

The Polyketide Synthase Gene *pkS4* of *Trichoderma reesei* Provides Pigmentation and Stress Resistance

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Species of the fungal genus *Trichoderma* (Hypocreales, Ascomycota) are well-known for their production of various secondary metabolites. Nonribosomal peptides and polyketides represent a major portion of these products. In a recent phylogenomic investigation of *Trichoderma* polyketide synthase (PKS)-encoding genes, the *pkS4* from *T. reesei* was shown to be an orthologue of pigment-forming PKSs involved in synthesis of aurofusarin and bikaverin in *Fusarium* spp. 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 cell wall stability and the antagonistic abilities of *T. reesei* against other fungi, including formation of inhibitory metabolites. In addition, deletion of *pkS4* significantly influences the expression of other PKS-encoding genes of *T. reesei*. To our knowledge, this is the first indication that a low-molecular-weight pigment-forming PKS is involved in defense, mechanical stability, and stress resistance in fungi.

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 nonribosomal peptides with antimicrobial and plant defense-stimulating activities (4). However, the role of polyketide synthases (PKSs) in *Trichoderma* ecophysiology is not well studied. *Trichoderma* spp. polyketides are produced by iterative PKSs, multifunctional enzymes consisting of several active sites capable of catalyzing the fusion of variable numbers of coenzyme A (CoA)-linked acyl monomers, such as acetyl-CoA and malonyl-CoA, into polymers known as polyketides. They can be further grouped into nonreducing (NR) and reducing (RD) PKSs according to their domain organization (5). Recently, Baker et al. (6) used a phylogenomic approach to study the PKS repertoire in *T. reesei*, *T. atroviride*, and *T. virens*, and their findings enabled the putative *in silico* prediction of some of the respective products. A total of 11 PKS-encoding genes were found in the *T. reesei* genome, among which 2 occur only in *T. reesei* and 9 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 nonreducing type (clade I), has been shown to have orthologues in other fungi, i.e., PKSs 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* spp. (15, 16, 20). In this study, we used a reverse genetic approach to examine the functions of *pkS4* in the ecophysiology of *T. reesei*. We found that PKS4 is indeed responsible for the pigmentation of conidia and the non-melanized structures of fruiting bodies, but its loss of function also impacts the stability of the 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 changes the expression of other PKS-encoding genes of *T. reesei*. To our knowledge, this is the first indication that low-molecular-weight pigments can be involved in defense, mechanical stability, and stress resistance in fungi.

MATERIALS AND METHODS

Deletion of the *pkS4* gene in *T. reesei*. The *pkS4* gene was deleted by 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 (CAATGGCCGAATGTTC TAGC and GGAACAAGTTGAGCCAGAGC, respectively), the 3'-flanking region was amplified with primers F4 and R6 (GCAATACCGGTG

Received 22 April 2013 Accepted 1 September 2013

Published ahead of print 13 September 2013

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Supplemental material for this article may be found at <http://dx.doi.org/10.1128/EC.00103-13>.

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doi:10.1128/EC.00103-13

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AGAACGA and TGCGGAGGATCGAGACTATT, respectively), and the hygromycin B sequence was amplified with the primers hygF and hygR (GCTGGAGCTAGTGGAGGTCA 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 (AGGTACGCATGGAGACA and TACACACGCACTCACGCATA, respectively), leaving F1 and R6 available for downstream verification. Similar to previously published methods (22), a protoplast polyethylene glycol-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 *pk4* replacement with the hygromycin cassette by PCR amplification using primer pairs F1/R6 and F2/R5.

Verification of the *pk4* absence in the genome of *T. reesei* Δ *pk4* was tested by a specific quantitative PCR (qPCR) with primers for *pk4* (Table 1) and the following amplification protocol: initial denaturation step for 3 min at 95°C, followed by 40 cycles of denaturation for 15 s at 95°C, annealing for 20 s at 54°C, and extension at 72°C for 15 s.

Somatic incompatibility. The somatic compatibility of Δ *pk4* mutants and the parental strain was tested in confrontation assays on 2.4% potato dextrose agar (PDA; BD Difco, Germany) at 28°C for 7 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, which indicate somatic incompatibility between the opponents.

Morphological observations. The parental strain QM 6a and both deletion mutants were cultivated in darkness and a 12-h light cycle on 2.4% PDA (BD Difco, Germany) at 28°C for 7 days. The spore density was measured quantitatively per cm² of the developed colony. For this purpose, three 6.2-cm² agar fragments were cut from cultures pregrown at 28°C for 7 days and were rinsed separately in 15 ml of water containing 0.1% Tween 80 until visually all conidia were washed out. The optical density at 590 nm (OD₅₉₀) of the suspension was measured in a Biolog (Hayward, CA) turbidimeter calibrated to the Biolog standard for filamentous fungi. The final concentration value was calculated based on the calibration curve inferred from serial dilutions of the standard suspension. Furthermore, conidial size was assessed by measuring the length of 40 conidia per each strain under 400× magnification with a 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 by Druzhinina et al. (23) and Atanasova et al. (24). Briefly, the strains were cultivated on 2.4% PDA for 5 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 (by using a Biolog standard turbidimeter; 4×10^7 spores in 10 ml of phytigel). A total of 90 μ l of the conidial suspension was dispensed into each of the wells of the Biolog FF microplates and incubated at 28°C in darkness and with a 12-h light-dark cycle. The OD₇₅₀ (for detection of mycelial growth) was measured after 18, 24, 42, 48, 66, 72, 90, and 96 h using a microplate reader. The growth rate of each strain was assessed based on the averaged mycelial density, measured on all 95 carbon sources after 0, 24, 48, and 72 h 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 15-W/840 lamp) or in darkness at 28°C for 5 days. Mycelial density was measured at 750 nm. The growth rates and carbon utilization patterns were compared, and the data were statistically analyzed.

UV sensitivity. The tolerance to UV irradiation of the pigment-deficient spores was tested at 254 nm by using four HNS 15-W OFR UV lamps. The conidia collected after 5 days of growth on PDA plates were filtered through sterile glass wool to remove hyphal fragments. The volume of the spore suspension that contained 4×10^7 (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 μ l, which were then plated on four PDA plates per sample (QM 6a and both Δ *pk4* mutants) and four plates for each sample control. Open plates were then exposed to UV illumination for 5, 7, and 10 min, whereas the control was protected from UV light. Finally, the plates were incubated for 48 h at 28°C, the single-spore colonies observed under 10× magnification were counted, and the percentage of germinated spores was normalized to 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 5-day-old cultures on PDA plates were transferred to a carbon supporter and were coated with a 4-nm Au-Pd layer under vacuum conditions in a high-vacuum evaporation unit (Sputter Coater Quorum Q150T S; Quorum Technologies, Germany). Spores of QM 6a and both mutants were then separately studied, observing 10 ± 2 different fields for each strain under a FEI Quanta 200 field emission scanning electron microscope (FEGSEM) with 5 kV and high pressure under 25,000× magnification.

Mating tests. *In vitro* matings were carried out on PDA medium (BD Difco, Germany) at room temperature and cycling daylight in dual confrontation assays of QM 6a or *pk4* deletion mutants (*MAT1-2*) against *T. reesei* CBS 999.97 (*MAT1-1*). Plates were incubated for 10 to 20 days, until stromata formed. Single ascospore progeny were recovered from mature perithecia, and the obtained F₁ strains were then purified and tested for somatic incompatibility as described above. Strains that expressed the somatic incompatibility reaction were considered unique genets and thus were used further in this study. The obtained F₁ generations of the *MAT1-1* and *MAT1-2* *pk4* deletion progeny were further crossed as described above.

PCR verification of *MAT* loci and *pk4* inheritance. Mycelia were harvested after 2 to 4 days of growth on PDA (Difco, Germany) at 28°C, and genomic DNA was isolated by using a DNeasy plant minikit (Qiagen, Germany) following the manufacturer's protocol. The mating type of the progeny strains was determined by PCR amplification of *mat1-1-3* and *mat1-2-1* as described by Druzhinina et al. (25). Furthermore, the progeny were screened for inheritance of the *pk4* gene by using the PCR primers PK4-2fw (TCATTATACACGGACTTT) and PK4-1rev (TATAAGCCTGACTGTAGT) under the following conditions: 1 min of an initial denaturation step at 94°C, followed by 30 cycles of 1 min of denaturation at 94°C, 1 min of annealing at 50°C, and 90 s of extension at 72°C. Final extension was carried out at 72°C for 7 min. The results were verified by qPCR with the primer pair for the *pk4* gene listed in Table 1 under the conditions described above. The cycle thresholds obtained for *pk4* were compared to that for the *tef1* housekeeping gene.

Mycoparasitic potential. The mycoparasitic abilities of *T. reesei* QM 6a and the two *pk4* deletion mutants were assessed in dual confrontation tests against the plant pathogenic fungi *Rhizoctonia solani*, *Sclerotinia sclerotiorum*, and *Alternaria alternata* (the "hosts"). Agar plugs with fresh cultures of *Trichoderma* and the host fungus were each placed on opposite sides of a PDA plate, 1 cm from the edge. The cultures were incubated at 28°C for 10 days under a 12-h light-dark cycle. The antagonistic potential was quantified as the percent reduction of the host's growth, corrected for its growth when confronted with itself (the growth of antagonist against itself was set as the zero inhibition rate).

Production of VOCs. The impact of *pk4* gene on volatile organic compounds (VOCs) production was tested in "sandwich" culture assays. *T. reesei* strains were first cultivated on a PDA plate for 2 days at 28°C, and then they were sealed together with a plate containing a fresh plug of a host

TABLE 1 *Trichoderma reesei* repertoire of PKS-encoding genes, their most closely related orthologs for which products or functions have been identified, and the primers and annealing temperatures used for the expression analysis

Protein ID ^a	Name ^b	R or NR and clade(s)	No. of amino acids	PKS	Identity (%)	E-value	Accession no.(s)	NCHI Bispap identification	Reference(s) or source	Forward/reverse primers (5'--3')	T _m (°C)
Tr82208	PKS4	NR clade I	2,146	Yellow-green conidial pigment polyketide synthase	47	0.0	ACJ13039	<i>alb1</i> from <i>Aspergillus fumigatus</i>	28	CCAGGACGACGATACAAC/ AACAGTCCAGGCTCATTA	54
Tr81964	PKS8	NR clade I, II	1,863	Uncharacterized protein	47	0.0	JN257714	<i>adda</i> from <i>A. niger</i> or <i>vita</i> from <i>Penicillium aethiopicum</i> involved in anthracenone and naphthalenedione production, respectively	29, 30	ATAACAGTGGCGTCACAT/ CAATGACCCAGCAATCTC	54.7
Tr105804	PKS3	NR clade III	2,116	PKS involved in lipid metabolism	54	0.0	XP_003719468	PKS16 from <i>Borytrichia fuchikiana</i>	<i>Botrytis cinerea</i> T4 Genome Consortium	CCGTAATCTCTGCTGTATC/ GTGAACCATCTTGAAGGA	NA ^c
Tr73621	Singlet, PKS1S	NR clade III	2,633	Uncharacterized protein	37	0.0	EGE04288	Phenolphiocerol synthase (ppSB) from <i>Trichophyton equinum</i>	<i>Trichophyton equinum</i> CBS 127.97 Genome Consortium	AGCATTAAGGGGAATAGATC/ AGCCTGAGAAAGAGTTGAT	50.2
Tr65172	PKS1	RD clade I, lovastatin/citrinin diketide	2,598	Uncharacterized lovastatin/citrinin-like diketide synthase	35/40	0.0/2E-93	BAC20566/AAP32477	Mcb from <i>P. citrinum</i> synthesizes diketide portion of lovastatin/PKS for ochratoxin A from <i>A. ochraceus</i>	31, 32	AAACATCAATCTCAACATC/ ACACATTCGGTATTAAGTATA	57
Tr65891	PKS2		2,374		37	0.0	ELQ36243	6-Methylsalicylic acid synthase from <i>Magnaporthe oryzae</i>	33	GGACATATTCACAGGATTTCTC/ GGTGGCAACATCTTCAAG	54
Tr60118	PKS6		2,415		46	0.0	AAR90259	Uncharacterized PKS from <i>Cochliobolus heterostrophus</i>	5	TCAAGTGGTCTCTCTATTT/ AAMTGGTGTCTCAATCC	54.7
Tr106272	PKS9		2,612		54	0.0	AAR92209	Uncharacterized PKS2 from <i>Gibberella morilliformis</i>	5	CCGTATCTCTGCTGTATC/ ATCGTCTGTGATGAAGTG	54.7
Tr73618	Singlet, PKS2S		2,567		41/40	0.0	BAC20566/AAD34559	Mcb from <i>P. citrinum</i> and LovF from <i>A. terreus</i> synthesize diketide portion of lovastatin and citrinin, respectively	31, 34	TACCATTACACAGACTTG/ AGCAATTCACAAACATCATA	50.2
Tr59482	PKS5	RD clade III, T-toxin	2,205	Uncharacterized T-toxin-like synthase	34	0.0	ABR76806	PKS1 and PKS2 of <i>C. heterostrophus</i> required for synthesis of T-toxin	35	TCTCATTTGATGCGTGTAT/ GCTTGGACTCTCATTCATATC	57
Tr65116	PKS7	RD clade IV, fumonisins	2,434	Uncharacterized fumonisins-like synthase	40	0.0	ELQ64206	Myceroosin acid synthase from <i>M. oryzae</i>	33	AAGAAGACTTCCGCAACT/ AAGCAGCTCATACACAACCT	NA

^a According to the JGI genome portal (<http://genome.jgi-psf.org/Trre2/Trre2.home.html>).^b Based on the PKS grouping of Baker et al. (6).^c NA, not available.

fungus, so that the host fungus was facing the *T. reesei* culture from the top. No hyphal contact was established between the two confronted fungi. The plates were cocultivated under the same conditions for 4 days. The colony radii of the host fungi were measured every 24 h.

Production of water-soluble compounds. Water-soluble compounds (WSCs) were assessed by growing *Trichoderma* strains on the PDA plates covered by cellophane, which was removed together with *Trichoderma* mycelium after 60 h. Agar plugs of the host fungi were then put in the middle of the plates and were cultivated for a further 4 days under 12-h light-dark cycling at 28°C. The colony radii of the fungi were measured every 24 h. Additionally, the same experiment was performed when *T. reesei* was pregrown 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 plate and was sealed on 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 for *T. reesei* QM 6a and both $\Delta pks4$ strains against *Rhizoctonia solani* were carried out on potato dextrose agar plates covered with cellophane at 28°C and with a 12-h illumination cycle. To compensate for the effects caused by nutrient depletion, self-confrontation experiments were included as controls. Peripheral hyphal zones from each confrontation stage were sampled and 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 by using the RNeasy extraction kit (Qiagen, Germany). For cDNA synthesis, RNA (5 μ g) that was DNase treated (DNase I, RNase-free; Fermentas, Germany) was reverse transcribed by using 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 (Bio-Rad, Germany). For the reaction mixture, IQ SYBR green supermix (Bio-Rad, Germany) was prepared for 25- μ l assay mixtures with the standard MgCl₂ concentration (3 mM) and final primer concentrations of 100 nM each. The 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 s at 95°C), annealing (20 s at the temperature given in Table 1 for each primer), and extension (72°C for 15 s). The *tef1* (elongation factor 1 α -encoding) gene, whose expression remained constant under all conditions tested (\pm 20 relative percent), was used as a reference. Determination of the PCR efficiency was performed using triplicate reaction mixtures 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 under nonantagonistic conditions (confrontation to itself) was measured by using QM 6a as a control. The mathematical model used for the expression analysis was based on correction for exact PCR efficiencies and the mean crossing-point deviation between the sample group and the control group (26).

RESULTS

Generation of *pks4* knockout strains of *T. reesei* QM 6a. To generate *pks4* knockout strains, a linear DNA construct was designed to replace the reading frame of *pks4* with 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 Meth-

ods. Two verified deletants, $\Delta pks4-1$ and $\Delta pks4-2$, were selected for further studies.

Biolog phenotype microarray analyses verified that the phenotypes of both candidate strains were consistent and similar to the parental strain (see Fig. S1 in the supplemental material). Furthermore, the genetic identity of the mutants and the influence of the *pks4* knockout on the recognition of the QM 6a genotype were tested in a plate confrontation assay. Both mutants expressed somatic incompatibility reactions (a flat zone) to the parental QM 6a strain but not to each other, and no anastomoses were observed in contact with QM 6a. These findings suggested that the two deletion strains are genetically identical. In contrast, the parental strain recognized the mutants as nonself and thus reacted antagonistically, which eventually led to the partial overgrowth of the mutants.

$\Delta pks4$ mutants are devoid of conidial pigmentation. The default hypothesis of this work was that PKS4 is 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 under 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, 5 of which are shown in Fig. 1b.

The $\Delta pks4$ strains did not show any statistically significant difference in the intensity of conidia production on PDA: after 7 days, QM 6a, $\Delta pks4-1$, and $\Delta pks4-2$ produced on average 9.03×10^6 , 9.16×10^6 , and 9.94×10^6 spores per cm², respectively (analysis of variance [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 $\Delta pks4$ 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. A total of 21, 10, and 3 cultures contained purely white, green, or yellowish conidia, respectively. All strains from the first-generation progeny (F₁) were screened by PCR for mating types, and there was a nearly equal distribution (15 *MAT1-2* and 18 *MAT1-1*) that was independent of the phenotype (see Fig. S2 in the supplemental material). Specific *pks4* primers were designed (Table 1) and used to test the F₁ progeny for the presence of the *pks4* gene. We found that all the strains with green conidia had indeed inherited the wild-type gene, whereas the yellow and albino conidia phenotype conidia did not contain the *pks4* gene (see Fig. S2). This proved that the loss of the conidial green pigmentation is directly caused by the *pks4* deletion and that this gene is involved in its production.

Loss of green pigmentation caused reduced resistance to UV. The lack of green conidia pigmentation was also reflected in an increased sensitivity to UV light: after 7 min of UV exposure (see Materials and Methods for details) sixty-four percent of green spores survived, whereas only 8 to 20% of white conidia were able to germinate (Table 2). Prolongation of exposure time (to 10 min) led to a ca. 92% reduction of germination for both $\Delta pks4$ mutants (Table 2), while germination of QM 6a was decreased by only 60%.

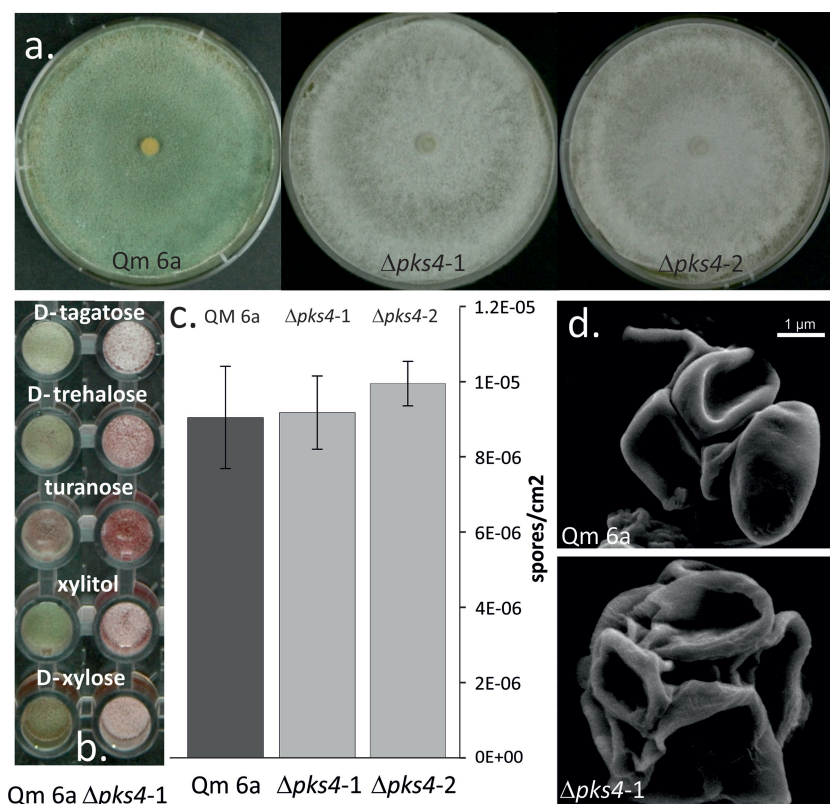


FIG 1 Conidiation morphology of QM 6a and the *pk4* deletion mutants. (a) Plate macromorphology and conidial pigmentation of QM 6a and $\Delta pk4$ cultures. (b) Conidial pigmentation of QM 6a and $\Delta pk4$ mutants on selected carbon sources of the Biolog FF microplate phenotype microarrays. (c) Conidial density of QM 6a and $\Delta pk4$ mutants' spores produced after 7 days of cultivation on 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 FEGSEM after application of vacuum conditions and high pressure, under 25,000 \times magnification.

***pk4* contributes to the development of the reddish-brown color of stroma.** The wild-type morphology of the *T. reesei* teleomorph is conspicuous, with perithecial openings that appear as dark brown dots against the reddish-brown to brown background of stroma (Fig. 2a and b) (36). Crossing of the two $\Delta pk4$ mutants with the *MAT1-1* tester strain CBS 999.79, as described above, produced stroma with fruiting bodies that were normally pigmented (see Fig. S3 in the supplemental material) and which ejected viable and fertile ascospores. Crossings between the albino strains from the F₁ progeny described above showed that the pigmentation of both the stroma surface and perithecial openings was altered (Fig. 2c to e, h, and i). Thus, the young stromata appeared to be white with slightly brownish dots of perithecial openings (Fig. 2c and d). However, the mature and overmature teleomorphs developed brown pigmentation of both the stroma

surface and perithecial walls and openings (Fig. 2d, e, and h). Importantly, the surfaces of mature stromata were covered by whitish cirri that originated from perithecial openings (Fig. 2e and h). Microscopic investigation showed that these structures were composed entirely of mature ascospores, as some of them started to germinate (Fig. 2g), indicating possible defects in the mechanism of ascospore discharge. The morphology of the asci was normal.

Loss of *pk4* slightly reduces the mycoparasitic potential of *T. reesei* and decreases the defense 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 $\Delta pk4$ mutants (Fig. 3a) by 37%, 13%, and 40%, respectively, for each host fungus. Interestingly, there were no significant differences in the abilities of QM 6a and the *pk4* mutants to overgrow their opponents, as can be observed for *A. alternata* and *R. solani* in Fig. 3a, and thus the mycoparasitic capacity was not affected. However, a difference was detected with respect to the ability to protect against the host's metabolites: in confrontations of QM 6a with *A. alternata*, *S. sclerotiorum*, or *R. solani*, a narrow antibiosis zone was observed, which is indicative of secretion of metabolites toxic for *T. reesei* (Fig. 3b). In the case of QM 6a, this zone was clear and thin, while it was considerably enlarged and displayed diffuse borders in the confrontations with the $\Delta pk4$ mutants,

TABLE 2 Spore germination after exposure to UV light for 7 and 10 min

<i>T. reesei</i> strain	% germinated spores (mean \pm SD) after UV exposure for:	
	7 min	10 min
QM 6a	63.6 \pm 10.1	39.4 \pm 6.4
$\Delta pk4-1$	19.7 \pm 6.5	5.9 \pm 8.4
$\Delta pk4-2$	7.7 \pm 6.5	7.7 \pm 6.5

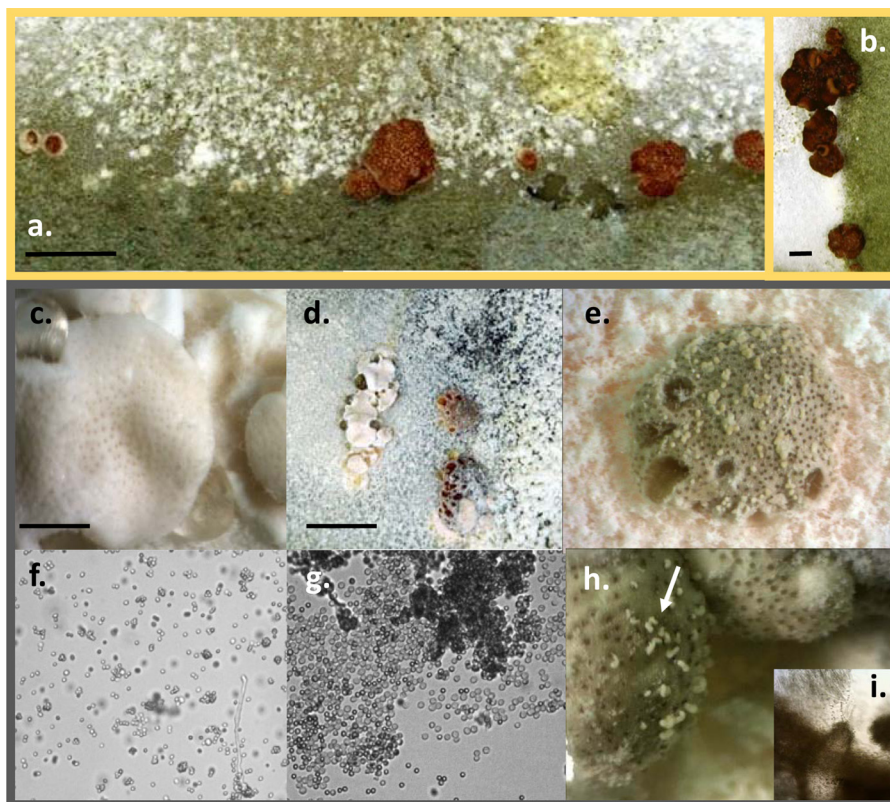


FIG 2 Teleomorph morphology of *T. reesei* QM 6a wild type and $\Delta pks4$ mutants. (a) Stromata of wild-type *T. reesei* strains Qm 6a and CBS 999.97, mated *in vitro*. (b) Fruiting bodies of strains $\Delta pks4-1$ and Qm 6a mated *in vitro*. (c) Young stromata and mature, partially melanized brownish fruiting bodies of F_1 $\Delta pks4$ strains mated *in vitro*. Bar, 2 mm. (d) Partially melanized overmature fruiting structures of F_1 $\Delta pks4$ strains with dark perithecia openings. Bar, 10 mm. (e) Magnified young white stromata of F_1 $\Delta pks4$ strains with melanized openings. (f) Conidia of F_1 $\Delta pks4$ strains. (g and h) Ascospores from the cirri of F_1 $\Delta pks4$ attached to overmature perithecia, indicated by the arrows in panel h. Magnification (panels f and g), $\times 200$. (i) Pigmented perithecium from the stroma shown in panel h. Magnification, $\times 200$.

suggesting that metabolites secreted by the other fungi had penetrated 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 $\Delta pks4$ 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. Compared to the effect caused by both of the *pks4* deletion mutants versus that of QM 6a, the growth of all four host fungi was strongly reduced by VOCs (Fig. 4; *R. solani* is shown as one example; see also Table S1 in the supplemental material), indicating that the *pks4* deletion mutants displayed enhanced production of VOCs compared to QM 6a.

In contrast, the formation of fungicidal WSCs (see Materials and Methods) by *T. reesei* was reduced in the $\Delta pks4$ strains (Fig. 4b). Interestingly, the secretion of WSCs by *T. reesei* QM 6a and the $\Delta pks4$ mutants was inhibited by the presence *R. solani* VOCs (no hyphal contact), and this effect was even enhanced in the $\Delta pks4$ strains (Fig. 4c; see also Table S2 in the supplemental material).

***pks4* influences the expression of other PKS-encoding genes during confrontation with other fungi.** Because of the impact of *pks4* on the production of components that inhibit fungal growth,

we were interested in 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 $\Delta pks4$ mutants when confronted with *R. solani*. Expression of *pks* genes was inspected both prior to 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 under the conditions of our experiments, which also included confrontations of *T. reesei* QM 6a and the $\Delta pks4$ strains with themselves.

Expression analysis of the remaining eight *pks* genes in the $\Delta pks4$ mutants and QM 6a prior to and at the time of contact, respectively, with *R. solani* revealed striking changes in the patterns of transcript formation that depended on the stage of the interaction. Before contact, both QM 6a and the mutants upregulated four *pks* genes from lovastatin/citrinin reducing clade I (*pks1*, *pks2*, *pks6*, and *pks9*; for protein ID numbers, see Table 1 and Fig. 5). Yet, contrary to *pks9* (Trire2:106272) and *pks2* (Trire2:65891), which were much highly expressed in QM 6a than in the mutants (Fig. 5a), *pks6* and *pks1* were strongly upregulated in the two mutant strains (Fig. 5a). The remaining two reducing genes, *pks5* (Trire2:59482) from the clade of fumonisins-like synthases and the singlet *pks2S* (Trire2:73618), as well as the nonreducing *pks1S* (Trire2:73621), were not influenced in QM 6a prior to contact with *R. solani* but showed upregulation in both mutants (Fig. 5a). At contact with *R. solani*, the pattern was different: QM 6a downregulated the majority of its *pks* genes, including *pks9* and

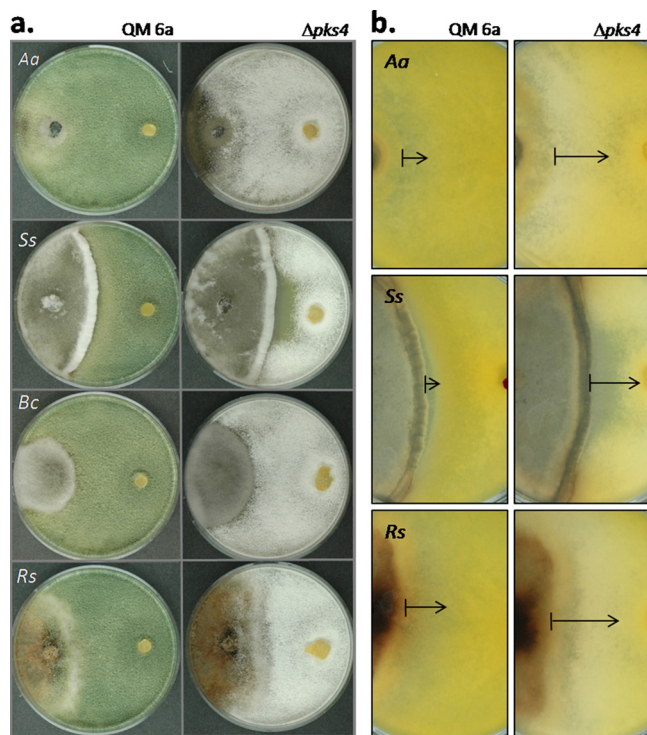


FIG 3 Antagonistic potentials of QM 6a and the *pk4-1* deletion mutant. Confrontations panels are shown only for $\Delta pk4-1$, but both mutants revealed identical mycoparasitic patterns. (a) Front sides of confrontation plates, with plant-pathogenic fungi always on the left side and *Trichoderma* QM 6a or the mutant always on the right. (b) Back sides of confrontation plates, with plant-pathogenic fungi always on the left side and *T. reesei* QM 6a or the mutant strain always on the right. Antibiosis zones for $\Delta pk4$ strains are indicated by the arrows. Aa, Ss, Bc, and Rs mark *Alternaria alternata*, *Sclerotinia sclerotiorum*, *Botrytis cinerea*, and *Rhizoctonia solani*, respectively.

pk2, which were strongly upregulated before the contact with *R. solani* (Fig. 5). Interestingly, the two singlet *pk*s genes (*pk1S* and *pk2S*) were differentially downregulated at the contact between the *pk4* mutants and *R. solani* (Fig. 5b), whereas they were strongly upregulated in QM 6a at this stage.

Most of the eight *pk*s genes were downregulated in the mutants, whereas two singlet genes, *pk2S*, a member of the lovastatin/citrinin clade, and *pk1S*, which belongs to nonreducing clade III, were upregulated (see Fig. S4 in the supplemental material).

DISCUSSION

In this study, we functionally characterized the role of the polyketide synthase PKS4 in *T. reesei*. *pk4* belongs to the nonreducing clade I of fungal *pk*s-encoding genes, which includes genes associated with pigment production, such as aurofusarin (7–9) and bikaverin (10, 11), but also DHN melanin (12–19). While the former comprise substances of relatively low molecular weights, 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 including 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 of *Trichoderma* spp. in general, have not been elucidated yet,

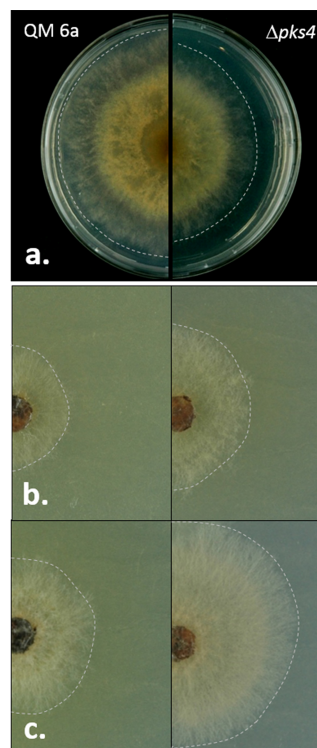


FIG 4 Effects of VOCs and WSCs from *pk4* deletion mutants and QM 6a on growth of *R. solani*. (a) Reduction of *R. solani* growth by the VOCs secreted by *T. reesei* QM 6a and $\Delta pk4$ mutants after 4 days of incubation. Both mutants consistently reduced the growth of *R. solani*. (b) *R. solani* growth on PDA medium containing WSCs of *T. reesei* and the *pk4* deletion mutants. (c) Growth of *R. solani* on medium with WSCs during the *pk4* mutants and QM 6a in the presence of VOCs from *R. solani* during growth of the *T. reesei* strains.

but due to their green and sometimes yellowish color they appear not to be melanins. Yellow pigments from *Aspergillus niger* have been shown to be dimeric linear naphtho- γ -pyrones (4, 7, 13). However, Benitez et al. (39) preliminarily characterized the conidial pigment of *Trichoderma* spp. (*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, are independent of PKS4, but the colorization is delayed in the early stages of fruiting body development. In the absence of *pk4*, the young stroma of *T. reesei* are colorless (white) with slightly darkened openings of perithecia, indicating some retained pigmentation. Mature and overmature stromata, however, showed some dark brown coloration on the surface and perithecia walls, indicating that melanin was still synthesized. The question of whether the pigments synthesized by PKS4 are melanins is important, because our data showed 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 indicating that *pk4* is upregulated during antagonism and mycoparasitic contact of *T. reesei* with *R. solani* (41). A role in defense, stress resistance, virulence, and me-

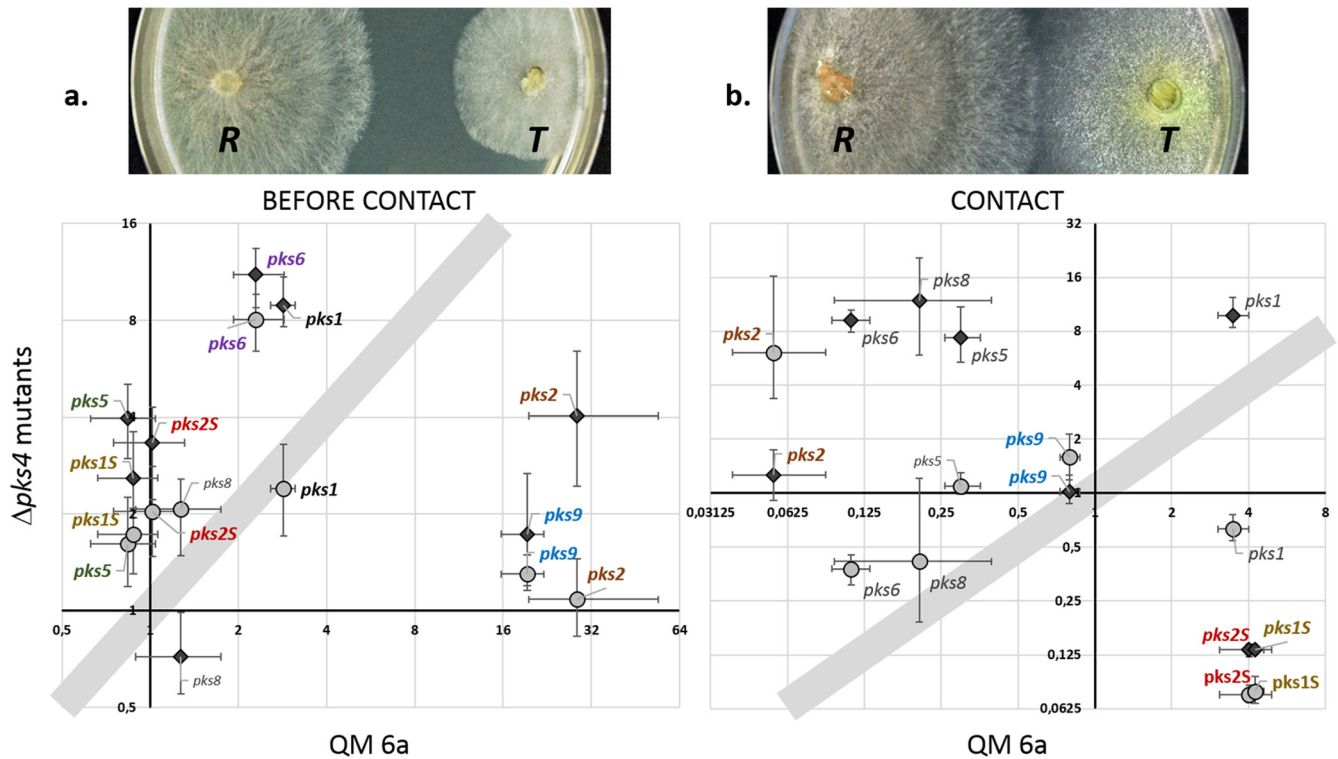


FIG 5 Expression of *pks* genes in $\Delta pks4$ mutants compared to QM 6a before contact (a) and at contact (b) with *Rhizoctonia solani*. The gray circles and black diamonds represent data for the $\Delta pks4-1$ and $\Delta pks4-2$ mutants, respectively. Shaded areas indicate that there was no difference in the regulation of the *pks4* deletion mutants compared to QM 6a. Protein numbers of *pks* genes in the genome of *T. reesei* are as follows (in parentheses): *pks1* (Trire2:65172), *pks2* (Trire2:65891), *pks5* (Trire2:59482), *pks6* (Trire2:60118), *pks8* (Trire2:81964), *pks9* (Trire2:106272), *pks1S* (Trire2:73621), *pks2S* (Trire2:73618). Those genes whose names are shown in color exhibited a consistent expression trend for both $\Delta pks4$ mutants. Vertical and horizontal bars indicate standard deviations for the wild type and the mutants, respectively. The images above the plots show the sampling stage of the *T. reesei* (T) strains under each condition of confrontation with *R. solani* (R).

chanical stability has so far been shown for melanins but not for the low-molecular-weight pigments formed by PKS4 orthologues mentioned above. In addition, some polyketides have been shown to be involved in sexual development (42–46) but none of them is a PKS4 orthologue, 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 *Aspergillus 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*, it 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 physical borders between genetically unique individuals are marked by thick melanized walls (bar-rage reaction) impermeable for competitive fungi, which could

also be the reason for the effect of PKS4 on vegetative compatibility for *T. reesei*.

An interesting consequence of *pks4* loss of function that has not been reported for any other PKS 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 downregulated 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, an antagonistic interaction with *R. solani* revealed that loss of *pks4* function influenced the expression of the other *pks* genes in different ways, with some being upregulated, some downregulated, and some not changed. From these data, we conclude that PKS4—or rather the function of its product in, for example, protection and defense against stress—is an important signal for the expression of these *pks* genes. This is definitely an area that requires further investigations.

Loss of function of *pks4* also led to a decreased synthesis of water-soluble inhibitory components by *T. reesei*, and it is tempting to speculate that they are 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 downregulated (see Fig. S4 in the supplemental material), it is, however, not possible to predict which ones are the responsible producers. In addition, these com-

ponents could also be products of other enzymes, such as non-ribosomal peptide synthases, of which *T. reesei* produces 10 (59).

Finally, it was interesting that deletion of *pk4* increased the production of VOCs by *T. reesei*. The chemical natures of the VOCs from *T. reesei* have not been identified yet, but their diversity compared to those from other *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-CoA.

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

ACKNOWLEDGMENTS

This study was supported by a grant of the Austrian Science Fund (FWF) to C.P.K. (P 21266). S.E.B. and B.P.K. were supported by funding from the U.S. DOE Biomass Program.

We thank Aldin Saracevic and Yuan Zhi-Lin for laboratory assistance and Bernhard Pummer for the SEM images.

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