Functional analysis of non-ribosomal peptide synthetases (NRPSs) in *Trichoderma virens* reveals a polyketide synthase (PKS)/NRPS hybrid enzyme involved in the induced systemic resistance response in maize

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Trichoderma virens genome harbours genes encoding 22 non-ribosomal peptide synthetases (NRPSs) with at least one complete module (containing adenylation, thiolation and condensation domains) and four PKS/NRPS (polyketide synthase/NRPS) hybrid enzymes. After a primary screen for expression of these 26 genes when mycelia of *T. virens* are in contact with maize roots, seven genes that are upregulated were selected for further study. Using homologous recombination, loss-of-function mutants in six of these were obtained (the seventh, tex2, was acquired from our previous studies). Plant assays in a hydroponics system revealed that all seven mutants retained the ability to internally colonize maize roots. However, a mutation in one of the PKS/NRPS hybrid genes impaired the ability of *T. virens* to induce the defence response gene pal (phenylalanine ammonia lyase), suggesting a putative role for the associated metabolite product in induced systemic resistance. Interestingly, the mutant retained its ability to induce another defence response gene aos (allene oxide synthase). We thus provide evidence that a PKS/NRPS hybrid enzyme is involved in *Trichoderma*–plant interactions resulting in induction of defence responses.

INTRODUCTION

*Trichoderma* spp. are a group of fungi whose application in agricultural settings results in significant economic impacts (Harman et al., 2010). As antagonists of other fungi, *Trichoderma* are the most widely used biofungicides (Verma et al., 2007; Lorito et al., 2010). They also produce numerous secondary metabolites, including potential antibiotics and anti-cancer compounds (Reino et al., 2008). Research in the past decade has revealed that *Trichoderma* spp. not only colonize the rhizosphere but also penetrate the roots (epidermis/outer cortex) and live as opportunistic symbionts (Harman et al., 2004). In this facultative/ atypical symbiosis, *Trichoderma* spp. derive nutrients from the plant host and, in return, provide protection against invading root and foliar pathogens and abiotic stresses (Shoresh et al., 2010; Harman et al., 2004; Vargas et al., 2009, 2011). The ability of *Trichoderma* spp. to boost resistance of plants is similar to that provided by plant-growth-promoting rhizobacteria (PGPR), such as *Pseudomonas fluorescens* (Bakker et al., 2007; Yedidia et al., 2003; Shoresh et al., 2010). During PGPR-mediated induced systemic resistance (ISR), the plants are ‘sensitized/primed’ (no major metabolic changes occur prior to challenge inoculation by a pathogen) in response to the presence of the bacteria in the root zone.

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Abbreviations: ET, ethylene; ISR, induced systemic resistance; JA, jasmonate; MAMP, microbe-associated molecular pattern; NRPS, non-ribosomal peptide synthetase; PGPR, plant-growth-promoting rhizobacteria; PKS, polyketide synthase; SA, salicylate; SAR, systemic acquired resistance.

Three supplementary figures are available with the online version of this paper.
(van Wees et al., 1999). In contrast, root colonization by *Trichoderma* per se induces both priming effects and transcriptomic/metabolic changes in plants, including the triggering of defence response genes encoding enzymes such as phenylalanine ammonia lyase (*pal*), hydroperoxide lyase (*hpl*) and allene oxide synthase (*aos*) (Shoresh & Harman, 2008; Djonović et al., 2007; Vargas et al., 2008; Viterbo et al., 2005; Shoresh et al., 2010; Mastouri et al., 2010). PGPR and *Trichoderma*-mediated ISR, is different from pathogen-triggered systemic acquired resistance (SAR), but both ISR and SAR require NPR1 (non-expressor of pathogenesis-related genes; Van der Ent et al., 2008). ISR is mediated by jasmonate (JA) and ethylene (ET) response pathways whereas SAR is dependent on and mediated by salicylate (SA) signalling. The ISR and SAR signalling diverge downstream of NPR1 because, unlike SAR, ISR is not marked by the transcriptional activation of pathogenesis-related genes (Van der Ent et al., 2008). In the case of pathogen–plant interactions, these systemic responses are the consequence of the local perception of microbe-associated molecular patterns (MAMPs) or other danger signals at the site of invasion (Boller & Felix, 2009). Perception of MAMPs (exogenous elicitors) or damage-induced molecular patterns by the pattern recognition receptors initiates an active defence response, called the basal immunity, in plants (Boller & Felix, 2009). This MAMP-triggered immunity (pathogen triggered immunity) is different from effector triggered immunity or R gene-based immunity (vertical immunity). This MAMP-triggered immunity is the local perception of microbe-associated molecular patterns (MAMPs) or other danger signals at the site of invasion (Boller & Felix, 2009). An earlier study suggests that *Trichoderma*-induced ISR is JA/ET signalling-dependent (Shoresh et al., 2005). Even though the details of signalling events are not known, a mitogen-activated protein kinase from *Trichoderma virens* and one from cucumber have been identified to be involved in ISR responses (Viterbo et al., 2005; Shoresh et al., 2006). The strong induction of *pal* in response to *Trichoderma* inoculation of roots is universal, and *pal* is considered to be activated by the JA/ET signalling (Shoresh et al., 2010). The plant phenylpropanoid pathway, of which PAL is a key enzyme, is responsible for the synthesis of a wide variety of secondary metabolic compounds, including lignins, salicylates, coumarins, hydroxycinnamic amides, flavonoid phytoalexins, pigments, UV light protectants and antioxidants (Mauch-Mani & Slusarenko, 1996; De Meyer et al., 1999). Root colonization by *Trichoderma* spp. also induces other components of the ISR mechanism, namely the genes *hpl*, *aos* and peroxidase (*prx*) (Viterbo et al., 2005, 2007; Vargas et al., 2008). *T. virens* induces ISR, but not SAR responses in maize; mutants of *T. virens* lacking the elicitor protein SM1 failed to induce ISR responses (Djonović et al., 2007).

With the identification of numerous MAMPs, effectors in plant pathogens, and receptors and R genes in plants (Boller & Felix, 2009; Bent & Mackey, 2007), the interaction biology of plants and pathogens is much better understood than the PGPR or *Trichoderma*–plant interactions. Even though the first report of induction of resistance by *Trichoderma* was some time ago (Bigirimana et al., 1997), we still do not have a clear understanding of the mechanisms of this interaction. *Trichoderma*-secreted xylanase, cellulase and swollenin induce localized plant reactions and necrosis (see Shoresh et al., 2010). SM1 is a hydrophobic, small, cysteine-rich, secreted protein and one of the most well-characterized elicitor-like proteins secreted by *T. virens* (Djonović et al., 2006, 2007; Vargas et al., 2008; Buensanteai et al., 2010). Two other well-characterized elicitors are the non-ribosomal peptides alamethicin (*T. viride*) and the 18-residue peptai-bols Tex1 (*T. virens*) (Leitgeb et al., 2007; Viterbo et al., 2007).

Induced resistance has the potential to revolutionize disease control in crops, and yet, after decades of research, it still remains outside the mainstream of crop protection (Walters, 2010). This is mainly due to inconsistency of performance and a lower level of protection compared with fungicides. Increased understanding of the pathways of activated and resistance mechanisms triggered by different agents may lead to the use of a cocktail of elicitors to provide more effective and reliable protection (Walters, 2010).

Fungi are known to produce a large number of secondary metabolites, and the role of these compounds in cross-kingdom communication is an active research area (Shank & Kolter, 2009). The genome sequences of many filamentous fungi have revealed large reserves of secondary metabolite-producing gene clusters, which are not expressed under standard laboratory conditions (Brakhage & Schroech, 2011). Presumably these secondary metabolites perform specialized functions in ecological niches. Many plant-pathogenic fungi produce phytotoxins such as HCToxin, victorin, sirodesmin PL, ergotamine and AM-toxin, by the activity of polyketide synthases (PKSs) and non-ribosomal peptide synthetases (NRPSs). These phytotoxins act as virulence factors (Walton et al., 2004; Hoffmeister & Keller, 2007; Walton, 2006; Johnson et al., 2000; Elliott et al., 2007). Additionally, a PKS/NRPS hybrid enzyme (Ace1) of *Magnaporthe grisea* acts as an avirulence factor, triggering resistance in selective rice cultivars harbouring the corresponding resistance gene Pi33 (Böhnert et al., 2004; Collemare et al., 2008a, b; Fudal et al., 2007; Khalid et al., 2008; Vergne et al., 2007). A recent comparative genomics study revealed that the genomes of the two biocontrol species *T. virens* and *Trichoderma atroviride* harbour a large number of NRPS and PKS genes, and the genome of *T. virens* contains more NRPSs than any other filamentous fungi studied so far (Kubicek et al., 2011). The diverse niches that *Trichoderma* spp. occupy (broad host mycoparasites, active colonizers of the rhizosphere and spermosphere, root endophytes, and saprophytes on decaying wood and soil organic fractions) suggest that some of these secondary metabolites are involved in communication with plant roots and interactions with other fungi. Recently, Velázquez-Robledo et al. (2011) demonstrated that the deletion of *ppt1*...
(gene encoding a 4-phosphopantetheinyltransferase) in *T. virens* resulted in mutants that were unable to synthesize non-ribosomal peptides and co-cultivation with *Arabidopsis* compromised SA and camalexin defence responses. Their results suggested that some of these secondary metabolites are involved in plant–*Trichoderma* interactions. Thus, our working hypothesis was that some fraction of the genes encoding NRPSs or PKSs in *T. virens* are involved in communication with plants, resulting in the induction of defence responses. Screening the expression of genes encoding 22 putative NRPSs and four putative PKS/NRPS hybrid enzymes demonstrated that seven genes were upregulated when the wild-type strain was in contact with maize roots. We report here a functional analysis of the seven genes encoding NRPSs of *T. virens* to determine their role in the induction of defence responses in maize.

**METHODS**

**Fungal strains and growth conditions.** The wild-type strain of *T. virens* (Gv29-8) isolated by our laboratory, has been sequenced ([http://genome.jgi-psf.org/TriviGv29_8_2/TriviGv29_8_2.home.html](http://genome.jgi-psf.org/TriviGv29_8_2/TriviGv29_8_2.home.html)) and the strain has been deposited at the Fungal Stock Center (Kansas City, MO). The Δtex2 mutant was obtained from our previous study ([Mukherjee et al., 2011](http://mic.sgmjournals.org/)). All the mutants were cultivated on either potato dextrose agar (PDA, Difco) with or without hygromycin B amendment or on Vogel's minimal medium supplemented with 1.5% sucrose (VMS). For root colonization studies, excised roots were plated on modified GVSM Vogel's minimal medium supplemented with 1.5% sucrose (VMS). For root colonization studies, excised roots were plated on modified GVSM (PDA supplemented with 50 mg rose Bengal, 50 mg streptomycin sulfate, 50 mg rifampicin, 500 mg sodium propionate, 200 µg Benomyl 50WP and 2.5 ml Triton; all per l) ([Park et al., 1992](http://mic.sgmjournals.org/)). The *Escherichia coli* strain Top10 was used for cloning experiments. All the cultures were stored at −80 °C in glycerol for genetic stability.

**Identification of the *T. virens* NRPSs and study of the modular structures, gene clusters and homology.** The putative NRPSs and the PKS/NRPS hybrid enzyme sequences were downloaded from the *T. virens* 29-8 genome site ([http://genome.jgi-psf.org/TriviGv29_8_2/TriviGv29_8_2.home.html](http://genome.jgi-psf.org/TriviGv29_8_2/TriviGv29_8_2.home.html)) and analysed for the presence of domains. The NRPS sequences of *T. atrorubens* and *T. reesei* were obtained from their respective genome sites ([http://genome.jgi-psf.org/Triat2/Triat2.home.html](http://genome.jgi-psf.org/Triat2/Triat2.home.html) or [http://genome.jgi-psf.org/Trir2/Trir2.home.html](http://genome.jgi-psf.org/Trir2/Trir2.home.html)). The homology-based relatedness of the selected proteins to the existing sequences in GenBank was analysed by [BLAST](http://blast.ncbi.nlm.nih.gov/Blast.cgi) algorithm on the NCBI site ([http://blast.ncbi.nlm.nih.gov/Blast.cgi](http://blast.ncbi.nlm.nih.gov/Blast.cgi)).

**Induction of NRPS genes by maize roots.** Root inducibility of the NRPSs was determined in a hydroponic system containing maize seedlings as described previously ([Djonović et al., 2006](http://mic.sgmjournals.org/)). Maize seeds (Silver Queen hybrid) were surface-disinfected with 10% H2O2 for 2 h, rinsed with distilled water, and placed in moist chambers for 3 days for germination. Seedlings with similar root lengths (approx. 5 cm) were selected for growth in hydroponic chambers containing 0.5 × Murashige and Skoog medium with vitamin supplements (Sigma) and 0.05% sucrose (MSGs). Two days after the seedlings were placed in the hydroponic chambers, they were inoculated with mycelia of *T. virens* (100 mg hyphal tissue harvested from a 48 h liquid culture per chamber) and incubated on a shaker at 25 r.p.m. under 14:10 h light/day cycles at 23–25 °C. Chambers with *T. virens* cultured in MSGs only served as controls. Three days after inoculation with fungal mycelia, the seedlings were removed from the hydroponic system along with any fungal growth on/in the roots. The roots and adhering mycelia of *T. virens* were flash frozen and ground in liquid nitrogen, and the RNA was extracted with TRIReagent. The relative abundance of *T. virens* in colonized roots or from axenic culture was determined by qRT-PCR with histone *h3* primers. The relative abundance of NRPS expression in mycelia of *T. virens* grown with or without roots was determined by RT-PCR. Real-time RT-PCR analysis of two apparently highly induced genes (*tex2* and *tex13*; Fig. 1) was performed (with three replicates) to confirm and quantify the mRNA induction in response to the presence of roots. The primers used for this and other experiments in this study are listed in Supplementary Table S1 ([available with the online version of this paper](http://mic.sgmjournals.org/)).

**Loss-of-function mutants and their growth and morphology.** The loss-of-function mutants for the NRPS genes that were differentially induced in the presence of maize roots were obtained by homologous recombination using the hygromycin B resistance gene *hph* expressed under the *Aspergillus nidulans* trpC promoter as a selectable marker. The *hygR* (hygromycin B resistance) cassette was taken from pAT-BS plasmid ([Mukherjee et al., 2003a](http://mic.sgmjournals.org/)). Based on the size of the ORFs, we performed gene disruption by either single ( tex5, 6, 7, 8 and 13 ) or double ( tex25 ) cross-over Fig. 2(a, c). Both of these strategies have been successful in previous functional studies of *T. virens* genes ([Mukherjee et al., 2003b](http://mic.sgmjournals.org/); Mukherjee & Kenerley, 2010; Djonović et al., 2007]. Disruption of tex2 was by single cross-over with selection for hygromycin B-resistant colonies ([Mukherjee et al., 2011](http://mic.sgmjournals.org/)). For each gene disrupted by single cross-over homologous recombination, a primer pair was designed to amplify part of the ORF (around the centre) with *Sal*I and *Kpn*I sites integrated in the primer sites. The amplified fragment was digested with *Sal*I and *Kpn*I, and ligated to pAT-BS predigested with these two enzymes. The resulting construct was used for transformation of Gv29-8 protoplasts as described previously ([Thomas & Kenerley, 1989](http://mic.sgmjournals.org/)). Stable transformants were tested for a homologous recombination event in the respective locus by PCR using M13R universal primer and a primer from outside the fragment used for making the knockout construct (designated ‘Outs’). The positive colonies (i.e. where a fragment was amplified) were further purified by single spore isolation; gene disruption was confirmed by PCR using a primer pair located outside the region used for homologous recombination. The clean (no amplification) mutants were used for further studies. For double cross-over homologous recombination, part of the ORF was deleted by replacing with the *hygR* cassette. The gene deletion was confirmed by amplification with internal (to the portion of the ORF deleted) primers. We obtained at least two mutants for each gene under study and all the experiments were performed independently with two different mutants. The colony growth of the mutants was compared to the wild-type inoculated at the edge of VMS agar plates over a period of 7 days. Southern hybridization was performed with the Δtex13 mutants to confirm that the phenotype obtained (see below) was not due to ectopic integration of the construct.

**Ability of the mutants to internally colonize roots and to induce defence responses in maize.** Ability of the mutants to internally colonize maize roots was studied in a hydroponic system as described above. Three days after inoculation with selected fungal strains, the seedlings were removed from the hydroponic system, and the roots were excised from the seedlings. The roots were rinsed with tap water to remove the fungal mycelia loosely adhering to the roots and surface-disinfected with 1% NaClO for 2 min. The roots were sectioned into 1 cm pieces and plated on modified GVSM medium ([Park et al., 1992](http://mic.sgmjournals.org/)). The number of colonies developing from roots was monitored over 3 days and expressed as colonies (cm root length)⁻¹. The leaves of the same plants were frozen in liquid nitrogen and used.
for extraction of RNA. The expression level of two defence-related genes, *pal* and *aos*, in maize seedlings was studied by RT-PCR as described previously (Buensanteai et al., 2010). The loss-of-induction of *pal* in *Dtex13*-inoculated plants was further confirmed by qRT-PCR as described previously (Vargas et al., 2008). The fold increase in mRNA was calculated relative to the glyceraldehyde 3-phosphate dehydrogenase gene (*gapc*) as a housekeeping control.

**Phylogenetic analysis of PKS/NRPS hybrid enzymes from *Trichoderma* spp. and other fungi.** The protein sequences of *T. virens*, *T. reesei* and *T. atroviride* PKS/NRPS hybrid enzymes were downloaded from the respective genome sites (http://genome.jgi-psf.org/). Other fungal orthologues, based on protein BLAST (BLASTP) analysis on the NCBI server, were downloaded from GenBank. The sequences corresponding to the 'A', 'PK' or 'KS' domains of PKS/NRPS hybrid enzymes were identified using the Pfam 25.0 server of the Sanger institute (http://pfam.sanger.ac.uk/). The putative secondary metabolism-related genes, such as cytochrome P450s, oxidoreductases, methyl-transferases etc., in the neighbourhood of the *tex13* PKS/NRPS gene in the genome of *T. virens* were identified on the corresponding genome site.

For phylogenetic analysis, sequences were submitted to the automated alignment in CLUSTAL_X (Thompson et al., 1997). Gblock server (http://molevol.cmima.csic.es/castresana/Gblocks_server.html; Castresana, 2000) with less stringent selection of options was used to retrieve unambiguous regions of each domain’s alignment. Of 452 aa residues of the A domain, 340 (75%) were used for the analysis; from 399 aa positions of the KS domains, 370 (92%) were used; and from 349 aa positions of the AT domains, 313 (89%) were used. The interleaved NEXUS file was formatted using PAUP*4.0b10 (Swofford, 2002). The resulting matrix was subjected to Bayesian analysis using MrBayes v 3.1.2 (http://mrbayes.sourceforge.net/) with 1 million generations of Metropolis-coupled Markov chain Monte Carlo. Bayesian posterior probabilities (PP) were obtained from the 50% majority rule consensus of trees sampled every 100 generations after removing the first 300 trees. PP values lower than 0.95 were not considered significant and thus are not shown on the resulting phylograms.

**RESULTS**

**T. virens harbours 22 NRPSs with at least one complete module and four PKS/NRPS hybrid enzymes**

The *T. virens* genome contained 22 NRPS enzymes with at least one complete module (Table 1). The size of the proteins ranged from 547 to 20,891 amino acids. *tex1* and *tex2* both encode peptaibol synthetases, which have been described previously (Wiest et al., 2002; Mukherjee et al., 2011). An additional gene predicted to be a peptaibol synthetase with seven complete modules arranged in a linear fashion (*tex3*) was homologous to *tex1*. The functions of most of the NRPSs could not be predicted based upon homology, except for ferrichrome synthetase (*Tex10*) and two putative siderophore synthetases (*Tex20* and *Tex21*). The *T. virens* genome harboured three putative ETP synthetases, related to gliotoxin (*Tex18*) and sirodesmin PL synthetases (*Tex17* and *Tex19*). There were four PKS/NRPS hybrid enzymes (*Tex11–14*) in the genome of *T. virens* compared with one in *T. atroviride* and two in *T. reesei*.

**NRPSs induced by maize roots.**

Of the 26 NRPS (including four hybrid enzymes) genes tested by RT-PCR for induction in the presence of maize roots, *tex2*, *tex5–8*, *tex13* and *tex25* were strongly upregulated (Fig. 1). The expression of two of these, *tex2* and *tex13*, was further confirmed by qRT-PCR. The results demonstrated that *tex2* and *tex13* were induced by the presence of maize roots to greater than 6- and 40-fold, respectively, compared with the induction levels when grown alone.
Isolation of loss-of-function mutants

Loss-of-function mutants were obtained for genes \( \text{tex5–8} \) and \( \text{tex13} \) using single cross-over homologous recombination and for \( \text{tex25} \) by using the double cross-over strategy (Fig. 2). Homologous recombination and gene disruption were confirmed by using a PCR-based strategy and Southern hybridization. All mutants, when grown on VMS agar, sporulated similar to the wild-type, but the growth rates varied; \( \Delta \text{tex7, 8 and 13} \) grew faster than the wild-type, whereas \( \Delta \text{tex5, 6 and 25} \) grew slower (Supplementary Fig. S1, available with the online version of this paper).

\( \Delta \text{tex13} \) mutants are defective in induction of \( \text{pal} \) in maize leaves

Mutants representing each of the disrupted genes were evaluated for their ability to colonize plant roots and induce the ISR-related defence-response genes \( \text{pal} \) and \( \text{aos} \). None of the mutants were defective in root colonization as the number of colonies (cm root length)\(^{-1} \) was not significantly different among the strains tested (Supplementary Fig. S2a). All mutants were able to upregulate \( \text{aos} \) in maize shoots, and all except \( \Delta \text{tex13} \) mutants were able to upregulate \( \text{pal} \) expression (Supplementary Fig. S2b). The differential upregulation of \( \text{aos} \) and \( \text{pal} \) by \( \Delta \text{tex13} \) mutants was further confirmed by qRT-PCR (Fig. 3a). The defect in upregulation of \( \text{pal} \) by \( \Delta \text{tex13} \) mutants was confirmed by qRT-PCR (Fig. 3b).

\text{Tex13} \) is phylogenetically distanced from other fungal PKS/NRPSs

A phylogenetic analysis of the A, KS and AT domains of fungal PKS/NRPS hybrid enzymes revealed that such
Table 1. The NRPSs of *T. virens* with at least one complete module

Tex13 and Tex14 belong to the same cluster. Tex16, Tex24 and Tex26 belong to the same cluster. A, Adenylation domain; T, thiolation domain; C, condensation domain; DH, dehydrogenase; NB4, nucleotide-binding 4 (male sterility protein); KS, keto-acyl synthase; AT, acyltransferase 1; MT12, methyltransferase 12; KR, keto-reductase. The proteins with at least one complete module (A, T and C domains) are listed here. The protein IDs are according to those listed here: [http://genome.jgi-psf.org/Trive1/Trive1.home.html](http://genome.jgi-psf.org/Trive1/Trive1.home.html). The orthology was derived by BLASTP analysis on the NCBI site.

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<th>Length (aa)</th>
<th>Modular structure/domain organization</th>
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<td>16510</td>
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</tbody>
</table>

Orthologues included in the table are from: AAM78457, AAX63399, AAX82227, AN7884, BAE56801, XP_001932133, CAA82227, CAG28797, XP_002486604, EED21789, CAO91861, CAG28797, AAT28740, EED43492, BAE56801, EED43492, BAE56801, EED43492, BAE56801, EED43492, BAE56801, EED43492, BAE56801, EED43492, BAE56801, EED43492, BAE56801, EED43492, BAE56801, EED43492, BAE56801, EED43492, BAE56801, EED43492, BAE56801, EED43492, BAE56801, EED43492.
proteins can be grouped into three statistically supported clades, typified by the equisetin synthase of *Fusarium heterosporum* (EqiS; Sims et al., 2005), *Gibberella moniliformis* fusarinC (FusS; Song et al., 2004) and *Cordyceps bassiana* tenellin toxin (TenS; Eley et al., 2007)/*Penicillium expansum* cytochalasin synthase CheA/CytS (Schümann & Hertweck, 2007) (Fig. 4). Tex12 from *T. virens* belongs to the clade of EqiS and was most related to *Magnaporthe grisea* Ace1 on phylograms for A and KS domains, while it was not observed for AT domains. *T. virens* Tex14 and *T. reesei* 59315 are closely related to *G. moniliformis* fusarinC synthase (FusS clade) which was confirmed on all three trees. Tex11 and *T. reesei* 58285 have TenS as the next phylogenetic neighbour, while Tex13, which is involved in ISR response, occupies the most basal position in this clade with no relatively related neighbours. The most interesting finding is the position of the only PKS/NRPS identified from *T. atroviride* (protein ID 51715); on the A domain tree it might be assigned to the EqiS clade, while on the AT domain tree it is basal to the TenS clade. The position of this protein on the KS domain is not resolved.

**tex13 is part of a large putative secondary metabolism gene cluster**

An examination of the *tex13* region on the *T. virens* genome site revealed that this gene was part of a large (83.4 kb) putative secondary metabolism gene cluster on scaffold 6 (Supplementary Fig. S3), which encompassed two PKS/NRPSs (*tex13* and *tex14*). The other members of this putative cluster were (in sequence) a lipase/thiolase-like protein (ID 10060), an NRPS-like protein (60181), a cytochrome P450 (60180), aldehyde dehydrogenase (29283), MFS transporter (28835), NmrA-like protein (29580), a protein with peptidase S15/glutathione S-transferase domains (53838), aspartic peptidase (53837), FAD-binding monooxygenase (60172), UbiA prenyltransferase (28484), a hypothetical protein with similarity to pyoverdine biosynthesis protein (28177), MFS (28108), short chain dehydrogenase/reductase SDR (60168), fungal-specific transcription factor (28724), MFS (28823), a serine/threonine protein kinase-related protein (29761), a hypothetical protein (60164), cytochrome P450 (29262), NAD(P) binding/dehydrogenase (60161), NAP-binding protein (53832), a hypothetical protein with similarity to a methyltransferase SirN of *A. fumigatus* (60159) and a copper amine oxidase (28132). Among all the fungal PKS/NRPS clusters examined, only the *Ace1* cluster of *M. grisea* and the putative *tex13/tex14* cluster of *T. virens* have two PKS/NRPS genes (data not shown).

**DISCUSSION**

Fungal mutualists confer a wide range of benefits to their hosts (Porras-Alfaro & Bayman, 2011), including enhanced host disease resistance (Djonić et al., 2007; Pozo & Azcón-Aguilar, 2007; Viterbo et al., 2007), drought tolerance (Hauge & Fester, 2005), improved nutrient uptake and
This gene is expressed specifically in the appressoria of the fungus M. grisea (Bohnert et al., 2004). The expression of induced defences is mediated by complex signalling networks in which the plant hormones JA and SA play key roles (Smith et al., 2009). The symbiotic fungus T. virens protects crop plants from infection by fungal and bacterial pathogens through induction of defence responses known as ISR (Shoresh et al., 2010; Djonović et al., 2007). Understanding the mechanisms of induced resistance and identifying novel elicitors would greatly enhance our capability to commercially exploit this eco-friendly approach to plant disease management (Lorito et al., 2010). Even though the phenomenon of Trichoderma-mediated induced resistance has been known for more than a decade, only a few elicitor molecules such as the 18-residue peptaibol (Tex1) and SM1 have been identified (Viterbo et al., 2007; Djonović et al., 2006). The recent genome sequencing of T. virens enabled the identification and high-throughput analysis of genes encoding NRPSs. Since secondary metabolites are known to be involved in interactions of plant pathogens with their host fungi, we hypothesized that some of these putative secondary metabolism-related genes would be involved in communication during root colonization by T. virens. The analysis of the NRPSs from T. virens using expression studies followed by evaluation of the loss-of-function mutants revealed that a PKS/NRPS, Tex13, is involved in upregulation of the defence gene pal, but not aos. The only other PKS/NRPS that is known to be involved in plant–fungus interactions is Ace1 of M. grisea. Ace1 putatively encodes an unidentified secondary metabolite that acts as an avirulence factor during interactions with rice cultivars harbouring the Pi33 ‘R’ gene (Böhnet et al., 2004; Collemare et al., 2008a, b; Fudal et al., 2007; Khaldi et al., 2008; Vergne et al., 2007). This gene is expressed specifically in the appressoria of M. grisea, and the metabolite produced by this gene cluster has not yet been identified. This gene cluster also includes another PKS/NRPS gene syn2. The T. virens genome harbours four PKS/NRPS genes compared with one in T. atroviride and two in T. reesei (Kubicke et al., 2011). Interestingly, only one of these (tex13) was induced (more than 40-fold) during interactions of T. virens with maize roots (Fig. 1). A Δtex13 strain failed to upregulate the key defence gene pal in maize shoots. tex13 is also part of a large putative secondary metabolism-related gene cluster that includes another PKS/NRPS, Tex14. However, phylogenetically, tex12 and not tex13 is related to M. grisea ace1 (Fig. 4). Perhaps there was no surprise that these two different gene clusters containing Ace1 or Tex13 PKS/NRPS as the core enzyme evolved independently in two fungi (M. grisea and T. virens), both belonging to the class Sordariomycetes, as these two fungi occupy different ecological niches (M. grisea is a hemibiotrophic leaf pathogen, whereas T. virens is a mycoparasite and a facultative root symbiont). While the ace1 product is an avirulence factor, the putative tex13 product (or the enzyme per se) is an elicitor-inducing molecule in maize. A careful observation of the phylogenetic trees (Fig. 4) also revealed that the phylogenetic relationship between the PKS/NRPSs does not follow the evolutionary relationships (e.g. Sordariomycetes vs Eurotiomycetes), even though all fungal PKS/NRPS hybrids fall into a single, well-supported monophyletic group, suggesting a single origin (Bushley & Turgeon, 2010).

Another interesting phenomenon observed in this study was that while Gv29-8 and its elicitor protein SM1 upregulate both aos and pal (deletion of sm1 results in loss of regulation of both the genes; Djonović et al., 2007), deletion of tex13 results in loss of upregulation of pal but not of aos (Fig. 3). This is indeed very significant and presents a new paradigm in ISR. PAL (the first enzyme in the phenylpropanoid pathway that produces secondary metabolites including SA), AOS and HPL (key enzymes of the oxylipin pathway known to be involved in the

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**Fig. 3.** Defense-related gene [(i) pal, (ii) aos] expression in maize in response to inoculation of roots with the wild-type (WT) or Δtex13 mutant. The expression was studied by RT-PCR (a) as well as qRT-PCR (b), using gapc as a loading control. Growth of the wild-type and Δtex13 mutant on VMS after 5 days (c). ‘C’ denotes control (maize seedlings inoculated without any Trichoderma inoculation).
production of green leaf volatiles and JA; Kessler et al., 2004; Matsui, 2006) are markers for ISR (Distefano et al., 2008). Our preliminary attempt to identify the Tex13-assembled product was not successful as we failed to determine any difference in secondary metabolite profiles between the wild-type and the Δtex13 mutant grown in the presence of roots. Thus, we are unable to say if the putative secondary metabolite acts as a MAMP that is recognized by plants triggering an immune response. Further studies, such as a mutational analysis of the catalytic domain would be necessary to ascertain whether the protein or the metabolite acts as an elicitor. The major hurdle in identifying the secondary metabolite catalysed by the Tex13 cluster is the very specific expression in the plant roots, which could be overcome by using the strategy of activating the cluster using novel genetic tools (Gressler et al., 2011; Brakhage & Schroeckh, 2011).

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