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Phosphopantetheinyl transferase (Ppt)-mediated biosynthesis of lysine, but not siderophores or DHN melanin, is required for virulence of Zymoseptoria tritici on wheat

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Zymoseptoria tritici is the causal agent of Septoria tritici blotch (STB) disease of wheat. Z. tritici is an apoplastic fungal pathogen, which does not penetrate plant cells at any stage of infection, and has a long initial period of symptomless leaf colonisation. During this phase it is unclear to what extent the fungus can access host plant nutrients or communicate with plant cells. Several important primary and secondary metabolite pathways in fungi are regulated by the post-translational activator phosphopantetheinyl transferase (Ppt) which provides an essential co-factor for lysine biosynthesis and the activities of non-ribosomal peptide synthases (NRPS) and polyketide synthases (PKS). To investigate the relative importance of lysine biosynthesis, NRPS-based siderophore production and PKS-based DHN melanin biosynthesis, we generated deletion mutants of ZtPpt. The ∆ZtPpt strains were auxotrophic for lysine and iron, non-melanised and non-pathogenic on wheat. Deletion of the three target genes likely affected by ZtPpt loss of function (Aar- lysine; Nrps1-siderophore and Pks1-melanin), highlighted that lysine auxotrophy was the main contributing factor for loss of virulence, with no reduction caused by loss of siderophore production or melanisation. This reveals Ppt, and the lysine biosynthesis pathway, as potential targets for fungicides effective against Z. tritici.

The Ascomycete fungus Zymoseptoria tritici (Desm.) (Quaedvlieg & Crous)1 causes Septoria tritici blotch (STB) disease of wheat in temperate growing regions worldwide2,3. This disease is widely considered, both in industry and academia, to be the most important wheat disease in Europe1. It is estimated that approximately 70% of the $1.7 billion a year European fungicide market for wheat is sold for the control of STB4. Extensive use of fungicides has led to numerous cases of resistance in Z. tritici, and new chemistries are needed for future disease control5,6. Knowledge of essential-for-life and important virulence genes in this fungus may aid the development of these new chemistries7.

Z. tritici is often considered a hemibiotrophic fungus, as it undergoes two distinct phases of plant colonisation – an initial symptomless infection lasting at least one week, which is subsequently followed by a necrotrophic phase associated with the formation of leaf lesions. During the symptomless phase, Z. tritici typically enters leaves

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through stomata and colonizes mesophyll tissue where it grows in the intercellular space, the "apoplast", without producing haustoria or any other form of plant cell penetrating structures. The onset of leaf necrosis is then rapid and begins with the appearance of chlorotic lesions. These eventually coalesce into necrotic blights bearing pycnidia, which are asexual fructifications containing rain splash-dispersed pycnidiospores. This entire infection cycle occurs without fungal penetration of host cells. For this reason, Z. tritici is also referred to as an ‘apoplastic’ pathogen, which delineates it from many other plant pathogenic fungi that do physically penetrate plant cells at some point during infection. However, the full repertoire of mechanisms that enable initial symptomless colonisation and the subsequent activation of leaf necrosis by Z. tritici have not been fully elucidated.

Several plant pathogenic fungi produce diffusible secondary metabolites (SMs) to induce host tissue necrosis, which supports colonisation. Many of these SMs are produced by non-ribosomal peptide synthase (NRPS) or polyketide synthase (PKS) enzymes frequently residing in genome clusters with other tailoring genes and transporters. Important examples include the victorin toxin of Cochliobolus victoriae and the T-toxin of C. heterocephalus. Victorin is a non-ribosomal peptide (NRP) that is produced by the NRPS enzyme TOX3. This toxin causes programmed cell death (PCD) in Arabidopsis thaliana by interacting with the nucleotide binding site leucine rich repeat (NB-LRR) receptor protein, LOV1. T-toxin is a polyketide (PK) produced by PKS enzymes encoded at two distinct loci, Tox1A and Tox1B. It interacts with mitochondria in Texas male sterile cytoplasmic corn to cause necrosis. Both T-toxin and victorin are essential for virulence in their respective pathogens.

As bioactive molecules, SMs are often involved in a variety of other processes such as microbial competition, and melanin and siderophores are two key examples of metabolites frequently found to be produced by most fungi. In the citrus pathogen Alternaria alternata, disruption of the NRPS siderophore synthetase-encoding gene NPS6 leads to low iron availability and reactive oxygen species (ROS), which leads to reduced virulence. This reduction in virulence is likely due to the iron cofactor requirement of enzymes that quench host derived ROS. In Z. tritici several studies have predicted the role of a specific PKS (Pks1) in melanin biosynthesis but this gene has, to date, not been functionally characterised.

Despite diverse biological roles, all NRPSs and PKSs share a single point of posttranslational activation. To function, they must be 4′-phosphopantetheinylated at conserved serine residues within the acyl carrier protein (ACP) domain. The enzyme that carries out this process is 4′-phosphopantetheinyl transferase (PPT), which was first described for filamentous fungi in Aspergillus nidulans. In addition to its role in the biosynthesis of SMs and siderophores, Ppt is essential for 4′-phosphopantetheinylation of alpha aminoadipate reductase (Aar), an enzyme crucial for lysine biosynthesis. The Saccharomyces cerevisiae Ppt homologue was first described as the enzyme LY55, for which cognate mutants were auxotrophic for lysine.

Following its discovery in model fungi, Ppt homologues were characterised in several plant pathogens with the aim of identifying links to pathogenicity via production of SMs. These include Colletotrichum graminicola, Magnaporthe oryzae, Fusarium fujikuroi and Cochliobolus sativus. In all these species, Ppt mutant strains were shown to be auxotrophic for lysine and unable to melanise or produce siderophores. In addition, a Ppt homologue with similar functions has also been investigated in the endophytic fungus Trichoderma virens. The auxotrophy for lysine of Ppt mutants has led to its consideration as a potential antifungal target. For example, in the human pathogens Candida albicans and Aspergillus fumigatus, high throughput screens have been developed with the aim of identifying inhibitors of this enzyme.

Though some components of the interaction between Z. tritici and Triticum aestivum have been elucidated, the biological role of SMs in Z. tritici and key genes implicated in the regulation of SMs have not been characterised. To gain an insight into the functions of Z. tritici SMs and to ascertain the importance of lysine biosynthesis, siderophore biosynthesis and melanisation for the apoplastic lifestyle and virulence, the Z. tritici Ppt homologue ZtPpt was functionally characterised by gene deletion. In addition, we generated and characterised deletion mutants for key downstream targets of Ppt that are directly responsible for melanisation (Pks1), siderophore (Nps6) and lysine (Aar) biosynthesis. Further gene deletion strains were generated and characterised for the recently described Z. tritici transcriptional regulator StuA, and two additional Polyketide synthases (PKS7 and PKS8) which previous studies had shown to be highly expressed during leaf infection.

Results

Identification of Zymoseptoria tritici homologues of Ppt and genes implicated in regulating biosynthesis of lysine, siderophore and DHN-melanin. A BLASTp analysis was conducted to determine whether Z. tritici contains clear homologues of Ppt and associated genes previously characterised in C. sativus. This showed that Z. tritici possesses a single Ppt homologue, a single Pks1 homologue, a single Aar homologue and two homologues for Nps6 (Supplementary Table 1). We performed additional Blastp analyses using characterised sequences from the A. fumigatus siderophore biosynthesis pathway to more accurately ascribe the best predicted functional homologue of the Aspergillus fumigatus protein Nps6, which revealed the protein ZtNps1 (Supplementary Table 1). The protein encoded by ZtNps1 was homologous to both the sidC and sidD protein families but not to other siderophore biosynthetic proteins that are not generated by NRPSs. ZtNps1 was in fact most similar to sidD, which is involved in the biosynthesis of an extracellular fusarin C siderophore (Supplementary Table 2).

Z. tritici Ppt deletion mutants are lysine auxotrophs, hypersensitive to iron depletion and ROS, and are deficient in melanisation. ZtPpt, ZtPks1, ZtNps1 and ZtAar gene deletion strains were all generated (Supplementary Figure 1) and firstly assessed for their responses to different stresses in vitro. This revealed that ΔZtPpt strains were hypersensitive to iron depletion, as were the ΔZtNps1 mutants (Fig. 1A,B). This was evident in the observation that neither of these strains grew in the presence of the iron chelating agent BPS but were rescued with further addition of an exogenous siderophore compound, desferriferrichrome (DF). ΔZtPpt strains were also auxotrophic for lysine biosynthesis, as was also observed for the AAR mutant ΔZtAar.
Finally, \( \Delta ZtPpt \) strains were unable to melanise when exposed to UV light for six days or longer, a phenotype also observed for \( \Delta ZtPks1 \) (Fig. 1A,D). This same phenotype was also recently observed for \( ZtStuA \) mutants (Supplementary Figure 2A,B) highlighting that both StuA and Ppt functions are required for melanisation in this fungus. This was further supported by our additional data on \( ZtStuA \), which demonstrated it to be a nuclear localised protein (Supplementary Figure 2C), and to positively influence the expression of several key genes for the DHN-melanin biosynthesis pathway, including the \( Pks1 \) gene itself (Supplementary Figure 2B and Supplementary Table 3).

**Zymoseptoria tritici** filamentous growth on nutrient limiting agar requires lysine biosynthesis but not Nrps1-derived siderophores or melanin. To evaluate whether \( \Delta ZtPpt \) strains were affected in filamentous growth *in vitro*, mutant strains were spot inoculated onto water agar, a nutrient depleted substrate which stimulates the formation of hyphal filaments from \( Z. \) tritici spores. This showed that, in the absence of exogenous lysine, \( \Delta ZtPpt \) and \( \Delta ZtAar \) could not extend hyphal filaments (Fig. 2 and Supplementary Figure 3). When lysine was added, filamentous growth was rescued in these strains but not to wild type (WT) levels (Fig. 2). Both the \( \Delta ZtNrps1 \) and \( \Delta ZtPks1 \) strains grew to at least WT levels whether supplemented with lysine or not. One of each of the \( \Delta ZtNrps1 \) and \( \Delta ZtPks1 \) strains grew significantly faster than the WT without supplements, though this was not consistent across both strains assayed (Fig. 2).

Lysine biosynthesis is a key requirement for \( Z. \) tritici infection of wheat but the Nrps1-derived siderophore and melanin are not. We next assayed the virulence of \( \Delta ZtPpt \) and the \( \Delta ZtPks1 \) and \( \Delta ZtAar \) downstream gene deletion mutants, on a susceptible wheat cultivar. This showed that \( \Delta ZtPpt \) was completely avirulent both with and without lysine supplementation in the starting inoculum on wheat seedlings (Fig. 3A,C; Supplementary Figure 4). The same phenotype was also observed for the \( \Delta ZtAar \) strain. In contrast, the \( \Delta ZtNrps1 \) strains were not affected in virulence, nor were the \( \Delta ZtPks1 \) strains (Fig. 3A,C). However, \( \Delta ZtPks1 \) generated non-melanised pycnidia at the end of the infection period (Fig. 3A,B). \( ZtStuA \) deletion mutants were recently shown to be attenuated in virulence on wheat and our data provided further support for this, which manifested itself in a delayed onset of leaf necrosis and lesions lacking asexual spores (Supplementary Figure 2D).
Two additional Zymoseptoria tritici PKS genes strongly up-regulated during leaf infection are not required for virulence. To assess the importance of two previously identified genes, ZtPks7 and ZtPks8, whose gene clusters were transcriptionally up-regulated during infection\(^4\), deletion strains were generated and inoculated onto a susceptible wheat cultivar to test for altered virulence. For the single \(\Delta ZtPks7\) strain and the three \(\Delta ZtPks8\) strains tested, no effects on virulence were observed (Fig. 4). After the full course of infection at 21 days, necrotic symptoms indistinguishable from the WT were observed in all cases (Fig. 4A). The number of spores retrieved from infected leaves for each of the mutant strains was also not significantly different to that for the WT strain (Fig. 4B,C).

Discussion

In this study we analysed the relative importance of particular primary and secondary metabolites for virulence and other processes in the apoplastic wheat pathogen *Z. tritici*. We functionally characterised several *Z. tritici* genes, some of which were homologues of those known in other fungi to regulate lysine biosynthesis, melanisation and siderophore biosynthesis, amongst other processes. A summary overview of the deletion strains generated, the downstream processes they regulate, and their contributions (or not) to virulence is presented in Fig. 5. The schematic also incorporates data from the recently characterised StuA mutants of *Z. tritici*\(^4\) as well as our own novel insights presented here which demonstrated StuA to have a direct impact on the expression of Pks1 and melanisation separately, or they may form part of, an as yet undescribed signalling pathway controlling *Pks1* expression. These two possibilities require further testing.

Alike the \(\Delta ZtStuA\) and \(\Delta ZtPks1\) mutants, \(\Delta ZtPpt\) mutants were also unable to melanise suggesting that the protein does indeed function to provide the essential co-factor for PKS and NRPS enzymes, including Pks1. This would indicate that the disrupted PPT gene functions similarly to those in other fungi to phosphopantetheinylate PKS and NRPS enzymes, including ZtPks1. \(\Delta ZtPpt\) mutant strains were also hypersensitive to iron depletion and ROS, and auxotrophic for lysine. This further supports the idea that ZtPpt is a functional homologue of the PPT genes characterised in other fungi.

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**Figure 2.** *In vitro* growth assays of Ppt-associated deletion strains on water agar with and without supplementation with lysine and iron. Measurement is radial growth after 20 days. From left to right portions of figure: the two WT strains and all PPT associated strains, \(\Delta ZtPpt-1\), \(\Delta ZtPpt-2\), \(\Delta ZtPks-1\), \(\Delta ZtPks-2\), \(\Delta ZtNrps-1\), \(\Delta ZtNrps-2\) and \(\Delta ZtAar\) on water agar with no supplements; the two PPT mutant strains \(\Delta ZtPpt-1\) and \(\Delta ZtPpt-2\) and the two WTs on water agar with both added available iron (FeSO₄) and lysine; The lysine auxotrophic PPT associated strains \(\Delta ZtPpt-1\), \(\Delta ZtPpt-2\) and \(\Delta ZtAar\) and the two WTs on water agar supplemented with lysine; the siderophore mutant strains \(\Delta ZtNrps-1\) and \(\Delta ZtNrps-2\), the PPT mutant strains \(\Delta ZtPpt-1\) and, \(\Delta ZtPpt-2\) and the two WTs on water agar supplemented with FeSO₄. Horizontal black bars represent median values, boxes represent second and third quartiles and whiskers represent interquartile range. Asterisks represent significant differences in radial growth relative to the WT that constituted the background strain at \(\alpha < 0.05\).
**ZtNrps1** mutant strains were hypersensitive to iron depletion, which would indicate that this gene encodes an enzyme that synthesises an important siderophore in a non-redundant manner under the conditions tested. Growth was restored when extracellular siderophores were added to the medium. In the model fungus *A. fumigatus* two major NRPS genes involved in siderophore biosynthesis have been characterised. These are *sidC*, which is required for biosynthesis of intracellular ferricrocin siderophores; and *sidD*, which is required for biosynthesis of extracellular fusarin C siderophores. Intracellular siderophores are required for iron storage and transport, whereas extracellular siderophores are required for chelation and uptake of unavailable iron. *ZtNrps1* was more similar to *sidD* than *sidC*, supporting the hypothesis that it produces an extracellular siderophore. However, *ZtNrps1* was not necessary for wheat infection by *Z. tritici*. This is in contrast to previous reports on other fungi which have shown homologues of *sidD* (*ZtNrps1*) to be important for plant pathogenicity. A notable exception to this was *Ustilago maydis*, where targeted disruption of biosynthesis of all siderophores in this fungus did not reduce virulence. Since *U. maydis* is a biotrophic fungus and *Z. tritici* exhibits an extensive biotrophic/symptomless phase, it may be that the fungus has sufficient easily accessible iron, which overrides the need for extracellular siderophore production during leaf infection. Alternatively, *Z. tritici* may be able to use stored iron during filamentous growth. This is supported by the ability of **ΔZtNrps1** mutant strains to produce WT levels of hyphal filaments on iron-free water agar.

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**Figure 3.** *In planta* inoculation assays for Ppt-associated gene deletion strains highlights the key role of lysine biosynthesis for virulence. (A) Upper panel: All PPT associated disruption strains, **ΔZtPpt-1**, **ΔZtPpt-2**, **ΔZtPks1-1**, **ΔZtPks1-2**, **ΔZtNrp1-1, 2**, **ΔZtAar**, and the two wild type strains IPO323 and **ΔZtku70** after 22 days post inoculation (dpi) of the susceptible wheat cv, Riband; lower panel: the *Z. tritici* lysine auxotrophic PPT associated strains **ΔZtPpt-1, ΔZtPpt-2** and **ΔZtAar**, and the two wildtype strains after 22 (dpi) with lysine added to starting inoculum. (B) The WT **ΔZtku70**, and **ΔZtPks1-1** and **ΔZtPks1-2** melanin synthetase mutants viewed under a light microscope after 22 dpi. White arrows mark pycnidia, in which the melanin synthetase mutants are unpigmented. (C) Spores collected from infected leaves after 22 dpi. Left portion of plot: all PPT associated strains and the WTs without lysine added to fungal inoculum; right portion of plot: lysine auxotrophic PPT associated strains and the WTs with lysine added to fungal inoculum. Asterisks represent significant differences in spore counts relative to the WT that constituted the background strain (either IPO323 for **ΔZtAar** or **ΔZtku70** for the rest) at α < 0.05. Black horizontal bars represent median values, boxes represent second and third quartiles and whiskers represent interquartile range.
In contrast to the ∆ZtNrps1 and ∆ZtPks1 strains, ∆ZtPpt and ∆ZtAar mutants were unable to grow hyphal filaments in vitro or undergo budding growth on minimal medium without lysine supplementation. This indicates that both filamentous and budding growth in Z. tritici require a fully functioning lysine biosynthetic pathway. This is consistent with the lysine auxotrophy observed in Ppt mutant strains in various filamentous fungi studied to date22,23,25. The virulence data on wheat leaves demonstrated that both ∆ZtNrps1 and ∆ZtPks1 exhibited WT virulence, but both ∆ZtPpt and ∆ZtAar were strongly reduced in virulence. Together this suggests that lysine availability (or lack of) forms a major barrier to infection by the latter two mutant strains, most probably as a result of restricting hyphal growth, and it is the loss of ZtAar function which is largely responsible for the loss of virulence in ∆ZtPpt. Hence, both ZtPpt and ZtAar would appear to be good targets for putative fungicides for the control of STB.

In a recent study37 it was shown that Z. tritici exhibits differential expression of several other large putative SM clusters during infection. Several of these gene clusters contained PKSs highly upregulated coincident with the first appearance of necrotic lesions on wheat leaves. However, functional studies reported here have now shown that two of these PKSs are not essential for infection of the susceptible host cultivar tested, although we cannot rule out possible interactions with other cultivars. Ablation of individual PKS enzymes often has no discernible effect on virulence in plant pathogens. For example, in Fusarium graminearum, 15 PKS genes were disrupted in a single study and none affected virulence 42. Hence, the functional roles of individual SM clusters in Z. tritici remain unknown. Besides their roles in host necrosis as toxins, SMs may be involved in, for example, microbial competition. In T. reesei, mutants for a specific PKS were less able to inhibit the growth of competing microbes, including the plant pathogens Alternaria alternata, Sclerotinia sclerotiorum, Botrytis cinerea and Rhizoctonia solani43. It is possible that Z. tritici specifically produces these energetically costly compounds, at a time when necrosis appears, to compete with other microbes that may wish to utilise the dead tissue. However, this, and the roles of several other predicted secondary metabolite clusters in the biology of Z. tritici, require further testing.

Methods

Strains, media and growth conditions. The fully sequenced Z. tritici reference strain IPO3238 and its derivative (ΔZtku70), in which the Ku70 gene has been disrupted to reduce non-homologous end joining (NHEJ)44, were employed as wild type (WT) and recipient strains for gene deletion and gene disruption. Both strains are highly virulent on the susceptible wheat cultivars Taichung 29 and Riband. The WT and all

Figure 4. In planta inoculation assays for the two PKS deletion mutant strains ΔZtPks7 and ΔZtPks8 reveal them to be dispensable for virulence. (A) Symptoms after 22 dpi on the susceptible wheat cv. Riband. Left: The WT (ΔZtku70), the sole ΔZtPks7 strain and one of the ΔZtPks8 strains, ΔZtPks8-1; right: The WT (ΔZtku70) and three ΔZtPks8 mutant strains, ΔZtPks8-1, ΔZtPks8-2 and ΔZtPks8-3. (B) Spores retrieved from leaves after 22 dpi. Horizontal bars represent median values, boxes represent second and third quartiles and whiskers represent interquartile range. No significant differences were detected.
generated strains were stored at −80 °C and re-cultured on potato dextrose agar (PDA) or yeast peptone dextrose (YPD) agar (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 conditions but at 24 °C. To assess lysine auxotrophy, 100 mM Lysine was added to the minimal medium Czapek-Dox (CD) agar. To induce filamentous growth *in vitro* strains were grown on 10% sterile distilled water (SDW) agar.

**Bioinformatic analyses.** Phylogenetic analysis of *ZtStuA* with homologues from other fungal pathogens was performed using the CLC genomics workbench package (Aarhus, Denmark). All StuA fungal proteins were retrieved from public databases and aligned with 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 support for the tree was assessed with 1000 bootstraps.

Additionally, the genome sequence of *Z. tritici* IPO323 (Goodwin et al., 2011) was queried in a BLAST search using the characterised sequences of PPT, NPS6 (a siderophore biosynthetic enzyme), PKS1 (the fungal melanin biosynthetic enzyme) and AAR (the lysine biosynthesis enzyme) from *C. sativus* (Leng and Zhong, 2012). This was done using the BLASTp program over the NCBI BLAST server (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins) with default settings. The PKS sequences *ZtPks7* and *ZtPks8* first identified as having a potential role in infection were also identified using the *Z. tritici* IPO323 genome sequence.
A further BLASTp analysis was conducted against the Z. tritici annotations using the A. fumigatus accessions XP_753088.1, XP_746662.1, XP_748660.1 and XP_748685.1, which correspond to the amino acid predictions for genes sidC, sidD, sidF and sigG, respectively. The former, sidC and sidD, encode NRPSs that produce intracellular ferricrocin and extracellular fusarin C siderophores, respectively. The latter, sidF and sigG, encode non-NRPS siderophore synthesis-associated enzymes.

**Generation of gene replacement, complementation and GFP fusion constructs.** To generate the ZtStuA deletion construct, pZtStuAKO, the USER friendly cloning method was used with minor modifications as described previously. Briefly, ZtStuA-F3 and R3, as well as ZtStuA-F4 and R4 primer pairs were used to amplify about 2,000 bp upstream and downstream of ZtStuA using PfuTurbo® CX Hotstart DNA polymerase (Stratagene, Cedar Creek, TX, US). In parallel, the pRF-HU2 vector possessing the hygromycin phosphotransferase (hph) gene as a selection marker was digested with two restriction enzymes, PciI 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 DH15α and subsequently cultured on kanamycin-selective medium.

To identify bacterial colonies carrying the construct with the insertions in the expected positions, colony PCR was performed using User-F and User-R primers (located in the middle of the hph gene) in combination with ZtStuA-F1 and ZtStuA-R1, respectively.

To produce the ZtStuA complementation construct and ZtStuA fused to GFP, the vectors pCZtStuA.com and pCZtStuA.GFP were generated by in vivo recombination in the yeast Saccharomyces cerevisiae DS94 (MATa, ura3-52, trp1-1, leu2-3, lys2-801, 52, trp1-1, leu2-3, his3-111, and neo2-101) following published procedures. The vector pCZtStuA.com contains the ZtStuA ORF under the control of the native promoter (1,025 bp) and terminator sequences (495 bp) for random ectopic integration into the genome of Z. tritici by using carboxin as the selection agent. A 9,760 bp fragment of pCGEN-YR (digested with Xbal and Zral1), 1,410 bp fragment covering the BASTA resistance cassette bar (amplified with MG-Sep-106 and MG-Sep-107), 3,146 bp fragment covering the 1025 bp of ZtStuA promoter, 1,626 bp pf ZtStuA gene and 495 bp of ZtStuA terminator (amplified with MG-Sep-113 and MG-Sep-116) were recombined in yeast S. cerevisiae to obtain the vector pCZtStuA.com. A 16,597 bp fragment of pCZtGFPSpa2 (digested with PshAI), 1,626 bp full-length ZtStuA gene (amplified with SK-Sep-346 and SK-Sep-347) were recombined in yeast S. cerevisiae to obtain the vector pCZGFPSpStuA.

To produce the disruption constructs for the Ppt-associated genes ZtNps1 and ZtPks1, and the PKs genes ZtPks7 and ZtPks8, primers with added 5’ restriction sites and two 5’ adenines were designed for the amplification of the selected flanking sequences using Geneious version 8.1. Flanking sequences were approximately 1 Kb either side of these genes. Flanking regions were amplified in PCR reactions from genomic DNA of isolate IPO323 using RedTaq mastermix according to the manufacturer’s protocol. Thermocycler settings were 95 °C 2 min, then 30 cycles of 95 °C 30 sec, 60 °C 30 sec, 72 °C 2 min, and 10 °C hold. Flanking sequences were cloned into pCHYG either side of hph under the trpC promoter. For all strains generated in this study, primers and added restriction sites are in Supplementary Table 4.

The ΔZtAar strain was generated in Ali et al. Briefly, pCambia 0380 was digested with SacII, the 2-micron + ura3 region was amplified by PCR from pYES2 using 2 µmURA3 FP and 2 µmURA3 RP was used to repair the plasmid in yeast recombination. The left and right flanks for ZtAar were amplified from genomic DNA by PCR and recombined into BamH1-digested pCambia0380YA along with the hygromycin resistance cassette. Plasmids were purified from yeast using the Zymoprep ITM yeast plasmid extraction kit and rescued into E. coli then construction confirmed by restriction digest, PCR and sequencing across recombination sites.

**Fungal transformation.** The gene deletion construct as well as the replacement construct were first cloned into A. tumefaciens strain AGL1 via electroporation. Agrobacterium mediated transformation was then carried out to delete ZtStuA in the ΔZtKu70 strain as previously described. For the complementation assay, the same procedure was applied but the putatively complemented strains were selected on plates with 50 µg mL−1 BASTA. All disrupted PKS and Ppt-associated mutant strains except ΔZtAar were also generated in the ΔZtKu70 background. ΔZtAar was disrupted in a wild type IPO323 background. For the Ppt and Aar mutants, 100 µM Lysine was added to the selection plates to ensure survival of transformed cells, which were expected to be lysine auxotrophs. Emerging colonies were then subjected to two rounds of selection on hygromycin amendedYPD agar plates. Genomic DNA of stable transformants was extracted and used in PCR screens to confirm homologous recombination in the correct positions.

**Epi-fluorescence microscopy.** Fluorescence microscopy was performed as previously described. In brief, IPO323 CzIGFPStuA cells were inoculated in YG media and grown at either 18°C with 200 rpm to induce the yeast-like cell growth or 24°C with 100 rpm to induce the hyphal growth for 24 h and placed onto a 2% agar cushion for direct observation using a motorized inverted microscope (IX81; Olympus, Hamburg, Germany), equipped with a PlanApo 100_/1.45 Oil TIRF objective (Olympus, Hamburg, Germany). Fluorescent tags and dyes were exited using a VS-LMS4 Laser Merge System with solid-state lasers (488 nm/50 mW and 75 mW; Visitron Systems, Puchheim, Germany) and single images or z-Stacks, using an objective piezo (Piezosystem Jena GmbH, Jena, Germany), over 6 µm depth with a z resolution of 0.2 µm were captured with 150 ms exposure. In addition a DIC image was taken for each cell using a CoolSNAP HQ2 camera (Photometrics/Roper Scientific, Tucson, USA). Overlays of the fluorescent and DIC images were generated using MetaMorph (Molecular Devices, Wokingham, UK). All parts of the system were under the control of the software.
package MetaMorph (MolecularDevices, Wokingham, UK). The localisation of ZtGFPStuA in the nucleus was confirmed by counterstaining the nucleus with DAPI (Sigma-Aldrich Company Ltd, Dorset, England). To this end, cells of strain IPO323_ ZtGFPStuA were grown for 24 h in YG medium at 18 °C with 200 rpm. DAPI was added the cell culture to a final concentration of 1 µg/ml and incubated for 10 minutes at 18 °C with 200 rpm followed by direct observation using a motorized inverted microscope (IX81; Olympus, Hamburg, Germany). The DAPI as well as the ZtGFP were exited using a standard mercury burner and imaged at 500 ms exposure time.

**RNA isolation and q-RT-PCR.** In *in vitro* and *in planta* expression profiling of ZtStuA was performed using quantitative real-time PCR (q-RT-PCR). For *in planta* analyses, the wheat cv. Taichung 29 was inoculated with ΔZtKu70 as described previously⁴⁸ 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 RNAeasy plant mini kit (Qiagen, location, USA) and subsequently DNA contamination was removed using the DNasefree 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 to the constitutively expressed *Z. tritici* β-tubulin gene⁴²,⁴³ and then calculated based on the comparative C(t) method described previously⁴⁸. Primers used in the expression profiling analysis are detailed in Supplementary Table 5.

**Analysis of sensitivity to different stressors in vitro.** To identify in *in vitro* defects caused by the disrupted PPT-associated genes, all mutant strains were 10 µl spot-inoculated from 10⁶ spores per ml suspensions in SDW onto YPD and/or CD minimal medium agar plates either with or without supplementation or incubation under UV light. To assess for increased ROS sensitivity, YPD plates were supplemented with 7 mM H₂O₂ to assess for melanisation deficiency YPD plates were incubated after an initial 6 day period for a further 6 days under UV light at 20 °C; to assess for lysine auxotrophy CD plates were supplemented with 100 µM lysine; and to assess for hypersensitivity to iron depletion YPD plates were supplemented with 100 µM bathophenanthroline disulphate (BPS) or 100 µM BPS and 30 µM desferriferichrome (DFF). BPS is an iron chelating agent that reduces iron availability whereas DFF, a siderophore purified from *U. sphaerogena*, is an iron chelating agent that restores iron availability.

When placed onto a medium containing zero nutrients such as water agar, *Z. tritici* spores undergo filamentous growth. This contrasts the budding growth observed on nutrient replete media such as YPD agar. To assess defects in filamentous growth, the two WT controls and all targeted disruption strains were spot inoculated onto water agar as described in the previous paragraph. Water agar plates were either amended with 100 µM lysine, 100µM FeSO₄, a combination of both or neither. This was to assess the impact of different PPT-associated phenotypes on filamentous growth. After 20 days, radial growth of spot inoculations was measured vertically and horizontally and the mean value of these measurements was taken.

**Statistical analysis of infection assays and in vitro stress response tests for the Ppt-associated and Pks mutant strains.** R version 3.2.3 was used for all statistical tests. For plant infection assays with the Ppt-associated and PKS strains, individual leaves if different seedlings were treated as replicates. Spore count data for leaves inoculated with each independent strain were assessed visually for how well they fit a normal distribution using quantile quantile plots and statistically using the Shapiro test for normality (Supplementary Figure 5). Two independent strains were not transformed as they were adequately normally distributed for ANOV A (Supplementary Figure 3). The same statistical test was carried out for the radial filamentous growth data from the spot inoculations of water agar. In this instance data were not transformed as they were adequately normally distributed for ANOVA (Supplementary Figure 3).

**Pathogenicity assays.** The susceptible wheat cultivars Taichung 29 and Riband were grown in a greenhouse until the first leaves were fully unfolded or for 17 days, respectively. Inoculum of all strains was produced in YGB (yeast extract 10 g/L, Glucose 30 g/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 followed by direct observation using a motorized inverted microscope (IX81; Olympus, Hamburg, Germany). The DAPI as well as the ZtGFP were exited using a standard mercury burner and imaged at 500 ms exposure time.

**References**


