The Sfp-Type 4′-Phosphopantetheinyl Transferase Ppt1 of *Fusarium fujikuroi* Controls Development, Secondary Metabolism and Pathogenicity

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**Abstract**

The heterothallic ascomycete *Fusarium fujikuroi* is a notorious rice pathogen causing super-elongation of plants due to the production of terpene-derived gibberellic acids (GAs) that function as natural plant hormones. Additionally, *F. fujikuroi* is able to produce a variety of polyketide- and non-ribosomal peptide-derived metabolites such as bikaverins, fusarubins and fusarins as well as metabolites from yet unidentified biosynthetic pathways, e.g. moniliformin. The key enzymes needed for their production belong to the family of polyketide synthases (PKSs) and non-ribosomal peptide synthases (NRPSs) that are generally known to be post-translationally modified by a Sfp-type 4′phosphopantetheinyl transferase (PPTase). In this study we provide evidence that the *F. fujikuroi* Sfp-type PPTase FfPpt1 is essentially involved in lysine biosynthesis and production of bikaverins, fusarubins and fusarins, but not moniliformin as shown by analytical methods. Concomitantly, targeted Ffppt1 deletion mutants reveal an enhancement of terpene-derived metabolites like GAs and volatile substances such as α-acorenol. Pathogenicity assays on rice roots using fluorescent labeled wild-type and Ffppt1 mutant strains indicate that lysine biosynthesis and iron acquisition but not PKS and NRPS metabolism is essential for establishment of primary infections of *F. fujikuroi*. Additionally, FfPpt1 is involved in conidiation and sexual mating recognition possibly by activating PKS- and/or NRPS-derived metabolites that could act as diffusible signals. Furthermore, the effect on iron acquisition of Ffppt1 mutants led us to identify a previously uncharacterized putative third reductive iron uptake system (FFt3/FFet3) that is closely related to the FtrA/FetC system of *A. fumigatus*. Functional characterization provides evidence that both proteins are involved in iron acquisition and are liable to transcriptional repression of the homolog of the *Aspergillus* GATA-type transcription factor SreA under iron-replete conditions. Targeted deletion of the first *Fusarium* homolog of this GATA-type transcription factor-encoding gene, *Ffsre1*, strongly indicates its involvement in regulation of iron homeostasis and oxidative stress resistance.

**Introduction**

Filamentous fungi of the genus *Fusarium* are notorious pathogens of economically relevant crops. They produce a variety of bioactive secondary metabolites (Fig. 1) that pose a potential threat to animals and humans when consumed. In particular, the well known rice pathogen *F. fujikuroi* is able to produce *ent*-kaurene-derived gibberellins (GAs) [1], bikaverin [2], neurosporaxanthin [3], fusarin C [4], fusaric acid [5], moniliformin [6], fumonisins [7], α-acorenol [8], and fusarubins [9] (Fig. 1). Some of these substances have harmful effects on human cell lines, e.g. bikaverin, fusarins, and fumonisins [10–13] and in animal models, e.g. moniliformin [14]. Other metabolites play a role as virulence factors in fungal-plant interaction, e.g. fusaric acid, fumonisins, and GAs [15–17]. The latter belong to a class of isoprenoid phytohormones that are secreted by the fungus thereby causing the *bakanae* or “foolish seeding” disease of rice. The afflicted plants are visibly etiolated and chlorotic, do not produce edible grains, and are incapable of supporting their stem weight at late stages of the disease [18]. Beside this disease-causing action, some GAs are used in agriculture, viticulture, and horticulture as important plant growth regulators which are largely produced by submerged fermentation of the fungus *F. fujikuroi* on an industrial scale [19].

The secondary metabolites produced by filamentous fungi can be classified into distinct chemical groups as polyketides, non-ribosomal peptides, chimeric molecules composed of a polyketide and a non-ribosomal peptide moiety, terpenes, and (prenylated) alkaloids. Production of secondary metabolites of each group involves specific key enzymes, hence named polyketide synthases (PKSs), non-ribosomal peptide synthetases (NRPSs), PKS/NRPS hybrids, terpene cyclases (TCs), and prenyl transferases (PTs) [20]. The recently sequenced genome of *F. fujikuroi* strain IMI58289...
identified the existence of genes encoding 13 type I PKSs, 1 type III PKS, 11 NRPSs, 3 PKS/NRPS hybrids, 8 TCs, and 1 PT (B. Tudzynski and coworkers, unpublished data). Up to date, only five secondary metabolites produced by \textit{F. fujikuroi} could be assigned to a specific key enzyme. The polyketide pigments bikaverin and fusarubins are produced by the PKSs Bik1 (former Pks4) [21,22] and Fsr1 [9], respectively, and Fus1 is the hybrid PKS/NRPS involved in fusarin formation (E.-M. Niehaus and B. Tudzynski, unpublished data). The bifunctional TC \textit{ent}-copalyl diphosphate/\textit{ent}-kaurene synthase (CPS/KS) is the key enzyme for \textit{ent}-kaurene biosynthesis, the first step in GA formation [23], whereas CarRA is the TC involved in neurosporaxanthin production [24].

From a mechanistic point of view PKSs closely resemble fatty acid synthases (FASs). Similar to FASs, these multidomain enzymes contain acyl-carrier-proteins (ACPs) that covalently bind the growing acyl chain during PKS assembly. For functionality the ACP domains need to be post-translationally modified by 4\(^9\)PPT transferases (PPTases). These Mg\(^{2+}\)-dependent enzymes catalyze the covalent linkage of the 4\(^9\)PPT moiety of coenzyme A to a conserved serine residue within the ACP domains, where this 4\(^9\)PPT linker functions as the carrier for the growing acyl chain. Similarly, NRPSs contain peptidyl-carrier-proteins (PCPs) for binding of the growing peptidyl chain that are also modified by 4\(^9\)PPTylation of a conserved serine residue [25,26]. In contrast to this post-translational modification of PKSs and NRPSs, TCs and PTs do not harbor a prosthetic group that is essential for full functionality.

In the yeast \textit{Saccharomyces cerevisiae}, three PPTases have been identified. One is an integral part of the cytoplasmic type I FAS alpha-subunit (Fas2p) providing intrinsic catalytic activity only [27]. The second PPTase, Ppt2p, belongs to the AcpS-type PPTases and exclusively activates the low molecular weight ACP of the mitochondrial type II FAS [28]. The third PPTase, Lys5p, is a member of the Sfp-type PPTases and is essentially involved in lysine biosynthesis where it transfers 4\(^9\)PPT to the \(\alpha\)-amino acid reductase Lys2p [29]. In filamentous fungi all three yeast PPTases have been identified. Apart from the integral domain of the cytoplasmic FAS alpha-subunit PPTase, the PPTase PptB required for targeting the mitochondrial ACP (AcpA) was recently characterized in \textit{Aspergillus fumigatus} [30]. The first homolog of the \textit{S. cerevisiae} Sfp-type PPTase has been described in \textit{A. nidulans} by two independent research groups who identified the genes responsible for the “null pigmented” and “cross-feedable white” phenotype of mutants, respectively. The gene loci were designated \textit{npgA} and \textit{cfwA}, respectively [31,32]. Later both loci were identified to be identical and encode a Sfp-type PPTase [33,34] that is responsible for penicillin, siderophore (extracellular triacetyl fusarimine C and intracellular ferriocin), emericellin, shamixanthone, dehydroaustinol, and lysine production [33–35]. Other examples for altered secondary metabolite spectra were found in \textit{npgA/ppt1} mutants of \textit{Colletotrichum graminicola} [36], \textit{Penicillium chrysogenum} [37], \textit{A. niger} [38] and \textit{Trichoderma viride} [39]. Furthermore, in \textit{A. fumigatus}, the homologous PptA was shown to pantetheinylate the NRPS Pes1 [40]. Similarly to the \textit{npgA/ppt1} mutant of \textit{A. nulans}, deletion mutants of the homologous genes in \textit{C. graminicola}, \textit{Magnaporthe oryzae} [36], \textit{P. chrysogenum} [37], \textit{A. fumigatus} [40], \textit{A. niger} [38], \textit{Cochliobulus sativus} [41] and \textit{T. viride} [39] are lysine auxotrophic. Recent studies of the cereal pathogens \textit{C. graminicola} and \textit{C. sativus} have shown that Ppt1 is required for establishment of full virulence on rice and barley leaves, respectively. Addition of lysine did not restore wild-type-like virulence indicating the involvement of PKS- and/or NRPS-derived products in necrotrophic growth [36,41]. Interestingly, \textit{ppt1} mutants of \textit{T. viride} are not affected in root colonization but
cause attenuation of specific plant defense responses and hence an attenuated resistance against the fungal pathogen Botrytis cinerea [39].

Apart from its lysine auxotrophy the A. nidulans npgA mutant was unable to grow without the addition of NRPS-derived siderophores [35]. This dependency on siderophore-mediated iron uptake was not reported in any other species lacking the respective Sfp-type PPTase, most likely due to the existence of alternative reductive iron uptake systems. These alternative uptake systems are represented by ferroxidases and iron perases that are missing in A. nidulans [42]. In A. fumigatus the only reductive iron uptake system which can be specifically inhibited by the iron chelator bathophenanthroline disulfonate (BPS) is represented by the ferroxidase FetC and the iron permease FtrA that are arranged in a small cluster sharing one promoter [43]. From seminal work in A. fumigatus it is known that several genes that are involved in iron homeostasis (including fetC and ftrA) are controlled by a complex regulatory network that centers around the GATA-type transcription factor SreA [42–45]. In other fungal species iron-dependent regulation also involves SreA homologs called Urb1s in Ustilago maydis [46,47], SreP in P. chrysogenum [48] and Sre1 in Histoplasma capsulatum [49]. In F. graminearum, additionally to Nps6 which is the NRPS responsible for production of the extracellular siderophore [50] two ferroxidases (Fet1 and Fet2) and two iron perases (Ftr1 and Ftr2) were recently identified to be involved in iron acquisition [51]. It was shown that Fet1 and Ftr1 are associated within the plasma membrane, whereas Fet2 and Ftr2 reside in the vacuolar membrane [51]. Whether transcriptional control of the encoding genes is mediated by a SreA homolog is yet unclear.

In this work, we report on the characterization of the first Sfp-type PPTase mutant generated in a species of the genus Fusarium, i.e. the rice pathogen F. fujikuroi. The work focuses on general growth characteristics regarding the dependency of the deletion mutant on lysine and iron, as well as developmental features (asexual and sexual differentiation) and pathogenicity on rice plants. Of special interest was the comparison of secondary metabolite profiles of the wild type and the Ffppt1 mutant regarding the ability to produce PKS and PKS/NRPS-derived versus terpene-derived products. Furthermore, we show that the deletion of Ffppt1 affects not only the biosynthesis of the PKS-, PKS/NRPS- and terpene-derived secondary metabolites but also the expression of genes coding for the respective key enzymes. Comparison of Ffppt1 deletion mutants in different F. fujikuroi strains with their respective wild-type strains points to a distinctive role of PKS and/or NRPS-derived products during sexual and asexual development. The ability of the Ffppt1 mutants to grow on medium containing lysine and only trace amounts of iron (CD minimal medium without addition of lysine (Fig. 2A)). These data suggest that siderophore-assisted iron uptake allows the F. fujikuroi wild type to grow in the presence of BPS, and that loss of siderophore production affects sensitivity against H2O2 in the ftr1 mutant. The ability of Ffppt1 to grow on medium containing lysine and only trace amounts of iron (CD minimal medium containing BPS/lactate and H2O2/lysine, respectively (Fig. 2A)). Additionally, pathogenicity assays on rice roots with fluorescently labeled Ffppt1 mutant strain showed wild-type-like growth on all media, whereas the Ffppt1 strains did not grow on medium without lysine (Fig. 2A).

Ffppt1 contributes to a functional iron uptake system that is controlled by the GATA-type transcription factor FfSre1

In addition to the growth defect on lysine-deficient medium, growth of the Ffppt1 strain was severely restricted on medium containing lysine in the presence of the iron chelator BPS and H2O2, respectively (Fig. 2A). This restriction was overcome when 2 μM of the siderophore ferrichrome (FEC) was added to the medium containing BPS/lactate and H2O2/lysine, respectively (Fig. 2A). These data suggest that siderophore-assisted iron uptake allows the F. fujikuroi wild type to grow in the presence of BPS, and that loss of siderophore production affects sensitivity against H2O2 in the fpppt1 mutant. The ability of Ffppt1 to grow on medium containing lysine and only trace amounts of iron (CD minimal medium containing BPS/lactate and H2O2/lysine, respectively (Fig. 2A)). All three F. fujikuroi ferroxidase encoding genes revealed the presence of an adjacent iron permease encoding gene (designated FfFtr1, FfFtr2, and FfFtr3, respectively) each sharing its promoter region with the adjacent Fet-encoding gene. Searching the available Fusarium genome sequences revealed that F. verticilloides and F. oxysporum each possess three of these pairs similar to F. fujikuroi. Phylogenetic analysis showed that the A. fumigatus FtrA/FetC cluster groups together with FfFtr3/FfFet3, but no respective homologs seem to be present in the F. graminearum genome (Fig. 2B). To test whether the respective genes are expressed in an iron-dependent manner, we grew the wild type and the Ffppt1 mutant under iron deficient conditions before addition of ferric chloride (FeCl3) or water. Northern analyses revealed that all of the genes coding for putative F. fujikuroi ferroxidases and iron perases are induced by iron starvation in the wild type and the Ffppt1 mutant (Fig. 2C and D).

Results

Identification and characterization of the Sfp-type PPTase gene Ffppt1 reveals involvement in lysine biosynthesis

In order to identify the NpgA/CfwA-encoding homolog in the F. fujikuroi genome, a BlastP analysis was performed using the A. fumigatus PptA sequence. One protein sequence with 32% identity to PptA (e-value = 2.6 x 10^-20) was found and designated Ffppt1 (GenBank accession number HE614113). RT-PCR revealed an open reading frame of 876 bp spanning one intron of 50 bp.
Furthermore, the expression of the \( F. fujikuroi \) gene \( Ff \text{nps6} \) coding for the homolog of the \( F. graminearum \) NRPS Nps6 responsible for extracellular siderophore production [50] revealed an identical expression pattern (Fig. 2C and D). Expression signals of the SreA-encoding homologous gene \( Ff \text{sre1} \) in the wild type and the \( Ff \text{ppt1} \) mutant were found under iron excess only (Fig. 2C).

Since in \( F. graminearum \) the proteins Ftr1/Fet1 were shown to constitute a \textit{bona fide} plasma membrane iron uptake system [51,52] and Ftr2/Fet2 were suggested to function as a vacuolar iron transport system due to their localization [51], we wanted to investigate the role of newly identified \( Ff \text{Ftr3/Fet3} \) in the wild type and the \( Ff \text{ppt1} \) mutant were found under iron excess only (Fig. 2C).

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Diagnostic PCR revealed three of eleven transformants to have lost the ORFs of both genes, respectively (Fig. S2B). These transformants were designated \( \text{DD} Ff \text{ftr3/fet3} \) (T3, T6 and T7) and \( \text{DDD} Ff \text{ppt1/ftr3/fet3} \) (T1, T2 and T3) of which \( \text{DD} Ff \text{ftr3/fet3} \) T3 and \( \text{DDD} Ff \text{ppt1/ftr3/fet3} \) T1 were arbitrarily chosen for further investigation. To learn more about the regulation of the genes putatively involved in iron acquisition in \( F. fujikuroi \) we also deleted the gene \( Ff \text{sre1} \), encoding the homolog of the the GATA-type transcription factor Sre1 from \( A. fumigatus \). This transcription factor was shown to be involved in regulation of iron homeostasis [44]. Of the eleven transformants obtained, seven were proven to have lost the ORF by diagnostic PCR (Fig. S2B) and designated \( \text{DD} Ff \text{sre1} \) (T1, T2, T3, T4, T6, T10, T11) of which T1 was arbitrarily chosen for further experiments.

Figure 2. Influence of \( Ff \text{ppt1}, Ff \text{sre1} \) and \( Ff \text{Ftr3/Fet3} \) on lysine biosynthesis, iron homeostasis and oxidative stress. A: Growth ability of the indicated strains on solidified Czapek Dox (CD) medium supplemented as indicated. Representative pictures were taken after 3 days of incubation at 28°C in the dark. B: Phylograms of ferroxidases and iron permeases from \( F. fujikuroi \) (Ff), characterized proteins from \( F. graminearum \) (Fg) and \( A. fumigatus \) (Af), as well as homologous sequences from \( F. oxysporum \) (FOXG) and \( F. verticillioides \) (FVEG) obtained from the Broad Institute database were created as described in Methods. Scale bars represent character changes. C and D: Northern blot analysis using indicated genes as probes and rRNA visualization as loading control. The indicated strains were grown as described in Methods. (–): addition of water, (+): addition of \( \text{FeCl}_3 \) to a final concentration of 1 mM.

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As expected, the Ffpt1/ftr3/fet3 triple mutant was unable to grow without the addition of lysine and showed restricted growth in the presence of H₂O₂ (Fig. 2A). Interestingly, the Ffjfr3/fet3 double and the Ffpt1/ftr3/fet3 triple mutant exhibited a slightly less restricted growth in the presence of H₂O₂ compared to the wild type and the Ffjfr3 single mutant, respectively (Fig. 2A). When additional 2 μM of the siderophore FEC was present, the growth defect of the Ffpt1 single mutant could partially be overcome whereas the Ffjfr3/fet3 double and the Ffpt1/ftr3/fet3 triple mutant were restored to wild-type-like growth (Fig. 2A). In contrast, in the presence of BPS the Ffpt1/ftr3/fet3 triple mutant showed a more severe growth defect compared to the Ffpt1 single mutant (Fig. 2A). The overall picture of growth ability of the mutants on the different media indicates that Ffpt1, FfFtr3 and FfFet3 participate in iron acquisition and mediate H₂O₂ tolerance in F. fujikuroi. The FfSre1 deletion mutant showed restricted growth compared to the wild type on all media tested (Fig. 2A) and no growth when 1 mM FeCl₃ was present (Fig. S3). To learn more about the role FfSre1 plays in regulation of genes involved in iron metabolism and to investigate whether transcriptional deregu- lation can be observed when the genes encoding FfFtr3/FfFet3 are missing, we performed northern blot analyses of the wild type and the ΔΔFfpt1/ftr3/fet3, ΔFfpt1, Δftr3, and Δfet3 strains. Similarly to the observations from the previous iron-shift experiment, signals of Ffppt1, FfFtr3, FfFet1, FfFet2 and FfFet2 were only visible under nitrogen starvation conditions in the Ffpt1/ ftr3/fet3 mutants and the parental strains (Fig. 2D). The fact that signals for Ffppt1, FfFtr3, FfFet1, FfFet2, FfFet3 and FfFet2 were detectable in the FfSre1 mutant even when 1 mM FeCl₃ was present (Fig. 2D) indicates that FfSre1 acts as a repressor of genes involved in iron metabolism in F. fujikuroi.

Ffpt1 is involved in conidiosgenesis and sexual development

A. nidulans ngsA/jfsA mutants showed delayed and reduced spore formation with an altered morphology at a range of 32°C to 37°C, but could be restored when contiguously grown to the wild type [34]. Since deletion mutants of the respective homologs in several filamentous fungi were also affected in conidiosgenesis or conidia morphology, we investigated the effect of the Ffpt1 deletion in F. fujikuroi. As to our knowledge none of the previous studies on PPTase mutants in any filamentous fungus compared sporulation ability to that of an α-aminoacidate reductase mutant, we included the FfFtr1 mutant in our analysis. Similarly to the observations in other fungi, sporulation was severely reduced, but morphologically unaltered, in the Ffpt1 mutant compared to the wild type, the ΔFfpt1C and ΔFfFtr1 strains (Fig. 3A and B). However, when the Ffpt1 mutant was grown contiguously to the wild type separated by water-permeable cellophane membrane sporulation was partially restored (Fig. 3A). Interestingly, this partial restoration was not observed when the individually cultivated Ffpt1 mutant was supplemented with FEC indicating that iron limitation is not responsible for the sporulation defect (Fig. 3A).

To study the influence of Ffpt1 on sexual development, a gene replacement mutant of the Ffpt1 locus was generated in the F. fujikuroi strain C-1995 that carries the MAT1-2 idiomorph using the same targeted deletion strategy. Similarly to the Ffpt1 mutant in the wild-type strain IMI58289, the deletion mutant in C-1995 was lysine auxotrophic (Fig. S4) and exhibited drastically reduced sporulation ability (Fig. 3A). When performing sexual crosses, the wild-type strain IMI58289 and all mutants strains generated in this background (ΔFfpt1, ΔFfpt1C and ΔFfFtr1) carrying the MAT1-1 idiomorph were able to interact with the wild-type strain C-1995 resulting in the formation of dark purple perithecia (Fig. 3C and S5). However, when the Ffpt1 locus was missing in strain C-1995 carrying the MAT1-2 idiomorph, no recombination took place when contiguously grown with any of the strains of the opposite mating type (wild-type IMI58289, ΔFfpt1C and ΔFfFtr1A). Subsequently, colonies from both mating partners did not come in close contact resulting in a lack of perithecia (Fig. 3C and S5). Supplementation with FEC did not restore formation of sexual structures (Fig. S5).

Deletion of Ffpt1 results in loss of PKS- and PKS/NRPS-derived products and in transcriptional down-regulation of distinct secondary metabolite cluster genes

Since deletion of Sfp-type PPTase-encoding genes in several fungal organisms resulted in absence of PKS-, NRPS- and PKS/ NRPS-derived metabolites, we assessed production of F. fujikuroi metabolites in the Ffpt1 mutant. The wild type and the Ffpt1 mutant were cultivated under bikaverin-, fusarubin-, and fusarin-stimulating conditions, respectively. The wild type exhibited the typical coloration for each of the three metabolites in the respective induction medium, whereas the Ffpt1 mutant appeared colorless in all three experiments (Fig. 4). Analysis of the culture filtrates using high-performance liquid chromatography (HPLC) coupled to a diode array detector (DAD) for bikaverin and fusarubins and to an ultraviolet light (UV) detector for fusarin detection, respectively, confirmed the absence of bikaverins, fusarubins and fusarins in the Ffpt1 mutant in contrast to the wild type (Fig. 4). Surprisingly, northern blot analyses revealed that genes encoding the PKS key enzymes for bikaverin and fusarubin production, respectively, are negatively affected on transcriptional level when Ffpt1 is deleted (Fig. 4). Furthermore, other bikaverin and fusarubin cluster genes are affected in the same manner in the Ffpt1 mutant in contrast to the wild type, the FfFtr1 and the Ffpt1 add-back strains (Fig. 4 and S6A). As expected, deletion of Ffpt1 does not affect the expression of all PKS- or NRPS-encoding genes tested: expression of jfsA, encoding the PKS/ NRPS hybrid responsible for fusarin production, was not repressed in the Ffpt1 mutant although no fusarins could be detected in the culture filtrate (Fig. 4). Similarly, expression of the NRPS-encoding Ffppt1C and PKS-encoding Ffppt1C genes was not affected in the Ffpt1 mutant compared to the wild type (Fig. 2 and Fig. S6B).

Some F. fujikuroi strains are able to produce the mycotoxin moniliformin, which was assumed to be a PKS-derived metabolite [53]. To test this hypothesis we deleted the jfsA locus in the highly moniliformin-producing wild-type strain MRC2276. Analyses using HPLC coupled to Fourier transformation mass spectrometry (FTMS) showed that the wild-type strain as well as the Ffpt1 deletion mutant was able to form moniliformin in detectable amounts (Fig. S6C), suggesting that no Sfp-type PPTase activity is required for moniliformin production.

Influence of Ffpt1 on the production of sesqui- and diterpenes

To show if the loss of PKS- and NRPS-derived products in the Ffpt1 deletion mutants have an effect on biosynthesis of terpenes, strains IMI58289 (GA high-producing) and C-1995 (GA low-producing) were investigated for their production of diterpenoid GAs, the GA precursor ent-kaurene and the sesquiterpene alcohol α-acorein. The latter was recently identified as the main volatile sesquiterpene produced by F. fujikuroi [8]. Quantification of GA in culture extracts by HPLC-DAD revealed no significant increase of GAs in the IMI58289/ΔFfpt1 mutant compared to IMI58289.
while deletion of Ffppt1 in the strain C-1995 resulted in a dramatic increase of GA production compared to the parental strain C-1995. Accordingly, GC-MS analysis of headspace extracts obtained by use of a closed loop stripping apparatus (CLSA) demonstrated that deletion of Ffppt1 in strain C-1995 led to a significant increase in ent-kaurene and a-acorenol production (Fig. 5B). These findings on the secondary metabolite level coincided with the expression levels for the genes ggs2 and cps/ks encoding the first two enzymes of GA biosynthesis: the signals were dramatically increased in the Ffppt1 mutant of strain C-1995 compared to the wild type, but only marginally altered in the IMI58289 Ffppt1 mutant compared to its parental strain (Fig. 5C). Surprisingly, a significantly decreased production of GAs was obtained in the Ffaar1 deletion mutant. In full agreement with this finding, expression signals for cps/ks and ggs2 were also significantly reduced compared to the Ffpp1 mutant and the wild-type IMI58289 (Fig. 5C).

**FfPpt1 is a pathogenicity factor in hydroponic rice cultures**

To investigate whether the loss of PKS- and NRPS-derived compounds by deleting Ffpp1 affects rice root infections, the wild-type strain and the Ffpp1 mutant were transformed with the vector pHphDsRed conveying constitutive expression of the red fluorescent protein (DsRed). The DsRed-tagged wild type and Ffpp1 mutant were inoculated onto roots of germinated rice seedlings in the presence or absence of lysine and lysine plus BPS, respectively. Fluorescence microscopy showed that the wild type was able to penetrate and infect rice root cells under all conditions tested (Fig. 6). The Ffpp1 mutant established infection patterns in the presence of lysine only. No interaction of the Ffpp1 mutant with...
the rice roots was observed in the absence of lysine or the presence of lysine when BPS was supplemented concurrently. The hyphae show a non-oriented growth on the root surface and do not penetrate (Fig. 6).

Discussion

FfPpt1 is essential for lysine biosynthesis and involved in iron acquisition

In S. cerevisiae it was proven that the Sfp-type PPTase Lys5p is essential for activating the apo α-aminoadipate reductase Lys2p by 4’phosphopantetheinylation and therefore the enzyme is essentially involved in lysine biosynthesis [29]. As expected, targeted deletion of the homologous gene ppt1 in F. fujikuroi resulted in lysine auxotrophic mutants demonstrating that FfPpt1 is essential...
for lysine biosynthesis. The high degree of amino acid conservation of FfPpt1 to Lys5p makes it very likely that FfPpt1 activates the F. fujikuroi ε-aminoadipate reductase Aar1 in the same mode of action as Lys5p activates Lys2p. The involvement of FfPpt1 in lysine biosynthesis is consistent with the observations made in several filamentous ascomycetes [30,33,36–39,41]. Since Ffppt1 mutants were not viable without lysine supplementation it is suggested that FAAar1 cannot be post-translationally modified by the mitochondrial AcpS-type PPTase homolog. 

A. nidulans npgA/cfwA mutants exhibit an iron uptake deficiency additional to a lysine auxotrophy since the production of NRPS-derived intra- and extra-cellular siderophores is abrogated and no additional reductive iron uptake system is present [35]. Similarly, the production of the NRPS-derived siderophores of C. graminicola is also dependent on Sfp-type PPTase activity, but respective PPTase mutants are able to grow without siderophore addition indicating the existence of a reductive iron uptake system under iron sufficient conditions [36]. In fact, absence of reductive iron acquisition systems has not been reported for any other fungal species other than A. nidulans [42]. Accordingly, the FfPpt1 mutants were able to grow without the addition of siderophores in the presence of lysine indicating the existence of such reductive iron uptake system also in F. fujikuroa. Furthermore, the ability of the F. fujikuroa wild type strain to grow in the presence of the iron chelator BPS, which specifically inhibits the reductive iron uptake system, strongly indicates the existence of NRPS-derived siderophores in F. fujikuroa. Since in the closely related species F. graminearum two NRPS-encoding genes were recently shown to be responsible for production of the extracellular siderophore triacetyl fusarine C [30] and the intra-cellular siderophore ferriocin [54], respectively, the homologous genes in F. fujikuroa, Ffnp6 and Ffnp5, are very likely required for siderophore production. However, the nature of the F. fujikuroa siderophores has to be elucidated in future experiments. Nevertheless, the involvement of FfPpt1 in siderophore biosynthesis is evident since respective mutants were severely restricted in growth when grown in the presence of BPS and lysine, resembling the situation in A. nidulans and C. graminicola [35,36]. This growth defect could be restored when FEC, which functions as intra- and extra-cellular siderophore in Schizosaccharomyces pombe [55], was supplemented, suggesting that it can be utilized by F. fujikuroa. Similar observations of FEC utilization were made in A. nidulans [56].

Ftr3/Fet3 are involved in iron metabolism of F. fujikuroa

Since Ffppt1 mutants were able to grow without siderophore addition in the presence of lysine but not when additional BPS was present, the existence of reductive iron uptake systems was suggested in F. fujikuroa. In F. graminearum two reductive iron uptake systems were identified, each consisting of a ferroxidase and an iron permease [51,52]. Blast searches performed in the recently obtained F. fujikuroa genome database revealed the existence of three gene pairs each encoding an iron permease and a ferroxidase that share one promoter region. Phylogenetic analysis shows that two of them, FFtr1/FFe11 and FFtr2/FFet2, are closely related to the characterized proteins from F. graminearum, and that the newly identified proteins FFtr3 and FFet3 group together with FtrA and FetC from A. fumigatus, respectively. Similarly to the fta deletion mutant of A. fumigatus [43], F. fujikuroa FFtr3/FFet3 deletion mutants were not restricted in growth compared to the wild type, most likely due to the production of siderophores. However, when FFtr3/FFet3 were deleted in a Ffppt1 mutant background the growth defect in the presence of BPS was more dramatic, indicating that FFtr3/FFet3 are involved in iron acquisition but can be complemented by another iron uptake system. Generation of triple mutants of Ffppt1 and FFtr3/FFet3 together with either FFtr1/FFet1 or FFtr2/FFet2 could resolve the question if the identified putative reductive iron uptake systems have redundant functions. Furthermore, it would be interesting to investigate if FFtr3/FFet3, which are not present in F. graminearum, could restore iron transport in Fftr1/Ffet1 mutants.

FfSre1 controls iron metabolism

Similarly to the expression of fta and ftc observed in A. fumigatus [44] and genes involved in reductive iron uptake in H. capsulatum [49], all of the six genes putatively involved in reductive iron uptake in F. fujikuroa were shown to be expressed under iron deficient conditions and repressed upon iron addition, strongly suggesting their role in iron metabolism. Furthermore, expression of Ffnp6 encoding the homolog of the F. graminearum NRPS, responsible for extracellular siderophore production [50], revealed the same expression pattern thereby indicating a role of Ffnp6 in iron homeostasis. The homolog in A. fumigatus, sidD, was also shown to be repressed by the addition of iron [44] underlying our hypothesis. In A. fumigatus and H. capsulatum the GATA-type transcription factor-encoding genes sidE and sidG, respectively, are expressed under iron sufficiency and act as repressors of genes involved in iron acquisition [44,49]. The F. fujikuroa homologous gene FfSre1 is also expressed under iron sufficient conditions. Deletion resulted in deregulation of all six genes putatively involved in reductive iron uptake as well as Ffnp6 when iron was supplemented, indicating that FfSre1 executes comparable repressing functions as SreA/Sre1 in A. nidulans, A. fumigatus and H. capsulatum, respectively [44,49,57,58]. Interestingly, FfSre1 deletion mutants were not viable in the presence of constant iron excess, which is likely due to a lack of repression, leading to increased iron
flux into the cells. The increased intracellular iron pool might function in Fenton/Harber Weiss chemistry generating oxidative stress to a toxic level. However, under physiological iron concentrations FfSre1 mutants exhibited a decreased sensitivity to H$_2$O$_2$ compared to the wild type, which might either be due to an increased intracellular siderophore concentration possibly scavenging free iron from Fenton/Harber Weiss chemistry and/or by specifically providing more intracellular iron as cofactor for the iron-dependent catalase known to detoxify H$_2$O$_2$. Supporting this hypothesis, addition of FEC to Ffpp1 mutants increases H$_2$O$_2$ resistance. This hypothesis is consistent with findings in A. nidulans and A. fumigatus where loss of intracellular siderophores increases the liable intracellular iron pool leading to a decreased oxidative stress resistance [59,60]. Future studies focusing on the involvement of FfSre1 in iron metabolism and oxidative stress responses in Fusarium species will help to elucidate this complex context. Here, investigating the role of the homolog of the bZIP transcription factor HapX will be of special interest. In A. fumigatus HapX was shown to be a transcriptional repressor of genes involved in iron consuming pathways and an activator of genes involved in iron acquisition under iron deficiency. The HapX encoding gene itself is transcriptionally repressed by SreA under iron replete conditions [61].

**Ppt1 controls secondary metabolism in F. fujikuroi**

The findings that putatively NRPS-derived siderophore biosynthesis is affected in Ffpp1 mutants led us to investigate a broader PKS- and PKS/NRPS-derived secondary metabolite spectrum of F. fujikuroi. Similarly to the findings in several filamentous ascomycetes [33–39], F. fujikuroi pp1 mutants were not able to produce any PKS-derived products such as bikaverins, fusarubins or PKS/NRPS-derived fusarins. This is in agreement with the fact that Sfp-type PPTases are essential for 4'-phosphopantetheinylation of ACPs and PCPs in PKSs and NRPSs, respectively [25,26]. However, production of moniliformin, that was suggested to be induced by a diffusible signal that involves the activity of the glutamine synthetase-like enzyme FluG [64]. The agp1/lac and fluG mutants showed a similar restoration of sporulation in contiguous growth experiments [34,64]. Recently it was shown that the sporulation defect of A. nidulans fluG mutants could be rescued by the concomitant addition of specific TC- and PKS-derived products [65]. Further evidence for the existence of diffusible sporulation signals comes from Ascoscyta psii, where the metabolite P310/mycosporine was shown to induce sporulation [66,67]. Mycosporines are produced by many fungal and marine organisms [68]. In cyanobacteria it was proven that this group of metabolites involves a NRPS during biosynthesis [69]. From the data obtained in this study it is intriguing to speculate that sporulation in F. fujikuroi also involves either a mycosporine-like NRPS product similar to A. psii and/or a distinct mixture of secondary metabolites as reported in A. nidulans.

Apart from the defect in conidiation of F. fujikuroi pp1 mutants, we also observed a defect in formation of sexual structures when Ffpp1 was deleted in the MAT1-1 mating partner and crossed with a wild-type MAT1-1 strain, whereas the vice versa situation led to formation of dark purple perithecia. In several ascomycetes, two NRPS-independent peptide pheromone/receptor systems that underlie transcriptional control of the MAT1-1 and MAT1-2 idiomorphs are a prerequisite for mating recognition in heterothallic species [70]. Although the MAT1-1 and MAT1-2 idiomorphs as well as the genes encoding the pheromone/receptor systems have recently been identified in heterothallic Fusarium species including F. fujikuroi [71] an involvement in mating recognition awaits experimental proof. Interestingly, in the homothallic species F. graminearum, which contains both MAT idiomorphs, deletion of one idiomorph prevents self-fertilization but mutants maintain the ability to outcross [72]. However, recent studies in F. graminearum revealed that the genes coding for the pheromone/receptor systems are not essential for self-fertilization and outcrossing [73,74]. Our results suggest that FfPpt1 is involved in sexual recognition, disclosing the possibility that the MAT1-2 idiomorph specifically controls a PKS- and/or NRPS-derived metabolite or its receptor. And although differential cDNA screening and microarray analyses of MAT1-2 deletion mutants of

FfPpt1 is involved in asexual and sexual development most likely by inactivating PKS and/or NRPS pathways

We noted that Ffpp1 mutants revealed a significant reduction of conidiation that was independent from FEC supplementation, but could be restored when the mutant was grown contiguously to the wild-type strain. These data suggest that possible PKS- and/or NRPS-derived metabolites can function as diffusible conidiation signals in F. fujikuroi. In A. nidulans, sporulation is also suggested to be induced by a diffusible signal that involves the activity of the glutamine synthetase-like enzyme FluG.
F. verticillioides and F. graminearum did not reveal any apparent Sfp-type PPTase target or putative receptor-encoding gene to be transcriptionally controlled by the MAT1-2 idiomorph [75,76], our hypothesis should thoroughly be investigated in F. fujikuroi.

FFppt1 is a pathogenicity factor during rice root infection of F. fujikuroi

The data obtained from pathogenicity assays of fluorescently labeled F. fujikuroi ppt1 mutants in hydroponic rice cultures indicate that lysine production and iron uptake are essential for the establishment of infection of the rice roots. However, when lysine was supplemented, wild-type-like infection structures of the ppt1 mutant could be observed, indicating that the reductive iron uptake systems of F. fujikuroi are sufficient for iron acquisition during infection. An essential role for reductive iron uptake was reported in the smut fungus U. maydis during maize infection [77], whereas ftr4 mutants of A. fumigatus were not affected in virulence in a murine model of invasive aspergillosis [43]. Interestingly, in F. graminearum, which lacks the Fet3/Pt3 homologous system, siderophore-assisted iron uptake is essential for full pathogenicity on wheat [52]. Our data further suggest that no PKS- and NRPS-derived metabolites of F. fujikuroi are essential for primary invasion of rice roots. This stands in contrast to the observations reported for Sfp-type PPTase mutants of the hemibiotrophic plant pathogens C. gramincola and M. oryzae that were unable to cause primary infections [36], and C. sativus that showed strongly reduced primary infections on unwounded host plant leaves, respectively [41]. However, T. virens Sfp-type PPTase mutants were able to colonize Solanum lycopersicum roots in a wild-type-like manner when lysine was supplemented [39]. The theory that plant pathogenic fungi and bacteria need PKS- and NRPS-derived secondary metabolites for establishment of full virulence is reported in several species [78-83]. Whether this is also true for F. fujikuroi needs to be investigated in a more sophisticated pathogenicity assay in the future. It is possible that the species-specific production of GAs in F. fujikuroi is the main determinant of host specificity and is essential for primary infection of rice roots. The fact that F. fujikuroi strains lacking the global regulator velvet are defective in both GA production and virulence support our suggestion on the role of TC-derived GAs for the infection of rice roots [84]. Examples for the essential role of other TC-derived secondary metabolites during infection come from the gray mold fungus B. cinerea [85] and the more closely related species F. graminearum [86]. Analyses of the roles different F. fujikuroi secondary metabolites play during primary infection of rice will be a major task in the future.

Summarizing, our studies describe the first investigations centering on a Sfp-type PPTase in the genus Fusarium. We show unequivocally that FFppt1 is essentially involved in primary metabolism of lysine biosynthesis and in PKS-, PKS/NRPS- and NRPS-derived secondary metabolites such as bikaverins, fusarubins, fusarins, and most likely siderophores. Surprisingly, moniliformin production was not affected indicating that the biosynthetic pathway does not involve a Sfp-type PPTase-dependent PKS. Furthermore we provide strong evidence that deletion of FFppt1 causes re-channelling of carbon flux into the terpene metabolism which results in increased GA and -acorenol production. Therefore, FFppt1 mutants provide a reasonable strain improvement strategy for terpene-derived secondary metabolic production. Pathogenicity assays using hydroponic rice cultures revealed that lysine biosynthesis and iron acquisition, but not PKS and NRPS secondary metabolism is essential for establishing primary infections of F. fujikuroi on rice roots. Additionally, the results obtained disclose the possibilities that PKS- and/or NRPS-derived metabolites might function as diffusible conidiation signals and also might play a crucial role in mating recognition in dependency on a specific MAT1 idiomorph in heterothallic Fusarium species.

Furthermore, our studies revealed the existence of a third previously unidentified putative reductive iron uptake system consisting of FFTn1 and FFTn2 that is closely related to the only reductive system, FtaA/FtaC, in A. fumigatus. Functional characterization using targeted deletion of both genes, FFTn1/FFTn2, provides strong evidence that they are involved in iron acquisition and under transcriptional repression of the GATA-type transcription factor FfSre1 under iron-replete conditions. Deletion of the first Fusarium homolog of this GATA-type transcription factor strongly indicates its involvement in regulation of iron homeostasis and oxidative stress resistance, providing evidence for conserved regulation mechanisms between Fusarium and Aspergillus species in this respect.

Methods

Fungal strains and culture conditions

The wild-type strains F. fujikuroi IMI58289 (Commonwealth Mycological Institute, Kew, UK), F. fujikuroi C-1995 (kindly provided by J.F. Leslie, Kansas State University), and the moniliformin-producing strain F. fujikuroi MRC2276 (kindly provided by W. F. O. Marasas, Research Institute for Nutritional Diseases, South Africa) were used for ppt1 knock-out experiments. For all cultures, F. fujikuroi was preincubated at 28°C for 48 h in 500 ml Erlenmeyer flasks with 100 ml Darken medium (DVK) [87] on a rotary shaker at 180 rpm. For RNA isolation and secondary metabolite analyses, 0.5 ml DVK were used to inoculate synthetic ICI (Imperial Chemical Industries Ltd., UK) media [88] containing either 6 mM glutamine (GAs and bikaverins), 60 mM glutamine (fusarins) or 6 mM NaNO3 (fusarubins). For cultivation including FFppt1 mutants, lysine was added to all media to give a final concentration of 1 mM. The cultures were incubated at 28°C on a rotary shaker at 190 rpm for 3, 5, 7 or 10 days. For iron shift experiments FeCl3 was added to a final concentration of 1 mM and incubated for 2 h. For moniliformin analyses, the strains were grown at 28°C for 14 days on cracked corn as previously described [89]. Headspace analyses were performed with agar plate cultures grown on complete medium (CM) [90] amended with 1 mM lysine after three days of incubation. For protoplasting, 0.5 ml of the starter culture was transferred into Erlenmeyer flasks with 100 ml ICI medium containing 6 mM (NH4)2SO4 and 10 g/L fructose instead of sucrose and incubated at 28°C on a rotary shaker at 190 rpm for 18 h. For DNA extraction, fungal strains were grown for 3 days at 28°C on cellophane sheets (Alba Gewürze, Bielefeld, Germany) placed on solidified CM. For sporation assays solidified 20% (v/v) vegetable juice (V8) (Campbell Foods, Puurs, Belgium) containing 30 mM CaCO3 and 10 mM lysine was used. Additionally solidified V8 contained 2 lM ferrichrome (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) as indicated. After 10 days incubation under constant light spores were washed of the plates, filtered and counted using a hemocytometer. For growth tests 5% (w/v) Czapek Dox (CD) medium (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) was used and complemented with 10 mM lysine, 100 μM BPS, 2 μM FEC as indicated. For additional growth test, solidified CM without iron was used containing 10 mM lysine and 1 mM FeCl3 when indicated. Sexual crossings were performed on carrot agar containing 10 mM lysine plus 2 μM FEC when indicated as described by Klüttich and Leslie [91]. Microscopy of perithecia was performed using a SteREO Dis-
covery.V20™ microscope equipped with an AxioCam MRc (Carl Zeiss MicroImaging GmbH, Jena, Germany). Spores were visualized using an Axio Imager.M2 (Carl Zeiss MicroImaging GmbH, Jena, Germany). Differential interference contrast (DIC) was used for bright field images captured with uniform exposure time using an AxioCam MRM. Images were processed uniformly using AxioVision Rel. 4.8 (Carl Zeiss MicroImagingGmbH, Jena, Germany).

Standard molecular methods

DNA and RNA analysis used standard techniques [92]. Fungal DNA or RNA was prepared by first grinding lyophilized mycelium into a fine powder with a mortar and pestle and then dispersing it in extraction buffer as described by Cenis [93]. DNA for Southern hybridization experiments was prepared following the protocol of Doyle and Doyle [94]. For Southern blot analysis, genomic DNA was digested with the indicated restriction enzymes (Fermentas GmbH, St. Leon-Rot, Germany), fractionated in 1% (w/v) agarose gels, and transferred to Nytran® nylon transfer membranes (Whatman Inc., Sanford, ME, USA) by downward blotting [95]. 32P-labelled probes were prepared using the random oligomer-primer method and membranes were hybridized according to the protocol of Sambrook et al. [92].

Total F. fujikuroi RNA was isolated using the RNAgentstotal RNA isolation kit (Promega GmbH, Mannheim, Germany). Samples of 20 μg of total RNA were transferred to Hybond-N+ membranes after electrophoresis on a 1% (w/v) agarose gel containing 1% (v/v) formaldehyde, according to Sambrook et al. [92]. Northern blot hybridizations were accomplished by the method of Church and Gilbert [96]. CDNA was synthesized from 1 μg of total RNA and the SuperScript II reverse transcriptase (Invitrogen, Groningen, The Netherlands) according to the manufacturer’s instructions.

All primers used for PCR were obtained from Eurofins GmbH (Ebersberg, Germany) (Table S1). PCR reactions contained 25 ng DNA, 5 pmol of each primer, 200 nM dNTPs, and 1 unit of BioTherm™DNA polymerase (GENECRAFT GmbH, Ludingshausen, Germany) and were initiated with a 4 min soak at 94°C followed by 36 cycles of 1 min at 94°C, 1 min at 56 to 65°C, 1–3 min at 70°C, and a final soak for 10 min at 70°C. PCR products were cloned into pCR®2.1-TOPO® vector using the TOPO TA Cloning® kit (Invitrogen, Groningen, The Netherlands) and transformed into Escherichia coli (Invitrogen) Plasmid DNA from E. coli was extracted using the GenJetTM Plasmid MiniPrep Kit (Fermentas GmbH, St. Leon-Rot, Germany) and sequenced using the BigDye® Terminator v3.1 Cycle Sequencing Kit and the ABI Prism® 3730 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) according to the manufacturer’s instructions. DNA and protein sequence alignments were done with DNA STAR (Madison, WI, USA). Sequence homology searches were performed using the NCBI database server. Protein homology was based on BlastX searches [97]. Phylogenetic analysis was performed using the web-based tool at www.phylogeny.fr [98]. The nucleotide and protein sequences were deposited in GenBank under accession number HE614115 (ppt1), HE614114 (aar1), HE614115 (feit), HE614116 (fb1), HE614117 (fb2), HE614118 (feit2), HE614119 (feit3), HE614120 (feit3), HE614121 (nps2), HE614122 (nps6) and HE614123 (see1), respectively.

Plasmid construction

The putative F. fujikuroi ppt1 gene and flanking regions were amplified using the primer pairs ppt1-F and ppt1-R which were based on the putative F. verticillioides ppt1 (FVEG_01894.3) sequence. The F. fujikuroi ppt1 knock-out plasmid pAAPpt1 was created by sequentially cloning the 550 bp 5’ Fppt1 flank (generated with primers ppt1-5F/ppt1-3R) and the 770 bp 3’ Fppt1 flank (generated with primers ppt1-3F/ppt1-3R) into pNR1 using SacI/XbaI and HindIII/Sall restriction sites, respectively [99], such that the nourseothricin resistance cassette was flanked by F. fujikuroi genomic sequence. For generating a complementation construct, a 1.8 kb fragment including 3’ and 3’ non-coding regions was amplified with primers ppt1-Prm-F/ppt1-Term-R. The plasmids pAAar1, pAftr3/fet3 and pAsre1 were assembled using yeast recombinational cloning as essentially described for Neurospora crassa deletion vectors [100] and recently established for F. fujikuroi vectors [84]. The 5’ and 3’ flanks of Ff532/fet3 and Ffser1 were amplified using primer pairs “gene”-5’-F1-R1 and “gene”-5’-F1/-R1, respectively. Plasmid DNA from S. cerevisiae was extracted using the GenJet™ Plasmid MiniPrep Kit (Fermentas GmbH, St. Leon-Rot, Germany) with slight modifications: cells were resuspended in 300 μL Resuspension Solution plus 100 μL glass beads, lysed by addition of 600 μL Lysis Solution and neutralized with 450 μL Neutralization Solution. DNA fragments used for deletion of Ff533/fet3 and Ffser1 were prepared by PCR using primers “gene”-5’-F1 and “gene”-5’-R1 and 1 μL of pAftr3/fet3 or pAsre1, respectively, as template. The plasmid pHPD5-Red was constructed by ligating the HindIII/XbaI fragment of pChap-GFP [101] containing the hygromycin resistance cassette into HindIII/XbaI restricted pPgpD5-Red [102].

Fungal transformations

Preparation of protoplasts from F. fujikuroi mycelium was carried out as described [103]. Briefly, 107 protoplasts of F. fujikuroi strains were transformed with 10 μg of the replacement cassette of the vector pAAPpt1 or PCR products obtained of pAFtr3/fet3 and pAsre1, respectively, as described above. Transformed protoplasts were regenerated for 6–7 days at 28°C in a complete regeneration agar (0.7 m sucrose, 0.05% yeast extract) containing 100 μg/ml nourseothricin and 1 μM lysine in case of targeted Fppt1 deletion (Werner-Bioagents, Jena, Germany) or 100 μg/ml hygromycin and 1 μM lysine (Calbiochem, Darmstadt, Germany) in case of targeted Faar1, Ffser3/fet3 and sre1 deletion. For complementation of AFppt1 strains, 10 μg of the genomic Fppt1 PCR fragment was used for transformation as described above, but without addition of lysine in the regeneration media. Protoplasts of the IM58289 wild-type strain and the Fppt1 mutant were transformed with 20 μg pHPD5-Red and transformed protoplasts were regenerated in complete regeneration agar containing 100 μg/ml hygromycin and 1 μM lysine.

The homologous integration events in transformants targeting replacement of Fppt1 with the nourseothricin resistance marker were verified by PCR using primers ppt1- F and ppt1- R targeting outside the replacement fragment in combination with pLOF-OH1 and Tub-T, respectively. In case of hygromycin resistant transformants, targeted replacement was verified using a primer outside the replacement fragment in combination with a primer targeting the hygromycin resistance cassette (“gene”-F1/-R1).

Virulence assays

Infection assays of single plants of Oryza sativa spp. japonica c.v. Nipponbare were performed as described previously [84]. Gamborg B5 Medium (Duchefa Biochemie, Haarlem, The
Netherlands) solution was supplemented with 10 mM lysine and 10 mM BPS when indicated. Microscopy was performed using an Axio Imager.M2 (Carl Zeiss Microlmaging GmbH, Jena, Germany). DIC was used for bright field images and DsRed fluorescence was detected using filtered set 30 (excitation band pass 470/40 nm, color splitter 495, emission band pass 525/50 nm). Images were captured with uniform exposure time using an AxioCam MRm and were processed uniformly using AxioVision Rel. 4.8 (both Carl Zeiss Microlmaging GmbH, Jena, Germany).

Chemical analysis

Gibberellic acids GA₃ and GA₄/₇ were extracted from 20 mL culture filtrate after 7 days of incubation in ICI medium containing 6 mM glutamine. Extraction was performed using Sep Pak C18 cartridges (Waters GmbH, Eschborn, Germany) from which GA₃ was eluted with 2 mL 20% acetonitril (ACN) [LGC/ Promocem GmbH, Wesel, Germany] and GA₄/₇ were eluted with 2 mL 55% ACN. GA₃ and GA₄/₇ amounts were measured by HPLC-DAD analysis using a Merck-Hitachi System (Merck KGaA, Darmstadt, Germany) consisting of a gradient pump (L-7100), an autosampler (L-7200) and a Diode Array Detector (L-245). As column a Lichrospher 100 RP-18 column (5 μm; 250 mm×4 mm; Merck KGaA) was applied. HPLC conditions were as follows: solvent A: 0.05% H₃PO₄ (Merck KGaA), pH 3; solvent B: ACN. The subsequent gradient was applied: 15 min 15% B; in 20 min to 40% B; in 2 min to 15% B. Data analysis was carried out using EZChrom Elite Version 3.3.2 SP1 (Scientific Software, Inc.). Quantification of GAs was performed by generating a calibration line using different dilutions of 1 μg/mg GA₃ and GA₄/₇ standards (DKSH GmbH Hamburg, Germany). GA amounts were calculated per 1 L culture filtrate and 1 g dry weight applying peak areas of the different samples and the gradient of the calibration line.

Fusarin, bikaverin and fusarubin production was analyzed as previously described [9,104]. For moniliformin analyses fungal cultures were extracted as previously described [105] for 1 h on a rotary shaker at 190 rpm at 28°C. For analyses 1 mL of extract was evaporated under a stream of nitrogen at 40°C, dissolved in 150 μl 5% Methanol (v/v) and analyzed by HPLC-DAD using chromatographic conditions as previously described [106].

The volatiles released by agar plate cultures were collected and analyzed as previously described [8]. Briefly, the volatiles emitted by the fungal cultures on solidified CM were collected by use of a closed loop stripping apparatus (CLSA). Therefore, a circulating air flow was directed through a charcoal filter (Chromtech GmbH, Idstein, Precision Charcoal Filter, 5 mg) in a closed apparatus containing the fungal culture for 24 h. The charcoal filter was extracted with 30 μL of analytically pure dichloromethane and the obtained solutions were immediately analyzed by GC-MS and stored at -80°C. GC-MS analyses were carried out on a HP6890 GC system connected to a HP5973 Mass Selective Detector fitted with a HP-5 fused silica capillary column (25 m×0.22 mm, 0.25 μm film, SGE Inc.). Conditions were as follows: inlet pressure: 77.1 kPa; He 23.3 μl min⁻¹; injection volume: 1 μl; injector: 250°C; transfer line: 300°C; electron energy: 70 eV. The GC was programmed as follows: 50°C (5 min isothermic), increasing at 10°C min⁻¹ to 320°C, and operated in splitless mode (60 s valve time); carrier gas (He): 1.0 ml min⁻¹. Quantification was carried out by peak integration with the MSD Chem Station software (Agilent) of three replicate samples and is given as arithmetic means ± standard deviations, normalized to 100% for the production of the C-1995 wild-type strain.

Supporting Information

Figure S1  Deletion strategy of Ffppt1 and Southern blot analysis. A: Gene replacement of Ffppt1. Physical maps of the SacI/Apal gene replacement fragment from the plasmid pAppt1, the Ffppt1 locus from the wild-type strain IMI58289 and the gene locus from a Ffppt1 knock-out mutant showing the nourseothricin resistance cassette (grey). Small arrows indicate positions of primers used for cloning the replacement vector and for the PCR analysis of replacement mutants. Dotted lines and Roman numerals represent primer combinations used for the diagnostic PCR shown in B. B: Diagnostic PCR results of the analyzed Ffppt1 replacement transformants and the wild type (WT) as well as the complemented strains ΔFfppt1. Roman numerals represent primer combinations as schematically drawn in A. M: marker in kb, C: For the Southern blot analysis the genomic DNA of the wild type and ΔFfppt1 strains was digested with EcoRI, blotted and hybridized with the HindIII/SalI flank of the replacement vector pAppt1 as probe (heavy line with asterisks). In three mutants the wild-type fragment with a size of 18.5 kb is replaced by a 4.8 kb fragment, resulting from an additional EcoRI restriction site in the nourseothricin resistance cassette. M: marker in kb. (TIF)

Figure S2 Diagnostic PCR results of gene replacement transformants. A: Diagnostic PCR results of the analyzed Fas1 replacement transformants and the wild type (WT). Roman numerals represent primer combinations as schematically drawn. M: marker in kb, C: Diagnostic PCR results of the analyzed Ffpr5/ pr6 replacement transformants in wild-type and ΔFfppt1 background, respectively. Roman numerals represent primer combinations as schematically drawn. M: marker in kb. C: Diagnostic PCR results of the analyzed Fis1 replacement transformants and the wild type (WT). Roman numerals represent primer combinations as schematically drawn. M: marker in kb. (TIF)

Figure S3 Influence of FfPpt1, FfSre1 and FfFt3/FfFet3 on growth on extreme iron conditions. Growth of indicated mutants on solidified complete medium (CM) without iron (10 mM Lys) and 1 mM FeCl₃. Representative pictures were taken after 3 days of incubation at 28°C in darkness. (TIF)

Figure S4 Influence of FfPpt1 on growth on lysine-deficient media. Representative photographs of indicated strains on solidified CD media supplemented with or without lysine as indicated. (TIF)

Figure S5 Influence of FfPpt1 on sexual mating recognition. A, C: Representative photographs of sexual crossings of indicated strains as described in Methods. Scale bar represents 1 cm. B: Representative magnifications of sexual crossings seen in A showing produced perethcia. Scale bar represents 1 cm. D: Representative photographs of sexual crossings of indicated strains as described in Methods on media supplemented with FEC. Scale bar represents 1 cm. (TIF)

Figure S6 Influence of FfPpt1 on secondary metabolite gene expression and moniliformin production. A: Northern blot analysis of all six bikaverin cluster genes in the designated strains and rRNA visualization as loading control. B: Northern blot analysis of Ffppt1 in the designated strains and rRNA as loading control. C: Extracted ion chromatogram of moniliformin
detected by HPLC-FTMS as described in Methods. Black: MRC2276; blue: Fjpp1 mutant in MRC2276.

(ThF)

Table S1 Primer used in this study.

(DOCX)

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Author Contributions

Conceived and designed the experiments: PW BT. Performed the experiments: PW SA EMN LS KWvB NLB. Analyzed the data: PW SA EMN LS KWvB NLB HUH JSD BT. Contributed reagents/materials/analysis tools: BT JSD HUH. Wrote the paper: PW.

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