THE POLYKETIDE SYNTHASE GENE PKS4 OF TRICHODERMA REESEI PROVIDES PIGMENTATION AND STRESS RESISTANCE

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Running title: Function of polyketide synthase 4 (pks4) from Trichoderma reesei
Abstract

Species of the fungal genus *Trichoderma* (Hypocreales, Ascomycota) are well known for their production of various secondary metabolites. Non-ribosomal peptides and polyketides represent a major portion of these products. In a recent phylogenomic investigation of *Trichoderma* polyketide synthase encoding gene, *pks4* from *T. reesei*, was shown to be an orthologue of pigment forming PKSs involved in synthesis of aurofusarin and bikaverin in *Fusarium* sp. In this study we show that deletion of this gene in *T. reesei* results in loss of green conidial pigmentation and in pigmentation alteration of teleomorph structures. It also has an impact on conidial wall stability, and the antagonistic abilities of *T. reesei* against other fungi, including formation of inhibitory metabolites. In addition, deletion of *pks4* significantly affects the expression of other PKS-encoding genes of *T. reesei*. To our knowledge this is the first indication that a pigment-forming PKS is involved in defense, mechanical stability and stress resistance in fungi.
Introduction

The economically important genus *Trichoderma* (Hypocreales, Ascomycota, Dikarya) is well known for its mycotrophic lifestyle and for the broad range of biotrophic interactions with plants and animals. Moreover it contains several cosmopolitan species characterized by their outstanding environmental opportunism. These properties have given rise to the use of several species in agriculture as biopesticides and biofertilizers while *T. reesei* is utilized for production of bioenergy-related enzymes (1).

The molecular basis for the opportunistic success of *Trichoderma* is not yet well understood. While there is some evidence for a role of some secreted proteins (2, 3), less is known about a possible role(s) of secondary metabolites. In this respect *Trichoderma* spp. are probably best known for production of peptaibols, which are non-ribosomal peptides with antimicrobial and plant defense-stimulating activities (4). However, the role of polyketide synthases (PKS) in *Trichoderma* ecophysiology is not well understood. *Trichoderma* spp. polyketides are produced by iterative PKSs, multifunctional enzymes consisting of several active sites, capable of catalyzing the fusion of variable numbers of CoA linked acyl monomers, such as acetyl-CoA and malonyl-CoA, into polymers known as polyketides. They can be further grouped into non-reducing (NR) and reducing (RD) PKSs according to their domain organization (5). Recently Baker et al. (6) have taken a phylogenomic approach to study the PKS repertoire in the genomes of *T. reesei*, *T. atroviride* and *T. virens*, which enabled the putative *in silico* prediction of some of their products. In total 11 PKS encoding genes were found in *T. reesei* genome, among which two occur only in *T. reesei* and nine have orthologues in *T. virens* or/and *T. atroviride* PKSs (6). *Pks4* (Trire2:82208, Triat2:79 and Trive2:77826 in *T. reesei*, *T. atroviride* and *T. virens*, respectively), which encodes an enzyme of the...
non-reducing type (clade I), has been shown to have orthologues in other fungi i.e. PKS associated with synthesis of aurofusarin in *Fusarium graminearum* (7-9), bikaverin in *F. fujikuroi* (10, 11) and DHN melanin in *Aspergillus* spp. (12-19). It was therefore hypothesized that PKS4 would likewise be involved in the production of the characteristic green pigment of *Trichoderma* (6).

Pigment forming PKSs are known to have functions beyond providing the color of conidia. For example, DHN-melanin is involved in virulence in *Aspergillus* sp. (15, 16, 20). In this study we used a reverse genetic approach to examine the functions of *pks4* in ecophysiology of *T. reesei*. We will show that PKS4 is indeed responsible for the pigmentation of conidia and the non-melanised structures of fruiting bodies, but its loss of function also impacts stability of conidial wall and the antagonistic abilities of *T. reesei* against other fungi, including formation of inhibitory metabolites.

In addition, we demonstrate that deletion of *pks4* significantly affects the expression of other PKS-encoding genes of *T. reesei*. To our knowledge, this is the first indication that low-molecular pigments can be involved in defense, mechanical stability and stress resistance in fungi.

**Material and methods**

**Deletion of *pks4* gene in *T. reesei***

The *pks4* gene was deleted utilizing a double joint PCR method as described by Yu et al. (21). Briefly, DNA fragments of 5’ and 3’ flanking regions of *pks4* were fused with a hygromycin B (*hyg*) selection marker. Amplification of the 5’ flanking sequence was done using primers F1 and R3 (CAATGGCGAATGTTCTAGC and GGAACAAGTTGAGCCAGAGC, respectively), the 3’ flanking region was amplified with primers F4 and R6 (GCAATACACGGTGAGAACGA and TGCGGAGGATCGAGACTATT, respectively), and the hygromycin B sequence amplified with *hygF*...
and hygR (GCTGGAGCTAGTGGAGGTC A and CGGTCGGCATCTACTCTATT, respectively). In a second PCR, the fragments were assembled into a single linear construct (21). The third PCR amplification of the final construct was performed using nested primers F2 and R5 (AGGTACGCATGGAGACAACA and TACACACGCACTCACGCATA, respectively) leaving F1 and R6 available for downstream knockout verification. Similar to previously published methods (22), a protoplast PEG-mediated transformation and selection scheme was utilized for introduction of the linear transforming DNA construct and subsequent selection on hygromycin. Albino transformants were selected and examined for double crossover-mediated pks4 replacement with the hygromycin cassette by PCR amplification using primer pairs F1/R6 and F2/R5.

Verification of the pks4 absence in the genome of T. reesei Δpks4 was tested by a specific qPCR primers for pks4 (Table 1) with the following amplification protocol; initial denaturation step for 3 min at 95 °C, followed by 40 cycles of denaturation for 15 sec at 95 °C, annealing for 20 sec at 54 °C and extension at 72 °C for 15 sec.

Somatic incompatibility

Somatic compatibility of Δpks4 mutants and the parental strain was tested in confrontation assays on 2.4 % PDA (BD Difco, Germany) at 28 °C for seven days in darkness. All three strains were cultivated together on multiple plates which were then micro- and macroscopically screened for flat zones, barrage zones and anastomoses that indicate somatic incompatibility between the opponents.
Morphological observations

The parental strain QM 6a and both deletion mutants were cultivated in darkness and 12 h cyclic light on 2.4 % PDA (BD Difco, Germany) at 28 °C for seven days. Spore density was measured quantitatively per cm² of developed colony. For this purpose three 6.2 cm² agar fragments were cut from cultures pre-grown at 28 °C for seven days and were rinsed separately in 15 ml of water containing 0.1 % of Tween-80 until visually all conidia were washed out. The OD₅₉₀ of the suspension was measured in a BIOLOG (BIOLOG Inc., Hayward, CA) turbidimeter calibrated to the BIOLOG standard for filamentous fungi. The final concentration value was calculated based on the calibration curve inferred from the serial dilutions of the standard suspension. Furthermore, conidial size was assessed by measuring the length of 40 conidia per each strain under the 400 × magnification using light microscope.

Mycelial growth rate and carbon source utilization

Growth rates and carbon utilization profiles of the strains were analyzed using a Phenotype microarray system with BIOLOG FF microplates for filamentous fungi (BIOLOG Inc., Hayward, CA) as described in Druzhinina et al. (23) and Atanasova et al. (24). Briefly, the strains were cultivated on 2.4 % PDA for five days in darkness and conidial inocula were prepared by rolling a sterile, wetted cotton swab over conidiating areas of the plates. The conidia were then suspended in sterile BIOLOG FF inoculating fluid (0.25 % phytigel, 0.03 % Tween-40), gently mixed, and adjusted to a transmission of 75 % at 590 nm (using a BIOLOG standard turbidimeter; 4 × 10⁷ spores in 10 ml of phytigel). A total of 90 µl of the conidial suspension was dispensed into each of the wells of the BIOLOG FF microplates and incubated at 28 °C in darkness and at 12 h cyclic light. The OD₇₅₀
(detection of mycelial growth) was measured after 18, 24, 42, 48, 66, 72, 90 and 96 hours using a microplate reader. The growth rate of each strain was assessed by the averaged mycelial density measured on all 95 carbon sources after 0, 24, 48 and 72 hours of incubation in darkness. Statistical analyses were performed using the Statistica software package (version 6.1; StatSoft Inc., Tulsa, OK).

Response to illumination

All three strains were inoculated in the BIOLOG FF Phenotype microarray plates containing 95 carbon sources and water as described above, incubated in light (20 cm distance to a Master TLD 15W/840 lamp) or in darkness at 28 °C for five days. Mycelial density was measured at 750 nm. The growth rates and carbon utilization patterns were compared to those incubated in darkness and the data were statistically analyzed.

UV sensitivity

The tolerance to UV irradiation of the pigment-deficient spores was tested at 254 nm using four HNS 15W OFR UV lamps. The conidia collected after five days of growth on PDA plates were filtered through sterile glass wool to remove hyphal fragments. The volume of the spore suspension that contained $4 \times 10^7$ (75 % turbidity) was determined using a BIOLOG turbidimeter at 590 nm. The suspensions were then diluted several times to obtain ca. 20 spores per 100 µl which were then plated on four PDA plates per sample (QM 6a and both Δpks4 mutants) and four for each sample control. Open plates were then exposed to the UV illumination for five, seven and ten minutes, whereas the control was protected from UV light. Finally, the plates were incubated for 48 h at 28 °C and the single spore colonies observed under 10-times magnification were counted.
and the percentage of germinated spores were normalized by the spore numbers obtained from control plates. Statistical analyses were performed using the Statistica software package (version 6.1; StatSoft Inc., Tulsa, OK).

**Mechanical stability of** *T. reesei* **conidial cell walls**

Spores of the mutants and the parental strain collected from five days old PDA plates were transferred to a carbon supporter and were coated with 4 nm Au-Pd layer under vacuum conditions in the high vacuum evaporation unit, type Sputter Coater Quorum Q150T S (Quorum Technologies, Germany). Spores of QM 6a and both mutants were then separately studied each at 10±2 different fields under a FEI Quanta™ 200 Field Emission Scanning Electron Microscope (FEGSEM) using 5kV and high pressure under 25 000 × magnification.

**Mating tests**

*In vitro* matings were carried out on PDA (BD Difco, Germany) medium at room temperature and cycling day light as dual confrontation assays of QM 6a or *pks4* deletion mutants (*MAT1-2*) against *T. reesei* CBS 999.97 (*MAT1-1*). Plates were incubated for 10 - 20 days until stromata were formed. Single ascospore progenies were recovered from mature perithecia and the obtained F1 strains were then purified and tested for somatic incompatibility as described above. Strains which expressed somatic incompatibility reaction were considered unique genets and thus were further used in this study. So obtained F1 generations of the *MAT1-1* and *MAT1-2* *pks4* deletion progeny was further crossed as described above.

**PCR verification of MAT loci and pks4 inheritance**
Mycelia were harvested after two to four days of growth on PDA (Difco, Germany) at 28 °C and genomic DNA was isolated using DNeasy Plant Mini Kit (Qiagen, Germany) following the manufacturer’s protocol. The mating type of the progeny strains was determined by PCR-amplification of \textit{mat1-1-3} and \textit{mat1-2-1} as described in Druzhinina et al. (25) respectively. Furthermore, the progeny was screened for inheritance of \textit{pks4} gene using PCR primers PKS4-2fw (TCATTATACACGGACTTT) and PKS4-1rev (TATAAGCCTGACTGTAGT) at following conditions: 1 min of initial denaturation step at 94 °C followed by 30 cycles of 1 min denaturation at 94°C, 1 min of annealing at 50 °C and 90s of extension at 72 °C. Final extension was carried out at 72 °C for 7 min. The results were verified by qPCR with primer pair for \textit{pks4} gene listed in Table 1 under the conditions described above. The cycle thresholds obtained for \textit{pks4} were compared to the \textit{tef1} housekeeping gene.

**Mycoparasitic potential**

The mycoparasitic ability of \textit{T. reesei} QM 6a and the two \textit{pks4} deletion mutants was assessed by dual confrontation tests against the plant pathogenic fungi \textit{Rhizoctonia solani, Sclerotinia sclerotiorum} and \textit{Alternaria alternata} (further named as ‘hosts’). Agar plugs with fresh cultures of \textit{Trichoderma} and the host fungus were each placed on opposite sides of a PDA (BD Difco, Germany) plate, 1 cm from the edge. The cultures were incubated at 28 °C for 10 days under the 12 h cyclic light and darkness. The antagonistic potential was quantified as a percentage reduction of the host’s growth corrected for its growth when confronted with itself (the growth of antagonist against itself was set up as a zero inhibition rate).

**Production of volatile compounds (VOCs)**
The impact of pks4 gene on VOCs production was tested by “sandwich” culture assays. T. reesei strains were first cultivated on the PDA plate for two days at 28 °C and were then sealed together with a plate containing fresh plug of a host fungus so that host fungus was facing T. reesei from the top. No hyphal contact was established between the two confronted fungi. The plates were co-cultivated at the same conditions for four days. The colony radii of the host fungi were measured every 24h.

Production of water soluble compounds (WSCs)

WSCs were assessed by growing Trichoderma strains on the PDA covered by cellophane, which was removed together with Trichoderma mycelium after 60 hours. Agar plugs of the host fungi were then put in the middle of the plates and were cultivated for further four days under 12 h cycling light and darkness and 28 °C. The colony radii of the fungi were measured every 24 h. Additionally, the same experiment was performed when T. reesei was pre-grown under the same conditions as described above under the influence of volatile compounds of the host fungus. The latter was plated on a fresh PDA and was sealed on the top of the T. reesei plate. The plate with the host fungus was then removed simultaneously with Trichoderma and the cellophane. The same host fungus was then plated on this plate and its growth was observed for 4 days.

Gene expression analysis

Confrontation assays of T. reesei QM 6a and both Δpks4 strains against Rhizoctonia solani were carried out on potato dextrose agar plates (BD Dicfo, Germany) covered with cellophane at 28 °C and 12 hours cyclic illumination. To compensate for effects caused by nutrient depletion, self-confrontation experiments were used as control. Peripheral hyphal zones from each confrontation
stage were sampled and were shock frozen in liquid nitrogen. Mycelia of five replicate plates were harvested and pooled together before the RNA extraction when (i) the hyphae were ca. 20 mm apart (before the contact) and (ii) at contact of Trichoderma and host hyphae. The biomass was ground to a fine powder under liquid nitrogen and total RNA was isolated using the RNeasy extraction Kit (Qiagen, Germany). For cDNA synthesis, DNase treated (DNase I, RNase free; Fermentas, Germany) RNA (5 μg) was reverse transcribed with the RevertAid™ First Strand cDNA Kit (Fermentas, Germany) according to the manufacturer’s protocol with a combination of the provided oligo(dT) and random hexamer primers.

All real-time PCR experiments were performed in an iCycler IQ (Biorad, Germany). For the reaction the IQ SYBR Green Supermix (Bio-Rad, Germany) was prepared for 25 μl assays with standard MgCl₂ concentration (3 mM) and a final primer concentration of 100 nM each. Primers used are given in Table 1. The amplification protocol consisted of an initial denaturation step (3 min at 95 °C) followed by 40 cycles of denaturation (15 sec at 95 °C), annealing (20 sec at the temperature given in the Table 1 for each primers separately) and extension (72 °C for 15 sec). The tef1 (elongation factor 1-alpha encoding) gene, whose expression remained constant under all conditions tested (± 20 relative %), was used as a reference. Determination of the PCR efficiency was performed using triplicate reactions from a dilution series of cDNA (0.1, 10⁻², 10⁻³ and 10⁻²). Amplification efficiency was then calculated from the given slopes in the IQ5 Optical system Software v2.0. The qPCR were performed with the cDNA of 5 pooled biological replicates for each species and condition separately. Expression ratios were calculated by the Pfaffl test model implemented in the Relative expression software tool (REST) (26). The expression of pks-genes in the mutants in a non-antagonistic condition (confrontation to itself) was done using QM 6a as a
control. The mathematical model used for the expression analysis is based on the correction for exact PCR efficiencies and the mean crossing point deviation between sample group and control group (26).

Results

**Generation of pks4 knockout strains of T. reesei QM 6a**

To generate pks4 knock out strains, a linear DNA construct was designed to replace the reading frame of pks4 by a hygromycin B (hyg) selection marker under the T. reesei pki1 promoter via homologous recombination (21). Transformants with the expected albino phenotype were picked from plates and the deletion of pks4 was verified by PCR and by qPCR as described in Materials and Methods. Two verified deletants, Δpks4-1 and Δpks4-2 were selected for further studies.

BIOLOG Phenotype microarray analyses verified that the phenotypes of both candidate strains are consistent and similar to the parental strain (Fig. S1). Furthermore, the genetic identity of the mutants and the influence of the pks4 knock-out on the recognition of QM 6a genotype were tested by a plate confrontation assay. Both mutants expressed somatic incompatibility reactions (flat zone) to the parental strains QM 6a but not to each other, no anastomoses were observed in contact with QM 6a. It suggested that the two deletion strains are genetically identical. In contrast, the parental strain recognized the mutants as non-self genotype and thus reacted antagonistically, what eventually led to the partial overgrowth of the mutants.

**Δpks4 mutants are devoid of conidial pigmentation**

The default hypothesis of this work was that PKS4 would be involved in pigment formation. This was confirmed: morphological examination on PDA plates revealed that both pks4 deletion
mutants lost their green conidial pigmentation (Fig. 1a) while formation of the yellow pigment and
its secretion into the medium, a characteristic of T. reesei, remained unaffected. No green conidia
were formed during any of the cultivation conditions in either light or darkness, and the color was
not recovered by cultivation on any of 95 carbon sources of the BIOLOG Phenotype Microarrays,
five of which are shown in Fig. 1b.

The Δpks4 strains did not show any statistically significant difference in the intensity of conidia
production on PDA: after seven days QM 6a and Δpks4-1 and Δpks4-2 produced in average 9.03,
9.16 and 9.94 × 10^6 spores per cm^2, respectively (ANOVA p > 0.05; Fig. 1c). Spore size remained
unchanged (ANOVA p > 0.05; data not shown), however, the conidia of the deletion mutants
showed less mechanical stability against reduced air pressure (Fig. 1d).

In order to test whether pks4 is solely responsible for conidial pigmentation, we crossed the Δpks4
mutants (which due to their QM 6a background possess MAT1-2; 27) with the T. reesei MAT1-1
strain CBS 999.97. In total 34 pure single spore strains were isolated from mature ascospores. 21,
10 and 3 cultures contained purely white, green and yellowish conidia, respectively. All strains
from the first generation progeny (F1) were screened by PCR for the mating types, and revealed
equal distribution (15 MAT1-2 and 18 MAT1-1) which was independent of the phenotype (Fig. S2).
Specific pks4 primers were designed (Table 1) and used to test the F1 progeny for the presence of
pks4 gene. It was thereby found that all the strains with green conidia had indeed inherited the
wild-type gene, whereas the yellow and albino conidia phenotype did not contain pks4 gene (Fig.
S2). This proves that the loss of the conidial green pigmentation indeed is the direct cause of the
pks4 deletion and that this gene is involved in its production.
Loss of green pigmentation caused reduced resistance to UV light. The lack of green conidia pigmentation was also reflected in an increased sensitivity to UV light: after seven minutes of UV exposure (see Materials and Methods for details) 64% of green spores survived, whereas only 8-20% of white conidia were able to germinate (Table 2). Prolongation of exposure time (to 10 min) led to ca. 92% reduction of germination for both Δpks4 mutants (Table 2) while germination of QM 6a was decreased only by 60%.

Pks4 contributes to the development of the redish-brown color of fruiting bodies and stroma

The wild-type morphology of the T. reesei teleomorph is conspicuous with perithecial openings appearing as dark brown dots against the reddish-brown to brown background (Fig. 2a-b; 36). Crossing of the two Δpks4 mutants with the MAT1-1 tester strain CBS 999.79, as described above, produced stroma with fruiting bodies that were normally pigmented (Fig. S3), and which ejected viable and fertile ascospores. Crossings between the albino strains from progeny F1 described above showed that the pigmentation of both stroma surface and perithecial openings was also altered (Fig. 2c-e, h-i). Thus, the young stromata appeared to be white with slightly brownish dots of perithecial openings (Fig. 2c, d). However, the mature and over-mature teleomorphs developed brown pigmentation of both stroma surface and perithecial walls and openings (Fig. 2d, e, h). Importantly, the surface of mature stromata was covered by whitish cirri that originated from perithecial openings (Fig. 2e, h). Microscopic investigation showed that these structures were entirely composed of mature ascospores as some of them started to germinate (Fig. 2g) indicating possible loss defects in mechanism of ascospore discharge. Morphology of the asci was normal.
Loss of *pks4* slightly reduces the mycoparasitic potential of *T. reesei* and decreases the defence against other fungi.

Dual confrontation assays with *Rhizoctonia solani*, *Sclerotinia sclerotiorum* and *Alternaria alternata* showed that the ability of *T. reesei* QM 6a to attack and inhibit growth of these fungi was reduced in the Δ*pks4* mutants (Fig. 3a) by 37, 13 and 40 % respectively, for each host fungus. Interestingly, there were no significant differences in the ability of QM 6a and the *pks4* mutants to overgrow their opponents, as can be observed for *A. alternata* and *R. solani* on the Figure 3a, and thus the mycoparasitic ability was not affected. However, the difference was detected in respect to the ability to protect against host's metabolites: in confrontations of QM 6a with *A. alternata*, *S. sclerotiorum* and *R. solani* a narrow antibiosis zone was observed which is indicative of secretion of metabolites toxic for *T. reesei* (Fig. 3b). In case of QM 6a this zone was clear and thin while it was clearly enlarged and displayed diffused borders in confrontations with the Δ*pks4* mutants suggesting that metabolites secreted by other fungi penetrate the colonies of the deletion mutants.

*Pks4* affects the production of water soluble and volatile inhibitory compounds.

In order to test whether the loss of *pks4* is also reflected in an alteration of water soluble and volatile metabolites produced by *T. reesei*, strain QM 6a and the two Δ*pks4* strains were grown in sealed “sandwich” cultures (see Materials and Methods) with *R. solani*, *S. sclerotiorum* and *A. alternata* so that the host fungi were facing *Trichoderma* cultures from the top. No hyphal contact was established between the two fungi. When compared to the effect caused by the both *pks4* deletion mutants with that of QM 6a, the growth of all four host fungi was strongly reduced by
VOCs (Fig. 4 showing *R. solani* as one example and Table S1), thus indicating that the *pks4* deletion mutants display an enhanced production of VOCs compared to QM 6a.

In contrast, the formation of fungicidal water soluble compounds (WSCs) by *T. reesei* was reduced in the Δ*pks4* strains (Fig. 4b). Interestingly, the secretion of WSCs by *T. reesei* QM 6a and the Δ*pks4* mutants was inhibited by the presence *R. solani* VOCs (no hyphal contact), and this effect was even enhanced in the Δ*pks4* strains (Fig. 4c; Table S2).

**Pks4 is influences the expression of other PKS-encoding genes during confrontation with other fungi**

Because of the impact of *pks4* on the production of components inhibiting fungal growth, we were interested whether the loss of PKS4 would impact other PKS-encoding genes in *T. reesei*. To this end, we assessed their expression in QM 6a and the two Δ*pks4* mutants when confronted with *R. solani*. Expression of *pks* genes was inspected both prior the contact and at contact of the hyphae (Fig. 5). The expression of two *pks* genes, *pks3* (Trire2:105804) and *pks7* (Trire2:65116), was not detectable in the conditions of our experiments, also including confrontations of *T. reesei* QM 6a and the Δ*pks4* strains to themselves.

The expression analysis of the remaining eight *pks* genes in Δ*pks4* mutants and QM 6a prior and at the contact with the *R. solani* revealed striking changes in the patterns of transcript formation depending on the stage of the interaction, respectively. Before contact, both QM 6a and the mutants up-regulated four *pks* genes from the lovastatin/citrinin reducing clade I (*pks1, pks2, pks6* and *pks9*; for protein ID see Table 1 and Fig. 5). Yet, contrary to *pks9* (Trire2: 106272) and *pks2* (Trire2: 65891), which were much higher expressed in QM 6a than in the mutants (Fig. 5a), *pks6*
and pks1 were strongly up-regulated in the two mutant strains (Fig. 5a). The remaining two reducing genes pks5 (Trire2:59482) from the clade of fumonisin–like synthases and the singlet pks2S (Trire2:73618) as well as the non-reducing pks1S (Trire2:73621) were not influenced in QM 6a prior the contact with R. solani, but showed up-regulation in both mutants (Fig. 5a). At contact with R. solani the pattern was different: QM 6a down-regulated the majority of its pks genes including pks9 and pks2 that were strongly up-regulated before the contact with R. solani (Fig. 5b and 6a). Interestingly, the two singlet pks genes (pks1S and pks2S) were differentially down-regulated at the contact between the pks4 mutants and R. solani (Fig. 5b), whereas they were strongly up-regulated in QM 6a at this stage.

Most of the eight pks genes were down-regulated in the mutants, whereas two singlet genes, pks2S that is a member of the lovastatin/citrinin clade and pks1S belonging to the non-reducing clade III were up-regulated (Fig. S4).

Discussion

In this paper, we functionally characterized the role of the polyketide synthase PKS4 in T. reesei. pks4 belongs to the non-reducing clade I of fungal pks-encoding genes, which includes genes associated with pigment production such as aurofusarin (7-9) or bikaverin (10, 11), but also DHN melanin (12-19). While the former comprise substances of relatively low molecular weight, melanins - the dark to black pigments - are of high molecular mass that derive from oxidative polymerization of phenolic compounds (20, 37). Melanins are pigments that occur in all biological kingdoms, and serve many functions such as defense against environmental stresses as UV light, oxidizing agents and ionizing radiation, and they also contribute to fungal pathogenesis (38). The
chemical structures of the conidial pigments of *T. reesei*, and *Trichoderma* spp. in general, have not been elucidated yet, but due to their green and sometimes yellowish color would appear not to be melanins. Yellow pigments from *Aspergillus niger* were shown to be dimeric linear naphtho-γ-pyrones (7, 13, 4). However, Benitez et al. (39) preliminarily characterized the conidial pigment of *Trichoderma* sp. (*T. viride* at that time) as a non-indolic melanin-like polyphenol. Consistent with these data, Csiktusnádi Kiss et al. (40) identified the main pigment fractions of *T. harzianum* as oxidation polymers originating from monomer molecules containing polar substructures and double bonds in the alkyl chain. Here we showed that the final dark brown component of the fruiting body and the stroma color, which likely represents melanin, is independent on PKS4, but its colorization is delayed in the early stages of fruiting body development. In the absence of *pks4* the young stroma of *T. reesei* are colorless (white) with slightly darkened openings of perithecia indicating some remained pigmentation. Mature and overmature stromata, however, show some dark brown coloration of the surface and perithecia walls, indicating that melanin is still synthesized. The question of whether or not the pigments synthesized by PKS4 are melanins is important because our data show that PKS4 is involved in antagonism and defense against other fungi, and in the mechanical stability of the conidium. A role in antagonism is also supported by earlier findings that *pks4* is up-regulated during antagonism and mycoparasitic contact of *T. reesei* with *R. solani* (41). A role in defense, stress resistance, virulence and mechanical stability has so far been shown for melanins but not for the low-molecular weight pigments formed by PKS4 orthologs mentioned above. In addition, some polyketides have been shown to be involved in sexual development (42-46), but none of them was a PKS4 ortholog and the mechanism of involvement is still only poorly understood. In most cases this may be related to cell-wall stabilization (46). *T. reesei* PKS4 therefore exhibits a biological function otherwise typical for melanin synthesizing PKSs:
in human pathogenic fungi, such as *Cryptococcus neoformans* (47-59), *Sporothrix schenckii* (51, 52), *Paracoccidioides brasiliensis* (53), *Histoplasma capsulatum* (54), and the opportunistic pathogen *A. fumigatus* (20), melanin is involved in virulence, probably because of resistance against oxidative stress. Also, it contributes to resistance against antifungal drugs in *H. capsulatum* (55). Melanins have also been demonstrated to play crucial roles in plant pathogenic fungi: in *Magnaporthe grisea*, melanin accumulates between the plasma membrane and the cell wall of the appressorium and creates the turgor pressure needed for penetration (56). In addition, expression of an *A. alternata* melanin biosynthetic PKS in the insect pathogen *Metarhizium anisopliae* resulted in increased virulence (57). In wood decaying Basidiomycota such as *Phellinus weirii*, (58) and *Pleurotus ostreatus* (I. S. Druzhinina, unpublished data), melanin is crucially important in reactions of somatic incompatibility when genet borders are marked by thick melanised walls (barrage-reaction) impermeable for competitive fungi, which could also be the reason for the effect of PKS4 on vegetative compatibility in *T. reesei*.

An interesting consequence of *pks4* loss of function that has not been reported with any other PKS before was its effect on the expression of the other eight *pks* genes of *T. reesei*. During normal growth in the absence of a competing fungus, all but two of these eight genes were significantly down-regulated in the *pks4* mutants. Since growth of the mutant strains and the wild type occurred at the same rate, these differences are not the consequence of variable rates of nutrient uptake. Furthermore, antagonistic interaction with *R. solani* revealed that loss of *pks4* function affected the expression of the other *pks* genes in different ways, some being up-regulated, some down-regulated and some not affected. From these data we conclude that PKS4 – or rather the
function of its product in e.g. protection and defense against stress – is an important signal for the expression of these \textit{pks} genes. This is definitely an area that requires further investigations.

Loss of function of \textit{pks4} also led to a decreased synthesis of water soluble inhibitory components by \textit{T. reesei}, and it is tempting to speculate that they would be formed by one or more of the affected PKSs. Since their expression was tested in the absence of other fungi, and they were all found to be down-regulated (Fig. S4), it is however not possible to predict which ones are the responsible producers. In addition, these components could also be products of other enzymes, such as non-ribosomal peptide synthases of which \textit{T. reesei} has ten (59).

It was finally interesting to note that deletion of \textit{pks4} increased the production of volatile organic compounds (VOCs) by \textit{T. reesei}. The chemical nature of the VOCs from \textit{T. reesei} has not been identified yet, but their diversity from other \textit{Trichoderma} spp. showed that they are composed mainly of long aliphatic acids; and that they have alcohols and esters (60, 61), which are usually products of fatty acid catabolism (62). Biosynthesis of these compounds may be favored by the lack of PKS activity which results in an increased access to the cellular pools of acetyl- and malonyl-

\textit{CoA}.

The function of PKSs has so far mainly been investigated with respect to the role of their products in human or plant pathogenesis. Our data show that PKS4 also influences several biological functions in \textit{T. reesei} which are not only related to the interaction with other organisms. Transcriptomic analyses of \textit{T. reesei} have recently shown that many \textit{pks} genes are maximally expressed during rapid vegetative growth (63-65), which is not a pattern that would be expected for genes whose function is traditionally viewed as unrelated to growth (“secondary metabolites”).
It will thus be worthwhile to perform a deeper investigation of the regulation and role of pks genes in fungal physiology.

Acknowledgments

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References


58. **Li CY.** 1981. Phenoloxidase and peroxidase activities in zone lines of *Phellinus weirii*. Mycologia **73**:811-821.


Legend to figures

FIG 1 Conidiation morphology of QM 6a and the pks4 deletion mutants. a. Plate macromorphology and conidial pigmentation of QM 6a and Δpks4 cultures. b. Conidial pigmentation of QM 6a and Δpks4 mutants on selected carbon sources of the BIOLOG FF Microplate Phenotype MicroArrays. c. Conidial density of QM 6a and Δpks4 mutants’ spores produced after seven days of cultivation PDA. No statistically significant difference in the intensity of conidia production was observed (ANOVA p > 0.05). d. Conidial mechanical stability observed under a FEI Quanta™ 200 Field Emission Scanning Electron Microscope (FEGSEM) after applied vacuum conditions and high pressure under 25 000 × magnification.

FIG 2 Teleomorph morphology of T. reesei QM 6a wild type and Δpks4 mutants’. a. Stromata of wild-type T. reesei strains Qm 6a and CBS 999.97 mated in vitro. b. Fruiting bodies of Δpks4-1 strain and Qm 6a mated in vitro c. Young stromata and mature, partially melanised brownish
fruiting bodies of F1 Δpks4 strains mated in vitro. The scale annotates 2 mm. d. Partially melanised over-mature fruiting structures of F1 Δpks4 strains with dark perithecia openings. The scale annotates 10 mm.  

d. Partially melanised over-mature fruiting structures of F1 Δpks4 strains with dark perithecia openings. The scale annotates 10 mm.  e. Magnified young white stromata of F1 Δpks4 strains with melanised openings. f. Conidia of F1 Δpks4 strains. g. Ascospores from the cirri of F1 Δpks4 attached to over-mature perithecia indicated by arrows on figure h. f - g imaged under 200 × magnification.

Pigmented perithecium from h stroma under 200 × magnification.

**FIG 3** Antagonistic potential of QM 6a and pks4-1 deletion mutant. Confrontations panels are shown only for Δpks4-1, both mutants revealed identical mycoparasitic pattern. a. Front sides of confrontation plates with plant pathogenic fungi always on the left side and *Trichoderma* QM 6a and the mutants always on the right. b. Back sides of confrontation plates with plant pathogenic fungi always on the left side and with *T. reesei* QM 6a and the mutants always on the right.  

Antibiosis zones for Δpks4 strains are indicated by the arrows.

**FIG 4** Effect of VOCs and WSCs from pks4 deletion mutant and QM 6a on growth of *R. solani*. a. Reduction of *R. solani* growth by the VOCs secreted by *T. reesei* QM 6a and Δpks4 strain after four days of incubation. Both mutants consistently reduced the growth of *R. solani*. b. *R. solani* growth on the PDA medium containing WSCs of *T. reesei* and pks4 deletion mutant. c. Growth of *R. solani* on the medium with WSCs secreted by the pks4 mutant and QM 6a in the presence of VOCs from *R. solani* during the growth of *T. reesei* strains.

**FIG 5** Expression of pks genes in Δpks4 mutants compared to QM 6a before the contact (a) and at the contact (b) with *Rhizoctonia solani*. The □ and ○ indicate the data for the Δpks4-1 and Δpks4-2 mutants respectively. Shadowed area indicates no differences in regulation of pks4 deletion.
mutants compared to QM 6a. Protein numbers of \textit{pks} genes in the genome of \textit{T. reesei}: \textit{pks1} (Trire2:65172), \textit{pks2} (Trire2:65891), \textit{pks5} (Trire2:59482), \textit{pks6} (Trire2:60118), \textit{pks8} (Trire2:81964), \textit{pks9} (Trire2:106272), \textit{pks1S} (Trire2:73621) and \textit{pks2S} (Trire2:73618). Those genes marked with color exhibited consistent expression trend between both \textit{Δpks4} mutants. The figures above the plots show the sampling stage of the \textit{T. reesei} (T) strains at each condition of confrontation with \textit{R. solani} (R). Vertical and horizontal bars indicate standard deviations for the wild type and the mutants respectively.
Table 1: *Trichoderma reesei* repertoire of PKS encoding genes, their most related orthologs for which products or functions are identified and the primers and annealing temperatures used in the expression analysis.

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<th>R or NR</th>
<th>Nr. of amino acids</th>
<th>PKS Identity [%]</th>
<th>E-value</th>
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<th>NCBi blastp identification</th>
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RD - reducing, NR - non-reducing PKS; * JGI genome portal (http://genome.jgi-psf.org/Trire2/Trire2.home.html); after PKS grouping of Baker et al. (6)
Table 2: Percentage of germinated spores after the exposure to UV light for seven and ten minutes. Sd annotate standard deviation values.

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