Identification and functional characterization of putative (a)virulence factors in the fungal wheat pathogen *Zymoseptoria tritici*

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Chapter 1

General introduction and outline of the thesis

Host-microbe interactions

In all natural environments, plants are attacked by a wide variety of pathogens including viruses, bacteria, fungi, oomycetes and nematodes. To efficiently defend themselves against these threats, they have evolved sophisticated mechanisms to recognize and respond to pathogen attacks (Chisholm *et al*., 2006). The first line of defense against invading microorganisms is achieved by recognition of invariant molecular patterns that are commonly known as pathogen-associated molecular patterns (PAMPs) through pattern recognition receptors (PRRs) located on the surface of the plant cell that mediate PAMP-triggered immunity (PTI) and prevent further colonization of the host (Dodds and Rathjen, 2010; Jones and Dangl, 2006). One of the earliest PTI responses of plants after PAMP recognition is a rapid generation of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), which affects the protein and lipid content and quality of cells at high concentrations and functions as a signal molecule activating additional defense responses at low concentrations (Macho and Zipfel, 2014). In turn, fungal pathogens secrete proteins, referred to as effectors that suppress PTI resulting in host susceptibility, a phenomenon that is called effector-triggered susceptibility (ETS). Co-evolutionary interactions between plants and pathogens resulted in the development of resistance proteins encoded by resistance (*R*) genes in plants that recognize these effectors either directly or indirectly resulting in effector-triggered immunity (ETI), the second line of defense that is often accompanied by local cell death at the attempted site of infection, which is also known as the hypersensitive response (HR) (Dodds and Rathjen, 2010; Jones and Dangl, 2006). Typically, *R* gene-mediated host resistance against obligate biotrophic pathogens can be adequately explained by PTI, ETS and ETI, while interactions between apoplastic fungal pathogens and their host plants require expanded theoretical frameworks. Hence, Stotz et al. (2014) introduced effector-triggered defense (ETD), which is initiated by *R* genes encoding extracellular cell surface-localized receptor-like protein (RLPs) wherase ETI is triggered by *R* genes that encode intracellular nucleotide-binding leucine-rich repeat (NLR) receptors (Dangl *et al*., 2013). Moreover, ETI is associated with HR and ETD with fortifications of host cell walls, including cell wall appositions after a relatively long symptomless colonization phase (Stotz *et al*., 2014).

Fungal plant pathogens have developed diverse lifestyles in order to retrieve nutrients from invaded plant tissues. Biotrophic fungi require living cells to complete their lifecycle and deploy small-secreted proteins (effector proteins) to modulate plant defense systems and facilitate infection. For example, the biotrophic fungal tomato pathogen *Cladosporium fulvum* secretes multiple effector proteins, that facilitate the infection process (de Wit *et al.*, 2009; Mesarich *et al*., 2014; Stergiopoulos and de Wit, 2009). For some of the *C. fulvum* effectors the

matching Cf- protein receptors have been identified and the encoding *Cf* genes have been cloned and are therefore also called avirulence (Avr) proteins (Dixon *et al*., 1998; Jones *et al*., 1994; Thomas *et al*., 1997). Interactions between biotrophs and their host plants comply with the gene-for-gene (GFG) model in which an Avr protein from the pathogen interacts either directly or indirectly with the R protein from the host as demonstrated in various pathosystems (Flor, 1947; Chisholm *et al*., 2006; Joosten and de Wit, 1999).

In contrast, necrotrophic pathogens do not require living cells, but kill host cells for nutrition through the combined actions of cell wall-degrading enzymes (CWDEs) and toxins (Horbach *et al.*, 2011). It is extensively documented that necrotrophs generate phytotoxic metabolites and toxic peptides as virulence factors (Stergiopoulos *et al.*, 2013). For example, the fungal wheat pathogen *Parastagonospora nodorum* secretes a suite of toxic peptides or host selective toxins (HSTs) that induce necrosis on wheat genotypes harbouring the corresponding HST sensitivity genes (Oliver *et al.*, 2012), which fits the proposed inverse GFG (iGFG) (Friesen *et al*., 2008; Oliver *et al.*, 2012; Wolpert *et al.*, 2002).

Finally, hemibiotrophic fungi combine biotrophy and necrotrophy. They deploy effector proteins to suppress host recognition or programmed cell death (PCD) during early stages of infection. This initial biotrophic phase is followed by a rapid switch to necrotrophy that is associated with cell death and the manifestation of extensive necrotic lesions likely caused by the (over)production of toxic compounds such as HSTs or CWDEs that kill host tissue and provide the growing fungus with sufficient nutrients to complete its lifecycle. For instance, the hemibiotrophic rice blast fungus *Magnaporthe oryzae* secretes SLP1, a protein with LysM domains that is pivotal for supressing PTI initiated by the chitin fragments released from the fungal cell wall during early biotrophy (Mentlak *et al.*, 2012). However, the molecular mechanisms and signalling pathways that mediate the transition from biotrophy to necroptrophy are only partially understood, but many new studies employing advanced genetic and genomic technologies will contribute to a better understanding of the infection process(es) employed by hemibiotrophic fungal plant pathogens (Horbach *et al.*, 2011).

The *Zymoseptoria tritici*-wheat interaction

Z. tritici (Quaedvlieg *et al.*, 2011) previously known as *Mycosphaerella graminicola* is the causal agent of septoria tritici blotch (STB), one of the economically most important diseases of bread wheat (*Triticum aestivum*) and durum wheat (*T. durum*) threatening global food security. Under favourable environmental conditions for disease development, STB causes significant yield losses and also reduces grain quality (Eyal, 1999). Disease management is

generally obtained through fungicide application and breeding for resistance. Control of STB by applying fungicides is common practice, but has resulted in frequent development of fungicide-resistant strains, often cross-resistant to different types of fungicides, over the last decades (Cools and Fraaije, 2008; Fraaije *et al*., 2007). Due to environmental considerations and overall governmental regulations aiming at producing environmentally safe synthetic crop protection agents (Gullino and Kuijpers, 1994), development of new fungicides has become very expensive and less attractive for chemical companies. Fortunately, breeding for resistance has become increasingly successful, not the least through the identification of resistance genes in wild accessions and deploying them in commercial cultivars (McDowell and Woffenden, 2003). To date, eighteen resistance genes against STB (*Stb* genes) have been genetically mapped (Brown *et al.,* 2015), but the efficacy of the majority is of limited value under natural conditions at least partly due to the versatility of the organism with its active sexual cycle and enormous potential to overcome adverse environmental (fungicide resistance) and biological (host resistance) conditions (Cowger *et al.*, 2000; Wittenberg *et al.*, 2009). Only one resistance gene, *Stb16q,* has thus far not been overcome by the fungus (Tabib Ghaffary *et al*., 2012).

Although *Z. tritici* is generally considered as a typical hemibiotroph with two distinct invasion phases comprising both biotrophy and necrotrophy (Fig. 1), it was proposed that being a late necrotroph is a more appropriate term to describe its lifestyle (Hammond-Kosack and Rudd, 2008; Sánchez-Vallet *et al*., 2015). The initial phase of the infection process employed by *Z. tritici* is typical biotrophic. The fungus penetrates wheat leaves through stomata and subsequently colonizes the extracellular space surrounding the mesophyll cells without apparent major damage to host cells. After approximately 7-10 days, depending on the environmental conditions, a sudden switch follows this stealth and symptomless phase to necrotrophic growth coinciding with the manifestation of macroscopically visible chlorotic lesions that eventually coalesce into larger necrotic blotches bearing numerous pycnidia, the asexual fructifications that contain the pycnidiospores that can easily be released by a splash-borne mechanism and re-infect healthy leaves of neighbouring plants (Duncan and Howard, 2000; Kema *et al.*, 1996c).

Although in recent years substantial progress has been made in the development of new genetic tools for deciphering the *Z. tritici*-wheat pathosystem (Kilaru and Steinberg, 2015; Mehrabi *et al.*, 2015; Sidhu *et al.*, 2015), the molecular mechanisms underlying the host-pathogen interaction are not well understood and require more in depth studies. In order to successfully establish infection, *Z. tritici* must overcome early host defense responses, including circumventing or neutralizing the host surveillance system and the generation of host-derived H_2O_2. For instance, *Z. tritici* secretes an effector protein designated as Mg3LysM at the early

stage of colonization that is pivotal in preventing host recognition by scavenging chitin fragments released from fungal cell walls as demonstrated by the attenuated phenotypes of *ΔMg3LysM* strains (Marshall *et al.*, 2011). The silencing of either *CERK1* or *CEBiP*, plasma membrane receptors in wheat involved in chitin perception, restored the pathogenicity of the *ΔMg3LysM* strains (Lee *et al*., 2014). Additionally, it was shown that pretreating susceptible wheat cultivars with purified β-1, 3-glucan fragments from *Z. tritici* cell walls acted as PAMPs and provided complete resistance against *Z. tritici* through PTI activation, including callose deposition and the up-regulation of genes encoding β-1, 3-glucanases (Shetty *et al.*, 2009).

The role of H_2O_2 in the *Z. tritici*-wheat interaction was investigated by infiltrating H_2O_2 into wheat leaves prior to challenging them with *Z. tritici*, which reduced fungal colonization and biomass production and extended the latent period rendering plants more resistant (Shetty *et al*., 2007). The current hypothesis is that H_2O_2 is harmful to *Z. tritici* throughout its lifecycle, but that the fungus can cope with it at various phases of pathogenesis, particullarly at the transition from biotrophy to necrotrophy, which is a crucial phase for *Z. tritici* to counreact the massive accumulation of H_2O_2 that is geneated by the host (Shetty *et al.*, 2003; Shetty *et al.*, 2007). These preliminary data called for a functional analysis of genes involved in H_2O_2 modulation by *Z. tritici* (Shetty *et al.*, 2003; Shetty *et al.*, 2007; Yang *et al.*, 2013).

Also, it remains unclear how *Z. tritici* acquires nutrients upon landing and germinating on the plant surface and during the symptomless biotrophic growth in the intercellular space of wheat leaves. However, recent RNA-seq data indicated that genes involved in the beta oxidation of fatty acids and lipids are specifically up-regulated at one day post inoculation (dpi), suggesting that the enzymes attacking these organic molecules might provide the primary source of nutrients during this early stage of infection, which may explain the limited increase in biomass during the biotrophic phase (Rudd *et al.*, 2015). The large number of proteases expressed by *Z. tritici* during the biotrophic growth phase supports this hypothesis and suggests that *Z. tritici* obtains the most required nutrients through protease processing of host proteins located in the apoplast or cell wall, rather than by processing of carbohydrates in this environment (Goodwin *et al.*, 2011). Some of these expressed proteases might also target host defense enzymes, including chitinases and eventually contribute to virulence of *Z. tritici* in a similar way as shown for the *Fusarium oxysporum* f.sp. *lycopersici*-tomato interaction (Karimi Jashni *et al*., 2015). Comparative genome analyses of *Z. tritici* to other sequenced plant pathogens showed the strikingly reduced number of genes encoding plant cell wall-degrading enzymes (CWDEs) in the *Z. tritici* genome. CWDE expression profiling revealed that they are differentially expressed throughout the *Z. tritici* lifecycle. Typically, the CWDEs of *Z. tritici* are

produced during the asymptomatic phase at low concentration in order to avoid recognition through the plant surveillance system and probably are involved in degrading plant cell wall componnets to release nutrients without causing significatnt damage to the plant cell (Brunner *et al.*, 2013; Goodwin *et al.*, 2011; Rudd *et al.*, 2015). Early chloroplast deformations in wheat mesophyll cells without apparent physical contact also suggests an active role of diffusible toxic compounds secreted by this fungus (Kema *et al.*, 1996c), but the identification and biological function of these toxic compounds still awaits elucidation.

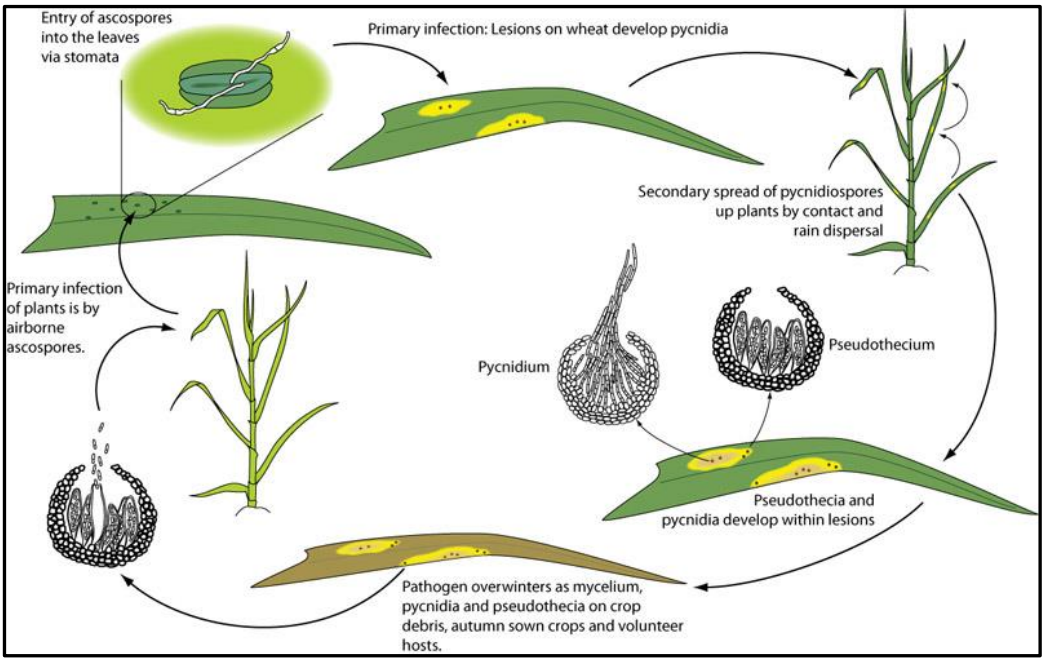

Figure 1. Life cycle of the fungal wheat pathogen *Zymoseptoria tritici* (Ponomarenko *et al.*, 2011).

Z. tritici, like necrotrophic pathogens, probably produces toxins or small-secreted proteins (SSPs) triggering a necrotrophic phase that facilitates the completion of the infection process (Kema *et al.*, 1996c). Several genes encoding putative effector proteins are specifically expressed during the necrotrophic phase of infection. However, deletion of top candidate effector genes, highly expressed during this phase, could not confirm that they are required for virulence (Rudd *et al.*, 2015; this thesis).

Although transition from the biotrophic to necrotrophic phases plays a key role in the pathogenesis and initiates the destructive phase of the *Z. tritici* infection process, the molecular basis underlying this developmental switch is poorly understood. Ultrastructural studies revealed that this switch has features reminiscent of programmed cell death (PCD) coinciding with a loss of cell-membrane integrity, dramatic increases in apoplastic metabolites and a sharp increase in fungal biomass in infected leaves (Keon *et al.*, 2007). However, Rudd et al. (2008) demonstrated

that resistance in the *Z. tritici*-wheat pathosystem is not associated with PCD, which is an effective resistance mechanism toward biotrophic fungi. More recently, the role of functional chloroplasts in regulating host PCD was investigated through virus-induced gene silencing (VIGS). Two central genes in the carotenoid and chlorophyll biosynthesis, *PDS* and *ChlH*, were silenced in leaves of the susceptible wheat cv. Riband that were subsequently inoculated with *Z. tritici*. This study showed that the silenced leaves underwent more rapid PCD triggered by *Z. tritici* compared to the non-silenced leaves but silenced ones were remarkably less able to support the subsequent asexual proliferation of *Z. tritici.* This indicates that chloroplasts are important for temporally regulating host PCD, which occurs prior to the initiation of necrotrophic growth (Lee *et al*., 2015). Therefore, Hammond-Kosack and Rudd (2008), suggested that *Z. tritici* exploits host defense responses such as PCD, which are commonly deployed against biotrophs, to facilitate the overall infection process.

(A)virulence factors of *Zymoseptoria tritici*

Specificity in the *Z. tritici*-wheat interactions has been previously reported in several studies (Brading *et al.*, 2002; Kema *et al.*, 1996a; Kema *et al.*, 1996b; Kema and van Silfhout, 1997; Kema *et al.*, 2000) indicating that this pathosystem complies with a GFG model. Map-based cloning of the first putative *Avr* gene in *Z. tritici* started with genetic analyses and phenotyping of a range of segregation populations, including the standard mapping population originating from a cross between reference isolate IPO323 (avirulent) and *Z. tritici* IPO94269 (virulent) (Kema *et al.*, 2000). This resulted in several quantitative trait loci (QTL) involved in induction of necrosis and formation of pycnidia (Ware, 2006). Mapping these QTLs on the *Z. tritici* genome demonstrated that a particular region of chromosome 5 covered putative *Avr* genes that are responsible for cultivar and host specificity (this thesis). However, bioinformatic analyses to prioritize candidate *Avr* genes for subsequent functional characterization including *in planta* expression profiling failed to identify the genes controlling specificity in the *Z. tritici*-wheat pathosystem (Rudd *et al.*, 2015; this thesis). Alternative routes for gene discovery, including analyses of crude and partly purified culture filtrates of *Z. tritici* using fast protein liquid chromatography (FLPC) followed by mass spectrometry, resulted in the identification of two novel SSPs that differentially induced necrosis in a range of wheat cultivars (Ben M'Barek *et al*., 2015).

Virulence factors of *Zymoseptoria tritici*

Before the release of the reference *Z. tritici* genome of the IPO323 isolate, several

pathogenicity and virulence genes, mostly encoding conserved signaling proteins, had been functionality characterized using gene replacement strategies (Cousin *et al.*, 2006; Mehrabi and Kema, 2006; Mehrabi *et al.*, 2006a; Mehrabi *et al.*, 2006b). For example, *MgHog1* encodes a mitogen-activated protein (MAP) kinase that regulates transition from yeast-like to filamentous growth of *Z. tritici*; hence disruption of this gene disabled the pathogen to penetrate its host plant (Mehrabi *et al.*, 2006b). *MgSlt2* encodes another MAPK that is required for colonization and fungal cell wall integrity (Mehrabi *et al.*, 2006a). Up to now, 20 pathogenicity or virulence genes have been described in *Z. tritici* (Table 1) that mostly play a role in cell signaling pathways or function as global metabolic regulators. Compared with other fungal pathogens such as *Magnaporthe oryzae*, the number of identified virulence factors in *Z. tritici* is relatively low (Rudd, 2015). However, the recent development of new genetic tools will accelerate functional analyses of genes in *Z. tritici* (Kilaru and Steinberg, 2015; Mehrabi *et al.*, 2015; Sidhu *et al.*, 2015).

Scope of thesis

The research presented in this PhD thesis aims at understanding the biological functions of (a)virulence factors playing an essential role in the infection process of wheat by *Z. tritici*.

Chapter 1 describes lifestyle features of *Z. tritici* with emphasis on events occurring during the biotrophic and necrotrophic stages of plant infection. In addition, a brief overview of (a)virulence factors of *Z. tritici* is presented and discussed.

Chapter 2 describes the generation of 22 entry constructs that form a new molecular toolbox based on gateway technology facilitating the rapid construction of binary vectors for fungal transformations.

Chapter 3 describes the functional characterization of *ZtWor1*, a transcriptional regulator of *Z. tritici*. The biological role of *ZtWor1* in transcriptionally regulating *Z. tritici* genes encoding small-secreted proteins (SSPs) is studied and shows that this gene is essential for *Z. tritici* pathogenicity.

Chapter 4 describes methods to identify and map candidate SSPs in *Z. tritici* through combined bioinformatics and map-based cloning approaches to prioritize putative *ZtSSPs* to be studied. Subsequent functional analyses, however, showed that despite the overall merit of this approach, two top candidate *ZtSSPs* appeared dispensable for virulence, questioning the method of candidate effector discovery as opposed to unbiased map-based cloning strategies to identify effector genes from this fungal pathogen.

Table 1. Functionally characterized pathogenicity/virulence genes of *Zymoseptoria tritici* (adapted from (Rudd, 2015)).

Gene name	Gene category	Reference
MgAtr4	ABC Transporter	(Stergiopoulos *et al.*, 2003)
MgFus3	Mitogen-activated protein kinase (MAPK)	(Cousin *et al.*, 2006)
MgSlt2	MAPK	(Mehrabi *et al.*, 2006a)
MgHog1	MAPK	(Mehrabi *et al.*, 2006b)
MgSTE11	MAPK kinase kinase	(Kramer *et al.*, 2009)
MgSTE50	Scaffold protein for MAPK signaling	(Kramer *et al.*, 2009)
MgSTE12	Transcription factor target of MAPK signaling	(Kramer *et al.*, 2009)
MgSTE7	MAPK kinase	(Kramer *et al.*, 2009)
MgAlg2	Protein N-glycosylation	(Motteram *et al.*, 2011)
MgGpa1	G-protein alpha subunit	(Mehrabi *et al.*, 2009)
MgGpa3	G-protein alpha subunit	(Mehrabi *et al.*, 2009)
MgGpb1	G-protein beta subunit	(Mehrabi *et al.*, 2009)
MgTpk2	Protein kinase A catalytic subunit	(Mehrabi and Kema, 2006)
MgBcy1	Protein kinase A regulatory subunit	(Mehrabi and Kema, 2006)
Mg3LysM	Chitin binding effector protein	(Marshall *et al.*, 2011)
MCC1	c-type cyclin	(Choi and Goodwin, 2011)
ZtWor1	Transcription factor	(Mirzadi Gohari *et al.*, 2014)
Zt80707	Unknown/Secreted	(Poppe *et al.*, 2015)
Zt89160	Unknown	(Poppe *et al.*, 2015)
Zt103264	Unknown	(Poppe *et al.*, 2015)

Chapter 5 describes the functional characterization of *ZtCpx1* and *ZtCpx2* encoding extracellular and intracellular catalase-peroxidases, respectively. Our results show that, albeit temporally and differentially regulated, both genes contribute to protecting *Z. tritici* against host-derived H_2O_2 and hence, to virulence.

Chapter 6 discusses the experimental data obtained in the previous chapters and puts them in a broader context. In a summarizing discussion I highlight the importance of the identified virulence factors and how they have increased our understanding of the *Z. tritici* infection process. Suggestions for future directions in research on this pathosystem will be discussed.

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Chapter 2

Flexible gateway constructs for functional analyses of genes in plant pathogenic fungi

Mehrabi*, R., Mirzadi Gohari*, A., da Silva, G.F., Steinberg, G., Kema, G.H.J. and de Wit, P.J.G.M. (2015) Flexible gateway constructs for functional analyses of genes in plant pathogenic fungi. *Fungal Genetics and Biology* **79**, 186-192.

***The first and the second author contributed equally to this work.**

Summary

Genetic manipulation of fungi requires quick, low-cost, efficient, high-throughput and molecular tools. In this paper, we report 22 entry constructs as new molecular tools based on the gateway technology facilitating rapid construction of binary vectors that can be used for functional analysis of genes in fungi. The entry vectors for single, double or triple gene-deletion mutants were developed using hygromycin, geneticin and nourseothricin resistance genes as selection markers. Furthermore, entry vectors containing green fluorescent (GFP) or red fluorescent (RFP) in combination with hygromycin, geneticin or nourseothricin selection markers were generated. The latter vectors provide the possibility of gene deletion and simultaneous labeling of the fungal transformants with GFP or RFP reporter genes. The applicability of a number of entry vectors was validated in *Zymoseptoria tritici*, an important fungal wheat pathogen.

Introduction

Filamentous fungi are diverse eukaryotic microorganisms that are important for various reasons in industry, medicine, agriculture, and basic sciences. Many of them are important plant pathogens and cause severe losses in agricultural production. A wide range of filamentous fungi is used in industry for production of commercially valuable proteins and metabolites that are of considerable interest to market. Some of the filamentous fungi like *Aspergillus nidulans* and *Neurospora crassa* are among the first-rate model organisms and have been widely used in fundamental research. The genomes of many filamentous fungi, including plant pathogenic fungi, have been sequenced and are publicly available which opens tremendous possibilities for future functional research of genes and their roles in pathogenesis (Marthey *et al.*, 2008). In addition, advances in genome annotation as well as comparative genomics have revealed an ever-increasing number of interesting and novel genes that require high throughput functional tools for analysis. To date a number of genetics tools has been developed intending to lower the cost of such analyses, addressing biological questions. This requires the construction of vectors to generate knock-out strains, overexpression strains and fluorescently labelled strains to analyse and monitor the function of genes in different biological processes. However, the construction of vectors for fungal transformation has always been an important obstacle slowing down the efficiency of functional analyses. Generating constructs using traditional

approaches like digestion/ligation is labour intensive, time-consuming, relatively expensive as it requires several cloning steps. Hence, recently several studies have been conducted to improve or develop new genetic tools for large-scale functional analyses (Abe *et al.*, 2006; Nakagawa *et al.*, 2007; Shafran *et al.*, 2008; Zhu *et al.*, 2009; Paz *et al.*, 2010). Among these, the gateway® cloning technology has attracted molecular biologists from different disciplines due to its amenability and robustness (Schoberle *et al.*, 2013). To date, a few methods or constructs have been developed using this technology for the functional analyses of genes in plant pathogenic fungi (Abe *et al.*, 2006; Nakagawa *et al.*, 2007; Shafran *et al.*, 2008; Zhu *et al.*, 2009; Paz *et al.*, 2010). For instance, the One Step Construction of Agrobacterium-Recombination-ready-plasmids (OSCAR) has been developed to create deletion constructs for *Agrobacterium tumefaciens* mediated transformation (Paz *et al.*, 2010). Two gateway vectors, pCBGW and pGWBF, were generated for expression of genes under control of the P*gpdA* promoter and T*trpC* terminator (Zhu *et al.*, 2009). The gateway RNAi vector was also developed allowing gene silencing in a high-throughput manner (Shafran *et al.*, 2008).

These data indeed confirm the enormous potential of the gateway cloning strategy and, therefore, new gateway constructs for different purposes need to be developed. We have generated and described 22 entry vectors based on the gateway three-fragment vector methodology. They represent a user-friendly tool in the demanding field of molecular biology and will accelerate progress in the functional analyses of genes in plant pathogenic fungi. As an example, the application of a number of entry vectors was validated through the transformation of *Zymoseptoria tritici,* the septoria leaf blotch pathogen that is among the most destructive foliar blights in global wheat production.

Results and discussion

Description of method

To understand the function of genes in plant pathogenic fungi and their roles in biology and disease establishment, robust and feasible functional genomics tools are required. To date a number of molecular tools for genetic manipulation in fungi have been described (Catlett *et al.*, 2003; Abe *et al.*, 2006; Geu-Flores *et al.*, 2007; Frandsen *et al.*, 2008; García-Pedrajas *et al.*, 2008; Shafran *et al.*, 2008; Paz *et al.*, 2010). However, development of high-throughput approaches is still one of the

challenges for the functional genomics in filamentous fungi. One of the main limiting factors is the generation of constructs with different selection markers for fungal transformation. The process of vector construction through general digestion/ligation procedures is laborious, time-consuming and inefficient. Moreover, in some cases the vectors are incompatible with multiple-cloning sites for the cloning of foreign genes (Zhu *et al.*, 2009). The gateway® recombination cloning technology, invented and commercialized by Invitrogen since the late 1990s, circumvents traditional restriction enzyme-based cloning limitations, enabling users to generate appropriate constructs regardless of DNA sequences to be cloned in just a few simple steps. Here, we describe new molecular tools based on Invitrogen's gateway technology facilitating the rapid construction of various vectors that can be used for the functional analyses of fungal genes. We have generated a number of entry vectors that can be potentially used for gene deletion, overexpression, generation of GFP- or RFP-labelled transformants and double or triple gene deletions (Fig. 1).

Entry vector for gene deletion, complementation and overexpression

One of the most important and frequently used approaches to determine gene function is gene deletion (Zhu *et al.*, 2009). A general scheme of gene deletion constructs consists of a selection marker flanked by upstream and downstream sequences of the targeted gene. In our system, upstream and downstream stretches of the gene of interest can be cloned by BP reaction in pDONRTM-P4-P1R and pDONRTM-P2R-P3, respectively. Several new entry vectors derived from pDONRTM-221 have been developed enabling the selection of entry vectors containing one of the three selection markers hygromycin (pRM250), geneticin (pRM251) and nourseothricin (pRM249). Additionally, the complementation of deleted genes is crucial to ascertain that the obtained phenotypes are the consequence of the deletion of the targeted gene. Once the given gene is deleted using a selection marker such as Hph, another selection marker should be used for complementation as it was shown for the functional analysis of *ZtWor1* (Mirzadi Gohari *et al*., 2014). To generate a fungal transformation construct, the entry vector containing upstream sequences of gene of interest, one of the entry vectors containing the selection marker and the entry vector containing downstream sequence of gene of interest is subjected to the LR reaction against the destination vector (pPm43GW) generating the required fungal transformation construct. We have successfully used this quick approach to

delete more than eight genes in *Z. tritici* (data not shown). It is worth noting that these three selection markers allow the construction of making double or triple gene deletion mutants.

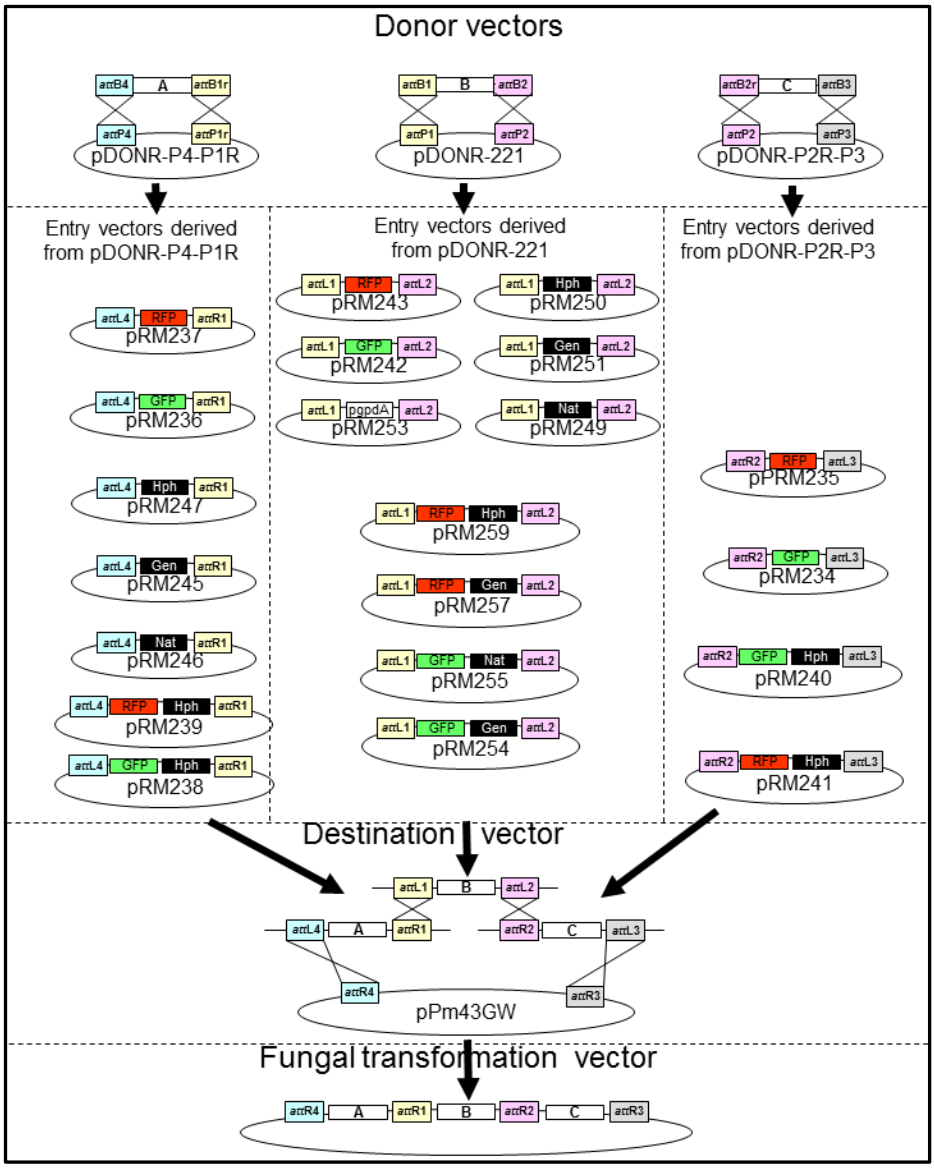

Figure 1. A schematic overview of the gateway cloning strategy. The entry vector developed in this study is derived from the donor vectors, pDONR^{TM}-P4-P1R, pDONR^{TM}-221 and pDONR^{TM}-P2R-P3. To generate a fungal transformation vector, three entry vectors can be selected for different purposes to recombine the three fragments into the binary destination vector, pPm43GW. Specifications of the entry vectors are described in materials and methods.

Furthermore, we have developed entry vectors containing RFP in combination with the hygromycin (pRM259), or geneticin (pRM257) and GFP with geneticin (pRM254) and nourseothricin (pRM255) selection markers. This provides the

possibility of gene deletion as well as simultaneous labeling of fungal transformants with GFP or RFP for high level microscopical monitoring. Heterologous overexpression of the desired genes is an alternative powerful tool to identify pathway components that might remain undetected using traditional loss-of-function analysis (Prelich, 2012). We generated an entry vector (pRM253) derived from pDONR^{TM}-221 containing the strong, constitutive fungal promoter P*gpdA* (Frandsen *et al.*, 2008). The gene of interest can be inserted into pDONR^{TM}-P2R-P3 by the BP reaction. Hygromycin (pRM247), geneticin (pRM245) or nourseothricin (pRM246) selection markers can be selected from the derivative pDONR^{TM}-P4-P1R entry vectors. In addition, the GFP-Hph and the RFP-Hph cassettes presented in entry vectors pRM238 and pRM239 allow labeling the resulting fungal transformants with GFP or RFP. Similarly, pRM240 and pRM241 derived from pDONR^{TM}-P2R-P3 might be used for labeling of fungal transformants with GFP or RFP, respectively.

Vector validation by examining the expression of GFP and RFP reporter genes

To validate the applicability of entry vectors developed in this study, a number of fungal transformation constructs was generated and used for *Z. tritici* transformation. We used GFP and RFP as the reporter genes and the resulting fungal transformants were examined using fluorescence microscopy. The first FT vector, pFT1 (Fig. 2A') was developed to express GFP. After transformation, the fungal colonies were selected on hygromycin containing medium and the resulting hygromycin resistant transformants were screened for GFP expression. All the transformants expressed GFP as shown in Fig. 3, indicating that the GFP-expressing vector was functionally active. In the same way, the pFT2 vector (Fig. 2B') was generated containing the RFP reporter gene and the hygromycin selection marker. Again, *Z. tritici* IPO323 strain was subjected to transformation and the resulting transformants were examined by fluorescence microscopy. All transformants expressed RFP indicating that the vector was functional (Fig. 3). In order to validate the geneticin selection marker, pFT3 and pFT4 were generated to express GFP and RFP, respectively. Both constructs were used to transform *Z. tritici* strains deleted for *Gpb1* (Mehrabi *et al.*, 2009) and *Wor1* (Mirzadi Gohari *et al.*, 2014). These gene deletion mutants have been previously generated using the hygromycin as a selection marker.

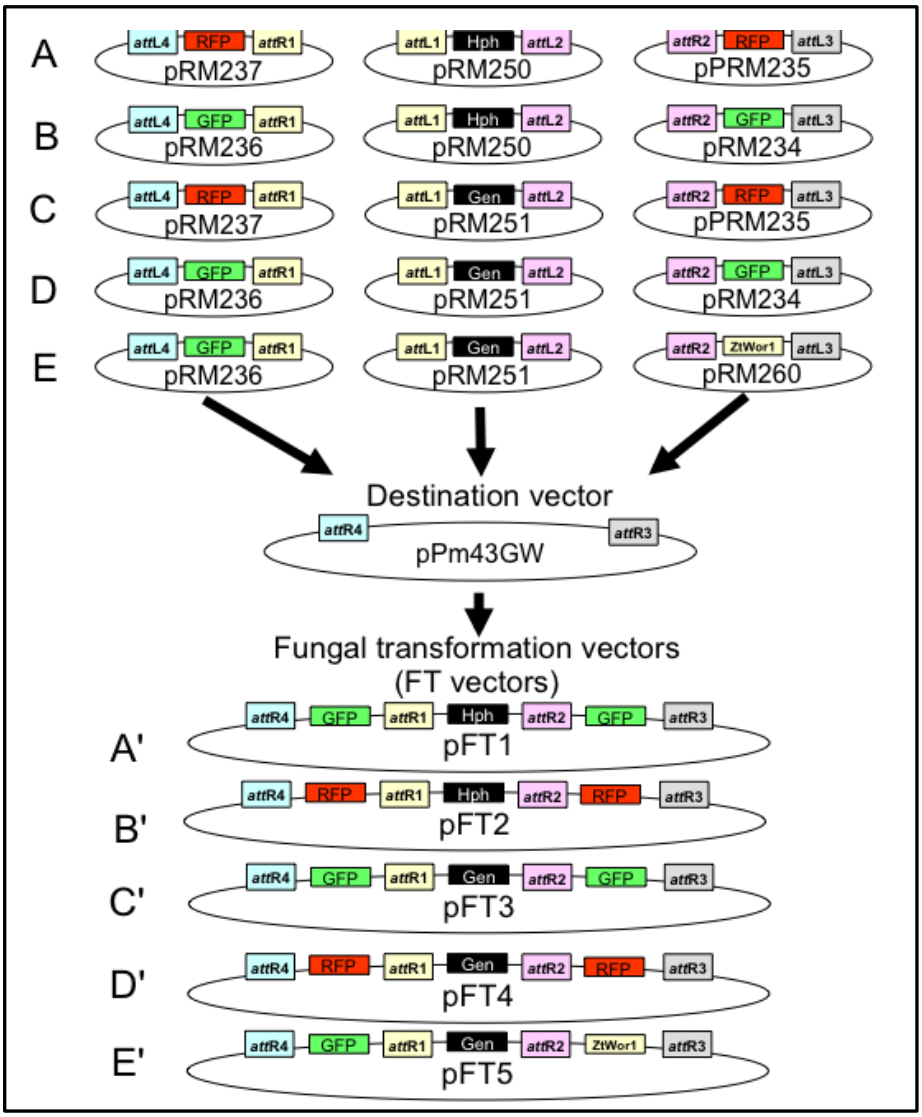

Figure 2. Construction of fungal transformation vectors (FT vectors). To generate each FT vector, three entry vectors were used to recombine the three fragments into the binary destination vector, pPm43GW, generating pFT1 (A'), pFT2 (B'), pFT3 (C'), pFT4 (D') and pFT5 (E').

After transformation, selection was performed on media containing geneticin. Fungal colonies generated using pFT3 and pFT4 did express GFP and RFP reporter genes, respectively, indicating that both vectors were functional in both fungal strains. Furthermore, a complementation construct (pFT5) was generated in which full-length *ZtWor1* was cloned into pDONR^{TM}-P2R-P3 (pRM260) in combination with the GFP expressing entry vector (pRM236) as well as the geneticin selection entry vector (pRM251). The pFT5 was generated by LR reaction of these vectors to incorporate three DNA fragments into pPm43GW followed by transformation of *Z. tritici* strains

mutated for *ZtWor1*. The results show that pFT5 was functional as the transformants could be selected on geneticin-supplemented media, expressed GFP and could complement the *ZtWor1* phenotypes (Mirzadi Gohari *et al.*, 2014).

Conclusions

In conclusion, we have confirmed the elegance of the gateway technology for the high-throughput generation of vectors destined for functional analyses of virtually any fungal gene. The method was validated using *Z. tritici* as a model and applying four entry vectors with two different antibiotics selection markers as well as two fluorescence markers (GFP and RFP). We showed that the technology advances the efficiency of gene cloning, which is a crucial step in the functional analysis of candidate genes in fungal biology.

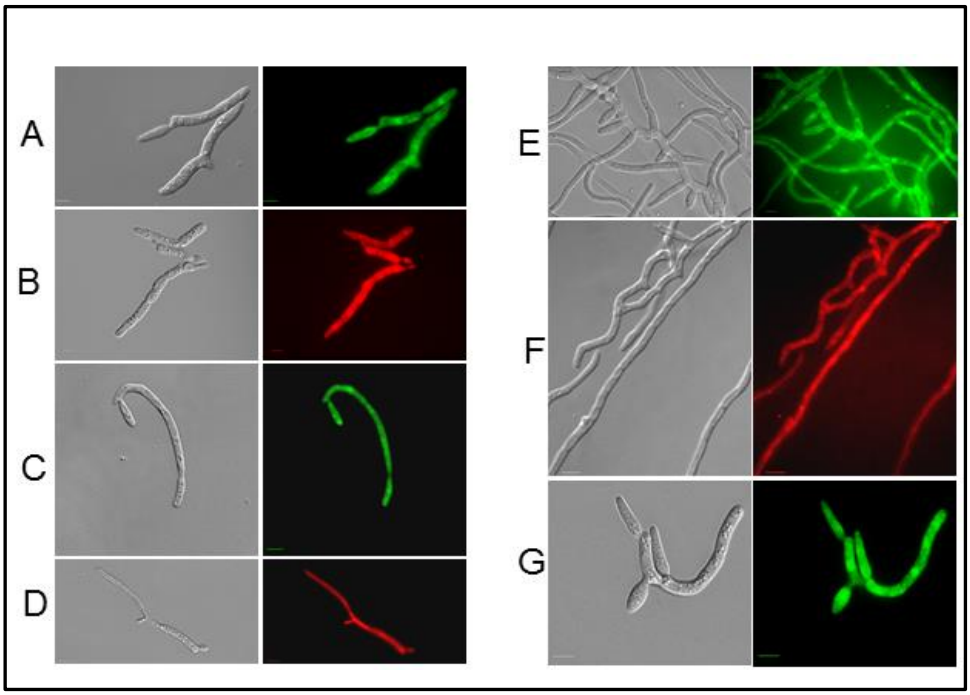

Figure 3. Vector validation by examining the expression of GFP and RFP reporter genes in *Zymoseptoria tritici* strains. Left and right panels show light microscopic and fluorescence microscopic images, respectively. (A) *Z. tritici* wild-type strain (IPO323) transformed by the pFT1 vector expressing GFP. (B) *Z. tritici* wild-type strain (IPO323) transformed by the pFT2 vector expressing RFP. (C) *Z. tritici Gpb1* mutant transformed by the pFT3 vector expressing GFP. (D) *Z. tritici Gpb1* mutant transformed by the pFT4 vector expressing RFP. (E) *Z. tritici Wor1* mutant transformed by the pFT3 vector expressing GFP. (F) *Z. tritici Wor1* mutant

transformed by the pFT4 vector expressing RFP. (G) *Z. tritici Wor1* mutant transformed by complementation the pFT5 vector expressing GFP. Scale bars, 5 μm.

Experimental Procedures

Bacterial, fungal strains and growth conditions

Z. tritici IPO323 (Goodwin *et al.*, 2011) was used throughout this study. The fungus was grown in YGM (1% yeast extract, 3% glucose) in an orbital shaker (Innova 4430; New Brunswick Scientific, Nijmegen, The Netherlands) at 18 ºC for five days to produce yeast-like spores, which were collected by centrifugation and subsequently used for fungal transformation or stored at –80 ºC (Kema and van Silfhout, 1997). *Escherichia coli* DH5α was used for general plasmid transformation. *E. coli* was grown in or on Luria Bertani (LB) medium amended with appropriate antibiotics. *E. coli* DB3.1 (Invitrogen) was used for propagation of plasmids containing the ccdB gene that is lethal for most *E. coli* strains. *Agrobacterium tumefaciens* strain AGL-1 was used for all fungal transformations.

DNA manipulation and analysis

Basic DNA manipulations were according to standard protocols (Sambrook *et al.*, 2001). DNAs were purified using QIA quick PCR Purification. PCR products were extracted from agarose gels and purified using the Illustra GFX PCR DNA and Gel Band Purification kit (GE Healthcare, Life Sciences). Plasmid DNA was isolated using the plasmid Prep Purification Mini Spin Kit (GE Healthcare, Life Sciences). Fungal genomic DNA of *Z. tritici* IPO323 was prepared from freeze-dried spores using the DNeasy Plant kit (Qiagen). DNA sequences were obtained on an ABI-prism 3100 capillary automated sequencer using the Amerdye terminator reaction mix (GE Healthcare). Primers used in this study are listed in Table 1.

Construction of entry vectors

The donor vectors, pDONR^{TM}-P4-P1R, pDONR^{TM}-221, pDONR^{TM}-P2R-P3, were used as the backbone to construct the gateway entry vectors (Invitrogen) (Fig. 1). To construct the entry vectors, BP reactions were performed to clone DNA fragments into donor vectors according to the manufactures instructions (Invitrogen). The promoter, pgpdA, was amplified from plasmid pRF-HU2E (Frandsen *et al.*,

2008) using primer pair GW-pgpdA-F1/GW-pgpdA-R1 and inserted into pDONR^{TM}-221 generating pRM253.

Table 1. Primers used in this study.

Primer name	Primer sequence 5'-3'
Gen-GRFP-F1	TCCTATTCCGAAGTTCCTATTCTCTCAGTTAACGTCGACGGTATCGATT
Gen-R1	AGAGAATAGGAACTTCGGAATAGGA
Nat-GRFP-F1	AGCTTGGCACTGGCCGTCGTTTTACAGTTAACGTCGACGGTATCGATT
Nat-R1	AAAACGACGGCCAGTGCCAAG
GW-Gen-R1	GGGGACCACTTTGTACAAGAAAGCTGGGTAGAGAATAGGAACTTCGGAATAGGA
GW-Gen-F1	GGGGACAAGTTTGTACAAAAAAGCAGGCTACGCTTACAATTTCCATTCGCCAT
GW-PgpdA-F1	GGGGACAAGTTTGTACAAAAAAGCAGGCTGGGTGATGTCTGCTCAAGCGG
GW-PgpdA-R1	GGGGACCACTTTGTACAAGAAAGCTGGGTGAATTCCCTTGTATCTCTACACAC
GW-GRFP-F1	GGGGACAAGTTTGTACAAAAAAGCAGGCTCAGTTAACGTCGACGGTATCGATT
GW-GRFP-R1	GGGGACCACTTTGTACAAGAAAGCTGGGTCGCGCAATTAACCCTCACTAAAG
GW-hph-F1	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGACAGAAGATGATATTGAAGGAG
GW-hph-R1	GGGGACCACTTTGTACAAGAAAGCTGGGTAAGAAGGATTACCTCTAAACAAGTGT
GW-Nat-R1	GGGGACCACTTTGTACAAGAAAGCTGGGTTAAAAACGACGGCCAGTGCCAAG
GW-Nat-F1	GGGGACAAGTTTGTACAAAAAAGCAGGCTCGGTACCCGGGGATCCTCTA
Hph-P4-F	GGGGACAACTTTGTATAGAAAAGTTGTCGACAGAAGATGATATTGAAGGAG
Hph-P4-R	GGGGACTGCTTTTTTGTACAAACTTGAAGAAGGATTACCTCTAAACAAGTGT
Gen-P4-F	GGGGACAACTTTGTATAGAAAAGTTGACGCTTACAATTTCCATTCGCCAT
Gen-P4-R	GGGGACTGCTTTTTTGTACAAACTTGAGAGAATAGGAACTTCGGAATAGGA
Nat-P4-F	GGGGACAACTTTGTATAGAAAAGTTGCGGTACCCGGGGATCCTCTA
Nat-P4-R	GGGGACTGCTTTTTTGTACAAACTTGTAAAAACGACGGCCAGTGCCAAG
GRFP-P4-F	GGGGACAACTTTGTATAGAAAAGTTGCAGTTAACGTCGACGGTATCGATT
GRFP-P4- R	GGGGACTGCTTTTTTGTACAAACTTGTGGAGCTCCACCGCGGTGGC
GRFP-P2-F	GGGGACAGCTTTCTTGTACAAAGTGGCAGTTAACGTCGACGGTATCGATT
GRFP-P2-R	GGGGACAACTTTGTATAATAAAGTTGTGGAGCTCCACCGCGGTGGC
GRFP-221-R	GGGGACCACTTTGTACAAGAAAGCTGGGTTGGAGCTCCACCGCGGTGGC
GRFP-221-F	GGGGACAAGTTTGTACAAAAAAGCAGGCTGGTACCGGGCCCCCCCTCGA
Hph-GRFP-P4-F	GGGGACAACTTTGTATAGAAAAGTTGGGTACCGGGCCCCCCCTCGA
Hph-GRFP-P4-R	GGGGACTGCTTTTTTGTACAAACTTGTGGAGCTCCACCGCGGTGGC
GRFP-P2-F	GGGGACAGCTTTCTTGTACAAAGTGGGGTACCGGGCCCCCCCTCGA
GRFP-P2-R	GGGGACAACTTTGTATAATAAAGTTGTGGAGCTCCACCGCGGTGGC

The hygromycin phosphotransferase gene (*Hph*) was amplified from pRF-HU2 (Frandsen *et al.*, 2008) using primer pairs GW-hph-F1/GW-hph-R1 and Hph-P4-F/Hph-P4-R. The resulting PCR products were inserted into pDONR^{TM}-221 and pDONR^{TM}-P4-P1R, generating pRM250 and pRM247, respectively. The geneticin resistance gene (neomycin phosphotransferase) was amplified from pSM334 (Hou *et*

al., 2002) using primer pairs GW-Gen-F1/GW-Gen-R1 and Gen-P4-F/Gen-P4-R, and inserted into pDONR^{TM}-221 and pDONR^{TM}-P4-P1R, generating pRM251 and pRM245, respectively. The nourseothricin resistance gene (*Nat*) was amplified from pNR3 (Zhang *et al.*, 2011) using primer pairs GW-Nat-F1/GW-Nat-R1 and Nat-P4-F/Nat-P4-R inserted into pDONR^{TM}-221 and pDONR^{TM}-P4-P1R, generating pRM249 and pRM246, respectively. To construct entry vectors containing GFP, pSC001 (Armesto *et al.*, 2012) was used as a template to amplify GFP using primer pairs GW-GRFP-F1/GW-GRFP-R1, GRFP-P4-F/GRFP-P4-R and GRFP-P2-F/GRFP-P2-R. The resulting PCR amplicons were inserted into pDONR^{TM}-221, pDONR^{TM}-P4-P1R, and pDONR^{TM}-P2R-P3, generating pRM242, pRM236 and pRM234, respectively. The RFP cassette was amplified from pSC002 using primer pairs GW-GRFP-F1/GW-GRFP-R1, GRFP-P4-F/GRFP-P4-R and GRFP-P2-F/GRFP-P-R and inserted into pDONR^{TM}-221, pDONR^{TM}-P4-P1R and pDONR^{TM}-P2R-P3 generating pRM243, pRM237 and pRM235, respectively.

To construct entry vectors containing the Hph-GFP cassette, pSC001 was used as template to amplify the Hph-GFP cassette using primer pairs Hph-GRFP-P4-F/Hph-GRFP-P4-R and GRFP-P2-F/GRFP-P2-R. The resulting PCR amplicons were inserted into pDONR^{TM}-P4-P1R and pDONR^{TM}-P2R-P3 generating pRM238 and pRM240, respectively. Likewise, to construct entry vectors containing Hph-RFP, pSC002 was used as template in PCR reactions along with primer pairs Hph-GRFP-P4-F/Hph-GRFP-P4-R, GRFP-P2-F/GRFP-P2-R and GRFP-221-F/GRFP-221-R to amplify the Hph-RFP cassette. The resulting PCR products were inserted into pDONR^{TM}-P4-P1R, pDONR^{TM}-P2R-P3 and pDONR^{TM}-221, generating pRM239, pRM241 and pRM259, respectively.

To generate the geneticin-GFP cassette (Gen-GFP), geneticin and GFP fragments were amplified separately and fused by an overlapping PCR. To this aim, the geneticin resistance gene was amplified from pSM334 (Hou *et al.*, 2002) using primers GW-Gen-F1 and Gen-R1. The GFP fragment was amplified from pSC001 using Gen-GRFP-F1/GW-GRFP-R1. An overlapping PCR was performed using GFP and geneticin fragments (as templates) and GW-Gen-F1 and GW-GRFP-R1 primers. The resulting PCR (Gen-GFP cassette) was purified and introduced into pDONR^{TM}-221 generating pRM254. The same procedure was used to generate the Gen-RFP entry vector. Geneticin was amplified from pSM334 using primers GW-Gen-F1 and Gen-R1. RFP was amplified from pSC002 using Gen-GRFP-F1 and GW-GRFP-R1.

An overlapping PCR was performed using GW-Gen-F1 and GW-GRFP-R1 and the purified products of RFP and geneticin as template and the resulting PCR (Gen-RFP) were introduced into pDONR^{TM}-221 generating pRM257.

To construct the Nat-GFP entry vector (pRM255), an overlapping PCR was used to generate the Nat-GFP cassette. To this aim, the nourseothricin resistance gene was amplified from pNR3 using primers GW-Nat-F1 and Nat-R1. GFP was amplified from pSC001 using Nat-GRFP-F1 and GW-GRFP-R1. The purified products of GFP and Nat were used as a template in a PCR reaction using primer pair GW-Nat-F1/GW-GRFP-R1; and the resulting PCR product (Nat-GFP cassette) was introduced into pDONR^{TM}-221, generating pRM255.

Construction of fungal transformation vectors (FT vectors)

To generate FT vectors, three entry vectors including entry vector derived from pDONR^{TM}-P4-P1R, pDONR^{TM}-221 and pDONR^{TM}-P2R-P3 were used and the LR reaction was performed to recombine the fragments into the binary destination vector, pPm43GW. To generate the FT vector for GFP expression in *Z. tritici* wild type strain (IPO323), the pRM236, pRM250 and pRM234 were incorporated into pPm43GW generating pFT1 (Fig. 2A'). To transform and express RFP in *Z. tritici* wild type strain (IPO323), pRM237, pRM250 and pRM235 were incorporated into pPm43GW generating pFT2 (Fig. 2B'). To generate the FT vector for GFP expression in *Z. tritici Gpb1* mutant (Mehrabi *et al.*, 2009) and the *Z. tritici Wor1* mutant (Mirzadi Gohari *et al.*, 2014), pRM236, pRM251 (geneticin entry vector) and pRM234 were incorporated into pPm43GW generating pFT3 (Fig. 2C'). For RFP expression in fungal strains already resistant to hygromycin including *Z. tritici Gpb1* mutant (Mehrabi *et al.*, 2009) and *Z. tritici Wor1* mutant (Mirzadi Gohari *et al.*, 2014), pRM237, pRM251 (geneticin entry vector) and pRM235 were incorporated into pPm43GW generating pFT4 (Fig. 2D'). To express GFP and complement *Z. tritici Wor1* mutant, pRM236 (GFP entry vector), pRM251 (geneticin entry vector) and pRM260 containing a full length *ZtWor1* were incorporated into pPm43GW generating pFT5 (Fig. 2E').

Fungal transformation and microscopy

The FT constructs were cloned into *A. tumefaciens* strain AGL1 by electroporation. *A. tumefaciens*-mediated transformation was performed according to

Zwiers and de Waard (2001) and Mehrabi et al. (2006). After three weeks, individual *Z. tritici* transformant colonies were collected and transferred to PDA containing 200 µg cefatoxime/mL and either 100 µg hygromycin/mL or 250 µg geneticin/mL. The yeast-like spores or mycelia of each sample were placed on a glass slide and covered with a cover slip. The samples were examined using an Olympus IX81 microscope (Olympus, Hamburg, Germany), equipped with a 100X/1.45 Oil TIRF or 60x/1.35 Oil objective and a VS-LMS4 Laser-Merge-System with solid state lasers (488 nm 70mW and 561 nm/70 mW, Visitron System, Munich, Germany). The images were taken using a Photometrics Cool SNAP HQ2 camera (Roper Scientific, location, Germany) and processed by MetaMorph (Molecular Devices, Downingtown, USA) software.

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Chapter 3

Molecular characterization and functional analyses of *ZtWor1*, a transcriptional regulator of the fungal wheat pathogen *Zymoseptoria tritici*

Mirzadi Gohari, A., Mehrabi, R., Robert, O., Ince, I.A., Boeren, S., Schuster, M., Steinberg, G., de Wit, P.J.G.M. and Kema, G.H.J. (2014) Molecular characterization and functional analyses of *ZtWor1*, a transcriptional regulator of the fungal wheat pathogen *Zymoseptoria tritici*. *Molecular Plant Pathology* **15,** 394-405.

Summary

Zymoseptoria tritici causes the major fungal wheat disease septoria tritici blotch, and is increasingly being used as a model for transmission and population genetics, as well as host–pathogen interactions. Here, we study the biological function of *ZtWor1*, the orthologue of *Wor1* in the fungal human pathogen *Candida albicans*, as a representative of a superfamily of regulatory proteins involved in dimorphic switching. In *Z. tritici*, this gene is pivotal for pathogenesis, as *ZtWor1* mutants were nonpathogenic and complementation restored the wild-type phenotypes. *In planta* expression analyses showed that *ZtWor1* is up-regulated during the initiation of colonization and fructification, and regulates candidate effector genes, including one that was discovered after comparative proteome analysis of the *Z. tritici* wild-type strain and the *ZtWor1* mutant, which was particularly expressed *in planta*. Cell fusion and anastomosis occur frequently in *ZtWor1* mutants, reminiscent of mutants of *MgGpb1*, the β-subunit of the heterotrimeric G protein. Comparative expression of *ZtWor1* in knock-out strains of *MgGpb1* and *MgTpk2*, the catalytic subunit of protein kinase A, suggests that *ZtWor1* is downstream of the cyclic adenosine monophosphate (cAMP) pathway that is crucial for pathogenesis in many fungal plant pathogens.

Introduction

Co-evolution of plants and their pathogens has resulted in complex interactions where both pathogens and hosts evolved elaborate mechanisms resulting in either compatible interactions where pathogens successfully invade plants or incompatible interactions where host defenses restrict pathogen growth (Dodds and Rathjen, 2010). Plant pathogenic fungi secrete a repertoire of effector proteins that facilitate infection by interfering with host defense mechanisms, whereas most host plants have developed receptors that mediate resistance against these fungi after recognition of these effectors (de Wit *et al.*, 2009). Unravelling molecular networks involved in pathogenicity provides crucial information that might lead to the development of effective disease control strategies (Lucas, 2011).

Zymoseptoria tritici (Desm.) Quaedvlieg & Crous (Quaedvlieg *et al.*, 2011), formerly known as *Mycosphaerella graminicola*, the causal agent of septoria tritici blotch (STB) of wheat, is one of the most destructive fungal wheat diseases. Currently, disease management is achieved mainly through fungicide applications, but this is a costly and unsustainable strategy due to the development of fungicide resistance in the pathogen (Cools and Fraaije, 2008; Fraaije *et al.*, 2007; Stergiopoulos *et al.*, 2003). Introgression of resistance genes into commercial wheat cultivars is considered to be a cost-effective and environmentally safe

alternative for the application of fungicides. However, relatively few resistance genes have been characterized (Arraiano *et al.*, 2007; Tabib Ghaffary *et al.*, 2012; Tabib Ghaffary *et al.*, 2011) and provide limited efficacy to the complex natural *Z. tritici* populations. Moreover, *Z. tritici* has the potential to rapidly evolve new virulence patterns that reduce the durability of resistance as exemplified by the cultivar Gene, carrying *Stb4*, and Madsen, with partial resistance, whose resistance declined within five years after their release in Oregon (US) (Wittenberg *et al.*, 2009; Cowger *et al.*, 2000). A better understanding of *Z. tritici* biology and the molecular mechanisms underlying the infection process are crucial to design novel effective approaches for STB management. Availability of the genome sequence of *Z. tritici* (Goodwin *et al.*, 2011) provides an excellent opportunity for gene discovery and functional analyses elucidating developmental networks and pathogenicity processes in this fungal pathogen.

Z. tritici is a model pathogen to study hemibiotrophy and is considered to be among the top ten most important plant pathogens worldwide (Dean *et al.*, 2012). Unlike other fungal plant pathogens, such as *Magnaporthe oryzae* (Dean *et al.*, 2005), the fungus does not form appressoria or specialized structures to penetrate the foliage, but enters the leaves through stomata and subsequently colonizes the mesophyll tissue where it grows in the intercellular space without producing haustoria. The initial biotrophic phase is followed by a rapid switch to necrotrophy resulting in chlorotic lesions that eventually coalesce into larger necrotic blotches bearing numerous pycnidia, the asexual fructifications that contain the splash-borne pycnidiospores. The switch from biotrophy to necrotrophy is not well understood, but an active role of toxic compounds has been suggested (Cohen and Eyal, 1993; Duncan and Howard, 2000; Kema *et al.*, 1996).

A suite of genes that is involved in virulence or pathogenicity has been functionally characterized by a variety of targeted gene replacement approaches (Orton *et al.*, 2011). Some of them belong to MAP kinase pathways that affect among others penetration and host colonization. For instance, *MgSlt2* encodes a MAP kinase that is essential for colonization and fungal cell wall integrity (Mehrabi and Kema, 2006), whereas *MgSte12* regulates the ability to form filaments on the plant surface, which is crucial for successful infection (Kramer *et al.*, 2009). *MgGpb1* regulates the cAMP pathway, is required for pathogenicity and negatively controls anastomosis, a phenomenon that is rare in *Z. tritici* (Mehrabi *et al.*, 2009).

Recently, the transcription factor Wor1 that regulates phase specific gene expression and controls the white-opaque switch in the human fungal pathogen *Candida albicans*, has been functionally analysed as a representative of the WOPR superfamily (Huang *et al.*, 2006;

Lohse *et al.*, 2010; Srikantha *et al.*, 2006; Zordan *et al.*, 2006). Members of this transcriptional regulator family have also a role in the transition from yeast-like to filamentous growth in *Histoplasma capsulatum* (Nguyen and Sil, 2008). In both pathogens, this morphological transition is correlated with pathogenicity (Cain *et al.*, 2012). Furthermore, targeted gene deletion of the *Wor1* orthologs *Sge1* and *Reg1* in the fungal plant pathogens *Fusarium oxysporum* f.sp. *lycopersici* (Fol) and *Botrytis cinerea*, respectively, revealed their involvement in pathogenicity or virulence, conidiogenesis and the expression of phase-specific genes, including effectors and genes implemented in the production of secondary metabolites such as mycotoxins (Michielse *et al.*, 2011; Michielse *et al.*, 2009). This indicates that the WOPR gene family may specifically target a cellular function required for different biological and developmental processes in fungal plant pathogens.

In this study we investigated the role of the *Wor1* ortholog *ZtWor1* in *Z. tritici* and our results show that it is involved in pathogenicity, regulates the expression of small-secreted proteins and is most likely part of the cAMP signalling pathway that plays a pivotal role in many cellular processes.

Results

Identification and characterization of *ZtWor1*

A BLASTp search of the *Z. tritici* genomic database using *C. albicans* Wor1 (CaWor1) as query resulted in the identification of two significant hits; Mycgr3_46572 and Mycgr3_72926, with E-values $1.43E^{-28}$ and $1.52E^{-12}$, which are located on chromosomes 8 and 6, respectively. Amino acid alignments revealed that Mycgr3_46572 had the highest homology with the CaWor1 ortholog (Lohse *et al.*, 2010) and phylogenetic tree analysis displayed that Mycgr3_46572 was clustered in the same clade as CaWor1. We, therefore, designated it as *ZtWor1* and studied it in more detail (Fig. 1A). *ZtWor1* has an open reading frame of 1,614 bp, without introns as verified by reverse-transcription polymerase (RT-PCR), encoding a protein of 537 amino acids (aa) (Fig. 1B). The aforementioned phylogenetic analysis grouped *ZtWor1* with FoSge1 from Fol (Fig. 1A), suggesting that it may play a role in the regulation of effector encoding genes in the *Z. tritici*-wheat pathosystem. Similar to other members of the WOPR superfamily, the *ZtWor1* N-terminus is more conserved than its C-terminus. Amino acid alignment of ZtWor1 with the four characterized orthologs from Fol (FoSge1), *B. cinerea* (BcReg1), *C. albicans* (CaWor1) and *H. capsulatum* (HcRyp1) revealed the presence of a gluconate transport-inducing protein domain called Gti1_Pac2 (Pfam09729), which is present across these fungal lineages. Finally, ZtWor1 contains a potential protein

kinase A (PKA) phosphorylation site (KRWTDS/G) and a nuclear localization site (+94 to +101), which are also conserved among members of WOPR (Fig. 1B), suggesting that the ZtWor1 protein is localized in the nucleus as has also been demonstrated for Sge1 and Ryp1 (Michielse *et al.*, 2009; Nguyen and Sil, 2008).

Deletion and complementation of *ZtWor1*

In order to evaluate the biological function of *ZtWor1* during the infection process, gene knock-out and complementation mutants were generated based on homologous recombination (Supplementary Fig. S1A). Three independent transformants with similar morphology, IPO323*ΔZtWor1* #1, #29 and #26, were obtained. The latter, coded *ΔZtWor1-26*, was used for all subsequent analyses. Since *ΔZtWor1-26* was unable to produce yeast-like spores, *Agrobacterium tumefaciens*–mediated transformation (ATMT) was performed on fragmented mycelial tissue using a construct harbouring the *ZtWor1* wild type allele, which resulted into *ΔZtWor1-com7* (Supplementary Fig. S1B).

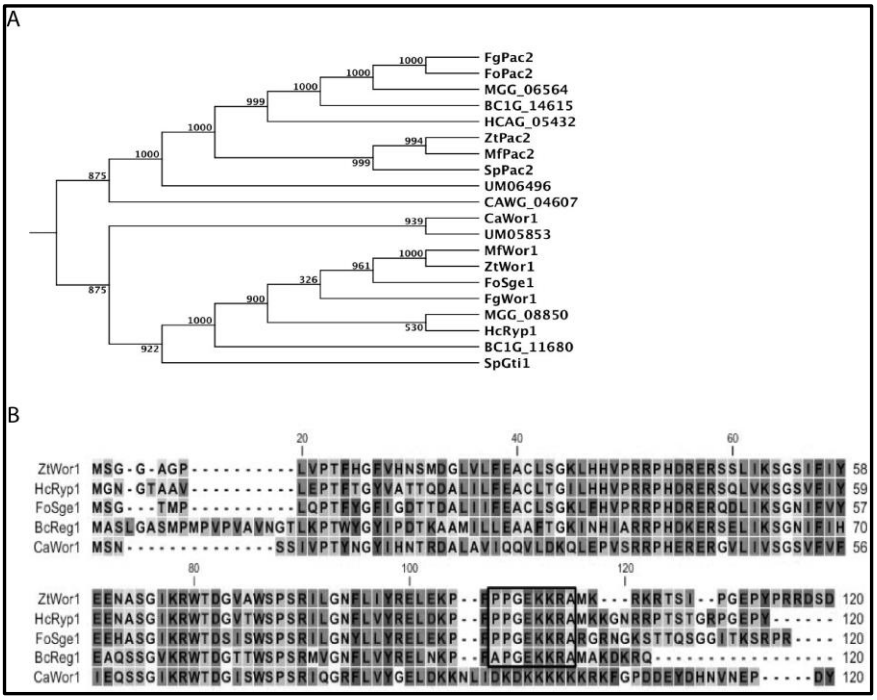

Figure 1. Phylogenetic comparison of *Zymoseptoria tritici* Wor1 (ZtWor1) with members of the WOPR superfamily based on amino acid sequence alignments. **A,** The tree shows the phylogenetic relationship of ZtWor1 with Wor1 and Pac2 orthologs in other fungi including BcReg1, BC1G_14615, HcRyp1, HCAG_05432, CaWor1, CAWG_04607, SpGti1, SpPac2, MGG_08850, MGG_06564, FgWor1, FgPac2, FoSge1, FoPac2, MfWor1, MfPac2, UM05853, UM06496 from *Botrytis cinerea*, *Histoplasma capsulatum*, *Candida albicans*, *Schyzosaccharomyces pombe*, *Magnaporthe grisea*,

Fusarium graminearum, Fusarium oxysporum f. sp. *lycopersici*, *Mycosphaerella fijiensis* and *Ustilago maydis*, respectively, using CLC genomics software. The bootstrap values (1000 replicates) are exhibited above the branches. **B**, Alignment of the first 120 deduced amino acid sequences of ZtWor1 and its ortholog members of the WOPR superfamily in other fungi. The nuclear localization motif is boxed.

ZtWor1 regulates fungal development

In order to assess the role of *ZtWor1* in fungal growth and development, *Z. tritici* IPO323 (WT) strain, *ΔZtWor1-com7* and *ΔZtWor1-26* were compared in liquid YGB medium and on the solid media PDA, *Aspergillus nidulans* minimal medium (MM) and V8 over a period of 10 days at 20 °C. In YGB, the WT and *ΔZtWor1-com7* produced abundant yeast-like cells (Fig. 2A(a,b)), but *ΔZtWor1-26* did not produce any spores and exclusively produced a dense, extensive mycelial network with abundant abnormally swollen-cell structures (Fig. 2A(c)). Microscopic comparison showed uncontrolled cell fusions or anastomosis in *ΔZtWor1-26* that was rare in the WT (Fig. 2B(d), Fig. 3 and Supplementary Fig. S2).

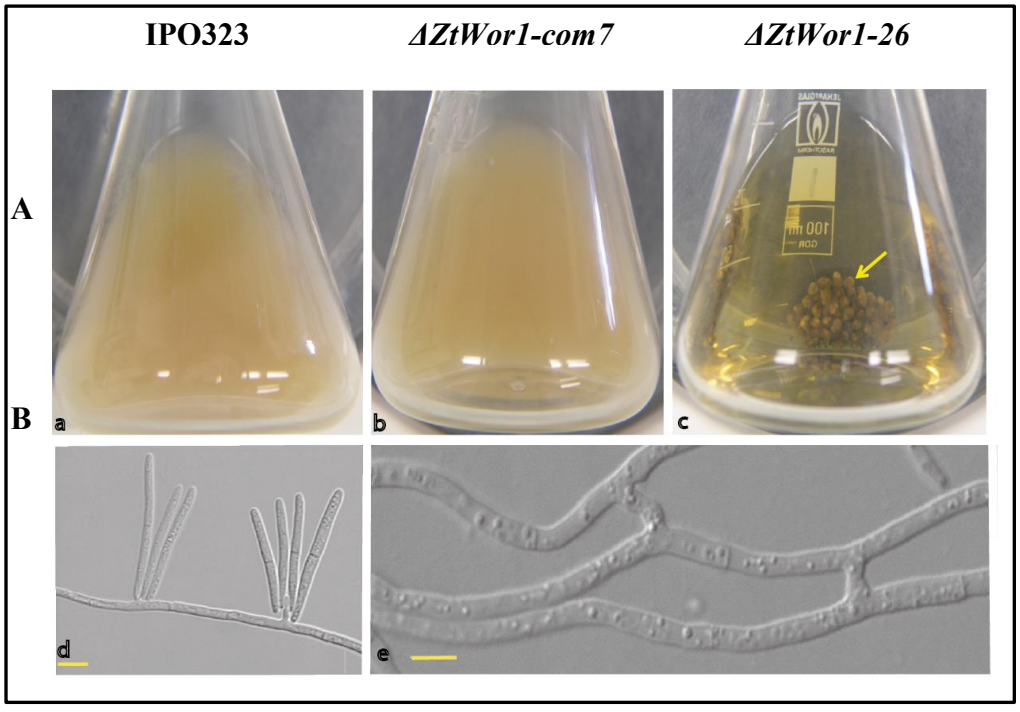

Figure 2. Deletion of *Zymoseptoria tritici Wor1* (*ZtWor1*) affects yeast-like cell production and early development. **A (a and b)**, The *Z. tritici* WT strain and *ΔZtWor1-com7* produce abundant yeast-like cells resulting from blastic conidiogenesis in yeast glucose broth medium. **A (c)**, *ΔZtWor1-26* is

blocked in sporulation and exclusively produces compact hyphal networks resulting in a distinct bulbous mycelial mass (marked via yellow arrow). **B,** Comparative light microscopy of early *Z. tritici* development; **B** (**d**) production of yeast-like cells on the hyphae of the WT on water agar; **B**(**e**) unique cell fusion or anastomosis events that occur frequently in *ΔZtWor1-26*. Scale bars are 5µm.

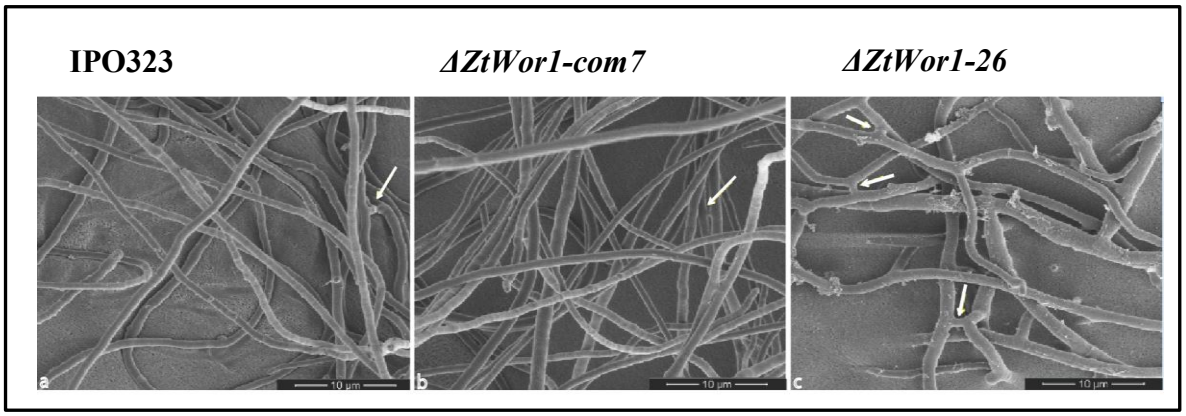

Figure 3. Comparative scanning electron micrographs of hyphae of the *Zymoseptoria tritici* WT strain, *ΔZtWor1-com7* and *ΔZtWor1-26* growing on MM at 10 days after inoculation at 20 °C; Infrequent cell fusion/anastomosis events occur in the *Z. tritici* WT strain and *ΔZtWor1-com7* (a-b; arrows), but cell fusion/anastomosis frequently happens in *ΔZtWor1-26* (c; arrows). Scale bars are 10µm.

On PDA, we did not observe any effect of the deletion of *ZtWor1* on germination and early colony development during the first 48 hrs after inoculation (data not shown). On MM, the growth pattern started to differ at five dai as *ΔZtWor1-26* grew significantly slower than the WT as well as *ΔZtWor1-com7,* resulting in more compact colonies. Comparative scanning electron microscopy revealed significant differences ($P < 0.05$) in hyphae diameter between the WT, *ΔZtWor1-com7* and *ΔZtWor1-26* (Supplementary Fig. S3). On V8, the WT and *ΔZtWor1-com7* strains abundantly produced yeast-like spores, whereas *ΔZtWor1-26* hardly produced any spores, even after prolonged incubation (>14 days). At five days after inoculation (dai) the WT and *ΔZtWor1-com7* turned black, likely due to melanisation, but the *ΔZtWor1-26* produced an additional mass of aerial hyphae covering the dark colonies that were absent in the WT (Fig. 4).

ZtWor1 expression relies on *MgGpb1* and *MgTpk2*

Overall, signal transduction pathways including the MAP kinase and cAMP-dependent protein kinase A (PKA) pathways play a crucial role in sensing and responding to environmental stimuli and represent important cascades in the regulation of development in eukaryotes. Previously, we showed that these pathways are also involved in pathogenicity and development of *Z. tritici* (Cousin *et al.*, 2006; Mehrabi *et al.*, 2006a; Mehrabi *et al.*, 2006b). Since the *ΔZtWor1-26* showed abundant anastomosis, we tested whether the cAMP signaling pathway that controls a similar phenotype in *Z. tritici* Gβ mutants (Mehrabi *et al.*, 2009), is also involved in *ZtWor1* regulation, and determined the relative expression level of *ZtWor1* in *MgGpb1* and *MgTpk2* mutants (Mehrabi and Kema, 2006; Mehrabi *et al.*, 2009).

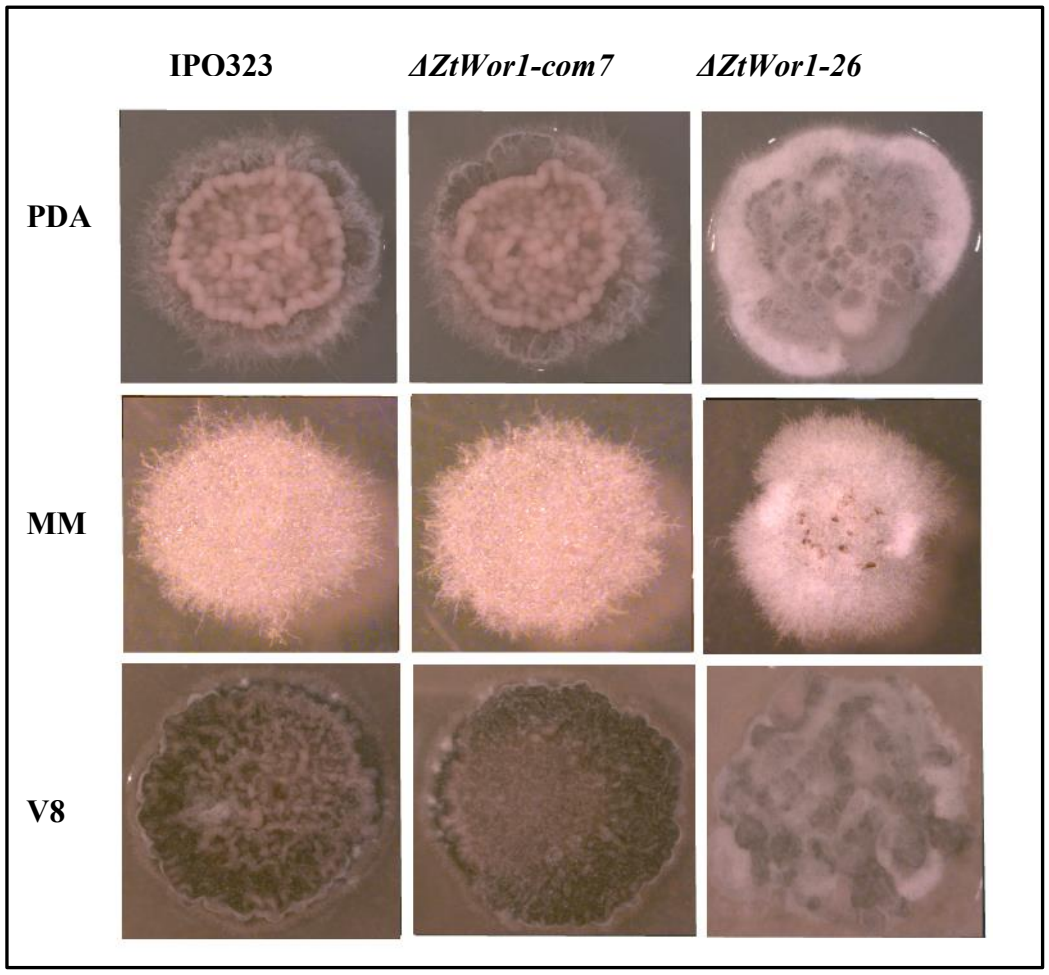

Figure 4. The *in vitro* effect of *ZtWor1* deletion and complementation in *Zymoseptoria tritici* at 10 days after inoculation on three different media at 20 ºC. Comparison of the WT strain and *ΔZtWor1-26* shows that the latter exclusively produces strongly melanised mycelial cultures without any spores. This is particularly evident on V8, but also on MM, *ΔZtWor1-26* exclusively produces hyphae. The WT phenotype is restored in *ΔZtWor1-com7*.

In both mutants, *ZtWor1* expression was severely reduced compared to the WT (Fig. 5A) while the expression levels of *MgGpb1* and *MgTpk2* in *ΔZtWor1-26* were the same as WT strain (Fig. 5B). Taken together, it can be concluded that ZtWor1 and the PKA pathways function in parallel to regulate various developmental processes such as cell fusion in *Z. tritici.* Alternatively, *ZtWor1* is downstream of the β-subunit of the heterotrimeric G protein and the cAMP pathway as shown by the expression analysis.

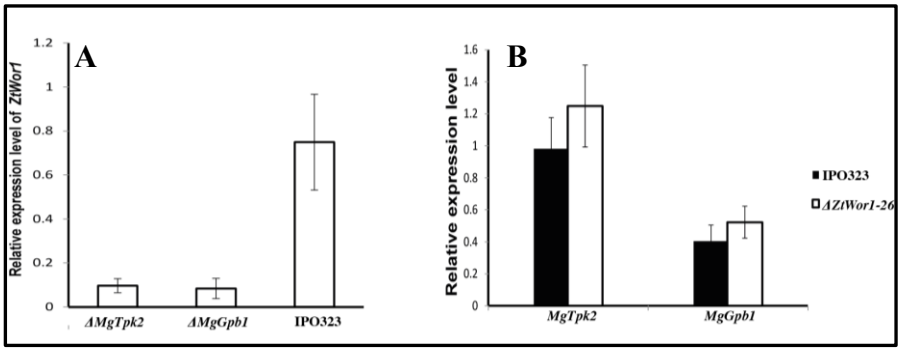

Figure 5. Expression analysis of *Zymoseptoria tritici Wor1* (*ZtWor1*) in the disrupted mutants *MgGpb1* and *MgTpk2*. **A,** *In vitro* expression level of *ZtWor1* in disruption mutants of *MgGpb1* and *MgTpk2* compared with expression in the *Z. tritic*i WT strain. **B**, Comparative *in vitro* expression of *MgGpb1* and *MgTpk2* in the *Z. tritici* WT and *ΔZtWor1-26* strains.

ZtWor1 is up-regulated at early and late stages of infection

Since orthologs of *ZtWor1* in other fungal plant pathogens are implicated in pathogenicity we analysed the expression levels of *ZtWor1 in vitro* and *in planta* (Fig. 6). *ZtWor1* has a bimodal expression profile and is up-regulated during the early stage of infection (2 dai), then gradually down-regulated until 16 dai and significantly up-regulated again at 20 dai, the stage of infection that coincides with pycnidial formation. The expression level of *ZtWor1* in an axenic mycelial culture was comparable with the *in planta* expression at 2 dai, while the expression level in yeast-like cells was similar to the *in planta* expression at 20 dai during abundant asexual fructification (Fig. 6).

ZtWor1 is required for pathogenicity

To assess the biological function of *ZtWor1* during pathogenesis, WT strain, *ΔZtWor1-com7* and *ΔZtWor1-26* were used to inoculate the susceptible wheat cv. Obelisk and disease development was monitored over time. Small chlorotic flecks appeared at 9 dai, especially at the leaf tips, which over time expanded and eventually coalesced into large necrotic blotches

containing numerous pycnidia in the control strains. Occasionally a few chlorotic and necrotic lesions were observed after inoculations with the deletion mutant, that, sometimes, contained a few immature pycnidia in a limited number of lesions (Fig.7).

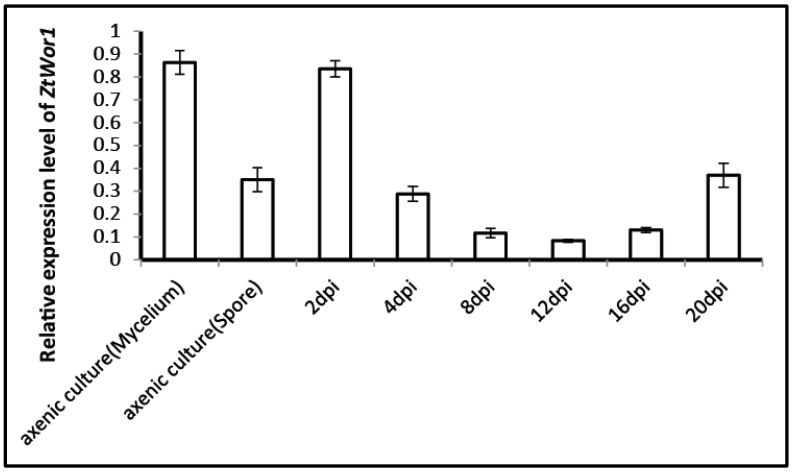

Figure 6. *In vitro* and *in planta* expression levels of *Zymoseptoria tritici Wor1*. *In vitro* conditions (18 °C and 25 °C to induce yeast-like cells and mycelia formation, respectively) were chosen to compare the expression levels of *Ztwor1* in mycelial and conidial cultures with *in planta* conditions. The susceptible cv. Obelisk was inoculated with the *Z. tritici* WT strain and infected leaves were harvested 2, 4, 8, 12, 16 and 20 days after inoculation followed by RNA isolation and cDNA synthesis, which showed that *Ztwor1* is particularly expressed at the onset of colonization (switch from yeast-like spores to hyphae) and conidiogenesis (pycnidia production at the later phase of pathogenesis). The expression of *Ztwor1* was normalized with the constitutively expressed *Z. tritici* beta-tubulin gene.

ZtWor1 regulates the expression of specific small-secreted proteins

Considering that *ΔZtWor1-26* was significantly reduced in pathogenicity and that orthologs in other fungal plant pathogens regulate the expression of effector genes, we hypothesized a similar role for *ZtWor1* in *Z. tritici.* First, q-RT-PCR was used to determine the role of *ZtWor1* in the *in vitro* expression of several small-secreted proteins (SSPs) that are candidate effectors in the *Z. tritici*– wheat pathosystem based on bioinformatics analyses (Morais do Amaral *et al.*, 2012) (Supplementary Table 1) and *in planta* expression profiling (Kema *et al*., unpublished data). We determined that ZtWor1 either positively or negatively regulates SSPs. For instance, the expression level of SSP60 was down-regulated more than 20-fold compared to the WT (Fig. 8 and Supplementary Table 2). Secondly, we compared the *in vitro* proteome of WT strain and *ΔZtWor1-26*. Overall, 125 *Z. tritici* proteins were identified from all conditions (three minimal media including MM, B5 and dextrose broth) of

which 18 proteins were unique to *Z. tritici*, for no homologs could be identified in fungal databases. Hundred fourteen proteins possess a SignalP motif indicating that they are secreted and only one (SSP127) of the 114 proteins was not expressed in *ΔZtWor1-26* (Fig. 9). *In vitro* expression on MM indicates that SSP127 might have an important role during the early stages of infection. This was confirmed by the relative *in planta* expression of SSP127 that was highly up-regulated until four dai. (Fig. 10).

Figure 7. The effect of *Zymoseptoria triici Wor1* (*ZtWor1*) deletion on disease development in the susceptible wheat cv. Obelisk. First leaves were inoculated with the *Z. tritici* WT strain (2), *ΔZtwor1-com7* (3) and *ΔZtwor1-26* (4) with water as a control (1). Pictures were taken at 21 days after inoculation.

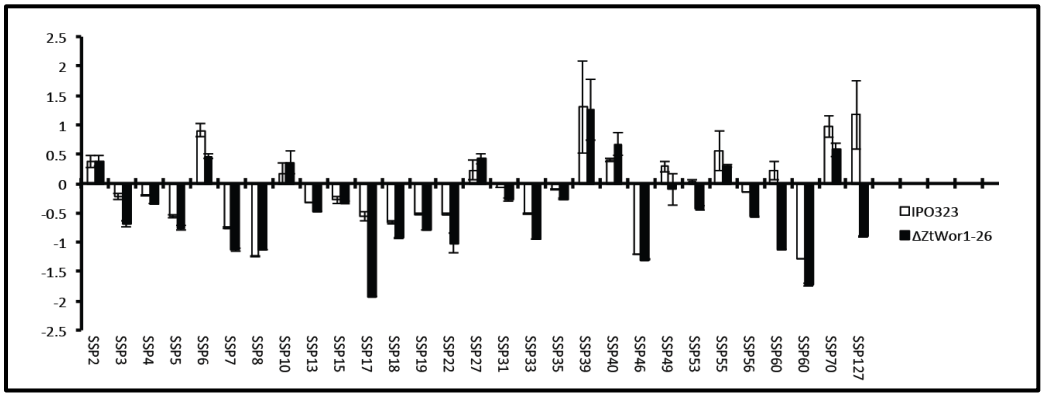

Figure 8. Comparative *in vitro* expression analysis of 29 small-secreted proteins in the *Zymoseptoria tritici* WT strain and *ΔZtwor1-26* grown in YG medium for seven days at 18 °C. Expression levels were normalized with the constitutively expressed *Z. tritici* beta-tubulin gene and plotted on a Log10 scale.

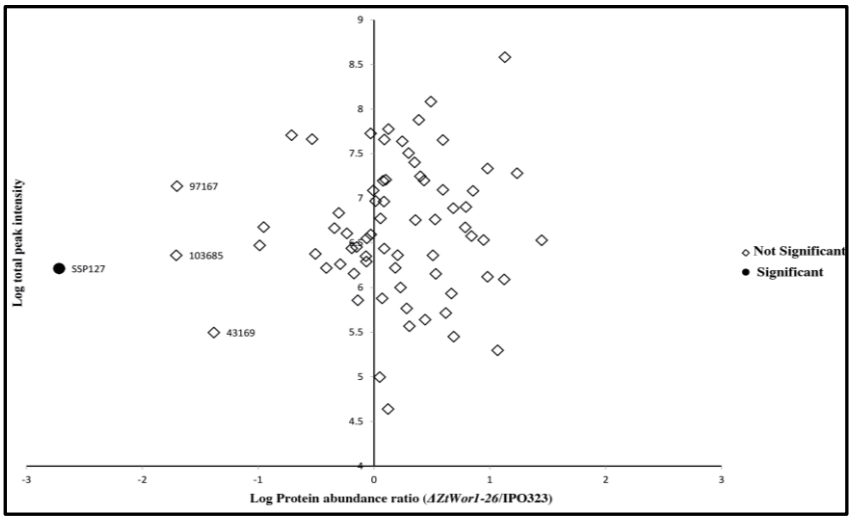

Figure 9. Comparative discriminative *in vitro* proteome analysis of the *Zymoseptoria tritici* WT strain and *ΔZtWor1-26.* Plot of the normalized *ΔZtWor1-26*/IPO323 intensity ratio against the total measured protein intensity. Proteins not significantly different between mutant and WT are depicted in white while the significantly different one is shown in a black circle. SSP127 is the only identified protein that showed significant expression differences between the two strains (ratio=0.002, P-value=3.1.10-5). Proteins 43169, 97167 and 103685 showed P-values larger than 0.05 and are considered non-significant by the Perseus software, which takes both the ratio and P-value into account.

Subsequently, two independent knock-out strains of SSP127 were generated and phenotyped on a range of 12 unrelated wheat cultivars that are parents of mapping populations as well as the suite of wheat cultivars with mapped resistance (*Stb*) genes (Tabib Ghaffary, 2011), but, surprisingly, no significant differences in disease development were observed between the knock-out strains and the WT (Supplementary Fig. S4). In summary our data suggest that *ZtWor1* is much more involved in developmental processes rather than being a specific regulator of effector genes.

Discussion

For successful infection and completion of its lifecycle on wheat, *Z. tritici* employs a variety of mechanisms to penetrate, colonize and kill host tissue. To date, several

pathogenicity factors, such as *MgSlt2* and *MgGpb1*, have been identified and to some extend it has been shown how they contribute to the infection process, evade host defense responses and enable disease establishment (Orton *et al.*, 2011).

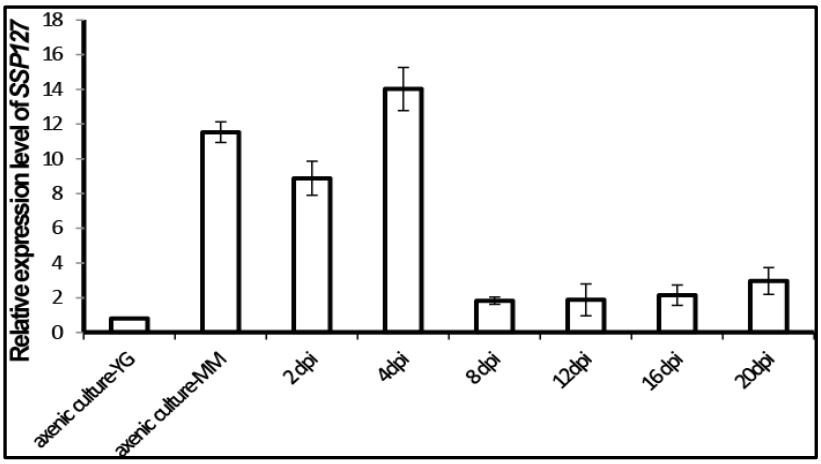

Figure 10. *In vitro* and *in planta* expression levels of small-secreted protein (SSP) 127 of *Zymoseptoria tritici*. *In vitro* conditions represented mycelium production (on MM) and blastic conidiogenesis (in YG broth medium). *In planta* expression profiles were measured during a time course (2-20 days after inoculation) experiment, using the susceptible wheat cv. Obelisk. The relative expression of SSP127 was normalized with the constitutively expressed *Z. tritici* beta-tubulin gene.

Here, we analysed the biological function of the regulatory gene *ZtWor1* and show that it is required for pathogenicity of *Z. tritici*, possibly through the regulation of effector genes as it controls the expression levels of a suite of genes encoding SSPs *in vitro*, which should be corroborated in future experiments. Members of the conserved WOPR family of regulatory proteins, such as the master regulator Wor1, are involved in the dimorphic switch of the human fungal pathogen *C. albicans*. Similar to other well characterized family members in both fungal human and plant pathogens such as HcRyp1, FoSge1, BcReg1, *S. pombe* (GtiI) and *F. graminearum* (Fgp1) (Caspari, 1997; Jonkers *et al.*, 2012; Michielse *et al.*, 2011; Michielse *et al.*, 2009; Nguyen and Sil, 2008), this putative transcriptional regulator possesses two globular domains, the WOPR box1 (amino acids 16 to 107) and the WOPR box2 (amino acids 160 to 250) located at the N-terminal region, which is highly conserved across fungal lineages. In contrast, the C-terminus regions are rich in glutamine amino acids and very divergent among family members. Another common feature of WOPR family members is the presence of a highly conserved amino acid motif (PPGEKKRA) that was

shown to be involved in nuclear localization of Ryp1 and Sge1. This motif (+94 to +101) is also present in ZtWor1, and likely serves the same role in *Z. tritici*.

C. albicans Wor1 is a master regulator of "white to opaque switching", which refers to the development and transition between two distinctive *in vitro* cell types. Strains deleted for *Wor1* cannot form opaque cells, but this phenotype can be rescued by ectopic expression of *Wor1* (Huang *et al.*, 2006; Ohara and Tsuge, 2004; Srikantha *et al.*, 2006; Zordan *et al.*, 2006). Furthermore, it was shown that Wor1 regulates white-opaque switching through phase specific expression of the genes *Wor2*, *Czf1*, and *Efg1* (Huang *et al.*, 2006; Morschhäuser, 2010). The *Efg1* ortholog in Fol is required for conidiogenesis (Ohara and Tsuge, 2004). In *H. capsulatum*, Ryp1 is a master transcriptional regulator that controls the transition from filamentous growth to the pathogenic budding-yeast form. Nguyen and Sill (2008) showed that Ryp1 is involved in the expression of yeast-specific genes including two genes that are linked to virulence. In both aforementioned human pathogens the up-regulation of Wor1 (45x) and Ryp1 (4x) is correlated with dimorphism and with pathogenicity through the regulation of cell type specific genes (Nguyen and Sil, 2008; Tsong *et al.*, 2003) .

Our analyses showed that *ZtWor1* expression oscillates with distinct phases of pathogenesis; up-regulation during initial disease establishment (2 dai), down-regulation during colonization (until 12 dai) and again up-regulation during conidiogenesis (20 dai). In addition, we showed that *in vitro ZtWor1* expression in WT strain correlates with the transition from yeast-like cells to filamentous growth that occurs during the early stage of infection (~2 dai). During these respective *in planta* and *in vitro* conditions *Z. tritici* undergoes extreme morphological changes (Goodwin *et al.*, 2011). Thus, the inability of *ΔZtWor1-26* to develop or differentiate the required appropriate cell types may abolish pathogenicity. However, in addition we provide evidence that ZtWor1 regulates a suite of genes encoding SSPs *in vitro* that likely have effector functions by acting as virulence or avirulence determinants in the *Z. tritici*–wheat pathosystem. In Fol, Sge1 regulates the expression of *Six* (secreted in xylem) effectors during the colonization of the vascular tissue of tomato plants (Michielse *et al.*, 2009). Recently, Jonkers and colleagues showed that the *ZtWor1* ortholog *Fgp1* in *F. graminearum* is required for the infection process and the *in vitro* and *in planta* expression of genes involved in the trichothecene biosynthetic (TRI) pathway (Jonkers *et al.*, 2012). Thus, besides the role of *ZtWor1* in morphological changes that possibly affect pathogenicity, it is probable that ZtWor1 globally regulates various virulence factors, which requires further investigation.

The dimorphic switch involved in *ZtWor1* expression and the comparison with knock-out strains in other fungi demonstrate its global involvement in developmental morphogenesis. Functional analysis of *BcReg1* in *B. cinerea* revealed that knock-out strains produce aberrant non-conidia bearing conidiophores during pathogenesis (Michielse *et al.*, 2011), and *Sge1* and *Fgp1* also affect conidia formation in Fol (Michielse *et al.*, 2009) and Fg, respectively (Jonkers *et al.*, 2012). We observed that *ZtWor1* mutants do not sporulate *in vitro.* Each and every effort to induce sporulation of *ZtWor1* mutants using different conditions and (liquid) media was not successful, but complementation of *ΔZtWor1-26* restored the WT, hence *in vitro* and *in planta* conidiogenesis, suggesting that Wor1 is a crucial factor in the yeast-like cell formation.

Complementation also restores pathogenicity, whereas heterologous complementation with *Sge1* from Fol did not (data no shown), indicating that Wor1 orthologs in various fungal human and plant pathogens have evolved divergently to regulate pathogenicity through different mechanisms as has been shown in Fg where interchanging the N- and C- terminal portions of the Wor1 homologs from Fol and Fg did not mutually restore loss of function (Jonkers *et al.*, 2012).

The current study suggests that ZtWor1 may be positioned downstream of two important components of the cAMP pathway, *MgGpb1* and *MgTpk2*, which play important roles in cell differentiation and pathogenicity (Mehrabi and Kema, 2006; Mehrabi *et al.*, 2009). This is a unique hypothesis that requires further investigation, but interestingly the phenotypes of the *ZtWor1*, *MgTpk2* and *MgGpb1* mutants share several common features. Firstly; they are hampered in pathogenicity, secondly; *MgTpk2* and *MgGpb1* mutants penetrate the host and colonize the mesophyll, but cannot differentiate cells towards fructification and thirdly; *ZtWor1* and *MgGpb1* mutants show an intriguing cell fusion or anastomosis phenotype that is unique in *Z. tritici.* MgGpb1 negatively regulates anastomosis, and this gene is upstream of MgTpk2 and positively regulates the cAMP pathway as exogenous cAMP restored the WT phenotype (Mehrabi *et al*., 2009). Our current data suggest that the previously characterized cAMP genes (*MgTpk2 and MgGpb1*) and *ZtWor1* might be three components of the cAMP pathway controlling different aspects of differentiation and infection. Interestingly, all family members of WOPR contain a protein kinase A (PKA) phosphorylation site and the functionality of this well conserved motif was determined by mutation resulting in non-pathogenic phenotypes in Fol, indicating that FoSge1 is pivotal for pathogenicity (Michielse *et al.*, 2009). Furthermore, it was demonstrated that the CaWor1 protein was phosphorylated by Tpk2, thus regulating the "white to opaque" switch (Huang *et*

al., 2010). Our data suggest that ZtWor1 is regulated by the *Z. tritici* homolog of Tpk2, MgTpk2, as shown by the conducted expression analyses.

In summary, we conclude that *ZtWor1* is a putative transcriptional regulator in the dimorphic fungal plant pathogen *Z. tritici* and plays an essential role in differentiation, asexual fructification, conidiogenesis as well as regulation of SSPs that might act as putative effector genes. In addition, we suggest that *ZtWor1* might be regulated by two upstream key genes, *MgGpb1* and *MgTpk2*, indicating that the functionality of ZtWor1 is controlled through the cAMP pathway and, hence, ZtWor1 could be considered as a key transcriptional regulator downstream of this pathway. Further research into the cAMP signalling network and the exact role of ZtWor1 in this pathway is required to elucidate how these components regulate morpho-pathogenic behaviour of *Z. tritici*. The presented data show that a comprehensive understanding of the regulatory function of ZtWor1 may lead to the identification of key pathogenicity factors or effector proteins, which will contribute to the further understanding of the complex *Z. tritici* – wheat interaction.

Experimental Procedures

Strains, media and growth conditions

The sequenced *Z. tritici* reference strain IPO323, which is highly virulent on the susceptible wheat cv. Obelisk, was used as wild type (WT) and recipient strain for gene deletion. The WT and all developed strains were stored at -80 °C and recultured on potato dextrose agar (PDA) (Sigma-Aldrich Chemie, Steinheim, Germany) at 18 °C. Yeast-like spores were produced on V8 juice medium (Campbell Foods, Puurs, Belgium) or in yeast glucose broth (YGB) medium (yeast extract 10 g/L, glucose 20 g/L) placed in an orbital shaker (Innova 4430; New Brunswick Scientific, Nijmegen, The Netherlands) at 18 °C. To induce mycelial growth, all *Z. tritici* strains were grown under the same condition but at 25 °C. *Aspergillus nidulans* minimal medium (MM) and *Cladosporium fulvum* B5 (B5) medium were prepared and used for morphological charecterization experiments and proteomic assays, respectively (Ackerveken *et al.*, 1994; Barratt *et al.*, 1965). Nucleotide sequence data of *ZtWor1* are available at GenBank under accession number BK008803. *Zymoseptoria tritici* strain IPO323 is available at the Centraal Bureau voor Schimmelcultures, Utrecht, the Netherlands: http://www.cbs.knaw.nl/.

Phylogenetic tree construction

Phylogenetic analysis of ZtWor1 with homologues from other fungal pathogens was conducted using the CLC genomics workbench package (Aarhus, Denmark). All Wor1 and Pac2 fungal proteins were retrieved from public databases and aligned using the aforementioned software, considering a gap opening cost and gap extension penalty of 10 and 1, respectively. The phylogenetic tree was constructed based on the unweighted pair group method with arithmetic average (UPGMA) algorithm, and the statistical accuracy of the tree was tested by bootstrap analysis (1000 repetitions).

Generation of gene deletion and complementation constructs

To generate the *ZtWor1* deletion construct, p*ZtWor1*KO, the USER friendly cloning method was used with minor modifications (Frandsen *et al.*, 2008). Briefly, ZtWor1-PRF-F1, R1 as well as ZtWor1-PRF-F2, R2 primer pairs were used to amplify about 2,000 bp upstream and down-stream of *ZtWor1* using PfuTurbo® Cx Hotstart DNA polymerase (Stratagene, Cedar Creek, TX, US). In parallel, the pRF-HU2 vector possessing the *hph* gene as a selection marker was digested with two restriction enzymes, PacI and a nicking enzyme Nt.BbvCI, to generate a compatible overhang with the PCR amplicons. Subsequently, the PCR amplicons and the digested vector were mixed and treated with the USER enzyme mix (New England Biolabs, Ipswich, USA) and incubated at 37 °C for 30 min followed by 25 °C for 30 min. The resulting reaction was directly transformed into *Escherichia coli* strain DH5α and was subsequently cultured on selective kanamycin media. In order to identify bacterial colonies carrying the construct with the insertions in the expected positions, colony PCR was conducted using User-F and User-R primers (located on the middle of *Hph* gene) in combination with ZtWor1-R and ZtWor1-F, respectively (Table 1).

To generate the *ZtWor1* complementation construct, p*ZtWor1*com, the multisite gateway® three-fragment vector construction kit was used enabling us to clone three fragments into the destination vector, which was compatible with the *A. tumefaciens*–mediated transformation (ATMT) procedure. The full open reading frame of *ZtWor1* including 1,200 bp upstream as its promoter and 500 bp downstream as terminator were cloned into pDONR™P2R-P3 (Invitrogen, CA, USA) resulting in the generation of p2-*ZtWor1*com. Furthermore, the green fluorescent protein (GFP) gene and neomycin phosphotransferase gene (known as geneticin selection marker) were cloned into pDONR™P4-P1R and pDONR™221, resulting in p4-GFP and p221-geneticin, respectively. Finally, three entry vectors were used to clone these three fragments into the destination vector, pPm43GW, through the LR reaction

Fungal transformation

The gene deletion construct, p*ZtWor1*KO, was cloned into *A. tumefaciens* strain LBA1100 via electroporation. ATMT was carried out to delete *ZtWor1* in WT strain as previously described (Zwiers and de Waard, 2001). Genomic DNAs of stable transformants were extracted using a KingFisher robot (Thermo Scientific, Hudson, NH, USA) and used in PCR screens.

For complementation the same procedure was applied with minor modifications. Due to the lack of spore production in *ΔZtWor1-26*, small pieces of hyphal fragments - adjusted to 10^5 per mL - were used in ATMT and putatively complemented strains were selected on plates with 250 µg mL^{-1} geneticin.

Table 1. Primers used in this study.

Name	Sequence (5-3)	Location
ZtWor1-PRF-F1	GGTCTTAAUTGGACGGGCACCTGTACTATTGGCCG	Upstream of *ZtWor1*
ZtWor1-PRF-R1	GGCATTAAUGAGAGATCGAACACACAGCGGCGCAC	Upstream of *ZtWor1*
ZtWor1-PRF-F2	GGACTTAAUCCGAGCACTACGCCATTGACGGCC	Downstream of *ZtWor1*
ZtWor1-PRF-R2	GGGTTTAAUGTTTCGCCTGCCTGCGTTGCCGAG	Downstream of *ZtWor1*
ZtWor1-F1	ATGAGCGGGGGAGCCGGA	*ZtWor1*
ZtWor1-R1	CTCCTCAACCGGCGCGC	*ZtWor1*
ZtWor1-F2	GTGCTCACCGCCTGGACGACTAAAC	Middle of *hph* gene
ZtWor1-R2	ACCTTGCTA ATAACCCAAACGCC	Downstream of *ZtWor1*

Cell biology assay

Cell biology assays were performed using mycelial fragments as starting material that were generated in YGB at 25 °C for 12 days and subsequently blended and passed through a miracloth filter (Merck Millipore, location, Germany) and finally adjusted to 10^5 hyphal fragment per millilitre to monitor anastomosis in WT strain and *ΔZtWor1-26*. Approximately 10 µl of each sample were spotted on 1% water agar plugs, which were placed on a glass slide and covered with a cover slip. The samples were kept in Petri plates containing a piece of wet cotton to maintain high humidity and were incubated at 20°C for seven days. The samples were monitored using an Olympus IX81 microscope (Olympus, Hamburg, Germany), equipped with a 100X/1.45 Oil TIRF or 60x/1.35 Oil objective and a VS-LMS4 Laser-Merge-System with solid state lasers (488 nm 70mW and 561 nm/70 mW, Visitron System, Munich, Germany). The images were taken using a Photometrics CoolSNAP HQ2 camera (Roper

Scientific, location, Germany) and processed by MetaMorph (Molecular Devices, Downingtown, USA) software.

Pathogenicity assay

The susceptible wheat cv. Obelisk was grown in a greenhouse until the first leaves were fully unfolded. Since *ΔZtWor1-26* did not sporulate, we used mycelial fragments for all strains. Inoculum was produced by blending mycelia 24 hrs before inoculation that were subsequently maintained in YGB at 25 °C for cell recovery, then passed through Miracloth to remove large mycelial fragments and adjusted to 10^5 hyphal fragments/mL for spray inoculation. Knock-out strains of *SSP127* and WT strain were inoculated on a wide variety of wheat germplasm that was grown and inoculated according to standard procedures (Tabib Ghaffary, 2011). Inoculated plants were incubated in black plastic bags for 48 hrs and then transferred to a greenhouse compartment (22 °C, relative humidity > 90% and 16 hrs light). Disease development was monitored every three days and final scoring was performed at 20 dai.

RNA isolation and q-RT-PCR

In vitro and *in planta* expression analyses of selected genes were conducted using quantitative real-time PCR (q-RT-PCR). Plants of cv. Obelisk were inoculated with WT strain as described previously (Mehrabi *et al.*, 2006a) and leaf samples were collected in three biological replications, flash frozen and ground in liquid nitrogen using a mortar and pestle. Total RNA was extracted either from ground leaves or fungal biomass produced in YGB using the RNeasy plant mini kit (Qiagen, location, USA) and subsequently DNA contamination was removed using the DNAfree kit (Ambion, Cambridgeshire, U.K.). First-strand cDNA was synthesized from 2 µg total RNA primed with oligo(dT) using the SuperScript III according to the manufacturer's instructions. One µl of resulting cDNA was used in 25 µl PCR reaction using a QuantiTect SYBR Green PCR Kit (Applied Biosystems, Warrington, UK), and run and analysed using an ABI 7500 Real-Time PCR System. The relative expression level of each gene was initially normalized with the constitutively expressed *Z. tritici* beta-tubulin gene (Keon *et al.*, 2007; Motteram *et al.*, 2009) and then calculated based on a comparative C(t) method described previously (Schmittgen and Livak, 2008).

Secretome analysis of WT strain and *ΔZtWor1-26*

The WT and *ΔZtWor1-26* strains were grown in YGB (125 rpm, 25 °C, for 5 days) to obtain adequate fungal biomass. Afterwards, fungal mycelia were passed through Miracloth and washed three times with sterile water to remove residual medium. Subsequently, the resulting mycelial fragments were inoculated in three minimal media including MM, B5 and dextrose broth (30 gr dextrose/L) in four biological replications for 48 hrs. After recovery from these media, mycelia were removed by centrifugation (Beckman, Pleasanton, USA) at 10,000 rpm and the supernatants were applied to filters (0.45 µm). Proteins were precipitated with 10% trichloroacetic acid (TCA) and dissolved in 1 M Tris pH 8.3. Two-hundred µl of the crude protein extracts were applied to Nanosep 3K Omegacentrifuge filters (Pall Corporation, Ann Arbor, MI) and centrifuged at 5000 g for 30 min at room temperature (20 °C). Hereafter, the Filter Aided Sample Preparation (FASP) method (Manza *et al.*, 2005; Wisniewski *et al.*, 2009) was used to generate tryptic peptides for LC-MS/MS analysis. The peptide solutions were acidified by adding 3.5 ul 0.1 % trifluoro-acetic acid and analysed by LC-MS/MS as described previously (Lu *et al.*, 2011). LCMS runs with all MSMS spectra obtained were analyzed with MaxQuant 1.1.1.36 (Cox and Mann, 2008) using default settings for the Andromeda search engine (Cox *et al.*, 2011) except that extra variable modifications were set for de-amidation of N and Q. The *Z. tritici* database stored at the JGI Genome Portal (genome.jgi.doe.gov/Mycgr3/Mycgr3.home. html) was used together with a database that contains sequences of common contaminants such as for instance: BSA (P02769, bovin serum albumin precursor), Trypsin (P00760, bovin), Trypsin (P00761, porcin), Keratin K22E (P35908, human), Keratin K1C9 (P35527, human), Keratin K2C1 (P04264, human) and Keratin K1CI (P35527, human). The "label-free quantification" as well as the "match between runs" (set to 2 minutes) options were enabled. De-amidated peptides were allowed to be used for protein quantification and all other quantification settings were kept default. Filtering and further bioinformatic analysis of the MaxQuant/Andromeda workflow output and the analysis of the abundances of the identified proteins were performed with the Perseus module (available at the MaxQuant suite) as described previously (Kariithi *et al.*, 2012). Accepted were peptides and proteins with a false discovery rate (FDR) of less than 1% and proteins with at least 2 identified peptides of which one should be unique and one should be unmodified. Reversed hits and contaminants were deleted from the MaxQuant result table. The relative protein quantitation of WT to mutant was done with Perseus by applying a two sample T-test using the "LFQ intensity" columns obtained with threshold 0.05 and S0=1. The normal logarithm was taken from normalised label free quantitation protein MS1 intensities

(LFQ) as obtained from MaxQuant. Zero values for one of the two LFQ columns were replaced by a value of 2.4 to make sensible ratio calculations possible.

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Supporting Information

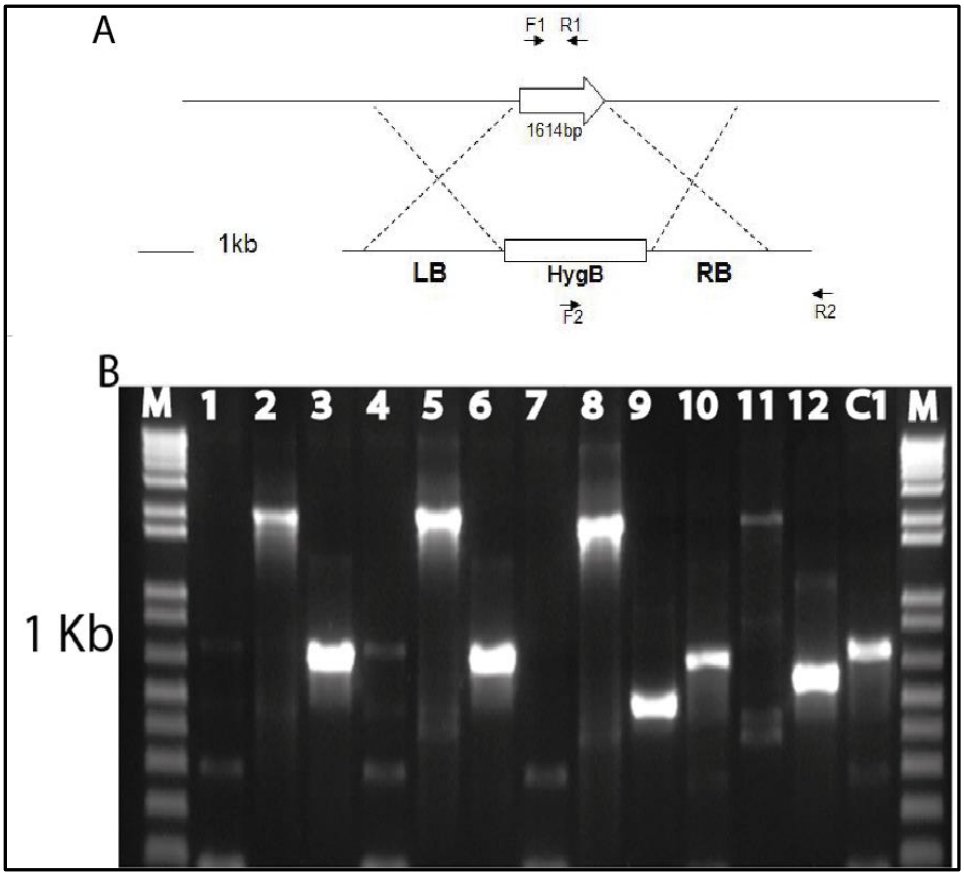

Figure S1. Replacement strategy for *ZtWor1* in *Zymoseptoria tritici.* **A**, Diagram showing the replacement by the hygromycin phosphotransferase (*Hph*) resistance cassette through homologous recombination. The dotted line depicts the flanking regions used for homologous recombination. **B,** Identification of replacement mutants by PCR; Lane M, 1-kb-plus ladder marker. Lanes 1, 4 and 7 show three independent replacement mutants (Δ*ZtWor1-1*, Δ*ZtWor1*-26 and *ΔZtWor1-29*) with no amplicon by using primers ZtWor1-F1 and ZtWor1-R1, while the WT strain (lane C1) and the complemented strain, Δ*ZtWor1-com7* (lane10), show the expected amplicon of 650 bp with the same primers. Primers ZtWor1-F2 and R2 that are located in the middle of the *Hph* gene and downstream of the *ZtWor1* ORF produced an amplicon of 2,000 bp (lanes 2, 5 and 8), but did not result in amplification in the wild type strain (C1) and the complemented strain (lane 11).

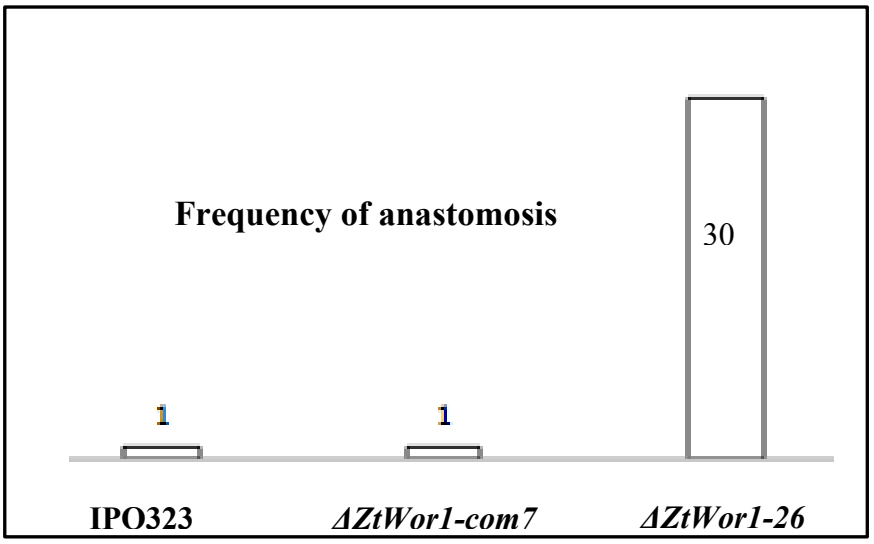

Figure S2. The number of cell fusion events, counted in 0.016 mm^2, in colonies of *Zymoseptoria tritici* WT strain, *ΔZtWor1-com7* and *ΔZtWor1-26*, grown on MM for 10 days at 20 °C.

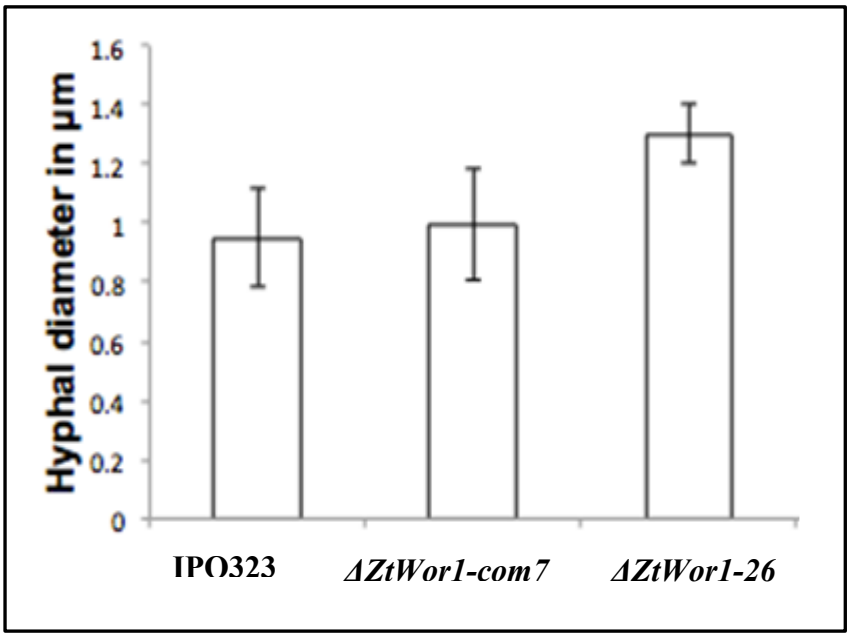

Figure S3. The differences in hyphal diameters of *Zymoseptoria tritici* WT strain, *ΔZtWor1-com7* and *ΔZtWor1-26*, grown on MM for 10 days. N=100 for each strain. The difference is significant at $P < 0.05$

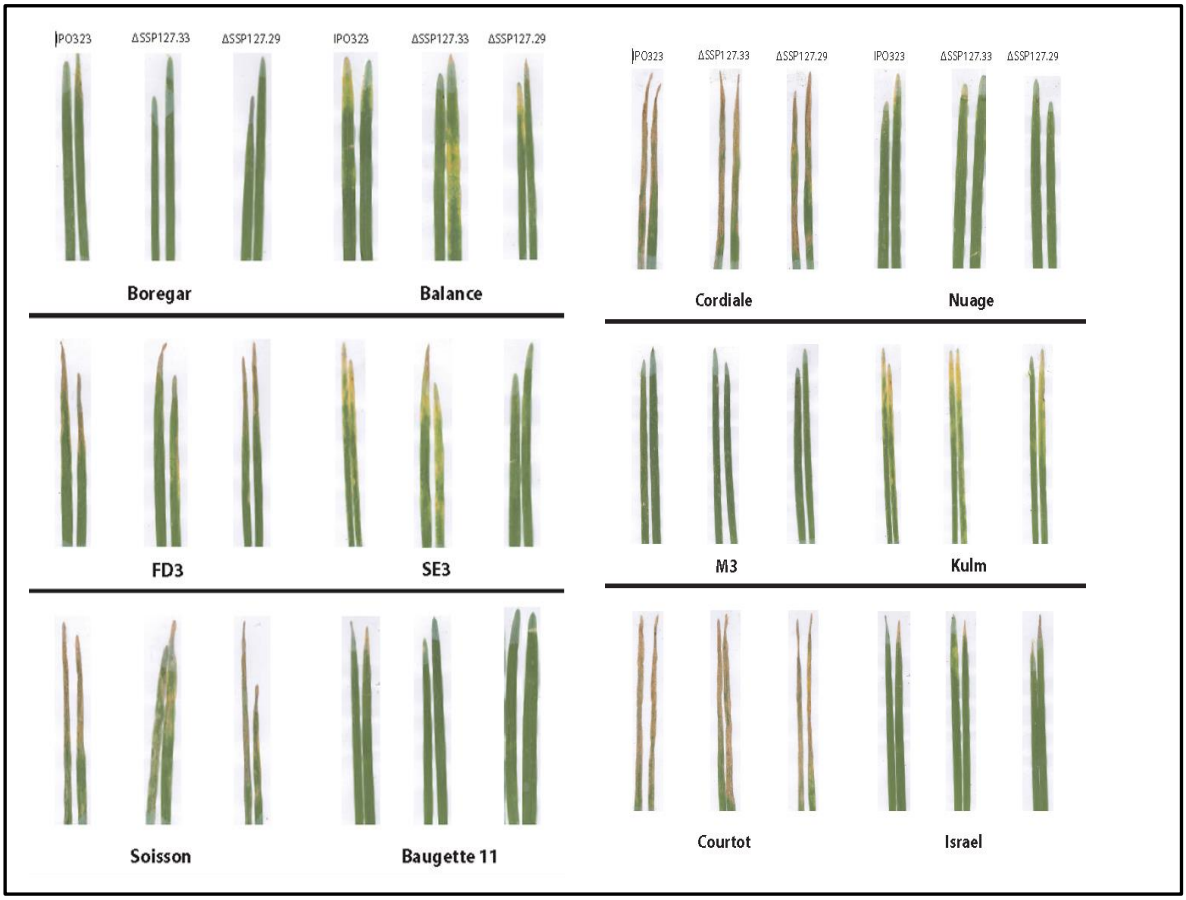

Figure S4. Disease development in 12 wheat cultivars that are parents of mapping populations after inoculation with *Zymospetoria tritici* WT strain compared with two independent knock-out strains of *SSP127*.

Table S1. Putative small-secreted proteins (SSPs) and their corresponding primers used in this study.

Protein	JGI Id	Primers used in q-PCR	
SSP2	79161	Q-SSP2-F	GAAGACTTCCAATCCGAACG
		Q-SSP2-R	TTGCAGATGATAACGCCTTG
SSP3	79286	Q-SSP3-F	TGATCTCCCGGTGTGTTTTG
		Q-SSP3-R	CACAACCTCCGTCATTTTCG
SSP4	80332	Q-SSP4-F	CGCCAATAACTCACCATGC
		Q-SSP4-R	ACGATTGGTTGAAGCAGAGC
SSP5	82029	Q-SSP5-F	AGAGGTTCAATTGCGACAGC
		Q-SSP5-R	TTGTTGATCAGGTCGGTCTTC
SSP6	82925	Q-SSP6-F	AATCTCGGCGTGTATCGAAG
		Q-SSP6-R	CATCATCAACGAACCTGCAC
SSP7	83081	Q-SSP7-F	CGCTTTCAACAAACCTTGC
		Q-SSP7-R	ATGTGCCGGTGTGGTAGTTG
SSP8	87205	Q-SSP8-F	CAACTTCATCTGCCTCATGG
		Q-SSP8-R	GATCTCCACCACAGCAGAATC
SSP10	99124	Q-SSP10-F	AAGGGCATATGTTGCCTTCC
		Q-SSP10-R	TCAACAAGGGAAGACGTTGG
SSP13	100649	Q-SSP13-F	AGCTTTCGGTGCTTCTTAGC
		Q-SSP13-R	CGAAAGCAGTACATCGAAGG
SSP15	102792	Q-SSP15-F	TGAAGATCTCGGTCATGCTG
		Q-SSP15-R	CTTGAACGCTGTCGCATATC
SSP17	102996	Q-SSP17-F	GTCAATCACGGCATGAACTG
		Q-SSP17-R	AGAGGAAGGTTTGGCACATC
SSP18	103572	Q-SSP18-F	TGATGGCTGCTACTGCTTTC
		Q-SSP18-R	TTGCACTCGTGATAGTGGTG
SSP19	103713	Q-SSP19-F	ACCAGGATGTTGCTCCAAAC
		Q-SSP19-R	CGAGCGGGTATTTTGAAGAG
SSP22	104383	Q-SSP22-F	GTTCTCCAACGTCGTCCTTC
		Q-SSP22-R	TCCTAGTCACGTCGCATTTG
SSP27	106125	Q-SSP27-F	TTCCCTGGCTGTACCTCAAC
		Q-SSP27-R	CGTGGCATTTGAAGTTGGTC
SSP31	106502	Q-SSP31-F	CAATGCCTGAAGGATTGTCC
		Q-SSP31-R	CGGTTTTGGCAGTAATCAGG

SSP33	108877	Q-SSP33-F	AGCTCTCCATGGCATTCATC
		Q-SSP33-R	AATGCCGTTTGGGTATTCG
SSP35	110386	Q-SSP35-F	CTGGATTTGCGCCTACAAAC
		Q-SSP35-R	GGATTGGGGATTTGAGGTTG
SSP39	104283	Q-SSP39-F	AAGCCCAATGCCCTCTAATC
		Q-SSP39-R	TGGGAGTACTCGGAGATGTTG
SSP40	104404	Q-SSP40-F	TTTGGCCTCAAGTCATGCTC
		Q-SSP40-R	GAATGACAGCGATGCTGAAG
SSP46	105478	Q-SSP46-F	TGCAATGTGCACTGCTGTAG
		Q-SSP46-R	CTGCCATAACCCGAAGAAAC
SSP49	105677	Q-SSP49-F	AACAACAACGTCCTCCAAGG
		Q-SSP49-R	GTCTTTGCCGATCCAGAATG
SSP53	111505	Q-SSP53-F	TAAACTCGATGCCGGAACTG
		Q-SSP53-R	AAGTCGGAACATGGAGCTTG
SSP55	109137	Q-SSP55-F	ACAACACGGAGGACAATGAG
		Q-SSP55-R	ACGAAGTTGTCGTAGGTCGTG
SSP56	107904	Q-SSP56-F	ACAAGGGACCACGTCAAATC
		Q-SSP56-R	AGCCATTCGGAAACTGGTC
SSP60	109710	Q-SSP60-F	CACCAACCTTGAGACAATCG
		Q-SSP60-R	TGTGTATGGAATCCCAGCAG
SSP69	111203	Q-SSP69-F	TCAAGAGACAGCACGATTGC
		Q-SSP69-R	CTTGATTTCGGTCAGTCCATC
SSP70	85504	Q-SSP70-F	ACGGTCTCATCACCAGATACG
		Q-SSP70-R	CGCATTGATCGCTGTTGTAG
SSP2	79161	Q-SSP2-F	GAAGACTTCCAATCCGAACG
		Q-SSP2-R	TTGCAGATGATAACGCCTTG
SSP127	101740	Q-SSP127-F	TTCCCCATCAAGTTCACCTC
		Q-SSP127-R	TTGAGGCCGAAGTCGTAGTAG

Table S2. Comparative expression profiling of small-secreted proteins (SSPs) in the *Z. tritici* WT strain versus *ΔZtWor1-26*.

Protein	Expression in the *Z. triici* strain	Expression in *ΔZtWor1-26*	Up-regulated (Folds)	Down-regulated (Folds)	No effect
SSP2	2.35	2.39			----------
SSP3	0.59	0.20		2.5 fold	
SSP4	0.63	0.45		1.4 fold	
SSP5	0.27	0.17		1.5 fold	
SSP6	8.05	2.90		2.7 fold	
SSP7	0.17	0.07		2.4 fold	
SSP8	0.05	0.07		0.71 fold	
SSP10	1.47	2.27			----------
SSP13	0.47	0.33		1.42 fold	
SSP15	0.27	0.17			----------
SSP17	0.27	0.01		20 fold	
SSP18	0.21	0.11		1.9 fold	
SSP19	0.30	0.16		1.8 fold	
SSP22	0.30	0.09		3.3 fold	
SSP27	1.6	2.6			----------
SSP31	0.85	0.52		1.6 fold	
SSP33	0.30	0.11		2.7 fold	
SSP35	0.78	0.53		1.4 fold	
SSP39	20.43	18.38			----------
SSP40	2.48	4.69	1.9 fold		
SSP46	0.06	0.05		1.2 fold	
SSP49	1.9	0.80			----------
SSP53	1.09	0.38		5 fold	
SSP55	3.59	1.99			----------
SSP56	0.71	0.27		2.6 fold	
SSP60	1.6	0.07		22 fold	
SSP69	0.05	0.01		5 fold	
SSP70	9.4	3.8		2.4 fold	
SSP127	0.84	0.005		168 fold	

Chapter 4

Effector discovery in the fungal wheat pathogen *Zymoseptoria tritici*

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Summary

Fungal plant pathogens such as *Zymoseptoria tritici* (formerly known as *Mycosphaerella graminicola*) secrete repertoires of effectors facilitating infection or triggering host defense mechanisms. Discovery and functional characterization of effectors renders valuable knowledge that contributes to designing new and effective disease management strategies. Here, we combined bioinformatics approaches with expression profiling during pathogenesis to identify candidate effectors of *Z. tritici*. Additionally, a genetic approach was conducted to map quantitative trait loci (QTL) carrying putative effectors enabling the validation of both complementary strategies for effector discovery. *In planta* expression profiling revealed that candidate effectors were up-regulated in successive waves corresponding with consecutive stages of pathogenesis, contrary to candidates identified by QTL mapping that were overall lowly expressed. Functional analyses of two top candidate effectors (SSP15 and SSP18) showed their dispensability for *Z. tritici* pathogenesis. These analyses reveal that generally adopted criteria such as protein size, cysteine residues and expression during pathogenesis may preclude an unbiased effector discovery. Indeed, genetic mapping of genomic regions involved in specificity render alternative effector candidates that do not match the aforementioned criteria, but should nevertheless be considered as promising new leads for effectors that are crucial for the *Z. tritici*-wheat pathosystem.

Introduction

Zymoseptoria tritici (Desm.) Quaedvlieg & Crous (Quaedvlieg *et al.*, 2011), the causal agent of septoria tritici blotch (STB) disease, is a major threat for global wheat production (Eyal, 1999). This foliar blight frequently occurs in many countries throughout the world, but particularly in regions with high rainfall and moderate temperatures where the disease is responsible for significant yield losses causing very high direct and indirect costs representing millions of Euro's for disease control (Eyal, 1987). Over the last decade *Z. tritici* emerged as a genetic model for the Dothideales (Goodwin *et al.*, 2004) due to its finished genome sequence (Goodwin *et al.*, 2011) and detailed genetic studies (Kema *et al.*, 2002; Kema *et al.*, 1996c; Linde *et al.*, 2002; Mirzadi Gohari *et al.*, 2014; Wittenberg *et al.*, 2009) and was recently placed in the top ten of the most important global plant pathogens (Dean *et al.*, 2012).

Z. tritici has a hemibiotrophic lifestyle with two distinct colonization phases, a stealth biotrophic and ramifying necrotizing pathogenesis, in which various aspects of growth and differentiation can be studied in detail using a range of biological and molecular tools.

Following stomatal penetration, the initial symptomless biotrophic phase where hyphae colonize the extracellular space lasts for about 10 days post infection (dpi). The transition to necrotrophy coincides with the formation of small chlorotic lesions that gradually expand and coalesce into large necrotic blotches bearing abundant pycnidia, the asexual fructifications that contain the splash-born pycnidiospores (Orton *et al.*, 2011). An array of pathogen derived toxic compounds is suggested to be actively secreted into the apoplast but the accurate events and mechanisms underlying this complex phase are poorly understood (Cohen and Eyal, 1993; Kema *et al.*, 1996d). The genetic diversity in natural populations of the fungus is driven by the sexual process that comprises several cycles within a single growing season and results in extraordinary diverse airborne inoculum (Chen and McDonald, 1996; Wittenberg *et al.*, 2009; Zhan *et al.*, 2003). In addition, *Z. tritici* produces splash-dispersed asexual conidia during the growing season that disseminate over shorter distances and results in largely clonal foci (Hunter *et al.*, 1999; Kema *et al.*, 1996c; Suffert and Sache, 2011; Zhan *et al.*, 2003).

Z. tritici is pathogenic on both hexaploid bread wheat (*Triticum aestivum* L., AABBDD, 2n=42) and tetraploid durum wheat (*T. turgidum* L. (Thell.) subsp. *durum* L., AABB, 2n=28) (Kema *et al.*, 1996a) as well as various grass species (Stukenbrock *et al.*, 2007). Interestingly, isolates of *Z. tritici* exhibit a high degree of both host species specificity and cultivar specificity (Kema *et al.*, 1996a; Kema *et al.*, 1996b; Kema and van Silfhout, 1997). These are hierarchical levels of pathogenicity. Host species specificity in *Z. tritici* refers to avirulence to the vast majority of wheat cultivars of a wheat species. Thus, the majority of durum wheat cultivars is highly resistant to the majority of *Z. tritici* isolates originating from bread wheat, whereas the majority of bread wheat cultivars is highly resistant to the majority of isolates originating from durum wheat cultivars. Cultivar specificity is at a lower hierarchical level and refers to avirulence on particular cultivars within these wheat species (Eyal *et al.*, 1973; Saadaoui, 1987; van Ginkel and Scharen, 1988). A gene-for-gene interaction for cultivar specificity in *Z. tritici* to bread wheat has been proven where host resistance and pathogenicity were controlled by complementary single genetic loci (Brading *et al.*, 2002; Kema *et al.*, 2002). However, the genes controlling host species and cultivar specificity have thus far not been identified.

Fungal effector molecules are small-secreted proteins (SSP) that modulate physiological and morphological processes in the plant hosts, thus promoting infection or triggering defense responses (Rep, 2005). This dual biological activity of effectors that can function as virulence or avirulence factors has been widely accepted to determine the eventual

outcome of interactions between pathogens and their associated hosts (Bent and Mackey , 2007; Stergiopoulos and de Wit, 2009). Hence, the discovery and functional characterization of effectors can principally render valuable knowledge that eventually will contribute to designing new and effective disease management and resistance breeding strategies (Vleeshouwers and Oliver, 2014). The majority of characterized effectors in plant pathogenic fungi share similar structural features that can be used for their identification. Candidate effectors are usually small proteins (less than 300 amino acids, aa) containing cysteine residues and an N-terminus signal peptide that is required for extracellular secretion, here collectively called small-secreted proteins (SSPs). It is well documented that some effectors are broadly present in different taxa, such as Ecp6 (de Jonge and Thomma, 2009) whereas others are unique and specific to an individual fungal species, such as AVRPiz-t of *Magnaporthe oryzae* (Park *et al.,* 2012). Despite their polymorphism, homologs of some effector proteins such as Ecp6, Ecp2 and *Avr4* - small-secreted proteins of the tomato pathogen *Cladosporium fulvum* - were found in *Z. tritici* as well as the banana black leaf streak pathogen *Mycosphaerella fijiensis* (Bolton *et al*., 2008; Stergiopoulos *et al*., 2010, 2012, 2014). Nevertheless, the identification of fungal effectors through homology analyses is complicated due to their low conservation as compared to the identification of resistance genes analogs (Chisholm *et al.*, 2006; Dangl and Jones, 2001). Hence, several complementary approaches have been employed to successfully identify functional SSPs in plant pathogenic fungi, including genetic analyses, bioinformatic cataloguing and functional genomics. For instance, a combined bioinformatic and RNA sequencing approach resulted in the discovery of *Avr5* in the fungal tomato pathogen *C. fulvum* (Mesarich *et al.,* 2014). For others map-based strategies were used to clone effector genes, such as *AvrLm1, AvrLm6* and *AvrLm11*, in the oilseed rape pathogen *Leptosphaeria maculans* (Balesdent *et al.*, 2013; Fudal *et al.*, 2007; Gout *et al.*, 2006; Van de Wouw *et al.*, 2014).

In *Z. tritici* two effectors were identified by the functional analyses of homologues of the well-known effector proteins MgNLP and Mg3LysM, which were functionality analyzed by knock-out and heterologous protein expression strategies. MgNLP belongs to the necrosis and ethylene-inducing peptide 1 (Nep1)-like protein family (NLP), but it is not instrumental for virulence of *Z. tritici.* However, its expressed protein in *Pichia pastoris* triggered cell death and the activation of defense-related genes in *Arabidopsis* leaves (Motteram *et al.*, 2009), and Mg3LysM plays an essential role in establishing the initial symptomless biotrophic phase of *Z. tritici* (Marshall *et al.*, 2011).

We have previously developed a robust protocol to cross *Z. tritici* isolates providing an excellent tool for generating mapping populations and their analyses and deployment in genome assembly (Goodwin *et al.*, 2011; Wittenberg *et al.*, 2009). Here, we report on the cataloguing of SSPs, subsequent expression profiling during pathogenesis and eventually a complementary quantitative trait locus (QTL) mapping approach to identify whether candidate effectors map to these regions on the *Z. tritici* genome. The analyses result in a list of promising SSPs that remain to be further explored in future studies. However, they also indicate an intriguing ambiguity between bioinformatics and expression profiling driven SSPs identification and characterization versus map based strategies, thereby questioning the potential of unbiased sequence based strategies for effector discovery.

Results

Identification of candidate effectors

In order to build a comprehensive list of conceivable SSPs, we followed two strategies. First, we mined the genome of *Z. tritici* that resulted in identification of 266 secreted proteins with size of ≤300 aa and ≥4 cysteine residues. Twenty-four were predicted to possess TM (transmembrane) domains outside the signal peptide sequence and were therefore excluded from the list. Subsequently, the EST database, which is accessible via JGI genome browser (http://genome.jgi-psf.org/Mycgr3/Mycgr3.home.html), was used to further narrow down the list to 68 SSPs with transcript support (Table 1). Secondly, we used the *in vitro* secretome of *Z. tritici* (Mirzadi Gohari *et al.*, 2014) for another round of independent SSP identification. This resulted in the identification of 114 extracellular proteins of which 94 were supported by EST analyses and eventually - after using the abovementioned criteria - we narrowed this number down to 30 candidates. Interestingly, both strategies resulted in two largely complementing sets of candidates as the overlap was only nine SSPs. Eventually, we selected the entire set of 68 candidates from the *in silico* bioinformatics approach and supplemented this with 10 randomly selected candidates from the secretome analysis, resulting in a total of 78 SSPs for expression profiling (Table 1).

Z. tritici SSPs show expression profiles that correspond with infection stages

Ninety-three percent of the selected SSP-encoding genes were supported by EST data generated either under *in vitro* or *in planta* conditions (Kema *et al.*, 2008; Keon *et al.*, 2005). Here, we used reverse-transcription quantitative polymerase chain reaction (RT-qPCR) analysis to determine their *in planta* expression profiles during pathogenesis. All genes, including those without previous

EST support, were transcribed *in planta* and their profiles corresponded remarkably well with the (i) early stage of infection or biotrophic phase (2 and 4 dpi), (ii) the transition from biotrophy to necrotrophy (8 dpi) and (iii) the necrotrophic phase (>8dpi) (Fig. S1, see Supporting Information). For example SSP42 was strongly induced (around 20x) during biotrophy at 2dpi (Fig. 1A). The SSPs specifically expressed during the transition phase, usually accompanied by early macroscopic chlorosis, included SSP15, whose expression at 8dpi was 120x (Fig. 1B).

Table 1. Overall characterization of the *Zymoseptoria tritici* small-secreted proteins (SSPs) used in this study.

Protein	Protein ID	EST support	Location on Chromosome	Length	No. Cys	PFAM domain	*Z. tritici* specific	Approach to identify
SSP1	73448	Y	6	180	8	N	Y	Bioinformatics analysis
SSP2	79161	Y	1	69	7	N	Y	Bioinformaticsanalysis
SSP3	79286	Y	2	64	8	N	Y	Bioinformatics analysis
SSP4	80332	Y	4	77	6	N	Y	Bioinformatics analysis
SSP5	82029	Y	9	67	7	N	Y	Bioinformatics analysis
SSP6	82925	Y	12	58	6	N	Y	Bioinformatics analysis
SSP7	83081	Y	13	54	6	N	Y	Bioinformatics analysis
SSP8	87205	Y	8	56	9	N	Y	Bioinformatics analysis
SSP9	98580	Y	1	271	8	CFE domain	N	Bioinformatics analysis
SSP10	99124	Y	2	113	7	N	Y	Bioinformatics analysis
SSP11	99161	Y	2	165	18	N	N	Bioinformatics analysis
SSP12	99676	Y	3	169	5	N	N	Bioinformatics analysis
SSP13	100649	Y	7	76	8	N	Y	Bioinformatics analysis
SSP14	102617	Y	1	159	13	N	Y	Bioinformatics analysis
SSP15	102792	Y	1	115	8	N	Y	Bioinformatics analysis
SSP16	102849	Y	1	286	6	N	N	Bioinformatics analysis
SSP17	102996	Y	1	164	8	N	Y	Bioinformatics analysis
SSP18	103572	Y	3	67	7	N	Y	Bioinformatics analysis
SSP19	103713	Y	3	104	4	N	Y	Bioinformatics analysis
SSP20	103792	Y	3	197	16	PAN domain	N	Bioinformatics analysis
SSP21	104000	Y	4	181	6	N	N	Bioinformatics analysis
SSP22	104383	Y	5	75	8	N	Y	Bioinformatics analysis
SSP23	104444	Y	5	80	10	N	Y	Bioinformatics analysis
SSP24	104758	Y	6	119	10	N	Y	Bioinformatics analysis
SSP25	105182	Y	7	144	6	N	N	Bioinformatics analysis
SSP26	105826	Y	10	99	8	N	Y	Bioinformatics analysis
SSP27	106125	Y	11	71	6	N	Y	Bioinformatics analysis

SSP28	106127	Y	11	98	6	N	Y	Bioinformatics analysis
SSP29	106260	Y	12	110	16	N	N	Bioinformatics analysis
SSP30	106445	Y	13	120	10	N	Y	Bioinformatics analysis
SSP31	106502	Y	13	90	8	N	Y	Bioinformatics analysis
SSP32	107286	Y	1	117	6	N	Y	Bioinformatics analysis
SSP33	108877	Y	3	112	6	N	Y	Bioinformatics analysis
SSP34	110220	Y	7	132	6	N	Y	Bioinformatics analysis
SSP35	110386	Y	8	195	10	LysM domain	N	Bioinformatics analysis
SSP38	103900	Y	3	130	8	N	Y	Bioinformatics analysis
SSP39	104283	Y	4	227	12	N	N	Bioinformatics analysis
SSP40	104404	Y	5	180	4	Ecp2	N	Bioinformatics analysis
SSP41	104697	Y	5	150	4	N	N	Bioinformatics analysis
SSP42	104794	Y	6	158	4	N	N	Bioinformatics analysis
SSP43	104867	Y	6	171	4	N	N	Bioinformatics analysis
SSP44	105223	Y	7	189	5	N	N	Bioinformatics analysis
SSP45	105265	Y	7	200	10	N	N	Bioinformatics analysis
SSP46	105478	Y	8	136	9	N	N	Bioinformatics analysis
SSP47	105487	Y	8	98	4	LysM domain	N	Bioinformatics analysis
SSP48	105659	Y	9	183	4	N	Y	Bioinformatics analysis
SSP49	105677	Y	9	199	4	N	N	Bioinformatics analysis
SSP50	106106	Y	11	157	6	N	N	Bioinformatics analysis
SSP51	106335	Y	12	239	5	N	N	Bioinformatics analysis
SSP52	106345	Y	12	164	4	N	N	Bioinformatics analysis
SSP53	111505	Y	12	198	4	N	N	Bioinformatics analysis
SSP54	108976	Y	4	201	6	N	N	Bioinformatics analysis
SSP55	109137	Y	4	155	4	N	N	Bioinformatics analysis
SSP56	107904	Y	2	171	6	Ecp2	N	Bioinformatics analysis
SSP57	108329	Y	2	193	6	N	N	Bioinformatics analysis
SSP58	108482	Y	3	109	10	N	N	Bioinformatics analysis
SSP59	110756	Y	9	247	7	Cerato-platanin	N	Bioinformatics analysis
SSP60	109710	Y	6	201	5	SCP domain	N	Bioinformatics analysis
SSP61	111636	Y	13	160	4	Ecp2	N	Bioinformatics analysis
SSP62	111352	Y	11	165	6	N	Y	Bioinformatics analysis
SSP63	111008	Y	10	220	4	N	N	Bioinformatics analysis
SSP64	111027	Y	10	152	4	N	N	Bioinformatics analysis
SSP65	67799	Y	2	273	7	N	N	Bioinformatics analysis
SSP66	70376	Y	3	203	4	N	N	Bioinformatics analysis
SSP67	76021	Y	10	280	7	Peptidase-M43	N	Bioinformatics analysis
SSP68	71724	Y	5	274	6	Glyco-hydro	N	Bioinformatics analysis
SSP69	111203	Y	11	155	6	N	Y	Bioinformatics analysis

SSP70	85504	N	4	150	4	Cerato-platanin	N	Bioinformatics analysis
SSP126	110887	Y	9	284	14	N	Y	Secretome analysis
SSP128	86778	Y	7	132	5	N	Y	Secretome analysis
SSP134	106446	Y	13	120	10	N	Y	Secretome analysis
SSP138	100429	Y	6	164	1	N	Y	Secretome analysis
SSP139	41491	N	5	82	8	N	N	Secretome analysis
SSP140	104441	Y	5	285	6	N	Y	Secretome analysis
SSP142	103877	Y	3	169	5	N	N	Secretome analysis
SSP146	91995	N	3	168	5	N	N	Secretome analysis
SSP147	96868	N	11	157	4	N	N	Secretome analysis
SSP160	92365	N	4	107	8	PI-PLC-X	N	Secretome analysis
SSP71	90699	N	2	154	6	N	N	QTL analysis
SSP74	103274	N	2	177	3	N	Y	QTL analysis
SSP77	79484	Y	2	73	4	N	Y	QTL analysis
SSP84	102982	Y	1	107	2	N	Y	QTL analysis
SSP85	102983	Y	1	55	0	N	Y	QTL analysis
SSP89	41315	Y	4	196	6	N	N	QTL analysis
SSP91	71216	Y	4	197	1	N	N	QTL analysis
SSP100	104754	Y	6	164	1	N	Y	QTL analysis
SSP101	93741	N	6	160	0	N	N	QTL analysis
SSP103	72923	Y	6	113	0	N	N	QTL analysis
SSP112	94597	N	7	163	1	N	N	QTL analysis
SSP114	83064	Y	13	75	6	N	Y	QTL analysis
SSP116	97500	Y	13	138	10	N	Y	QTL analysis
SSP118	97526	N	13	206	12	N	Y	QTL analysis
SSP125	93501	N	5	106	0	N	N	QTL analysis
SSP150	71681	Y	5	410	4	Cellulase	N	QTL analysis
SSP151	58567	Y	5	251	2	Adh-short	N	QTL analysis
SSP152	41969	N	5	545	11	PLA2B	N	QTL analysis
SSP153	104341	Y	5	146	1	N	Y	QTL analysis
SSP154	92954	N	5	363	2	N	N	QTL analysis
SSP155	42164	Y	5	302	8	HRXXH	N	QTL analysis
SSP156	100094	Y	5	303	0	ZIP	N	QTL analysis

Cys, cysteine; EST, expressed sequence tag; QTL, quantitative trait locus.

The group expressed during necrotrophy included for instance SSP44. Its expression started at 8 dpi, peaked at 12 dpi and subsequently dropped-off until 20 dpi (Fig. 2A), whereas the expression of other SSPs peaked at 8 dpi and then steadily decreased until 20 dpi (Fig. 2B). In addition to these profiles only four SSPs were particularly up-regulated at the very end of pathogenesis (20 dpi), a phase that is characterized by the development of abundant pycnidia, the asexual fructifications of *Z. tritici* (Fig. S2, see Supporting Information).

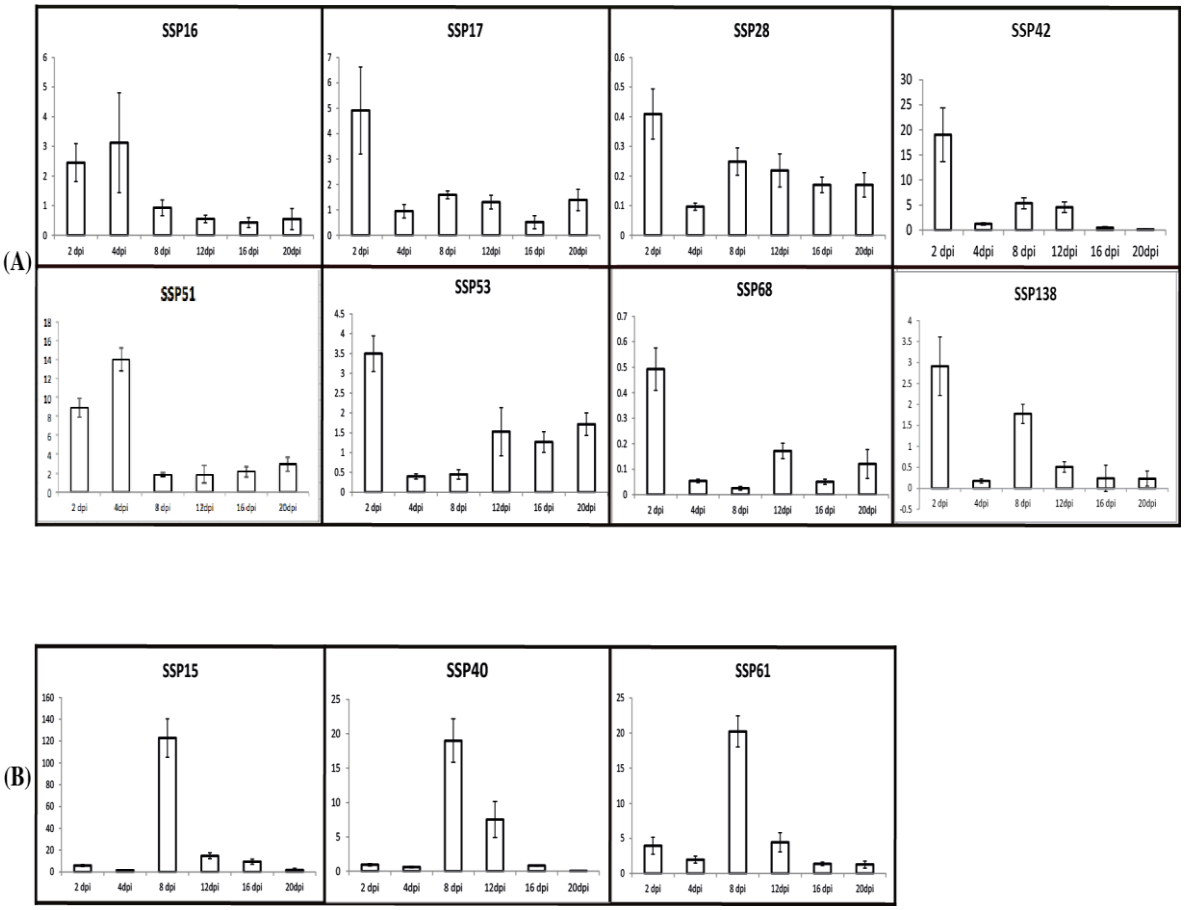

Figure 1. (A) Relative *in planta* expression profiling of *Zymoseptoria tritici* small-secreted proteins (SSPs) that are up-regulated during biotrophy. (B) The transcription levels of the *Z. tritici* SSPs that are exclusively up-regulated during the transition to necrotrophy. dpi, days post-infection.

In summary, 42% of the selected SSPs was specifically expressed during necrotrophy (Figs 2, 3; Fig. S3, see Supporting Information) and 21% was expressed during biotrophy and necrotrophy (Fig. 3; Fig. S4, see Supporting Information). Finally, 18% of selected SSPs was lowly transcribed (less than 1x) throughout the entire infection process (Fig. 3; Fig. S5, see Supporting Information).

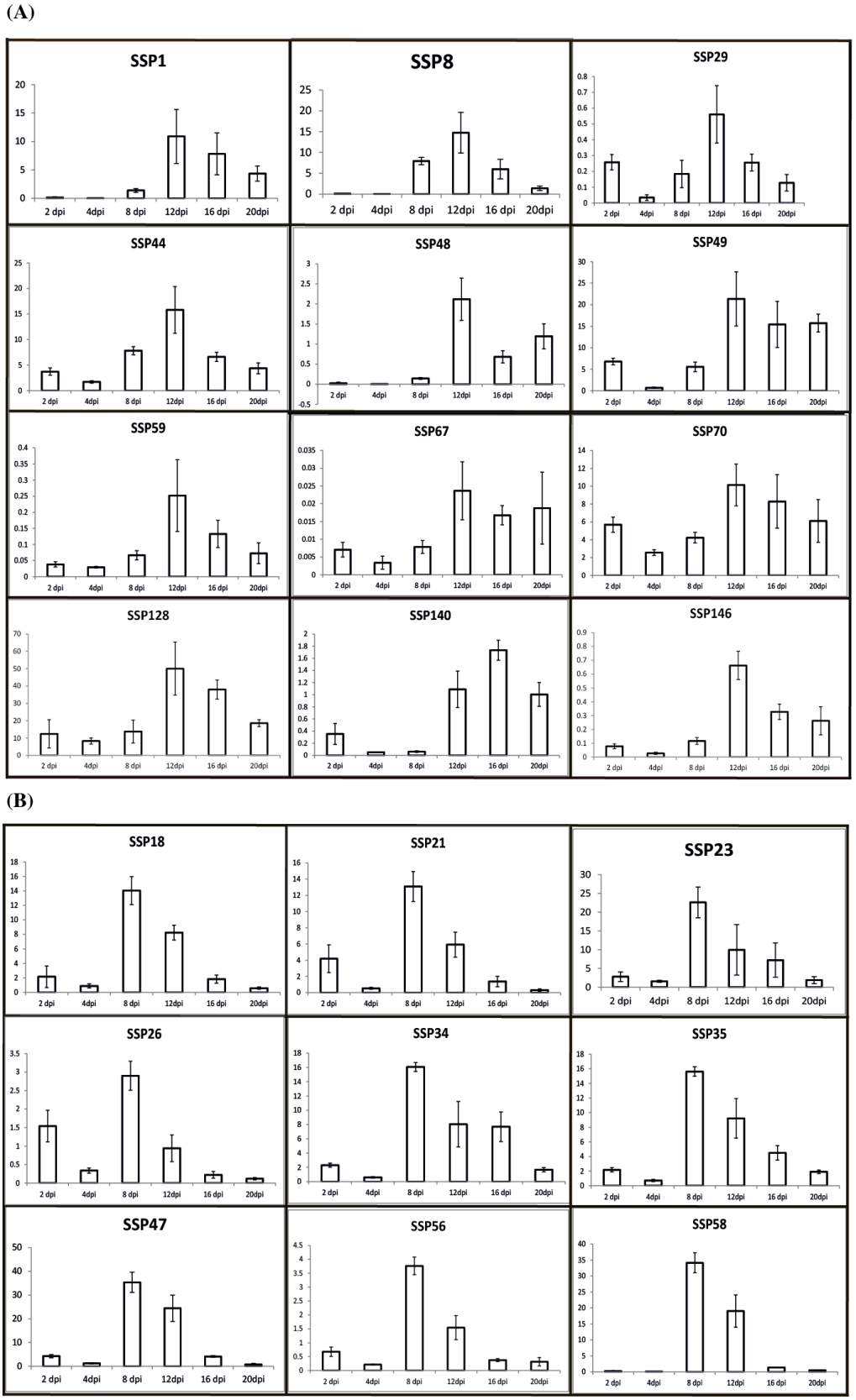

Figure 2. Relative *in planta* expression profiling of *Zymoseptoria tritici* small-secreted proteins (SSPs) that are specifically up-regulated during necrotrophy: (A) SSPs that peak at 12 days post-infection (dpi); (B) SSPs that peak at 8 dpi and subsequently decrease steadily towards 20 dpi.

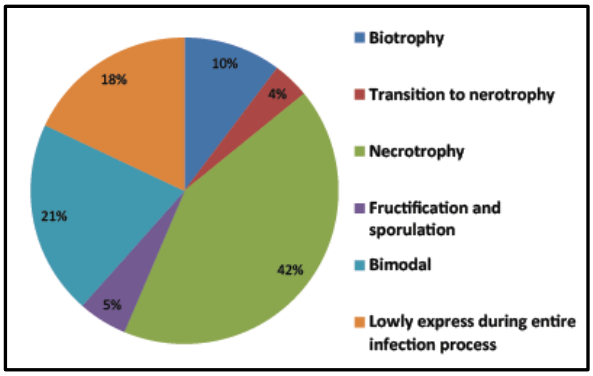

Figure 3. The majority of studied *Zymoseptoria tritici* small-secreted proteins (SSPs) were highly expressed during necrotrophy. Each section corresponds to the percentage of studied SSPs that are up-regulated during biotrophy [0–4 days post-infection (dpi)], the transition phase to necrotrophy (4–8 dpi) and necrotrophy (>8 dpi). Also shown are sections with SSPs particularly important during fructification and sporulation (20 dpi), with a bimodal expression pattern (during biotrophy and necrotrophy), and SSPs that are expressed at low levels throughout the entire infection process.

Functional analyses of *Z. tritici* SSP15 and SSP18 reveal their dispensability for pathogenicity

Since SSP15 and SSP18 were highly expressed at 8 dpi (120x) and (14x), respectively, we generated three independent knock-out mutants for each gene by homologous recombination to determine their role during pathogenesis. At 21 dpi the developed phenotypes of the knock-out strains and the WT were similar on each evaluated wheat cultivar, indicating that SSP15 and SSP18 are dispensable for pathogenesis (Fig. S6, see Supporting Information). However, daily comparison of symptom development between the *SSP15ΔIPO323* and the WT strains showed a slight delay in pathogenesis in wheat accession FD3 between 14-16 dpi (necrosis and pycnidia differences), suggesting a quantitative effect of SSP15 during the late phase of infection, but only in that specific wheat accession that is also used as a parent in a *Stb* mapping population (Goudemand *et al.*, 2013).

An unbiased quantitative trait loci approach results in SSPs that are lowly expressed during pathogenesis

As the bioinformatics- and proteomics-driven SSP approach did not reveal SSPs with a clear function in pathogenesis, despite their unique expression profiles, we alternatively considered a genetic approach to map candidate effectors. An existing mapping populations from a cross between the *Z. tritici* reference strain IPO323 and the Algerian durum wheat field strain IPO95052 (Goodwin *et al.*, 2011; Wittenberg *et al.*, 2009) resulted in a progeny of

163 isolates that was phenotyped on a suite of durum wheat and bread wheat cultivars (Table S1, see Supporting Information). Isolate IPO323 developed less than 1% leaf area covered by pycnidia (P) on the durum wheat cvs., but was highly pathogenic on the bread wheat cvs. (P values ranging between 56-71%). As expected, the other parental isolate IPO95052 showed opposite responses, as it was highly pathogenic on the durum wheat cvs. (P values ranging between 49-62%), but avirulent on the bread wheat cultivars (<1%P; Fig. 4A). Despite both parental isolates are avirulent on cv. Shafir, we included this cultivar in the phenotyping to study the independent segregation of host species and cultivar specificity. The progeny strains clearly showed a highly diverse range of pathogenicity with large qualitative and quantitative differences for host species and cultivar specificity (Fig. 4B). Among 163 progeny, 150 isolates represented recombinant phenotypes (Fig. 4B for examples), including isolates that were virulent or avirulent on all tested cultivars. Interestingly, nine isolates were virulent on bread wheat cv. Shafir despite the avirulence of both parental isolates on this cultivar.

(A)

	Durum wheat cultivars			Bread wheat cultivars			
	Volcani 447	Zenati Bouteille	Bidi 17	Taichung 29	Obelisk	Gerek 79	Shafir
IPO323	0.08±0.09	0.08±0.09	0.83±0.87	70.83±6.36	60.00±6.17	55.83±5.22	0
IPO95052	62.08±6.79	49.17±5.67	53.75±6.00	0.21±0.15	0	0.08±0.09	0

(B)

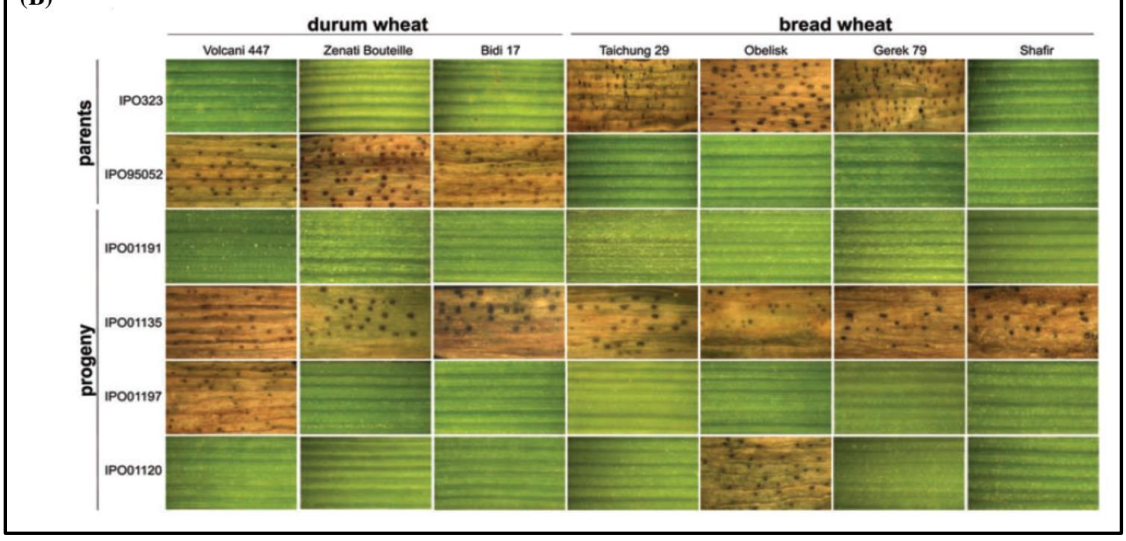

Figure 4. Phenotyping of *Zymoseptoria tritici* on wheat. (A) Percentages of primary leaf area covered with pycnidia averaged over 11 individual experiments. Grey highlighting indicates incompatible interactions. (B) Screening of the parental *Z. tritici* isolates IPO323 and IPO95052 and four progeny isolates from a cross between these strains on three durum wheat and four bread wheat cultivars.

Data analyses revealed a major QTL on chromosome 5 (LOD=17.56), covering genes controlling specificity for the durum wheat cvs. Volcani 447, Zenati Bouteille, and Bidi 17, as well as to the bread wheat cv. Shafir that explained up to 47% of the observed variation on these cultivars (Fig. 5, Table S1, see Supporting Information).

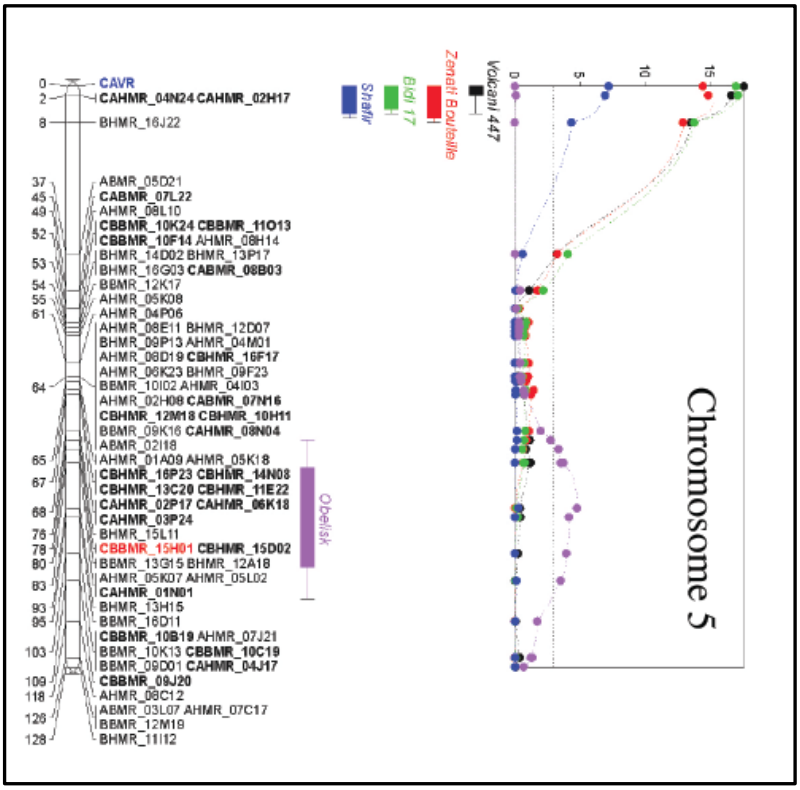

Figure 5. Quantitative trait loci (QTLs) for *Zymoseptoria tritici* pathogenicity measured by the percentage of foliage covered by pycnidia, the asexual fructifications that are positioned in the substomatal cavities, mapped on chromosome 5. The subtelomeric region carries a QTL with a logarithm of the odds (LOD) value of 17.56 that covers genes involved in cultivar or host specificity.

In addition, eight additional QTLs with lower, though significant LOD values were detected on seven other chromosomes (Fig. S7, see Supporting Information). Five of these eight QTLs control specificity for bread wheat cultivars and three for durum wheat cultivars (Table S1, see Supporting Information). None of the identified QTLs mapped to the dispensable chromosomes 14-21. Finally, we mapped the identified QTLs to the *Z. tritici* genome sequence and determined that the identified QTLs cover a total of 2795 genes (Table 2), comprising 64 secreted proteins containing signal peptides.

Table 2. Overview and characterization of mapped quantitative trait loci for P parameters (leaf area covered by pycnidia) *in Zymoseptoria tritici* with their physical boundaries on the genome and the protein families covered.

Chromosome number	QTL locus	QTL specificity and LOD value	QTL explained variance (%)	QTL position*	Number of predicted genes
Chromosome 2	QTL-P1	BW Taichung 29 (LOD ≥ 5.70)	15.1	1468524–3025189	586
				1703574–2109386 (LOD ≥ 4.6)	154
Chromosome 3	QTL-P2	BW Shafir (LOD ≥ 3.22)	8.6	3313278–3502029	69
Chromosome 4	QTL-P3	DW Volcani 447 (LOD ≥ 7.64)	12.4	703405–2293727	581
				1913310–2242122 (LOD ≥ 6)	120
Chromosome 5	QTL-P4.1	BW Shafir (LOD ≥ 7.20)	23.5	146–252047	84
	QTL-P4.2	DW Zenati Bouteille (LOD ≥ 14.83)	45.2	146–252047	84
	QTL-P4.3	DW Volcani (LOD ≥ 16.60) 447	47.9	146–252047	84
	QTL-P4.4	DW Bidi 17 (LOD ≥ 17.12)	48.5	146–252047	84
	QTL-P5	BW Obelisk (LOD ≥ 4.80)	16.7	2308292–2709039	140
Chromosome 6	QTL-P6	DW Bidi 17 (LOD ≥ 3.33)	6.2	527868–942291	158
Chromosome 7	QTL-P7	BW Obelisk (LOD ≥ 3.76)	10.9	977135–2038435	391
	QTL-P8.1	DW Bidi 17 (LOD ≥ 2.91)	5.4	37079–95788	24
	QTL-P8.2	DW Zenati Bouteille (LOD ≥ 3.89)	7.7	37079–95788	24
Chromosome 13	QTL-P9.1	BW Gerek (LOD ≥ 3.06)	14.1	901651–1184505	106
	QTL-P9.2	BW Taichung 29 (LOD ≥ 7.61)	28.3	901651–1184505	106

LOD, logarithm of the odds; QTL, quantitative trait locus.

*Sequence markers above the LOD threshold of 3.0 were used to determine the boundaries. If no sequences were available which mapped in the LOD > 3 interval, the first flanking marker with a mapped sequence was taken to determine the chromosomal location.

Eventually, 15 SSPs were excluded because they contained GPI anchors (2 SSPs) and trans-membrane domain(s) (TM) outside of the signal peptide (13 SSPs), respectively, resulting in a final number of 49 SSPs under the identified QTLs that were partially supported by expressed sequence tags (http://genome.jgi-psf.org/Mycgr3/Mycgr3.home.html). We, therefore, performed a RT-PCR of these SSPs in pooled c-DNA of several dpi's to check for *in planta* expression (data not shown) and finally selected 22 SSPs as candidate effector for further analyses (Table 1). Overall, the protein length for the selected SSPs ranged from 55 to 545 aa and the number of cysteine residues varied between 0-12. Twelve SSPs have a predicted role

associated with polysaccharide degradation, seven in protein modification and two in lipid degradation (Table S2, see Supporting Information). Expression profiling of these SSPs, surprisingly revealed that all SSPs were less than 1x expressed throughout pathogenesis except SSP114, which was ≤35x expressed during necrotrophy (Fig. 6; Fig. S8, see Supporting Information).

Functional annotation of 100 ZtSSPs

Gene ontology analyses of the studied 100 ZtSSPs provided an overview of their putative biological and molecular functions. This revealed that the *in planta*–expressed ZtSSPs are not clearly attributed to a specific biological process (Fig. S9a, see Supporting Information), whereas more than 50% is specifically considered to be involved in either catalytic activity or hydrolase activity (Fig. S9B, see Supporting Information).

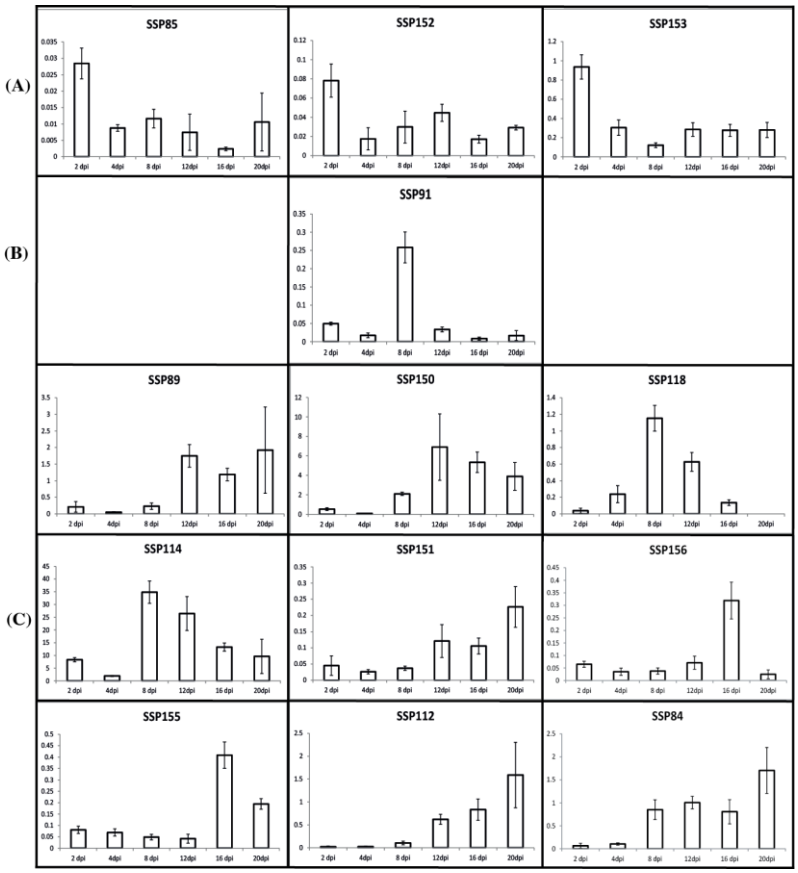

Figure 6. Relative *in planta* expression profiling of *Zymoseptoria tritici* small-secreted proteins (SSPs) that are positioned under the mapped quantitative trait loci (QTLs) on the *Zymoseptoria tritici* genome. (A) SSPs up-regulated during biotrophy. (B) SSPs expressed during the transition to necrotrophy. (C) SSPs primarily expressed during necrotrophy, fructification and sporulation. dpi, days post-infection.

Discussion

Septoria leaf blotch caused by *Z. tritici* is one of the most economically devastating wheat diseases around the world and globally impacts food security. The *Z. tritici*–wheat pathosystem currently represents a model to investigate the molecular mechanisms involved in pathogenicity and the infection process of hemibiotrophic fungal plant pathogens (Brading *et al*., 2002; Goodwin *et al*., 2004; Kellner *et al*., 2014; Rudd *et al*., 2015; Yang *et al*., 2013). The identification and characterization of effectors have contributed significantly to our understanding of the molecular mechanisms underlying pathogenesis, which are increasingly being used in the design of effector-driven breeding programmes (Rietman *et al*., 2012; Vleeshouwers and Oliver, 2014; Vleeshouwers *et al*., 2008). To date, several strategies, including biochemical (Rose *et al*., 2002) and genetic (Fudal *et al*., 2007; Gout *et al*., 2006) approaches have been implemented to discover effector genes from plant pathogens, but primarily next-generation sequencing (NGS) technologies have provided broad sets of genome sequence data from diverse microorganisms, including oomycete and fungal plant pathogens, that have resulted in a massive amount of putative effector genes (Dean *et al*., 2005; Haas *et al*., 2009; Kämper *et al*., 2006; Lévesque *et al*., 2010; Rouxel *et al*., 2011). The application of high-performance bioinformatics tools, such as pexfinder (Torto *et al*., 2003), has enabled the mining of fungal genome sequences for effector proteins (Orsomando *et al*., 2001; Stergiopoulos *et al*., 2012) and, indeed, has resulted in the identification of key effectors playing major roles in host–pathogen interactions and other biological processes (de Jonge *et al*., 2012).

Despite the functional analyses of a range of genes that play key roles during pathogenesis in the *Z. tritici*—wheat pathosystem, key effectors have thus far not been identified (Cousin *et al*., 2006; Marshall *et al*., 2011; Mehrabi *et al*., 2006a, b; Motteram *et al*., 2009; Rudd *et al*., 2015). Here, we focused on a wide approach to identify and functionally analyse putative effector proteins by taking advantage of the completed genome sequence of *Z. tritici* (Goodwin *et al*., 2011). We primarily identified SSPs through a funnel strategy, starting with bioinformatics cataloguing, followed by expression profiling and functional analyses and, eventually, the short-listing of candidates by linkage mapping.

First, we built a comprehensive list of 78 SSPs from *in silico* and *in vitro* proteomic analyses (Mirzadi Gohari *et al*., 2014) adopting protein size (less than 300 amino acids) and protein secretion as key qualifiers for candidate discovery (Rep, 2005). In addition, we included cysteine richness, the presence of GPI anchors and TM domains as important characteristics (Rep, 2005; Stergiopoulos and de Wit, 2009). Finally, we analysed expression

profiles, enabling the identification of SSP15 and SSP18, two top candidate SSPs that were uniquely expressed during a specific and defined phase of pathogenesis. However, subsequent functional analyses revealed that these SSPs were dispensable for pathogenicity. We therefore concluded that an unbiased approach indeed results in a range of effector candidates, but does not suffice in the conclusive identification of key effectors. Moreover, each and every candidate needs to be functionally analysed, which is very time consuming. Hence, discovery criteria should be redefined or, alternatively, additional strategies should support candidate discovery. For instance, recently, MoCDIP1, a secreted protein of *Magnaporthe oryzae*, was discovered which encodes a homologue of ricin B lectin inducing cell death in both monocot and dicot species (Songkumarn, 2013). This protein has a size of 355 amino acids, suggesting that a borderline of 300 amino acids for SSPs is too narrow a qualification. Furthermore, recent transcriptome analysis of *Colletotrichum higginsianum* candidate effector (ChEC) genes has revealed successive waves of expression that correspond with the phases of infection. The first wave included genes that were particularly expressed in appressoria before penetration, and the second wave contained genes transcribed before and during penetration (O'Connell *et al*., 2012). Giraldo and Valent (2013) also suggested that plant pathogens express effector proteins during distinct stages of pathogenesis, probably based on delicately regulated and fine-tuned requirements. Hence, sample preparations at defined phases of pathogenesis—such as, in our case, at 2, 4, 8, 12, 16 and 20 dpi—can still miss crucial SSPs with unique expression profiles. Our expression analysis of ZtSSPs is generally consistent with the abovementioned considerations, as we identified SSPs with distinct and/or high expression profiles (e.g. SSP42, SSP39 and SSP15), as well as those with similar expression profiles throughout pathogenesis (e.g. SSP2 and SSP6) or with remarkably low expression profiles (e.g. SSP5 and SSP20). In general, we observed three major *in planta* SSP expression waves: at biotrophy, when *Z. tritici* commences the invasion of the extracellular space of the mesophyll tissue; at the transition to necrotrophy, which is accompanied by the sudden appearance of chlorosis and necrosis and; at necrotrophy, when the largest group of candidate SSPs is up-regulated, apparently to facilitate the further destruction of host cells and the access to nutrients supporting fructification. Indeed, these observations could suggest that *Z. tritici* deploys distinct SSPs at different pathogenic stages, e.g. to suppress host defenses, facilitate colonization and, finally, induce host necrosis and survival by massive fructification (Kema *et al*., 1996d). Previous histological analyses have suggested that the switch from biotrophy to necrotrophy is associated with massive changes in mesophyll cell content, which are actually already underway from the moment *Z. tritici* hyphae colonize the apoplast at early

phases of infection (Kema *et al*., 1996d). Therefore, we were particularly interested in SSPs with distinct expression patterns during this switch. Nevertheless, the functional analyses of two of the most highly expressed SSPs (SSP15 and SSP18) showed their dispensability for *Z. tritici* pathogenicity. Intriguingly, Rudd et al. (2015), following a RNAseq strategy, selected five candidate SSPs, including those functionally analysed in this study, and concluded that all of these candidates were dispensable for the pathogenicity of *Z. tritici* in wheat.

A search of the *Z. tritici* genomic database also resulted in the discovery of homologues of well-known effector proteins, including two LysM effectors (SSP35 and SSP47) which have been functionally characterized previously (Marshall *et al*., 2011) and are highly transcribed during necrotrophy. This result accords with a recent transcriptomic analysis of the wheat—*Z. tritici* interaction by Yang *et al*. (2013). It has also been suggested that LysM effector proteins play a role in the suppression of chitin-mediated wheat defense responses during the entire infection cycle, and may be essential for disease development because of the enhanced level of chitin/biomass during the necrotrophic phase (Lee *et al*., 2013; Marshall *et al*., 2011). We also identified cerato-platanin, a protein that has been implicated as a phytotoxin or pathogen-associated molecular pattern (PAMP) triggering host defense mechanisms, such as the salicylic acid (SA) pathway (Frías *et al*., 2011, 2013; Yang *et al*., 2009). Expression analysis of the *Z. tritici* cerato-platanin protein, which we designate as *ZtCP* (SSP70), revealed that it is highly expressed during the necrotrophic phase. This suggests a possible role during the transition phase to necrotrophy or in ascertaining fungal proliferation during the end phase of pathogenesis, as has been proposed in the necrotrophic plant pathogen *Botrytis cinerea* (Frías *et al*., 2013). We therefore generated a knock-out strain of *ZtCP* and tested its pathogenicity on 20 wheat cultivars. However, none of these *IPO323ΔZtCP* strains were attenuated in pathogenicity or virulence (data not shown). Hence, the exact biological role of *ZtCP* in the *Z. tritici*–wheat interaction remains to be elucidated in subsequent studies.

Eventually, essentially as a result of the failure of the aforementioned approaches to identify SSPs that are crucial for pathogenesis, we decided to explore the map-based identification of SSP candidates. We mapped nine QTLs explaining between 5.4% and 47.9% of the observed variation on durum wheat cultivars Volcani 447, Zenati Bouteille and Bidi 17, as well as the bread wheat cultivars Shafir, Taichung 29, Gerek 79 and Obelisk. Subsequently, we catalogued SSPs that were placed under the mapped QTLs and showed that none of these overlapped with any of the SSPs identified by the other strategies. We subsequently

determined their expression profiles and observed that all of these SSPs, except SSP114, were expressed at low levels throughout pathogenesis. This was contrary to our expectations and may indicate that an unbiased map-based approach for effector discovery is the way forward to uncover essential components in the host–pathogen interaction. For instance, we identified SSP115 (302 amino acids) positioned under QTL5 with a LOD value of 17.56 explaining 47% of the observed variation, which is a homologue of BEC1019, recently characterized as a new class of biotrophic fungal effectors notably present in 97 of 271 sequenced fungal genomes (Whigham, 2013).

In summary, our data show that predetermined key qualifiers, including protein size, cysteine residues and expression patterns or magnitudes (Rep, 2005), so far have not revealed any useful links in the *Z. tritici*—wheat pathosystem. This corroborates the recent findings of Rudd et al. (2015). Clearly, any sampling strategy will exclude candidates that peak (transiently) at other stages of pathogenesis. Moreover, redundancy may also significantly affect the determination of individual SSPs and their role in pathogenesis, which is experimentally complicated to address, requiring double or triple knock-out strains (Bakkeren and Valent, 2014; Gijzen *et al*., 2014). Furthermore, incomplete or incorrect annotations of the genomic stretches carrying the QTLs significantly hampers the discovery of essential SSPs. Therefore, we will pursue our strategy to fine map QTL windows in the regions of interest, followed by functional analyses of the QTL-based SSPs, in order to determine their contribution to cultivar and wheat species specificity, which is crucial for effector-driven wheat breeding programs.

Experimental Procedures

Identification and bioinformatics analyses of SSPs

The genome of *Z. tritici*, which is publicly available at the JGI website (http://genome.jgi-psf.org/Mycgr3/Mycgr3.home.html), was mined and all SSPs ≤ 300 amino acids were retrieved. We followed the same pipeline as Morais do Amaral et al. (2012) to predict the secretome of *Z. tritici* IPO323 with minor modifications. *In silico* prediction of extracellular proteins was performed using SignalP to determine the presence of signal peptides. The number of cysteine residues inside the mature proteins was manually computed and the number of selected SSPs was narrowed down to those with four or more cysteine residues. The TargetP program was used to identify and remove SSPs with either a chloroplast transit peptide or a mitochondrial targeting peptide. Tmhmm software was then utilized to identify and remove the TM-containing SSPs, except those with one TM in the N-

terminal signal peptide. The remaining SSPs were screened for the presence of GPI anchor proteins using big-PI (http://mendel.imp.ac.at/gpi/fungi_server.html). The EST databases were used to further narrow down the SSPs to those that were transcribed in at least one of the three *in planta* or seven *in vitro* EST libraries. Finally, the selected SSPs were subjected to PFAM analysis using the PFAM database (ftp://ftp.ncbi.nih.gov/pub/mmdb/cdd/). In addition, BlastP analysis was conducted to determine whether SSPs were either conserved or unique for *Z. tritici* (e-value of 10^{-6}). Finally, we performed gene ontology analyses using Blast2go (Conesa *et al*., 2005).

Inoculations, RNA isolation and SSP expression profiling during pathogenesis in wheat

Wheat cv. Obelisk was used throughout these experiments. Ten-day-old plants were inoculated with a spore suspension of *Z. tritici* IPO323 using previously reported protocols (Mehrabi *et al*., 2006a). The infected leaves were collected in three biological replications and flash frozen in liquid nitrogen and ground manually using a mortar and pestle. Total RNA was isolated with the RNeasy plant mini kit (Qiagen, Valencia, CA, USA) and residual DNA was subsequently removed using the DNAfree kit (Ambion, Huntingdon, Cambridgeshire, UK). First-strand cDNA was obtained from 2 µg of total RNA primed with oligo(dT) using SuperScript III (Invitrogen, Carlsbad, CA 92008, USA) according to the manufacturer's instructions. Expression analyses of selected SSPs were performed using RT-qPCR. One microlitre of the resulting cDNA was used in a 25-µL PCR employing a QuantiTect SYBR Green PCR Kit (Applied Biosystems, Warrington, UK), and run and analysed using an ABI 7500 Real-Time PCR machine (Applied Biosystems, Foster City, CA 94404, USA). The relative expression levels of each gene were initially normalized by the constitutively expressed *Z. tritici* β-tubulin gene (Keon *et al*., 2007; Motteram *et al*., 2009) and then calculated based on the comparative *C*(*t*) method described previously (Schmittgen and Livak, 2008).

Functional analyses

The gene deletion constructs, p∆*MgSSP15* and p∆*MgSSP18*, were generated using the USER friendly cloning method with minor modifications, as described previously (Mirzadi Gohari *et al*., 2014). The constructs were then transformed into *Agrobacterium tumefaciens* strain LBA1100 via electroporation and, subsequently, *A. tumefaciens*-mediated transformation (ATMT) was performed to delete *MgSSP15* and *MgSSP18* in *Z. tritici* IPO323, as described previously (Mehrabi *et al*., 2006a). All knock-out strains and the WT strain

Z. tritici IPO323 were compared for pathogenicity on 12 wheat cultivars (Table S3, see Supporting Information) following regular protocols (Mehrabi *et al*., 2006a).

Zymoseptoria tritici crosses, and selection and analyses of mapping populations

We used the *Z. tritici* reference IPO323 and isolate IPO95052, an Algerian field strain originating from durum wheat, for *in planta* crosses (Kema *et al*., 1996c). Two F1 progenies were generated on either the bread wheat cv. Obelisk (n = 103) or the durum wheat cv. Inbar (n = 60) which, after initial molecular and *in planta* analyses, were not significantly different and therefore bulked for further analyses. Each progeny isolate was used to inoculate (30 mL of 10^7 spores/mL) a set of bread and durum wheat cultivars (Table S1) in at least two biological repetitions, and disease severity was scored as the percentage primary leaf area covered by pycnidia (P_p) at 21 dpi following the protocols and conditions reported previously (Kema *et al*., 1996c). Histograms with frequency distributions of progeny using log-transformed P_p data were generated for each cultivar using bins (classes) in intervals of 0.1 after logarithmic transformation to evaluate segregation distributions. The P_p scores from 147 of the 163 progeny were used to map QTLs on a genetic linkage map previously generated with this population (Goodwin *et al*., 2011; Wittenberg *et al*., 2009). This subset includes 23 twin pairs of isolates for which P_p data were merged after averaging. These twin pairs are genetically identical isolates resulting from mitosis after meiosis in an ascus, leading to four pairs of genetically identical ascospores (Wittenberg *et al*., 2009). Because histograms did not reveal normal distributions of virulence data, QTL mapping based on a continuous scale of P_p was necessary. Mapping with the average of the log-transformed P_p data [log(average P_p) + 1] from each isolate–cultivar result, versus mapping with the average raw P_p data, yielded higher LOD values; hence, we therefore continued with log-transformed data. The software program MapQTL 5.0 (van Ooijen, 1992) was used to detect QTLs with both the interval mapping (Lander and Botstein, 1989) and Multiple-QTL Mapping (MQM) mapping (Jansen, 1994) methods. First, interval mapping was performed to detect QTLs. Subsequently, co-factors were determined using the automatic co-factor selection option, followed by MQM mapping of the same trait with the selected co-factor(s) to identify new QTLs. The LOD profiles and the percentage of explained variance were obtained with the MQM mapping approach when co-factors were selected. When only one QTL was detected, the LOD profile of the interval mapping procedure was shown. Permutation tests were performed to determine QTL significance, which resulted in a genome-wide significance threshold of LOD = 3.0 for all traits. LOD profiles were graphically displayed using MapChart version 2.2 (Voorrips, 2002),

including the LOD − 1/LOD − 2 support interval to approximate a 95% confidence interval (van Ooijen, 1992).

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Supporting Information

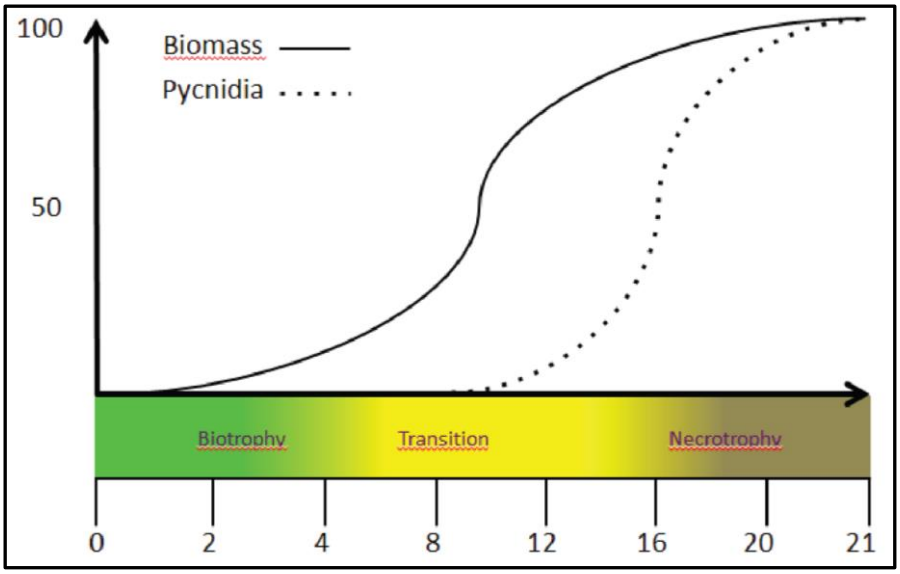

Figure S1. Schematic representation of symptom development accompanied by foliar discoloration and fructification/sporulation of *Zymoseptori tritici* in wheat foliage. Sampling of leaves for RNA extraction rarely represents a very clear event during pathogenesis, but rather covers zones of different stage s of symptom development.

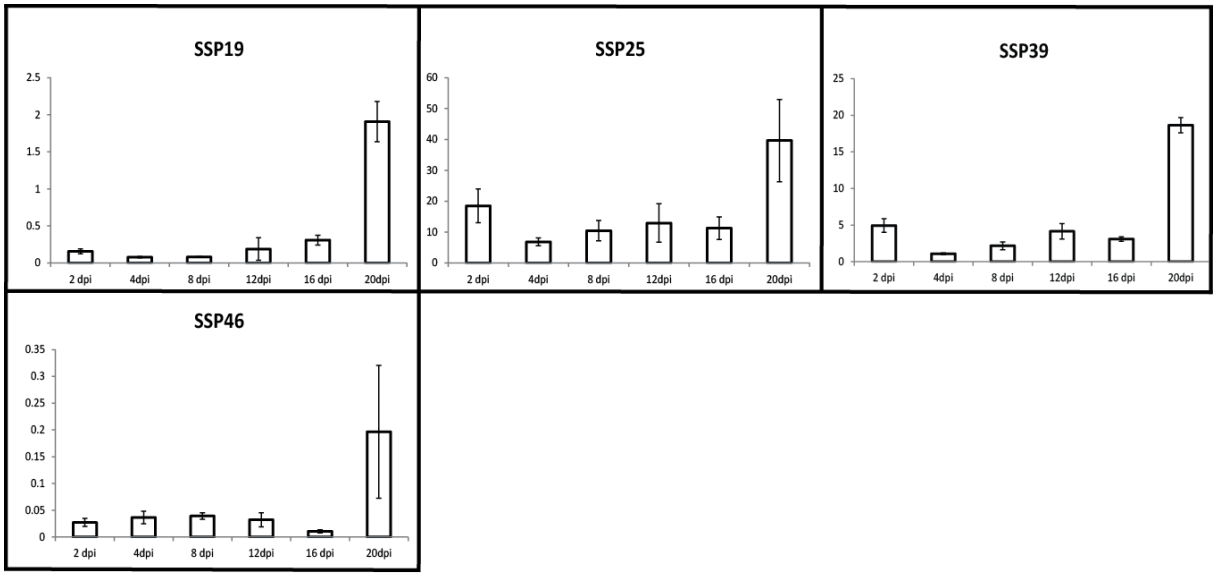

Figure S2. Relative *in planta* expression profiling of *Zymoseptoria tritici* SSPs that are specifically up-regulated during fructification/sporulation.

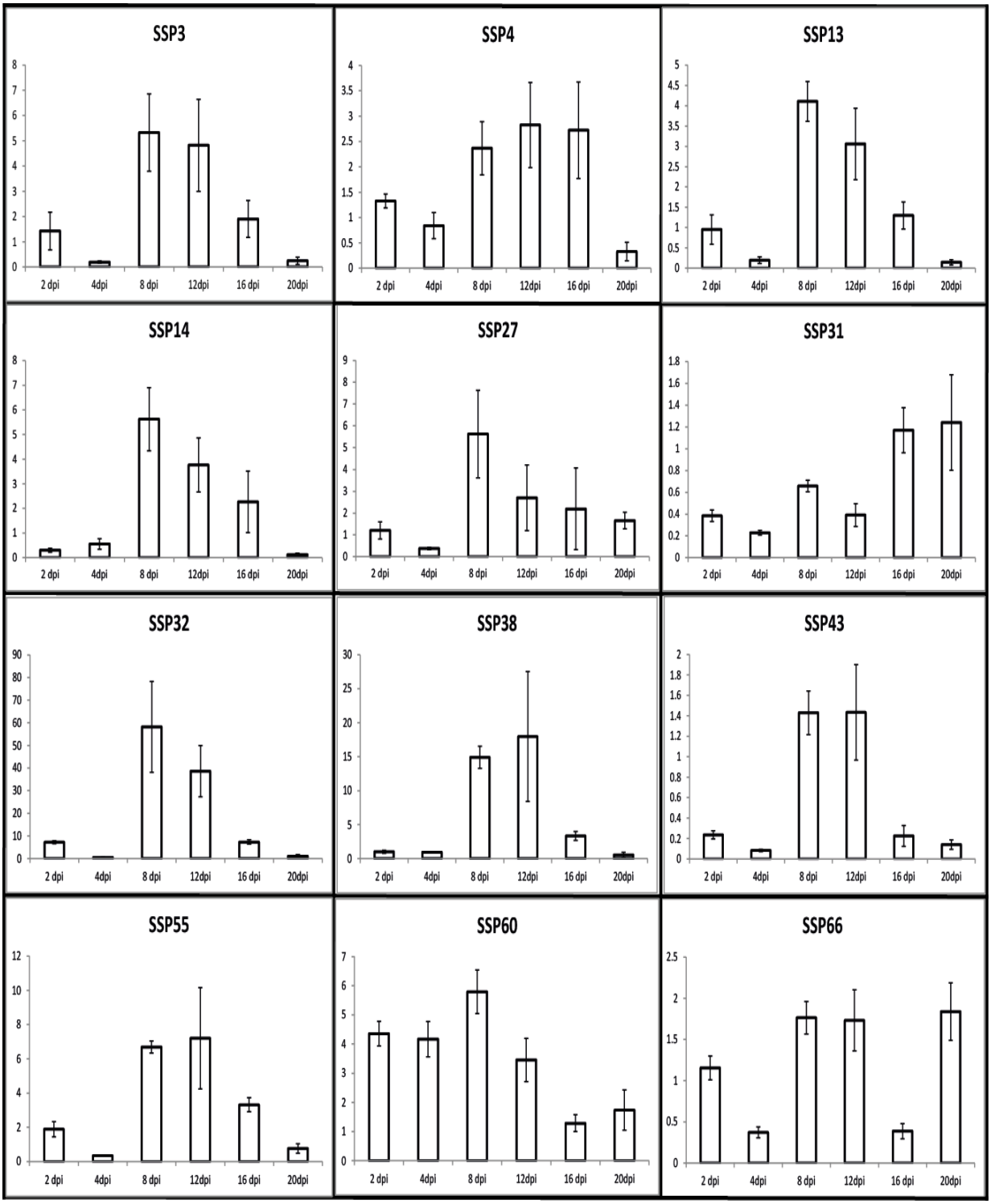

Figure S3. Relative *in planta* expression profiling of *Zymoseptoria tritici* SSPs that are up-regulated during the transition/necrotrophic phase of pathogenesis but do not show a particular pattern.

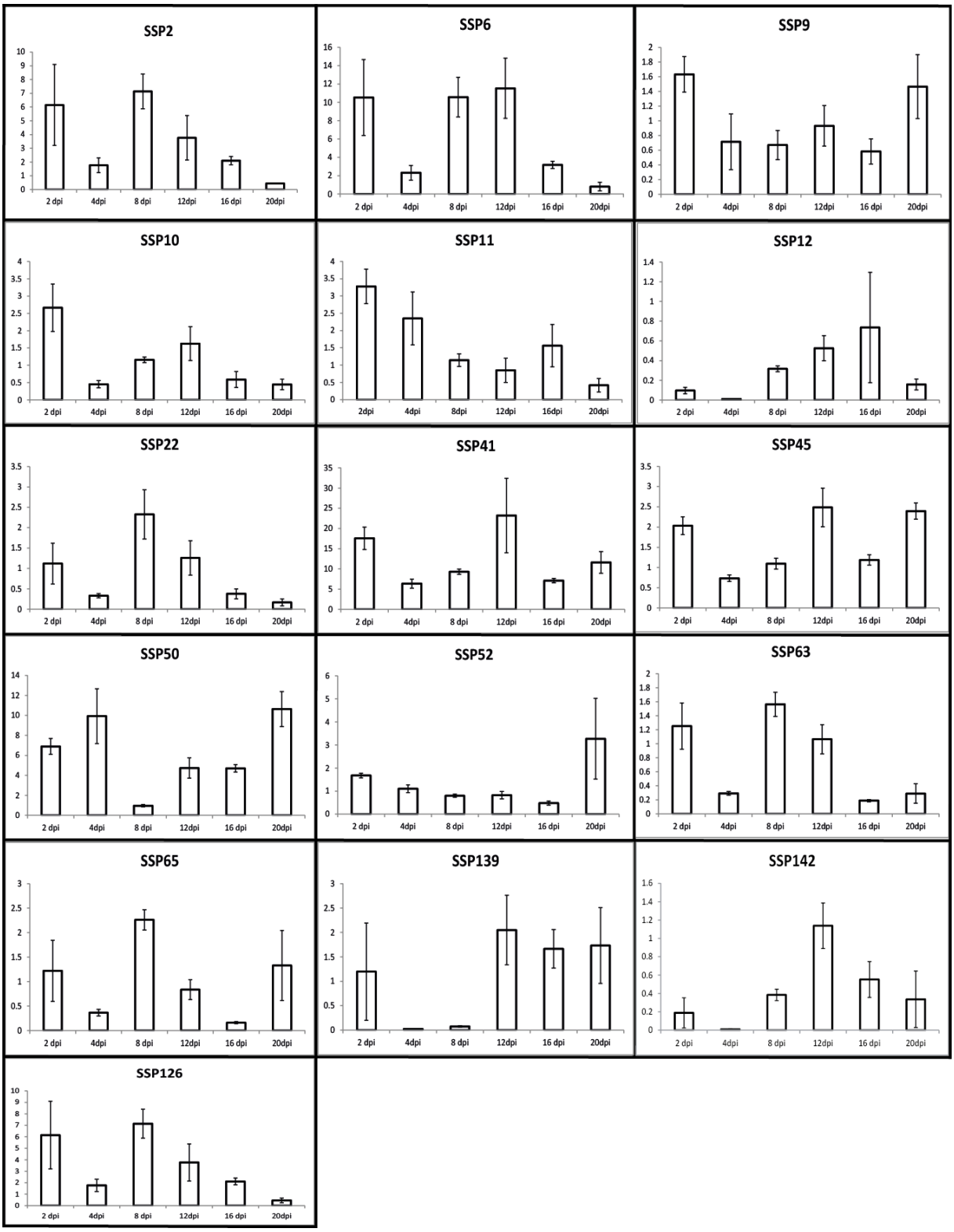

Figure S4. Relative *in planta* expression profiling of *Zymoseptoria tritici* SSPs with a bimodal pattern (biotrophy and necrotrophy).

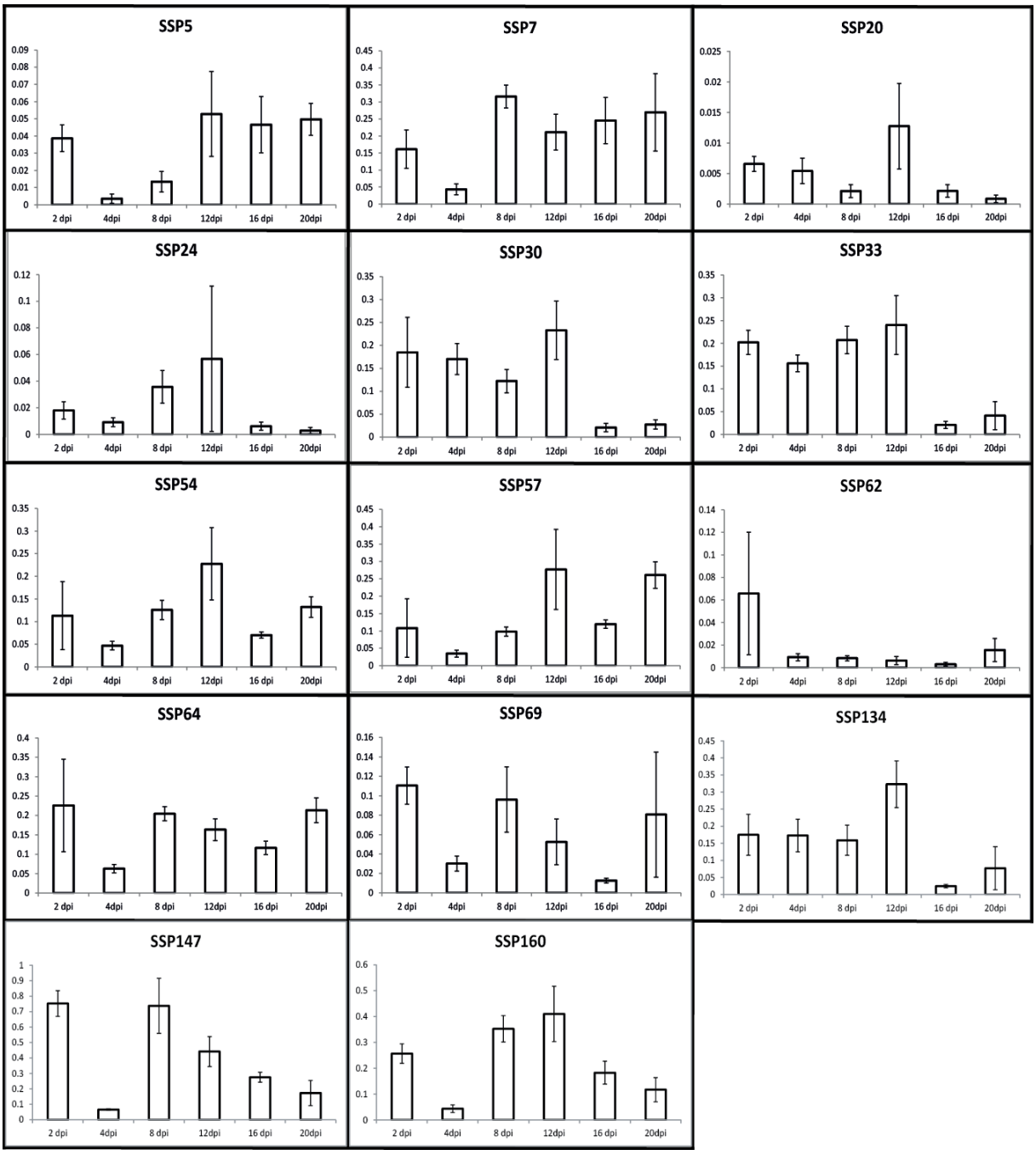

Figure S5. Relative *in planta* expression profiling of *Zymoseptoria tritici* SSPs that are overall very lowly expressed throughout pathogenesis.

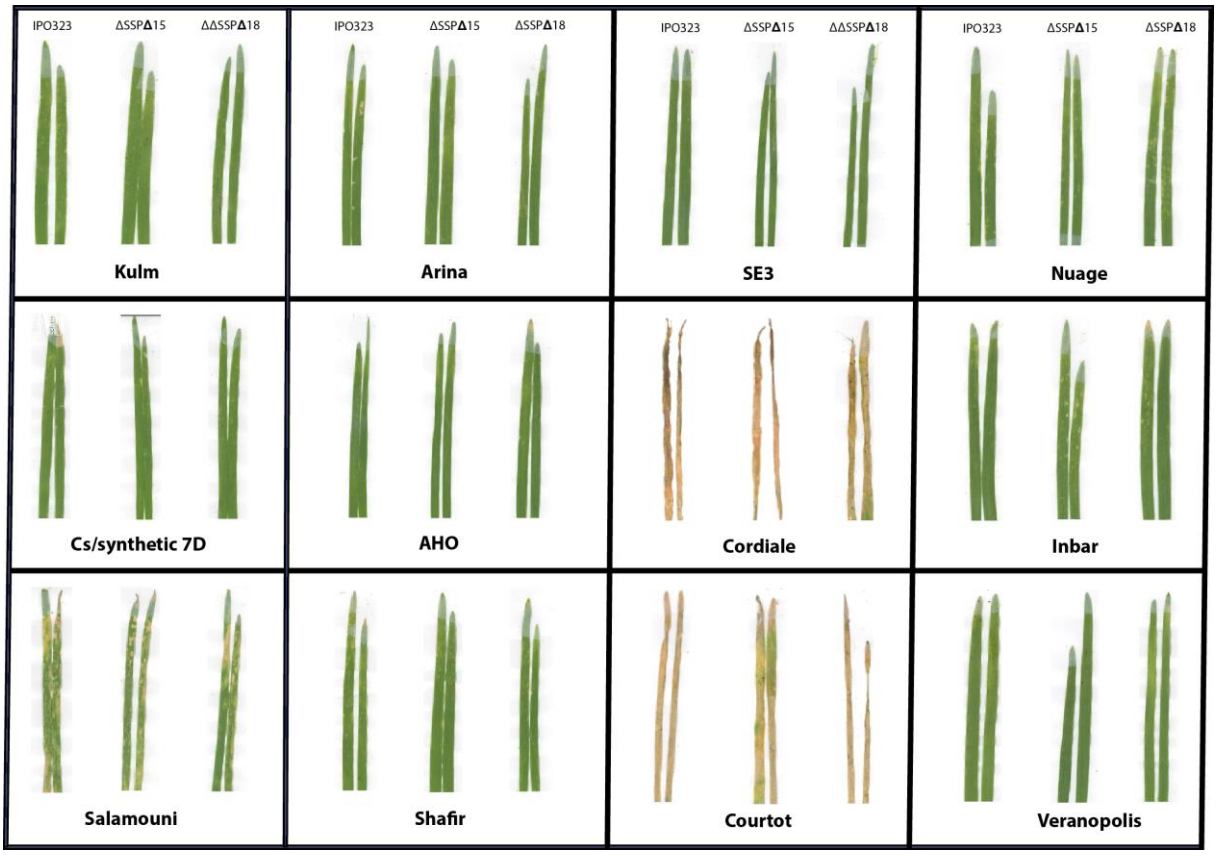

Figure S6. Disease development at 21 days after inoculation with the *Zymoseptoria tritici* WT strain IPO323 and the *IPO323ΔSSP15* and *IPO323ΔSSP18* knock-out strains in 12 wheat cultivars that are parents of mapping populations and/or used in differential sets for *Z. tritici* phenotyping.

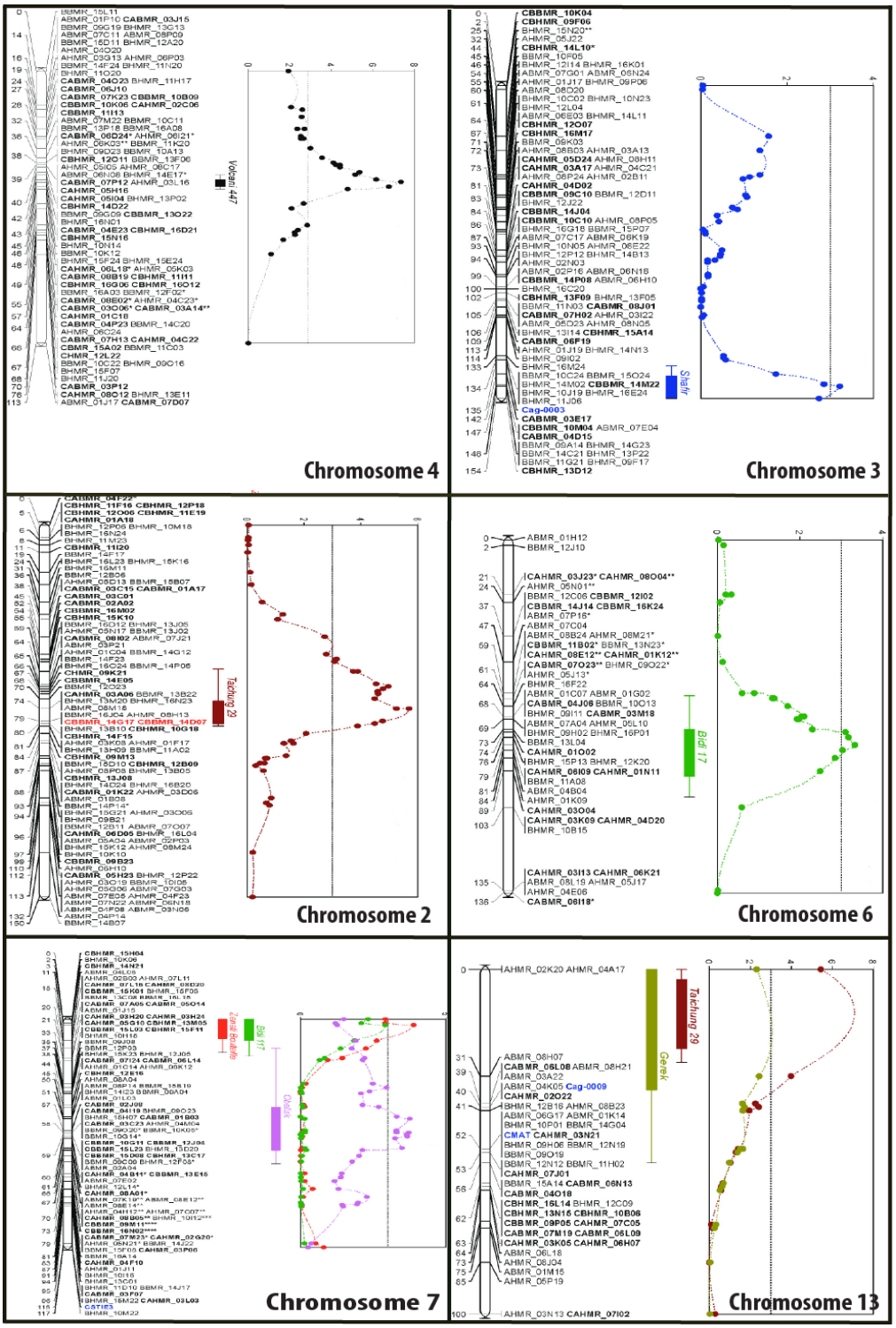

Figure S7. Quantitative trait loci (QTL) for *Zymoseptoria tritici* pathogenicity measured by the percentage of foliage covered by pycnidia, the asexual fructifications that are positioned in the substomatal cavities, mapped on six *Z. tritici* chromosomes.

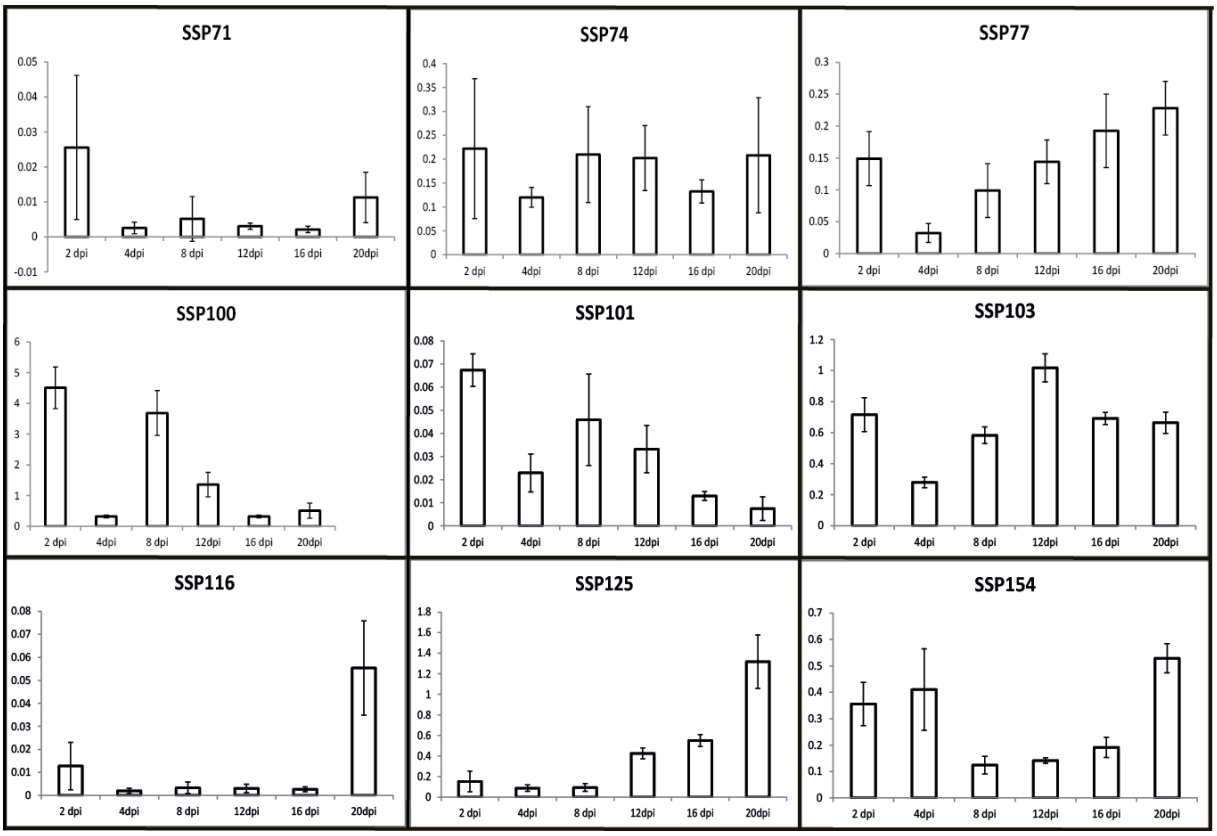

Figure S8. Relative *in planta* expression profiling of *Zymoseptoria tritici* SSPs that are positioned under mapped QTLs and overall very lowly expressed throughout pathogenesis.

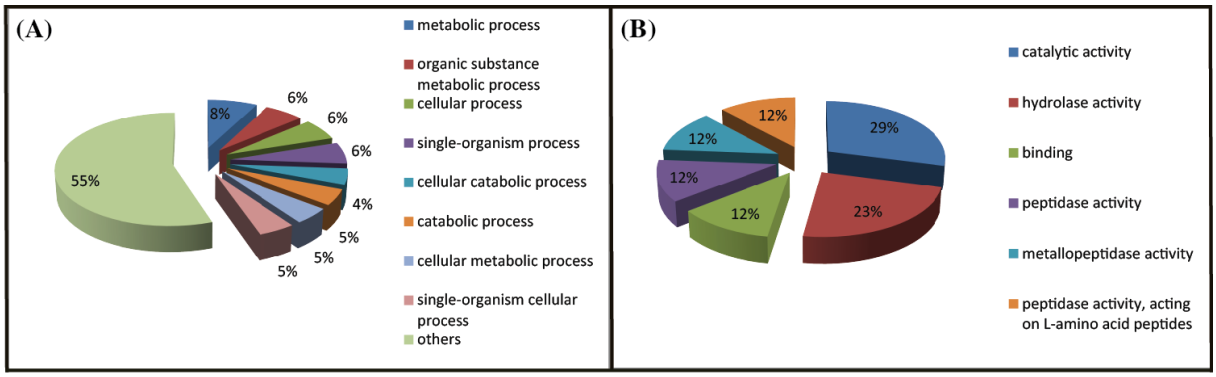

Figure S9. Gene ontology (GO) analyses of 100 *Zymoseptoria tritici* SSPs into different functional categories based on their biological process (A) and molecular function (B).

Table S1. Mapped quantitative trait loci involved in (a)virulence in progeny isolates of *Zymoseptoria tritici* from a cross between isolates IPO323 and IPO95052 tested for virulence on the durum wheat cvs. Volcani 447, Zenati Bouteille and Bidi 17 and the bread wheat cvs. Taichung 29, Obelisk, Gerek 79 and Shafir.

Species	Cultivar	Chromosome	LOD-value	Explained overall variance (%)
Durum wheat	Volcani 447	5	17.56	47.9
		4	7.35	12.4
	Zenati Bouteille	5	15.26	45.2
		7	3.89	7.7
	Bidi 17	5	17.5	48.5
		6	3.33	6.2
		7	2.91	5.4
Bread wheat	Taichung 29	13	7.61	28.3
		2	5.70	15.1
	Obelisk	5*	4.80	16.7
		7**	3.76	10.9
	Gerek 79	13	3.06	14.1
	Shafir	5	7.2	23.5
		3	3.22	8.6

*different position than the other locus on Chromosome5.

**different position than the other locus on Chromosome7.

Table S2. The *Zymoseptoria tritici* SSPs positioned under the QTLs that are involved in the degradation of polysaccharides, proteins and lipids.

Annotation	Protein ID (amino acid size)	Functional annotation	PFAM
Glycoside Hydrolase Family 79	31582(481)	Hydrolysis of glycosidic bonds	PF03662
Glycoside Hydrolase Family 18	86391(404)	Hydrolysis of glycosidic bonds	PF00704
Glycoside hydrolase family 3	45540(804)	Hydrolysis of glycosidic bonds	PF00933
Glycoside hydrolase family 32	74191(565)	Hydrolysis of glycosidic bonds	PF00251
Glycoside hydrolase family 27	110289 (813)	Hydrolysis of glycosidic bonds	PF02065
Glycoside hydrolase family 15	42503(610)	Hydrolysis of glycosidic bonds	PF00723
Glycoside hydrolase family 5	71681(410)	Hydrolysis of glycosidic bonds	PF00150
Glycoside hydrolase family 13	71695(850)	Hydrolysis of glycosidic bonds	PF02903
Glycoside hydrolase family 13/Alpha amylase	72646 (2407)	Hydrolysis of glycosidic bonds	PF00128
Serine hydrolase	104337(251)	Hydrolysis of glycosidic bonds	PF03959
haloacid dehalogenase-like hydrolase	9953(1280)	Hydrolysis of glycosidic bonds	PF00702
Pectate lyase	42327(343)	Cleavage of pectate	PF00544
Metallo-peptidase family M12	67183(829)	Hydrolysis of peptide bonds	PF13688
Serine carboxypeptidase S10	109759(547)	Hydrolysis of peptide bonds	PF00450
Peptidase_A4	105030(270)	Hydrolysis of peptide bonds	PF01828
Aspartic protease	110047(442)	Hydrolysis of peptide bonds	PF00026
Peptidase family M20/M25/M40	45102(408)	Hydrolysis of peptide bonds	PF1546
Peptidase_S8	72659(401)	Hydrolysis of peptide bonds	PF00082
Serine carboxypeptidase	42709((552)	Hydrolysis of peptide bonds	PF00450
Carboxylesterase Familly	74078(713), 72632(579)	Hydrolysis of carboxylic ester	PF00135

Table S3. Cultivars used in this study

Cultivar	Origin	*Stb* gene
Kulm	USA	Susceptible parent
Arina	Switzerland	Stb15 +Stb6
SE3	France	----------
Nuage	France	----------
Cs Synthetic (6x)7D	China/USA	Stb5+Stb6
AHO	France	----------
Cordiale	France	----------
Inbar	France	----------
Salamouni	Canada	Stb13 + Stb14
Shafir	Israel	Stb6
Courtot	France	Stb9
Veranopolis	Brazil	Stb2+Stb6

Chapter 5

Functional characterization of extracellular and intracellular catalase-peroxidases involved in virulence of the fungal wheat pathogen *Zymoseptoria tritici*

Mirzadi Gohari, A., Mehrabi, R., Zamani, E., de Wit, P.J.G.M. and Kema, G.H.J. (Manuscript to be submitted)

Summary

In order to investigate how the hemibiotrophic fungal wheat pathogen *Zymoseptoria tritici* copes with H_2O_2 generated by the host upon infection, we functionality analyzed two catalase-peroxidase (CP) genes designated *ZtCpx1* and *ZtCpx2*. Expression analyses revealed that *ZtCpx1* is up-regulated during the biotrophic growth phase and during asexual spore formation *in vitro*, whereas *ZtCpx2* is up-regulated during the switch from the biotrophic to the necotrophic growth phase and during *in vitro* vegetative growth. Deletion of the *ZtCpx1* gene increased the *in vitro* sensitivity of the mutant to exogenously added H_2O_2 and significantly reduced its virulence, as shown by reduced severity of the septoria tritici blotch symptoms as well as fungal biomass production. All phenotypes were restored after reintroducing the wild type allele of *ZtCpx1* driven by its native promoter. Although, *ZtCpx2* was dispensable for full virulence of *Z. tritici*, disruption significantly reduced fungal biomass development during the switch from biotrophic to necrotrophic growth. We also showed that both CP genes act synergistically, as the double knock-out mutant strain was significantly more reduced in virulence than the Δ*ZtCpx1* strain.

Introduction

Plants have evolved different basal defense mechanisms in response to pathogen attack, including the generation of reactive oxygen species (ROS), such as superoxide anion radicals (O_2–), hydroxyl radicals ($HO^{\cdot}$), and hydrogen peroxide (H_2O_2) at the site of penetration, a phenomenon known as the oxidative burst (Bolwell, 1999; Doke *et al.*, 1996; Lamb and Dixon, 1997). In general, high levels of ROS cause an imbalance between radical-generating and radical-scavenging systems, a condition called oxidative stress, which is deleterious for living cells as this can lead to oxidation of DNA, proteins and lipids, consequently leading to damage and malfunctioning of cells (Heller and Tudzynski, 2011; Sies, 1997). During plant infection, increasing levels of ROS stimulate fungal pathogens to develop infection structures (Heller and Tudzynski, 2011; Samalova *et al.*, 2014). H_2O_2 is the most stable form of all three ROS and more amenable for experimental studies than O_2– and $HO^{\cdot}$ which both have a very short half-life and are extremely toxic (Costet *et al.*, 2002). However, H_2O_2 has anti-fungal activity and acts also as a signaling molecule in numerous biological processes, including phytoalexin production, activation of defense-related genes and programmed cell death (Apostol *et al.*, 1989; Gadjev *et al.*, 2008; Joseph *et al.*, 1998; Venturini *et al.*, 2002; Wu *et al.*, 1995). There is growing evidence that H_2O_2 exposure has bifunctional effects on plant pathogenic fungi; it reduces tissue colonization of biotrophic and hemibiotrophic fungal

pathogens, but in contrast increases ramification of host tissue by necrotrophs (Mellersh *et al.*, 2002; Shetty *et al.*, 2007; Able, 2003; Govrin and Levine, 2000; Tiedemann, 1997). In order to cope with the lethal effects of ROS, plant pathogens generate small molecules such as glutathione to scavenge or detoxify ROS using a number of antioxidant enzymes such as glutathione peroxidase (GSHPx), catalase and catalase-peroxidases (CPs) (Lehmann *et al.*, 2015; Nanda *et al.*, 2010; Huang *et al*., 2011). For instance, *MoHYR1* in *Magnaporthe oryzae* encodes a GSHPx that mediates the detoxification of host-derived ROS and it is required for full virulence (Huang *et al*., 2011). Catalases and peroxidase are well-known enzymes that are involved in protecting cells from oxidative stress through catalyzing the elimination of H_2O_2 (Vidossich *et al*., 2012), such as *MoPRX1* in *M. oryzae* that encodes a thioredoxin peroxidase (TPx), which modulates host-derived H_2O_2 during early stages of colonization and therefore contributes to virulence (Mir *et al*., 2015). CPs are unique bifunctional enzymes belonging to class I peroxidases with predominant catalase activity and considerable peroxidase activity. Phylogenetic analysis suggests that plant pathogenic fungi have acquired CP genes through lateral gene transfer (from *Negibacteria*), followed by gene duplication and diversification (Passardi *et al.*, 2007). Currently, CPs are classified in two well-separated clades: (i) one with cytoplasmic enzymes present in saphophytic and plant pathogenic fungi and (ii) one with extracellular enzymes detected exclusively in plant pathogenic fungi, which are distinct with respect to location, structure and function (Tanabe *et al.*, 2011; Zámocký *et al.*, 2010).

Biotrophic fungal pathogens obtain nutrients from living cells and need to have a robust antioxidant mechanism in order to overcome oxidative stress imposed by the host defense system during early stages of infection. It was speculated that *BghCatB* of *Blumeria graminis* f.sp. *hordei*, encoding a secreted catalase, might be involved in virulence by detoxifying H_2O_2 produced at the sites of invasion (Skamnioti *et al.*, 2007). In contrast, deletion mutants of a gene encoding secreted catalase CpCAT1 from *Claviceps purpurea* displayed no signification reduction in virulence indicating that the gene is not required for virulence of this fungus (Garre *et al.*, 1998). Functional analysis of CfCAT2, a cytoplasmic catalase of the biotrophic tomato pathogen *Cladosporium fulvum* demonstrated to be dispensable for virulence although it was preferentially expressed in response to exogenously applied H_2O_2 (Bussink and Oliver, 2001).

Unlike biotrophs, necrotrophic fungal pathogens kill host cells and are assumed to acquire nutrition from decaying host tissue. Consequently, they should be able to cope with increasing ROS levels during oxidative stress situations once a pathogen establishes infection

and proceeds with colonizing the host plant (Schouten *et al.*, 2002). Indeed, the growth of necrotrophic plant pathogens is stimulated in the presence of H_2O_2 (Kumar *et al.*, 2001; Govrin and Levine, 2000) and recent studies showed that deletion mutants of genes encoding both secreted and cytoplasmic catalases in *Cochliobolus heterostrophus* (*ChCAT3*), and *Botrytis cinerea* (*BcCAT2*) are pathogenic on their respective hosts but they become hypersensitive to exogenously added H_2O_2 during *in vitro* mycelial growth (Robbertse *et al.*, 2003; Schouten *et al.*, 2002; Yarden *et al.*, 2014).

Hemibiotrophs initially grow mostly intercellularly on their host plants as a biotroph followed by a necrotrophic phase at later stages of infection, where they need to neutralize host-generated H_2O_2. In *M. oryzae*, MgCATB, a secreted catalase plays a central role in the onset of infection of rice by maintaining the integrity of fungal cell walls and regulating appressorium function (Skamnioti *et al.*, 2007). So far, only one secreted fungal CP, *MgCPXB* of *M. oryzae*, has been functionally analyzed, which only protects the fungus against host-derived H_2O_2 during the early stages, but not during advanced stages of infection (Tanabe *et al.*, 2011).

Zymoseptoria tritici (Desm.) (Quaedvlieg & Crous) (Quaedvlieg *et al.*, 2011) is a major foliar pathogen of bread and durum wheat and causes septoria tritici blotch (STB) under temperate climatic conditions (at different altitudes and latitudes) in all wheat-growing areas worldwide (Eyal, 1999; Fones and Gurr, 2015). As a hemibiotroph, *Z. tritici* shows two distinct phases of colonization; initial biotrophy and advanced necrotrophy (Kema *et al.*, 1996. Yang et al. (2003) quantitatively showed that incompatible *Z. tritici*-wheat interactions are associated with a high and early accumulation of H_2O_2, whereas in compatible interaction, significantly lower amounts of H_2O_2 accumulated during the initial biotrophic phase. Moreover, a massive accumulation of H_2O_2 was detected during the switch to necrotrophy in compatible interactions, coinciding with the occurrence of severe disease symptoms. This suggests that *Z. tritici* is able to cope with different levels of H_2O_2 during the two phases of infection. Here, we demonstrate that *Z. tritici* produces a cytoplasmic and an extracellular catalase peroxidase, encoded by CP genes *ZtCpx1* and *ZtCpx2*, respectively, and show that both are crucial for virulence.

Results

Identification and characterization of *Z. tritici* catalases and catalase-peroxidases

The *Z. tritici* genome (Goodwin *et al.*, 2011) contains two bifunctional CPs; CP A (*ZtCpx1*; ID protein: 105409) and CP B (*ZtCpx2*; ID protein: 67250), as well as the mono-

functional catalase-encoding genes *ZtCat1* (protein ID: 85387) and *ZtCat2* (protein ID: 98331). *In silico* analysis revealed that only *ZtCpx2* contains a signal peptide (signalP) with a cleavage site at position 22 and 23, suggesting that this enzyme is secreted. Recently, both the secreted and non-secreted CPs were identified in apoplastic fluids extracted from compatible and incompatible interactions between *Z. tritici* and wheat (Ben M'Ben Barek *et al.*, 2015) and were selected for further detailed characterization. *ZtCpx1* has a 2,508 bp open reading frame (ORF) with one intron and encodes a 752 amino acid (aa) protein, whereas *ZtCpx2* has an 2,636 bp ORF with four introns and encodes a 797 aa secreted protein. Sequence analysis revealed that both *ZtCpx1* and *ZtCpx2* encode two-domain peroxidases (PF00141), and the domains are positioned between aa stretches 119–449/454-762 and 61-410/415-716, respectively. Comparison of the catalases and CPs of other plant pathogens allowed building of a phylogenetic tree with strong bootstrap support representing two distinct clades, one containing the two catalases and one the two CPs. ZtCpx1 aligns with the group of cytoplasmic CPs (Fig. 1) and is closely related to CpeA1 and CpeA2 of *Verticillium longisporum* (Singh *et al.*, 2012). ZtCpx2 aligns with the group of secreted CPs that have been predicted in other fungal plant pathogens (Fig. 1) and clusters closely with CP MgCPXB of *M. grisea*, and Cpx2 of *V. dahliae* (Tanabe *et al.*, 2011; Tran *et al.*, 2014).

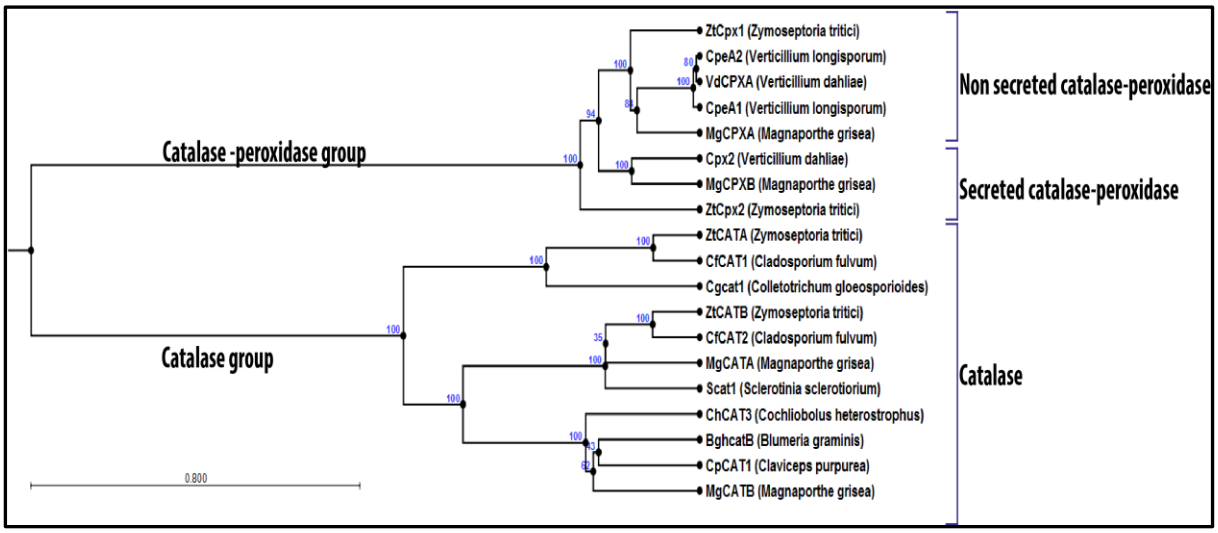

Figure 1. Phylogenetic analysis of *Z. tritici* catalases (CAT1 and CAT2) and catalase-peroxidases (Cpx1 and Cpx2) and their homologues in *Verticillium longisporum* (CpeA1-2), *Verticillium dahlia* (VdCPXA and Cpx2), *Magnaporthe grisea* (MgCPXA, MgCPXB, MgCATA and MgCATB), *Cladosporium fulvum* (CfCAT1-2), *Colletotrichum gloeosporioides* (Cgcat1), *Sclerotinia sclerotiorium* (Scat1), *Cochliobolus heterostrophus* (ChCAT3), *Blumeria graminis* f.sp. *hordei* (BghcatB), *Claviceps purpurea* (CAT1). The tree was constructed with CLC software using the

UPGMA method and 1000 bootstrap replicates. The statistical support values are displayed at the nodes of the tree branches.

ZtCpx1 and *ZtCpx2* disruption and complementation

In order to determine a possible role for *ZtCpx1* in virulence, gene disruption and complementation mutants were generated by homologous recombination. Three independent transformants showing similar morphological phenotypes were obtained (Fig. S1A, see Supporting Information). One of these (*ΔZtCpx1*) was chosen for complementation with the *ZtCpx1* wild-type allele, which resulted in *ΔZtCpx1*-C and homologous recombination was confirmed using PCR (Fig. S1A, see Supporting Information). The knock-out construct for *ZtCpx2* was generated through the USER-friendly protocol as described previously (Mirzadi Gohari *et al.*, 2014) and eventually one transformant, *ΔZtCpx2*, was obtained (Fig. S1B, see Supporting Information). Subsequently, the *ΔZtCpx2* strain was used to delete *ZtCpx1*, resulting in the double knock-out strain *ΔΔZtCpx1-Cpx2* (Fig. S1c, see Supporting Information).

ΔZtCpx1 and *ΔΔZtCpx1-Cpx2* are sensitive to exogenously added H_2O_2

In order to investigate the role of *ZtCpx1* and *ZtCpx2* in tolerance of *Z. tritici* to H_2O_2, we tested the single and double knock-out strains under various conditions, including a continuous exposure to H_2O_2 for 14 days. The *ΔZtCpx1*, *ΔZtCpx2* and *ΔΔZtCpx1-Cpx2* strains and both controls, including the *Z. tritici* IPO323 wild type (WT) and complemented strain (*ΔZtCpx1*-C) were plated on potato dextrose agar (PDA) supplemented with 0, 6, 8 and 10 mM H_2O_2. Both control strains as well as the *ΔZtCpx2* strain were able to grow under these conditions similar to the WT, but the *ΔZtCpx1* and *ΔΔZtCpx1-Cpx2* strains were clearly affected and unable to grow (Fig. 2). This shows that *ZtCpx1* is essential for tolerating or degrading H_2O_2 under *in vitro* conditions.

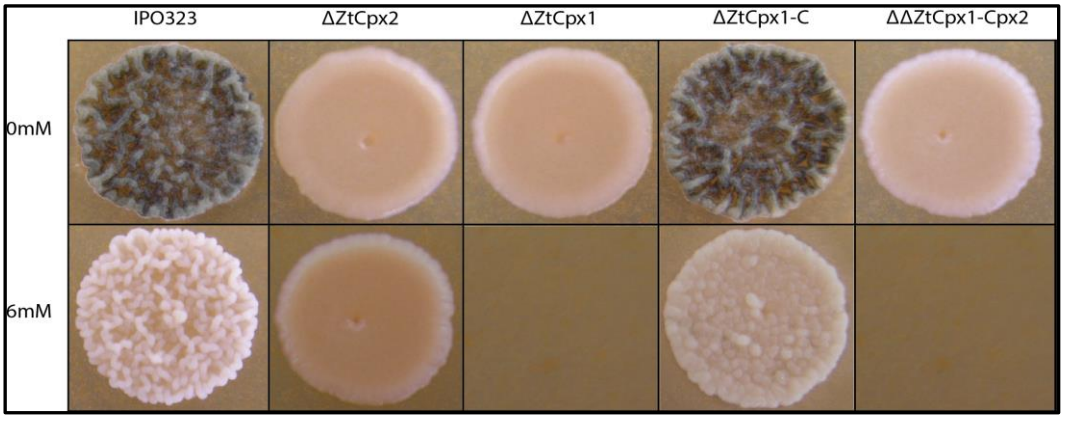

Figure 2. Sensitivity assay of *Zymoseptoria tritici* strains to H_2O_2. The spore suspensions of *Z. tritici* IPO323 (WT), disruptant strains *ΔZtCpx2*, *ΔZtCpx1*, *ΔΔZtCpx1-Cpx2* and the complementation strain *ΔZtCpx1*-C were plated on PDA amended with 6 mM H_2O_2 and subsequently maintained at 18 °C. Pictures were taken 14 days post incubation. Note that *ΔZtCpx1* and *ΔΔZtCpx1-Cpx-2* are unable to grow at 6 mM H_2O_2.

Reduced spore germination of *ΔZtCpx1* and *ΔZtCpx2* strains

All mutant strains and the controls were inoculated on PDA medium amended with different H_2O_2 concentrations, and the spore germination frequency was recorded at 24 and 48 hours after inoculation (hai). At 24 hai in 4 mM H_2O_2 spore germination of the *ΔZtCpx1* and *ΔΔZtCpx1-Cpx2* strains was decreased to 34% and 4%, respectively, whereas the germination of *ΔZtCpx2* was not different from the control strain (94%). At increased H_2O_2 concentrations (6 mM), however, none of the *ΔZtCpx1* and *ΔΔZtCpx1-Cpx2* spores germinated, whereas the germination frequency of the *ΔZtCpx2* strain was decreased to 30% (Fig. 3A). At 48 hai, all strains germinated on PDA medium in the absence of H_2O_2. These results indicate that H_2O_2 inhibits spore germination of the *ΔZtCpx1* and *ΔΔZtCpx1-Cpx2* strains (Fig. 3B).

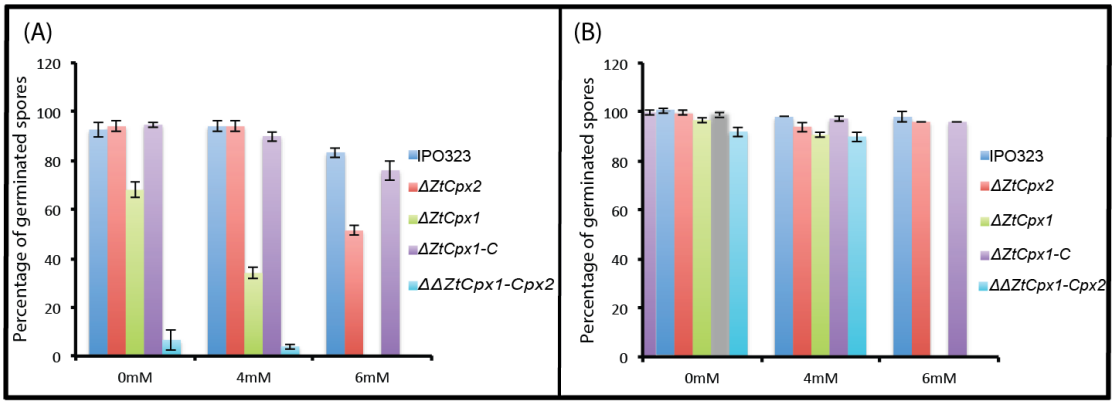

Figure 3. Spore germination frequencies of *Zymoseptoria tritici* IPO323 (WT) after treatment with H_2O_2. Spores of the disruptant strains *ΔZtCpx2* and *ΔZtCpx1* and the double disruptant *ΔΔZtCpx1-Cpx2* and the complemented strain *ΔZtCpx1*-C were plated on PDA amended with 4 and 6 mM H_2O_2 and the germination of the spores was determined at 24 hours (A) and 48 hours after treatment (B). For each strain 50 spores were analyzed in three biological replicates.

Expression profiling of the *Z. tritici* catalase and catalase-peroxidase genes

To study the *in planta* expression levels of *ZtCpx1*, *ZtCpx2*, *ZtCat1* and *ZtCat2*, we inoculated the susceptible wheat cv. Taichung 29 with the WT and sampled every four days

after inoculation until 20 dpi and compared gene expression with *in vitro* conditions under nutrient-rich (yeast glucose) and nutrient-poor (minimal medium) growing conditions using quantitative real-time PCR (q-RT-PCR). Expression of *ZtCpx1* showed a bimodal pattern with peaks at 4 dpi and 16/20 dpi, which correspond with activation during the initial biotrophic stage and the late necrotrophic stage. In contrast, the expression of *ZtCpx2* peaked at eight dpi and then gradually decreased, suggesting that *ZtCpx2* expression might be related to the switch from biotrophic to necrotrophic growth when H_2O_2 production is triggered, accumulates and subsequently decreases due to cell death (Fig. 4A). Eventually, the expression levels of *ZtCpx1* and *ZtCpx2* were analyzed under *in vitro* condition during vegetative growth and asexual spore formation (Fig. 4B). *ZtCpx1* was mainly expressed in spores, whereas *ZtCpx2* was exclusively expressed in vegetative mycelium, consistent with the massive increase in fungal biomass *in planta* at eight dpi and onwards (Fig. 4B) (Kema *et al.*, 1996).

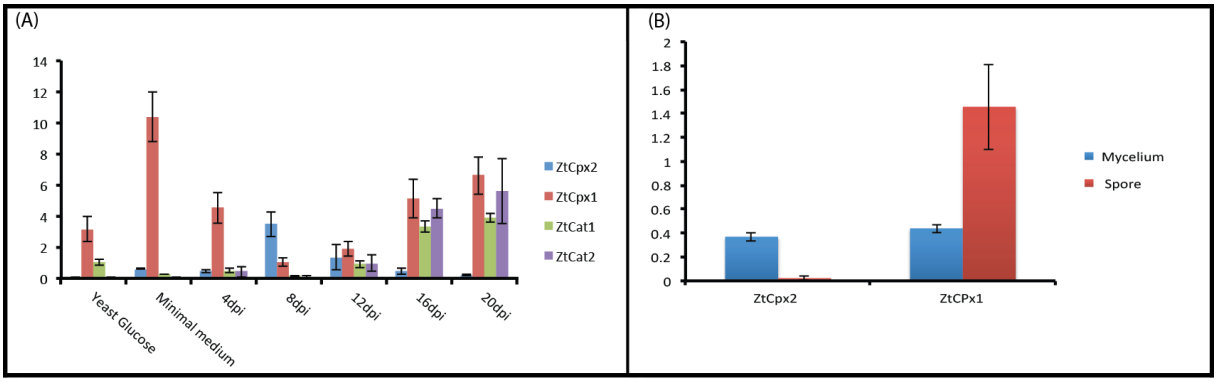

Figure 4. *In planta* (A) and *in vitro* (B) expression levels of the *Zymoseptoria tritici* catalase genes *ZtCat1* and *ZtCat2* and catalases-peroxidase genes *ZtCpx1* and *ZrCpx2*. Leaves of cv. Taichung 29 were inoculated with the WT strain and harvested 4, 8, 12, 16 and 20 days post inoculation (dpi) Under *in vitro* growth conditions expression of *ZtCpx1* and *ZtCpx2* was profiled in mycelium and spore. Data were normalized with the constitutively expressed *Z. tritici* β-tubulin gene.

Loss of function of *ZtCpx1* modulates expression patterns of related antioxidant genes

Disruption of *ZtCpx1* affects the transcription of *ZtCpx2*, *ZtCat1* and *ZtCat2* during infection. Profiling of these genes in the *ΔZtCpx1* strain showed that the expression of *ZtCpx2* was delayed (12 dpi) compared to the WT (8 dpi). *ZtCat1* was specifically up-regulated at 4 dpi, which is different from its expression pattern in the WT, whereas *ZtCat2* showed a similar expression profile in both *ΔZtCpx1* and WT strains (Fig. 5A). Finally, we monitored the relative expression levels of *ZtCat1* and *ZtCat2* in the *ΔΔZtCpx1-Cpx2* double mutant,

and showed that the former was only expressed at 12 dpi, while the latter had a variable expression pattern over the entire time course (Fig. 5B).

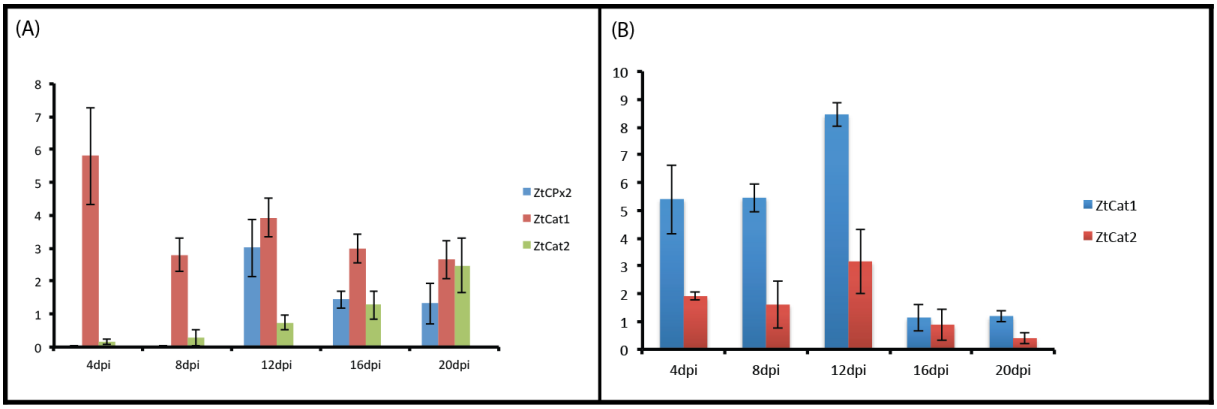

Figure 5. Relative *in planta* transcription of *ZtCpx2*, *ZtCat1* and *ZtCat2* in the *ΔZtCpx1* disruptant background (a) and of *ZtCat1* and *ZtCat2* in the *ΔΔZtCpx1-Cpx2* double disruptant background (b). RNA was isolated from leaf samples of cv. Taichung 29 at 4, 8, 12, 16 and 20 days post inoculation (dpi).

ZtCpx1 and *ZtCpx2* are required for full virulence

In addition, we investigated the effect of individual and combined disruption of *ZtCpx1* and *ZtCpx2* on virulence. The WT and all disruptants (*ΔZtCpx1*, *ΔZtCpx2* and *ΔΔZtCpx1-Cpx2*) as well as the complemented strain *ΔZtCpx1*-C were used to inoculate cv. Taichung 29 and assayed as described before (Mehrabi *et al*., 2006). The WT and *ΔZtCpx2* strains produced typical symptoms with small chlorotic flecks around 7-8 dpi that expanded into larger chlorotic lesions at 10-12 dpi and eventually coalesced into typical necrotic STB blotches bearing numerous pycnidia at 14-16 dpi (Fig. 6), indicating that *ZtCpx2* is dispensable for full virulence. In contrast, the expression of disease symptoms was significantly delayed and not uniformly distributed over the inoculated leaf area after inoculation with the *ΔZtCpx1* strain, whereas the WT phenotype was completely restored in the complemented *ΔZtCpx1*-C strain. Finally, the double disruptant *ΔΔZtCpx1-Cpx2* showed severely attenuated symptoms and only caused a limited number of necrotic lesions with few pycnidia at 21 dpi (Fig. 6). To further substantiate these observations we quantified fungal biomass in all abovementioned interactions using a TaqMan assay (Fig. 7). The fungal biomass of the WT and *ΔZtCpx1*-C strains started to increase at 8 dpi, which significantly differed from the *ΔZtCpx2, ΔZtCpx1* and *ΔΔZtCpx1-Cpx2* strains. Fungal biomass increase of the *ΔZtCpx2* strain was delayed and at 12 dpi eventually reached to a level comparable with

that of WT at 8dpi, indicating that *ZtCpx2* is dispensable for virulence but may play a role in the switch from biotrophic to necrotrophic growth, possibly involving the modulation of host-derived H_2O_2 levels. Fungal biomass of the *ΔZtCpx1* strain developed slower and remained significantly lower during the entire infection process and, as expected, biomass of the *ΔΔZtCpx1-Cpx2* strain hardly developed throughout the infection process (Fig. 7).

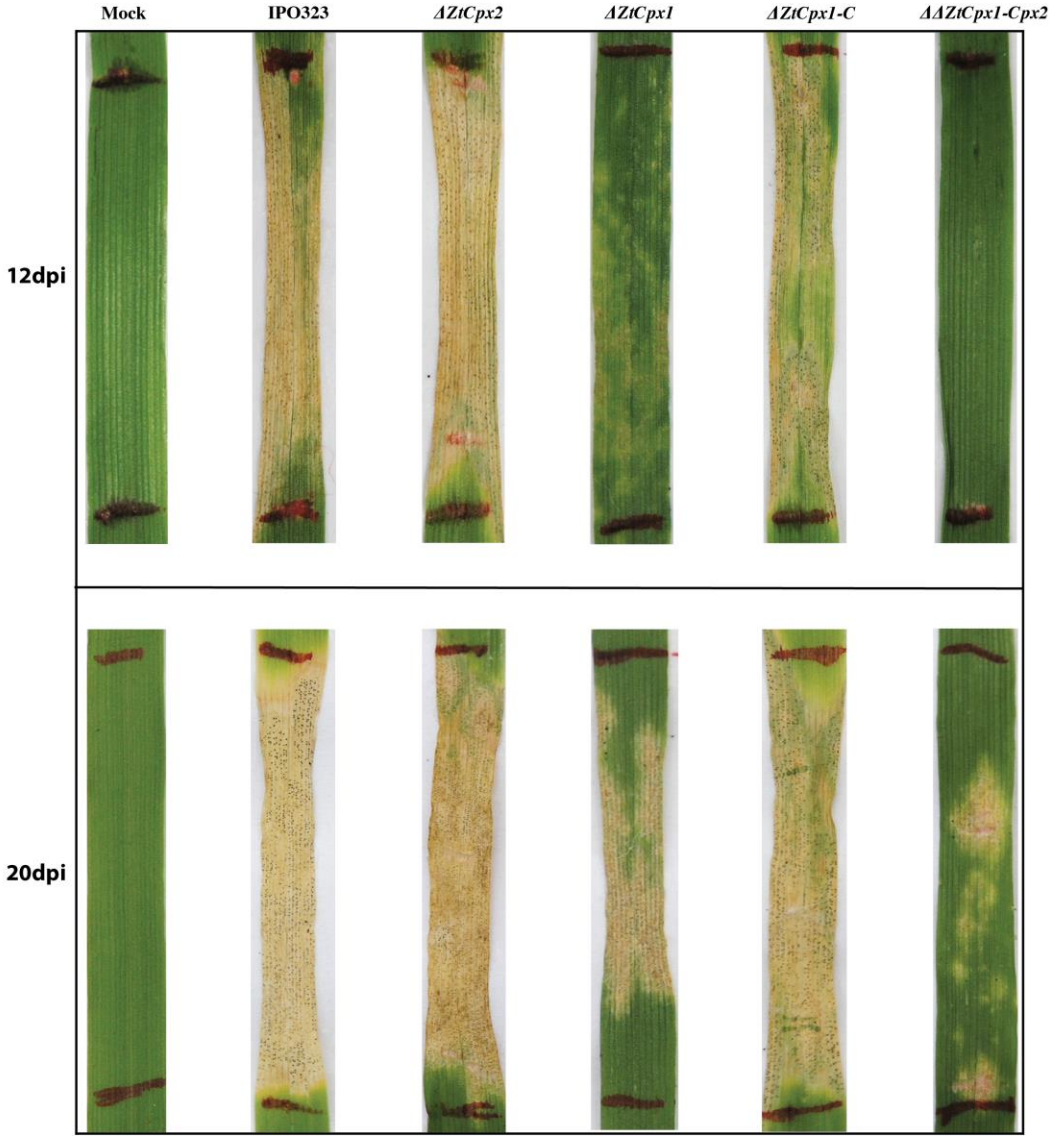

Figure 6. The effect of *ZtCpx1* and Zt*Cpx2* disruption on pathogenicity of *Zymoseptoria tritici.* Leaves of susceptible wheat cv. Taichung 29 were inoculated between the marked lines with *Z. tritici* IPO323 (WT), the single disruptants *ΔZtCpx2*, *ΔZtCpx1*, the double disruptant *ΔΔZtCpx1-Cpx2* and the complemented strain Δ*ZtCpx1*-C. Experiments were repeated *in triplo* and photographs were taken at 12 and 20 days post inoculation (dpi).

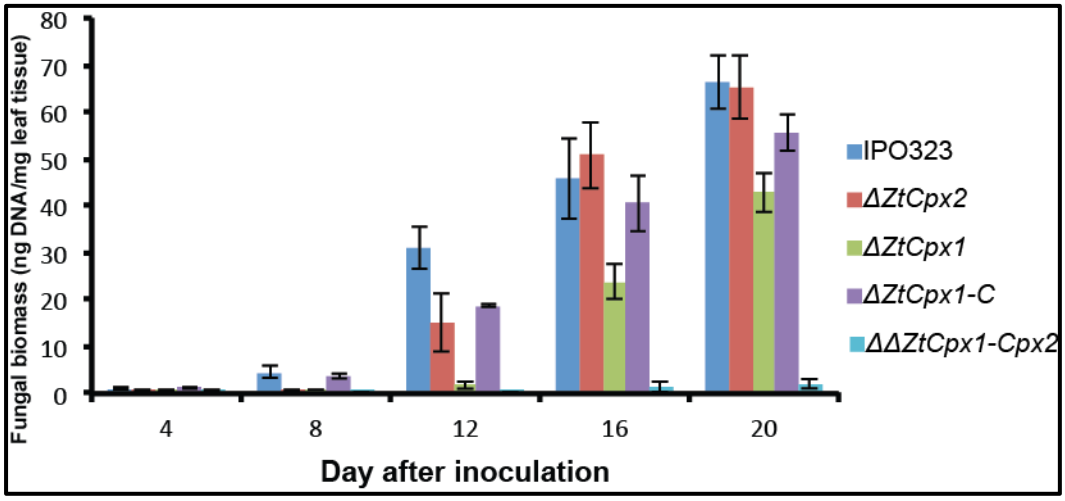

Figure 7. Fungal biomass quantifications in the susceptible wheat cv. Taichung 29 at 4, 8, 12, 16 and 20 days post inoculation. *Z. tritici* IPO323 (WT), the single disruptant strains *ΔZtCpx2*, *ΔZtCpx1*, the double disruptant *ΔΔZtCpx1-Cpx2* and the complemented strain *ΔZtCpx1*-C.

Discussion

For successful colonization of host tissues, fungal plant pathogens have evolved sophisticated mechanisms to overcome physical and chemical host defense barriers. One of the most rapid and earliest host defense responses is the generation of ROS to prevent or slow down fungal invasion (Heller and Tudzynski, 2011). However, a balance between radical-generating and radical-scavenging systems is required for proper physiological function of plant cells. Thus generation and degradation of ROS is critical to avoid deleterious effects on plant cells (Nanda *et al*., 2010). Various families of enzymes are involved in ROS production and ROS degradation in plant cells during infection by pathogens. NADPH oxidases, which are found in all kingdoms (NOx/RBOH), are associated with ROS production (Sumimoto, 2008), whereas glutathione peroxidases (Bae *et al*., 2009; Huang *et al*., 2011) and peroxiredoxins (Tripathi *et al*., 2009) are involved in ROS scavenging or detoxification. During interactions between host plants and fungal pathogens, ROS levels are highly elevated as an early defense response. Fungal pathogens, in turn, are equipped with several ROS detoxifying enzymes, such as CPs, in order to overcome the deleterious effects of ROS (Tanabe *et al*., 2010).

Several studies have attempted to elucidate the role of detoxifying enzymes in necrotrophic and biotrophic pathosystems. It has been suggested that ROS scavenging enzymes may play an important role in disease establishment for biotrophic pathogens (Bussink and Oliver, 2001; Garre *et al*., 1998), while necrotrophic pathogens are reported to benefit from ROS production (Govrin and Levine, 2000). However, little is known about how

hemibiotrophic pathogens deal with increased ROS levels during interactions with their hosts and how fungal detoxifying enzymes facilitate host infection.

To address this question we identified and functionally analyzed the biological role of two CP genes (dessignated *ZtCpx1* and *ZtCpx2*) in *Z. tritici*, a fungal wheat pathogen with a distinct hemibiotrophic lifestyle. Phylogenetic analysis revealed that ZtCpx1 grouped with CpeA1-2 of *V. longisporum* (Singh *et al*., 2012), which points at a presumed role of ZtCpx1 in protecting the fungus against oxidative stress generated by the host plant. Additionally, ZtCpx2 clusters closely with *M. oryzae* CPXB (Tanabe *et al*., 2011) and *V. dahliae* Cpx2 (Tran *et al*., 2014), suggesting a potential role of *ZtCpx2* in the scavenging or detoxification of host-derived H_2O_2. Our results showed that deletion of the *ZtCpx1* gene resulted in enhanced sensitivity of the mutant to H_2O_2 and significantly reduced its virulence. In contrast, *ZtCpx2* was dispensable for full virulence of *Z. tritici*, albeit that disruption significantly reduced fungal biomass development during the switch from biotrophic to necrotrophic growth. Interestingly, by generation of double mutants of both genes we showed a synergistic mode of action of both CPs to facilitate wheat infection. Similar to our findings, deletion of *MgCat2*, encoding a secreted catalase, in the hemibiotroph *M. grisea* severely affected virulence by partly impaired appressorium formation and reduced sporulation. Additionally, conidia melanization was impaired, which is an important metabolic process to fortify fungal cell walls. Overall virulence was reduced by 60% and 65% compared with the controls on barely and rice, respectively. Finally, *MgCat2* proofed to be essential for cell wall strength (Skamnioti *et al*., 2007). Another *M. oryzae* MgCPXB, which encodes a secreted CP, is required for neutralizing host-produced H_2O_2 during initial colonization, but not for full virulence (Tanabe *et al*., 2010). Later, Singh et al. (2012) reported that CpeA1-2 from *V. longisporum*, which encodes a cytoplasmic CP, plays an important role during late phases but not during the initial phases of infection of oilseed rape, Recently, Tran et al. (2014) showed that *Vta2*, a transcription activator of adhesion in *V. dahliae* and the secreted CP *Cpx2* are required to detoxify extracellular ROS. These results show that hemibitrophs need mechanisms to cope with host-derived H_2O_2 throughout the infection cycle.

When a pathogen enters leaves and colonizes the apoplast surrounding the host mesophyll cells or enters the cells, the host responds with an oxidative burst as shown in several pathosystems (Mellersh *et al*., 2002; Shetty *et al*., 2003). At that particular stage, neutralizing ROS is critical to successfully initiate biotrophy. Beyond that stage, a transition from biotrohy to necrotrophy takes places, which is associated with host cell collapse and a second wave of defensive responses, including massive generation of ROS. Hence,

hemibitrophic fungi need these mechanisms to cope with ROS during both phases of the infection process.

Hemibiotrophic fungi like *Z. tritici* may behave similar to biotrophic fungal pathogens. Since, biotrophic fungi thrive on nutrients released from living plant cells, they need a strong antioxidant machinery to deal with responsive oxidative stress upon plant invasion and further colonization. However, in contrast to this hypothesis, targeted gene replacement of both secreted and cytoplasmic catalase genes in biotrophic fungi, including *C. purpurea* and *C. fulvum* showed that these genes are dispensable for virulence (Bussink and Oliver, 2001; Garre *et al*., 1998). One possible explanation for these unexpected observations might be functional redundancy of catalases or activation of other related antioxidant enzymes that compensate the disruption. Indeed, when mining the genomes of *C. fulvum* and *C. purpurea* (Amselem *et al*., 2011; de Wit *et al*., 2012) we noted that both fungi contain four genes encoding catalases, suggesting that the effect of disruption of single catalase genes might be masked by the enzymes encoded by the other encoding genes. However, we previously deleted *ZtCat1* in *Z. tritici* and we showed that this gene is not required for virulence (un-published data). Additionally, we do not expect that *ZtCat2* plays an important role in modulating host-derived H_2O_2 as its expression levels in the *ΔΔZtCpx1-Cpx2* hardly changed. Collectively, it can be concluded that both catalases could not compensate the loss of function of either or both investigated CPs.

Nevertheless, in necrotrophs ROS-degrading enzymes do not seem to play a critical role in virulence (Robbertse *et al.*, 2003; Schouten *et al.*, 2002; Yarden *et al.*, 2014). For example in *C. heterostrophus*, deletion of all monofunctional catalase-encoding genes demonstrated that only ChCAT3 that encodes a secreted catalase, is involved in protecting the fungus from oxidative stress during vegetative growth as its deletion resulted in sensitivity to exogenously applied H_2O_2, but all the generated CAT mutant strains were not essential for virulence (Robbertse *et al*., 2003). Similarly, BcCAT2-deficient mutants of *B. cinerea* are hypersensitive to extracellular H_2O_2, but were not affected in virulence on tomato (Schouten *et al*., 2002).

One of the technical limitations for detecting slightly reduced virulence of mutants compared to WT strains might be due to the largely quantitative nature of symptom expression, where small variation can be easily overlooked. Therefore, it was suggested that more sensitive monitoring tools are required to detect small changes in virulence in generated disrupted strains (Robbertse *et al*., 2003; Singh *et al*., 2012). Quantification of fungal DNA at

various time points was therefore proposed to reveal such slight changes during the infection process. In this study, we used real-time quantitative PCR in order to determine small changes in fungal biomass in infected leaf tissues at different time points. Our results showed that this technique is sensitive and reliably enables the precise measurement of fungal biomass, even at very early stages infection when no disease symptoms are observed. Interestingly, although we could not detect disease symptom differences between the *ΔZtCpx2* mutant and the WT strain, we were able to determine the profile of fungal biomass development of the *ΔZtCpx2* strain, which differed from the WT. The growth of *ΔZtCpx2* mutant was slowed down and at 12 dpi eventually reached to a level comparable with that of the WT at 8dpi, indicating that *ZtCpx2* is dispensable for infection, but may play a role in the switch from biotrophic to necrotrophic growth. Similarly, Singh et al. (2012) were able to show that CpeA1-2 of *V. longisporum* is not involved in launching the initial phase of plant infection whilst the examined CP played an important role in advanced stages of infection, but the overall *V. longisporum* DNA content in plants infected by the *CpeA1-2* mutant was significantly lower than that of the WT at 35 dpi .

Z. tritici is commonly categorized as a hemibiotrophic fungus with a long symptomless phase that is considered to be biotrophic (Kema *et al*., 1996). Accumulation of H_2O_2 in wheat response to *Z. tritici* penetration has been reported and its deleterious effects have been reported (Shetty *et al*., 2003; Yang *et al*., 2013). Infiltration of wheat leaves with H_2O_2 increased the latency period but also decreased stomatal penetration and mesophyll colonization, suggesting that H_2O_2 is harmful during this stealth phase of colonization (Shetty *et al*., 2007; Goodwin *et al*., 2013). Consequently, *Z. tritici* needs genes enabling it to efficiently initiate biotrophic growth. Here, we show that ZtCpx1, the only cytoplasmic CP, plays a central role in protection of *Z. tritici* against host-generated H_2O_2 during the initial phases of host colonization. Expression analyses revealed that *ZtCpx1* was up-regulated during the biotrophic phase (4 dpi) as well as during *in vitro* spore production, suggesting that *ZtCpx1* probably plays an essential role in the establishment of the biotrophic stage. Furthermore, loss of *ZtCpx1* attenuated virulence and fungal DNA quantifications showed a significant reduction of fungal biomass of the *ΔZtcpx1* strain throughout the infection process. Collectively, we showed that *ZtCpx1* is pivotal for managing host-generated H_2O_2 during the initial and final phases of colonization enabling *Z. tritici* to complete its lifecycle and sporulate in infected tissues. However, after 8-10 dpi, a transition from biotrophic to necrotrophic growth occurs (Kema *et al*., 1996), which likely requires additional mechanisms to cope with varying waves of ROS during infection (Shetty *et al*., 2003; Yang *et al*., 2013).

Initially, it was hypothesized that *Z. tritici* would benefit from plant defense responses similar to necrotrophic pathogens such as *B. cinerea* to facilitate host colonization (Govrin and Levine, 2000). However, Shetty et al. (2007) showed that scavenging H_2O_2 through catalase during the biotrophy/necrotrophy switch enhanced its growth and symptom expression, demonstrating that the regulation of H_2O_2 accumulation during this specific stage is crucial to continue colonization, survive cell death and eventually allow sporulation (Shetty *et al*., 2007). In agreement with these findings, functional characterization of ZtCpx2, the only secreted CP in the *Z. tritici* genome, demonstrated that it is involved in regulation H_2O_2 levels during the transition from biotrophy to necrotrophy, which is supported by the *in planta* expression analyses of *ZtCpx2* that clearly peaked at 8 dpi. Disruption of *ZtCpx2* indeed significantly reduced fungal biomass at this crucial stage, indicating that *ZtCpx2* is important for pathogen survival during the metabolic *in planta* switch. Finally, analysis of the double knock-out strain *ΔΔZtCpx1-Cpx2* showed that both *ZtCpx1* and *ZtCPx2* are required for pathogenicity and likely also for regulation by *Z. tritic* of ROS waves throughout plant colonization.

Taken together, these data provide strong evidence how specific genes contribute to the modulating versatility of a plant pathogen in dealing with adverse *in planta* and environmental conditions. To our knowledge, this is the first demonstration that two CPs collectively contribute to pathogenicity in a fungal plant pathogen.

Experimental Procedures

Strains, media and growth conditions

The fully sequenced *Z. tritici* reference strain IPO323, which is highly pathogenic on wheat cv. Taichung 29, was used as wild type (WT) and recipient strain for gene replacement. The WT and all deletion strains were kept at -80 °C and were re-cultured on potato dextrose agar (PDA) (Sigma-Aldrich Chemie, Steinheim, Germany) at 18 °C once desired for experimentation. Yeast-like spores were produced in yeast glucose broth (YGB) medium (yeast extract 10 g/L, glucose 30 g/L) after placement in an orbital shaker (Innova 4430; New Brunswick Scientific, Nijmegen, The Netherlands) at 18 °C. *Aspergillus nidulans* minimal medium (MM) was used for *in vitro* expression analyses (Barratt *et al.*, 1965). *Escherichia coli* DH5a and DH10β were used for general plasmid transformation and *Agrobacterium tumefaciens* strain AGL-1 was used for all fungal transformations.

Phylogenetic tree construction

Phylogenetic analyses of *Z. titici* catalases and CP enzymes with their homologues from other fungal plant pathogens were conducted using the CLC genomics workbench package (Aarhus, Denmark). All fungal proteins were retrieved from public databases and aligned using CLC software, considering a gap opening cost and gap extension penalty of 10 and 1, respectively. The phylogenetic tree was constructed based on the unweighted pair group method with arithmetic average (UPGMA) algorithm, and the statistical accuracy of the tree was tested by bootstrap analysis with 1000 repetitions.

Generation of gene replacement and complementation constructs

A full-length 2.5 kb cDNA clone (ZtEST2P12K00276) containing *ZtCpx1* (named pSport1-*ZtCpx1*) was identified in the cDNA libraries of *Z. tritici* IPO323 (Kema *et al.*, 2008). The *ZtCpx1* insert was excised from the pSport1-*ZtCpx1* plasmid using KpnI/XbaI and ligated into the binary vector pCGN1589 generating pCGN*ZtCpx1*. The GPS-Mutagenesis system (New England Biolabs, Leusden, The Netherlands) was used to make the disruption construct of *ZtCpx1*. A customized donor construct, pGPS3HygKan (Mehrabi *et al*., 2006) was used for transposition. The target construct pCGN*ZtCpx1* was transposed by the donor construct pGPS3HygKan and the resulting transposition mixture was cloned into *E. coli* DH10β. A colony PCR was performed to identify clones carrying a construct with the insertion of the transposon into the *ZtCpx1* gene. We selected a construct (named pCGN*ΔZtCpx1*) in which the transposon was inserted almost in the middle of the *ZtCpx1* open reading frame (ORF). This construct was used to disrupt *ZtCpx1* in *Z. tritici* IPO323 through *A. tumefaciens*–mediated transformation (ATMT) according to Mehrabi et al. (2006). In order to generate the *ZtCpx1* complementation construct (p*ZtCpx1*com), the multisite gateway® three-fragment vector construction kit (Invitrogen, CA, USA) was used enabling us to clone three fragments into the destination vector, which was compatible with the ATMT procedure. The full ORF of *ZtCpx1* including 994 bp upstream as its promoter and 498 bp downstream as terminator were cloned into pDONR™P221 (Invitrogen, CA, USA) resulting in the generation of p221-*ZtCpx1*com. The p221-*ZtCpx1*com as well as two entry vectors pRM245 and pRM234 (Mehrabi *et al.*, 2015) were used to clone these three fragments into the destination vector, pPm43GW, through the LR reaction. To generate the *ZtCpx2* deletion construct, a 2 kb upstream and downstream sequence of *ZtCpx2* was cloned in pDONR™P4-P1R and pDONR™P2R-P3. The generated constructs along with pRM250 (Mehrabi *et al.*, 2015) containing the hygromycin phosphotransferase (*Hph*) gene as a selection marker were

cloned into the destination vector, pPm43GW, via the LR reaction. To make the double knock-out construct, p*ZtCpx1-2*, approximately 1.2 kb upstream and downstream of *ZtCpx1* sequence was cloned in pDONR™P4-P1R and pDONR™P2R-P3, respectively. These two resulting entry vectors, along with pRM251 (Mehrabi *et al.*, 2015) (containing geneticin as a selectable marker) (Mehrabi *et al.*, 2015) were used to clone these three fragments into the destination vector, pPm43GW. Primers used in this study are listed in Table 1.

Table 1. Primers used in this study.

Name	Sequence (5'–3')	Location
PrimerE	ATGTCTGCAAACGGTTGCCCAA	*ZtCpx1*
PrimerF	CTACAACTTCGCGCTGCTGAC	*ZtCpx1*
PrimerG	ATGAAGGGTTGTCTCAATCATCTG	*ZtCpx2*
PrimerH	CTACTGGACATCGTTCTGAGGA	*ZtCpx2*
PrimerK	GTGCTCACCGCCTGGACGACTAAAC	Middle of *hph* gene
PrimerL	GATGAGACCCGGCGACAAGT	Downstream of *ZtCpx2*
PrimerM	TTCGACCGTGGCTTGACACC	Upstream of *ZtCpx1*
PrimerN	TCCACCCAAGCGGCCGGA	Beginning of geneticin gene
Q-ZtCpx1-F	ACAACGCCAATCTCGACAAG	*ZtCpx1*
Q-ZtCpx1-R	GACTCAATGGCGACATTTCC	*ZtCpx1*
Q-ZtCpx2-F	TCCTAAATCCGAGCCTTTCC	*ZtCpx2*
Q-ZtCpx2-R	TCAACCCACTGCCAAGAATC	*ZtCpx2*
Q-ZtCat1-F	GATGCACATGAAGAGCAAGC	*ZtCat1*
Q-ZtCat1-F	GAAACTCGCCCTTCTCAATG	*ZtCat1*
Q-ZtCat2-F	ACTTTGGCGTTCAGGTCATC	*ZtCat2*
Q-ZtCat2-F	ACTCCGACGTTCTGAATTGG	*ZtCat2*

Fungal transformation

All transformations were performed using ATMT as described previously (Mehrabi *et al.*, 2006; Zwiers and de Waard, 2001). Genomic DNA of stable transformants was extracted according to standard protocols (Sambrook and Russell, 2006). For complementation and double knock-out strategies, the same procedure was utilized with minor modifications, including the use of 250 µg mL^{-1} geneticin for selection of mutants.

In vitro oxidative stress assays

Sensitivity of *Z. tritici* strains to continuous exposure of H_2O_2 was conducted in PDA with various concentrations of H_2O_2. Autoclaved PDA was cooled down to 40 °C, and H_2O_2

(Sigma) was added to final concentrations of 6, 8 and 10 mM. Five µl of spore suspension of each strain with concentrations of 10^8 spores/mL were spotted in the centre of the Petri dishes that were subsequently incubated in an incubator at 20 °C for 7 days. Photographs were taken with an Olympus camera.

Germination frequency assays

PDA plates supplemented with 4, and 6 mM H_2O_2 were prepared and cut into one cm^2 plugs placed on glass slides that were subsequently inoculated with 10 µl of a yeast-like spore suspensions (10^4 spores/mL) that were then covered with a cover slip. The samples were kept in Petri plates containing a piece of wetted cotton wool to maintain a high relative humidity and were incubated at 20 °C for two days. The germination frequency of each strain was calculated based on the number of germinated spores of a 50 randomly selected spores using a light microscope (Zeiss, Munich, Germany) at 40x magnification. The experiments were conducted in three replicates and the percentage of germinated spores was recorded as shown in Fig. 3.

RNA isolation and q-RT-PCR

In vitro and *in planta* expression profiling of *ZtCpx1, ZtCpx2, ZtCat1* and *ZtCat2* was performed using quantitative real-time PCR (q-RT-PCR). For i*n planta* analyses, the wheat cv. Taichung 29 was inoculated with the WT as described previously (Mehrabi *et al.*, 2006) and leaf samples were collected in three biological replicates, flash frozen and ground in liquid nitrogen using a mortar and pestle. Total RNA was extracted either from ground leaves or fungal biomass produced in YGB using the RNeasy plant mini kit (Qiagen, location, USA) and subsequently DNA contamination was removed using the DNAfree kit (Ambion, Cambridgeshire, U.K.). First-strand cDNA was synthesized from approximately two µg of total RNA primed with oligo(dT) using the SuperScript III according to the manufacturer's instructions. One µl of the resulting cDNA was used in a 25 µl PCR reaction using a QuantiTect SYBR Green PCR Kit and run and analyzed using an ABI 7500 Real-Time PCR System. The relative expression of each gene was initially normalized with the constitutively expressed *Z. tritici* beta-tubulin gene (Keon *et al.*, 2007; Motteram *et al.*, 2009) and then calculated based on the comparative C(t) method described previously (Schmittgen and Livak, 2008).

Pathogenicity assays

The wheat cv. Taichung 29 was grown in the greenhouse until the first leaves were fully unfolded. Inoculum of all strains was produced in YGB (yeast extract 10g/L, Glucose 30g/L) at 18 °C for 7 days in an orbital shaker (Innova 4430; New Brunswick Scientific, Nijmegen, The Netherlands) and yeast-like spores were obtained after centrifugation at 3000 rpm and two washing steps to remove residual medium. Subsequently, the spore concentrations were adjusted to 10^7 spores mL^{-1} and the resulting suspension was supplemented with 0.15% Tween 20 as a surfactant. A five cm fragment of each leaf was marked and inoculated with a cotton swab containing spores and inoculated plants were incubated in closed transparent plastic bags for 48 hours to maximize humidity and then transferred to a greenhouse compartment at 22 °C, with a relative humidity >90% and 16 hours light. Disease development was monitored and recorded every three days. Fungal DNA quantifications were conducted in leaves of cv. Taichung 29 that were harvested at 4, 8, 12, 16 and 20 dpi. Genomic DNA was extracted from approximately 100 mg of infected leaves using standard phenol/chloroform DNA extraction (Sambrook and Russell, 2006) q-RT-PCR was performed in order to quantify the fungal biomass in infected leaf tissues as described previously (Shetty *et al*., 2007, Waalwijk *et al.*, 2002).

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Supporting Information

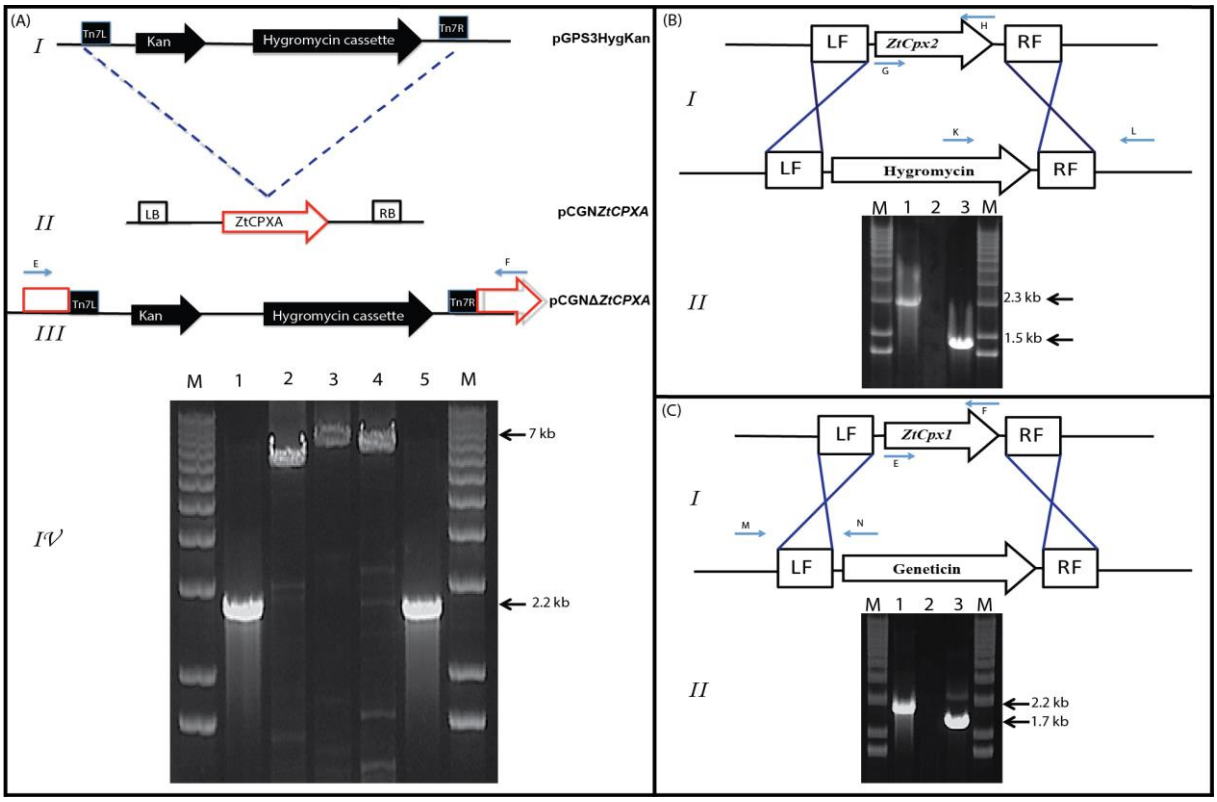

Figure S1. Scheme of construct generation for *ΔZtCpx1* (A), *ΔZtCpx2* (B), and *ΔΔZtCpx1-Cpx2* (c). A (I) customized donor construct (pGPS3HygKan) containing a kanamycin and hygromycin cassette was used for transposition into A (II), the target construct pCGN*ZtCpx1*, resulting in a (III), the disruption construct pCGNΔZtCpx1. A (IV), PCRs performed to confirm homologous recombination. The three independent disruptant strains were used for large size PCR amplification using primers E and F; Lane M, 1-kb-plus ladder marker. Lane 1, *Z. tritici* IPO323. Lanes 2, 3 and 4, *ΔZtCpx1* ≠5, ≠8 and ≠9. Lane 5, *ZtCpx1*-C. B (I), Diagram showing the replacement of *ZtCpx2* by the hygromycin phosphotransferase (*hph*) resistance cassette through homologous recombination. The lines depict the upstream (LF) and downstream (RF) fragments used for homologous recombination. B (II), Identification of *ΔZtCpx2* by PCR; Lane M, 1-kb-plus ladder marker. Lane 1 shows the amplification of *ZtCpx2* in *Z. tritici* IPO323 using primers G and H whereas no amplicon of *ZtCpx2* was observed with primers G and H in *ΔZtCpx2*. Lane 3 shows the expected band of 1.5 kb in *ΔZtCpx2* amplified by using primers K and L located in the middle of the *hph* gene and downstream of the *ZtCpx2* ORF. C (I), Diagram descripting the generation of *ΔZtCpx1-B.48*. The *ZtCpx1* ORF was deleted in the *ΔZtCpx2* disruptant through homologous recombination. Lane 1 shows the amplification of *ZtCpx1* in the WT using primers E and F, whereas no amplicon of *ZtCpx1* was observed with primers E and F in *ΔΔZtCpx1-Cpx2* . Lane 3 shows the expected band of 1.7 kb in *ΔΔZtCpx1-Cpx2* generated by primers M and N that are located upstream of the *ZtCpx1* ORF and the start of the geneticin gene.

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Chapter 6

General discussion

The interaction between plants and their pathogens represents a complicated research area with a long and intriguing history in science, particularly as the subject is closely linked with concerns about food production. The pioneering research on flax and the flax rust fungus *Melampsora lini* resulted in the gene-for-gene (GFG) hypothesis (Flor, 1947). Since then many plant pathologists have tried to confirm GFG in other pathosystem and to identify the molecular evidence for (in)direct interactions between host and pathogen molecules. Currently, we know that fungal pathogens are equipped with an array of effector proteins – usually called small-secreted proteins (SSPs) - enabling them to attack and/or circumvent host defense responses that eventually lead to a successful colonization. Effectors are SSPs that principally have intrinsic virulence function, but can act also as (a)virulence factors when (in)directly recognized by the plant immune system represented by the corresponding resistance proteins and the associated regulatory networks (Dangl *et al*., 2013). For necrotrophs, an inverse GFG (iGFG) has been proven, where secreted effector proteins are recognized by corresponding host receptor proteins produced by host susceptibility genes (Faris *et al*., 2010). These interacting fungal effectors are also known as host-selective toxins (HSTs), which act as virulence or pathogenicity factors (Friesen *et al.*, 2008a). One of the best-known examples for such an interaction is SnToxA, a small proteinaceous HST produced by *Parastagonospora nodorum*. The proteinaceous toxin induces necrosis on wheat lines carrying the corresponding toxin sensitivity genes *Tsn1* (Friesen *et al.*, 2006). In addition to effector proteins, plant pathogens produce a wide variety of other proteins such as plant cell wall degrading enzymes (CWDEs), proteases and protease inhibitors, detoxifying enzymes and secondary metabolites, which are commonly termed pathogenicity or virulence factors. Host defense responses have to be suppressed or circumvented for the colonizing pathogen to eventually complete its sexual or asexual life cycle. Evidently, identifying and a thorough understanding of the role of these (a)virulence factors and their distribution in natural populations will contribute to more sustainable forms of host resistance and an overall better disease management. For example, the importance of the melanin pathway for the rice blast fungus *Magnaporthe oryzae* has resulted in antifungal compounds that interfere with melanin metabolism and disable the pathogen to produce the required turgor for appressorium production and hence plant penetration. The tricyclazol that inhibits melanin synthesis in *M. oryzae* is applied for managing rice blast disease in the field (Howard and Valent, 1996).

The aim of the current thesis is to develop a better understanding of the molecular mechanisms underlying virulence and pathogenicity of the fungal wheat pathogen *Zymoseptoria tritici* that causes a foliar blight in bread and durum wheat also known as

septoria tritici blotch (STB). Here, we identified many SSPs that potentially can act as (a)virulence factors in the *Z. tritici*-wheat pathosystem. Using various technologies, including transcriptomics, proteomics, bioinformatics and mapping approaches an array of SSPs has been identified and await further functional characterization. However, new strategies should support either narrowing down the number of candidates or enable a much higher efficiency in (simultaneous) screening of such candidates. Our data as well as those of others have shown that many of the identified candidates are dispensable for virulence, or once required for virulence, they do not explain specificity. In retrospect, much of the candidate SSP identification might be erroneous due to the poor annotation of genes in the *Z. tritici* genome sequence. Nevertheless, we showed the biological functions of two pivotal virulence factors and mapped many others that will be the subject of subsequent studies. In this chapter, we reflect on the major findings of this study and discuss future strategies to identify effectors in *Z. tritici*.

Functional genomics toolbox for *Zymoseptoria tritici*

The finished genome sequence of the *Z. tritici* reference strain IPO323 (reference isolate) contains approximately 12,000 genes. In order to investigate biological roles of candidate genes in the infection process of *Z. tritici*, a high throughput functional analysis procedure is crucial, primarily focussing on the generation of knock-out, disruption or knock-down strains. We have invested in developing a routine high throughput gene disruption protocol by designing a suite of vectors for manipulating *Z. tritici* that replace contemporary methods using cumbersome digestion and ligation protocols (Adachi *et al.*, 2002; Marshall *et al.*, 2011; Roohparvar *et al.*, 2007; Zwiers and de Waard, 2001). We developed 22 entry constructs as new molecular tools based on the gateway technology, which facilitate the swift construction of binary vectors that can be used for functional analysis of genes in *Z. tritici* (**Chapter 2).** The functionality of these entry vectors was validated through *Agrobacterium tumefaciens*-mediated transformations (ATMT) of *Z. tritici*. For example, in **chapter 3**, pRM236 and pRM251 containing the green fluorescent protein (GFP) gene and neomycin phosphotransferase gene (known as the geneticin selection marker), respectively, were used to complement *ΔZtWor1* strains. In **chapter 5,** pRM250 and pRM251 carrying the hygromycin phosphotransferase (*Hph*) gene and the geneticin gene as markers, respectively, were employed to either delete the *ZtCpx2* gene or for generating the double disruptant strain *ΔΔZtCpx1-Cpx2*. However, development and application of other techniques such as virus-induced gene silencing (VIGS) or heterologous expression system would be beneficial to

rapidly and routinely test the potential candidate effector genes in *Z. tritici* (Kombrink, 2012; Mascia *et al*., 2014).

Effector discovery in *Zymoseptoria tritici*

Using proteome analyses of the infected host apoplast to identify candidate effectors

Fungal effector proteins modulate host immunity resulting in various levels of disease severity depending on a range of regulatory molecular switches that enable or disable the pathogen to infect and colonize host tissues. Currently, effectors are classified as apoplastic or cytoplasmic effectors, depending on their host targets (Kamoun, 2006; Stergiopoulos and de Wit, 2009). Apoplastic effectors are secreted and accumulate in the plant extracellular space where they interact with host targets or membrane receptors. The plant apoplast, therefore, is a rich resource for discovery research into virulence or (a)virulence proteins (de Jonge *et al.*, 2010; Houterman *et al.*, 2009; Kamoun, 2006; Thomma *et al.*, 2005). Cytoplasmic effectors target various subcellular compartments probably through specialized delivery structures such as haustoria or distinct secretion systems as shown in *M. oryzae* (Giraldo *et al.*, 2013; Kamoun, 2006). Indeed, the majority of effector proteins from the tomato fungal pathogens *Cladosporium fulvum* and *Fusarium oxysporum* f. sp. *lycopersici* (Fol) were identified by analyzing the host apoplast and vascular systems of infected plants (Bolton *et al.*, 2008; Houterman *et al.*, 2009; Rep *et al.*, 2004; van Kan *et al.*, 1991). Most recently, Kim et al. (2013) developed a screening method for the secretome of *M. oryzae* using proteome analyses of *in vivo* apoplastic fluids, which resulted in proteins belonging to the glycosyl hydrolase protein (GH) family and four GH genes could actually be new apoplastic effectors.

We also isolated apoplastic fluids (AFs) from resistance and suceptibile wheat cultivars during colonization by *Z. tritici* and analyzed them using SDS-PAGE gel electrophoresis and liquid chromatography/mass spectrometry (LC–MS/MS), which resulted in a plethora of fungal proteins, including SSPs, CWDE and proteases. Clearly, this is a rich resource for detailed studies aiming at deciphering the role of these proteins and their encoding genes during colonization of wheat by *Z. tritici* (Ben M'Barek *et al.*, 2015b). For example, in **Chapter 5**, we functionality characterized two catalase-peroxidase (CPs) genes that we discovered in AFs and which are the first CPs that were associated with disease development by a fungal wheat pathogen, possibly through modulating H_2O_2 levels at various phases of pathogenesis.

Culture filtrates of in vitro-grown fungi as a resource for effector discovery

It is widely documented that culture filtrates (CFs) of many Dothideomycete necrotrophic fungal pathogens contain phytotoxic peptides or HSTs that differentially induce cell death on cultivars harboring sensitivity gene (Stergiopoulos *et al.*, 2013). In *Pyrenophora tritici-repentis* and *P. nodorum*, fractionation of CFs combined with fast protein liquid chromatography (FPLC) resulted in the discovery of this novel class of effectors (Friesen *et al.*, 2007; Friesen *et al.*, 2008b; Lamari *et al.*, 2003; Tomas *et al.*, 1990). Similar approaches identified proteinaceous effectors in the *Z. tritici*-wheat pathosystem (Ben M'Barek *et al.*, 2015a). Two necrosis-inducing proteins (NIPs) were subsequently heterologously expressed in *Pichia pastoris* and infiltration assays demonstrated that they induce necrosis in a variety of wheat cultivars (Ben M'Barek *et al.* 2015a). However, so far this approach has not resulted in the discovery of major HSTs similar to well-known necrotrophic proteinaceous effectors.

Searching effectors by genome mining

Next-generation sequencing of fungal genomes provides a huge amount of data that are a valuable resource for discovery research. The initial analysis usually involves BLAST searches for effector genes that are shared among different fungal species. For instance, *ZtWor1*, the homologue of *FoSge1*, which is a conserved transcriptional regulator governing the expression of effector genes in Fol (Michielse *et al.*, 2009), was identified through BLAST analysis and expectations were high for comparative *Z. tritici* effector discovery. However, albeit that *ZtWor1* is required for pathogenicity of *Z. tritici*, possibly through the demonstrated regulation of effector genes, it is - contrary to Fol - more involved in developmental processes - such as conidiation - rather than being a specific regulator of effector genes **(Chapter 3).**

Obviously, the genome sequences of *Z. tritici* that currently comprise several hundred genomes (JGI 100 ready, 100 to come, Thierry Marcel INRA 200 to come) render an unparalleled starting position for candidate effector discovery. Several computational pipelines were developed to filter for candidate effector genes, but these are under continuous development, also depending on the used DNA sequencing strategies that are also in a constant state of development and throughput efficiency (van Dijk *et al*., 2014) . A typical functional genomics pipeline follows two distinct stages: firstly, cataloguing candidate effectors based on various data mining tools such as Signal P, and secondly, validations of the selected genes by functional assays (Kamoun, 2006). In **chapter 4,** we mined the genome of *Z. tritici* IPO323 and built a database with promising effector candidates with the following key features: (1) the presence of an N-terminus signal peptide for secretion, (2) relatively

small protein sizes (less than 300 amino acids), (3) the presence of at least four cysteine residues, (4) the absence of transmembrane domains outside the signal peptide, and (5) the absence of glycosylphosphatidylinositol (GPI) anchors. This resulted in a catalogue of 68 putative *Z. tritici* effectors and subsequently their expression levels were monitored at various stages of infection. Two outstanding candidate effectors - SSP15 and SSP18 - were selected for functional characterization, which were exclusively up-regulated during the transition from biotrophy to necrotrophy and during necrotrophic growth, respectively. However, the final result is that these genes are dispensable for *Z. tritici* virulence. Interestingly, Rudd et al. (2015) using RNA-seq approaches shortlisted the same candidate SSPs, including those functionally analyzed in **chapter 4**, and also determined their dispensability for pathogenicity of *Z. tritici*. The current status is that, despite tremendous efforts at various laboratories, thus far no major effectors have been discovered in the *Z. tritici*-wheat pathosysteem. Our results (**Chapter 4**) indicate that the aforementioned predetermined key qualifiers seem apparently inappropriate for prioritizing candidate effector genes. However, functional redundancy may of course also hamper the identification of individual SSPs and their role in pathogenesis, which can be addressed by generating double or triple knock-out strains as corroborated in several other pathosystems (Manning and Ciuffetti, 2015; Tan *et al.*, 2015).

Map-based identification of effector genes

Positional cloning has been successfully applied to discover and clone effector genes from several fungal pathogens, including *Leptosphaeria maculans*, *Peronospora parasitica* and *M. oryzae* (Fudal *et al.*, 2007; Gout *et al.*, 2006; Li *et al.*, 2009; Rehmany *et al.*, 2005), but it typically requires the generation of mapping populations and that is of course not possible for many pathogens that lack a functional sexual cycle.

Z. tritici, however, is one of the best studied organisms with respect to classical and population genetics (Linde *et al.*, 2002; Zhan *et al.*, 2003). Hence, we decided eventually and complementary to the previous strategies to explore a map-based identification of SSP candidates. Firstly, an existing mapping population from a cross between the Dutch *Z. tritici* reference strain IPO323, which is exclusively pathogenic on bread wheat and the Algerian durum wheat strain IPO95052 that causes disease in durum wheat resulted in 163 progeny isolates that were phenotyped on a suite of durum wheat and bread wheat cultivars. Since the studied parameters – necrosis development and pycnidia formation – have a continuous distribution and are largely quantitatively expressed, we mapped quantitative trait loci (QTLs) on the genome and one of those was positioned on the distal part of chromosome 5 and

showed high LOD values for specificity towards bread as well as durum wheat. Bioinformatic analyses were applied to catalogue SSPs that were located under these mapped QTLs. We subsequently determined their *in planta* expression profiles and unexpectedly observed that none of these SSPs, except SSP114, were expressed at low levels at all sampling points. This indicates that an unbiased map-based approach for effector discovery might be required to uncover essential components in the host–pathogen interaction between *Z. tritici* and wheat, similar to the approach of Lendenmann et al. (2014, 2015) to identify genes associated with melanization and fungicide sensitivity.

However, there is one very significant underlying assumption that needs to be scrutinized and that is the quality of the *Z. tritici* genome annotation. Whatever technique is being used, a poor annotation hampers efficient discovery of any candidate gene. Recent RNAseq data (Grandaubert *et al*., 2015) have now been used and resulted in an additonal 2,000 genes in the finished *Z. tritici* reference genome and –compared to the automated JGI annotation – many genes have been manually curated (Hesham Gibriel *et al*., unpublished). This has not altered our stretegy but contributed significantly to its accuracy and hence efficacy. Together with ongonig DArTseq fine mapping strategies we expect that new candidate genes explaining specificity in the *Z. tritici* – wheat pathosystem will be identified in the near future. This will then enable their dissemination in natural populations, which is the foundation for future disease management and *Stb* gene deployment strategies.

Weaponry of *Zymoseptoria tritici* to support its hemibiotrophic lifestyle

Plant pathogenic fungi have developed elaborated strategies in order to successfully colonize and infect host tissues. These mechanisms have been described at morphological levels, including the formation of highly specialized infection structures such as appressoria or haustoria and at molecular levels, comprising the secretion of toxins or secondary metabolites that facilitate the infection process (Horbach *et al.*, 2011). Based on nutritional behaviour fungal pathogens are defined as biotrophs, necrotrophs and hemibiotrophs (Horbach *et al.*, 2011). Biotrophic fungi thrive on living cells and they establish intimate relationships with their hosts (Mendgen and Hahn, 2002). It is generally accepted that biotrophic pathogens have a variety of means to avoid host recognition or suppress host defense response. For instance, *Ecp6* from *C. fulvum* encodes a protein with LysM domains (Bolton *et al.*, 2008) and is pivotal in scavenging chitin fragments that are released from the fungal cell wall during the infection of tomato, which prevents triggering basal defense signaling (de Jonge *et al.*, 2010). Biotrophic fungi have also to manage the generation of

highly toxic compounds such as ROS that are produced upon host attack (Wojtaszek, 1997). For example, *Yap1* encoding a transcription factor in the biotrophic maize pathogen *Ustilago maydis*, regulates the detoxification of host-derived H_2O_2 generated at the early stage of maize colonization (Molina and Kahmann, 2007).

In contrast, necrotrophic fungi thrive on dying host tissues and induce cell death to acquire the necessary nutrients from their host plants to continue colonization (Deller *et al.*, 2011). It is well documented that necrotrophs secrete either toxic peptides acting as HSTs or non-HSTs or secondary metabolites functioning as phytotoxins that play important roles in killing host tissues and thereby facilitate the infection process (Stergiopoulos *et al.*, 2013). For example, HC-toxin is a secondary metabolite toxin produced by *Cochliobolus carbonum* and plays a key role in host specificity and virulence of this fungus (Walton, 2006), whereas PtrToxA is a HST generated by *P. tritici-repentis* (Ballance *et al.*, 1989) that causes necrotic lesions on the leaves of susceptible wheat cultivars harboring the *Tsn1* sensitivity gene (Anderson *et al.*, 1999). Additionally, necrotrophs have many CWDE-encoding genes that support penetration and access to carbohydrates (Horbach *et al.*, 2011). In contrast to biotrophs, necrotrophs induce high ROS levels, which contribute to cell death (Glazebrook, 2005). For instance, it was demonstrated that the necrotrophic gray mold fungus *Botrytis cinerea* benefits from host cell death, which is initiated by host-generated ROS in order to increase its pathogenicity in Arabidopsis (Govrin and Levine, 2000).

Hemibiotrophic fungi, exhibit distinct morphological microscopic phases of pathogenesis, including an early symptomless biotrophic phase that switches to late necrotrophic growth, which is characterized by tissue collapse and expression of disease symptoms (Münch *et al.*, 2008) and likely requires a massive regulatory and metabolic switch. Like other fungal pathogens, hemibiotrophs must overcome plant defense responses upon invasion using an array of mechanisms. To efficiently establish biotrophic growth, the hemibiotrophic rice blast fungus *M. oryzae* secretes SLP1 a protein with LysM domains that plays a central role in suppressing PAMP-triggered immunity (PTI) mediated by chitin fragments recognized by a CEBiP receptor-like protein (Mentlak *et al.*, 2012). In addition, *M. oryzae* produces several enzymes and transcription factors that are required for the regulation of ROS generated by the host during early colonization (Guo *et al.*, 2011; Huang *et al.*, 2011; Skamnioti *et al.*, 2007; Tanabe *et al.*, 2011). The biotrophic growth switches to necrotrophy, which is accompanied by cell death and elicitation of host defense response such as the accumulation of host-derived H_2O_2. However, the underlying molecular mechanisms mediating the transition from biotrophy to necrotrophy are not well understood. Recently,

however, it was demonstrated that PPT1 from *Colletotrichum graminicola* that encodes the Sfp-type 4′-phosphopantheteinyl transferase is involved in this switch as Δ*ppt1* strains were able to colonize wounded maize leaves, but failed to generate typical anthracnose disease symptoms (Horbach *et al.*, 2009). Additionally, it was hypothesized that the shift from biotrophy to necotrophy in the hemibiotrophic oomycete *Phytophthora infestans* is regulated by the secretion of effector proteins that suppress early programmed cell death (PCD) in order to establish biotrophic growth and support massive necrosis at the late stage of infection (Lee and Rose, 2010). This was described as the "accelerator and brake" strategy in which *Sne1* that encodes the secreted hydrophilic protein at the early stage of colonization blocks PCD induced by necrosis-inducing effectors (Nep1-like proteins) and later during the infection process - once the pathogen proliferates through the host tissues - Nep-like proteins such as PiNPP1.1 that are expressed exclusively during necrotrophy induce rapid cell death and tissue necrosis (Kelley *et al.*, 2010; Lee and Rose, 2010).

The lifestyle of *Z. tritici* includes two distinct colonization phases, a stealth biotrophic phase and a ramifying necrotizing phase, in which various aspects of growth and differentiation can be studied in detail using a range of biological and molecular tools. Following stomatal penetration, the initial symptomless biotrophic phase, where hyphae remain strictly extracellular and grow in close contact with the mesophyll tissue, last approximately 10 days post infection (dpi). Like other fungal pathogens, *Z. tritici* must overcome multilayered host defense responses upon infection or suppress their activation, including ROS generation. To avoid host recognition *Z. tritici* secretes a plethora of proteins like Mg3LysM that blocks the elicitation of chitin-induced plant defenses. *ΔMg3LysM* strains were unable either to efficiently colonize the mesophyll and were blocked in asexual fructification and sporulation, indicating that *Mg3LysM* plays a major role in the virulence of *Z. tritici* (Marshall *et al.*, 2011). Quantitative proteomics and phosphoproteomics approaches were employed to investigate the accompanying signaling events and defense responses occurred upon recognition of *Z. tritici* during the compatible interaction. Perceiving *Z. tritici* through receptors such as receptor-like kinase on the plasma membrane leads to activation of several early signaling cascades, including of ROS, Ca^{++}, MAPKs, nitric oxide (NO) and sugars. Activation of these cascades triggers sucrose non-fermenting-related kinase (SNF) and several transcription and translation regulators such as WRKY transcription factors through phosphorylation, controlling defense-related and carbohydrate metabolic gene expression (Yang *et al.*, 2013). Additionally, the suppression of photosynthesis due to a decrease in chlorophyll *a* or an increase in sugar content was observed as early event in the *Z. tritici*-

wheat interaction. Interestingly, this was in agreement with observations on chloroplast condensation at 48 hours post inoculation (Kema *et al.*, 1996). Collectively, Yang et al. (2013) proposed a model describing early signaling networks and downstream defense responses occurring during the initial biotrophic phase of infection by *Z. tritici* (Fig. 1).

Several lines of evidence obtained from a variety of experimental investigations corroborated that the generation of H_2O_2 is a typical host response during *Z. tritici* infection, which occurs particularly during the biotrophic phase and the transition to necrotrophy. This event was visualized by DAB staining as well as by using the Amplex Red Hydrogen Peroxide/Peroxidase Assay quantification Kit (Shetty *et al.*, 2003; Yang *et al.*, 2013). Yang et al. (2013) measured the concentration of H_2O_2 during biotrophy (3 and 7 dpi) and the transition to necrotrophy (11 dpi) and found that the concentration of H_2O_2 gradually increased from 3 dpi onward and continued until the appearance of disease symptoms at 11 dpi, suggesting that *Z. tritici* requires to manage H_2O_2 accumulation during biotrophy and the subsequent switch to necrotrophy In **chapter 5**, we functionality characterized CPs that are involved in modulating oxidative stress during early and late phases of *Z. tritici* infection.

We clearly showed that the regulation of host-derived H_2O_2 is an essential process for *Z. tritici* pathogenicity. It was demonstrated that *ZtCpx1* encoding a cytoplasmic catalase-peroxidase, is upregulated during early pathogenesis. Additionally, we showed that *ZtCpx2*, which encodes a secreted catalase-peroxidase, has an important role in scavenging H_2O_2 accumulation during the switch from biotrophy to necrotrophy. Collectively, in **Chapter 5**, we demonstrated that the contribution of both genes is required for full virulence of *Z. tritici*

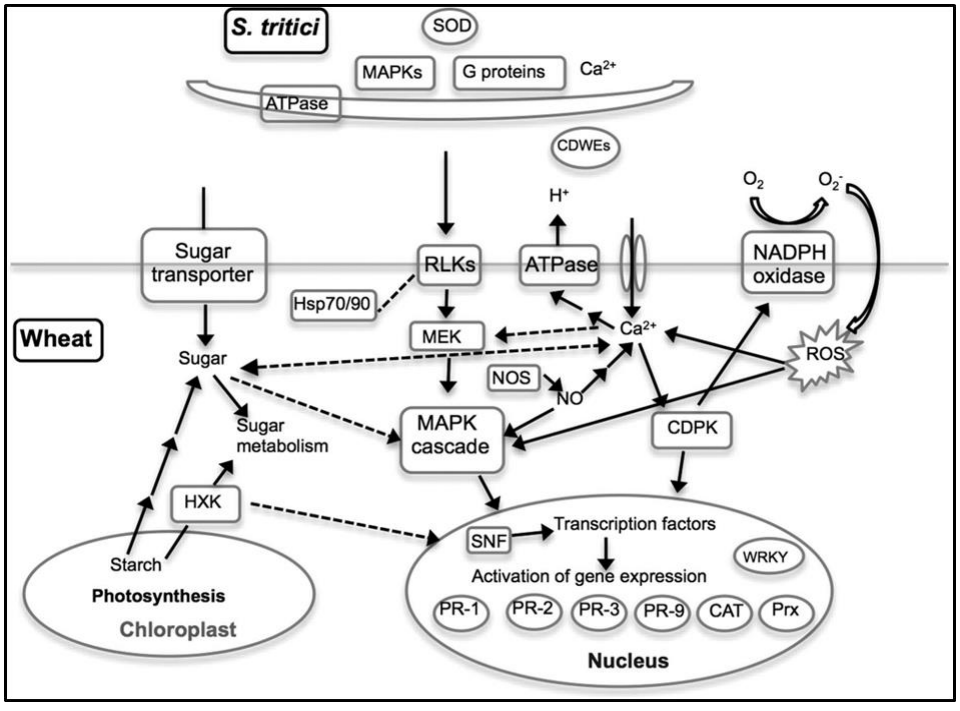

Figure 1. Hypothesized model of crosstalk among signaling networks, as well as defense responses and fungal symptomless growth, during the *Z. tritici* –wheat interaction. The pathogen is sensed by receptors such as receptor-like kinase on the host plasma membrane that subsequently initiate early signal transduction, comprising increases in NO and cytosolic Ca^{2+} concentration, the production of ROS, the activation of MAPKs and CDPKs, and increases in sugar levels. MAPK cascades and CDPK trigger transcription factors such as WRKY that control the expression of defense-related genes such as PR genes. SNF kinase can subsequently phosphorylate transcription factors to regulate the expression of genes that are required for sugar metabolism. Sugar signalling is synchronized with the suppression of photosynthesis and alterations of sugar metabolism (adapted from Yang *et al.*, 2013)).

The destructive necrotrophic lifestyle of *Z. tritici* is strongly associated with rapid growth and the onset of asexual proliferation. Presently, molecular mechanisms and environmental signaling such as the light mediated transition from biotrophy to necrotrophy remains unclear (Sánchez-Vallet *et al.*, 2015). It is originally assumed that *Z. tritici* secretes effector proteins during the biotrophy/necrotrophy switch or during necrotrophy to induce cell death and initiate the necrotrophic phase (**Chapter 4**). Expression profiling of putative ZtSSPs demonstrated that the majority is specifically expressed during necrotrophy, suggesting that *Z. tritici* might secrete SSPs to facilitate wheat infection similar to other necrotrophs, such as *P. tritici-repentis* (**Chapter 4**). Disruption of two ZtSSPs that are highly expressed during the necrotrophic phase confirmed that they are not required for virulence of *Z. tritici* (**Chapter 4**). However, our study mainly focused on mining the *Z. tritici* genome in order to discover functional ZtSSPs (**Chapter 3** and **Chapter 4**). Therefore, it is worthwhile to also look for other molecular key players such as secondary metabolites that were identified to function as effectors in several necrotrophic fungi (Stergiopoulos *et al.*, 2013).

Several attempts using an array of approaches attempted to elucidate the biochemical and molecular events occurring during the transition from the biotrophic to the necrotrophic lifestyle of *Z. tritici* (Keon *et al.*, 2007; Rudd *et al.*, 2008). Keon et al. (2007) identified an increase in the availability of a variety of sugars and amino acids released from wheat cells during the onset of lesion formation. Other observed cellular phenomena include DNA laddering, translocation of cytochrome *c* from mitochondria to the cytosol and electrolyte leakage. These metabolic changes are reminiscent of PCD, which is commonly associated with disease resistance against biotrophic fungi (Keon *et al.*, 2007). Additionally, it was found that there is a strong correlation between the appearance of PCD, which is linked with the activation of *TaMPK3*, the wheat homolog of Arabidopsis *AtMPK*, and symptom expression

during the compatible interaction (Rudd *et al*., 2008). Eventually, the abovementioned studies supported the hypothesis that *Z. tritici* hijacks the host resistance responses such as PCD that are commonly deployed against biotrophs in order to facilitate the infection process and resulted in a model showing how *Z. tritici* interacts with its host (Fig. 2) (Hammond-Kosack and Rudd, 2008).

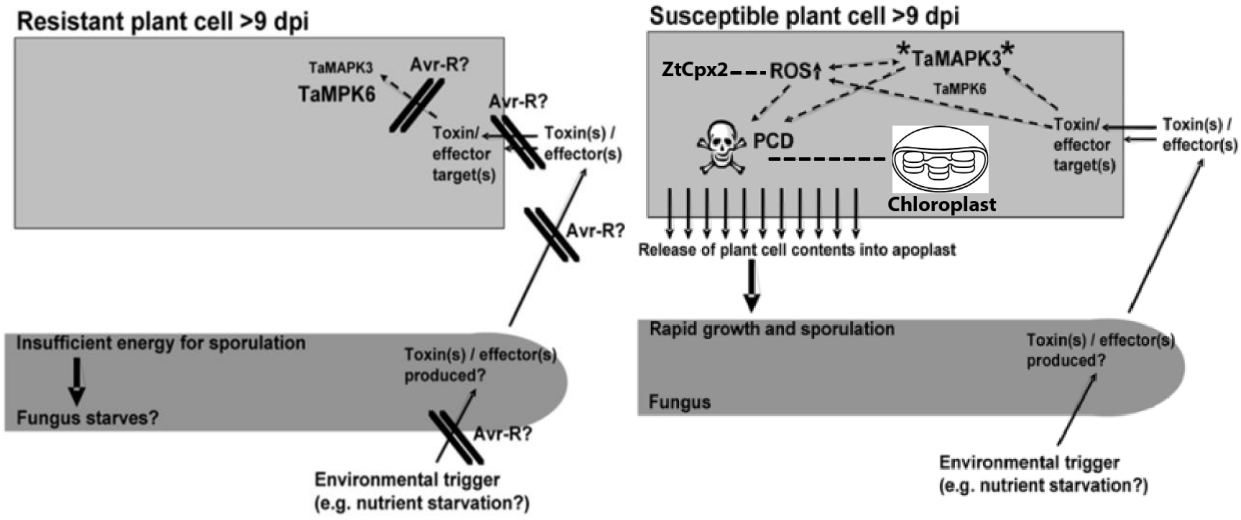

Figure 2. Schematic model illustrating the events occurring in wheat leaf cells during compatible and incompatible interactions between the hemibiotrophic fungal pathogen, *Zymoseptoria tritici* and wheat. In a compatible interaction, about nine days post inoculation (dpi), inverse changes in the relative levels of the two MAPK proteins are observed. During the transition phase, *Z. tritici* is stimulated to produce, as yet un-identified, toxin(s) and/or effector(s) that eventually induce the post-translational activation (**) of *TaMPK3*. These events happen in parallel with the activation of programmed cell death (PCD) signaling, which may lead to the generation of reactive oxygen species (ROS). The generated ROS could be tolerated via the action of ZtCpx2 that encodes a secreted catalase-peroxidase (This thesis). The clear effect is loss of host membrane integrity – also observed in histological studies (Kema *et al.*, 1996), and the release of nutrients from dying plant cells, which facilitates increased fungal growth and asexual reproduction. Chloroplasts play a crucial role to temporally regulate host PCD, which occurs prior to the initiation of necrotrophic growth (Lee *et al*., 2015). None of these responses occur during an incompatible interaction. The feasible positions for protective role of corresponding Avr-R protein combinations are signified as parallel cross lines. The absence of plant cell reactions during host resistance is a limitation on the nutrients obtainable to the fungus that inhibits its further colonization (adapted from Hammond-Kosack and Rudd (2008)).

Concluding remarks

Fungal effectors acting as virulence or (a)virulence factors are key players in host-pathogen interactions. Traditionally, biochemical and genetic approaches are employed to discover effector proteins in different microorganisms. Based on our investigation as presented in **Chapter 4** and additional recent reports on effector discovery (Rudd *et al.*, 2015), it is evident that the identification of functional effectors in *Z. tritici* remains a big challenge requiring complementary and novel approaches for future progress. We conclude that fine-mapping of previously identified QTLs in combination with other genetic and molecular approaches might lead to discovery of effectors that control specificity in the *Z. tritici*-wheat pathosystem. The identification of effector proteins, including toxins, in *Z. tritici* will elucidate their crucial roles in pathogenicity and eventually enable breeders to upscale phenotyping under controlled and field conditions.

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Summary

Zymoseptoria tritici (Desm.) Quaedvlieg & Crous (previously known as *Mycosphaerella graminicola*) is the causal agent of septoria tritici blotch (STB), which is a devastating foliar wheat disease worldwide. It is responsible for significant yield losses occurring annually in all major wheat-growing areas and threatens global food security. *Z. tritici* is a hemi-biotrophic fungal pathogen that, after stomatal penetration, establishes a stealthy biotrophic and symptomless relation with its host plant that is followed by a sudden switch to a necrotrophic growth phase coinciding with chlorosis that eventually develops in large necrotic blotches containing many pycnidia producing asexual splash-borne conidia. Under natural conditions - once competent mating partners are present and conditions are conducive- pseudothecia are formed producing airborne ascospores. Disease management of STB is primarily achieved through fungicide applications and growing commercial cultivars carrying *Stb* resistance genes. However, the efficacy of both strategies is limited as strains resistant to fungicides frequently develop and progressively dominate natural populations, which hampers disease management; also the deployed *Stb* genes are often overcome by existing or newly developed isolates of the fungus. Hence, there is a need for discovery research to better understand the molecular basis of the host-pathogen interaction that enables breeders to identify and deploy new *Stb* genes, which will eventually contribute to more sustainable disease control.

Chapter 1 introduces the subject of the thesis and describes various aspects of the lifestyle of *Z. tritici* with emphasis on dissecting the various stages and physiological processes during pathogenesis on wheat. In addition, it includes a short summary and discussion of the current understanding of the role of (a)virulence factors in the *Z. tritici*–wheat pathosystem.

Chapter 2 describes new gateway technology-driven molecular tools comprising 22 entry constructs facilitating rapid construction of binary vectors for functional analyses of fungal genes. The entry vectors for single, double or triple gene deletion mutants were developed using hygromycin, geneticin and nourseothricin resistance genes as selection markers. Furthermore, these entry vectors contain the genes encoding green fluorescent (GFP) or red fluorescent (RFP) protein in combination with the three selection markers, which enables simultaneous tagging of gene deletion mutants for microscopic analyses. The functionality of these entry vectors was validated in *Z. tritici* and described in **Chapters 3, 4 and 5**.

Chapter 3 describes the functional characterization of *ZtWor1*, the orthologue of *Wor1* in the fungal human pathogen *Candida albicans*. *ZtWor1* is up-regulated during initiation of colonization and fructification, and regulates expression of candidate effector genes, including one that was discovered after comparative proteome analysis of *Z. tritici* wild-type and *ΔZtWor1* strains. Cell fusion and anastomosis occurred frequently in *ΔZtWor1* strains, which is reminiscent of mutants of *MgGpb1*, the β-subunit of the heterotrimeric G protein. Comparative expression profiling of *ΔZtWor1, ΔMgGpb1* and *ΔMgTpk2* (the catalytic subunit of protein kinase A) strains, suggests that *ZtWor1* is downstream of the cyclic adenosine monophosphate (cAMP) pathway that is crucial for pathogenicity of many fungal plant pathogens.

Chapter 4 describes combined bioinformatics and expression profiling studies during pathogenesis in order to discover candidate effectors of *Z. tritici* important for virulence. In addition, a genetic approach was followed to map quantitative trait loci (QTLs) in *Z. tritici* carrying putative effectors. Functional analysis of two top effector candidates, small-secreted proteins SSP15 and SSP18, which were selected based on their expression profile *in planta*, showed that they are dispensable for virulence of *Z. tritici*. These analyses suggest that generally adopted criteria for effector discovery, such as protein size, number of cysteine residues and up-regulated expression during pathogenesis, should be taken with caution and cannot be applied to every pathosystem, as they likely represent only a subset of effector genes.

Chapter 5 describes the functional characterization of *ZtCpx1* and *ZtCpx2* encoding a secreted and a cytoplasmic catalase-peroxidase (CP) in *Z. tritici*, respectively. Gene replacement of *ZtCpx1* resulted in mutant strains that were sensitive to exogenously added H_2O_2 and *in planta* phenotyping showed they are significantly less virulent compared to wild-type. All mutant phenotypes could be restored to wild-type by complementation with the wild-type allele of *ZtCpx1* driven by its native promoter. Additionally, functional analysis of *ZtCpx2* confirmed that this gene encodes a secreted CP and is, however, dispensable for virulence of *Z. tritici* on wheat. However, we showed that both genes act synergistically, as the generated double knock-out strain showed a significantly stronger reduction in virulence than the individual single knock-out strains. Hence, both genes are required by *Z. tritici* for successful infection and colonization of wheat.

In **Chapter 6** I discuss and summarize the genetic approaches used in this study, reflect on the major findings and bottlenecks encountered, and propose new strategies to identify effectors of *Z. tritici* in the future.

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With the writing of these acknowledgements, the scientific journey of my PhD study is nearly completed. The PhD thesis is the biggest scientific achievement of my life, which kept me busy the last six years.

Performing the PhD study abroad was a big challenge for me and it truly changed my scientific and personal life. To conduct this study would have been impossible without the assistance and support from so many people.

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Although we were far away from our family, we had a nice Iranian community in Wageningen, which made me feel at home. I would like to thank all friends in Wageningen for creating the joyful times during our gatherings, parties and BBQs. Additionally, I would like to extend my deepest gratitude to all present and former Iranian friends who joined the weekly football games, for creating a friendly atmosphere and funny moments. I wish you all the best in your personal and scientific life.

Curriculum vitae

Amir Mirzadi Gohari was born in 1980 in Kerman, Iran. After completing high school in biological sciences in 1998, he continued his studies at Shahid Bahonar University of Kerman and obtained a BSc degree in Plant Protection in 2004. Subsequently, he was nominated as an elite student in the overall entrance MSc exam in Iran and completed his MSc degree at the department of Plant Pathology, University of Tehran, with his thesis on "Genetic diversity of *Fusarium verticillioides* isolates from maize in Iran based on vegetative compatibility grouping". After graduation, he was appointed by the University of Rafsanjan, Kerman, to teach courses on Plant Pathology and Mycology. In 2009, he was awarded with a full PhD scholarship by the Ministry of Science, Research and Technology of Iran for continued studies at Wageningen University and Research Center (WUR), Plant Research International. In August 2009, he started his PhD program in the group of Dr. Gert H.J. Kema with Prof. Pierre J.G.M. de Wit (WUR) as promotor and Dr. Rahim Mehrabi (WUR and Seed and Plant Improvement Institute, Karaj, Iran) as second co-promotor. His main focus was on effector identification and virulence determinants in *Zymoseptoria tritici*. This thesis presents the results of his PhD program on the characterization of (a)virulence factors in *Z. tritici*. He will join the department of Plant Pathology of the University of Tehran as a faculty member to continue his career.

List of publications:

1) Mirzadi Gohari, A., Ware, S.B., Wittenberg, A.H., Mehrabi, R., Ben M'Barek, S., Verstappen, E.C., van der Lee, T.A., Robert, O., Schouten, H.J., de Wit, P.J.G.M. and Kema, G.H.J. (2015) Effector discovery in the fungal wheat pathogen *Zymoseptoria tritici*. *Molecular Plant Pathology* **16,** 931-945.

2) Mirzadi Gohari, A., Mehrabi, R., de Wit, P.J.G.M. and Kema, G.H.J. (2015) Functional characterization of extracellular and intracellular catalase-peroxidases involved in virulence of the fungal wheat pathogen *Zymoseptoria tritici*. (Manuscript to be submitted).

3) Mehrabi*, R., **Mirzadi Gohari*,** A., da Silva, G.F., Steinberg, G., Kema, G.H.J. and de Wit, P.J.G.M. (2015) Flexible gateway constructs for functional analyses of genes in plant pathogenic fungi. *Fungal Genetics and Biology* **79**, 186-192.

4) Ben M'Barek, S., Cordewener, J.H., van der Lee, T.A., America, A.H., **Mirzadi Gohari, A.,** Mehrabi, R., Hamza, S., de Wit, P.J.G.M. and Kema, G.H.J. (2015) Proteome catalog of *Zymoseptoria tritici* captured during pathogenesis in wheat. *Fungal Genetics and Biology* **79**, 42-53.

5) Ben M'Barek, S., Cordewener, J.H., Tabib Ghaffary, S., van der Lee, T.A., Liu, Z., **Mirzadi Gohari, A.,** Mehrabi, R., America, A.H., Friesen, T., Hamza, S., Stergiopoulos, I., de Wit, P.J.G.M. and Kema, G.H.J. (2015) FPLC and liquid-chromatography mass spectrometry identify candidate necrosis-inducing proteins from culture filtrates of the Fungal wheat pathogen *Zymoseptoria tritici*. *Fungal Genetics and Biology* **79**, 54-62.

6) Mirzadi Gohari, A., Mehrabi, R., Robert, O., Ince, I.A., Boeren, S., Schuster, M., Steinberg, G., de Wit, P.J.G.M. and Kema, G.H.J. (2014) Molecular characterization and functional analyses of *ZtWor1*, a transcriptional regulator of the fungal wheat pathogen *Zymoseptoria tritici*. *Molecular Plant Pathology* **15,** 394-405.

7) Quaedvlieg, W., Kema, G.H.J., Groenewald, J., Verkley, G., Seifbarghi, S., Razavi, M., **Mirzadi Gohari, A.,** Mehrabi, R. and Crous, P.W. (2011) *Zymoseptoria* gen. nov.: a new genus to accommodate Septoria-like species occurring on graminicolous hosts. *Persoonia: Molecular Phylogeny and Evolution of Fungi* **26**, 57-69.

8) Mehrabi, M., Bahkali, A.H., Abd-Elsalam, K.A., Moslem, M., Ben M'Barek, S., **Mirzadi Gohari, A.,** Karimi Jashni, M., Stergiopoulos, J., Kema, G.H.J. and de Wit, P.J.G.M. (2011) Horizontal gene and chromosome transfer in plant pathogenic fungi affecting host range. *FEMS Microbiology Reviews* **35**, 542–554.

9) Rahjoo, V., Zad, J., Javan-Nikkhah, M., **Mirzadi Gohari, A.,** Okhovvat, S.M. and Bihamta, M.R., Razzaghian, J. and Klemsdal, S.S. (2008) Morphological and molecular identification of *Fusarium* isolated from maize ears in Iran. *Journal of Plant Pathology* **90**, 463-468.

10) Mirzadi Gohari, A., Javan-Nikkhah, M., Hedjaroude, G.A., Abbasi, M., Rahjoo, V. and Sedaghat, N. (2008) Genetic diversity of *Fusarium verticillioes* isolates from maize in Iran based on vegetative compatibility grouping. *Journal of Plant Pathology* **90**, 113-116.

11) Mirzadi Gohari, A., Sedaghat, N., Javan-Nikkhah, M. and Saberei-Riseh, R. (2007) Mycoflora of wheat grains in the main production area in Kerman Province, Iran. *International Journal of Agriculture and Biology* **9**, 635-637.

12) Rahjoo, V., Zad, J., Javan-Nikkhah, M., Bihamta, M.R., Okhovvat, S.M., **Mirzadi Gohari, A.,** Elameen, A.H. and Klemsdal, S.S. (2008) Study of genetic variation in Fusarium verticillioides isolates, the causal agent of Fusarium ear rot of corn using AFLP markers. *Seed and Plant* **24**, 457-474.

*Equal contribution

Education Statement of the Graduate School
Experimental Plant Sciences

The Graduate School EXPERIMENTAL PLANT SCIENCES

Issued to: Amir Mirzadi Gihari
Date: 15 December 2015
Group: Laboratory of Phytopathology & BU Biointeractions and Plant Health
University: Wageningen University & Research Centre

1) Start-up phase	date
► **First presentation of your project**	
Identification and molecular characterization of effectors in the wheat Mycosphaerella graminicola pathosystem	Jun 28, 2010
► **Writing or rewriting a project proposal**	
Identification and molecular characterization of effectors in the wheat Mycosphaerella graminicola pathosystem	Feb 2010
► **Writing a review or book chapter**	
► **MSc courses**	
► **Laboratory use of isotopes**	
Subtotal Start-up Phase	*7.5 credits**

2) Scientific Exposure	date
► **EPS PhD student days**	
EPS PhD student day, Utrecht University	Jun 01, 2010
EPS PhD student day, Leiden University	Nov 29, 2013
► **EPS theme symposia**	
EPS Theme 2 Symposium 'Interactions between Plants and Biotic Agents' and Willie Commelin Scholten day, Utrecht University	Jan 15, 2010
EPS Theme 2 Symposium 'Interactions between Plants and Biotic Agents' and Willie Commelin Scholten day, University of Amsterdam	Feb 03, 2011
EPS Theme 2 Symposium 'Interactions between Plants and Biotic Agents' and Willie Commelin Scholten day, Wageningen University	Feb 05, 2012
EPS Theme 2 Symposium 'Interactions between Plants and Biotic Agents' and Willie Commelin Scholten day, Utrecht University	Jan 24, 2013
► **NWO Lunteren days and other National Platforms**	
ALW meeting 'Experimental Plant Sciences', Lunteren (NL)	Oct 15-16, 2009
ALW meeting 'Experimental Plant Sciences', Lunteren (NL)	Oct 14-15, 2010
► **Seminars (series), workshops and symposia**	
First workshop INRA/WUR on Septoria diseases, Versailles, France	Sep 16-17, 2010
First Joint MPI-Marburg Phytopathology meeting, Wageningen, The Netherlands	Oct 28-29, 2010
ExPectationS day (EPS Career day)	Nov 19, 2010
2nd Joint workshop INRA/WUR on Septoria, Wageningen	Jun 07-08, 2011
Mini symposia on writing of world-class paper	Oct 26, 2011
Invited seminar Prof. Regine Kahmann "Effectors of the plant-pathogen fungus Ustilago maydis"	Oct 29, 2011
2nd joint Wageningen – Marburg Meeting on Plant-Fungal Interactions	Jan 30-31, 2012
Workshop on Mycosphaerella graminicola, Versailles, France	Jul 19-20, 2012
Symposium 'Intraspecific pathogen variation -implications and opportunities', Wageningen, NL	Jan 22, 2013
"Mycosphaerella graminicola / Zymoseptoria tritici European Workshop"	Aug 05-06, 2013
Invited seminar Dr. Jos Raaijmakers ('Back to the Roots')	Jan 07,2014
Iplant workshop	Mar 03-04, 2014
Pathogen-Informed Crop Improvement	Apr 08-10, 2014
Invited seminar Prof. Sophein Kamoun	May 28, 2014
Plant-fungus interactions symposium	Jun 05, 2014
► **Seminar plus**	
► **International symposia and congresses**	
10th European Conference on Fungal genetics, Leiden, NL	Mar 29-Apr 01, 2010
8th International Symposium on Mycosphaerella and Stagonospora Diseases of Cereals	Sep 11-14, 2011
11 th European Conference on Fungal genetics, Marburg, Germany	Mar 30-Apr 02, 2012
10th Conference of the European Foundation for Plant Pathology (EFPP), Wageningen, NL	Oct 01-05, 2012
The 27th Fungal Genetics Conference at Asilomar	Mar 12-17, 2013
12 th European Conference on Fungal genetics, Seville, Spain	Mar 23-27, 2014
The 2014 Mycosphaerella Research Community Meeting, Exeter, UK	Sep 11-12, 2014
Zymoseptoria tritici meeting, Paris, France	Sep 10-11, 2015
► **Presentations**	
First workshop INRA/WUR on Septoria diseases, Versailles, France (Talk)	Sep 16-17, 2010
8th International Symposium on Mycosphaerella and Stagonospora Diseases of Cereals (2x Talk)	Sep 11-14, 2011
2nd Joint workshop INRA/WUR on Septoria, Wageningen, The Netherlands (Talk)	Jun 07-08, 2011
Workshop on Mycosphaerella graminicola, Versailles, France (Talk)	Jul 19-20,2012
Dothideomycete Workshop,Asilomar, USA (Talk)	Mar 12, 2013
Mycosphaerella graminicola / Zymoseptoria tritici European Workshop,Rothamsted, UK (Talk)	Agu 05-06, 2013
EPS Theme 2 Symposium & Willie Commelin Scholten day (Talk)	Jan 24, 2013
The 2014 Mycosphaerella Research Community Meeting, Exeter, UK (Talk)	Sep 11-12, 2014
Dothideomycete Workshop,Sevilla, Spain (Talk)	Mar 23, 2014
Pathogen---Informed Crop Improvement Workshop,Wageningen, Netherlands (Talk)	Apr 08, 2015
11 th European Conference on Fungal genetics, Marburg, Germany (Poster)	Mar 30-Apr 02, 2012
10th Conference of the European Foundation for Plant Pathology (EFPP) (Poster)	Oct 01-05, 2012
12 th European Conference on Fungal genetics, Sevilla, Spain (Poster)	Mar 23-27, 2014
► **IAB interview**	
Interview with Prof. Harro Bouwmeester	Nov 14, 2012
► **Excursions**	
59242 Cappelle-en-Pe´ve`le, France	May 15, 2012
Cimmyt Field visit, breeding for resistance to Septoria and Fusarium, Mexico city, Mexico	Sep 12, 2011
Subtotal Scientific Exposure	*33.1 credits**

3) In-Depth Studies	date
► **EPS courses or other PhD courses**	
Bioinformatics: A Users Approach (a practical course)	Aug 30-Sep 03, 2010
Spring School 'RNAi and the World of Small RNA Molecules'	Apr 14-16, 2010
Postgraduate course 'Molecular Phylogeny'	Oct 18-22, 2010
Advanced course 'Guide to Scientific Artwork'	May 07-08 2012
Advanced course 'Proteomics'	Apr 18-21,2011
Spring School 'Host-Microbe Interactomics'	Jun 02-04, 2014
Postgraduate course 'Genome Assembly'	Apr 28-29, 2015
► **Journal club**	
participation in literature discussion group at PRI	Aug 2009- Aug 2013
► **Individual research training**	
Working in Fungal Molecular Cell Biology Group in Exeter for three weeks	May 28-Jun 18, 2012
Subtotal In-Depth Studies	*13.4 credits**

4) Personal development	date
► **Skill training courses**	
Techniques for Writing and Presenting a Scientific Paper (WGS course)	Sep 04-07, 2012
Project and Time Management (WGS course)	Sep 10 and 24, 2013
► **Organisation of PhD students day, course or conference**	
► **Membership of Board, Committee or PhD council**	
Subtotal Personal Development	*2.7 credits**

TOTAL NUMBER OF CREDIT POINTS*	**56,7**

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