identity to the corresponding motifs in the JASPAR database [3]. The gene promoter subsets (differentially regulated in *pf2ko*) tested for enrichment (Fisher's test with Bonferroni Padj < 0.01) relative to the total occurrences in the wildtype SN15 promoters are outlined in the remaining columns [4-7]. Gene promoter subsets that were significantly enriched vs SN15 are highlighted blue.

5.3.3. PnPf2 localises to the nucleus

Epifluorescence microscopy was used for *in situ* verification of the PnPf2 NLS using a *P. nodorum* mutant strain constitutively expressing a PnPf2-GFP translational fusion protein (*Pf2-GFP_OE*). GFP signals were detected that were compartmentalised in the mycelia of germinated pycnidiospores. DAPI-counterstaining indicated these signals corresponded to the location of fungal nuclei (**Figure 5.6**). Together with the *in silico* domain analysis and the identification of the enriched 5‘-WMGGVCCGAA-3’ motif resembling a Zn2Cys6-binding site, these observations provided strong evidence that PnPf2 is active in the nucleus.

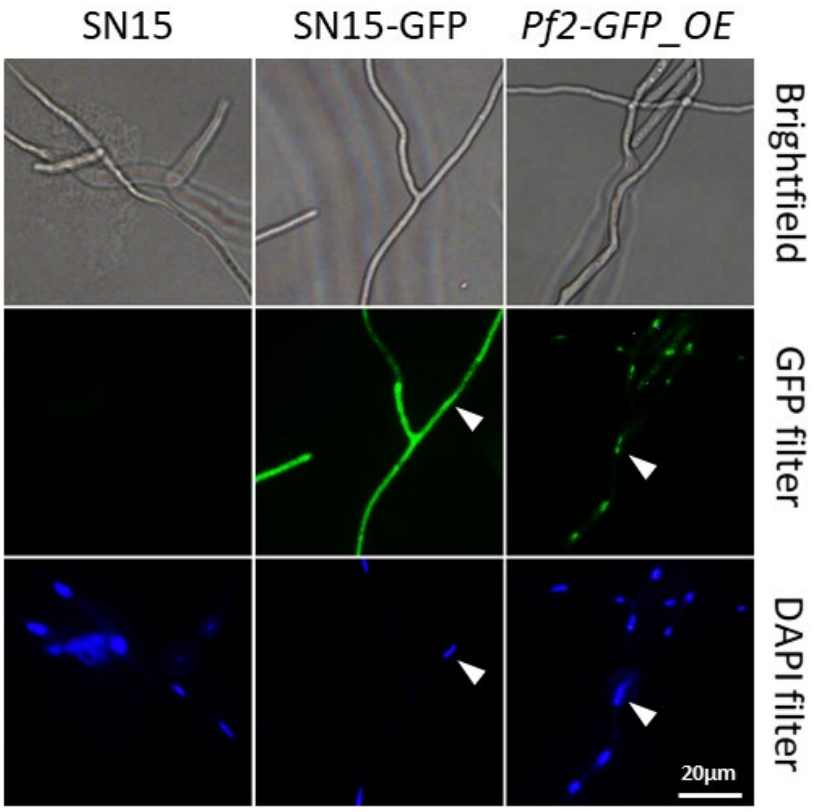


Figure 5.6 - Subcellular localisation of PnPf2 in the nucleus

Epifluorescence microscopy depicting the nuclear localisation observed for GFP-tagged PnPf2 specific to the *PF2-GFP_OE* 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 germinated pycnidiospores.

5.3.4. Chromatin immunoprecipitation analysis establishes the regulatory mechanisms of PnPf2

5.3.4.1. ChIP-seq datasets generated with PnPf2 under both native and overexpression promoters

A chromatin immunoprecipitation (ChIP) approach was used to determine the direct targets of PnPf2. The 3x hemagglutinin (HA) tagged PnPf2 mutant strain (*Pf2-HA*) was grown under the same *in vitro* conditions as previously described to ensure compatibility with the RNA-seq dataset (Jones et al., 2019). The proportion of fungal biomass under the *in planta* RNA-seq conditions was low for optimal ChIP-seq so an overexpression variant of the *Pf2-HA* strain (*Pf2-HA_OE*) was used to produce a complementary dataset designed to increase the scope of detectable binding sites.

Prior to ChIP-seq, a gene expression analysis demonstrated that *PnPf2* was expressed 2.7 fold higher in *Pf2-HA_OE* than *Pf2-HA* (**Figure 5.7.A)**. Interestingly, *PnPf2* was expressed 2.2 fold higher than the wildtype (SN15) in *Pf2-HA*. This was attributed to possible side-effects of incorporating the HA tag or trans-gene marker downstream (as depicted in **Figure 5.1.G**) as Sanger sequencing did not reveal inadvertent mutations at the gene promoter/coding regions. Nevertheless, *Tox3*, the NE known to be regulated by PnPf2 *in vitro* (Rybak et al., 2017), was expressed at a similar level to SN15 in both *Pf2-HA* and *Pf2-HA_OE*. This indicated the PnPf2-HA fusion protein was functional, but also that overexpression in *Pf2-HA_OE* did not correlate with greater *Tox3* expression, suggesting other limiting factors are involved in the regulation of this gene. A 75 kDa protein was then detected in both mutants, corresponding to the expected size of the PnPf2-HA fusion protein. This was not detected in SN15 and the negative control mutant *pf2-HA_KO*, indicating both *Pf2-HA* and *Pf2-HA_OE* were suited for ChIP-seq with the anti-HA antibody (**Figure 5.7.B**).

Moreover, the colony morphologies did not exhibit major differences and both *Pf2-HA* and *Pf2-HA_OE* produced comparable lesions on detached wheat leaves, suggesting that the 3xHA-PnPf2 fusion protein was functional (**Figure 5.7.C**).

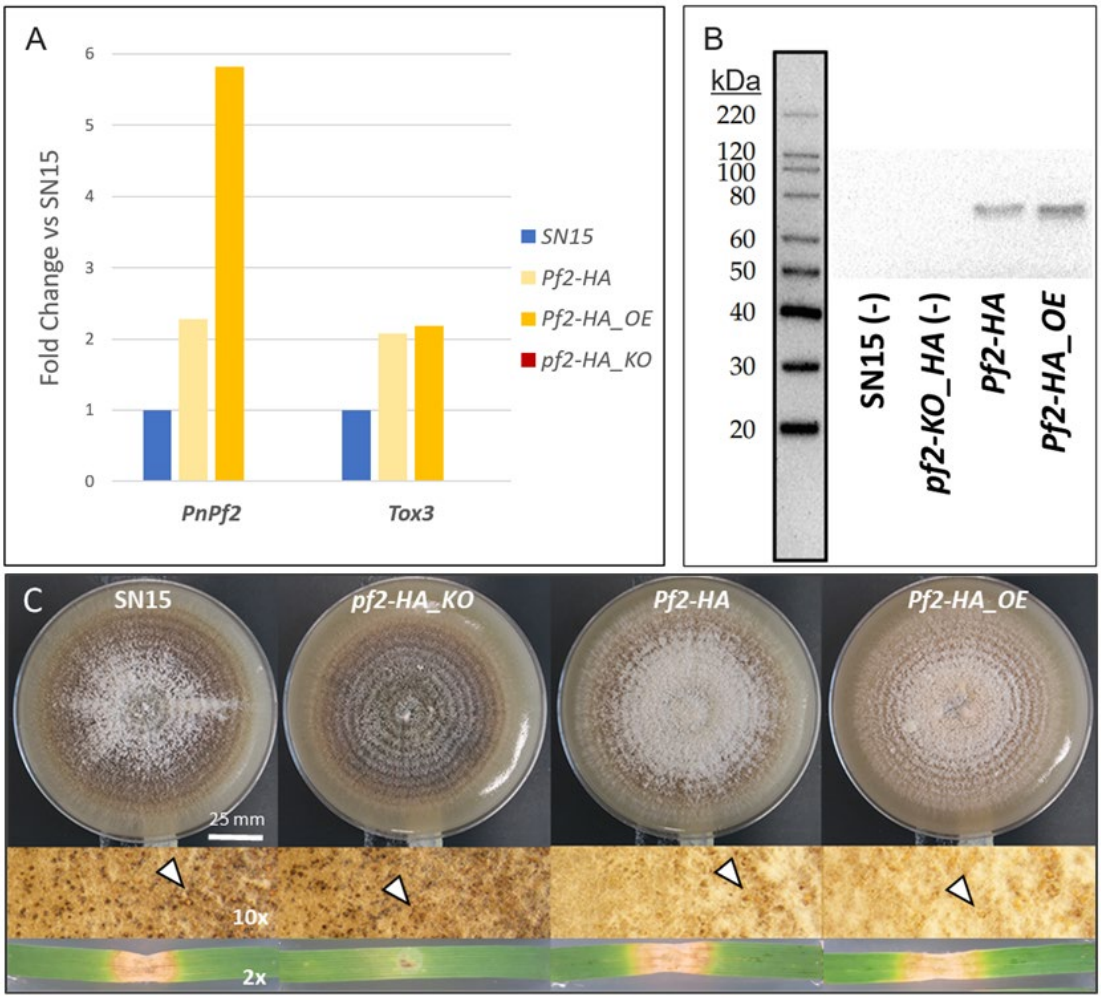


Figure 5.7 - PnPf2 3xHA-tagged mutants express functional PnPf2

Quality-control assessment of the 3xHA-tagged mutants. A) The relative gene expression vs SN15 of *PnPf2* and the PnPf2-regulated *Tox3* gene under the ChIP-seq conditions (Fries3 broth, three days growth). B) PnPf2-3xHA fusion protein corresponding to the expected size ~75 kDa detected by Western blot analysis of total-protein extracts. C) A phenotypic comparison for the respective mutants relative to the SN15 wildtype. The upper two images represent 12 days growth on V8PDA, with arrows indicating mature pycnidia. The lower images depict representative lesions, 12 days following inoculation on detached wheat leaves (cv. Halberd).

After quality control and mapping, ChIP-seq yielded 12,416,341 read-pairs for *Pf2-HA* and 17,697,729 for *Pf2-HA_OE* that were uniquely mapped to the SN15 genome. The input controls yielded 13,307,189 and 15,270,059 read-pairs, respectively (**Table 5.1**). The paired read lengths for each dataset were predominantly within the expected 100-300 bp size range, indicating that sample fragmentation and size-selection had been successful (**Figure 5.8**). In total, 997 summit loci for *Pf2-HA* and 2196 for *Pf2-HA_OE* were identified within the 740 and 1588 peak regions respectively. Summits corresponded to the best estimate of PnPf2 binding sites and multiple summits within a single peak region were also observed, indicative of adjacently bound loci. By merging the two datasets, 588 peak regions were identified that were common to *Pf2-HA* and *Pf2-HA_OE* samples (**Supplementary item 5.1**) indicating strong overlap between the predicted PnPf2 targeted genomic regions for the two datasets, but also that a large proportion were only detectable from the *Pf2-HA_OE* sample.

Table 5.1 - A summary of ChIP-seq reads for PnPf2 3xHA-tagged mutant samples

Summary of ChIP and input DNA samples prepared for the 3xHA-tagged mutant *Pf2-HA* (native promoter) and *Pf2-HA_OE* (overexpression promoter) [columns 1-2]. The remaining columns document the total raw ChIP-seq reads and reads mapped to the SN15 genome following quality-control (QC) filtering [3], the total uniquely mapped read-pairs that were used for peak/summit calling [4], the number of ChIP-seq peaks that were detected relative to the input control for *Pf2-HA* and *Pf2-HA_OE* [5] and the total summits called from those peak regions [6].

Sample ID	DNA	Raw reads (QC mapped)	Uniquely mapped	Peak regions	Peak summits
Pf2-HA	ChIP	22,740,293 (20,824,748)	12,416,341	740	997
	Input	21,762,558 (20,386,814)	13,307,189		
Pf2-HA_OE	ChIP	29,524,953 (27,391,018)	17,697,729	1588	2196
	Input	22,974,742 (21,826,313)	15,270,059		

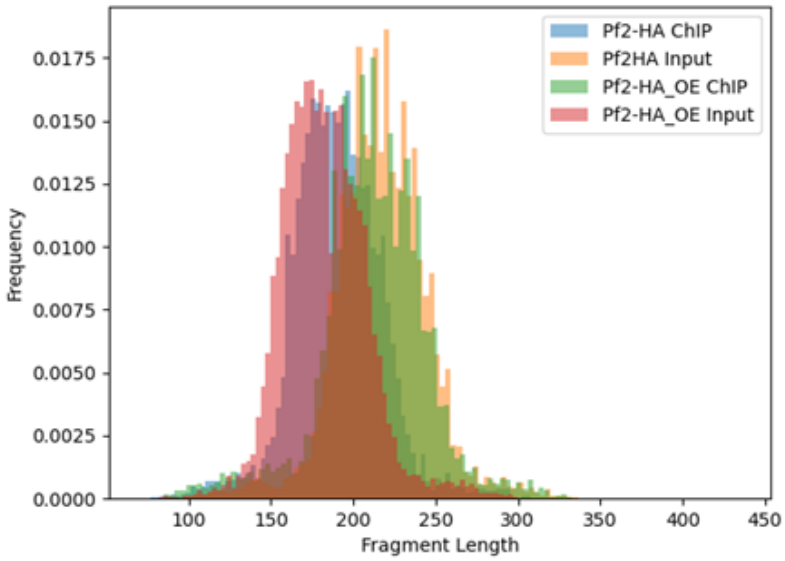


Figure 5.8 - ChIP-seq paired-read fragment lengths for the PnPf2 3xHA-tagged mutant samples used in this study

A frequency plot summarising the proportional frequency of read-pair lengths (in nucleotides) that were used for fragment pileup in the *Pf2-HA* (native promoter) and *Pf2-HA_OE* (overexpression promoter) strains that formed the basis of detecting enriched peak regions/summits.

5.3.5. PnPf2 directly regulates necrotrophic effectors and a broad set of target genes

A primary goal was to determine the nature of PnPf2 regulation on NE gene expression. The known NEs present in *P. nodorum* SN15 are ToxA, Tox1, Tox3 and Tox267 (Richards et al., 2021). Accordingly, their gene promoter regions were examined for evidence of PnPf2 binding to compare with their gene expression profile determined in the previous RNA-seq analysis (Jones et al., 2019). Inspection of the *Tox3* promoter revealed two distinct ChIP-seq summits in the *Pf2-HA* and *Pf2-HA_OE* datasets that indicated PnPf2 binding (**Figure 5.9**). This promoter is bidirectional, implying the upstream annotated gene SNOG_08982 (encoding a protein disulfide-isomerase; Interpro domain IPR005792) was simultaneously regulated by PnPf2. The expression of both genes is significantly down in the *pf2ko* mutant, suggesting they are both positively regulated by PnPf2 as a result of direct promoter binding. A ChIP-seq summit was also identified in the *Tox1* promoter, but only from the *Pf2-HA_OE* dataset (**Figure 5.9**). Unlike Tox3, Tox1 activity is still detected in the *pf2ko* mutant background (Rybak et al., 2017). This indicates the ChIP-seq summit represents a *Tox1* gene enhancer element not essential for expression. *ToxA* however did not exhibit a clear promoter summit, despite three instances of the putative PnPf2 consensus motif 5‘-WMGGVCCGAA-3’ being identified (**Figure 5.9**). This suggested that direct PnPf2 binding occurs, but is inhibited by other regulatory factors under the *in vitro* conditions used and was not detected by ChIP-seq. No distinct PnPf2 summit was observed in the promoter of *Tox267*, a NE gene where gene expression is not significantly altered in the *pf2ko* mutant. However, two instances of 5‘-WMGGVCCGAA-3’ were identified, indicating that other factors are required for PnPf2 to bind this motif under the conditions tested.

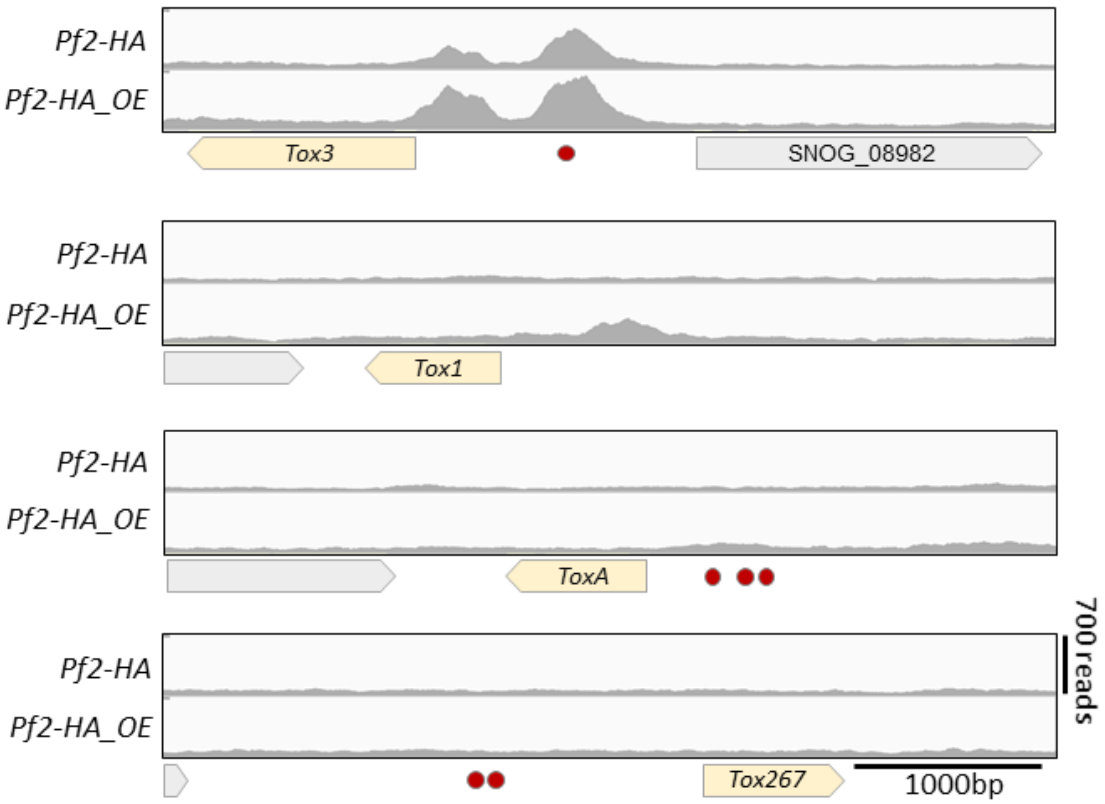


Figure 5.9 - PnPf2 targeting of cloned necrotrophic-effector (NE) genes in *P. nodorum* SN15

The *Pf2-HA* (native promoter) and *Pf2-HA_OE* (overexpression promoter) sample ChIP-seq reads at the *Tox3*, *Tox1*, *ToxA* and *Tox267* NE promoters. Peak summits were evident in the *Tox3* and *Tox1* promoters indicated by the elevation the read pileups. Red dots represent instances of the 5'-WMGGVCCGAA-3' consensus motif (where W=A/T and M=A/C) putatively targeted by PnPf2, which was modelled from the RNA-seq dataset enrichment in the *pf2ko* down-regulated gene promoters.

A broader range of PnPf2 direct targets was also identified in addition to the NEs. From the 1286 genes in total, 484 high-confidence targets were established that corresponded to a promoter summit in both the *Pf2-HA* and *Pf2-HA_OE* samples (**Figure 5.10**). A ChIP-qPCR analysis was then undertaken on independently prepared DNA samples to verify ChIP-seq summits using a subset of the genes targeted. The ChIP-qPCR fold enrichment was significantly correlated with ChIP-seq summit enrichment Q-values at the respective summit loci 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 (**Table 5.2**). The replicability across the separate methodologies provided confidence that the broad set of target genes identified were based on true instances of PnPf2 binding.

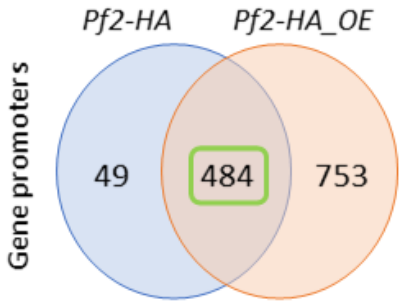


Figure 5.10 - Total direct PnPf2 gene targets detected by ChIP-seq in *P. nodorum*
The green box indicates the high-confidence targets that were identified in both the *Pf2-HA* and *Pf2-HA_OE* sample datasets from the 1286 putative targets in total.

Table 5.2 – ChIP-qPCR validation of PnPf2 ChIP-seq promoter summits

A summary table of several gene promoters assessed by ChIP-qPCR, that targeted the ChIP-seq summits or the negative control (-) summit-free loci [column 1]. The ChIP-qPCR fold-enrichment values vs the *pf2-HA_KO* control strain and the ChIP-seq -Log_{10}(Q-values) for the summits are given for the *Pf2-HA* and *Pf2-HA_OE* sample datasets respectively [2-3]. [A]Correlated values ($P < 0.01$) based on Pearson's correlation ($r = 0.77$). [B]Correlated values ($P < 0.01$) based on Pearson's correlation ($r = 0.74$).

Target	*Pf2-HA* [A]		*Pf2-HA_OE* [B]	
	qPCR	Summit	qPCR	Summit
Act1 exon (-)	1.0	-	1.0	-
TrpC terminator (-)	0.9	-	1.2	-
ToxA promoter	0.9	-	1.4	6.5
Tox3 promoter	2.2	325.9	3.7	550.7
Tox1 promoter	0.8	-	3.6	121.6
SNOG_03901 promoter	1.2	5.4	1.2	37.7
SNOG_04486 promoter	0.9	9.5	4.4	191.2
SNOG_12958 promoter	2.2	236.3	5.7	252.3
SNOG_15417 promoter	2.1	78.0	2.7	139.3
SNOG_15429 promoter	3.5	180.4	5.9	492.9
SNOG_15429 exon (-)	0.9	-	1.7	-
SNOG_16438 promoter	0.7	-	3.4	125.4
SNOG_20100 promoter	1.5	21.5	3.9	160.9
SNOG_30077 promoter	1.1	-	5.4	231.0

5.3.5.1. Two distinct PnPf2 binding elements underpin positive gene regulation

It was then determined if PnPf2 functions predominantly as a positive or negative direct regulator of target genes. To do this, the *pf2ko* RNA-seq expression patterns of the 484 high-confidence PnPf2 direct targets were compared to identify regulatory trends. The 484 high-confidence ChIP-seq targets were compared with the 665 DE genes from RNAseq analysis under the *in vitro* culture conditions. In total, 72 genes positively regulated by PnPf2 (*pf2ko*-down) were direct targets. This contrasted to just 6 for the repressed genes (*pf2ko*-up), suggesting PnPf2 regulates in a largely-positive fashion (**Figure 5.11**). For DE genes regulated by PnPf2 under *in vitro* or *in planta* conditions, 93 of the direct targets are positively regulated in contrast to 27 negatively regulated genes. Interestingly, the 364 remaining gene targets were not classed as PnPf2-regulated. This indicated that PnPf2 was either a redundant regulator, transiently bound or not active under the growth conditions tested at these loci. Furthermore, evidence of PnPf2 binding was missing for 1,214 of the total 1,334 genes that were DE under either condition. This suggested that indirect factors are a significant component of PnPf2 regulation.

Figure 5.11 – A comparison of PnPf2 direct targets with gene expression patterns in the *pf2ko* mutants

The PnPf2 positively regulated (*pf2ko*-down) and repressed (*pf2ko*-up) genes determined by RNA-seq. The *in vitro* used for ChIP-seq are presented on the left while the right presents the genes that were differentially expressed (DE) in either the *in vitro* or *in planta* conditions to capture the maximum possible scope of PnPf2-regulated genes. Both DE datasets were compared to the 484 high-confidence direct targets identified through ChIP-seq in this study.

The 5‘-WMGGVCCGAA-3’ consensus motif was already characterised as a candidate PnPf2 binding site from the promoters of PnPf2 positively regulated genes (**Section 5.3.2**). Here, the ChIP-seq dataset was used to assess this and to uncover any other PnPf2-regulatory elements. The genome-wide 1662 merged *Pf2-HA* and *Pf2-HA_OE* ChIP-seq peak regions (**Supplementary item 5.1**) were analysed to comprehensively explore sequence enrichment, from which two distinct motifs were identified. The first was 5’-RWMGGVCCGA-3’ (where R=A/G, W=A/T and V=A/C/G), which closely matched and further refined the 5‘-WMGGVCCGAA-3’ consensus RNA-

seq derived motif, suggesting this did represent a major PnPf2 positive-regulatory binding element (**Figure 5.12.A**). The second motif 5'-CGGCSBBWYYKCGGC-3 (where S=C/G, B=C/G/T, W=A/T, Y=C/T and K=G/T) was novel for PnPf2, encompassing two tandem copies of the canonical 'CGG' Zn2Cys6-binding triplets separated by eight nucleotides. This motif, like 5'-RWMGGVCCGA-3', was also specifically enriched in the *pf2ko*-down gene promoters (**Figure 5.12.A**). Both motifs were situated in close proximity to the ChIP-seq summits for the *Pf2-HA* and *Pf2-HA_OE* datasets (**Figure 5.12.B**), which indicated each reflected distinct PnPf2 binding elements. The alternative spacing and orientation of their CGG triplets raised the possibility that PnPf2 actively binds DNA in multiple conformations, either as a homodimer or a Zn2Cys6 homo-typic TF dimer.

Of the 484 high-confidence PnPf2 targets, the refined 5'-RWMGGVCCGA-3' consensus motif was identified in 209 gene promoters and 5'-CGGCSBBWYYKCGGC-3' in 207 promoters. This encompassed 84 high-confidence targets with both variants and 332 with at least one, which highlighted a strong association with PnPf2 binding. However, it demonstrated further that other regulatory factors must also play a considerable role in coordinating gene expression, since only 93 of the 484 targets are known to be positively regulated despite 332 harbouring at least one motif (**Figure 5.11**). Motif instances and ChIP-seq summit occurrences within individual gene promoters are detailed along with the corresponding gene functional annotations and relevant RNA-seq expression data (adapted from Jones et al., 2019) in **Supplementary item 5.2**.

A	PnPf2 ChIP-seq motif	Promoters screened	Total	≥ 1 motif	Padj
1		SN15	13577	1612	
		pf2ko-down	590	166	3.4E-23
		pf2ko-up	718	87	1
2		SN15	13577	2023	
		pf2ko-down	590	144	1.5E-07
		pf2ko-up	718	100	1

Figure 5.12 - PnPf2 positive-regulatory element motifs identified by ChIP-seq

A) The motifs 1 (5'-RWMGGVCCGA-3' where R=A/G, W=A/T and V=A/C/G) and 2 (5'-CGGCSBBWYYKCGGC-3' where S=C/G, B=C/G/T, W=A/T, Y=C/T and K=G/T) modelled from the combination of the *Pf2-HA* and *Pf2-HA_OE* sample peak regions [1-2]. Motif enrichment in PnPf2 positively (*pf2ko*-down) or negatively (*pf2ko*-up) regulated gene promoters vs the total occurrences in SN15 promoters (Fisher's test with Bonferroni Padj < 0.01) are also detailed [3-6]. B) The position of motif occurrences relative to ChIP-seq summits, demonstrating the higher likelihood at closer proximity to the summits for both motif 1 (green) and 2 (purple).

5.3.5.2. PnPf2 directly targets additional effector-like genes

Previous evidence had suggested that novel secreted virulence factors were also regulated by PnPf2 (Rybak et al., 2017) and 29 effector-like genes (i.e. secreted + EffectorP predictions) were shown to be positively regulated by PnPf2 either *in vitro* and/or *in planta* (Jones et al., 2019). From these, 11 effector-like genes showed evidence of direct PnPf2 regulation through promoter binding, which included the characterised NEs *ToxA*, *Tox1* and *Tox3* (**Table 5.3**). One target SNOG_13722 was annotated as a cerato-platanin phytotoxin and was conserved among several other plant-pathogenic Dothideomycete fungi. Two other effector-like targets, SNOG_12449 and SNOG_16438, encoded proteins lacking annotated functional domains but with homologues in other Pleosporales plant pathogens. The remaining candidate-effector genes that are directly regulated by PnPf2 did not harbour any known functional domains or obvious homologues. A further 12 effector-like genes without significant expression shifts based on RNA-seq data were also identified as direct-regulatory targets (**Table 5.3** and **Supplementary item 5.2**). One of these was SNOG_00200, which was annotated to contain an *Alternaria alternata* allergin 1 domain (IPR032382) while domain matches were not evident for the remainder.

Table 5.3 - Effector-like genes directly and positively regulated by PnPf2

The positively regulated (reduced expression in *pf2ko*) effector-like genes [column 1], their ChIP-seq summit position in each dataset and promoter motif matches relative to the annotated transcription start sites [2-3]. Also included are the relevant protein sizes, domain annotations and species IDs where homologues existed in the Uniprot database [4-6]. Putative PnPf2 direct targets that were not differentially expressed in *pf2ko* included SNOG_00200, SNOG_09147, SNOG_11853, SNOG_12214, SNOG_12819, SNOG_30701, SNOG_07039, SNOG_11632, SNOG_13486, SNOG_30026, SNOG_30316 and SNOG_30645.

Gene ID	Summit loci		Motif loci		Protein length (aa)	Protein annotation	Annotated homologues
	Pf2-HA	*Pf2-HA_OE*	RWMGGVCCGA	CGGCSBBWYYKCGGC			
Tox3	-185;-753	-189;-760	-680	-724; -713; -981	230	-	-
SNOG_13722	-663	-668	-	-	136	IPR010829 (Cerato-platanin); IPR009009 (RlpA-like protein, double-psi beta-barrel domain)	DOTSE, CERBE, PASFU, PSEFI, PYRTE, RAMCO, ZYMBR, ZYMTR
SNOG_20100	-708; -1294	-697; -1287	-70; -1303	-1304; -695	71	-	-
SNOG_08150	-	-204	-206	-196	124	-	-
SNOG_10510	-1410	-	-	-	346	-	-

SNOG_12449	-	-179	-	-984	113	-	BIPMA, BIPOR, BIPSO, BIPVI, BIPZE
SNOG_16438	-	-413	-507; -1241	-674	138	-	BIPMA, PYRTE
ToxA	-	-394	-216; -366; -409	-1330	178	IPR021635 (Proteinaceous host-selective toxin ToxA)	BIPSO, PYRTR
Tox1	-	-197; -578	-598	-	117	IPR044057 (Tox1, chitin binding-like domain)	-
SNOG_30077	-	-610	-609	-	67	-	-
SNOG_30352	-	-189	-	-	80	-	-

5.3.5.3. TFs are enriched among PnPf2 targets

A GO enrichment & network analysis was carried out to identify functional gene classes that are directly regulated by PnPf2. The high-confidence set of 484 PnPf2 target genes was assessed in addition to the 1284 genes with a promoter summit in either the *Pf2-HA* or *Pf2-HA_OE* samples, which provided a comparative group with increased statistical power to detect enriched GO terms. Five distinct groups representing TFs, redox molecules, CAZymes, cell-signalling molecules and nutrient transporters were enriched among the GO networks (**Figure 5.13**). Of these, the TFs, redox and CAZyme groups were enriched in both sample sets, which indicated these were prominent targets independent of PnPf2 expression.

The enrichment of CAZymes, redox molecules and nutrient transporters is consistent with enriched functional GO classes that were observed for *pf2ko* DE genes (Jones et al., 2019). This suggested PnPf2 indeed regulates metabolic activity and nutrient acquisition through the direct modulation of gene expression. In contrast, the TFs and cell-signalling molecules were not enriched in the genes with a significant expression change in *pf2ko*. This suggested the control of these types of cell-signalling/regulatory molecules could be tightly controlled by redundant pathways in addition to PnPf2. TF genes were significantly enriched in the high-confidence set of 484 PnPf2 direct targets (**Figure 5.13**). 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, suggesting a strong association with PnPf2 (**Table 5.4**). However, none of these TFs belonged to the C2H2 family that could provide a regulatory link to the 5‘-RTSYGGGGWA-3’ and 5‘-CTGYGCCGCA-3’ consensus motifs identified earlier from the promoters of the *pf2ko* DE genes (**Section 5.3.2**).

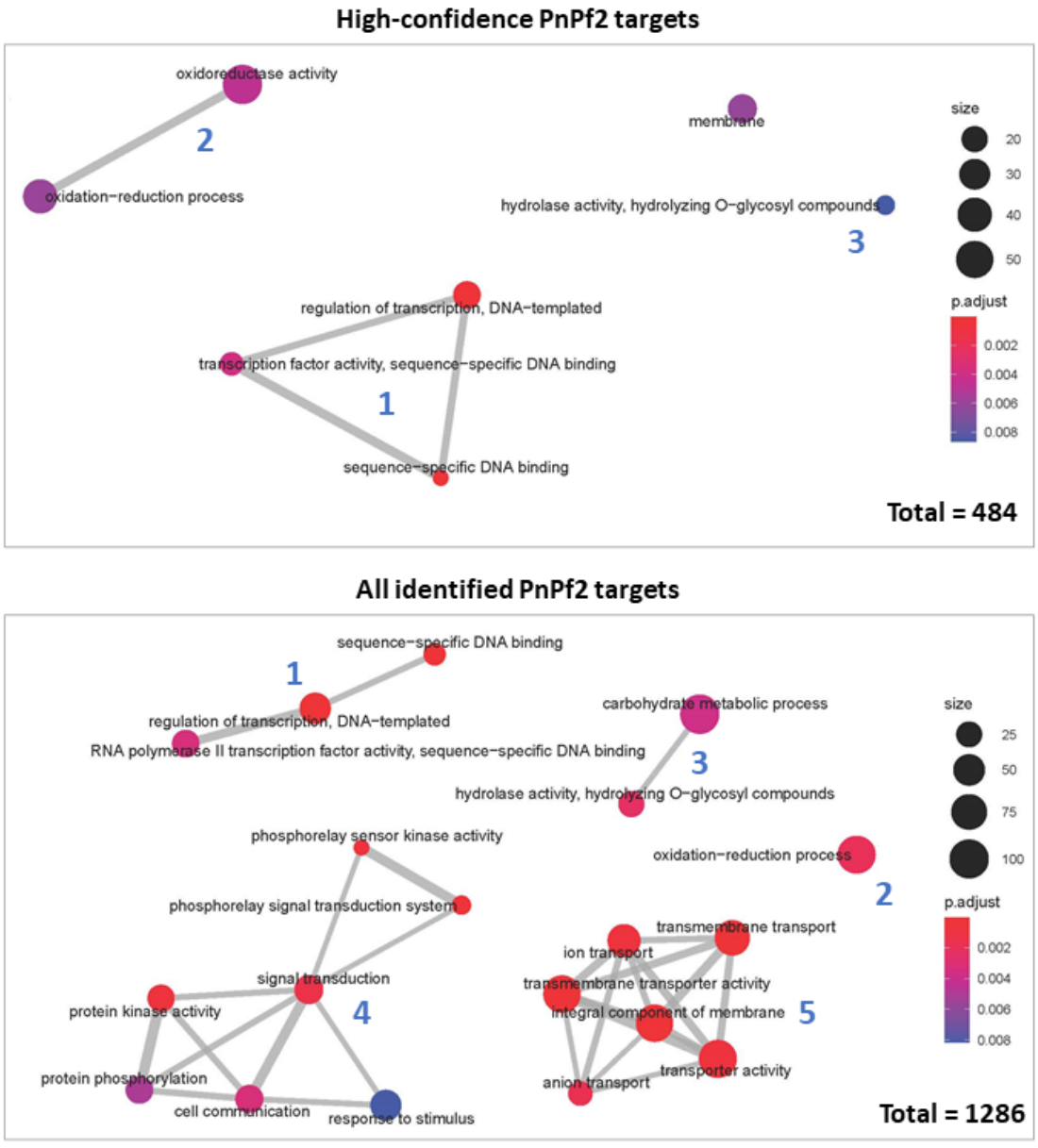


Figure 5.13 – Gene ontology (GO) enrichment/network analysis of PnPf2 direct targets

Only the GO terms that are significantly ($P < 0.05$) overrepresented among the PnPf2 targets identified by ChIP-seq are depicted. The high-confidence targets (top panel) and total identified targets (bottom) are displayed for comparison of the stringent and relaxed criteria respectively. The size of the bubbles represent the gene counts for each GO term and colours indicate the enrichment-test *P* values, while the lines

between bubbles are proportionate to the shared terms. Numbers in blue indicate connected gene networks representing transcription factors [number 1], redox molecules [2], carbohydrate-active enzymes [3], cell-signaling molecules [4] and trans-membrane transporters [5].

Table 5.4 - Transcription factor genes directly and positively regulated by PnPf2

The positively regulated (expressed down in the *pf2ko* mutant) annotated TFs [column 1], their ChIP-seq summit position in each dataset and promoter motif matches relative to the annotated transcription start sites [2-3]. Also included are the TF family [4], orthogroup [5] and gene IDs for the predicted orthologues.

Gene ID	Summit loci		Motif loci		TF family	TF orthogroup	Characterised pathogen orthologues
	Pf2-HA	*Pf2-HA_OE*	RWMGGVCCGA	CGGCSBBWYYKCGGC			
PnPro1 (SNOG_03490)	-353; -541	-270	-	-	Zn2Cys6	OG000165	AbPro1 (ALTBR), Pro1 (CRYPA), MoPRO1 (PYROR), GzZC232 (FUSGR), UvPro1 (USTVI)
PnAda1 (SNOG_04486)	-907	-903	-864	-887	bZIP	OG000202	*GzbZIP001 (FUSGR), FpAda1 (FUSPS), MobZIP10 (PYROR)*
SNOG_08237	-	-852	-762	-853	HD/Hox	OG000195	CoHox1 (COLOR), MoHox5 (PYROR), GzHOME004 (FUSGR)
SNOG_01243	-600	-556	-	-	SANT/Myb	OG000098	Myt1 (FUSGR)
SNOG_03674	-382	-434	-419	-	HMG box	OG000018	GzHMG021 (FUSGR)

5.3.6. Novel TFs characterised that regulate *P. nodorum* development and virulence

5.3.6.1. Selection and deletion of PnPf2-associated transcription factors

The distinct binding motifs with contrasting orientation of their CGG triplets suggested that PnPf2 forms a dimer with itself and/or other Zn2Cys6 TFs. This prompted the identification of candidate dimer-partners. One candidate was SNOG_08565, another Zn2Cys6 TF identified within the orthogroup OG0000017 (**Chapter 3**), which indicated SNOG_08565 shared a common evolutionary/functional origin with the PnPf2 orthologue clade. Two *PnPf2* co-expressed Zn2Cys6 TFs, SNOG_03067 (*PnEbr1*) and SNOG_07185, were also identified through a hierarchical cluster analysis of genome-wide SN15 expression data (**Supplementary item 5.3**) (Ipcho et al., 2012).

Direct ChIP-seq evidence for promoter binding was missing for a large proportion of the *pf2ko* DE genes (364 of the 484 high-confidence targets), which suggested intermediate TFs were important for PnPf2 regulation. The PnPf2 directly-regulated TF targets SNOG_03490 (PnPro1), SNOG_04486 (PnAda1) and SNOG_08237 also had virulence-associated roles established among their fungal orthologues (**Table 5.5**), suggesting an important role could be conserved. As such, these TFs, as well as the three Zn2Cys6 dimer candidates, were characterised through gene deletion alongside the *pf2ko* mutant to determine any shared virulence-related functions with PnPf2 (**Section 5.3.5.4**).

Table 5.5 - Transcription factors (TFs) targeted for gene deletion

The TFs deleted in *P. nodorum* [1] are detailed along with their association to PnPf2 that formed the rationale for their investigation [2]. The respective fungal TF orthogroups are also provided plus an indication of the orthologues with functionally-defined virulence regulatory roles [3]. The mutant IDs for the respective TFs are described in the final column [4]. *Indicates the complemented or ectopically integrated construct control mutant.

TF gene ID	PnPf2 association	Orthogroup; virulence orthologues	KO mutants
PnPro1	PnPf2 directly regulated	OG0000165; AbPro1 (ALTBR), MoPRO1 (PYROR), GzZC232 (FUSGR), UvPro1 (USTVI)	*pro1_11* *pro1_16* *Pro1_comp**
PnAda1	PnPf2 directly regulated	OG0000202; *GzbZIP001 (FUSGR), FpAda1 (FUSPS)*	*ada1_2* *ada1_11* *Ada1_comp**
SNOG_08237	PnPf2 directly regulated	OG0000195; CoHox1 (COLOR)	*08237_KO_8* *08237_KO_9* *08237_comp**
SNOG_08565	PnPf2 common orthogroup	OG0000017 Outside PnPf2 orthologue clade	*08565_KO_4* *08565_KO_5* *08565_comp**
PnEbr1	PnPf2 co-expressed Zn2Cys6 TF	OG0000121; EBR1 (FUSGR), EBR1 (FUSOX), MoCod2 and Cnf2 (PYROR)	*ebr1_10* *ebr1_11* *Ebr1_Ec**
SNOG_07185	PnPf2 co-expressed Zn2Cys6 TF	OG0000258	*07185_KO_7* *07185_KO_8* *07185_comp**

5.3.6.2. Novel phenotypes attributed for transcription factor gene deletion mutants

Gene deletion strains produced for the six TFs were phenotypically characterised in comparison to the wildtype SN15 and *pf2ko* (detailed in **Supplementary item 5.4**). Fungal culture filtrates derived from the novel mutants did not exhibit changes in necrotic activity on ToxA, Tox1 or Tox3 sensitive wheat lines, which indicated that NE secretion was largely unaffected. Furthermore, the mutants deleted in SNOG_08237, SNOG_08565 and SNOG_07185 were phenotypically indistinguishable from SN15 based on colony morphology, virulence on wheat and tolerance to osmotic, oxidative and cell wall stressors (summarised in **Supplementary item 5.4**). However, the deletion of *PnPro1*, *PnAda1* and *PnEbr1* produced clear phenotypic abnormalities which were therefore assessed in further detail relative to SN15 and *pf2ko* (**Figure 5.14**). This assessment also revealed that *pf2ko* displayed an increased sensitivity to H_2O_2 oxidative stress, a response previously undescribed, and that inoculation with *pf2ko* mycelial plugs could not induce disease lesions even on the highly susceptible wheat line Halberd. Relative to the wildtype SN15 and *pf2ko*, the following observations were made regarding the role of *PnPro1*, *PnAda1* and *PnEbr1*.

PnPro1

The *pnpro1* deletion mutants were abolished in their ability to form pycnidia and sporulate both during infection and on V8PDA. However, vegetative growth was expansive and aggressive in both conditions, suggesting PnPro1 acts to suppress hyphal development in *P. nodorum*. In addition, there was no evidence of increased sensitivity to the abiotic stressors tested. As a gene positively regulated by PnPf2,

these results did not reveal any clear similarities in general regulation since conidiation in *pf2ko* is relatively unperturbed (**Figure 5.14.D**).

PnAda1

The *pnada1* mutants displayed a significant reduction in virulence on detached leaves relative to SN15 (**Figure 5.14.B**). Dark brown discolouration at the site of infection suggested a hypersensitive response from the plant had contained the infection to a reduced size. This coincided with an increased susceptibility for *pnada1* mutants to oxidative (H_2O_2) stress, which was at a level comparable with *pf2ko*. The direct regulation of *PnAda1* by PnPf2 indicated a connected role could therefore exist in coordinating oxidative stress tolerance. In contrast to *PnPro1*, the *pnada1* mutants could still sporulate *in planta* and *in vitro*, albeit at a reduced rate relative to SN15.

PnEbr1

The *pnebr1* mutants exhibited vegetative growth defects on V8PDA (**Figure 5.14.A**). This was characterised by an uneven growth perimeter around the colony edges, a response also attributed to the hyphal branching defects produced following gene deletion of orthologues in *Fusarium* spp. (Zhao et al., 2011; Jonkers et al., 2014). Furthermore, pycnidia were abnormally developed *in vitro*, although still viable for the production of conidia, but weren't detected *in planta* (**Figure 5.14.D**). The vegetative growth defects were correlated with perturbed virulence on wheat, where lesions were contained within a small region akin to the hypersensitive response for *pnada1* mutants. Interestingly, the *pnebr1* mutants were also susceptible to osmotic stress at a level comparable to *pf2ko* and *pnada1* mutants. Whether this is connected or a side-effect of the perturbed hyphal development was unclear.

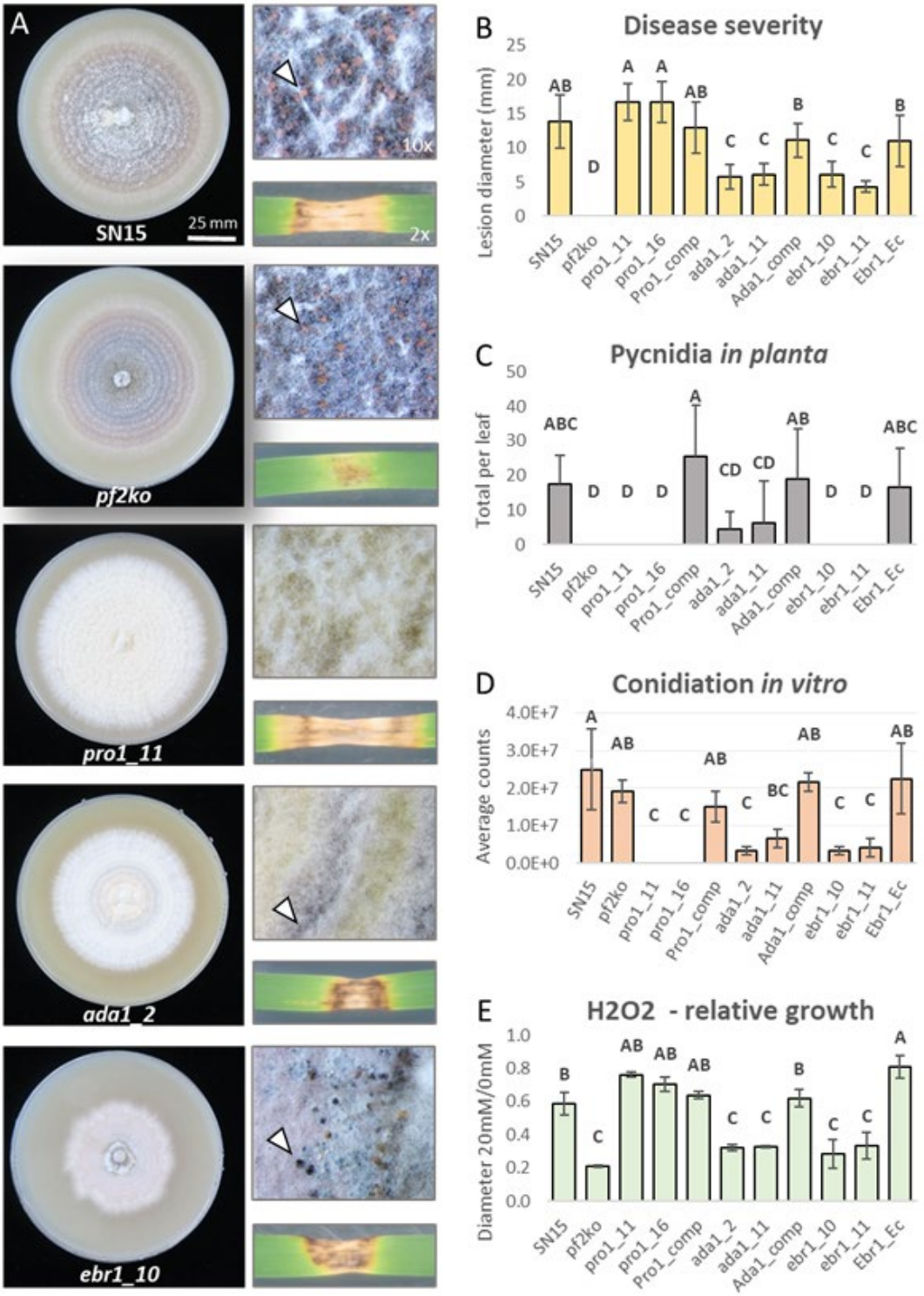

A
SN15
25 mm
10x
2x
pf2ko
pro1_11
ada1_2
ebr1_10
B
Disease severity
Lesion diameter (mm)
C
Pycnidia in planta
Total per leaf
D
Conidiation in vitro
Average counts
E
H2O2 - relative growth
Diameter 20mM/0mM
SN15
pf2ko
pro1_11
pro1_16
Pro1_comp
ada1_2
ada1_11
Ada1_comp
ebr1_10
ebr1_11
Ebr1_Ec

Figure 5.14 - Phenotypic assessment of transcription factor (TF) gene-deletion mutants in *P. nodorum* (previous page)

A phenotypic depiction of the *P. nodorum* TF deletion mutants targeting *PnPro1* (*pro1*), *PnAda1* (*ada1*) and *PnEbr1* (*ebr1*) relative to the wildtype (SN15) and *pf2ko* mutant. Panel A depicts the representative strains after 12 days growth on V8PDA or following the inoculation of mycelial plugs on wheat (cv. Halberd) leaves. Arrows indicate typical pycnidia. Panel B presents the average lesion sizes 12 days following infection by mycelial plugs on wheat (cv. Halberd) leaves, with the corresponding pycnidia counts in panel C. Conidia counts following 12 days growth on V8PDA are provided in panel D. The growth inhibition of each strain on 20mM H_2O_2 relative to 0mM is presented in panel E. Letters indicate statistical groupings by ANOVA with Tukey's-HSD ($P<0.05$) and error bars represent standard deviations in each graph.

5.4. Discussion

Prior to the research presented here, insights into the mechanisms of virulence regulation by *P. nodorum* PnPf2, or its taxonomic orthologues in other fungi, were limited to gene deletion and subcellular-localisation based analyses (Nitta et al., 2012; Cho et al., 2013; Chung et al., 2013; Oh et al., 2016; Rybak et al., 2017; Xiong et al., 2017; Jones et al., 2019; Habig et al., 2020; Han et al., 2020). This study first clarified the fungal PnPf2 orthologue lineage and conserved functional domains (**Figure 5.3** and **Figure 5.4**), then significantly expanded the current understanding through ChIP-seq. The results complemented the previous RNA-seq analysis in *P. nodorum* that had demonstrated a broad set of genes were under PnPf2 regulation, including both known or predicted virulence factors (Jones et al., 2019). By cross-referencing the datasets, 72 genes were identified as direct targets and positively regulated by PnPf2 under the same growth conditions. Since only six direct targets were under negative regulation, this provided strong evidence that functional PnPf2 binding (i.e. TF-DNA binding producing an expression change in the target gene) generally leads to greater gene expression. However, evidence of PnPf2 binding was also missing for a large proportion of the DE genes (**Figure 5.11**). This indicates that indirect regulation, either through direct intermediates, or pleiotropic effects in *pf2ko* are significant factors.

Among the genes directly regulated by PnPf2 was the NE *Tox3*; two clear peak summits were observed in the promoter region from both the *Pf2-HA* and *Pf2-HA_OE* ChIP samples (**Figure 5.9**). It is particularly interesting that this is a bi-directional promoter where the adjacent gene, SNOG_08982, encodes a disulphide isomerase (Interpro domain: IPR005792) which was also positively regulated by PnPf2. This class of protein catalyses cysteine-cysteine bond formation and a functional role in fungal effector protein production has already been attributed in other fungi (Marín-

Menguiano et al., 2019). Therefore, it would be worth exploring any involvement of SNOG_08982 in the post-translational modification of Tox3 and other NEs under PnPf2 control. Direct PnPf2 binding was also detected in the *Tox1* promoter centred at the locus of the refined ChIP-seq motif 5'-RWMGGVCCGA-3' (not previously detected with the 5'-WMGGVCCGAA-3' RNA-seq derived consensus motif), but from the *Pf2-HA_OE* sample only. A significant but incomplete reduction in *Tox1* expression was reported in *pf2ko* (Jones et al., 2019), which therefore suggests the PnPf2 binding site is a *Tox1* gene enhancer element, one which functions when PnPf2 is abundant, but is not essential for gene expression. Meanwhile, *ToxA* can only be expressed *in planta,* but in a PnPf2 dependent manner (Rybak et al., 2017). The absence of a strong PnPf2 ChIP-seq peak, despite multiple instances matching the 5'-RWMGGVCCGA-3' motif, indicates chromatin inaccessibility or the absence of essential cofactors inhibits *ToxA* promoter binding *in vitro*. Elsewhere, a yeast-1-hybrid assay had also failed to detect an interaction between PnPf2 and the AAGGACCGA *ToxA* promoter motif site (Jones et al., 2019). This indicates that additional cofactors are required for interaction or the yeast-1-hybrid system may not be a suitable conduit for PnPf2-DNA interaction studies. Another NE *Tox267* had recently been cloned in *P*. *nodorum* (Richards et al., 2021), although PnPf2 regulation was not evident from the ChIP-seq analysis. Nevertheless, several other effector-like genes were identified as direct PnPf2 targets (**Table 5.3**). Taken together, PnPf2 is a fundamental direct-regulator of multiple NE genes in *P*. *nodorum*, albeit with condition-dependent differences for *ToxA*, *Tox1* and *Tox3*, with promising candidates to characterise further for their role in pathogenicity on wheat.

It was previously concluded that PnPf2 also orchestrates a broad level of control over the necrotrophic lifestyle in *P. nodorum* (Jones et al., 2019). The ChIP-

seq analysis presented here supports the concept that PnPf2 is a master regulator, with 484 high-confidence targets identified. Two distinct regulatory elements bound by PnPf2 were also determined that were enriched in gene promoters that are positively regulated by PnPf2. The 5‘-RWMGGVCCGA-3’ consensus motif (**Figure 5.12**) was strikingly similar to another enriched in AbPf2 positively regulated gene promoters (Cho et al., 2013) and likely represents a conserved Pf2 binding mechanism in Pleosporales fungi. It would be worth exploring the presence of this motif in other plant-pathogenic fungi where the PnPf2 orthologues are also important for the establishment of disease. Interestingly, the second PnPf2 consensus motif (5’-CGGCSBBWYYKCGGC-3’) matches the AmyR regulatory response element that was modelled using an extensive SELEX procedure in *A. nidulans* (Kojima et al., 2016). AmyR was classed within the PnPf2 orthogroup OG0000017 (**Chapter 3**) but the TF exhibited distinct presence/absence polymorphisms relative to the PnPf2 orthologue clade, particularly within the first 150 AAs (**Figure 5.2**). These could be functionally prohibitive for an alternative binding mechanism and explain why the 5‘-RWMGGVCCGA-3’ motif was not detected as a target in AmyR (Kojima et al., 2016). Nevertheless, PnPf2 orthologues in other ascomycetes have still been associated with complex carbon metabolism similar to AmyR (Chung et al., 2013; Habig et al., 2020; Han et al., 2020; Oh et al., 2016). Therefore, some shared regulatory pathways likely exist given the evidence for at least one conserved binding mechanism.

An exploration of carbon substrates such as starch, maltose or cellulose as potential inducers of Pf2 activity has not been explored in *P. nodorum* or other Pleosporales. In these pathogens, Pf2 was originally identified by a systematic screen of TF knockout mutants for pathogenicity defects that did not significantly inhibit saprophytic growth (Cho et al., 2012; Cho et al., 2013). Hence, it would be interesting

to test whether such substrates are also triggers for the expression of Pf2 regulated virulence factors, for example through *PnPf2* promoter-reporter growth assays. An important consideration stems from recent analyses in *N*. *crassa*, which have explored in detail the transcriptional network linked to complex carbohydrate metabolism and the production of CWDEs (Craig et al., 2015; Wu et al., 2020). The C2H2 carbon catabolite repressor Cre-1 is a general suppressor in this system. The *N*. *crassa* 5′-TSYGGGG-3’ binding site is near-identical to the *P. nodorum* motif that was enriched gene promoters down-regulated in *pf2ko* during *in planta* growth (**Figure 5.5**). The SNOG_13619 annotated gene represented the nearest taxonomic orthologue in *P*. *nodorum* to both *N*. *crassa* and *A*. *nidulans*, where CreA represses enzymes that break down complex carbohydrates (David et al., 2005; Adnan et al., 2017; Assis et al., 2018). As such, the 5′-TSYGGGG-3’ consensus motif could represent a genetically linked repressor element in PnPf2-regulated gene promoters that is antagonistic to PnPf2. In the normalised (FPKM) RNA-seq data (**Supplementary item 5.2**), SNOG_13619 was expressed 2.3-fold higher in *pf2ko in planta* relative to SN15, which was not statistically significant (Jones et al., 2019), but does represent a measurable increase. It would therefore be of significant interest to test whether overexpression of SNOG_13619 in *P*. *nodorum* would mirror some of the virulence defects observed in *pf2ko*. A comparative exploration of fungal mutant viability on complex carbon substrates would also provide insight into the association between the CAZyme regulatory network and its relationship to pathogenicity in Pleosporales fungi. Currently, the identity of the other C2H2-type motif enriched in the *pf2ko* DE gene promoters (5‘-CTGYGCCGCA-3’) remains unclear, but could represent another TF target linked to PnPf2-regulated genes.

Three additional *P*. *nodorum* Zn2Cys6 TFs were characterised through gene deletion, based on the evidence that PnPf2 directly regulates through two distinct motifs with alternatively oriented 'CGG' triplets, a nucleotide sequence favoured by Zn2Cys6 monomers (MacPherson et al., 2006). The phenotypic screen did not identify any clear overlapping regulatory roles with PnPf2-mediated virulence. For SNOG_08565, this was despite the common lineage in OG0000017 (**Chapter 3**). It was also the case in *F. graminearum* and *M*. *oryzae* that none of the other TFs defined in this orthogroup lineage, besides the PnPf2 orthologues (i.e. Art1 and MoCod1 respectively), were involved in fungal virulence (Son et al., 2011; Lu et al., 2014). Hence, it is likely that the PnPf2 orthologues are the prominent virulence regulators among these evolutionarily related proteins. To further explore the possibility of PnPf2 homo-typic dimer formation, a co-immunoprecipitation/affinity purification analysis, or a yeast-2-hybrid screen would be of use. Nevertheless, despite no clear PnPf2 phenotypic coregulator being identified through the targeted gene deletion strategy, PnEbr1 was uncovered for the first time in *P. nodorum* as a key regulator that controls hyphal development and fungal proliferation on wheat.

PnPf2-intermediate regulators were also explored through gene deletion. A functional role could be described for two TFs that were directly and positively regulated by PnPf2; the Zn2Cys6 TF PnPro1 and the bZIP TF PnAda1. Paradoxically, *pnpro1* mutants exhibited vigorous mycelial growth both on nutrient rich media and *in planta*, suggesting the TF acts as a suppressor of hyphal proliferation to promote sporulation. Based on these observations it seems unlikely *PnPro1* is a single-major intermediate of PnPf2 regulation of virulence. On the other hand, the virulence of *pnada1* mutants was significantly perturbed. This was also correlated with increased susceptibility to oxidative stress, a novel phenotype that can now also be attributed to

pf2ko. Further exploration with alternative stress-inducing elements would be required to shed light on the exact nature of the relationship but it is possible that PnPf2 relies on PnAda1 to mitigate against a range of oxidative compounds encountered during plant infection. While it can not be ruled out that a major intermediate TF does exist, having characterised three prominent PnPf2 direct targets it is likely that PnPf2 is the major regulator coordinating the expression of host-specific virulence factors, and that a network of additional regulators is modulated to some degree.

In many instances of PnPf2 promoter binding at the high-confidence targets (364 of 484), it was not possible to confidently attribute a positive regulatory role for PnPf2. Such discrepancies between TF-binding and gene expression have also been observed in other ChIP-seq experiments on filamentous fungi (**Table 5.6**). One aspect to consider is that functional TF-binding requires specific cofactors/coregulators before gene expression is modulated (Slattery et al., 2014; Reiter et al., 2017). Hence, an unchanged basal gene expression rate in the *pf2ko* background can be explained by an absence of the respective PnPf2 coregulators under the conditions tested. Furthermore, TF-DNA interactions can be redundant or non-functional (Carey et al., 2012; Spivakov, 2014; Osterwalder et al., 2018). 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. Methods such as ChIP-seq (targeting histone modifications), ATAC-seq and/or methylation-sensitive sequencing would provide further insight into the chromatin accessibility for PnPf2 (Soyer et al., 2015; Hendrickson et al., 2018; Bewick et al., 2019). An exploration of chromatin topology in *P. nodorum,* for example using Hi-C or related technologies (Mishra and Hawkins, 2017; Winter et al., 2018), would also be

useful to identify potential regulatory targets which are spatially proximal to PnPf2 binding sites.

To conclude, this study has significantly advanced the understanding of the molecular mechanisms of PnPf2-mediated virulence in *P. nodorum*. A broad range of direct targets were identified, which included known NEs and candidate effector-like genes. PnPf2 positive regulatory elements were also characterised. The 5'-RWMGGVCCGA-3' motif may be distinct among PnPf2 orthologues while 5'-CGGCSBBWYYKCGGC-3' is conserved among other complex carbohydrate metabolic regulators. Avenues for characterising the nature of these elements and exploring associated regulators in *P. nodorum* and related fungi have also been presented as future research directions.

Table 5.6 – Transcription factor (TF) ChIP-seq in filamentous fungi

A comparison of TF ChIP-seq vs RNA-seq analyses conducted in filamentous fungi [1]. The reference and the TFs analysed are indicated [2-3] as well as the number of gene targets reported [4], the total RNA-seq DE genes for the respective KO mutants [5] and the number genes for which both is true [6], highlighting the discrepancies often observed.

TF studied	Reference	ChIP-seq targets	RNA-seq	Both
PnPf2 (PARNO)	This study (*in vitro*)	484	590	93
Clr1 (NEUCR)	(Craig et al., 2015)	164	117	39
Clr2 (NEUCR)		84	132	54
Xlr1 (NEUCR)		198	90	23
Tri6 (FUSGR)	(Nasmith et al., 2011)	198	1614	26
FgSR (FUSGR)	(Liu et al., 2019)	119	1790	Not reported
Ros1 (USTMA)	(Tollot et al., 2016)	1913	2006	790
MoCrz1 (PYROR)	(Kim et al., 2010)	346	346 (microarray)	140
CrzA (ASPFN)	(Castro et al., 2014)	102	3622	50
SrbA (ASPFN)	(Chung et al., 2014)	97	987	24

5.5. Supplementary items

Accessible via: https://figshare.com/s/b0bd50b709e823cbf43e

Supplementary item 5.1 - Genome-wide ChIP-seq peaks and summit loci

Summary of genomic coordinates for CHIP-seq peak regions [columns 1-4], the respective summit loci [5], the pileup height of the mapped reads [6] and their MACS summit -Log_{10}(Q-values) [7] for the *Pf2-HA* [1-7] and *Pf2-HA_OE* [8-14] samples. 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 [15-19].

Supplementary item 5.2 - Genome-wide summary of PnPf2 regulation in SN15

Summary spreadsheet for PnPf2 regulation data discussed in this study across the *P. nodorum* SN15 genome for the respective annotated genes [column 1]. Listed are whether ChIP-seq promoter summits were called from the *Pf2-HA* and *Pf2-HA_OE* samples [2-3], whether the enriched PnPf2 target motifs were present in the gene promoter regions [4-5] and whether the gene was also down-regulated in the *pf2ko* mutant [6]. Also listed are the functional annotations [7-12]; whether the gene was classed as effector-like [7], a TF [8], the associated GO IDs/terms [9-10] and Interpro domain information [11-12]. 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 RNA-seq vs ChIP-seq comparative purposes.

Supplementary item 5.3 - Hierarchical clustering of gene expression during infection

Depiction of *P. nodorum* SN15 gene clustering based on microarray expression data obtained from a previous study (Ipcho et al., 2012). Clusters were cut into the 10 most

distant groups to identify Zn2Cys6 TFs co-expressed with *PnPf2*, *ToxA*, *Tox1* and *Tox3*. Indicated are the *PnEbr1* (SNOG_03037) and SNOG_07185, selected from this cluster as total RNA-seq reads mapped were comparable to *PnPf2* during SN15 during infection (Jones et al. 2019).

Supplementary item 5.4 - Transcription factor gene deletion mutant phenotypic screening

A summary of the preliminary qualitative assessment of *P*. *nodorum* TF mutant [column 1] phenotypes relating to morphology [2], lesions on detached leaves [3], fungal culture filtrate necrosis-inducing activity on wheat leaves [4] and sensitivity to abiotic stressors [5-8].

5.6. References

Adnan, M., Zheng, W., Islam, W., Arif, M., Abubakar, Y.S., Wang, Z., et al. (2017) Carbon catabolite repression in filamentous fungi. *International Journal of Molecular Sciences*, 19. https://doi.org/10.3390/ijms19010048.

Andrews, S. (2010) Babraham Bioinformatics - FastQC a quality control tool for high throughput sequence data.

Armstrong, R.A. (2014) When to use the Bonferroni correction. *Ophthalmic & Physiological Optics: The Journal of the British College of Ophthalmic Opticians (Optometrists)*, 34, 502–508. https://doi.org/10.1111/opo.12131.

Assis, L.J. de, Ulas, M., Ries, L.N.A., Ramli, N.A.M.E., Sarikaya-Bayram, O., Braus, G.H., et al. (2018) Regulation of *Aspergillus nidulans* CreA-mediated catabolite repression by the F-Box proteins Fbx23 and Fbx47. *mBio*, 9. https://doi.org/10.1128/mBio.00840-18.

Bailey, T.L., Boden, M., Buske, F.A., Frith, M., Grant, C.E., Clementi, L., et al. (2009) MEME SUITE: tools for motif discovery and searching. *Nucleic Acids Research*, 37, W202-208. https://doi.org/10.1093/nar/gkp335.

Bailey, T.L. & Elkan, C. (1994) Fitting a mixture model by expectation maximization to discover motifs in biopolymers. *Proceedings. International Conference on Intelligent Systems for Molecular Biology*, 2, 28–36.

Bailey, T.L. & Machanick, P. (2012) Inferring direct DNA-binding from ChIP-seq. *Nucleic Acids Research*, 40, e128–e128. https://doi.org/10.1093/nar/gks433.

Bewick, A.J., Hofmeister, B.T., Powers, R.A., Mondo, S.J., Grigoriev, I.V., James, T.Y., et al. (2019) Diversity of cytosine methylation across the fungal tree of life. *Nature Ecology & Evolution*, 3, 479–490. https://doi.org/10.1038/s41559-019-0810-9.

Blum, M., Chang, H.-Y., Chuguransky, S., Grego, T., Kandasaamy, S., Mitchell, A., et al. (2020) The InterPro protein families and domains database: 20 years on. *Nucleic Acids Research*. https://doi.org/10.1093/nar/gkaa977.

Bursteinas, B., Britto, R., Bely, B., Auchincloss, A., Rivoire, C., Redaschi, N., et al. (2016) Minimizing proteome redundancy in the UniProt Knowledgebase. *Database: The Journal of Biological Databases and Curation*, 2016. https://doi.org/10.1093/database/baw139.

Bushnell, B. (2016) BBMap. *SourceForge*.

Carey, M.F., Peterson, C.L. & Smale, S.T. (2012) Confirming the Functional Importance of a Protein–DNA Interaction. *Cold Spring Harbor Protocols*, 2012, pdb.top070060. https://doi.org/10.1101/pdb.top070060.

Castro, P.A. de, Chen, C., Almeida, R.S.C. de, Freitas, F.Z., Bertolini, M.C., Morais, E.R., et al. (2014) ChIP-seq reveals a role for CrzA in the *Aspergillus fumigatus* high-osmolarity glycerol response (HOG) signalling pathway. *Molecular Microbiology*, 94, 655–674. https://doi.org/10.1111/mmi.12785.

Charron, M.J., Dubin, R.A. & Michels, C.A. (1986) Structural and functional analysis of the MAL1 locus of *Saccharomyces* cerevisiae. *Molecular and Cellular Biology*, 6, 3891–3899. https://doi.org/10.1128/mcb.6.11.3891.

Cho, Y., Ohm, R.A., Grigoriev, I.V. & Srivastava, A. (2013) Fungal-specific transcription factor *AbPf2* activates pathogenicity in *Alternaria brassicicola*. *The Plant Journal*, 75, 498–514. https://doi.org/10.1111/tpj.12217.

Cho, Y., Srivastava, A., Ohm, R.A., Lawrence, C.B., Wang, K.-H., Grigoriev, I.V., et al. (2012) Transcription factor *Amr1* induces melanin biosynthesis and suppresses virulence in *Alternaria brassicicola*. *PLOS Pathogens*, 8, e1002974. https://doi.org/10.1371/journal.ppat.1002974.

Chung, D., Barker, B.M., Carey, C.C., Merriman, B., Werner, E.R., Lechner, B.E., et al. (2014) ChIP-seq and *in vivo* transcriptome analyses of the *Aspergillus fumigatus* SREBP SrbA reveals a new regulator of the fungal hypoxia response and virulence. *PLOS Pathogens*, 10. https://doi.org/10.1371/journal.ppat.1004487.

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 Genetics and Biology*, 61, 133–141. https://doi.org/10.1016/j.fgb.2013.10.004.

Craig, J.P., Coradetti, S.T., Starr, T.L. & Glass, N.L. (2015) Direct target network of the *Neurospora crassa* plant cell wall deconstruction regulators CLR-1, CLR-2, and XLR-1. *mBio*, 6. https://doi.org/10.1128/mBio.01452-15.

David, H., Krogh, A.M., Roca, C., Åkesson, M. & Nielsen, J. (2005) CreA influences the metabolic fluxes of *Aspergillus nidulans* during growth on glucose and xylose. *Microbiology*, 151, 2209–2221. https://doi.org/10.1099/mic.0.27787-0.

Erdős, G. & Dosztányi, Z. (2020) Analyzing Protein Disorder with IUPred2A. *Current Protocols in Bioinformatics*, 70, e99. https://doi.org/10.1002/cpbi.99.

Feng, J., Liu, T., Qin, B., Zhang, Y. & Liu, X.S. (2012) Identifying ChIP-seq enrichment using MACS. *Nature Protocols*, 7, 1728–1740. https://doi.org/10.1038/nprot.2012.101.

Friesen, T.L., Stukenbrock, E.H., Liu, Z., Meinhardt, S., Ling, H., Faris, J.D., et al. (2006) Emergence of a new disease as a result of interspecific virulence gene transfer. *Nature Genetics*, 38, 953–956. https://doi.org/10.1038/ng1839.

Gaspar, J.M. (2018) Improved peak-calling with MACS2. *bioRxiv*, 496521. https://doi.org/10.1101/496521.

Grant, C.E., Bailey, T.L. & Noble, W.S. (2011) FIMO: scanning for occurrences of a given motif. *Bioinformatics*, 27, 1017–1018. https://doi.org/10.1093/bioinformatics/btr064.

Guindon, S., Dufayard, J.-F., Lefort, V., Anisimova, M., Hordijk, W. & Gascuel, O. (2010) New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. *Systematic Biology*, 59, 307–321. https://doi.org/10.1093/sysbio/syq010.

Gupta, S., Stamatoyannopoulos, J.A., Bailey, T.L. & Noble, W.S. (2007) Quantifying similarity between motifs. *Genome Biology*, 8, R24. https://doi.org/10.1186/gb-2007-8-2-r24.

Habig, M., Bahena-Garrido, S.M., Barkmann, F., Haueisen, J. & Stukenbrock, E.H. (2020) The transcription factor Zt107320 affects the dimorphic switch, growth and virulence of the fungal wheat pathogen *Zymoseptoria tritici*. *Molecular Plant Pathology*, 21, 124–138. https://doi.org/10.1111/mpp.12886.

Han, J.W., Kim, D.Y., Lee, Y.J., Choi, Y.R., Kim, B., Choi, G.J., et al. (2020) Transcription factor PdeR is involved in fungal development, metabolic change, and pathogenesis of gray mold <i>Botrytis cinerea<i>. *Journal of Agricultural and Food Chemistry*, 68, 9171–9179. https://doi.org/10.1021/acs.jafc.0c02420.

Hane, J.K., Paxman, J., Jones, D.A.B., Oliver, R.P. & Wit, P. de (2020) “CATAStrophy,” a genome-informed trophic classification of filamentous plant pathogens – how many different types of filamentous plant pathogens are there? *Frontiers in Microbiology*, 10. https://doi.org/10.3389/fmicb.2019.03088.

Hendrickson, D.G., Soifer, I., Wranik, B.J., Botstein, D. & Scott McIsaac, R. (2018) Simultaneous profiling of DNA accessibility and gene expression dynamics

with ATAC-Seq and RNA-Seq. In: Stechow, L. von and Santos Delgado, A. (Eds.) Computational Cell Biology: Methods and Protocols Methods in Molecular Biology. New York, NY: Springer, pp. 317–333.
Ipcho, S.V.S., Hane, J.K., Antoni, E.A., Ahren, D., Henrissat, B., Friesen, T.L., et al. (2012) Transcriptome analysis of *Stagonospora nodorum*: gene models, effectors, metabolism and pantothenate dispensability. *Molecular Plant Pathology*, 13, 531–545. https://doi.org/10.1111/j.1364-3703.2011.00770.x.
Jones, D.A.B., John, E., Rybak, K., Phan, H.T.T., Singh, K.B., Lin, S.-Y., et al. (2019) A specific fungal transcription factor controls effector gene expression and orchestrates the establishment of the necrotrophic pathogen lifestyle on wheat. *Scientific Reports*, 9, 1–13. https://doi.org/10.1038/s41598-019-52444-7.
Jonkers, W., Xayamongkhon, H., Haas, M., Olivain, C., Does, H.C. van der, Broz, K., et al. (2014) *EBR1* genomic expansion and its role in virulence of *Fusarium* species. *Environmental Microbiology*, 16, 1982–2003. https://doi.org/10.1111/1462-2920.12331.
Kim, S., Hu, J., Oh, Y., Park, J., Choi, J., Lee, Y.-H., et al. (2010) Combining ChIP-chip and expression profiling to model the MoCRZ1 mediated circuit for Ca2+/calcineurin signaling in the rice blast fungus. *PLOS Pathogens*, 6, e1000909. https://doi.org/10.1371/journal.ppat.1000909.
Kojima, T., Kunitake, E., Ihara, K., Kobayashi, T. & Nakano, H. (2016) A robust analytical pipeline forgGenome-wide identification of the genes regulated by a transcription factor: combinatorial analysis performed using gSELEX-Seq and RNA-Seq. *PLOS ONE*, 11, e0159011. https://doi.org/10.1371/journal.pone.0159011.
Kolde, R. (2015) pheatmap: Pretty Heatmaps. R package version 1.0.10.
Lefort, V., Longueville, J.-E. & Gascuel, O. (2017) SMS: Smart model selection in PhyML. *Molecular Biology and Evolution*, 34, 2422–2424. https://doi.org/10.1093/molbev/msx149.
Letunic, I. & Bork, P. (2019) Interactive Tree Of Life (iTOL) v4: recent updates and new developments. *Nucleic Acids Research*, 47, W256–W259. https://doi.org/10.1093/nar/gkz239.
Li, H. (2013) Aligning sequence reads, clone sequences and assembly contigs with BWA-MEM. *arXiv:1303.3997 [q-bio]*.
Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., et al. (2009) The sequence alignment/map format and SAMtools. *Bioinformatics*, 25, 2078–2079. https://doi.org/10.1093/bioinformatics/btp352.
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. https://doi.org/10.1186/1746-4811-8-48.
Liu, Z., Jian, Y., Chen, Y., Kistler, H.C., He, P., Ma, Z., et al. (2019) A phosphorylated transcription factor regulates sterol biosynthesis in *Fusarium graminearum*. *Nature Communications*, 10, 1–17. https://doi.org/10.1038/s41467-019-09145-6.
Lu, J., Cao, H., Zhang, L., Huang, P. & Lin, F. (2014) Systematic analysis of Zn2Cys6 transcription factors required for development and pathogenicity by high-throughput gene knockout in the rice blast fungus. *PLOS Pathogens*, 10. https://doi.org/10.1371/journal.ppat.1004432.
MacPherson, S., Larochelle, M. & Turcotte, B. (2006) A fungal family of transcriptional regulators: the zinc cluster proteins. *Microbiology and Molecular Biology Reviews*, 70, 583–604.

https://doi.org/10.1128/MMBR.00015-06.
Marín-Menguiano, M., Moreno-Sánchez, I., Barrales, R.R., Fernández-Álvarez, A. & Ibeas, J.I. (2019) N-glycosylation of the protein disulfide isomerase Pdi1 ensures full *Ustilago maydis virulence*. *PLOS Pathogens*, 15, e1007687. https://doi.org/10.1371/journal.ppat.1007687.
Martin, M. (2011) Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet.journal*, 17, 10–12. https://doi.org/10.14806/ej.17.1.200.
Mattern, I., Punt, P. & Hondel, C. (1988) A vector for Aspergillus transformation conferring phleomycin resistance. *Fungal Genetics Reports*, 35, 25. https://doi.org/10.4148/1941-4765.1533.
McDonald, M.C. & Solomon, P.S. (2018) Just the surface: advances in the discovery and characterization of necrotrophic wheat effectors. *Current Opinion in Microbiology*, 46, 14–18. https://doi.org/10.1016/j.mib.2018.01.019.
McDonald, M.C., Taranto, A.P., Hill, E., Schwessinger, B., Liu, Z., Simpfendorfer, S., et al. (2019) Transposon-mediated horizontal fransfer of the host-specific virulence protein ToxA between three fungal wheat pathogens. *mBio*, 10. https://doi.org/10.1128/mBio.01515-19.
Mishra, A. & Hawkins, R.D. (2017) Three-dimensional genome architecture and emerging technologies: looping in disease. *Genome Medicine*, 9. https://doi.org/10.1186/s13073-017-0477-2.
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. https://doi.org/10.1271/bbb.50694.
Nasmith, C.G., Walkowiak, S., Wang, L., Leung, W.W.Y., Gong, Y., Johnston, A., et al. (2011) Tri6 is a global transcription regulator in the phytopathogen *Fusarium graminearum*. *PLOS Pathogens*, 7, e1002266. https://doi.org/10.1371/journal.ppat.1002266.
Nguyen Ba, A.N., Pogoutse, A., Provart, N. & Moses, A.M. (2009) NLStradamus: a simple Hidden Markov Model for nuclear localization signal prediction. *BMC bioinformatics*, 10, 202. https://doi.org/10.1186/1471-2105-10-202.
Nitta, M., Furukawa, T., Shida, Y., Mori, K., Kuhara, S., Morikawa, Y., et al. (2012) A new Zn(II)2Cys6-type transcription factor BglR regulates β-glucosidase expression in *Trichoderma reesei*. *Fungal Genetics and Biology*, 49, 388–397. https://doi.org/10.1016/j.fgb.2012.02.009.
Oh, M., Son, H., Choi, G.J., Lee, C., Kim, J.-C., Kim, H., et al. (2016) Transcription factor ART1 mediates starch hydrolysis and mycotoxin production in *Fusarium graminearum* and *F. verticillioides*. *Molecular Plant Pathology*, 17, 755–768. https://doi.org/10.1111/mpp.12328.
Osterwalder, M., Barozzi, I., Tissières, V., Fukuda-Yuzawa, Y., Mannion, B.J., Afzal, S.Y., et al. (2018) Enhancer redundancy allows for phenotypic robustness in mammalian development. *Nature*, 554, 239–243. https://doi.org/10.1038/nature25461.
Ramírez, F., Ryan, D.P., Grüning, B., Bhardwaj, V., Kilpert, F., Richter, A.S., et al. (2016) deepTools2: a next generation web server for deep-sequencing data analysis. *Nucleic Acids Research*, 44, W160–W165. https://doi.org/10.1093/nar/gkw257.
Reiter, F., Wienerroither, S. & Stark, A. (2017) Combinatorial function of transcription factors and cofactors. *Current Opinion in Genetics & Development*, 43, 73–81. https://doi.org/10.1016/j.gde.2016.12.007.

Richards, J.K., Kariyawasam, G., Seneviratne, S., Wyatt, N.A., Xu, S.S., Liu, Z., et al. (2021) A triple threat: the *Parastagonospora nodorum* SnTox267 effector exploits three distinct host genetic factors to cause disease in wheat. *bioRxiv*, 2021.02.25.432871. https://doi.org/10.1101/2021.02.25.432871.

Rybak, K., See, P.T., Phan, H.T.T., Syme, R.A., Moffat, C.S., Oliver, R.P., et al. (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. *Molecular Plant Pathology*, 18, 420–434. https://doi.org/10.1111/mpp.12511.

Sexton, A.C. & Howlett, B.J. (2001) Green fluorescent protein as a reporter in the *Brassica–Leptosphaeria maculans* interaction. *Physiological and Molecular Plant Pathology*, 58, 13–21. https://doi.org/10.1006/pmpp.2000.0307.

Shao, Z., Zhang, Y., Yuan, G.-C., Orkin, S.H. & Waxman, D.J. (2012) MAnorm: a robust model for quantitative comparison of ChIP-Seq data sets. *Genome Biology*, 13, R16. https://doi.org/10.1186/gb-2012-13-3-r16.

Slattery, M., Zhou, T., Yang, L., Dantas Machado, A.C., Gordân, R. & Rohs, R. (2014) Absence of a simple code: how transcription factors read the genome. *Trends in Biochemical Sciences*, 39, 381–399. https://doi.org/10.1016/j.tibs.2014.07.002.

Son, H., Seo, Y.-S., Min, K., Park, A.R., Lee, J., Jin, J.-M., et al. (2011) A phenome-based functional analysis of transcription factors in the cereal head blight fungus, *Fusarium graminearum*. *PLOS Pathogens*, 7, e1002310. https://doi.org/10.1371/journal.ppat.1002310.

Soyer, J.L., Möller, M., Schotanus, K., Connolly, L.R., Galazka, J.M., Freitag, M., et al. (2015) Chromatin analyses of *Zymoseptoria tritici*: methods for chromatin immunoprecipitation followed by high-throughput sequencing (ChIP-seq). *Fungal Genetics and Biology*, 79, 63–70. https://doi.org/10.1016/j.fgb.2015.03.006.

Spivakov, M. (2014) Spurious transcription factor binding: non-functional or genetically redundant? *BioEssays: News and Reviews in Molecular, Cellular and Developmental Biology*, 36, 798–806. https://doi.org/10.1002/bies.201400036.

Syme, R.A., Tan, K.-C., Hane, J.K., Dodhia, K., Stoll, T., Hastie, M., et al. (2016) Comprehensive annotation of the *Parastagonospora nodorum* reference genome using next-generation genomics, transcriptomics and proteogenomics. *PLOS ONE*, 11, e0147221. https://doi.org/10.1371/journal.pone.0147221.

Tan, K.-C., Phan, H.T.T., Rybak, K., John, E., Chooi, Y.H., Solomon, P.S., et al. (2015) Functional redundancy of necrotrophic effectors – consequences for exploitation for breeding. *Frontiers in Plant Science*, 6. https://doi.org/10.3389/fpls.2015.00501.

Tani, S., Katsuyama, Y., Hayashi, T., Suzuki, H., Kato, M., Gomi, K., et al. (2001) Characterization of the amyR gene encoding a transcriptional activator for the amylase genes in *Aspergillus nidulans*. *Current Genetics*, 39, 10–15. https://doi.org/10.1007/s002940000175.

Tollot, M., Assmann, D., Becker, C., Altmüller, J., Dutheil, J.Y., Wegner, C.-E., et al. (2016) The WOPR protein Ros1 is a master regulator of sporogenesis and late effector gene expression in the maize pathogen *Ustilago maydis*. *PLOS pathogens*, 12, e1005697. https://doi.org/10.1371/journal.ppat.1005697.

Winter, D.J., Ganley, A.R.D., Young, C.A., Liachko, I., Schardl, C.L., Dupont, P.-Y., et al. (2018) Repeat elements organise 3D genome structure and mediate

transcription in the filamentous fungus Epichloë festucae. *PLOS Genetics*, 14, e1007467. https://doi.org/10.1371/journal.pgen.1007467.
Wu, V.W., Thieme, N., Huberman, L.B., Dietschmann, A., Kowbel, D.J., Lee, J., et al. (2020) The regulatory and transcriptional landscape associated with carbon utilization in a filamentous fungus. *Proceedings of the National Academy of Sciences*, 117, 6003–6013. https://doi.org/10.1073/pnas.1915611117.
Xiong, Y., Wu, V.W., Lubbe, A., Qin, L., Deng, S., Kennedy, M., et al. (2017) A fungal transcription factor essential for starch degradation affects integration of carbon and nitrogen metabolism. *PLOS Genetics*, 13, e1006737. https://doi.org/10.1371/journal.pgen.1006737.
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. https://doi.org/10.1089/omi.2011.0118.
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. https://doi.org/10.1093/bioinformatics/btv145.
Zambelli, F., Pesole, G. & Pavesi, G. (2014) Using Weeder, Pscan, and PscanChIP for the discovery of enriched transcription factor binding site motifs in nucleotide sequences. *Current Protocols in Bioinformatics*, 47, 2.11.1-2.11.31. https://doi.org/10.1002/0471250953.bi0211s47.
Zhao, C., Waalwijk, C., Wit, P.J.G.M. de, Lee, T. van der & Tang, D. (2011) EBR1, a novel Zn(2)Cys(6) transcription factor, affects virulence and apical dominance of the hyphal tip in *Fusarium graminearum*. *Molecular Plant-Microbe Interactions*, 24, 1407–1418. https://doi.org/10.1094/MPMI-06-11-0158.

Chapter 6

Variability in an effector gene promoter of a necrotrophic fungal pathogen dictates epistasis and effector-triggered susceptibility in wheat

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A signed co-author contribution statement demonstrating that the work contained herein is my own is provided in **Appendix 3**.

6.1. Background

The major virulence factors of *Parastagonospora nodorum* are proteinaceous necrotrophic effectors (NE)s, which contribute significantly to septoria nodorum blotch (SNB) disease of wheat (Friesen et al., 2007; Friesen and Faris, 2010; McDonald and Solomon, 2018). NEs subvert host-immune receptors in an 'inverse' gene-for-gene fashion that elicits 'effector-triggered susceptibility' (ETS), enabling the pathogen to proliferate. Genetic evidence has revealed at least 10 NE-host receptor interactions in the *P. nodorum*-wheat pathosystem. These are ToxA-*Tsn1* (Friesen et al., 2007; Friesen and Faris, 2010), Tox1-*Snn1* (Liu et al., 2012; Shi et al., 2016), Tox267-*Snn2*/*Snn6*/*Snn7* (Gao et al., 2015; Shi et al., 2015; Richards et al., 2021), Tox3-*Snn3B1*/*Snn3D1* (Liu et al., 2009; Zhang et al., 2021), Tox4-*Snn4* (Abeysekara et al., 2012), Tox5-*Snn5* (Friesen et al., 2012; Kariyawasam et al., 2021) and Tox2A-*Qsnb.cur–2AS1* (Phan et al., 2016), with ToxA, Tox1, Tox3, Tox267 and Tox5 having been clonally characterised for their roles in virulence. Many studies have sought to understand how these interactions determine the level of disease, making use of isolates with different complements of NEs and wheat lines with different complements of receptors (Friesen et al., 2008; Tan et al., 2015; Gao et al., 2016; Phan et al., 2016; Peters-Haugrud et al., 2019; Ruud et al., 2019; Lin et al., 2020a; Richards et al., 2021). This has included the use of fungal NE gene knockout mutants, genome-wide association analyses and both biparental and multi-parent wheat mapping populations.

SNB resistance is quantitatively inherited (Downie et al., 2020) and improvements in resistance have been, in part, mediated through the removal of dominant susceptibility genes in the host (Vleeshouwers and Oliver, 2014). However, the situation is complex. Multiple NEs expressed by *P. nodorum* afford the pathogen

functional redundancies on wheat varieties that possess multiple dominant susceptibility genes (Tan et al., 2014; Tan et al., 2015). For instance, it has been demonstrated that the deletion of *ToxA*, *Tox1* and *Tox3* in *P. nodorum* SN15 did not significantly alter virulence on a diverse collection of modern wheat varieties (Tan et al., 2015; Phan et al., 2018). A further complication in breeding for SNB resistance is that some NE-host gene interactions were not consistently detected in field trials using mapping populations with known susceptibility genes (Ruud et al., 2019; Lin et al., 2021).

Accumulating evidence suggests NE epistasis plays a significant role in shaping the variations observed in host resistance to SNB (**Table 6.1**). NE epistasis can be defined as interactions between NE genes where the effect conferred by one is masked by the presence of another (Tan and Oliver, 2017). NE epistasis in the *P. nodorum*-wheat pathosystem can be divided into two broad regulatory categories. Firstly, the disease contribution of one NE-host receptor interaction is suppressed by another, possibly through host-gene action or cross-talk among NE recognition pathways (Friesen et al., 2008; Friesen et al., 2012). Secondly, NE gene repression mediated by the expression of another (Phan et al., 2016; Peters-Haugrud et al., 2019; Richards et al., 2021). For the latter, a clear-cut example was revealed as part of a study that mapped SNB on a double-haploid (DH) wheat population derived from two major Australian commercial wheat cultivars (Phan et al., 2016). SNB quantitative trait loci (QTLs) detected during infection by the Australian *P. nodorum* reference isolate SN15 were compared with a *Tox1* deletion mutant (*tox1-6*). This revealed the Tox1-*Snn1* NE-host receptor interaction was epistatic to Tox3-*Snn3* and a major SNB QTL located on chromosome 2A in the DH population that confers sensitivity to a novel NE coined Tox2A. Quantitative PCR (qPCR) revealed *Tox3* expression was significantly

higher in *tox1-6* than in the wildtype, which presented a basis for NE epistasis mediated by gene repression (Phan et al., 2016).

Variation in the expression of *Tox1* is also observed among different *P. nodorum* isolates. This originally prompted the use of *P. nodorum* SN2000, an American (USA) isolate that produced sufficient Tox1 to enable its detection and functional characterisation (Liu et al., 2004; Liu et al., 2004; Chu et al., 2010; Liu et al., 2012). This contrasted other USA isolates such as SN4 and SN6 where *Tox1* expression is low relative to SN2000 (Gao et al., 2015). A recent study demonstrated *P. nodorum* SN15 also expressed *Tox1* significantly higher than SN4, which correlated with a greater SNB disease contribution mediated by the Tox1-*Snn1* interaction (Peters-Haugrud et al., 2019). The mechanisms behind this differential expression remain unexplored however, it was noted that the *Tox1* promoter region was polymorphic between these isolates (Peters-Haugrud et al., 2019). The current knowledge of *Tox1* regulation is limited. A chromatin immunoprecipitation analysis suggests the Zn2Cys6 transcription factor (TF) PnPf2 enhances *Tox1* expression through direct-promoter binding (see **Chapter 5** and Jones et al., 2019). At the same time, other prominent regulators must also induce *Tox1* expression, since PnPf2 is not essential for Tox1-mediated disease on *Snn1* wheat (Rybak et al., 2017). Therefore, novel regulatory factors targeting *Tox1* are yet to be identified.

Table 6.1 - Epistasis between *P. nodorum* necrotrophic effector (NE)-host receptor SNB disease interactions

A summary of reports describing NE-receptor interactions epistatically suppressed [column 1], indicating the suppressing interaction [2], the *P. nodorum* isolates and the infected wheat populations where epistasis was observed [3-4] and the reference to the corresponding study [5]. *RI = recombinant inbred, DH = doubled haploid, ITMI = International Triticeae Mapping Initiative.

Interaction suppressed	Suppressing interaction	Isolates	Wheat population*	Reference
Tox1-*Snn1*	ToxA-*Tsn1*	SN2000/SN4/SN5/SN15	Sumai 3 x CS-DIC-5B (RI)	(Peters-Haugrud et al., 2019)
	Tox3-*Snn3*	SN4/SN5/SN15	Sumai 3 x CS-DIC-5B (RI)	(Peters-Haugrud et al., 2019)
	Tox267-*Snn2/6*	SN4	ITMI (RI)	(Richards et al., 2021)
Tox267-*Snn2*	ToxA-*Tsn1* and/or Tox3-*Snn3*	SN15	Calingiri x Wyalkatchem (DH)	(Phan et al., 2016)
Tox3-*Snn3*	ToxA-*Tsn1*	SN15/SN1501	BR34 x Grandin (RI)	(Friesen et al., 2008)
	Tox1-*Snn1*	SN15	Calingiri x Wyalkatchem (DH)	(Phan et al., 2016)
	Tox2-*Snn2*	SN15/SN1501	BR34 x Grandin (RI)	(Friesen et al., 2008)
	Tox5-*Snn5*	SN1501	Lebsock x PI94749 (DH)	(Friesen et al., 2012)
	Tox267-*Snn2/6*	SN4	ITMI (RI)	(Richards et al., 2021)
Tox2A-*Qsnb.cur–2AS1*	Tox1-*Snn1*	SN15	Calingiri x Wyalkatchem (DH)	(This study, Phan et al., 2016)

The observations to date regarding Tox1 epistasis and gene expression prompted several key questions. What genetic polymorphisms exist in the promoter of *Tox1* in *P. nodorum* populations? Do these polymorphisms drive differential *Tox1* expression and affect the NE epistasis? Also, what other mechanisms remain to be defined in *Tox1* regulation? The study presented here was undertaken with the aim to characterise the gene regulatory elements controlling *Tox1* expression in *P. nodorum* and explore their role in SNB to improve our understanding of NE epistasis and disease management. Multiple avenues of investigation were pursued. An *in silico* exploration was first undertaken exploring the *Tox1* promoter variants in *P. nodorum* populations worldwide. *Tox1* promoter replacements were then made in *P. nodorum* followed by SNB disease mapping to assess differences in NE-mediated virulence by the genetic polymorphisms. Finally, a systematic deletion of the core *Tox1* promoter elements was performed, to identify regulatory binding sites targeted by factors other than the TF PnPf2.

6.2. Materials and methods

6.2.1. Compilation of *Tox1* promoter variants

The *P. nodorum Tox1* sequence was derived from the genome annotation SNOG_20078 for the Australian reference isolate SN15 (Syme et al., 2016). A BLAST database was built (Geneious Prime 20.2.5) from the other available published and assembled genomes for 33 *P. nodorum* and *Parastagonospora avenae* isolates (Richards et al., 2017; Syme et al., 2018). The corresponding *Tox1* loci, including the 1500 bp upstream regions, were retrieved using the SN15 *Tox1* nucleotide sequence. The 24 sequences retrieved were aligned using the Geneious aligner to detect polymorphisms in the promoter region. The 159 Australian isolates from a previous study (Phan et al., 2020) were then PCR screened (Tox1_screen_F/R) to detect product size shifts corresponding to a 401 bp element (PE401) presence/absence variant. Sanger sequencing was undertaken on the region for two Australian isolates with PE401 (WAC13443 WAC13072) and an additional isolate without (WAC13690). The *Tox1* locus and 1500 bp upstream regions for a worldwide set of 146 *P. nodorum* isolates were kindly provided by Prof. Daniel Croll (University of Neuchatel), which were derived from Illumina genome assemblies (Stukenbrock et al., 2006; Pereira et al., 2020). For the remaining USA *P. nodorum* isolates, the NCBI BLAST service (Johnson et al., 2008) was used to query the sequence read archives for 184 Illumina sequenced genomes published in a recent study under bioproject PRJNA398070 (Richards et al., 2019), which were non-redundant to the isolates already compiled. The *Tox1* nucleotide sequence and 1000 bp promoter from SN4 was used to query the respective archives for each isolate. Where continuous coverage of reads >95% identity was obtained across the corresponding region, the presence/absence of the PE401 was able to be determined. The isolate sources and metadata were compiled from the corresponding publications (summarised in **Supplementary item 6.1**).

6.2.2. *In silico* analyses of the *Tox1* promoter variants

A world distribution map was produced from the geographical information assembled for the isolates with or without *Tox1* PE401 (**Supplementary item 6.1**) using ggmaps (Kahle and Wickham, 2013). The SN4 *Tox1* promoter sequence was queried for matches in Repbase by submitting the 1500 bp region through the CENSOR portal using default settings (Kohany et al., 2006; Bao et al., 2015). The *Tox1* PE401 sequence was submitted to the Rfam portal (Kalvari et al., 2018). The NCBI BLAST service (Johnson et al., 2008) was used to scan PE401 against the SN15 genome, the NCBI nucleotide and the whole-genome shotgun collections. Dot plots and GC% graphs were produced using Geneious. The SNOG_30065 expression and gene annotation data were obtained from a previous study (Jones et al., 2019)**.**

6.2.3. Generating *Tox1* promoter replacement mutants

The molecular cloning strategy and an overview of the modified *Tox1* promoter locus in fungal transformants are depicted diagrammatically in **Figure 6.1**. The primers designed for fragment amplification and/or screening, plus descriptions of their use pertaining to these steps are outlined in **Supplementary item 4.1**. To summarise, promoter replacement constructs were assembled by attaching flanking regions to a *pTef1-HygR-tTef1* resistance marker using the 'Golden Gate' (GG) style cloning system (**Chapter 4**). The left flank was the same for all constructs, amplified from SN15 using pTox1_HR_FL_Bsa1_F/R. The right flanks were amplified from SN15 gDNA for the (-) PE401 construct variant using pTox1_873_HR_FR_Bsa1_F/pTox1_HR_FR_Bsa1_R. The same primers were used in combination with pTox1_Dom_BsaI_F/R to amplify the right flank from WAC13443 gDNA for the (+) PE401 constructs (which allowed fragment domestication for GG

cloning by the introduction of a point mutation). The (+) PE401 was also substituted in the previous construct by amplifying the GG plasmid with pTox1_indel_3_BbsI_F/pTox1_indel_5_BbsI_R and ligating in a spacer sequence (S) 401 bp element (provided in **Supplementary item 6.2**) which was amplified from the pGEM-T-Easy vector (Promega) using the primer pair spacer_BbsI_F/R. The three resulting homologous recombination (HR) constructs, were amplified and purified from these templates using pTox1_HR_FL_F/R, before polyethylene glycol (PEG) mediated transformation (see **Chapter 4**) into SN15, WAC13443 and the *pf2ko* mutant from a previous study (Rybak et al., 2017). Truncated versions of the *Tox1* promoter replacement construct were produced by modifying the forward primer used to amplify the right flank to incorporate either 86, 107, 157, 263, 310 (+/- PE401) or 598 bp (+/- PE401) regions upstream of the SN15 ATG. The resulting constructs were PEG transformed into SN15 only. All cloned constructs were verified through PCR screening and Sanger sequencing, while the fungal transformants were PCR screened using pTox1_HR_screen_F/tTox1_HR_screen_R and by qPCR (Tox1_qPCR_F/R vs Actin_qPCR_F/R) to confirm single-copy integration by the method detailed in **Chapter 4**. Two single copy mutants for all transformants were retained for downstream gene expression analysis.

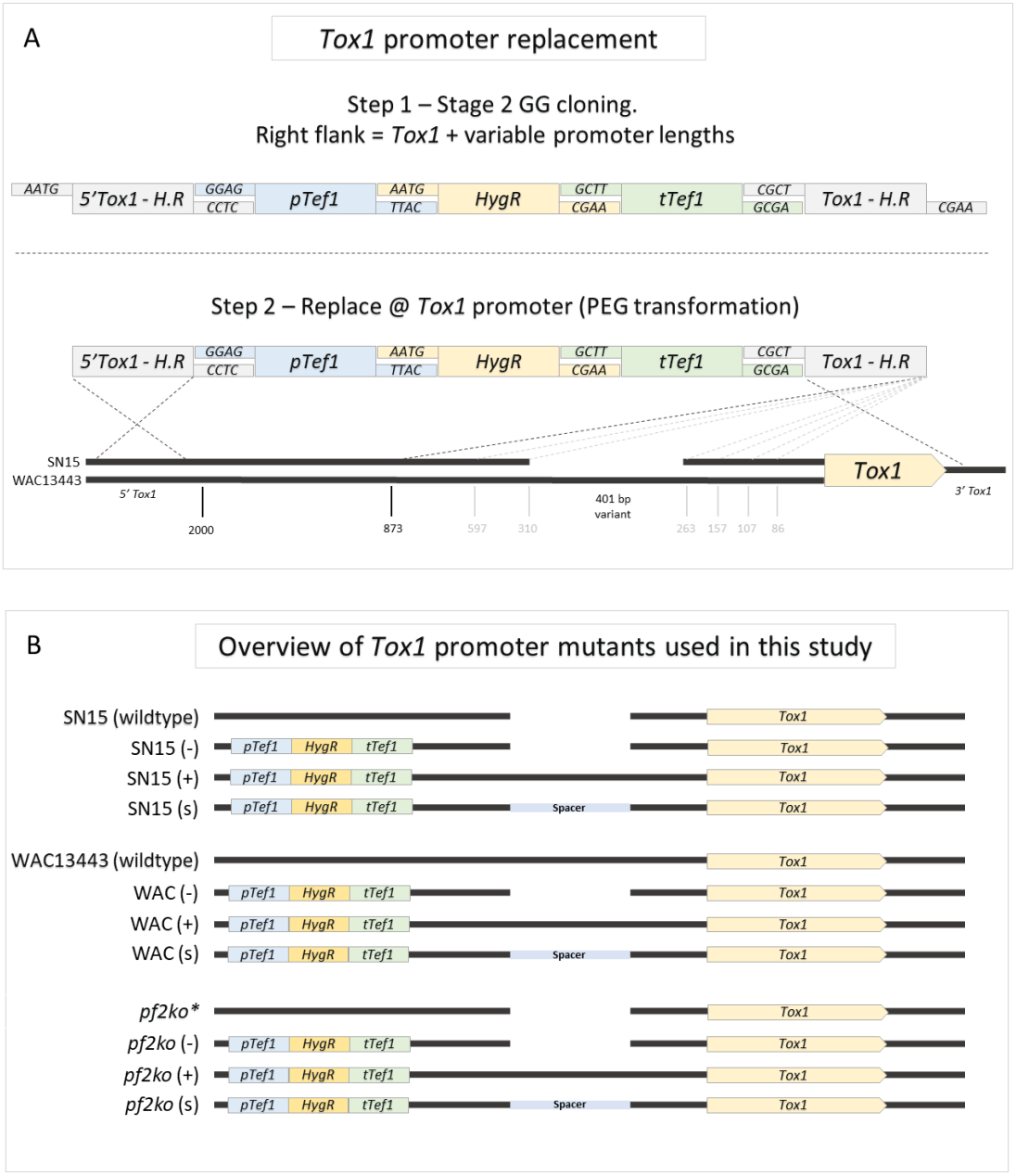

Figure 6.1 - *Tox1* promoter replacement strategy and corresponding *P. nodorum* mutants generated

A) The cloning procedure used to create the constructs subsequently used for promoter replacement by PEG-mediated transformation. B) The replacement mutants generated in the study, representing the *Tox1* gene locus in the respective background strains. *Indicates the *pf2ko* mutant from Rybak et al. (2017).

6.2.4. Gene expression analysis

P. nodorum cDNA was synthesised from the respective strains/mutants grown for three days in Fries3 media (detailed in **Chapter 4**) and subject to qPCR using Tox1_qPCR_F/R primers relative to Actin_qPCR_F/R (2^{dCt}). Fries3 growth medium was used as it is conducive for *Tox1* expression in SN15 and made it possible to assess SN4 in compliance with local quarantine requirements. For *in planta* time-series analysis of gene expression, lesions were harvested at three, five and seven days post-infection by the seedling spray method, from which cDNA was synthesised for qPCR (procedures detailed in **Chapter 4**). Tox1_qPCR_F/R and Tox3_qPCR_F/R were used to assess the respective gene expression vs *Act1* (2^{dCt}). A one-way ANOVA with Tukeys-HSD post-hoc test was used to test for differences ($p<0.05$) between isolates and/or mutants (SPSS version 27.0).

6.2.5. Population mapping and QTL analysis

The Calingiri (*Snn1*, *snn3*, *tsn1*) and Wyalkatchem (*snn1*, S*nn3*, *tsn1*) (CxW) DH wheat population consisting 177 lines (Intergrain Pty Ltd, Perth, Australia) was infected using the seedling spray method (**Chapter 4**), in line with a previous study (Phan et al., 2016). Two-week old seedlings were visually scored for SNB severity on a standardised scale of 1-9 (where 1 indicates no visible symptoms and 9 indicates a fully-necrotised plant) used previously (Phan et al., 2016; Phan et al., 2018) by an independent assessor (Huyen T. T. Phan) at seven days following inoculation. QTL mapping was undertaken using MultiQTL v. 2.6-Complete software (MultiQTL Ltd, Institute of Evolution, Haifa University, Israel). The QTL mapping was based on a genetic linkage map previously built for the CxW population using the Kosambi mapping function using MultiPoint v. 3.2 (MultiQTL Ltd, Institute of Evolution, Haifa University, Israel) from maximum recombination frequencies of 0.35 (Phan et al.,

2016). This included 385 markers polymorphic between the CxW parent lines. Seedling disease scores taken from the average of three completely randomised biological replicates were used for interval mapping to determine QTL linked to SNB. Markers with the logarithm of the odds (LOD) scores >= 2.5 were declared significant and used to construct an interval model for the corresponding QTL as previously described (Phan et al., 2016).

6.3. Results

6.3.1. Genetic variability in the *Tox1* promoter region

To explore why *Tox1* expression differed between *P. nodorum* isolates, the promoters were aligned from a geographically diverse set of 24 *Parastagonospora* spp. with coverage at the *Tox1* locus (Syme et al., 2016; Richards et al., 2017; Syme et al., 2018). The alignment revealed a 401 bp element was absent in four isolates within the collection (**Figure 6.2**). This element, henceforth called PE401, was notably absent in the Australian *P. nodorum* SN15 and USA SN2000, isolates that were associated with a higher level of *Tox1* expression (Liu et al., 2012; Gao et al., 2015; Peters-Haugrud et al., 2019). The size and proximity to the gene within the *Tox1* promoter suggested PE401 was the most plausible variant involved in gene repression, despite the presence of other smaller genetic polymorphisms.

PE401 was then examined for features of a mobile genetic element (MGE), as they are known to modulate the expression of proximal fungal genes (Soyer et al., 2014; van Dam and Rep, 2017; Krishnan et al., 2018; Mat Razali et al., 2019). The SN4 promoter sequence was queried against the Repbase or Rfam databases. There were no matches to known MGEs or expressed non-coding elements. BLAST analysis of PE401 identified a single region with 63% identity in the SN15 genome, partially overlapping the annotated gene SNOG_30065 (Syme et al., 2016). However, the SNOG_30065 predicted peptide sequence did not contain annotated protein domains, nor was the gene expressed in a previous study under *in vitro* conditions or during host infection (Jones et al., 2019). No further similar matches to PE401 were detected when queried against the NCBI nucleotide and whole-genome shotgun collections, suggesting the sequence was not a repetitive element. Terminal repeats characteristic of fungal MGEs were not identified, although the GC% was low (41.9%) relative to the

rest of the SN4 promoter region (48.9%) (Muszewska et al., 2017) (**Figure 6.3**). It was concluded that PE401 is unlikely to be a transposable element or functional gene in *P. nodorum*.

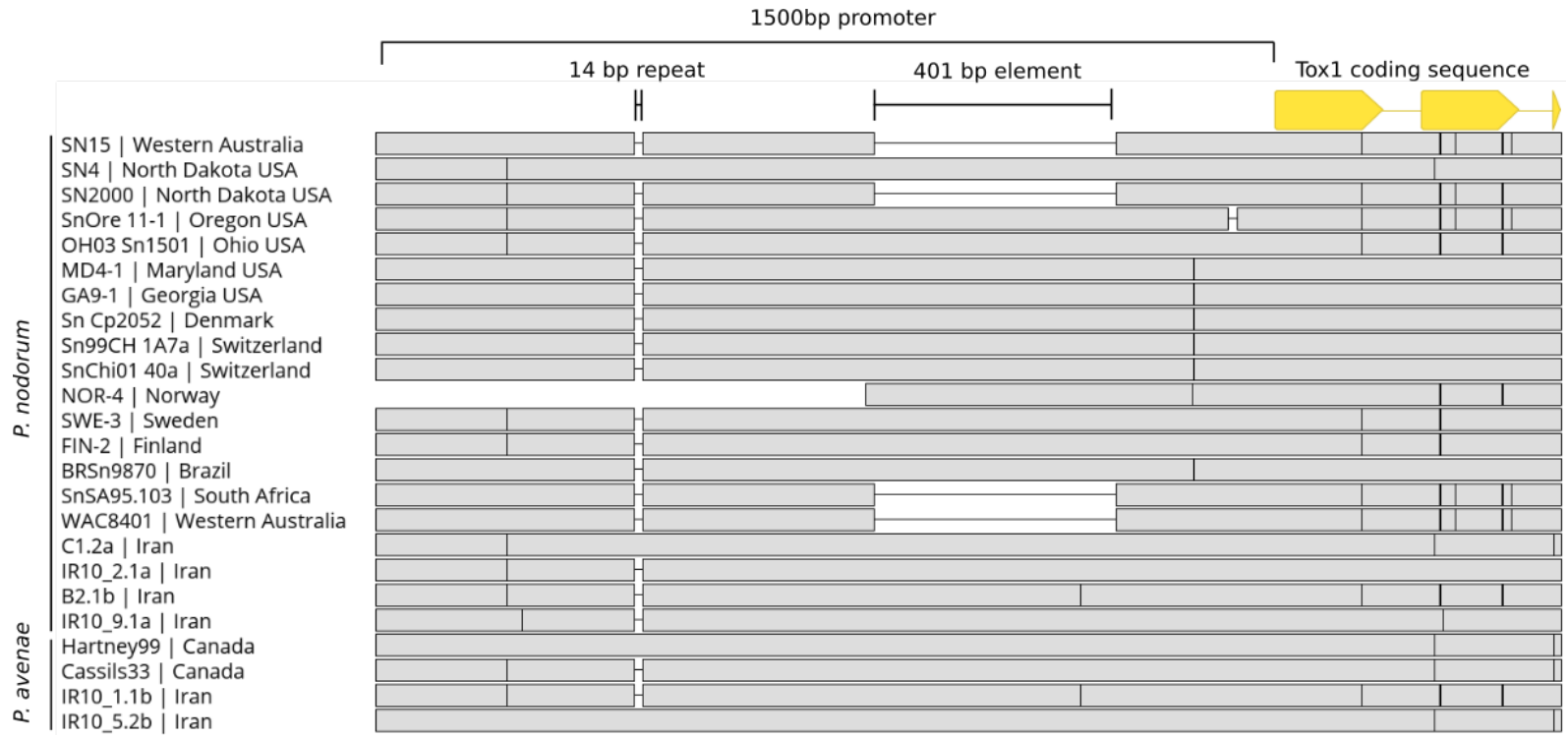

Figure 6.2 - Genetic variation in the *Tox1* promoter region in *Parastagonospora spp*.

An alignment highlighting promoter variants in a geographically diverse selection of 24 *Parastagonospora* spp. isolates that harbour the *Tox1* gene. Within the 1500bp promoter region, the PE401 and a 14 bp GTTTTCGGCCGTAT tandem repeat polymorphism are indicated. Genome coverage for the NOR-4 isolate is missing 5' of PE401.

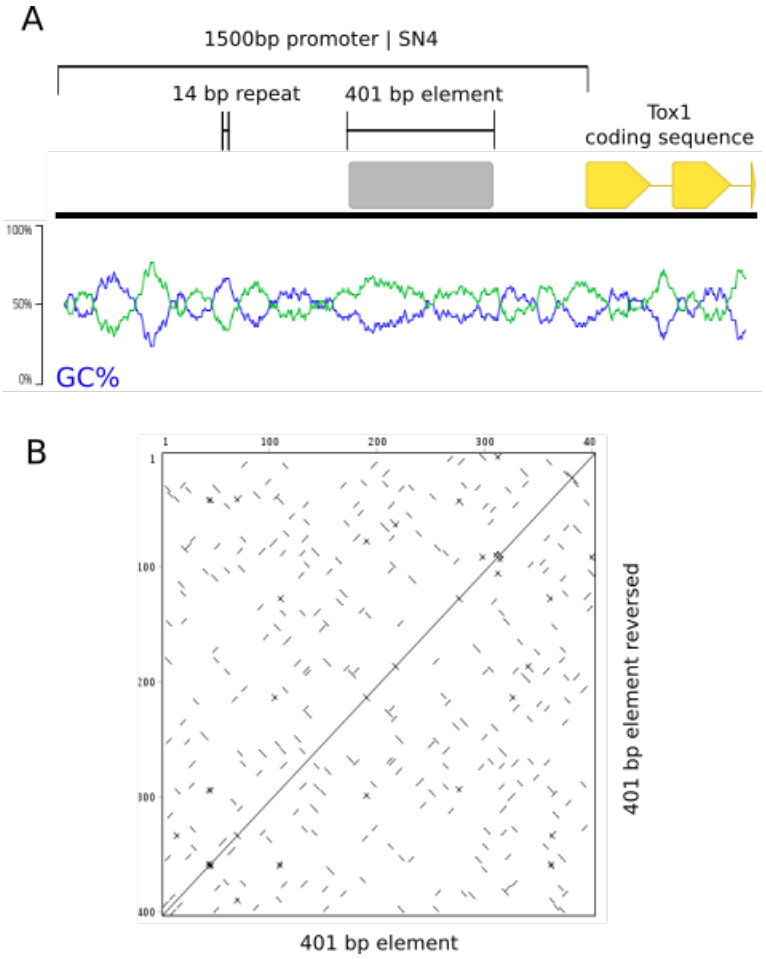

Figure 6.3 - Nucleotide composition of the *P. nodorum* PE401 variant

A) A GC% (blue) vs AT% (green) plot using a 50 bp average sliding window covering the *Tox1* promoter from the USA reference isolate SN4. The PE401 element has a low GC% relative to directly adjacent promoter regions. B) A dot plot of the 401 bp using a 5 bp sliding window demonstrating the paucity of tandem/inverted repeat stretches.

6.3.2. Evidence of regional distribution of PE401 in the global *P. nodorum* population

The distribution of PE401 was determined in a global panel of 489 *Tox1*-containing *P. nodorum* isolates to explore population variability (isolates are detailed in **Supplementary item 6.1**). It was observed that isolates carrying PE401 were ubiquitous in Europe (Switzerland, Denmark, Finland, Netherlands and Norway), most sampling sites in North America (USA and Canada) and also in Iran. The latter region was previously proposed as a centre of origin for *P. nodorum* NEs including Tox1 (McDonald et al., 2012; Ghaderi et al., 2020). Isolates that lacked the 401bp genetic element were dominant in the Australian (87% of 179 isolates) and South African (100% of 22 isolates) populations, while 27% of all isolates from North Dakota (USA) and a single isolate from Texas (USA) also lacked the element (**Figure 6.4**). Therefore, a major shift in the frequency of the element was identified between wheat-growing regions worldwide.

Figure 6.4 - The global distribution of PE401 in the *P. nodorum Tox1* promoter

Presence/absence of PE401 in a global collection of 489 *P. nodorum* isolates. Spheres represent individual populations based on the country of origin or, for the USA and Australia, states. % values represent the proportion of (-) PE401 isolates where it was identified in that population. Isolate details are provided in **Supplementary item 6.1**. Image from Google (CA, USA).

6.3.3. Functional exploration of PE401

6.3.3.1. PE401 functions as a repressor of *Tox1* expression

It was hypothesised that PE401 dictates *Tox1* expression based on the differential expression profile between SN4 (+PE401) and SN15 (-PE401) that carry the two versions of the promoter (Peters-Haugrud et al., 2019). Therefore, *Tox1* expression was assessed in SN4, SN15 and three representative Australian *P. nodorum* wildtype isolates with or without PE401 (**Figure 6.5.A**). Quantitative PCR analysis revealed the two Australian isolates carrying PE401, WAC13443 and WAC13072, shared a low expression profile with SN4 (**Figure 6.5.B**). On the other hand, *Tox1* expression was significantly higher in SN15 and WAC13690 which lacked PE401. Sequence alignment of the promoter region revealed these isolates did not carry the 14 bp tandem repeat found in SN4, indicating that PE401 was the variant responsible (**Figure 6.5.A**).

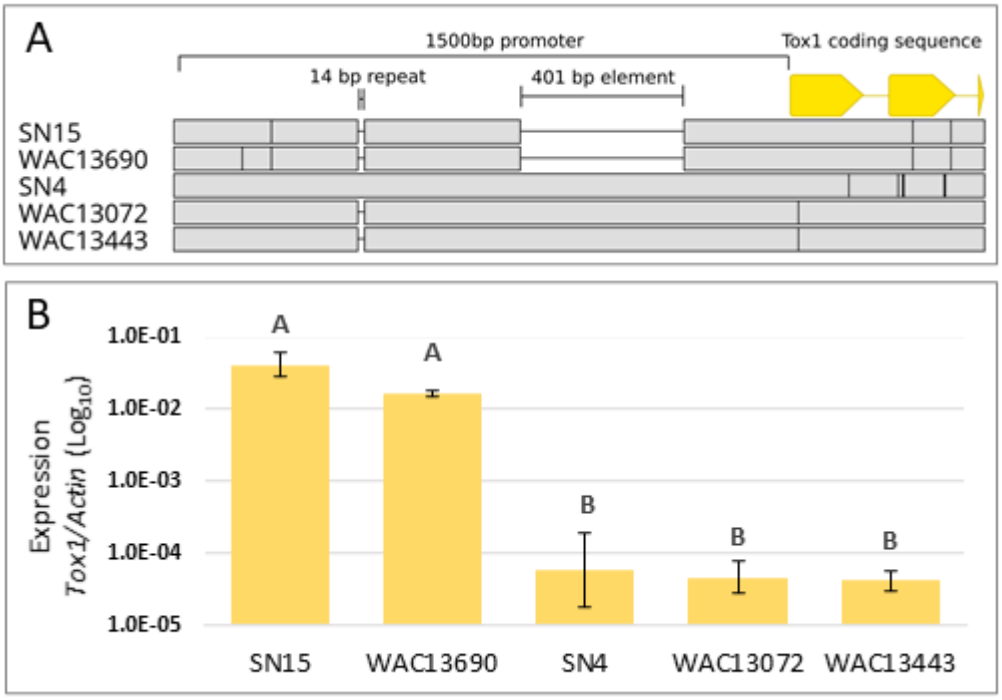

Figure 6.5 - *Tox1* expression is repressed in isolates carrying PE401

A) Alignment of the promoter regions comparing the USA reference isolate SN4 with four Australian isolates with (SN15 and WAC13690) or without (WAC13072 and WAC13443) the PE401 promoter element. B) *Tox1* expression ($2^{dCt(Tox1\text{-}Actin)}$) in the corresponding isolates grown in Fries3 liquid culture. Error bars indicate standard deviations from biological triplicates. Different letters indicate significant differences ($P<0.05$) based on ANOVA.

6.3.3.2. Promoter replacement PE401 is a sequence-specific *Tox1* repressor

Isogenic strains were then created with and without PE401 to eliminate the possibility of strain-specific genetic factors located outside of the *Tox1* locus involved in gene repression. The constructs used for promoter replacement included 873bp of the *Tox1* promoter either with PE401 (+), without PE401 (-), or with a 401 bp spacer (S) (**Figure 6.6**). The spacer had a similar GC content but unrelated sequence (**Supplementary item 6.2**). The three constructs were independently transformed into SN15 at the *Tox1* promoter locus. They were similarly introduced into WAC13443 (henceforth referred to as WAC) which weakly expressed *Tox1* to control for isolate-specific variables. Furthermore, the constructs were introduced into an SN15 PnPf2 knockout mutant (*pf2ko*) from a previous study (Rybak et al., 2017). This was to test whether enhancer activity of the predicted PnPf2 binding site (CGGTCCG; located 397bp 5' of the PE401 locus, **Chapter 5**) was affected by changes to the proximity of *Tox1*.

Quantitative PCR analysis of the mutants grown in Fries3 broth *in vitro* revealed a 40-fold (SN15) and 38-fold (WAC) reduction in *Tox1* gene expression occurred with PE401 present. This was not observed for mutants carrying the spacer sequence, suggesting the effect was specifically attributable to PE401 (**Figure 6.6**). Direct involvement by PnPf2 was also not evident since *Tox1* expression was comparably reduced across all *pf2ko* derived mutants vs the SN15 equivalents, irrespective of the *Tox1* promoter construct (**Figure 6.6**). It was concluded that PE401 is associated with *Tox1* repression, perhaps through an interaction with a trans-acting regulatory factor.

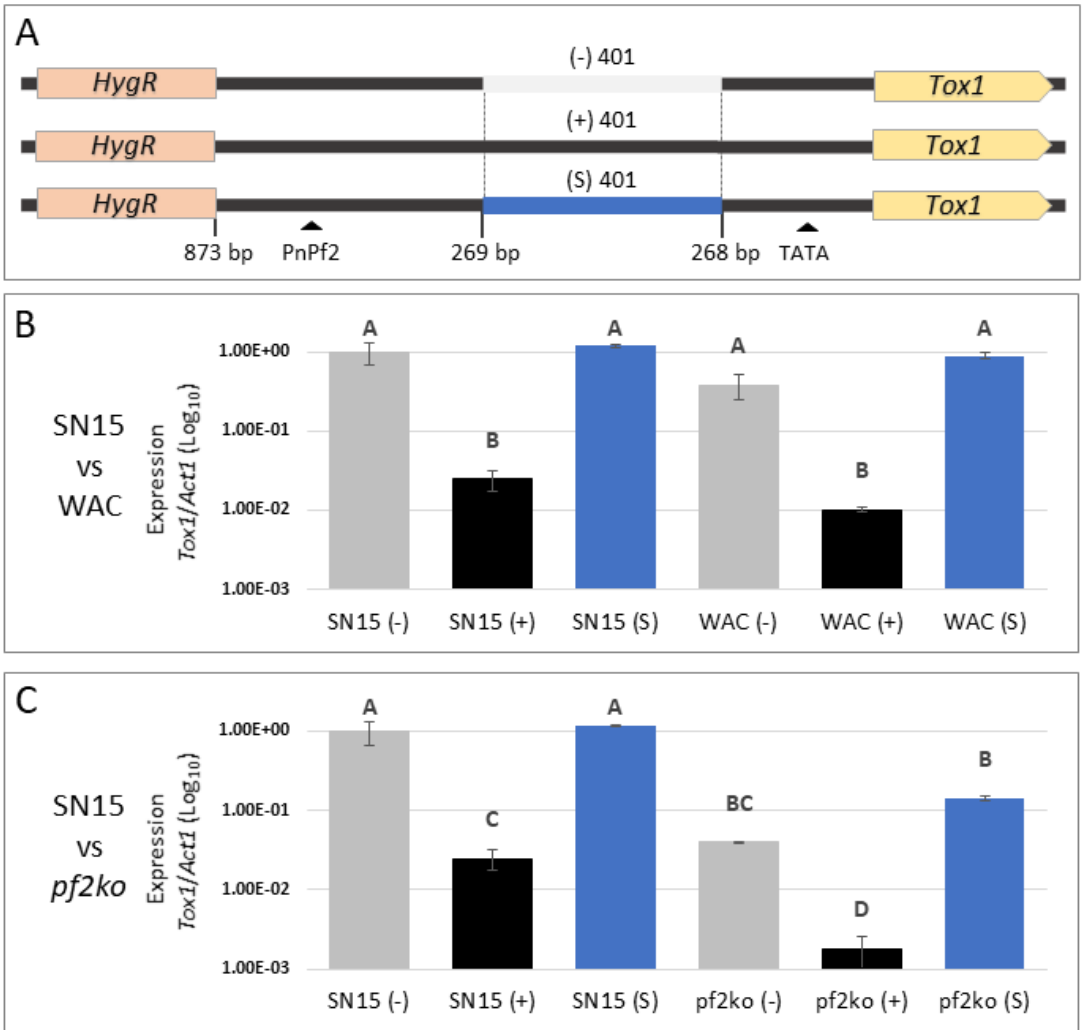

Figure 6.6 - Sequence specificity of *Tox1* promoter variant PE401 dictates *Tox1* repression in *P. nodorum*

A) Schematic overview of the three *Tox1* promoter replacement loci either with PE401 (+), without PE401 (-), or using a spacer (S) 401 bp sequence in the SN15, WAC13443 (WAC) and SN15 *PnPf2* knockout (*pf2ko*) backgrounds. B & C) *Tox1* expression ($2^{dCt(Tox1\text{-}Actin)}$) in the corresponding SN15 and WAC mutants (B), or SN15 and *pf2ko* mutants (C) during growth in Fries3 liquid culture. Error bars indicate standard deviations from the combined averages (n = 2) of independently verified isogenic promoter replacement mutants. Different letters indicate significant differences (P<0.05) based on ANOVA.

6.3.3.3. PE401 represses *Tox1* during host infection

The regulatory role of PE401 was then assessed during infection through qPCR. Infection time points were chosen to represent major stages of infection such as penetration, colonisation and sporulation (3, 5 and 7 days post-inoculation respectively). *Tox1* expression was reduced in both the SN15 and WAC mutants carrying PE401 relative to the mutants carrying the spacer or lacking PE401 throughout the infection period (**Figure 6.7**). This provided further evidence that PE401 specifically represses *Tox1 in planta* as well as *in vitro*.

It was previously observed that gene deletion of *Tox1* in SN15 resulted in higher *Tox3* expression, which established a basis for Tox1-*Snn1* being epistatic to Tox3-*Snn3* (Phan et al., 2016). Therefore, it was hypothesised that the inclusion of the PE401 would alleviate *Tox3* repression by *Tox1*. However, the decrease in *Tox1* expression conferred by the 401 bp element did not result in an increase in *Tox3* expression in the SN15 (+) or WAC (+) mutants (**Figure 6.7**). This indicated *Tox1* repression caused by the 401 bp promoter element was insufficient to alleviate epistasis of the Tox3-*Snn3* interaction and that full suppression is needed to achieve this.

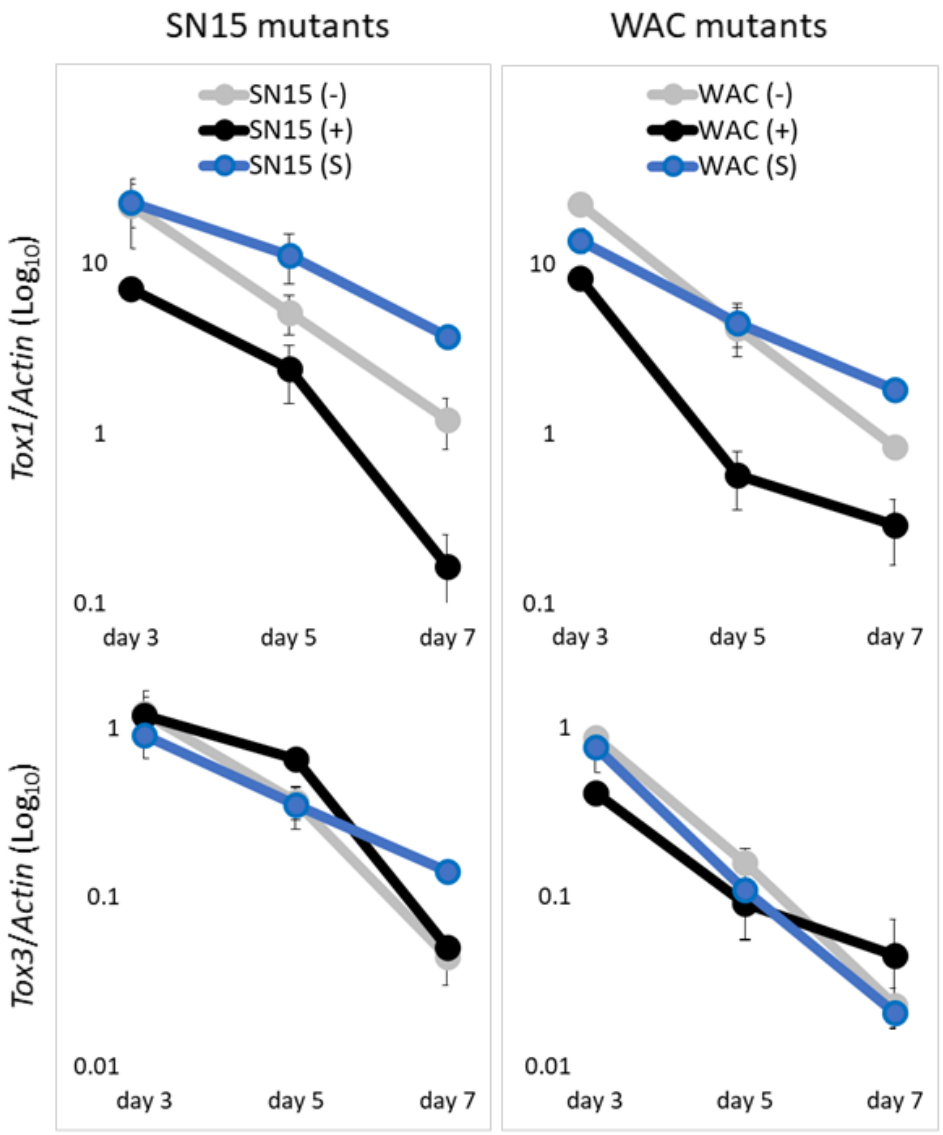

Figure 6.7 – Sequence specific *Tox1* promoter variant PE401 represses *Tox1* expression during *P. nodorum* infection of wheat

Quantitative PCR determination of *Tox1* and *Tox3* expression in promoter replacement mutants of SN15 and WAC13343 sampled at three (early penetration), five (colonisation) and seven (sporulation) days post-infection on wheat cv. Halberd. The average values ($2^{dCt(Target-Actin)}$) of two biological replicates were used. Error bars indicate standard deviations.

6.3.3.4. PE401 influences the Tox1-*Snn1* SNB interaction

The contribution of Tox1-*Snn1* to SNB symptoms caused by SN15 (+) and SN15 (-) mutant strains was determined using a population mapping approach. The CxW DH wheat population was infected with either SN15 (-) or SN15 (+) mutant spores at the seedling stage to directly compare the effect of PE401 repression on Tox1-*Snn1* and other important SNB interactions. Seedling resistance QTL can be detected in the CxW population on chromosomes 1BS (*Snn1*), 5BS (*Snn3*), 2A (*Qsnb.cur–2AS1*), 2D, 3A and 4B when infected with SN15 or its derivative NE deletion mutants (Phan et al., 2016). The average disease score on the 179 CxW wheat lines for the SN15 (+) mutant was 4.4, which was significantly lower than SN15 (-) which was 4.8 (**Table 6.2**; **Supplementary item 6.3**). SNB severity was then compared for SN15 (+) and (-) on genotype combinations that possess *Snn1* and it was observed that SN15 (+) was significantly less pathogenic. In contrast, no significant difference was observed on *snn1* wheat lines.

There was no difference in disease severity on *Snn3* vs *snn3* wheat for either mutant, which had previously been observed for the *tox1-6* mutant compared to SN15 (Phan et al., 2016). This indicated that *Tox1* remained epistatic to *Tox3* even when expressed at a lower level conferred by PE401. Reliable markers for the 2A, 2D, 3A and 4B resistance loci are not yet available which meant the disease severity could not be directly compared between the SN15 (-) and (+) mutants. Nevertheless, the results suggest that the Tox1-*Snn1* interaction contributed significantly to SNB severity, and that higher expression of *Tox1* conferred by the absence of PE401 produced a significantly higher level of disease on *Snn1* wheat.

Table 6.2 – Seedling SNB-disease rating of the Calingiri x Wyalkatchem doubled-haploid wheat population when infected by PE401 mutants, grouped by *Tox1* and *Tox3* sensitivity conferred by *Snn1* and *Snn3*, respectively

SN15 isogenic mutants with (+) and without (-) PE401 were used to assess seedling wheat infection. The wheat lines [columns 1-2], the mean SNB ratings/standard deviations (SD) [3-4] and the P values are included (calculated using the paired Student's T-test, *n* = 3). *Indicates significant differences between the mutants at the *P*<0.05 threshold. Disease scores for all lines are provided in **Supplementary item 6.3**.

Genotype	Lines (*n*)	SN15 (-) Mean / SD	SN15 (+) Mean / SD	*P* value
All lines	179	4.8 / 1.4	4.4 / 1.3	3.8E-07*
Snn1	91	5.7 / 1.0	5.0 / 1.1	7.4E-09*
snn1	88	3.9 / 1.3	3.8 / 1.2	2.0E-01
Snn3	76	4.8 / 1.3	4.5 / 1.2	7.0E-03*
snn3	103	4.8 / 1.4	4.4 / 1.3	1.4E-05*
Snn1/Snn3	31	5.6 / 1.1	5.0 / 1.1	2.6E-03*
Snn1/snn3	60	5.7 / 0.9	5.0 / 1.1	8.4E-07*
snn1/Snn3	45	4.2 / 1.1	4.1 / 1.1	3.5E-01
snn1/snn3	44	3.6 / 1.2	3.5 / 1.1	4.0E-01

6.3.3.5. Tox1 repression by PE401 alleviates epistasis of a major SNB QTL on chromosome 2A

Tox1 is epistatic to the SNB QTL *Qsnb.cur–2AS1* detected on chromosome 2A in addition to Tox3-*Snn3B1* (Phan et al., 2016). Since neither the NE or host receptor on chromosome 2A have been cloned, epistasis can only be studied through genetic mapping using wheat mapping populations that segregate for *Qsnb.cur–2AS1*. Therefore, interval mapping and QTL analysis were undertaken using the markers previously developed for the CxW population (Phan et al., 2016) to explore any differences in the disease interactions produced by the SN15 (-) and (+) PE401 mutant strains. The QTL detected on chromosome 1B, where *Snn1* is localised, explained the highest phenotypic contribution to SNB in the SN15 (-) mutant (47.9%) compared with SN15 (+) (30.1%) (**Table 6.3; Supplementary item 6.3**). However, the *Qsnb.cur–2AS1* SNB QTL was detected in the SN15 (+) mutant only where it contributed 12.1% to the disease. *Qsnb.cur–2AS1* was the most prominent QTL previously detected during infection with the *Tox1* knockout mutant *tox1-6* (**Figure 6.8**) (Phan et al., 2016). Despite Wyalkatchem being a donor of *Snn3B1* in the CxW population (Phan et al., 2016), association mapping did not detect the *Snn3B1* QTL on chromosome 5B. Therefore, it was concluded that *Tox1* repression by PE401 alleviated the epistatic effect on Tox2A-*Qsnb.cur–2AS1* but not Tox3-*Snn3*.

Table 6.3 - A summary of SNB quantitative-trait loci (QTLs) detected with the Calingiri x Wyalkatchem doubled-haploid wheat population

Details of the mutant used in the disease treatment [1], the chromosome mapped and the disease QTL with the respective flanking markers [2-4], logarithm of the odds (LOD) scores [4] and the phenotype contribution (R^2) explained by the QTL [5].

Treatment	Chromosome	QTL	Locus/QTL flanking markers	LOD	R^2
SN15 (-)	1BS	*QSnb.fcu–1BS*	*Snn1*	22.5	47.9
SN15 (+)	1BS	*QSnb.fcu–1BS*	*Snn1*	12.0	30.1
	2A	*Qsnb.cur–2AS1*	wmc453a-gwm294	4.6	12.1

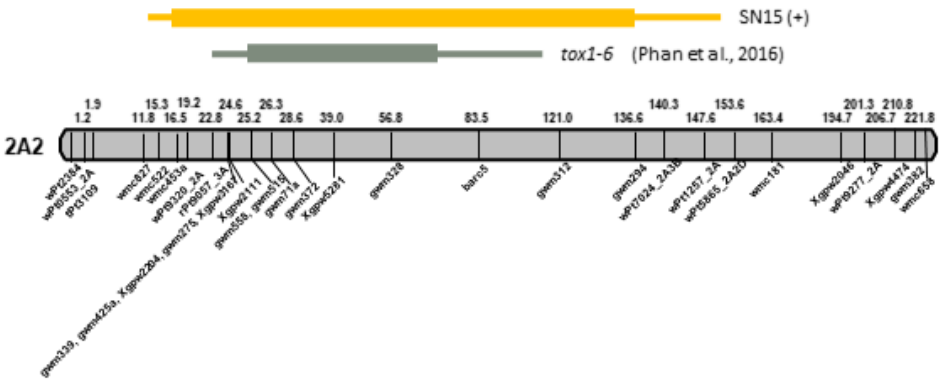

Figure 6.8 - The *Qsnb.cur–2AS1* QTL on the CxW DH wheat population

The chromosomal linkage-group map depicting genetic distances (cM) above and the molecular markers below. Box plots represent the *Qsnb.cur–2AS1* QTL interval detected for the *tox1-6* knockout mutant (Phan et al., 2016) and the corresponding QTL from SN15 (+) that was not detected in SN15 (-).

6.3.4. Promoter substitution identifies the positive regulatory regions of *Tox1*

SN15 promoter replacement mutants were generated with sequentially truncated *Tox1* promoter sequences to characterise the core regulatory regions involved in gene expression (**Figure 6.9**). The shortest truncation included only the *Tox1* 5' UTR (untranslated region) from the annotated genome which encompassed 86 bp upstream of the start codon (Syme et al., 2016). Further truncations were made at 107 bp and 157 bp upstream of the ATG, either side of a TATATAA sequence typical of a core eukaryotic TATA box element (Basehoar et al., 2004). Additional truncations were made at 263 bp and 310 bp upstream to exclude/include the site of the PE401 variant. A 598 bp upstream mutant was selected to exclude the putative PnPf2 binding site CGGTCCG (detailed in **Chapter 5**), while the 873 bp region corresponded to the entire sequence of the SN15 (-) mutant used in the preceding analyses. Furthermore, SN15 (+) mutants carrying PE401 were generated in each of the 310, 598 and 873 bp mutant backgrounds to assess any effect of the upstream elements on the repressor activity.

Gene expression analysis revealed that rather than any single element being essential, an additive effect on *Tox1* gene expression was observed by the inclusion of regions up to 310 bp, where the expression was maximal (**Figure 6.9**). In particular, significant increases were observed by the inclusion of the putative TATA box region (107 - 157 bp) and the upstream region (157 - 263 bp). PE401 acted as a strong repressor in each of the promoter deletion mutants tested. The inclusion of longer promoter regions, including the PnPf2 binding site, did not increase *Tox1* gene expression. This suggested that positive regulatory elements are located directly adjacent to the 3' end of the PE401 which functions as a repressor independent of

upstream sequences. It also suggested that sequences adjacent to the PnPf2 binding site in the 598 - 873 bp region are repressor elements that negate any PnPf2 enhancer activity.

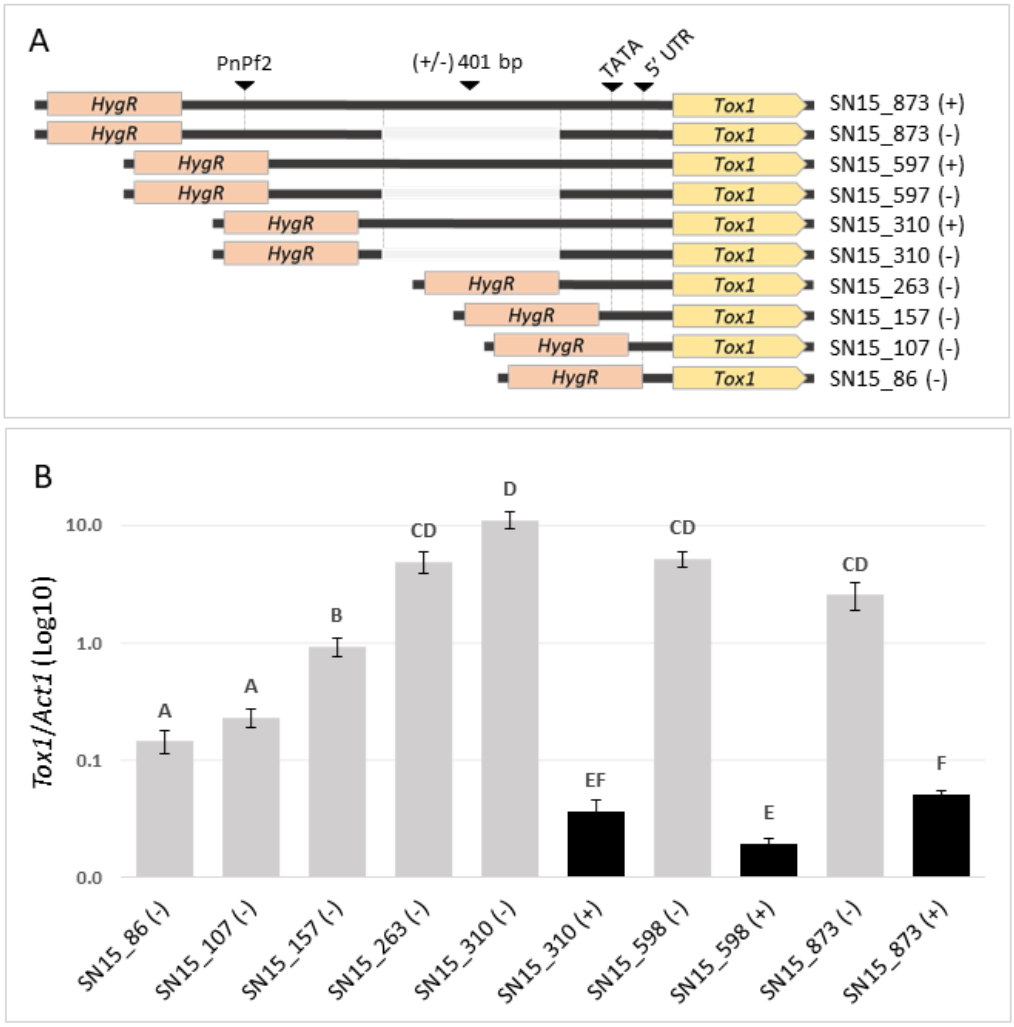

Figure 6.9 - Analysis of *Tox1* expression regulation through sequential promoter deletions

A) Schematic overview of the *Tox1* sequential promoter deletions in SN15 either with (+) or without (-) PE401. Indicated are the predicted PnPf2 binding site, the TATA box and 5' UTR derived from the reference genome. B) *Tox1* gene expression ($2^{dCt(Tox1-Ac1)}$) in the corresponding SN15 mutants after 72hrs growth in Fries3 liquid culture. Error bars indicate standard deviations from the combined averages (n = 2) of independently verified isogenic promoter replacement mutants. Different letters indicate significant differences (P<0.05) based on ANOVA.

6.4. Discussion

6.4.1. *Tox1* promoter regulatory elements in *P. nodorum*

This study sought to characterise the gene regulatory elements controlling *Tox1* expression and explore their role in NE epistasis and the virulence of *P. nodorum*. An important finding was the clear role in *Tox1* repression conferred by PE401 located 267 bp upstream of the start codon of *Tox1*. Analysis of the truncated promoter mutants indicated that PE401 could repress the transcriptional activators driving *Tox1* expression (**Figure 6.9**). The exact mechanism remains obscure, as there were no distinctive features of a non-coding or MGE, nor was it due to spatial variation affecting upstream promoter elements including the proposed PnPf2 binding site CGGTCCG. Nonetheless, we can conclude that a novel polymorphic NE regulatory element has been identified in *P. nodorum*, likely targeted by sequence-specific repressor proteins.

The most parsimonious explanation regarding the origins of PE401 is that the sequence was present in the ancestral *Tox1* gene promoter and was subsequently lost in a subset of isolates. This stems from the fact that it was only detected in isolates outside the proposed centres of origin for SNB (McDonald et al., 2012; Ghaderi et al., 2020). Populations carrying the highest proportion of isolates with PE401 absent were South Africa and Australia. There is little data regarding South African wheat but it is known that *Snn1* wheat in Australia has been widely sown throughout the period corresponding to the isolate collections assessed (**Supplementary item 6.4**) (Tan et al., 2014; Phan et al., 2020). This indicates either that selection was neutral throughout this period, or that the maintenance of some variation in the population is beneficial for the pathogen. The other major *P. nodorum* population where PE401 was absent is the North Dakota population, albeit at a reduced frequency (27%) compared to

Australia (87%) and South Africa (100%). In this region, it has been reported that *Snn1* is prevalent in widely grown durum wheat varieties (Liu et al., 2012; Cowger et al., 2020). Interestingly, *P. nodorum* population structure analyses have suggested the North Dakota population is unrelated to Australia and South Africa, which are closely related to each other (McDonald et al., 2012; Pereira et al., 2020). It is possible that PE401 was lost on separate occasions in these populations. On the other hand, it is also possible that genetic exchange has occurred between the populations followed by selection.

In Western Australia (WA), a region where SNB is endemic but PE401 frequency is low, *Snn1* has continually been maintained in the wheat varieties grown (Phan et al., 2020). However, the cv. Scepter, which lacks *Snn1*, is now the most widely grown in WA (52% area sown and increasing) (Shackley et al., 2021) and varieties with *Snn1* are being sown at a very low rate (www.caigeproject.org.au/germplasm-evaluation/bread/disease-screening/toxicity/). Without conferring a selective advantage for the pathogen, the maintenance of high *Tox1* expression can be bioenergetically taxing. Therefore, it will be interesting to monitor the impact that the decline in *Snn1* frequency will have on the frequency of PE401 within the Australian *P. nodorum* population over the next few years following the mass adoption of Scepter.

6.4.2. *Tox1* expression variation in SNB and NE epistasis

The infection assay using the CxW DH population demonstrated that higher expression of *Tox1* conferred a higher average level of virulence on *Snn1* wheat. While it is known that a fungal NE isoform/repertoire dictates pathogenic fitness (Tan et al., 2012), the role of NE gene expression has not been extensively examined. In the case

of the tan spot of wheat fungus *Pyrenophora tritici-repentis*, which has acquired *ToxA* through horizontal gene transfer (Friesen et al., 2006), *ToxA* is expressed much higher during infection relative to *P*. *nodorum* (Rybak et al., 2017). It was proposed this can compensate for the reduced potency of the PtrToxA isoform relative to *P*. *nodorum* variants (Rybak et al., 2017) and demonstrates an example in another pathosystem where NE expression change can have significant disease outcomes.

The genetic mapping results comparing SN15 (-) and SN15 (+) on the CxW DH wheat population suggested removal of PE401 did not affect Tox1-*Snn1* epistasis over Tox3-*Snn3*. However, the *Qsnb.cur–2AS1* disease resistance QTL was only detected by the inclusion of PE401. The physical interval for *Qsnb.cur–2AS1* is notably large (Phan et al., 2016). Nevertheless, several recent studies suggest the locus contains a gene conferring sensitivity to an undiscovered NE, that functions as an important determinant of both seedling, adult leaf and glume SNB resistance, since these QTLs overlap with *Qsnb.cur–2AS1* (Abeysekara et al., 2009; Phan et al., 2016; Lin et al., 2020a; Lin et al., 2021). In addition, a minor seedling resistance QTL on chromosome 2A was detected in a recombinant inbred (RI) population derived from Swiss winter wheat varieties Arina and Forno (Abeysekara et al., 2009). Therefore, the Tox2A-*Qsnb.cur–2AS1* interaction provides a layer of NE redundancy that maintains virulence on wheat, albeit at a lower level, in the absence of other interactions such as Tox1-*Snn1*. In addition to SNB, a co-localised *Qsnb.cur-2AS1* QTL is a major contributor to tan spot resistance in wheat (Cowger et al., 2020). Interestingly, tan spot is the most damaging necrotrophic fungal disease of wheat in Australia (Murray and Brennan, 2009) and the fungus frequently co-infects wheat with *P. nodorum* (Loughman et al., 1993; Abdullah et al., 2020; Justesen et al., 2021).

Regional *P. nodorum* populations differ greatly in NE gene frequency (McDonald et al., 2013; Lin et al., 2020b), possess high haplotype variability (Ghaderi et al., 2020), differential isoform activity (Tan et al., 2012), variable NE expression (Faris et al., 2011; Gao et al., 2015) and high evolutionary potential (McDonald and Linde, 2002). This makes SNB a difficult disease to eliminate using a common genetic resistance background in wheat across different cereal growing regions where SNB is endemic. Through population mapping a complex picture has emerged where some interactions are additive in SNB (Downie et al., 2020) while others are epistatic (**Table 6.1**). In some cases, differential NE gene expression underpins epistasis, but the regulatory elements were not resolved. In one example, the expression of *ToxA* was reported to be two-fold higher during infection in the *P. nodorum* isolate SN5 compared with SN4 (Faris et al., 2011). Higher *ToxA* expression correlated with a greater contribution of ToxA-*Tsn1* to the disease phenotype of SN5 on the BR34 x Grandin RI wheat population that segregates for *Tsn1* and *Snn2*. Conversely, the phenotypic contribution of Tox2-*Snn2* was reduced (Faris et al., 2011). Recently, a gene encoding the NE Tox267, which interacts with *Snn2*, was cloned and functionally characterised (Richards et al., 2021). The deletion of *Tox267* in *P*. *nodorum* SN4 resulted in up-regulation of the NEs *ToxA*, *Tox1*, and *Tox3*, demonstrating further connectivity between the expression of these genes. Another study that used an RI wheat population (ITMI) segregating for *Tsn1*, *Snn1* and *Snn3* reported that the presence of *ToxA* in SN2000 suppressed Tox1-*Snn1* ETS (Peters-Haugrud et al., 2019). Gene deletion of *ToxA* produced only a mild reduction in disease severity, which was compensated by increased *Tox1* expression. Collectively, these studies suggest gene repression as the basis for NE epistasis is common, for which the results presented here on PE401 have provided a novel mechanistic insight.

The phenomenon of NE epistasis is also observed in other fungal-plant pathosystems, dictating the contribution of effector-susceptibility/resistance gene interactions during disease (Tan and Oliver, 2017). When *ToxA* was deleted in *P. tritici-repentis* virulence increased on some *Tsn1* wheat lines, which unmasked the effect of a novel chlorosis-inducing factor (Moffat et al., 2014; Manning and Ciuffetti, 2015). In the *Leptosphaeria maculans-Brassica napus* pathosystem, *Rlm3*-mediated resistance through recognition of the avirulence effector protein AvrLm3 was suppressed by another avirulence effector AvrLm4-7 (Plissonneau et al., 2016).

6.4.3. The *Tox1* regulatory model and future perspectives

The identity of major trans-acting regulatory molecules that are associated with Tox1 regulation remains unknown at this stage. However, key results from this study allow a regulatory model to be proposed for *Tox1* and NE-*Snn* epistasis detected using the CxW population (**Figure 6.10**). An unidentified repressor binds to PE401 and inhibits the transcriptional activator(s) bound to the TATA-region, thereby repressing *Tox1* expression and the consequent epistatic effect on the Tox2A-*Qsnb.cur-2AS1* interaction during SNB (**Figure 6.10.A**). In the absence of the PE401, transcriptional activator(s) can drive high *Tox1* expression (**Figure 6.10.B**). As a result, Tox1-*Snn1* contributes strongly to SNB, while the *Qsnb.cur-2AS1* QTL is suppressed. PnPf2 also acts as an upstream enhancer of *Tox1* expression independent of PE401. Although the proposed regulatory mechanism remains speculative at this stage, it provides a framework that will allow future studies of regulatory components. As such, affinity purification assays using these sequences as bait represent a promising avenue to identify the regulators involved.

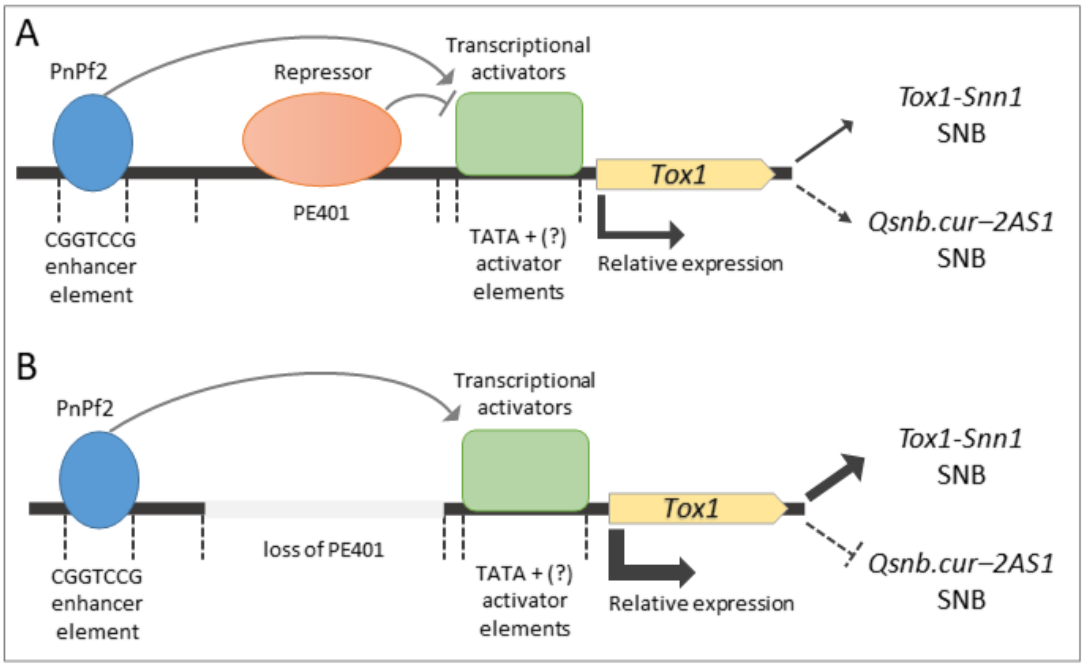

Figure 6.10 - The proposed regulatory model of the *P. nodorum Tox1* promoter

A) The proposed model of regulators interacting with the *P. nodorum Tox1* promoter in isolates carrying PE401 and (B) isolates where the element has been lost. The effect of PE401 on *Tox1* expression and the direct (solid arrows) or epistatic (dashed arrows) SNB-disease interactions (*Tox1-Snn1* and *Qsnb.cur-2AS1*) are depicted by the strength of the arrows. In this model, the PnPf2 enhancer element recruits PnPf2 independent of the presence/absence of PE401.

The findings presented here have advanced our knowledge regarding the control of *Tox1* expression in *P. nodorum* and its profound epistatic effect on SNB resistance in wheat. Although the proposed regulatory mechanism remains speculative at this stage, individual elements were defined within the promoter region of *Tox1*. This provides a framework for future studies into the components regulating *Tox1*. A DNA-protein bioassay approach would help to identify transcriptional activator and repressor proteins that are associated with the promoter region of *Tox1*. However,

underlying questions on the mechanism of the epistatic suppression of NE gene expression and phenotypic contributions of SNB remain. It is possible that small interfering RNA may be present within the fungus that regulate gene expression by silencing NE genes (Raman et al., 2017). Alternatively, NE-host gene interactions within the same system compete for common plant-signaling pathways or are antagonistic in the mechanisms by which ETS is triggered (Shi et al., 2016; Faris and Friesen, 2020).

The evidence presented highlights the general importance of understanding NE regulation, the outcomes of which extend beyond the individual NE-host receptor interactions. The evolution of changes in NE gene expression provides a mechanism for *P. nodorum* to adapt where the complement of host receptors shifts through resistance breeding efforts. More importantly, the identification of PE401 will help to formulate effective crop protection strategies against SNB of wheat. Specific recommendations include:

- Monitor for changes in the frequency of PE401 in regional *P. nodorum* populations.
- The maintenance of *Snn1* in wheat is a likely driver for *P. nodorum* to retain the PE401 polymorphism and evolve *Tox1*. Therefore, *Snn1* removal should be prioritised in cereal breeding programmes.
- Introduce the SNB/tan spot resistance allele for *Qsnb.cur-2AS1* alongside the Tox1 resistance allele *snn1*. The stacking of regionally relevant SNB resistance alleles has been demonstrated to provide optimal SNB disease resistance in wheat (Phan et al., 2018; Lin et al., 2020a).

It is anticipated that the outcome of this study will drive a greater level of research into the field of effector regulation and epistasis in other fungal-plant pathosystems to generate similar outcomes to improve existing crop protection strategies.

6.5. Supplementary items

Accessible via: https://figshare.com/s/b0bd50b709e823cbf43e

Supplementary item 6.1 - *Tox1* isolate associated metadata

A table describing the isolate ID [column 1], the species, country, region and host information (if available) where it was derived [2-5], the date it was isolated [6]. The presence of the 401 bp element PE401 in the *Tox1* promoter is indicated [7] along with the data source and reference from which this was determined [8-9].

Supplementary item 6.2 - PE401 and spacer sequence

The PE401 and 401 bp spacer sequences used to substitute the element in SN15 (S) control mutants. A dotplot is provided to demonstrate the sequence dissimilarity.

Supplementary item 6.3 - SNB disease-mapping data

A table indicating the CxW lines tested [column 1], the presence of the *Snn1* and *Snn3* marker (Phan et al., 2016) [2-3], the average disease scores for SN15 (-) and SN15 (+) mutants [4-5] as well as a summary of the disease score statistical analysis and the chromosomal linkage groups analysed.

Supplementary item 6.4 - Temporal assessment of PE401 frequencies in Australia

A frequency plot of Australian *P*. *nodorum* isolates assessed in this study with or without the 401 bp element PE401 and group by the year of collection.

6.6. References

Abdullah, A.S., Gibberd, M.R. & Hamblin, J. (2020) Co-infection of wheat by *Pyrenophora tritici-repentis* and *Parastagonospora nodorum* in the wheatbelt of Western Australia. *Crop and Pasture Science*, 71, 119–127. https://doi.org/10.1071/CP19412.

Abeysekara, N.S., Faris, J.D., Chao, S., McClean, P.E. & Friesen, T.L. (2012) Whole-genome QTL analysis of *Stagonospora nodorum* blotch resistance and validation of the SnTox4-Snn4 interaction in hexaploid wheat. *Phytopathology*, 102, 94–104. https://doi.org/10.1094/PHYTO-02-11-0040.

Abeysekara, N.S., Friesen, T.L., Keller, B. & Faris, J.D. (2009) Identification and characterization of a novel host–toxin interaction in the wheat–*Stagonospora nodorum* pathosystem. *Theoretical and Applied Genetics*, 120, 117–126. https://doi.org/10.1007/s00122-009-1163-6.

Bao, W., Kojima, K.K. & Kohany, O. (2015) Repbase Update, a database of repetitive elements in eukaryotic genomes. *Mobile DNA*, 6, 11. https://doi.org/10.1186/s13100-015-0041-9.

Basehoar, A.D., Zanton, S.J. & Pugh, B.F. (2004) Identification and distinct regulation of yeast TATA box-containing genes. *Cell*, 116, 699–709. https://doi.org/10.1016/s0092-8674(04)00205-3.

Chu, C.-G., Faris, J.D., Xu, S.S. & Friesen, T.L. (2010) Genetic analysis of disease susceptibility contributed by the compatible *Tsn1*-SnToxA and *Snn1*-SnTox1 interactions in the wheat-*Stagonospora nodorum* pathosystem. *TAG. Theoretical and applied genetics. Theoretische und Angewandte Genetik*, 120, 1451–1459. https://doi.org/10.1007/s00122-010-1267-z.

Cowger, C., Ward, B., Brown-Guedira, G. & Brown, J.K.M. (2020) Role of effector-sensitivity gene interactions and durability of quantitative resistance to Septoria nodorum blotch in eastern U.S. wheat. *Frontiers in Plant Science*, 11. https://doi.org/10.3389/fpls.2020.00155.

Dam, P. van & Rep, M. (2017) The distribution of *Miniature Impala* elements and SIX genes in the *Fusarium* genus is suggestive of horizontal gene transfer. *Journal of Molecular Evolution*, 85, 14–25. https://doi.org/10.1007/s00239-017-9801-0.

Downie, R.C., Lin, M., Corsi, B., Ficke, A., Lillemo, M., Oliver, R.P., et al. (2020) Septoria nodorum blotch of wheat: disease management and resistance breeding in the face of shifting disease dynamics and a changing environment. *Phytopathology*. https://doi.org/10.1094/PHYTO-07-20-0280-RVW.

Faris, J.D. & Friesen, T.L. (2020) Plant genes hijacked by necrotrophic fungal pathogens. *Current Opinion in Plant Biology*, 56, 74–80. https://doi.org/10.1016/j.pbi.2020.04.003.

Faris, J.D., Zhang, Z., Rasmussen, J.B. & Friesen, T.L. (2011) Variable expression of the *Stagonospora nodorum* effector SnToxA among isolates is correlated with levels of disease in wheat. *Molecular Plant-Microbe Interactions*, 24, 1419–1426. https://doi.org/10.1094/MPMI-04-11-0094.

Friesen, T.L., Chu, C., Xu, S.S. & Faris, J.D. (2012) SnTox5-*Snn5*: a novel *Stagonospora nodorum* effector-wheat gene interaction and its relationship with the SnToxA-*Tsn1* and SnTox3-*Snn3-B1* interactions. *Molecular Plant Pathology*, 13, 1101–1109. https://doi.org/10.1111/j.1364-

3703.2012.00819.x.
Friesen, T.L. & Faris, J.D. (2010) Characterization of the wheat-*Stagonospora nodorum* disease system: what is the molecular basis of this quantitative necrotrophic disease interaction? *Canadian Journal of Plant Pathology*, 32, 20–28. https://doi.org/10.1080/07060661003620896.
Friesen, T.L., Meinhardt, S.W. & Faris, J.D. (2007) The *Stagonospora nodorum*-wheat pathosystem involves multiple proteinaceous host-selective toxins and corresponding host sensitivity genes that interact in an inverse gene-for-gene manner. *The Plant Journal*, 51, 681–692. https://doi.org/10.1111/j.1365-313X.2007.03166.x.
Friesen, T.L., Stukenbrock, E.H., Liu, Z., Meinhardt, S., Ling, H., Faris, J.D., et al. (2006) Emergence of a new disease as a result of interspecific virulence gene transfer. *Nature Genetics*, 38, 953–956. https://doi.org/10.1038/ng1839.
Friesen, T.L., Zhang, Z., Solomon, P.S., Oliver, R.P. & Faris, J.D. (2008) Characterization of the interaction of a novel *Stagonospora nodorum* host-selective toxin with a wheat susceptibility gene. *Plant Physiology*, 146, 682–693. https://doi.org/10.1104/pp.107.108761.
Gao, Y., Faris, J.D., Liu, Z., Kim, Y.M., Syme, R.A., Oliver, R.P., et al. (2015) Identification and characterization of the SnTox6-*Snn6* interaction in the *Parastagonospora nodorum*–wheat pathosystem. *Molecular Plant-Microbe Interactions*, 28, 615–625. https://doi.org/10.1094/MPMI-12-14-0396-R.
Gao, Y., Liu, Z., Faris, J.D., Richards, J., Brueggeman, R.S., Li, X., et al. (2016) Validation of genome-wide association studies as a tool to identify virulence factors in *Parastagonospora nodorum*. *Phytopathology*, 106, 1177–1185. https://doi.org/10.1094/PHYTO-02-16-0113-FI.
Ghaderi, F., Sharifnabi, B., Javan-Nikkhah, M., Brunner, P.C. & McDonald, B.A. (2020) SnToxA, SnTox1, and SnTox3 originated in *Parastagonospora nodorum* in the Fertile Crescent. *Plant Pathology*, 69, 1482–1491. https://doi.org/10.1111/ppa.13233.
Johnson, M., Zaretskaya, I., Raytselis, Y., Merezhuk, Y., McGinnis, S. & Madden, T.L. (2008) NCBI BLAST: a better web interface. *Nucleic Acids Research*, 36, W5-9. https://doi.org/10.1093/nar/gkn201.
Jones, D.A.B., John, E., Rybak, K., Phan, H.T.T., Singh, K.B., Lin, S.-Y., et al. (2019) A specific fungal transcription factor controls effector gene expression and orchestrates the establishment of the necrotrophic pathogen lifestyle on wheat. *Scientific Reports*, 9, 1–13. https://doi.org/10.1038/s41598-019-52444-7.
Justesen, A.F., Corsi, B., Ficke, A., Hartl, L., Holdgate, S., Jørgensen, L.N., et al. (2021) Hidden in plain sight: a molecular field survey of three wheat leaf blotch fungal diseases in North-Western Europe shows co-infection is widespread. *European Journal of Plant Pathology*, 160, 949–962. https://doi.org/10.1007/s10658-021-02298-5.
Kahle, D. & Wickham, H. (2013) ggmap: Spatial visualisation with ggplot2. *The R Journal*, 5, 144–161.
Kalvari, I., Nawrocki, E.P., Argasinska, J., Quinones-Olvera, N., Finn, R.D., Bateman, A., et al. (2018) Non-coding RNA analysis using the Rfam database. *Current Protocols in Bioinformatics*, 62, e51. https://doi.org/10.1002/cpbi.51.
Kariyawasam, G.K., Richards, J.K., Wyatt, N.A., Running, K., Xu, S.S., Liu, Z., et al.

(2021) The *Parastagonospora nodorum* necrotrophic effector SnTox5 targets the wheat gene *Snn5* and facilitates entry into the leaf mesophyll. *bioRxiv*, 2021.02.26.433117. https://doi.org/10.1101/2021.02.26.433117.
Kohany, O., Gentles, A.J., Hankus, L. & Jurka, J. (2006) Annotation, submission and screening of repetitive elements in Repbase: RepbaseSubmitter and Censor. *BMC bioinformatics*, 7, 474. https://doi.org/10.1186/1471-2105-7-474.
Krishnan, P., Meile, L., Plissonneau, C., Ma, X., Hartmann, F.E., Croll, D., et al. (2018) Transposable element insertions shape gene regulation and melanin production in a fungal pathogen of wheat. *BMC Biology*, 16, 78. https://doi.org/10.1186/s12915-018-0543-2.
Lin, M., Corsi, B., Ficke, A., Tan, K.-C., Cockram, J. & Lillemo, M. (2020a) Genetic mapping using a wheat multi-founder population reveals a locus on chromosome 2A controlling resistance to both leaf and glume blotch caused by the necrotrophic fungal pathogen *Parastagonospora nodorum*. *TAG. Theoretical and Applied Genetics. Theoretische Und Angewandte Genetik*, 133, 785–808. https://doi.org/10.1007/s00122-019-03507-w.
Lin, M., Ficke, A., Cockram, J. & Lillemo, M. (2020b) Genetic structure of the Norwegian *Parastagonospora nodorum* population. *Frontiers in Microbiology*, 11. https://doi.org/10.3389/fmicb.2020.01280.
Lin, M., Stadlmeier, M., Mohler, V., Tan, K.-C., Ficke, A., Cockram, J., et al. (2021) Identification and cross-validation of genetic loci conferring resistance to Septoria nodorum blotch using a German multi-founder winter wheat population. *TAG. Theoretical and applied genetics. Theoretische und angewandte Genetik*, 134, 125–142. https://doi.org/10.1007/s00122-020-03686-x.
Liu, Z., Faris, J.D., Oliver, R.P., Tan, K.-C., Solomon, P.S., McDonald, M.C., et al. (2009) SnTox3 acts in effector triggered susceptibility to induce disease on wheat carrying the *Snn3* gene. *PLOS Pathogens*, 5, e1000581. https://doi.org/10.1371/journal.ppat.1000581.
Liu, Z., Friesen, T.L., Rasmussen, J.B., Ali, S., Meinhardt, S.W. & Faris, J.D. (2004) Quantitative trait loci analysis and mapping of seedling resistance to *Stagonospora nodorum* leaf blotch in wheat. *Phytopathology*, 94, 1061–1067. https://doi.org/10.1094/PHYTO.2004.94.10.1061.
Liu, Z., Zhang, Z., Faris, J.D., Oliver, R.P., Syme, R., McDonald, M.C., et al. (2012) The cysteine rich necrotrophic effector SnTox1 produced by *Stagonospora nodorum* triggers susceptibility of wheat lines harboring *Snn1*. *PLOS Pathogens*, 8, e1002467. https://doi.org/10.1371/journal.ppat.1002467.
Loughman, R., Wilson, R.E. & Thomas, G.J. (1993) The influence of disease complexes involving *Leptosphaeria* (Septoria) *nodorum* on detection of resistance to three leaf spot diseases in wheat. *Euphytica*, 72, 31–42. https://doi.org/10.1007/BF00023770.
Manning, V.A. & Ciuffetti, L.M. (2015) Necrotrophic effector epistasis in the *Pyrenophora tritici-repentis*-wheat interaction. *PLOS ONE*, 10, e0123548. https://doi.org/10.1371/journal.pone.0123548.
Mat Razali, N., Cheah, B.H. & Nadarajah, K. (2019) Transposable elements adaptive role in genome plasticity, pathogenicity and evolution in fungal phytopathogens. *International Journal of Molecular Sciences*, 20. https://doi.org/10.3390/ijms20143597.
McDonald, B.A. & Linde, C. (2002) Pathogen population genetics, evolutionary

potential, and durable resistance. *Annual Review of Phytopathology*, 40, 349–379. https://doi.org/10.1146/annurev.phyto.40.120501.101443.
McDonald, M.C., Oliver, R.P., Friesen, T.L., Brunner, P.C. & McDonald, B.A. (2013) Global diversity and distribution of three necrotrophic effectors in *Phaeosphaeria nodorum* and related species. *New Phytologist*, 199, 241–251. https://doi.org/10.1111/nph.12257.
McDonald, M.C., Razavi, M., Friesen, T.L., Brunner, P.C. & McDonald, B.A. (2012) Phylogenetic and population genetic analyses of *Phaeosphaeria nodorum* and its close relatives indicate cryptic species and an origin in the Fertile Crescent. *Fungal Genetics and Biology*, 49, 882–895. https://doi.org/10.1016/j.fgb.2012.08.001.
McDonald, M.C. & Solomon, P.S. (2018) Just the surface: advances in the discovery and characterization of necrotrophic wheat effectors. *Current Opinion in Microbiology*, 46, 14–18. https://doi.org/10.1016/j.mib.2018.01.019.
Moffat, C.S., See, P.T. & Oliver, R.P. (2014) Generation of a *ToxA* knockout strain of the wheat tan s pot pathogen *Pyrenophora tritici-repentis*. *Molecular Plant Pathology*, 15, 918–926. https://doi.org/10.1111/mpp.12154.
Murray, G.M. & Brennan, J.P. (2009) Estimating disease losses to the Australian wheat industry. *Australasian Plant Pathology*, 38, 558–570. https://doi.org/10.1071/AP09053.
Muszewska, A., Steczkiewicz, K., Stepniewska-Dziubinska, M. & Ginalski, K. (2017) Cut-and-paste transposons in fungi with diverse lifestyles. *Genome Biology and Evolution*, 9, 3463–3477. https://doi.org/10.1093/gbe/evx261.
Pereira, D., Ogenfuss, U., McDonald, B.A. & Croll, D. (2020) The population genomics of transposable element activation in the highly repressive genome of an agricultural pathogen. https://doi.org/10.1101/2020.11.12.379651.
Peters-Haugrud, A.R., Zhang, Z., Richards, J.K., Friesen, T.L. & Faris, J.D. (2019) Genetics of variable disease expression conferred by inverse gene-for-gene interactions in the wheat-*Parastagonospora nodorum* pathosystem. *Plant Physiology*, 180, 420–434. https://doi.org/10.1104/pp.19.00149.
Phan, H.T.T., Jones, D.A.B., Rybak, K., Dodhia, K.N., Lopez-Ruiz, F.J., Valade, R., et al. (2020) Low amplitude boom-and-bust cycles define the Septoria nodorum blotch interaction. *Frontiers in Plant Science*, 10. https://doi.org/10.3389/fpls.2019.01785.
Phan, H.T.T., Rybak, K., Bertazzoni, S., Furuki, E., Dinglasan, E., Hickey, L.T., et al. (2018) Novel sources of resistance to Septoria nodorum blotch in the Vavilov wheat collection identified by genome-wide association studies. *Theoretical and Applied Genetics*, 131, 1223–1238. https://doi.org/10.1007/s00122-018-3073-y.
Phan, H.T.T., Rybak, K., Furuki, E., Breen, S., Solomon, P.S., Oliver, R.P., et al. (2016) Differential effector gene expression underpins epistasis in a plant fungal disease. *The Plant Journal*, 87, 343–354. https://doi.org/10.1111/tpj.13203.
Plissonneau, C., Daverdin, G., Ollivier, B., Blaise, F., Degrave, A., Fudal, I., et al. (2016) A game of hide and seek between avirulence genes AvrLm4-7 and AvrLm3 in *Leptosphaeria maculans*. *New Phytologist*, 209, 1613–1624. https://doi.org/10.1111/nph.13736.
Raman, V., Simon, S.A., Demirci, F., Nakano, M., Meyers, B.C. & Donofrio, N.M. (2017) Small RNA functions are required for growth and development of

Magnaporthe oryzae. *Molecular Plant-Microbe Interactions*, 30, 517–530. https://doi.org/10.1094/MPMI-11-16-0236-R.
Richards, J.K., Kariyawasam, G., Seneviratne, S., Wyatt, N.A., Xu, S.S., Liu, Z., et al. (2021) A triple threat: the *Parastagonospora nodorum* SnTox267 effector exploits three distinct host genetic factors to cause disease in wheat. *bioRxiv*, 2021.02.25.432871. https://doi.org/10.1101/2021.02.25.432871.
Richards, J.K., Stukenbrock, E.H., Carpenter, J., Liu, Z., Cowger, C., Faris, J.D., et al. (2019) Local adaptation drives the diversification of effectors in the fungal wheat pathogen *Parastagonospora nodorum* in the United States. *PLOS Genetics*, 15, e1008223. https://doi.org/10.1371/journal.pgen.1008223.
Richards, J.K., Wyatt, N.A., Liu, Z., Faris, J.D. & Friesen, T.L. (2017) Reference quality genome assemblies of three *Parastagonospora nodorum* isolates differing in virulence on wheat. *G3: Genes, Genomes, Genetics*, 8, 393–399. https://doi.org/10.1534/g3.117.300462.
Ruud, A.K., Dieseth, J.A., Ficke, A., Furuki, E., Phan, H.T.T., Oliver, R.P., et al. (2019) Genome-wide association mapping of resistance to Septoria nodorum feaf blotch in a Nordic spring wheat collection. *The Plant Genome*, 12, 180105. https://doi.org/10.3835/plantgenome2018.12.0105.
Rybak, K., See, P.T., Phan, H.T.T., Syme, R.A., Moffat, C.S., Oliver, R.P., et al. (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. *Molecular Plant Pathology*, 18, 420–434. https://doi.org/10.1111/mpp.12511.
Shackley, B., Paynter, B., Bucat, J., Troup, B., Seymour, M. & Blake, A. (2021) 2021 Western Australian crop sowing guide. *Grains Research and Development Corporation*.
Shi, G., Friesen, T.L., Saini, J., Xu, S.S., Rasmussen, J.B. & Faris, J.D. (2015) The wheat *Snn7* gene confers susceptibility on recognition of the *Parastagonospora nodorum* necrotrophic effector SnTox7. *The Plant Genome*, 8, plantgenome2015.02.0007. https://doi.org/10.3835/plantgenome2015.02.0007.
Shi, G., Zhang, Z., Friesen, T.L., Raats, D., Fahima, T., Brueggeman, R.S., et al. (2016) The hijacking of a receptor kinase–driven pathway by a wheat fungal pathogen leads to disease. *Science Advances*, 2. https://doi.org/10.1126/sciadv.1600822.
Soyer, J.L., Ghalid, M.E., Glaser, N., Ollivier, B., Linglin, J., Grandaubert, J., et al. (2014) Epigenetic control of effector gene expression in the plant pathogenic fungus *Leptosphaeria maculans*. *PLOS Genetics*, 10, e1004227. https://doi.org/10.1371/journal.pgen.1004227.
Stukenbrock, E.H., Banke, S. & McDONALD, B.A. (2006) Global migration patterns in the fungal wheat pathogen *Phaeosphaeria nodorum*. *Molecular Ecology*, 15, 2895–2904. https://doi.org/10.1111/j.1365-294X.2006.02986.x.
Syme, R.A., Tan, K.-C., Hane, J.K., Dodhia, K., Stoll, T., Hastie, M., et al. (2016) Comprehensive annotation of the *Parastagonospora nodorum* reference genome using next-generation genomics, transcriptomics and proteogenomics. *PLOS ONE*, 11, e0147221. https://doi.org/10.1371/journal.pone.0147221.
Syme, R.A., Tan, K.-C., Rybak, K., Friesen, T.L., McDonald, B.A., Oliver, R.P., et al. (2018) Pan-*Parastagonospora* comparative genome analysis—effector

prediction and genome evolution. *Genome Biology and Evolution*, 10, 2443–2457. https://doi.org/10.1093/gbe/evy192.
Tan, K.-C., Ferguson-Hunt, M., Rybak, K., Waters, O.D.C., Stanley, W.A., Bond, C.S., et al. (2012) Quantitative variation in effector activity of ToxA isoforms from *Stagonospora nodorum* and *Pyrenophora tritici-repentis*. *Molecular Plant-Microbe Interactions*, 25, 515–522. https://doi.org/10.1094/MPMI-10-11-0273.
Tan, K.-C. & Oliver, R.P. (2017) Regulation of proteinaceous effector expression in phytopathogenic fungi. *PLOS Pathogens*, 13, e1006241. https://doi.org/10.1371/journal.ppat.1006241.
Tan, K.-C., Phan, H.T.T., Rybak, K., John, E., Chooi, Y.H., Solomon, P.S., et al. (2015) Functional redundancy of necrotrophic effectors – consequences for exploitation for breeding. *Frontiers in Plant Science*, 6. https://doi.org/10.3389/fpls.2015.00501.
Tan, K.-C., Waters, O.D.C., Rybak, K., Antoni, E., Furuki, E. & Oliver, R.P. (2014) Sensitivity to three *Parastagonospora nodorum* necrotrophic effectors in current Australian wheat cultivars and the presence of further fungal effectors. *Crop and Pasture Science*, 65, 150–158. https://doi.org/10.1071/CP13443.
Vleeshouwers, V.G.A.A. & Oliver, R.P. (2014) Effectors as tools in disease resistance breeding against biotrophic, hemibiotrophic, and necrotrophic plant pathogens. *Molecular Plant-Microbe Interactions*, 27, 196–206. https://doi.org/10.1094/MPMI-10-13-0313-IA.
Zhang, Z., Running, K.L.D., Seneviratne, S., Haugrud, A.R.P., Szabo-Hever, A., Shi, G., et al. (2021) A protein kinase-major sperm protein gene hijacked by a necrotrophic fungal pathogen triggers disease susceptibility in wheat. *The Plant Journal*, n/a. https://doi.org/10.1111/tpj.15194.

Chapter 7

Thesis outcomes and future directions

...and a return to a first-person perspective...

At the outset of this thesis, I outlined some of the diverse components by which virulence manifests in plant-pathogenic fungi. These included effector production, secondary metabolite (SM) biosynthesis, signal transduction/cellular metabolism, cellular trafficking/secretion systems and the channelling of non-coding RNAs. I emphasised a key challenge which remains; to better understand the regulatory factors that specifically control their expression and define pathogenic lifestyles. The *Parastagonospora nodorum*-wheat pathosystem that culminates in the disease septoria nodorum blotch (SNB), is a particularly good example. A multitude of proven virulence factors have been uncovered that includes the host-specific necrotrophic effectors (NEs), as well as non-specific phytotoxic SMs and general metabolic components (e.g. **Table 1.1**). However, there has been a significant knowledge gap in understanding how these components are regulated. This is typified by the phenomenon of NE redundancy and epistasis, concepts I introduced in **Chapter 1**, which have complicated efforts to breed resistance to SNB based on the removal of their cognate plant receptors. Therefore, I sought to establish novel insights into the transcriptional regulation of virulence in *P. nodorum* and advance scientific knowledge of the disease.

Recent work had demonstrated that the Zn2Cys6 transcription factor (TF) PnPf2 was a key regulator of virulence, and a transcriptome analysis highlighted the

control of a broad set of genes that included NEs and putative cell wall-degrading enzymes (Rybak et al., 2017; Jones et al., 2020). It was not yet understood whether this was through a direct mechanism, or how PnPf2 had evolved to acquire this virulence role. Defining these aspects became a primary aim of this thesis.

I first deemed it important to better classify TFs in plant-pathogenic fungi at large and provide much-needed context for exploring the role of PnPf2. The distinct aim was to systematically review and identify evolutionary trends in TFs that regulate fungal virulence. Significant outcomes were two important resources for fungal plant pathologists that I presented in **Chapter 2** and **Chapter 3**. The comprehensive literature review in **Chapter 2** clarified the major TF families in plant-pathogenic fungi and compiled the functional evidence that had accumulated until publication. I was able to highlight the diverse facets of fungal development and virulence that are now known to be under TF control. It also highlighted the need to better identify and characterise TFs that are primarily associated with phytopathogenicity. The compilation of fungal TF repertoires and the orthology analysis conducted in **Chapter 3** provided useful insights and a novel resource with which to trace TF origins among fungal pathogen lineages. A distinct trend was observed, where pathogens that exist in an obligate-host relationship have fewer TFs, suggesting the limited ecological-niche of these fungi lends itself to a reduced regulatory capacity. Furthermore, evidence for extensive TF orthologue copy-number expansion and loss was observed across fungal lineages. The analysis ultimately provided candidate targets to investigate as virulence regulators.

Building on the TF orthology, I then conducted an extensive analysis into the origin and mechanisms of PnPf2-mediated virulence in *P*. *nodorum* (**Chapter 5**). I demonstrated that PnPf2 orthologues are rooted in the regulation of polysaccharide metabolism, a function intimately connected with virulence in a range of species. Chromatin immunoprecipitation (ChIP) analysis provided direct evidence that PnPf2 binds two distinct positive-regulatory elements, 5‘-RWMGGVCCGA-3’ and 5’-CGGCSBBWYYKCGGC-3’. The former is distinct to PnPf2 and should be explored further as a binding mechanism for its taxonomic orthologues. This regulatory motif could then be a tool to explore the regulation of virulence factors in other pathogens and present PnPf2 orthologues as a distinct target for disease control. Further insights in **Chapter 5** were that PnPf2 directly regulates *Tox3*, as well as *Tox1*, for which it played a moderate role. The presence of three 5‘-RWMGGVCCGA-3’ promoter motifs suggested *ToxA* is also a direct target but would require an *in planta* ChIP-seq analysis where the gene can be expressed. As well as this evidence for direct NE control, TFs were themselves prominent PnPf2 targets. Gene deletion analysis revealed important functions for *PnPro1*, *PnAda1* and *PnEbr1*, but supported the conclusion that PnPf2 is the major coordinator of virulence-specific functions on wheat. Future analyses should involve a co-immunoprecipitation assay, to identify any co-regulatory factors that could explain why two distinct PnPf2 binding motifs exist? Another question remains, which signals activate or suppress PnPf2? This can be explored through reporter-based assays that screen for polysaccharide triggers of PnPf2 signalling pathways. The putative carbon-catabolite repressor CreA in *P*. *nodorum* (SNOG_13619) should also be functionally investigated. The conserved 5′-TSYGGGG-3’ motif identified in PnPf2-regulated gene promoters suggested CreA could act as a direct antagonist.

In **Chapter 6** I shifted the focus from the analysis of TFs, to a detailed investigation of the promoter elements that regulate *Tox1* expression. The aim was to identify and functionally characterise their role in SNB which was motivated for several reasons; major regulators remained unidentified, variation in *Tox1* expression and promoter polymorphisms had been reported, and the Tox1-*Snn1* interaction represented a model to explore the regulation of NE epistasis. The promoter replacement strategy I pursued demonstrated clearly that the PE401 directly repressed *Tox1* expression. The effect of PE401 in SNB was significant; Tox1-*Snn1* mediated disease was reduced, but this elicited another major disease interaction that demonstrated a clear example of NE epistasis. While the complexities of NE epistasis require further investigation, this analysis provided direct evidence for gene expression as a major contributing factor. By exploring the *P*. *nodorum* population distribution worldwide, the most plausible scenario was that PE401 was lost from the ancestral *Tox1* gene. The substantial differences in the prevalence of PE401 across populations should be continually monitored, particularly in the Australian context where there has been a shift to the cultivation of Tox1-insensitive Scepter wheat. While RNA-seq had indicated PnPf2 exerts some regulatory effect on *Tox1* (Jones et al., 2020), the sequential promoter deletions I undertook suggested the key positive regulators target elements 3' of PE401 that includes a putative TATA box. These regulatory factors, and any suppressors that target PE401, could be identified with an affinity purification assay that uses these regions as bait. Their identification would establish other important regulators that control virulence in *P*. *nodorum*.

In closing, the three primary aims outlined in **Chapter 1** have been addressed. In doing so, my research has made demonstrable scientific contributions to the field of molecular plant pathology and our scientific understanding of SNB. I presented a novel approach to studying TF regulation in virulence, contributed to new knowledge of effector biology and provided direct insights to improve current plant protection strategies. I have also proposed several pathways to build upon this research and further advance our scientific understanding in the future.

Appendices

Appendix 1 - Attribution statement for published material in Mol Plant Path.

To Whom It May Concern,

I, Evan John, contributed the writing compilation of "Transcription factor control of virulence in phytopathogenic fungi" *Molecular Plant Pathology*. 2021; 22: 858-881.

Assistance was provided by co-authors in the form of guidance and editing for the publication.

Evan John [redacted]

I, as Co-Author, endorse that this level of contribution by the candidate indicated above is appropriate:

Karam B. Singh

Richard P. Oliver

Kar-Chun Tan [redacted]

Appendix 2 - Attribution statement for published material in Sci Rep

To Whom It May Concern,

I, Evan John, contributed experimentation and writing of the Results section "Identification of DNA motifs enriched in the promoters of PnPf2-regulated genes" and the Methods section "Analysis of promoters for enriched motifs", as well as general editing to the publication entitled:

"A specific fungal transcription factor controls effector gene expression and orchestrates the establishment of the necrotrophic pathogen lifestyle on wheat" Scientific Reports. 2019; 9: 15884.

Evan John [redacted]

I, as Co-Author*, endorse that this level of contribution by the candidate indicated above is appropriate:

Darcy A. B. Jones** [redacted]

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**Current Curtin PhD student. This work not affiliated with work to be submitted for co-authors own thesis.

Appendix 3 - Attribution statement for published material in BioRxiv*

To Whom It May Concern,

I, Evan John, contributed the following to the publication: "Variability in an effector gene promoter of a necrotrophic fungal pathogen dictates epistasis and effector-triggered susceptibility in wheat" *BioRxiv*. 2021; doi.org/10.1101/2021.07.28.454099

- I planned and undertook the experimental work published except for:
  - "Droplet digital PCR quantification for fungal biomass quantification" (performed by Silke Jacques).
  - SNB disease scoring (which required a blind assessment by Huyen Phan).
  - Genome assemblies (provided by Danilo Pereira) used to extract/align *Tox1* promoter sequence information from a worldwide set of 146 *P. nodorum* isolates (provided by Daniel Croll).
- I undertook the data analyses and the compilation of all figures, tables and supplementary items with the exception of:
  - "Droplet digital PCR quantification for fungal biomass quantification, Fig 5" (performed by Silke Jacques).
  - The construction of the genetic maps (performed and published previously by Huyen Phan) which I used for "Association mapping and QTL analysis".
  - General editing support from Kar-Chun Tan.
- I wrote the manuscript with Kar-Chun Tan. Additional supervisory feedback and support was provided by Richard Oliver and Karam Singh. General editing/comments were provided by all other authors.

Any specific contributions not undertaken by me under supervision from Kar-Chun Tan, Richard Oliver and Karam Singh are made explicit or have been excluded from my thesis submission to Curtin University for the Doctor of Philosophy to which this statement will be attached.

Evan John [redacted]

I, as Co-Author, endorse that this level of contribution by the candidate indicated above is appropriate:

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Richard P. Oliver [redacted]

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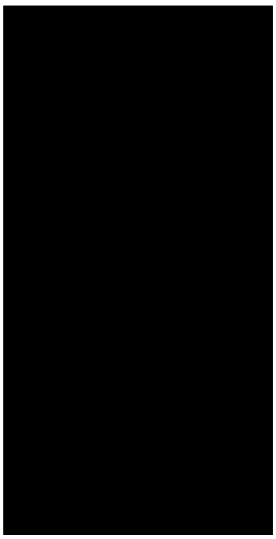

*Richard Oliver not available at time of submission; email evidence attached