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

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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

The co-evolution of plants and their pathogens has resulted in complex interactions in which both pathogens and hosts have evolved elaborate mechanisms resulting in either compatible interactions, in which pathogens successfully invade plants, or incompatible interactions, in which host defences restrict pathogen growth (Dodds and Rathjen, 2010). Plant pathogenic fungi secrete a repertoire of effector proteins that facilitate infection by interfering with host defence mechanisms, whereas most host plants have developed receptors that mediate resistance against these fungi after recognition of these effectors (De Wit *et al*., 2009). The unravelling of molecular networks involved in pathogenicity provides crucial information which may 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 because of 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 to the application of fungicides. However, relatively few resistance genes have been characterized (Arraiano *et al*., 2007; Tabib Ghaffary *et al*., 2011, 2012) and provide limited efficacy against 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 cultivars Gene, carrying *Stb4*, and Madsen, with partial resistance, whose resistances declined within 5 years after their release in Oregon (USA) (Cowger *et al*., 2000; Wittenberg *et al*., 2009). A better understanding of *Z. tritici* biology and the molecular mechanisms underlying the infection process is crucial to the design of novel effective approaches for STB management. The 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.

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Zymoseptoria tritici is a model pathogen to study hemibiotrophy and is considered to be among the top 10 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 mitogen-activated protein (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. *MgGpb1* regulates the cyclic adenosine monophosphate (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, which 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 also have 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* orthologues *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*., 2009, 2011). 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* orthologue *ZtWor1* in *Z. tritici*, and our results show that it is involved in pathogenicity, regulates the expression of small secreted proteins (SSPs) and is most likely part of the cAMP signalling pathway which 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 of $1.43E^{-28}$ and $1.52E^{-12}$, which are located on chromosomes 8 and 6, respectively. Amino acid alignments revealed that Mycgr3_46572 showed the highest homology with the CaWor1 orthologue (Lohse *et al*., 2010), and phylogenetic tree analysis revealed 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 1614 bp, without introns as verified by reverse-transcription polymerase chain reaction (RT-PCR), encoding a protein of 537 amino acids (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 orthologues 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 (Fig. S1A, see Supporting Information). 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. As *ΔZtWor1-26* was unable to produce yeast-like spores, *Agrobacterium tumefaciens*-mediated transformation was performed on fragmented mycelial tissue using a construct harbouring the *ZtWor1* wild-type (WT) allele, which resulted in *ΔZtWor1-com7* (Fig. S1B).

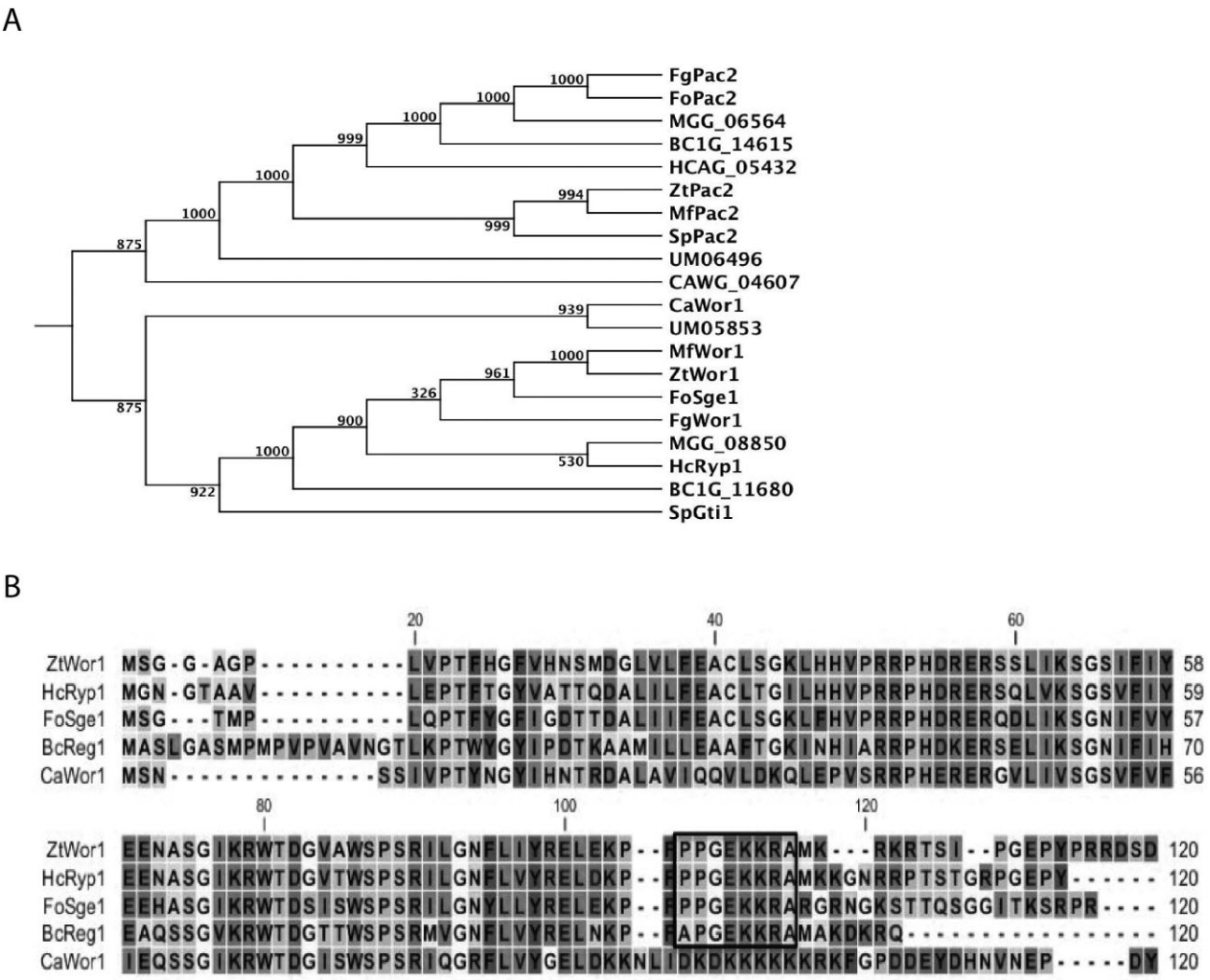

Fig. 1 Phylogenetic comparison of *Zymoseptoria tritici* Wor1 (ZtWor1) with members of the WOPR superfamily based on amino acid sequence alignments. (A) The tree shows the phylogenetic relationship of ZtWor1 with Wor1 and Pac2 orthologues in other fungi, including BcReg1, BC1G_14615, HcRyp1, HCAG_05432, CaWor1, CAWG_04607, SpGti1, SpPac2, MGG_08850, MGG_06564, FgWor1, FgPac2, FoSge1, FoPac2, MfWor1, MfPac2, UM05853 and UM06496 from *Botrytis cinerea*, *Histoplasma capsulatum*, *Candida albicans*, *Schizosaccharomyces 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 shown above the branches. (B) Alignment of the first 120 deduced amino acid sequences of ZtWor1 and its orthologue 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 yeast glucose broth (YGB) and on solid potato dextrose agar (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. 2Aa,Ab), but *ΔZtWor1-26* did not produce any spores and exclusively produced a dense, extensive mycelial network with abundant abnormally swollen cell structures (Fig. 2Ac). Microscopic comparison showed uncontrolled cell fusions or anastomosis in *ΔZtWor1-26* that was rare in the WT (Figs 2Bd, 3 and S2, see Supporting Information). On PDA, we did not observe any effect of the deletion of *ZtWor1* on germination and early colony development during the first 48 h after inoculation (data not shown). On MM, the growth pattern started to differ at 5 days after inoculation (dai), as *ΔZtWor1-26* grew significantly more slowly than the WT, as well as *ΔZtWor1-com7*, resulting in more compact colonies. Comparative scanning electron microscopy revealed significant differences ($P < 0.05$) in hyphal diameter between the WT, *ΔZtWor1-com7* and *ΔZtWor1-26* (Fig. S3, see Supporting Information). On V8, the WT and *ΔZtWor1-com7* strains abundantly produced yeast-like spores, whereas

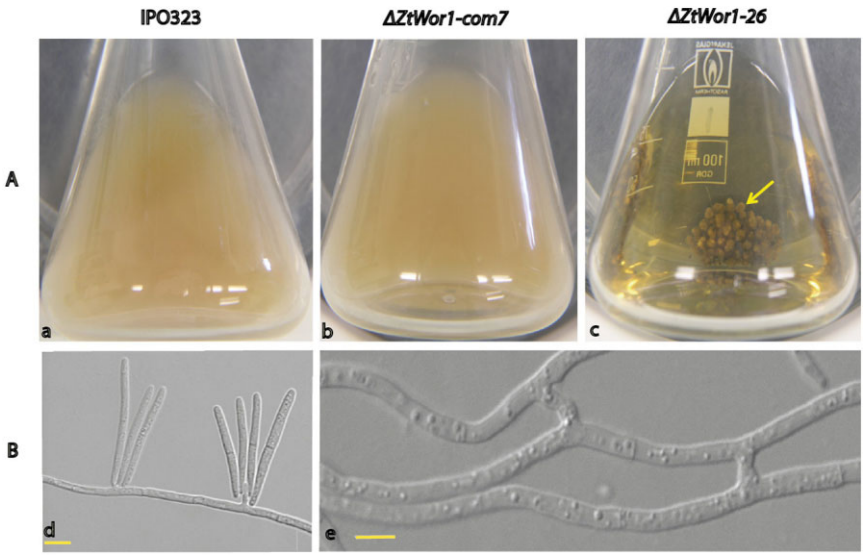

Fig. 2 Deletion of *Zymoseptoria tritici Wor1* (*ZtWor1*) affects yeast-like cell production and early development. (Aa, Ab) The *Z. tritici* wild-type (WT) strain and *ΔZtWor1-com7* produce abundant yeast-like cells resulting from blastic conidiogenesis in yeast glucose broth medium. **(**Ac) *ΔZtWor1-26* is blocked in sporulation and exclusively produces compact hyphal networks resulting in a distinct bulbous mycelial mass (marked with a yellow arrow). (B) Comparative light microscopy of early *Z. tritici* development. (Bd) Production of yeast-like cells on the hyphae of the WT on water agar. (Be) Unique cell fusion or anastomosis events that occur frequently in *ΔZtWor1-26*. Scale bars, 5 μm.

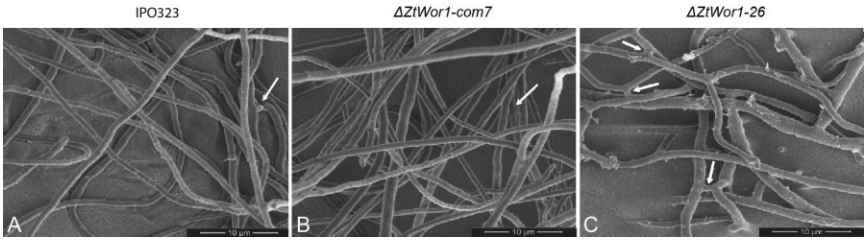

Fig. 3 Comparative scanning electron micrographs of hyphae of the *Zymoseptoria tritici* wild-type (WT) strain, *ΔZtWor1-com7* and *ΔZtWor1-26* growing on minimal medium (MM) at 10 days after inoculation at 20 °C. Infrequent cell fusion/anastomosis events occur in the *Z. tritici* WT strain and *ΔZtWor1-com7*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

ΔZtWor1-26 hardly produced any spores, even after prolonged incubation (>14 days). At 5 dai, the WT and *ΔZtWor1-com7* turned black, probably as a result of melanization, but *ΔZtWor1-26* produced an additional mass of aerial hyphae covering the dark colonies that was absent in the WT (Fig. 4).

ZtWor1 expression relies on MgGpb1 and MgTpk2

Overall, signal transduction pathways, including the MAP kinase and cAMP-dependent PKA pathways, play a crucial role in the sensing of and responding to environmental stimuli, and represent important cascades in the regulation of development in eukaryotes. Previously, we have shown that these pathways are also involved in the pathogenicity and development of *Z. tritici* (Cousin *et al*., 2006; Mehrabi *et al*., 2006a,b). As *ΔZtWor1-26* showed abundant anastomosis, we tested whether the cAMP signalling pathway, which 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). In both mutants, *ZtWor1* expression was severely reduced compared with the WT (Fig. 5A), whereas the expression levels of *MgGpb1* and *MgTpk2* in *ΔZtWor1-26* were the same as in the WT strain (Fig. 5B). Taken together, it can be concluded that ZtWor1 and the PKA pathway 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.

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

As the orthologues 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; it is up-regulated during the early stage of infection (2 dai), gradually down-regulated until 16 dai and then 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, whereas 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 expanded over time and eventually coalesced into large necrotic blotches containing numerous pycnidia in the control strains. Occasionally, a few chlorotic and necrotic lesions were observed after inoculation with the deletion mutant, which sometimes contained a few immature pycnidia in a limited number of lesions (Fig 7).

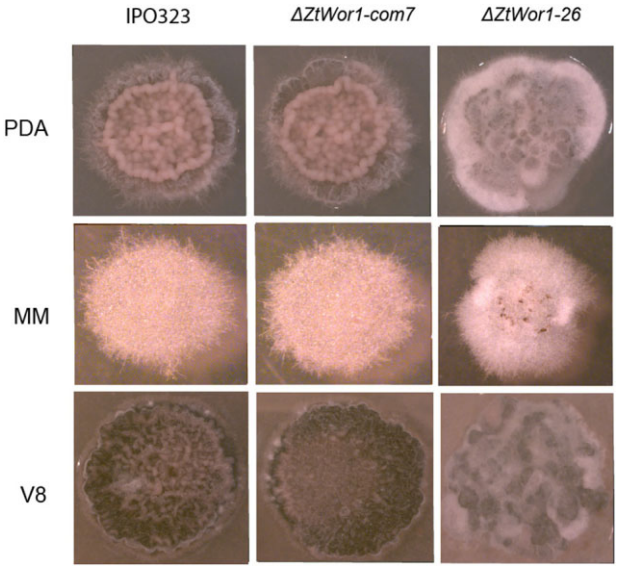

Fig. 4 The *in vitro* effect of *ZtWor1* deletion and complementation in *Zymoseptoria tritici* at 10 days after inoculation on three different media at 20 °C. Comparison of the wild-type (WT) strain and *ΔZtWor1-26* shows that the latter exclusively produces strongly melanized mycelial cultures without any spores. This is particularly evident on V8, but also on minimal medium (MM). *ΔZtWor1-26* exclusively produces hyphae. The WT phenotype is restored in *ΔZtWor1-com7*. PDA, potato dextrose agar.

ZtWor1 regulates the expression of specific SSPs

Considering that *ΔZtWor1-26* was significantly reduced in pathogenicity and that orthologues in other fungal plant pathogens regulate the expression of effector genes, we hypothesized a similar role for *ZtWor1* in *Z. tritici*. First, quantitative RT-PCR was used to determine the role of *ZtWor1* in the *in vitro* expression of several SSPs that are candidate effectors in the *Z. tritici*–wheat pathosystem based on bioinformatics analyses (Morais do Amaral *et al*., 2012) (Table S1, see Supporting Information) and *in planta* expression profiling (G. H. J. Kema *et al*., Plant Science Group, Plant Research International BV, Wageningen University, Droevendaalsesteeg, 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 with the WT (Fig. 8 and Table S2, see Supporting Information). Second, we compared the *in vitro* proteome of the WT strain and *ΔZtWor1-26*. Overall, 125 *Z. tritici* proteins were identified from all conditions [three minimal media including MM, *Cladosporium fulvum* B5 (B5) and dextrose broth], 18 of which were unique to *Z. tritici*, as no homologues could be identified in fungal databases. One hundred and fourteen proteins possessed a SignalP motif, indicating that they were secreted, and only one (SSP127) of the 114 proteins was not expressed in *ΔZtWor1-26* (Fig. 9). *In vitro* expression on MM indicated that SSP127 may have an important role during the early stages of infection. This

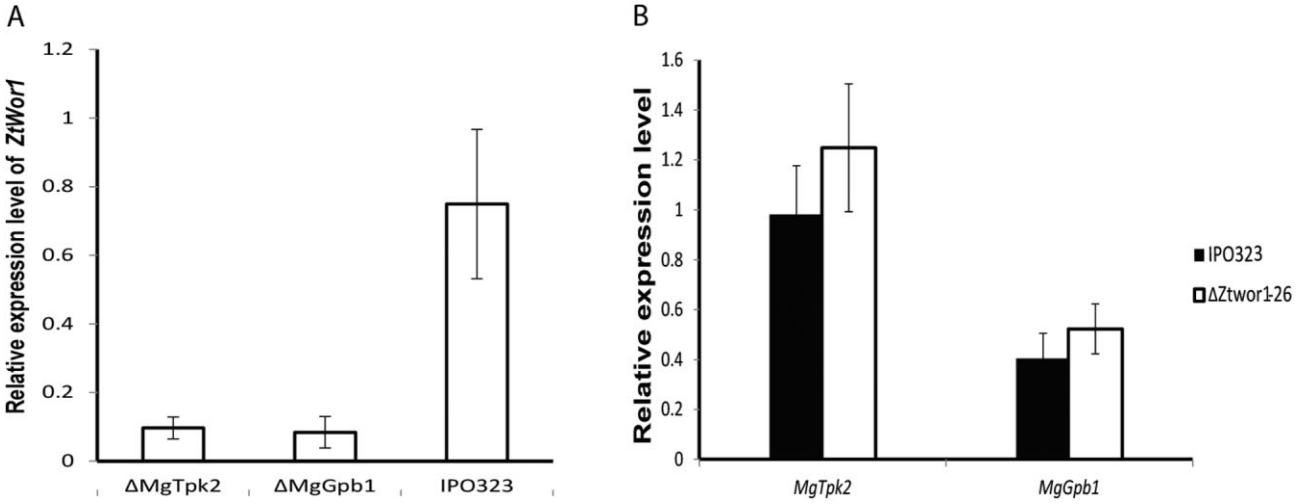

Fig. 5 Expression analysis of *Zymoseptoria tritici Wor1* (*ZtWor1*) in the disrupted mutants *MgGpb1* and *MgTpk2*. (A) *In vitro* expression level of *ZtWor1* in disrupted mutants *MgGpb1* and *MgTpk2* compared with expression in the *Z. tritici* wild-type (WT) strain. (B) Comparative *in vitro* expression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

was confirmed by the relative *in planta* expression of SSP127, which was highly up-regulated until 4 dai. (Fig. 10). 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 *et al*., 2011); however, surprisingly, no significant differences in disease development were observed between the knock-out strains and the WT (Fig. S4, see Supporting Information). In summary, our data suggest that *ZtWor1* is much more involved in developmental processes than as a specific regulator of effector genes.

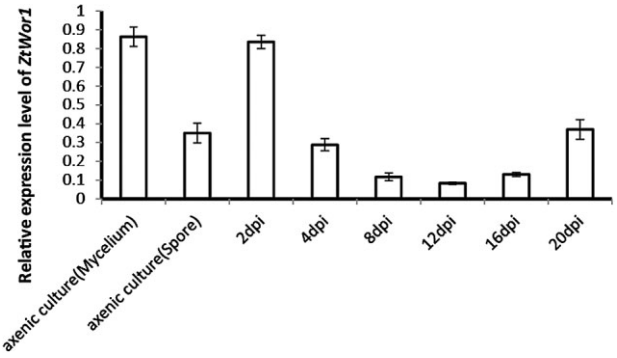

Fig. 6 *In vitro* and *in planta* expression levels of *Zymoseptoria tritici Wor1*. *In vitro* conditions (18 °C and 25 °C to induce yeast-like cells and mycelia formation, respectively) were chosen to compare the expression levels of *Ztwor1* in mycelial and conidial cultures with *in planta* conditions. The susceptible cv. Obelisk was inoculated with the *Z. tritici* wild-type (WT) strain and infected leaves were harvested at 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* β-tubulin gene.

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

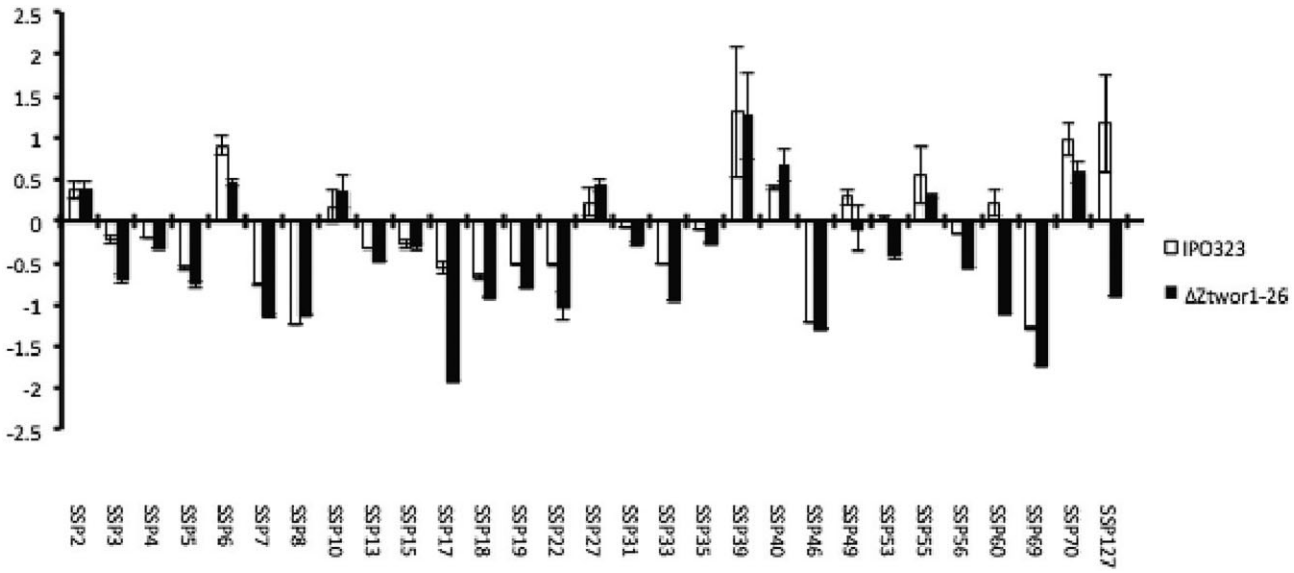

Fig. 8 Comparative *in vitro* expression analysis of 29 small secreted proteins (SSPs) in the *Zymoseptoria tritici* wild-type (WT) strain and *ΔZtwor1-26* grown in yeast glucose broth (YGB) medium for 7 days at 18 °C. Expression levels were normalized with the constitutively expressed *Z. tritici* β-tubulin gene and plotted on a $\log_{10}$ scale.

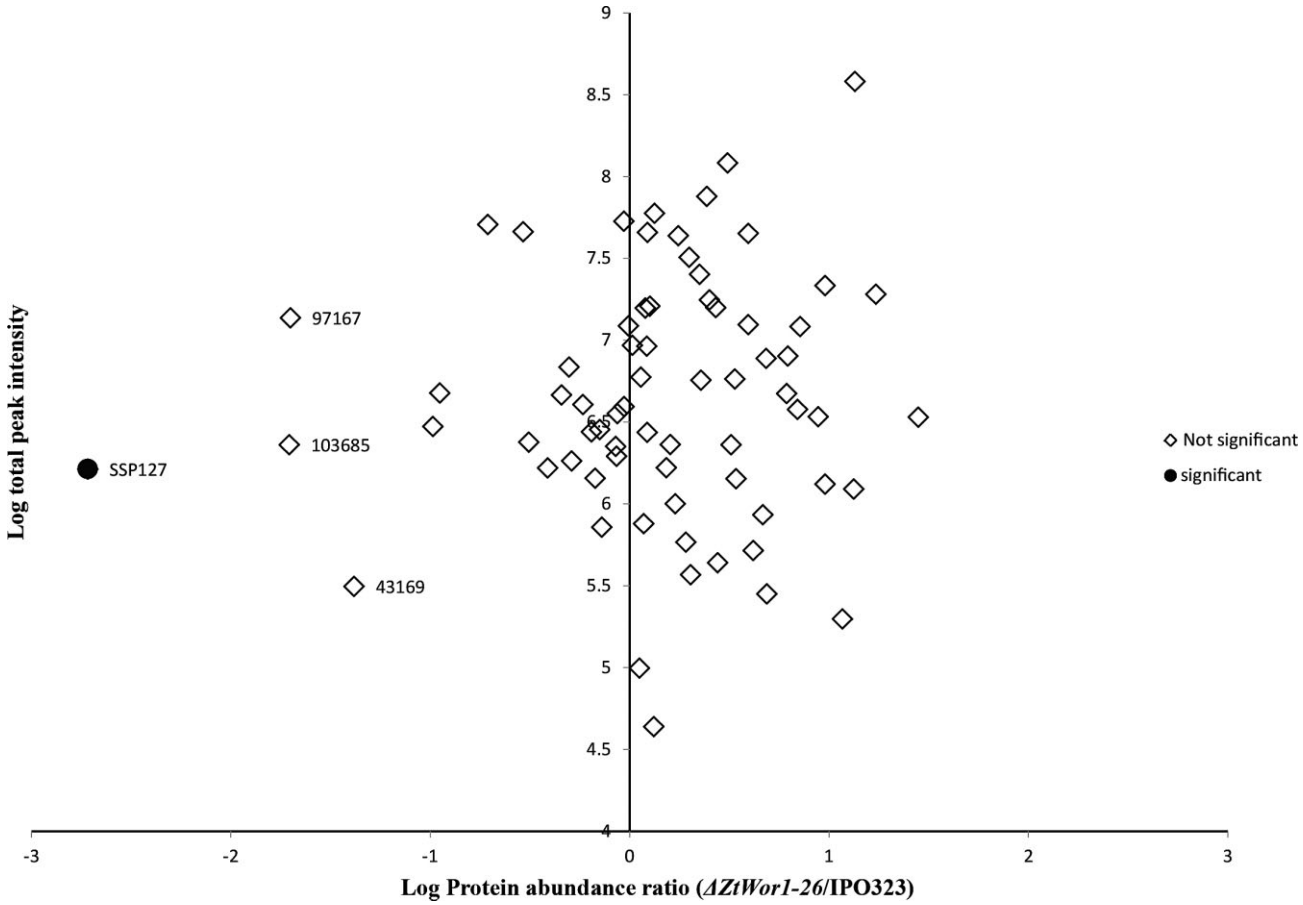

Fig. 9 Comparative discriminative *in vitro* proteome analysis of the *Zymoseptoria tritici* wild-type (WT) strain and *ΔZtWor1-26*. Plot of the normalized *ΔZtWor1-26*/IPO323 intensity ratio against the total measured protein intensity. Proteins not significantly different between mutant and WT are depicted in white, whereas the significantly different protein is shown as a black circle. SSP127 is the only identified protein that showed significant expression differences between the two strains (ratio = 0.002, $P = 3.1 \times 10^{-5}$). Proteins 43169, 97167 and 103685 showed *P* values larger than 0.05 and are considered to be nonsignificant by the Perseus software, which takes both the ratio and *P* value into account.

DISCUSSION

For successful infection and completion of its life cycle 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 extent, it has been shown how they contribute to the infection process, evade host defence responses and enable disease establishment (Orton *et al*., 2011).

Here, we analysed the biological function of the regulatory gene *ZtWor1* and showed that it is required for the 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, *Schizosaccharomyces pombe* (GtiI) and *Fusarium graminearum* (Fgp1) (Caspari, 1997; Jonkers *et al*., 2012; Michielse *et al*., 2009, 2011; Nguyen and Sil, 2008), this putative transcriptional regulator possesses two globular domains, the WOPR box1 (amino acids 16–107) and the WOPR box2 (amino acids 160–250) 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 has been shown to be involved in the nuclear localization of Ryp1 and Sge1. This motif (+94 to +101) is also present in ZtWor1, and probably serves the same role in *Z. tritici*.

Candida 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

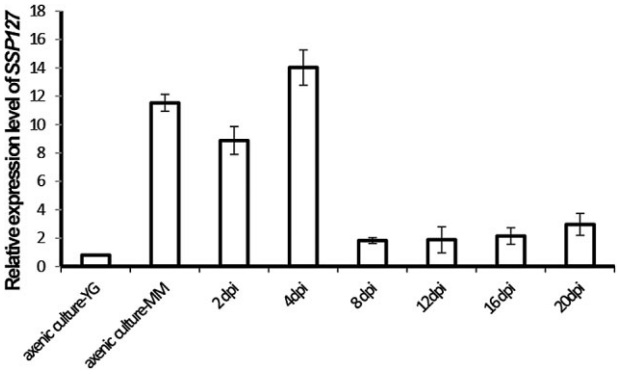

Fig. 10 *In vitro* and *in planta* expression levels of small secreted protein (SSP) 127 of *Zymoseptoria tritici*. *In vitro* conditions represented mycelium production [on minimal medium (MM)] and blastic conidiogenesis [in yeast glucose broth (YGB) medium]. *In planta* expression profiles were measured 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* β-tubulin gene.

by the ectopic expression of *Wor1* (Huang *et al*., 2006; Ohara and Tsuge, 2004; Srikantha *et al*., 2006; Zordan *et al*., 2006). Furthermore, it has been 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* orthologue 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 Sil (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 (45×) and Ryp1 (4×) 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 up-regulation again during conidiogenesis (20 dai). In addition, we showed that *in vitro ZtWor1* expression in the 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 probably 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 *et al*. (2012) showed that the *ZtWor1* orthologue *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. Thus, in addition to 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 nonconidia-bearing conidiophores during pathogenesis (Michielse *et al*., 2011), and *Sge1* and *Fgp1* also affect conidia formation in Fol (Michielse *et al*., 2009) and *F. graminearum* (Jonkers *et al*., 2012), respectively. 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 unsuccessful, but complementation of *ΔZtWor1-26* restored the WT, and hence *in vitro* and *in planta* conidiogenesis, suggesting that Wor1 is a crucial factor in yeast-like cell formation.

Complementation also restores pathogenicity, whereas heterologous complementation with *Sge1* from Fol did not (data not shown), indicating that Wor1 orthologues in various fungal human and plant pathogens have evolved divergently to regulate pathogenicity through different mechanisms; this has also been shown in *F. graminearum*, where the interchange of the N- and C-terminal portions of the Wor1 homologues from Fol and *F. graminearum* 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. First, they are hampered in pathogenicity; second, *MgTpk2* and *MgGpb1* mutants penetrate the host and colonize the mesophyll, but cannot differentiate cells towards fructification; third, *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 restores the WT phenotype (Mehrabi *et al*., 2009). Our current data suggest that the previously characterized cAMP genes (*MgTpk2* and *MgGpb1*) and *ZtWor1* may be three components of the cAMP pathway controlling different aspects of differentiation and infection. Interestingly, all family members of WOPR contain 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* homologue of Tpk2, MgTpk2, as shown by the expression analyses conducted.

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 and the regulation of SSPs, which may act as putative effector genes. In addition, we suggest that *ZtWor1* may be regulated by two upstream key genes, *MgGpb1* and *MgTpk2*, indicating that the functionality of ZtWor1 is controlled through the cAMP pathway; hence, ZtWor1 may 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 the morphopathogenic behaviour of *Z. tritici*. The data presented 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 WT and recipient strain for gene deletion. The WT and all developed strains were stored at −80 °C and recultured on PDA (Sigma-Aldrich Chemie, Steinheim, Germany) at 18 °C. Yeast-like spores were produced on V8 juice medium (Campbell Foods, Puurs, Belgium) or in 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 conditions, but at 25 °C. *Aspergillus nidulans* MM and 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 and ZtWor1-PRF-F2, R2 primer pairs were used to amplify about 2000 bp upstream and downstream of *ZtWor1* using PfuTurbo® Cx Hotstart DNA polymerase (Stratagene, Cedar Creek, TX, USA). In parallel, the pRF-HU2 vector possessing the *hygromycin phosphotransferase* (*hph*) gene as a selection marker was digested with two restriction enzymes, *Pac*I and a nicking enzyme *Nt.Bbv*CI, 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, MA, 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 subsequently cultured on selective kanamycin medium. 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 in the middle of the *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 procedure. The full open reading frame of *ZtWor1*, including 1200 bp upstream as promoter and 500 bp downstream as terminator, were cloned

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	ACCTTGCTAATAACCCAAACGCC	Downstream of *ZtWor1*

into pDONR™P2R-P3 (Invitrogen, Carlsbad, 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™221 and pDONR™P4-P1R, resulting in p221-GFP and p4-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. *Agrobacterium tumefaciens*-mediated transformation was carried out to delete *ZtWor1* in the WT strain, as described previously (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 screen.

For complementation, the same procedure was applied with minor modifications. As a result of the lack of spore production in *ΔZtWor1-26*, small pieces of hyphal fragments—adjusted to 10^5 per mL—were used in *A. tumefaciens*-mediated transformation, and putatively complemented strains were selected on plates with 250 μg/mL geneticin.

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, Darmstadt, Germany) and finally adjusted to 10^5 hyphal fragments/mL to monitor anastomosis in the WT strain and *ΔZtWor1-26*. Approximately 10 μL of each sample were spotted onto 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 7 days. The samples were monitored using an Olympus IX81 microscope (Olympus, Hamburg, Germany), equipped with a 100×/1.45 Oil TIRF or 60×/1.35 Oil objective and a VS-LMS4 Laser-Merge-System with solid state lasers (488 nm/70 mW and 561 nm/70 mW; Visitron System, Munich, Germany). The images were taken using a Photometrics CoolSNAP HQ2 camera (Roper Scientific, Planegg/Martinsried, Germany) and processed by MetaMorph (Molecular Devices, Downingtown, PA, USA) software.

Pathogenicity assay

The susceptible wheat cv. Obelisk was grown in a glasshouse until the first leaves were fully unfolded. As *ΔZtWor1-26* did not sporulate, we used mycelial fragments for all strains. Inoculum was produced by blending mycelia 24 h before inoculation, which were subsequently maintained in YGB at 25 °C for cell recovery, 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 the WT strain were inoculated on a wide variety of wheat germplasm that was grown and inoculated according to standard procedures (Tabib Ghaffary *et al*., 2011). Inoculated plants were incubated in black plastic bags for 48 h and then transferred to a glasshouse compartment (22 °C, relative humidity >90% and 16 h light). Disease development was monitored every 3 days and final scoring was performed at 20 dai.

RNA isolation and quantitative RT-PCR

In vitro and *in planta* expression analyses of selected genes were conducted using quantitative real-time RT-PCR. Plants of cv. Obelisk were inoculated with the 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, Valencia, CA, USA), and subsequently DNA contamination was removed using the DNAfree kit (Ambion, Huntingdon, Cambridgeshire, UK). First-strand cDNA was synthesized from 2 μg of total RNA primed with oligo(dT) using SuperScript III according to the manufacturer's instructions. One microlitre of resulting cDNA was employed in a 25-μL PCR 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* β-tubulin gene (Keon *et al*., 2007; Motteram *et al*., 2009) and then calculated on the basis of 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 g dextrose/L), in four biological replications for 48 h. After recovery from these media, mycelia were removed by centrifugation (Beckman, Pleasanton, CA, 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 microlitres of the crude protein extracts were applied to Nanosep 3 K Omegacentrifuge filters (Pall Corporation, Ann Arbor, MI, USA) and centrifuged at 5000 ***g*** for 30 min at room temperature (20 °C). Thereafter, the Filter Aided Sample Preparation (FASP) method (Manza *et al*., 2005; Wisniewski *et al*., 2009) was used to generate tryptic peptides for liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. The peptide solutions were acidified by adding 3.5 μL of 0.1% trifluoroacetic acid and analysed by LC-MS/MS as described previously (Lu *et al*., 2011). LC-MS runs with all MS/MS spectra obtained were analysed 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 (http://genome.jgi-psf.org/Mycgr3/Mycgr3.home.html) was used together with a database that contains sequences of common contaminants, such as, for instance, BSA (P02769, bovine serum albumin precursor), trypsin (P00760, bovine), trypsin (P00761, porcine), keratin K22E (P35908, human), keratin K1C9 (P35527, human), keratin K2C1 (P04264, human) and keratin K1CI (P35527, human). The 'label-free quantification' (LFQ) as well as the 'match between runs' (set to 2 min) options were enabled. De-amidated peptides were allowed to be used for protein quan-

tification and all other quantification settings were kept at 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). Peptides and proteins with a false discovery rate (FDR) of less than 1% and proteins with at least two identified peptides, one of which should be unique and one of which should be unmodified, were accepted. Reversed hits and contaminants were deleted from the MaxQuant result table. The relative protein quantification of WT to mutant was performed with Perseus by applying a two-sample *t*-test using the 'LFQ intensity' columns obtained with threshold 0.05 and S_0 = 1. The normal logarithm was taken from normalized LFQ 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

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1 Replacement strategy for *ZtWor1* in *Zymoseptoria tritici*. (A) Diagram showing the replacement by the *hygromycin phosphotransferase* (*hph*) resistance cassette through homologous recombination. The broken line depicts the flanking regions used for homologous recombination. (B) Identification of replacement mutants by polymerase chain reaction (PCR). Lane M, 1-kb-plus ladder marker. Lanes 1, 4 and 7 show three independent replacement mutants (*ΔZtWor1-1*, *ΔZtWor1*-26 and *ΔZtWor1-29*) with no amplicon using primers ZtWor1-F1 and ZtWor1-R1, whereas the wild-type (WT) strain (lane C1) and the complemented strain *ΔZtWor1-com7* (lane10) show the expected amplicon of 650 bp with the same primers. Primers ZtWor1-F2 and ZtWor1-R2, which are located in the middle of the *hph* gene and downstream of the *ZtWor1* open reading frame (ORF), produced an amplicon of 2000 bp (lanes 2, 5 and 8), but did not result in amplification in the WT strain (C1) and the complemented strain (lane 11).

Fig. S2 Number of cell fusion events, counted in 0.016 mm^2, in colonies of *Zymoseptoria tritici* wild-type (WT) strain, *ΔZtWor1-com7* and *ΔZtWor1-26* grown on minimal medium (MM) for 10 days at 20 °C.

Fig. S3 Differences in hyphal diameters of *Zymoseptoria tritici* wild-type (WT) strain, *ΔZtWor1-com7* and *ΔZtWor1-26* grown on minimal medium (MM) for 10 days. $n = 100$ for each strain. The difference is significant at $P < 0.05$.

Fig. S4 Disease development in 12 wheat cultivars that are parents of mapping populations after inoculation with *Zymoseptoria tritici* wild-type (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.

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