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A putative terpene cyclase, *vir4*, is responsible for the biosynthesis of volatile terpene compounds in the biocontrol fungus *Trichoderma virens*

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ABSTRACT

A putative terpene cyclase *vir4*, which is a member of a secondary metabolite cluster, has been deleted in *Trichoderma virens* to determine its function. The deletion mutants were compared for volatile production with the wild-type as well as two other *Trichoderma* spp. This gene cluster was originally predicted to function in the synthesis of viridin and viridiol. However, the experimental evidence demonstrates that this gene cluster is involved in the synthesis of volatile terpene compounds. The entire *vir4*-containing gene cluster is absent in two other species of *Trichoderma*, *T. atroviride* and *T. reesei*. Neither of these two species synthesizes volatile terpenes associated with this cluster in *T. virens*. We have thus identified a novel class of volatile fungal sesquiterpenes as well as the gene cluster involved in their biosynthesis.

1. Introduction

Secondary metabolites produced by filamentous fungi are extremely diverse in structure and function, providing a source of novel compounds for pharmaceutical, agricultural, and medical markets (Keller and Hohn, 1997; Reino et al., 2008; Osbourn, 2010). Compounds such as antibiotics (penicillin) and hormones (gibberellins) have yielded positive benefits in the areas of medicine and food production for many decades. However, mycotoxins, including fumonisins and aflatoxin, have substantial negative impacts on food production each year, and have the potential to negatively affect both humans and livestock. Other metabolites are important virulence factors for disease in mammals and plants, such as gliotoxin from *Aspergillus fumigatus* and the metabolite produced by ACE1 gene in *Magnaporthe oryzae*, respectively (Fudal et al., 2007; Reino et al., 2008; Osbourn, 2010).

Trichoderma spp. are considered to be an abundant source of secondary metabolites, as 373 different compounds have been identified according to the Antibase database, some shown to have medicinal or agronomic importance (Sivasithamparam and Ghisalberti, 1998; Laatsch, 2007; Reino et al., 2008). As a member of this genus of filamentous fungi, *Trichoderma virens* produces several secondary metabolites that have been suggested as playing a role in its ecological development (Howell et al., 1993). Examples of these compounds include gliotoxin, gliovirin, viridin and viridiol. Volatile organic compounds (VOCs) represented by several chemical classes (e.g. alcohols, aldehydes, esters and terpenes) are produced by many species of fungi including *Trichoderma* (Bruce et al., 2000; Vinale et al., 2008). VOCs are involved in biological processes such as plant defense against predators, parasites and pathogens during biocontrol interactions, competition with other microorganisms, and production of reproductive structures in species of *Trichoderma* (Sivasithamparam and Ghisalberti, 1998; Bruce et al., 2000; Humphris et al., 2002). Some *Trichoderma* spp. are commonly associated with rotting wood, and production of VOCs has been shown to inhibit the growth of competing basidiomycete wood decay fungi in this environment (Bruce et al., 2000; Humphris et al., 2001). An analysis of volatile compounds produced by *Trichoderma atroviride* demonstrated the presence of 25 fungal metabolites including alcohols, ketones, alkanes, furanes, pyrones, mono- and sesquiterpenes (Schmoll et al., 2009; Stoppacher et al., 2010). However, the genetics of the biosynthesis of these compounds has not been previously documented in any *Trichoderma* spp.

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Terpene cyclases, are a diverse family of enzymes found in both plants and microorganisms, that catalyze the cyclization of prenyl diphosphate chains and are the first committed steps in the formation of monoterpenes, sesquiterpenes, and diterpenes (Christianson, 2006; Davis and Croteau, 2000). Terpenoids are derived from the precursor isopentnyl diphosphate (IPP), which is altered by prenyltransferases to produce geranyl diphosphate (GPP), farnesyl diphosphate (FPP), and gernylgeranyl diphosphate (GGPP). These precursors function as the divergent branch points for the production of monoterpenes (from GPP), sesquiterpenes (from FPP), and diterpenes (from GGPP) through the action of terpene cyclases.

Previously, a partial gene cluster (designated as the *vir* cluster) was identified in the "P" strain IMI 304061 of *T. virens* by suppression subtractive hybridization with wild-type strain against a non-conidiating mutant deficient in secondary metabolite biosynthesis (Mukherjee et al., 2006). A cosmid clone containing a cyclase gene was sequenced and several full length genes were identified as members of a putative secondary metabolism related gene cluster. These genes included three cytochrome P450s and a terpene cyclase. The genes and organization of this cluster closely resembled that of an un-annotated gene cluster in *A. oryzae,* the *Gibberella fujikuroi* gibberellin cluster, and the *Fusarium graminearum* trichothecene cluster. To elucidate the function of this gene cluster and determine the compounds produced, we have deleted a putative terpene cyclase (*vir4*) and compared the volatile profiles of generated mutants with the wild-type *T. virens* and two additional *Trichoderma* spp. (*T. atroviride* and *Trichoderma reesei*) that do not harbor this cluster.

2. Materials and methods

2.1. Strains and culture conditions

Two strains of *T. virens* were used in this study: Gv29-8 (wild-type) and Tv10.4 (an arginine auxotrophic strain) (Baek and Kenerley, 1998). Strains of *Trichoderma* (*T. virens*, *T. atroviride*, and *T. reesei*) and transformants were maintained on potato dextrose agar (PDA) at 27 °C. Vogel's minimal medium (Vogel, 1956) supplemented with 1.5% sucrose (VMS) was used to screen for deletion mutants and PDA with 100 μg/mL hygromycin (PDA-H) was used to screen *vir4* complemented strains.

Escherichia coli XL1-Blue Supercompetent Cells (Stratagene) were grown on Luria Bertani agar (LBA) at 37 °C. Liquid cultures were grown at 250 rpm and ampicillin (Research Products, Inc.) was used as a selective agent.

2.2. Bioinformatic analysis

The full cluster was described previously for *T. virens* (Mukherjee et al., 2012). The genomes of *T. reesei* and *T. atroviride* (www.genome.jgi-psf.org) were surveyed for the presence of the *vir4* cluster. The full cluster in *Aspergillus* spp. was identified by walking on their respective scaffolds (http://www.aspgd.org/). A protein BLAST was performed at the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/) using the predicted amino acid sequence encoded by *vir4*. The tree was constructed with PhyML 3.0 (Guindon et al., 2010) after a sequence alignment using ClustalX (Larkin et al., 2007). A sh-like aLRT statistical test for computing branch support was used for the tree construction (Anisimova and Gascuel, 2006). A maximum likelihood phylogeny was constructed using PhyML software (http://www.atgc-montpellier.fr/phyml/) with a Blosum62 substitution model and a bootstrap of 100 (Guindon et al., 2010).

2.3. DNA manipulation

Genomic DNA from *T. virens* was extracted as previously described (Xu et al., 1996). Sequencing reactions were completed at the Texas A&M University Gene Technologies Lab. Southern blot analysis was performed according to a common protocol (Sambrook et al., 1989). Probes were labeled using a Random Primer DNA Labeling Kit (Takara) and Southerns were hybridized using Ultrahyb (Ambion) at 42 °C overnight. DNA amplified by PCR was purified using the Wizard® SV Gel and PCR Clean-up System (Promega). Plasmids were purified using the Wizard® *Plus* SV Miniprep kit (Promega).

2.4. Generation of vir4 deletion and complementation vectors

The sequence of the *vir4* gene (DQ456846) (Mukherjee et al., 2006) from *T. virens* strain IMI 304061 was used to BLAST against the *T. virens* Gv29-8 genome (http://genome.jgi-psf.org). One gene showed high nucleotide sequence similarity and was designated as *vir4*. The full sequence was obtained from the genome database and the *vir4* deletion vector (pFKC1) was constructed by cloning the upstream and downstream flanking regions of *vir4* into pJMB4 (Baek and Kenerley, 1998), which contains the *arg2* gene as a selectable marker for complementation of strain Tv10.4. The upstream region was inserted into the *EcoR*V site, and the downstream region inserted into the *Sma*I site using blunt end cloning. Thymine tags were added to the blunt digestion sites by the addition of 0.1 mM dTTPs, 2 units of Taq polymerase, and 1X PCR buffer to the digestion and incubated for twenty minutes at 72 °C. The pFKC1 vector was linearized at the *Apa*I restriction digest site before transformation into strain Tv10.4 (Baek and Kenerley, 1998).

To construct the *vir4* complementation vector (pFKC14), the *vir4* coding region along with 2000-bp of the promoter and 1000-bp of the terminator regions were amplified using Phusion High Fidelity Taq Polymerase (Finnzymes). Adenine overhangs were added to the amplified product by the following reaction. Four micrograms of purified DNA were combined with 0.2 mM dATP, DNA Taq polymerase (Promega), and 1X Taq polymerase buffer and incubated for twenty minutes at 72 °C. The reaction was used directly to clone into pGEM-T easy vector (Promega) (pFKC14). To obtain complementation strains, pFKC14 and pCSN43, a vector containing the *hygB* gene as a selection marker, were co-transformed into the deletion mutant Δvir4-137.

2.5. Transformation and screening of transformants

Protoplasts of *T. virens* were produced and transformed with linearized DNA by the method of Baek and Kenerley (1998). Stable transformants were selected by sequential transfer of conidia to agar slants of VMS, PDA, and VMS. Viable transformants on the last round of VMS were screened by PCR using a primer upstream of the *vir4* region cloned into pJMB4, Vir4-11 (5′-CAACACGGCTTAGATTGAGTCA-3′) and a primer within the *arg2* gene, Vir4-12 (5′-GGTGAGTCACGGTAACACC-3′). These primers were used to amplify a product specific for a homologous recombination event. PCR amplification was performed for 35 cycles with each cycle framed as 30 s at 95 °C, 30 s at 52 °C, and 2 min 30 s at 72 °C. Once positive transformants were identified from this screening, they were further analyzed with primers specific for *vir4* [Vir4F (5′-CAACACTGCCTGTCATCGCTA-3′) and Vir4R (5′-CAGCCATCTTGGAGT GAGTCA-3′) with 35 cycles (30 s at 95 °C, 30 s at 55 °C, and 1 min at 72 °C)] to verify the deletion of the gene. Strains containing the correct insert, but without the 300 bp fragment of *vir4* were further analyzed by Southern blotting. To identify stable complementation mutants, conidia of isolated colonies were transferred sequentially from PDA-H, PDA, to PDA-H. Resulting stable

transformants were initially screened by PCR using the primers Vir4F and Vir4R prior to verification by Southern blot.

2.6. Extraction of RNA and evaluation of cluster expression

Real-time quantitative PCR (qPCR) was performed to examine expression of genes within the *vir4* cluster and to observe putative changes in a *vir4* deletion mutant. Gv29-8 and Δvir4-137 were grown in potato dextrose broth (PDB) for 2 days. Mycelia was washed, ground in liquid nitrogen, and RNA extracted using TRIZOL® reagent (Gibco-BRL). The DNA-free™ DNase Treatment and Removal kit (Ambion) was used to remove any residual DNA. This RNA was used for qPCR of the gene cluster using actin (Vargas, 2008) as a control reference. All primers used for qPCR in this study are presented in Table S1. The difference in expression of the cluster was examined using the Thermo Scientific Verso SYBR 1-Step QRT + Rox Kit. The real-time reactions were performed in a 25 μL reaction containing 1X Verso SYBR mix and 200 nM primers. Each reaction contained 100 ng total RNA and was loaded on the 7500 Fast real time PCR system (Applied Biosystems). Data were analyzed using three biological replicates, and the absence of primer dimers was confirmed by creation of a dissociation curve. The expression of each gene was normalized using the formula $2^{-\Delta\Delta CT}$ per manufacturer's instructions (PE Applied Biosystems, 2001).

2.7. Measurement of non-volatile secondary metabolite production in vir4 transformants

The arginine deficient mutant Tv10.4 and Δvir4-137 were grown in malt extract broth (MEB) for 4 days, and culture filtrate was extracted using ethyl acetate. Samples were air dried by passive evaporation, resuspended in methanol, and 20 μL was spotted on a TLC Silica gel 60 F254 plate (EMD). A mobile phase of 70:30:0.5 chloroform:acetone:formic acid (v/v) was used to separate samples on the TLC plate. The plate was allowed to dry, viewed at a UV of 254 nm, and photographed. In addition, Gv29-8 and three *vir4* deletion mutants (Δvir4-61, Δvir4-87, Δvir4-137) were grown in PDB for three days, and culture filtrate was extracted as above. Resuspensions in methanol were analyzed by HPLC (USDA-ARS-Southern Plains Agricultural Research Center).

2.8. Chemicals and standards for volatile analysis

The chemical n-hexane (95%) was obtained from J.T. Baker (Deventer, The Netherlands) and PDA from Merck (Darmstadt, Germany). Following standards were purchased from Sigma–Aldrich (Vienna, Austria): 2-heptanone (99%), 3-octanone (>98%), 3-octanol (95%), 1-octen-3-ol (98%), 2-nonanone (99%), 2-undecanone (99%), trans-nerolidol (85%), alpha-terpinene (95%), phenylethyl alcohol (99%), 6-pentyl-alpha-pyrone (96%), alpha-phellandrene and the alkane standard solutions (mixture of C_8–C_{20} and C_{21}–C_{40} in n-hexane). For the production of the alkane standard mix C_5–C_{10}, pentane (99%), octane (99%) and nonane (99%) were obtained from Sigma–Aldrich (Vienna, Austria), n-hexane (99.5%) from Merck, (Darmstadt, Germany), n-heptane (99.5%) from J.T. Baker (Deventer, Netherlands) and n-decane p.a. from Promochem (Wesel, Germany).

2.9. Cultivation of fungi and time course experiments

The cultivation and GC–MS analysis of the different *Trichoderma* strains were performed as described earlier (Stoppacher et al., 2010; Kluger et al., 2012) with slight modifications. Fungi were pre-cultured on petri dishes with PDA as culture medium. After 4 days of pre-cultivation, all samples of a particular time series were prepared simultaneously in the following way: for each time point and replicate, an agar plug with fungal mycelium was excised from the petri dish and placed centrally on top of a layer of solidified PDA (5 mL) in a 20-mL headspace (HS) vial (4 time points, 3 replicates per time point, see below). The HS vials were closed with cotton plugs and incubated for 24 h, 48 h, 72 h and 96 h respectively (darkness, 28 °C, rel. humidity 70%). After cultivation for the respective time period, the culture vials were purged with synthetic air for 30 s, sealed with a screw-cap containing gas-tight silicone/Teflon septa and incubated for 6 h at 22 °C prior to GC–MS analysis. For each *Trichoderma* strain, three biological replicates per time point were analyzed. For GC–MS measurements a 65 μm PDMS/DVB fiber was used for HS extraction and chromatographic separation of the volatiles was carried on a HP5-MS column (30 m × 0.25 mm × 0.25 μm; Agilent, Waldbronn, Germany).

2.10. Measurement of the volatile metabolites by GC–MS

Automated extraction of volatiles was performed with the aid of a Gerstel MPS 2XL autosampler (Gerstel, Mülheim, Germany). Fungal cultures were equilibrated for 15 min at 30 °C and subsequently volatiles were extracted from the headspace for 30 min by exposure to an SPME fiber with 65 μm PDMS/DVB coating without agitation. Volatiles bound to the fiber were transferred to the GC–MS instrument, and further measured on an Agilent 6890N gas chromatograph coupled to an Agilent 5975 mass selective detector (MSD) (Agilent, Waldbronn, Germany). Analytes were desorbed for 2 min in a split-/splitless injector (splitless mode 250 °C, headspace glass liner, 1.5 mm i.d. (Supelco, Vienna, Austria), and separated on a HP5-MS 30 m × 0.25 mm × 0.25 μm (Agilent, Waldbronn, Germany). Helium (5.0) was used as carrier gas at a constant flow rate of 1 mL/min. The oven program consisted of 40 °C (hold 2 min), 10 °C/min to 200 °C, and 25 °C/min to 260 °C (hold 5 min). MSD parameters were electron impact ionization (EI) at 70 eV, source 230 °C, quadrupole 150 °C, solvent delay: 2.2 min, full scan (45–400 amu).

2.11. Compilation of an in-house Trichoderma library

GC–MS chromatograms, which showed the highest number of chromatographic peaks with highest intensities (one chromatogram per *Trichoderma* wild-type strain), were selected to build a "*Trichoderma*-library" containing the respective mass spectra and linear temperature programmed retention index (LTPRI) values of the detected volatile metabolites. To this end, chromatograms of *T. virens* Gv29-8 96 h-old cultures and *T. reesei* and *T. atroviride* 48 h-old cultures were evaluated using the Automated Mass Spectral Deconvolution and Identification System (AMDIS) software (version 2.69).

First, a "NIST database search" was performed comparing the measured and deconvoluted mass spectra of all peaks with the entries of the NIST 05a and Wiley 7n libraries. All volatiles, which showed mass spectra with a match factor ⩾90, were selected, and their mass spectra and LTPRI values (median from NIST Chemistry Webbook (NCWB) added to the "*Trichoderma*-library". Additionally, chromatograms were searched for different types of terpenes using extracted ion current (EIC) chromatograms at m/z 136 for monoterpenes, m/z of 202 and m/z 204 for sesquiterpenes, and m/z 272 for diterpenes. The deconvoluted mass spectra underneath these EIC peaks were inspected manually, and in case of typical terpene fragmentation patterns, the corresponding measured spectra and LTPRI values were added to the library. The putative terpenes were classified into mono- (MT), sesqui- (ST), diterpenes (DT), and named "unknown terpenes". The final *Trichoderma* library was subsequently used for the automated data evaluation of all samples applying the AMDIS software.

2.12. Annotation and identification of volatile metabolites

All chromatographic peaks/substances which were detected in blank samples (HS vials containing PDA without fungi) were eliminated from further data evaluation. Regarding compound identity four levels of confidence were defined. For identification of the highest level of confidence (marked with "superscript b" in Table 1), the MS match factor (MF) had to be ⩾90 and LTPRI ⩽ 2%,

Table 1
Volatile compounds produced by *T. virens*, *T. atroviride*, and *T. reesei*.

Number of compound	Compound # classification	Compound	CAS #	RI measured (HP5, median)	RI (median NCWB)
1	Others_01	1-Butanol, 3-methyl-[a]	123513	735	736
2	C8_01	1-Octene[b]	111660	791	789
3	C8_02	1,3-Octadiene[b]	1002331	826	829
4	C8_03	1,3-Trans,5-cis-octatriene[b]	40087614	880	880
5	Others_02	2-Heptanone[a]	110430	891	894
6	C8_04	1-Octen-3-ol[a]	3391864	980	980
7	C8_05	3-Octanone[a]	106683	988	987
8	Others_03	2-Pentyl furan[b]	3777693	992	991
9	MT_01	beta-Myrcene[a]	123353	993	991
10	C8_06	3-Octanol[b]	589980	995	995
11	Others_04	Hexanoic acid, ethyl ester[b]	123660	999	998
12	MT_02	Alpha-terpinene[b]	99865	1020	1017
13	MT_03	Alpha-limonene[a]	138863	1032	1029
14	MT_04	Beta-phellandrene[b]	555102	1032	1030
15	MT_05	Alpha-terpinolene[a]	586629	1091	1088
16	Others_05	2-Nonanone[b]	821556	1093	1091
17	Others_06	Phenylethyl alcohol[b]	60128	1118	1113
18	Others_07	2-n-Heptylfuran[b]	3777717	1194	1193
19	Others_08	2-Undecanone[b]	112129	1295	1291
20	ST_01	Unknown sesquiterpene_01[d]		1346	
21	ST_02	Unknown sesquiterpene_02[d]		1358	
22	ST_03	Unknown sesquiterpene_03[d]		1388	
23	ST_04	Unknown sesquiterpene_04[d]		1395	
24	ST_05	2,4-Diisopropenyl-1-methyl-1-vinylcyclohexane[b]	515139	1403	1391
25	ST_06	1,1,4,7-Tetramethyl-1A,2,3,4,4A,5,6,7B-octahydro-1H-cyclopropa(E)azulene[b]	489407	1424	1409
26	ST_07	Unknown sesquiterpene_07[d]		1429	
27	ST_08	Beta-caryophyllene[b]	87445	1435	1418
28	ST_09	Unknown sesquiterpene_09[d]		1439	
29	ST_10	Unknown sesquiterpene_10[d]		1444	
30	ST_11	Alpha-bergamotene[b]	17699057	1444	1430
31	ST_12	Unknown sesquiterpene_12[d]		1455	
32	ST_13	Trans-beta-farnesene[b]	18794848	1459	1457
33	ST_14	Unknown sesquiterpene_14[d]		1465	
34	Others_09	6-Pentyl-alpha-pyrone[a]	27593233	1471	1469
35	ST_15	Unknown sesquiterpene_15[d]		1478	
36	ST_16	Unknown sesquiterpene_16[d]		1487	
37	ST_17	Germacrene D[b]	23986745	1497	1480
38	ST_18	(Z,E)-Alpha-farnesene[b]	26560145	1498	1489
39	ST_19	Alpha-zingiberene[b] or beta-himachalene[b]	495603 or 1461036	1501	1495 or 1499
40	ST_20	Cyclopropanemethanol, 2-methyl-2-(4-methyl-3-pentenyl)-3-(2-methyl-1-propenylidene)-, cis-[c]	129286324	1503	
41	ST_21	Unknown sesquiterpene_21[d]		1512	
42	ST_22	Beta-bisabolene[b]	495614	1516	1508
43	ST_23	Unknown sesquiterpene_23[d]		1520	
44	ST_24	Naphthalene, 1,2,3,4,4a,5,6,8a-octahydro-7-methyl-4-methylene-1-(1-methylethyl)-, (1à,4aá,8aà)-[b]	39029419	1530	1513
45	ST_25	Beta-sesquiphellandrene[b]	20307839	1532	1525
46	ST_26	1-Isopropyl-4,7-dimethyl-1,2,3,5,6,8A-hexahydronaphthalene[b]	483761	1536	1524
47	ST_27	Naphthalene, 1,2,4a,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, [1S-(1à,4aá,8aà)]-[c]	24406051	1552	
48	ST_28	Cis, trans-nerolidol[b]	40716663	1568	1563
49	ST_29	1,1,4,7-Tetramethyldecahydro-4AH-cyclopropa(E)azulen-4A-ol[b]	5986492	1588	1561
50	ST_30	1,1,4,7-Tetramethyldecahydro-1H-cyclopropa(E)azulen-4A-ol[b]	552023	1613	1590
51	DT_01	Unknown diterpene_01[d]		1964	
52	DT_02	Unknown diterpene_02[d]		1992	
53	DT_03	unknown diterpene_03[d]		1998	
54	DT_04	Unknown diterpene_04[d]		2002	
55	DT_05	Unknown diterpene_05[d]		2023	
56	DT_06	Unknown diterpene_06[d]		2063	

[a] Identified by MS match factor ⩾90, DRI ⩽ 2% and authentic standard.
[b] Annotated by MS match factor ⩾90, DRI ⩽ 2%.
[c] Annotated by MS match factor ⩾90, RI not available in NCWB.
[d] Assigned according to molecular mass and typical appearance of ST- and DT-spectra, MT (monoterpene), ST (sesquiterpene), DT (diterpene), C8 (C8 compound), Others (other class of compound).

and additionally confirmed by measurement of authentic standard was required. Compound annotation was either based on both MF ⩾ 90 and LTPRI ⩽ 2% (“superscript c”, Table 1), MF ⩾ 90 (“superscript d”, Table 1) or just assigned according to molecular mass and typical appearance of mass spectra in case of DT and some ST (“superscript e”, Table 1).

2.13. Growth assays of transformants

Growth of transformants was determined on malt extract agar (MEA), PDA, VMS, Vogel’s minimal medium with 1.5% glucose (VMG) or 1.5% lactose (VML). Wild-type and transformant strains were grown on PDA and 3 mm plugs were removed from the actively growing edge of the colony and placed in the center of the test media. Measurements of the colony diameter were recorded at 24, 48, and 72 h. Diameters measured at the 48 h time point were analyzed, and the mean and standard deviation calculated for all conditions using Microsoft Excel.

2.14. Root colonization

The root colonization abilities of Gv29-8 and the *vir4* deletion and complementation strains were compared using qPCR analysis. Seeds of *Zea mays* (Silver Queen hybrid) were sterilized by treating with 70% ethanol for 5 min and 10% hydrogen peroxide for 2 h, and then rinsed with distilled water. Surface disinfected seeds were planted in an 18 × 150 mm culture tube containing a 3:2 mixture (by weight) of a Lufkin fine sandy loam and fine sand (approximately 10 g/tube). The sterile sand soil mix (100 g) was infested with 10 mL of water containing 1×10^7 conidia per mL. After 3 days of growth, roots were harvested, rinsed with distilled water, and ground in liquid nitrogen. DNA was extracted using the same method described for *T. virens*. The method for qPCR was the same as described earlier using 100 ng of DNA without the reverse transcriptase step. The primers for actin were used to measure fungal DNA and primers for phenylalanine ammonia lyase (*pal*) were designed to measure plant DNA (qpalF 5′ GCTCATGTTCGCGCAGTTC 3′ and qpalR 5′ GGTGAGCCCGTTGTTGTAGTACT 3′). Cycle threshold values were calculated for each of the primers and the amount of DNA was estimated using a standard curve. Three replicates were performed for each strain.

3. Results and discussion

3.1. Phylogenetic and cluster analysis

The full *vir4*-containing cluster was previously identified in the *T. virens* Gv29-8 genome (Mukherjee et al., 2012). A thorough survey of more than 50 filamentous ascomycete genomes revealed that homologs of this cluster or part of it are present in *A. oryzae*, *A. clavatus*, and *A. flavus* (Fig. 1), but absent in other *Aspergillus* species (*A. nidulans*, *A. fumigatus*, *A. terreus*, *A. niger*) queried. In *A. oryzae* most of the entire cluster was present (except *vir7*), though the individual genes have undergone re-arrangements, and thus the members of the cluster were not in the same order as in *T. virens* (Fig. 1). The cluster in *A. clavatus* appeared to have suffered deletion with a truncated version of *vir2* and *vir5*, and the absence of *vir1*. Only a remnant of the cluster was present in *A. flavus* genome, comprising the cyclase (*vir4*), in addition to *gapdh*. No genes with strong homology to *vir4* or other members of the gene cluster were found in *T. reesei* or *T. atroviride* (www.genome.jgi-psf.org).

A phylogenetic analysis of the class I terpene cyclases from all three *Trichoderma* spp. and orthologs from other ascomycetes revealed that the *vir4* cyclases of *T. virens*, *A. oryzae* (Gibbons et al., 2012), *A. clavatus* and *A. flavus* share very strong homology (Fig. 2). Additionally, the *T. virens* vir-*gapdh* is unique and does show homology to other *gapdh* genes from *T. virens* as well as from *T. atroviride* and *T. reesei* (Fig. S1). *A. flavus* and *A. oryzae* vir-*gapdh* are however, phylogenetically close to each other. In contrast, analysis of the P450s (*vir1*, *vir2*, *vir3*, and *vir5*) from *T. virens*, *A. clavatus* and *A. oryzae* demonstrated that *vir1* from *T. virens* and *A. oryzae* (absent in *A. clavatus*) group together. This was also observed for *vir2*, *vir3*, and *vir5*, showing that they are structurally conserved (Fig. S2).

The presence of homologous gene clusters within the genomes of distantly related fungi has been observed previously with other secondary metabolite clusters (Yu and Keller, 2005). The gene cluster responsible for the production of gliotoxin in *T. virens* (Kenerley, unpublished) is also found in *A. fumigatus* (Gardiner and Howlett, 2005; Kupfahl et al., 2006). Similar to the *vir* cluster in *T. virens* and *A. oryzae*, the genes in the gliotoxin cluster are in a different order on the chromosome and some of the genes are oriented in opposite directions. The observation of homologous gene clusters in distantly related fungal species has been predicted

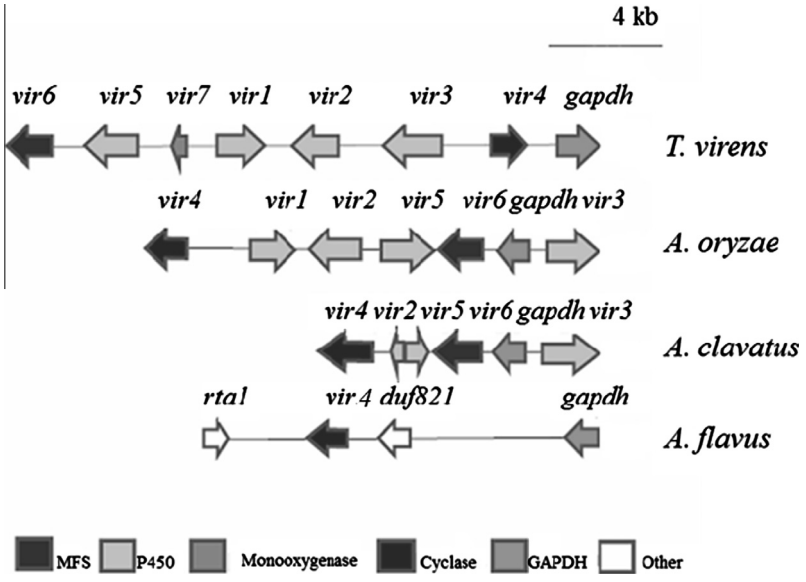

Fig. 1. Scheme representing gene cluster homology among *T. virens vir* cluster and clusters found in *Aspergillus*. The color of the arrow denotes predicted function. Arrow direction indicates 5′–3′ coding orientation.

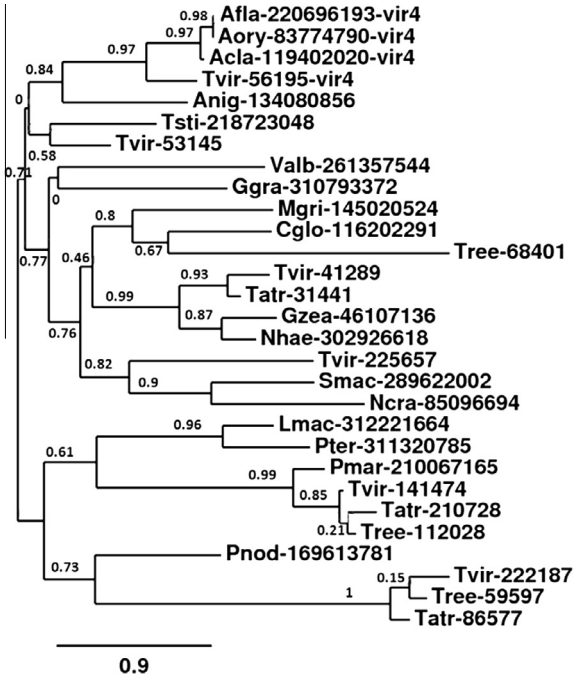

Fig. 2. Gene phylogeny of *vir4* cyclase homologs. Protein sequences gathered from an NCBI BLASTP search along with the class I cyclase protein sequences from *T. virens*, *T. atroviride*, and *T. reesei* were entered into a PhyML maximum-likelihood software with a bootstrap of 100. Fungal species used for the tree construction included *A. flavus* (Afla), *A. oryzae* (Aory), *A. clavatus* (Acla), *T. virens* (Tvir), *A. niger* (Anig), *Talaromyces stipitatus* (Tsti), *Verticillium albo-atrum* (Valb), *Glomerella graminicola* (Ggra) , *M. grisea* (Mgri), *Chaetomium globosum* (Cglo), *T. atroviride* (Tatr), *Gibberella zeae* (Gzea), *Nectria haematicocca* (Nhae), *Sordaria macrospora* (Smac), *Neurospora crassa* (Ncra), *Leptosphaeria maculans* (Lmac) , *Pyrenophora teres* (Pter), *T. reesei* (Tree), and *Phaeosphaeria nodorum* (Pnod). Numbers following abbreviated names correspond to protein ID numbers. The cyclase from *T.virens* is labelled Tvir-56195-vir4.

to be the effect of horizontal gene transfer (Rosewich and Kistler, 2000; Schmitt and Lumbsch, 2009). The homology between the clusters containing *vir4* and the genes within them allude to the possibility of a horizontal gene transfer as the mechanism for this similarity.

3.2. The vir4 gene is predicted to encode a terpene cyclase

The predicted 377 amino-acid sequence of *vir4* (protein ID: 56195) shows significant levels of similarity to fungal terpene cyclases. Most importantly, the amino acid sequence contains two highly conserved motifs experimentally demonstrated in other terpene cyclases to be involved in the binding of three Mg^{2+} cations (Christianson, 2006; Shishova et al., 2008). An aspartate rich sequence, DDEID, is found at amino acid 107 and an NSE/DTE motif, DMLSLRKE, is 150 amino acids downstream of the DDXXD region. These two regions predict the protein has the ability to catalyze the cyclization of farnesyl diphosphate (Christianson, 2006).

3.3. Generation of vir4 deletion and complementation transformants

The *vir4* deletion vector pFKC1 was used to replace the *vir4* open-reading frame with the arginine-specific carbamoyl-phosphate synthetase coding region (*arg2*) (Fig. 3A). One hundred and fourteen stable transformants were screened for insertion of the *arg2* cassette in place of the *vir4* coding region by PCR and seven were found to be positive for insertion of the deletion cassette (data not shown). Of these seven, only one transformant (137) did not contain the *vir4* gene in a heterokaryon state. Two other transformants (87 and 61) were purified by single-sporing, with transformant 61 requiring 12 rounds. Transformants with loss of the *vir4* coding region were further evaluated by Southern blot (Fig. 3B). The three transformants (61, 87, and 137) were all negative for the 1.3 kb band associated with the *vir4* coding region. All three did contain the 4.2 kb band from the deletion cassette. Only the *vir4* complements, vir4c12 and vir4c32, contained both bands. Multiple bands hybridized in 87 indicate both homologous recombination and multiple ectopic integrations of the deletion cassette occurred in the transformant. Even though 87 was identical to 137 and 61 in morphology, to avoid unexpected interference resulting from ectopic integration, in experiments where 87 was used, it was used in tandem with 137.

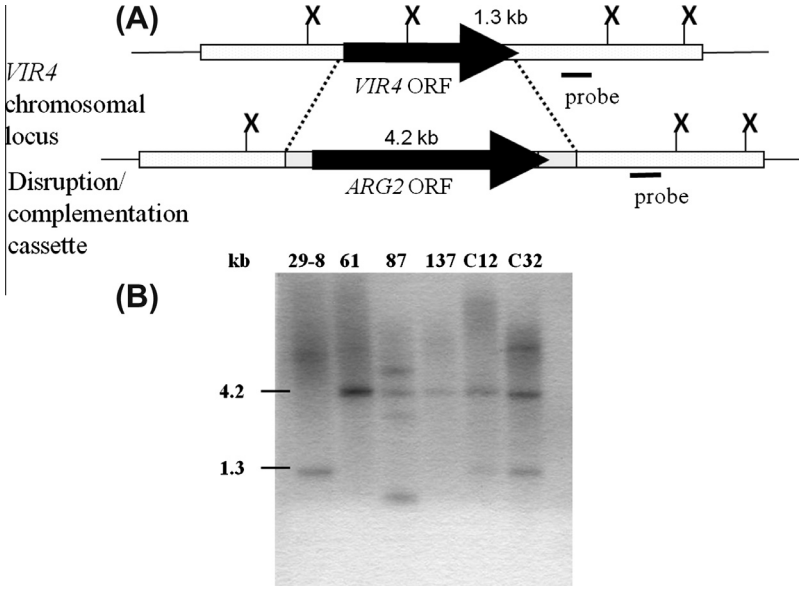

Fig. 3. Southern analysis and confirmation of vir4 transformants. (A) Scheme of the gene deletion strategy. The *vir4* open reading frame is replaced by a homologous integration event with a 3.0 fragment of the *arg2* gene from *T. virens*. A 1.3 kb band would result in *T. virens* wild-type strain and a 4.2 kb fragment in the deletion strains when digested with *Xba*I (restriction sites indicated by X). (B) Southern analysis of *T. virens* wild-type (Gv29-8) strain, VIR4-deletion mutants (61, 87 and 137) and VIR4-complement mutants (C12 and C32). Numbers along left side of panel indicate expected size in native and deletions events.

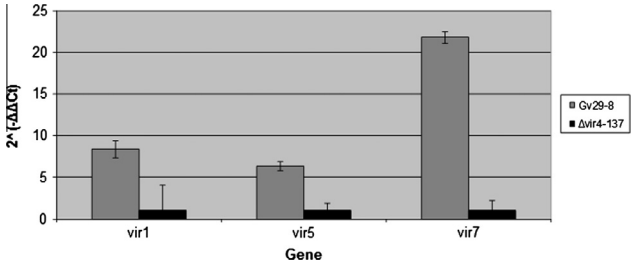

Fig. 4. Real time PCR analysis of *vir* cluster genes in the wild-type (Gv29-8) and the vir4 deletion mutant (Δvir4-137). The $2^{-(\Delta\Delta CT)}$ (indicating the difference in expression when the expression of the genes in the mutant were set at one) was significantly different between the wild-type and the mutant strain for each of the three genes (*vir1*, *vir5*, and *vir7*).

3.4. Vir4 deletion down-regulates the expression of three genes in the vir cluster

To determine the effect of the deletion of *vir4* on the other members of the *vir* cluster, quantitative real time PCR (qPCR) was performed to compare the change in expression of all the putative cluster genes between *T. virens* wild-type Gv29-8 and Δvir4-137. This analysis demonstrated that only the expression of two putative cytochrome P450s (*vir1* and *vir5*) and a putative oxidoreductase (*vir7*) was higher in Gv29-8 as compared to the deletion mutant (Fig. 4). These genes are located adjacent to each other along the chromosome (Fig. 1) and appear to be co-regulated. The insertion of *arg2* into the gene cluster is unlikely to have caused a disruption of gene expression, as the expression of *vir2* and *vir3* which lie between *vir4* and *vir1*, *vir5*, *vir7* was unaltered. However, metabolic feedback has been found to occur in secondary metabolite gene clusters (Cramer et al., 2009). When this feedback occurs, the presence of a particular metabolite will activate transcription of the genes responsible for this biosynthesis. This is a possible explanation for the disruption of gene expression in the Δvir4-137 transformant, but further study will be required to demonstrate whether metabolic feedback is responsible for the observed effects. The other genes within the *vir* cluster showed no significant difference in their transcription between the mutant and the wild-type (having a $2^{-(\Delta\Delta Ct)}$ of one in both wild-type and the transformant) and were therefore not included in Fig. 4.

3.5. A putative cyclase (vir4) is responsible for production of volatile terpenes in T. virens

Initially, *vir4* was predicted to be involved in the biosynthesis of viridin and viridiol, two steroid compounds produced by *T. virens* (Mukherjee et al., 2006). However, the evaluation of deletion and complement strains for production of the common secondary metabolites gliotoxin, dimethylgliotoxin, and viridin by thin layer chromatography (TLC) and high-pressure liquid chromatography

Table 2
Volatile profiling of *T. atroviride*, *T. reesei*, and *T. virens* mutant and complement strains.

Number of Compound	T. atroviride IMI 206040				T. reesei QM6a				T. virens GV29-8				Mutant Vir4-87				Mutant Vir4-137				Vir4-Compl. C12 [c]			Vir4-Compl. C32 [c]			
	d1[a]	d2	d3	d4	d1	d2	d3	d4	d1	d2	d3	d4	d1	d2	d3	d4	d1	d2	d3	d4	d1	d2	d3	d1	d2	d3	d4
1	3[a]	3	2		3	3	3	3																			
2										3	3	3		3	3	3		3	3	3							
3	3	3	3	2	3	2	3	3		3	3	3		3	3	3	1	3	3	3							
4										3	3	3															
5	3	3	3	3																							
6	3	3	3	3			1			3				3	1												
7					3	3	2	3		3	2	3		3	2	1		2		1							
8	3	3	3	3																							
9									3	3	3	3															
10	1	3	3	3			1	3																			
11							1	1																			
12										1		2															
13	3	3	3	3							3	3															
14										1			1	1													
15												3															
16	3	3	3	3																							
17					1	3	3	2																			
18		2	3	3																							
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26									1	3	3	3															
27									3	3	3	3															
28										3	3	3															
29											2	2															
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31											1	1															
32	3	3	3	3	1	3	1																				
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39	3	3	3	3	1							1															
40												1															
41												2															
42	2	3	3	3	3	3																					
43											3	3															
44									3	3	3	3															
45	1	3	2	3																							
46									3	3	2	3															
47									1	3	3	3															
48					3	3	3	3																			
49											3	3															
50									3	3	3	3															
51						1																					
52						1																					
53				1																							
54					1	3	3	3																			
55	3	3	3	3	3	3	3	3																			
56	3	3	3	3	3	3	3	3																			

[a] Columns d1, d2, d3 and d4 refer to cultivation duration in days.
[b] Numbers in cells refer to positive findings out of three biological replicates.
[c] One sample per day was measured from complement C12 (day 1–3) and C32 (day 1–4).

(HPLC) revealed normal production of all three compounds (data not shown). Volatile production in the strains was then analyzed by headspace solid phase microextraction coupled with gas chromatography-mass spectrometry (HS-SPME-GCMS).

HS-SPME-GCMS analysis demonstrated *T. virens* Gv29-8 produces three major categories of volatile compounds under the condition tested; 24 sesquiterpenes, five monoterpenes, and five C8 alkane compounds (Tables 1 and 2, and Fig. 5). When the profiles of the deletion mutants were compared with that of the wild-type, all 24 of the sesquiterpenes were absent in each of the *vir4* mutant cultures. These results were confirmed in all three independent biological replicates, indicating the direct involvement of *vir4* in sesquiterpene volatile biosynthesis. When the VOC profiles of both complementation transformants were compared to the wild-type, only 11 of the 24 sesquiterpenes were recovered indicating that complementation with the *vir4* gene did not completely restore VOC production. Partial complementation is not uncommon in filamentous fungi and *Trichoderma virens* is no exception (Mukherjee and Kenerley, 2010).

Four of the five monoterpenes produced in the wild-type Gv29-8 were not recovered in the Δ*vir4* mutants. Of these four, the production of three was observed in the complemented strains. This suggests that *vir4* may be responsible for the production of two different categories of terpene compounds. Monoterpenes and sesquiterpenes are produced by the cyclization of two different compounds (geranyl diphosphate and farnesyl diphosphate, respectively), and the respective enzymes are known for their high specificity (Davis and Croteau, 2000). Therefore it is also probable that the expression of the two different terpene cyclases is linked. *T. virens* contains a total of six putative terpene cyclases identified from the genome sequence (Fig. 2). Therefore, there are five possible cyclases that may be involved directly in monoterpene synthesis, and it is probable that volatile production in *T. virens* is highly regulated and interlinked.

Several of the volatiles produced in the wild-type, but missing in the deletion mutants, have been studied previously and may provide clues for their possible function in the biology of *T. virens*. For example, the monoterpene β-myrcene was shown to block the expression of a monooxygenase involved in the production of aflatoxin by *A. flavus* (De-Oliveira et al., 1997). Two sesquiterpenes have been shown to be involved in interactions with insects. Caryophyllene, when produced in rice, is regulated in response to methyl-jasmonate produced during insect feeding, and may be involved in the attraction of parasitoid wasps (Cheng et al., 2007). Another example of the importance of terpenes in insect attraction, is the compound germacrene, which is involved during insect attraction through stimulation of the antennae (Røstelien et al., 2000).

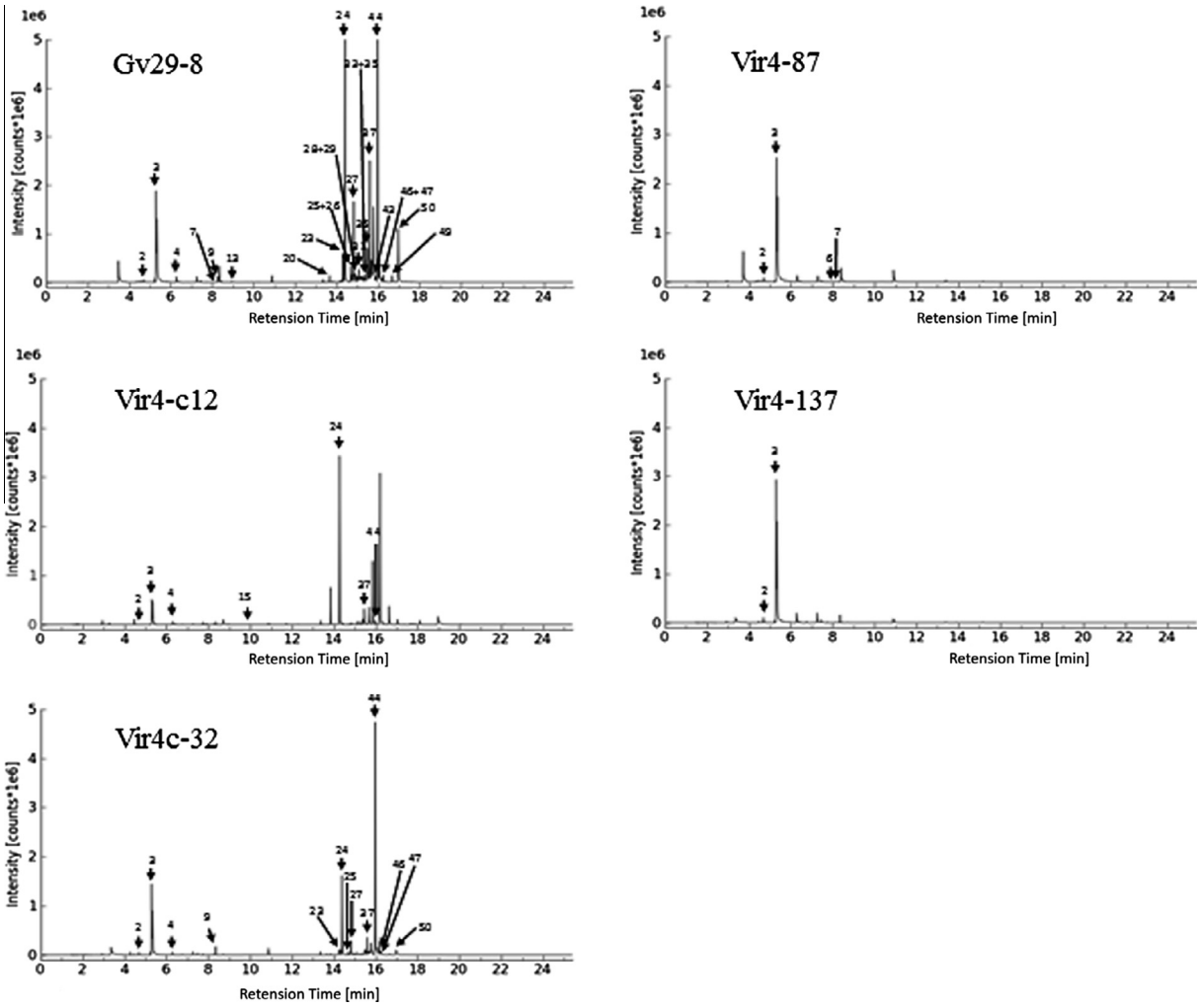

Fig. 5. HS-SPME-GC–MS total ion current chromatogram of MVOCs of wild-type (Gv29-8), complements (vir4-c12 and vir4-c32), and mutants (Δvir4-87 and Δvir4-137) after 72 h of cultivation on PDA. Peak numbering corresponds to numbers of metabolites in Tables 1 and 2.

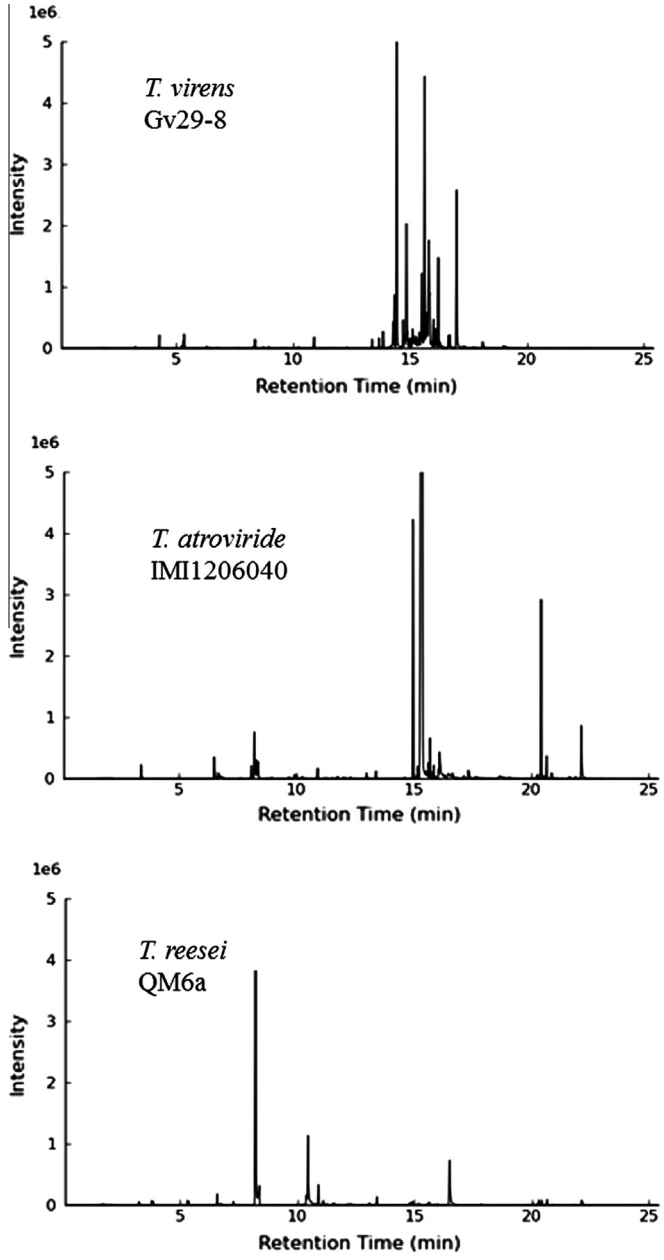

Fig. 6. HS-SPME-GC–MS total ion current chromatogram of MVOCs of *T. virens* Gv29-8, *T. atroviride* IMI1206040, and *T. reesei* QM6a after 72 h of incubation on potato dextrose agar (PDA).

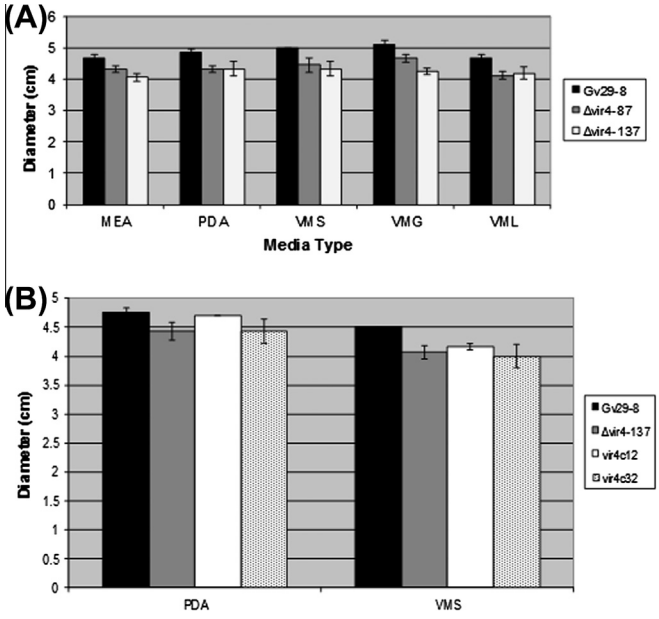

Fig. 7. Growth of cyclase deletion and complementation transformants. (A) Gv29-8, Δvir4-87, and Δvir4-137 were each plated on malt extract agar (MEA), potato dextrose agar (PDA), Vogel's minimal media plus sucrose (VMS), Vogel's minimal media plus glucose (VMG), and Vogel's minimal media plus lactose (VML). Cultures were grown for two days and measurements were taken. (B) Gv29-8, Δvir4-137, vir4c12, and vir4c32 were grown for 2 days on PDA and VMS and colony diameter was measured.

3.6. Volatile profiling of three species of Trichoderma

The *vir* cluster was only observed in *T. virens* and not in the other species of *Trichoderma* examined. To explore the possibility that other genes are involved in volatile production, two other *Trichoderma* spp. were assayed for volatile compounds. The volatile profiles of *T. virens, T. atroviride,* and *T. reesei* differ significantly (Tables 1 and 2 and Fig. 6). *T. virens* produces by far the most sesquiterpene volatiles, 24 total as compared to five for *T. atroviride* and six for *T. reesei*. Only *T. atroviride* and *T. virens* produced monoterpenes, while they were absent in *T. reesei*. Six unknown diterpenes were identified, but none were associated with *T. virens*. The C8 compounds were found among all three species, with different volatiles associated with different species of *Trichoderma*. Volatiles represented by the "others" category included alcohols, ketones, furans, and carboxylic acids and were associated with both *T. atroviride* and *T. reesei*, but not with *T. virens*.

The uniqueness of the volatiles produced by each species of *Trichoderma* corresponds with the previous observation that the majority of these compounds are associated with the *vir4* gene cluster in *T. virens*. All but two of the 24 sesquiterpenes absent in the mutant strains are unique to *T. virens.* All five monoterpenes, four of which are suspected to be associated with *vir4* were produced in *T. virens*, but only one was produced by *T. atroviride.*

A genomic comparison of *T. virens* with *T. atroviride* and *T. reesei* revealed that *T. virens* is genetically specialized for active predation of other fungi (Druzhinina et al., 2011; Kubicek et al., 2011). The use of antifungal secondary metabolites and cell wall degrading enzymes by *T. virens* is distinct from *T. atroviride*, which uses parasitism as the primary mechanism of biocontrol, and *T. reesei*, which essentially has lost the ability to parasitize other fungi (Druzhinina et al., 2011). The volatile profiles presented here demonstrate the uniqueness of *T. virens* as this species produces a larger number of volatile compounds and the most volatile terpenes (29) as compared to *T. atroviride* and *T. reesei* under the condition tested (9 and 11, respectively).

3.7. Preliminary phenotypic analyses of deletion and complementation transformants

To determine if growth among the deletion transformants (Δvir4-61, Δvir4-87, Δvir4-137) and wild-type are different, strains were grown on MEA, PDA, VMS, VMG, and VML. Both deletion mutants grew at a significantly slower rate than the wild-type (Fig. 7A). Complements were also tested for changes in growth rate (Fig. 7B). Both complements were intermediate between the growth of WT and Δvir4-137 on PDA indicating that there is partial complementation. The role of *vir4* in promotion of growth of *T. virens* is currently unknown. There is documentation that plant- and bacteria-derived terpenes can have effects on fungal growth (Akiy-

Table 3
Colonization of maize roots by *Trichoderma virens* wild-type Gv29-8 and the *Vir4* deletion and complementation transformants.[a]

Condition	Actin				Phenylalanine ammonia lyase				Ratio[d]
	Ct	SD	Qty[b]	SD	Ct	SD	Qty[c]	SD	
Gv29-8	26.73	1.58	0.46	0.53	26.03	3.36	99.38	122.22	0.15
Vir4-61	26.22	0.48	0.43	0.17	24.51	1.48	125.54	86.76	0.005
Vir4-87	27.22	0.37	0.19	0.05	24.69	1.85	128.6	119.07	0.003
Vir4-137	25.12	0.68	1.1	0.61	24.69	1.85	128.6	119.07	0.015
Vir4-c12	26.24	0.69	0.45	0.27	22.53	1.1	470.69	386.12	0.001

[a] Fungal DNA present on maize roots 72 hours after inoculation was quantified by real-time polymerase chain reaction. Three replicates were performed for each sample. Ct = threshold cycle and SD = standard deviation.
[b] Quantity of fungal DNA (ng) referred to by the *actin* gene in 100 μg of total DNA.
[c] Quantity of plant DNA (ng) referred to by the *phenylalanine ammonia lyase* gene in 100 μg of total DNA.
[d] Proportion of fungal DNA versus plant DNA per 100 μg total DNA.

ama et al., 2005; Kai et al., 2007), leading to the possibility that VOCs produced by *T. virens* may stimulate hyphal growth.

Root colonization of the deletion mutants and a complement was compared with the wild-type by qPCR analysis (Table 3). No significant difference was detected among the strains. Although volatile compounds have been known to act as intermediates between microbes and plants (Insam and Seewald, 2010; Stoppacher et al., 2010; Wenke et al., 2010), we were unable to demonstrate an influence of volatiles on the root colonization ability of *T. virens*.

This work represents the first report of a gene involved in the biosynthesis of volatile compounds in *T. virens*, and a gene cluster involved in terpenoid volatile synthesis in filamentous fungi. To date, the role of sesquiterpene volatiles in *T. virens* has yet to be determined. Future work will focus on characterizing the function of these individual terpenoid volatiles by the assessing the effect of purified compounds on the development of *T. virens* and its interactions with plants and insects. The cyclic and branched nature of sesquiterpene hydrocarbons make them interesting targets as biofuel alternatives and fungal VOCs equivalent to components in current fossil fuel were termed "myco-diesel" (Strobel et al., 2008). Further research on the VOC production capabilities of *Trichoderma* species and determination of the biosynthetic machinery may reveal further substances with potential applications as biofuels.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fgb.2013.05.003.

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