The MAP kinase TVK1 regulates conidiation, hydrophobicity and the expression of genes encoding cell wall proteins in the fungus *Trichoderma virens*

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Mitogen-activated protein (MAP) kinases modulate morphological and genetic processes, which determine cell fate. The *tvk1* gene encodes a MAP kinase of *Trichoderma virens* and its deletion promotes an unusual conidiation phenotype in submerged culture. Here, it is reported that the morphology, physiology and expression of genes encoding cell wall proteins from *Trichoderma* are significantly affected by Tvk1. Morphological changes were evident in the cell walls of aerial conidia produced by a MAPK null mutant when compared to those produced by the wild-type. Unexpectedly, conidia produced in submerged culture by the $\Delta tvk1$ strain were highly hydrophobic, whereas in aerial conidia hydrophobicity was severely reduced. In addition, the $\Delta tvk1$ strain was unable to break the liquid–air interface when the fungus grew in rich medium; however, when it grew in minimal medium the fungus produced large filaments which were much more efficient at breaking the interface than the wild-type. Through cDNA subtractive hybridization between the wild-type and $\Delta tvk1$ grown in submerged culture, five genes encoding hydrophobin-like proteins and two additional genes encoding cell wall proteins were identified. Four hydrophobin-encoding genes (*Tv-hfb1*, *Tv-srh1*, *tv-cfth1* and *Tv-qid3*) and a gene encoding a clock-controlled-like protein (*Tv-ccg14/TvSm1*) were upregulated in $\Delta tvk1$, whereas genes encoding a cell wall protein (*tv-qid74*) and an additional hydrophobin (*tv-hfb3*) were absent in the mutant strain. Clear differences in gene expression were shown during conidiation and emergence from the liquid–air interface, suggesting different functions of the corresponding proteins in these two phenomena. The results support a model in which Tvk1 regulates morphology and genes encoding cell wall proteins during development of *Trichoderma*.

Received 21 December 2006

Revised 16 February 2007

Accepted 20 February 2007

INTRODUCTION

Several species of the genus *Trichoderma* are considered effective biocontrol agents due to their ability to suppress the negative effects of phytopathogenic fungi (Herrera-Estrella & Chet, 2004). Like many other filamentous fungi, *Trichoderma* hyphae colonize a wide variety of substrates via apical extension. Once *Trichoderma* mycelium is established, aerial hyphae arise and develop a branched reproductive structure, the conidiophore, which forms asexual conidiospores. Due to the fact that the life cycle of many filamentous fungi starts and ends in the form of these spores, the conidiation process is highly regulated to ensure the effective dispersal and survival of the species.

Conidiation of fungi is rarely achieved during submerged culture. However, deletion or the constitutive activity of elements of the cyclic AMP-PKA or MAP kinase pathways results in inappropriate conidiation in submerged cultures in several filamentous fungi (Banno *et al.*, 2005; Kays *et al.*, 2000; Mendoza-Mendoza *et al.*, 2003; Rocha-Ramírez *et al.*, 2002). Deletion of MAP kinase components results in the production of conidia in shaken cultures, as in the case of deletion of the *nrc-1* gene, which encodes a MEK kinase in *Neurospora crassa* (Kothe & Free, 1998), and of the *tvk1* gene in *Trichoderma virens*, which encodes a MAP kinase (Mendoza-Mendoza *et al.*, 2003).

Conidiation and emergence from liquid–air interfaces represent two processes that are necessary for the survival of fungal species, the first for the propagation of the species and the second during the invasion of new environments. Filamentous fungi normally secrete small and moderately hydrophobic proteins called hydrophobins. These proteins

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Abbreviations: MAP, mitogen-activated protein; SEM, scanning electron microscopy; TEM, transmission electron microscopy.

are amphiphiles specific to filamentous fungi (Wösten, 2001), having hydrophilic and hydrophobic regions, and are among the most active surface proteins known (Linder *et al.*, 2005). Hydrophobins have several important roles in fungal physiology, such as adhesion, formation of protective surface coatings and the reduction of the surface tension of water, which allows growth of aerial structures (Wösten, 2001). They mediate escape of hyphae from hydrophilic environments (Wösten *et al.*, 1999) and are involved in formation of structures such as aerial hyphae and fruiting bodies (van Wetter *et al.*, 1996; Wösten *et al.*, 1999). These proteins are also involved in host–fungus interactions, and have been considered as virulence factors in phytopathogenic fungi (Talbot *et al.*, 1996; Wösten *et al.*, 1994a). Two classes of hydrophobins have been reported based on their different solubility and hydrophobicity profiles. Class II hydrophobin films are significantly less robust and lack the rodlet morphology of class I hydrophobins (Wessels, 1994; Wösten, 2001; Wösten & de Vocht, 2000).

The molecular mechanism by which the expression of hydrophobins is controlled is not clear. However, the expression of *mpg1*, which encodes a hydrophobin in *Magnaporthe grisea*, is regulated through the MAP kinase Pmk1 and the cAMP pathway (Soanes *et al.*, 2002). In this paper we describe for the first time the differential regulation of five putative hydrophobin genes through a MAP kinase. The expression of these genes and the morphology of *T. virens* mediated by *Tvk1* was influenced by the environmental conditions.

METHODS

Strains and media. *Trichoderma virens* Gv 29.8 (wild-type) and *Δtvk1* null mutant (*Δtvk24*, Mendoza-Mendoza *et al.*, 2003) strains were maintained on PDA (Potato Dextrose Agar, Difco) medium at 28 °C. *Escherichia coli* DH5 α (Invitrogen) was used for plasmid transformation using pCR2.1 vector (Invitrogen). All chemicals used in this study were purchased from Sigma-Aldrich.

Fungal growth conditions. To generate the cDNA libraries, spores of the wild-type and the *tvk1* null mutant (*Δtvk1*) of *T. virens* (1×10^6 spores per ml medium) were inoculated at 28 °C using an orbital shaker (170 r.p.m.) in 2 litre flasks containing 500 ml Vogel's minimal medium supplemented with 1.5% sucrose (VMS) (Mendoza-Mendoza *et al.*, 2003). Samples were collected by filtration, frozen in liquid nitrogen and stored at –70 °C. A small aliquot of each sample was fixed with formaldehyde (1.0%) and analysed by light microscopy (BX60, Olympus).

To perform the liquid–air interface breaking analysis we applied 50 μ l drops of conidia (1×10^6 ml $^{-1}$) suspended in PDB (Potato Dextrose Broth, Difco) or VMS media to Parafilm and incubated them at 28 °C. Emergence of hyphae was analysed by using a stereo microscope (Olympus) and the images were captured and modified by using the programs IMAGE-PRO PLUS 4.0 (Media Cybernetics) and PHOTOSHOP (Adobe Systems), respectively.

Gene expression in submerged culture was compared by cultivating 1×10^6 conidia ml $^{-1}$ of each strain of *T. virens* in 25 ml Vogel's medium in shaken flasks at 28 °C. To test the influence of carbon or nitrogen sources on gene expression we incubated the spores for 48 h

before transfer to fresh Vogel's medium with or without nitrogen or carbon sources. Samples were then collected at 0, 1, 2 and 6 h after transfer to the induction medium.

Subtraction hybridization and subtractive library construction.

Subtractive libraries were used to prepare a cDNA library specific for *T. virens* conidiation in submerged culture. From the different time points analysed we chose those that gave us the most representative genes for each differentiation stage: 58, 60 and 64 h post-inoculation. Total RNA was obtained using a RNeasy kit (Qiagen) following the manufacturer's instructions. Subtractive libraries were made using the PCR-Select cDNA subtraction kit (Clontech), according to the manufacturer's instructions. cDNA of the wild-type strain was subtracted from cDNA of the *Δtvk1* null mutant strain; the corresponding clones were designated k plus the clone number (Table 1). In addition cDNA from the *Δtvk1* strain was subtracted from cDNA of the wild-type; these clones were designated wt plus the clone number (Table 2). The cDNAs obtained by subtraction hybridization were ligated into pCRII-Topo vector using the Topo TA cloning kit (Invitrogen) and transformed into *E. coli* DH5 α . The presence of inserts in pCRII Topo was corroborated by PCR using primers AH-con1 (5'-GCCGCCAGTGTGATG-3') and AH-con2 (5'-GCCAGTGTGCTGGAATTC-3'). A total of 587 independent clones were sequenced using an ABI 377 DNA sequencer, and the sequences were compared with public sequence databases using the BLASTX algorithm (NCBI).

Expression analysis. *T. virens* mycelium for total RNA extraction was collected by filtration using a Millipore system, washed once with water, frozen in liquid nitrogen and stored at –70 °C. Total RNA was isolated by using standard procedures (Sambrook & Russell, 2001), except when indicated otherwise. To study the hydrophilic–hydrophobic interface between liquid and air environment, 50 μ l drops of conidia (1×10^6 conidia ml $^{-1}$) were applied to Parafilm. Twenty drops were scraped off the Parafilm and total RNA was extracted using Trizol reagent (Invitrogen) according to the manufacturer's instructions. Northern blot analyses were performed under high-stringency conditions (Sambrook & Russell, 2001). Fragments contained in the cDNA library were amplified by PCR using primers AH-con1 and AH-con2 and the resulting PCR products were labelled with [α^{32} P]dCTP by random priming, using the Readyprime kit (Amersham) according to the manufacturer's specification.

Surface hydrophobicity of aerial and submerged conidia.

Hydrophobicity of conidia was determined by the phase distribution test (Mozes & Rouxhet, 1987), assessing the distribution ratio of cells between water and an organic phase (toluene). Aerial conidia were collected from 7 day old plate cultures, and filtered through crossed layers of cleaning tissues (Kimwipes, Kimberly Clark). Submerged conidia were obtained from a 72 h old cultures in VMS. Aerial and submerged conidia were collected by centrifugation at 10 000 g at room temperature, washed once with water and suspended in Tris buffer (100 mM) at three different pHs (3, 6 and 9), to an OD₆₀₀ of 0.6. Samples (4 ml) of suspensions were transferred into 25 ml glass bottles and an equivalent volume of toluene was added. After vigorous vortexing for 10 s, the mixture was left to stand for 30 min. The OD₆₀₀ was measured and the results were expressed as a proportion of the conidia that were excluded from the aqueous phase according to the formula $100 \times (OD_i - OD_f) / OD_i$, where OD_i and OD_f are the initial and final optical densities of the aqueous phase.

Transmission electron microscopy (TEM). A PDB liquid culture of 72 h old *Δtvk1* strains was filtered using cleaning tissues (Kimwipes, Kimberly Clark), centrifuged for 10 min at 10 000 g at 4 °C and suspended in sterile distilled water. Suspensions of aerial conidia were prepared by adding sterile distilled water to the PDA plates of 7 day old cultures, agitating the culture surface with a rubber-tipped glass

Table 1. *T. virens* genes obtained from the wild-type after subtraction of genes expressed in the $\Delta tkv1$ null mutant

Clone name	Best BLASTX match	Cellular function	BLAST E
k2001-a03	Novel ubiquitin-like protein (<i>Danio rerio</i> gil27802769)	Protein fate	9.00×10^{-19}
k2001-a09	Ubiquitin-activating enzyme E1 (<i>Saccharomyces cerevisiae</i> gil6322639)	Protein fate	3.00×10^{-32}
k2001-a12	Putative mannosyltransferase (<i>Aspergillus niger</i> gil34484377)	Protein fate	6.00×10^{-43}
k2001-c02	Outer mitochondrial membrane protein porin (<i>Schizosaccharomyces pombe</i> gil19115573)	Metabolism	7.00×10^{-47}
k2001-c11	Related to glyoxal oxidase precursor (<i>Magnaporthe grisea</i> gil38110257)	Metabolism and oxidative stress	2.00×10^{-14}
k2001-d10	Probable proteasome subunit α type 2 (<i>Gibberella zeae</i> PH-1 gil425499481)	Protein fate	$<1 \times 10^{-100}$
k2001-e02	Protein disulphide isomerase (<i>Hypocrea jeroica</i> gil3288650)	Protein fate and oxidative stress	8.00×10^{-45}
k2001-e06	Aryl-alcohol oxidase precursor (<i>Pleurotus eryngii</i> gil3851524)	Oxidative stress	5.00×10^{-50}
k2001-e07	Serine/threonine protein phosphatase PP _i (<i>Emericella nidulans</i> gil130707)	Signal transduction	1.00×10^{-28}
k2001-f11	Possible glycogen phosphorylase (<i>Aspergillus nidulans</i> gil19577353)	Metabolism	6.00×10^{-51}
k2001-g11	Lipopolysaccharide and β -1,3-glucan binding protein (<i>Pacifastacus leniusculus</i> gil6689027)	Unknown	2.00×10^{-07}
k2001-h06	Histone H2B (<i>Gibberella zeae</i> PH-1 gil42555923)	DNA processing	2.00×10^{-53}
k6001-g07	Histone H2A (<i>Aspergillus niger</i> gil2632155)	DNA processing	
k2001-h09	QI74 protein (<i>Hypocrea lixii</i> gil3250920)	Cell wall	2.00×10^{-57}
k2001-h10	Hypothetical protein FG09286.1 (<i>Gibberella zeae</i> PH-1 gil42554084)	Unknown	6.00×10^{-70}
k2001-h12	Protein component of the small (40S) ribosomal subunit (<i>Saccharomyces cerevisiae</i> gil6320658)	Protein synthesis	3.00×10^{-54}
k6001-a03	Thiazole biosynthetic enzyme, mitochondrial precursor (stress-inducible protein sti35) (<i>Saccharomyces cerevisiae</i> gil134940)	Metabolism and oxidative stress	5.00×10^{-35}
k6001-a05	Probable 60S ribosomal protein 13 (rp13) (<i>Neurospora crassa</i> gil30580497)	Protein synthesis	$<1 \times 10^{-100}$
k6001-a09	Ribosomal protein L4-B embiCAB88236.11 rp14 (<i>Schizosaccharomyces pombe</i> gil19112671)	Protein synthesis	3.00×10^{-58}
k6001-a12	Mitochondrial carboxylate carrier (Yhm2p) (<i>Saccharomyces cerevisiae</i> gil6323897)	Metabolism	4.00×10^{-58}
k6001-b08	Aldehyde dehydrogenase ALDH (<i>Emericella nidulans</i> gil13309884)	Metabolism	$<1 \times 10^{-100}$
k6001-c07	Mitochondrial phosphate transporter (<i>Cicer arietinum</i> gil6469119)	Metabolism	4.00×10^{-11}
k6001-d09	Elongation factor 1 α (EF1 α) (<i>Trichoderma reesei</i> gil461990)	Protein synthesis	$<1 \times 10^{-100}$
k6001-d10	Ribosomal protein S7 (<i>Yarrowia lipolytica</i> gil6094184)	Protein synthesis	2.00×10^{-08}
k6001-e09	Citrate synthase, mitochondrial precursor (<i>Gibberella zeae</i> PH-1 gil42544554)	Metabolism	2.00×10^{-12}
k6001-e12	β -1,3 Exoglucanase (EC 3.2.1.-) precursor (<i>Trichoderma harzianum</i> gil11359357)	Metabolism	6.00×10^{-09}
k6001-f05	Glycogen (starch) synthase (<i>Gibberella zeae</i> PH-1 gil42553911)	Metabolism	1.00×10^{-30}
k6001-f07	Hypothetical protein related to Sls2 protein (<i>Neurospora crassa</i> gil32404694)	Unknown	8.00×10^{-18}
k6001-f09	Hypothetical protein MG02235.4 (<i>Magnaporthe grisea</i> 70-15 gil38108187)	Unknown	3.00×10^{-19}
k6001-f12	Annexin (<i>Schistosoma mansoni</i> gil4753140)		3.00×10^{-07}
k2001-h04	Phosphatidylethanolamine methyltransferase (<i>Coccidioides immitis</i> gil119191878)	Metabolism	4.00×10^{-78}
k6001-g02	Ribosomal protein L30 of the large (60S) ribosomal subunit (<i>Saccharomyces cerevisiae</i> gil6321587)	Protein synthesis	8.00×10^{-33}
k6001-d01	60S ribosomal protein L44 (L41) (<i>Gibberella zeae</i> PH-1)	Protein synthesis	3.00×10^{-56}
k6001-g04	Unnamed protein product (<i>Podospora anserina</i> gil27802989)	Unknown	1.00×10^{-13}
k6001-h07	Guanine nucleotide-binding protein β subunit-like protein (<i>Gibberella zeae</i> PH-1 gil46136709)	Signal transduction	2.00×10^{-33}
k6001-h08	Fumarylacetoacetate hydrolase (<i>Emericella nidulans</i> gil41017083)	Metabolism	6.00×10^{-28}
k2001-a11	Predicted protein (<i>Gibberella zeae</i> PH-1 gil42545608)	Unknown	6.00×10^{-22}
k2001-d11	Hypothetical protein AN5421.2 (<i>Aspergillus nidulans</i> gil40743391)	Unknown	7.00×10^{-33}
k2001-h07	Hypothetical protein FG08359.1 (<i>Hypocrea virens</i> gil91771559)	Unknown	2.00×10^{-64}
k2001-d12	Hypothetical protein FG1090.1 (<i>Gibberella zeae</i> PH-1 gil42552463)	Unknown	7.00×10^{-07}
k2001-f08	Hypothetical protein FG05620.1 (<i>Gibberella zeae</i> PH-1 gil42552348)	Unknown	1.00×10^{-49}
k6001-a01	Hypothetical protein FG04071.1 (<i>Gibberella zeae</i> PH-1 gil42547972)	Unknown	7.00×10^{-17}
k6001-c01	Hydrophobin 1 (<i>Trichoderma asperellum</i> gil86154173)	Cell wall	5.00×10^{-17}
k6001-f04	Hypothetical protein FG10021.1 (<i>Gibberella zeae</i> PH-1 gil42544839)	Unknown	4.00×10^{-14}

Table 1. cont.

Clone name	Best BLASTX match	Cellular function	BLAST E
k6001-h11	Cytochrome c549 (<i>Fusarium oxysporum</i> gil6116892)	Metabolism	9.00×10^{-23}
k6001-b12	No hits found	Unknown	–
k6001-c02	No hits found	Unknown	–
k6001-d06	No hits found	Unknown	–
k6001-d12	No hits found	Unknown	–
k6001-e08	No hits found	Unknown	–
k6001-g09	No hits found	Unknown	–
k6001-g12	No hits found	Unknown	–
k6001-h01	No hits found	Unknown	–
k6001-h02	No hits found	Unknown	–

rod and filtering the resulting suspension through crossed layers of Kimwipes). Fungal material was fixed with 3.0 % glutaraldehyde and 1.0 % OsO₄ for analysis by TEM as described previously (Puyesky *et al.*, 1999).

Scanning electron microscopy (SEM). Aerial and submerged conidia were analysed using a JEOL JSM-6700F microscope equipped with a cold-field-emission gun. Conidia from submerged cultures or pieces of PDA agar containing aerial conidia from the wild-type and the $\Delta tvk1$ strains were frozen using a Gatan Alto 2500 cryo-stage chamber. Samples were frozen in slush nitrogen and cryo-transferred under vacuum to the preparation chamber for ice sublimation and gold/palladium sputter coating. Material was transferred from the Gatan preparation chamber to the SEM stage for sample observation.

RESULTS

Morphological characteristics of conidia produced in aerial and submerged culture

We have previously shown that *T. virens* $\Delta tvk1$ conidiate abundantly in submerged culture, whereas the wild-type strain does not produce conidia under these conditions. In contrast, on solid medium the mutants yield fewer conidia than the wild-type and show a slight alteration of their characteristic green colour (Mendoza-Mendoza *et al.*, 2003). SEM revealed that cell walls from the aerial spores produced by the wild-type and $\Delta tvk1$ null mutant strains were covered with an extracellular material forming papilla-like structures; these were uniformly distributed in the wild-type strain but showed a heterogeneous arrangement in the $\Delta tvk1$ null mutant (Fig. 1A, B). Contrary to the papilla-like structures shown in aerial conidia, conidia generated in submerged culture showed a smooth phenotype (Fig. 1A). Analysis by TEM showed that cell walls of aerial conidia were composed of an electron-transparent inner layer (W1) and an electron-dense outer layer (W2). In contrast to the W1 layer, which was similar in both strains, W2 was thinner in the $\Delta tvk1$ strain than in the wild-type (Fig. 1C), particularly in the case of submerged culture (Fig. 1C, bottom panel).

Conidiation of the $\Delta tvk1$ strain was studied in Vogel's minimal medium with sucrose (VMS). Morphological changes became evident 60 h post-inoculation in the $\Delta tvk1$

mutant strain (Fig. 2A–E), whereas the wild-type strain did not show changes even after prolonged incubation (Fig. 2F–H). Pellets of the $\Delta tvk1$ strain were reduced in size (180 µm diameter) compared to the wild-type strain (420 µm diameter). This was accompanied by increasing branching of the mutant, which was followed by the development of a phialide in each newly formed hypha (Fig. 2B, C). During the next 8 h conidia differentiated (Fig. 2C, D), after which they were released into the medium (Fig. 2E). Interestingly, in several cases we observed the release of the conidium and phialide together, indicating that phialides were not strongly attached to the rest of the hyphae (Fig. 2E).

Genes encoding cell wall proteins in *T. virens* are highly conserved

To identify genes that were expressed specifically during conidiation, we constructed subtractive hybridization cDNA libraries using the wild-type and the $\Delta tvk1$ null mutant strains grown in submerged culture. A total of 567 clones were sequenced, representing 90 different genes. Classification of the matches obtained upon BLAST analysis of the sequences revealed similarities to proteins involved in a range of functions, including protein fate, primary metabolism, oxidative stress, signal transduction, DNA processing, protein biosynthesis, cell wall components and proteins with unknown function (see Tables 1 and 2).

Seven genes identified in the subtractive cDNA libraries encoded putative cell wall proteins. These genes were called *Tv-hfb1*, *Tv-srh1*, *Tv-qid3*, *Tv-cfth1*, *Tv-hfb3*, *Tv-qid74* and *Tv-ccg14/Sm1*. Eight cysteine residues were identified in five of the deduced proteins. We clearly identified two consensus patterns of cysteine residues: (1) C-X₇₋₁₀-CC-X₁₁-C-X₁₅₋₁₆-C-X₆₋₉-CC-X₁₀₋₁₁-C-X₇₋₈ and (2) C-X₆-CC-X₉-C-X₅-C-X₅-CC-X₈-C-X₁₆. Both patterns showed the hallmarks of hydrophobins (Wessels, 1994). Because the N-terminus is highly variable in hydrophobin-like proteins, we used only the sequences between the first and eighth cysteine residues. *Tv-hfb1*, *Tv-srh1*, *Tv-cfth1* and *Tv-qid3* belong to the class II hydrophobins, whereas *Tv-hfb3* belongs to the class I hydrophobins. *Tv-hfb1* showed the highest identity (64 %) to HFBI from *Trichoderma reesei*, whereas *Tv-srh1* was 56 %

Table 2. *T. virens* genes obtained from the $\Delta tvk1$ null mutant after subtraction of the genes expressed in the wild-type

Clone name	Best BLASTX match	Cellular function	BLAST E
wt2001-a09	Malate dehydrogenase (<i>Paracoccidioides brasiliensis</i> gil30961994)	Metabolism	5.00×10^{-17}
wt2001-e07	Protein component of the large (60S) ribosomal subunit (<i>Saccharomyces cerevisiae</i> gil6319444)	Protein synthesis	8.00×10^{-54}
wt2001-e11	Exochitinase (<i>Trichoderma harzianum</i> gil1839393)	Cell growth and mycoparasitism	6.00×10^{-16}
wt2001-h09	Hydrophobin II precursor (HFBII) (<i>Hypocrea jeroica</i> gil6647555)	Cell wall	3.00×10^{-16}
wt6003-c12	Probable t-complex protein 1, ϵ subunit (<i>Schizosaccharomyces pombe</i> gil19114189)	Protein synthesis	2.00×10^{-94}
wt6003-g11	SNODPROT1 Precursor (<i>Phaeosphaeria nodorum</i> gil6094315)	Cell wall	4.00×10^{-32}
wt2004-a12	Protein Ric1 (<i>Phytophthora infestans</i> gil15214248)		1.00×10^{-13}
wt2001-a05	Probable nickel-binding protein SPBC887.01 (<i>Schizosaccharomyces pombe</i> gil19113267)	Oxidative stress	2.00×10^{-41}
wt2001-e06	Hypothetical protein FG05601.1 (<i>Gibberella zeae</i> PH-1 gil42550933)	Unknown	3.00×10^{-35}
wt2001-h01	Hypothetical protein (<i>Aspergillus fumigatus</i> gil41581203)	Unknown	9.00×10^{-15}
wt6004-f09	Hypothetical protein MG09842.4 (<i>Magnaporthe grisea</i> gil38107749)	Unknown	2.00×10^{-08}
wt6004-d07	Hydrophobin I precursor (HFBII) (<i>Trichoderma reesei</i> gil1708378)	Cell wall	2.00×10^{-15}
wt2001-a08	CipC protein (<i>Emericella nidulans</i> gil20429042)	Unknown	6.00×10^{-20}
wt2001-c11	Cytochrome c549 (<i>Fusarium oxysporum</i> gil6116892)	Metabolism	9.00×10^{-23}
wt6004-e01	Flavo-haemoglobin (<i>Fusarium oxysporum</i> gil3551511)	Metabolism	2.00×10^{-07}
wt2001-d03	Cell wall protein QID3 (<i>Hypocrea lixii</i> embICA50728.1)	Cell wall	3.00×10^{-32}
wt2001-c07	No hits found	Unknown	—
wt2001-a10	No hits found	Unknown	—
wt2001-c02	No hits found	Unknown	—
wt2001-c08	No hits found	Unknown	—
wt2001-d01	No hits found	Unknown	—
wt2001-d10	No hits found	Unknown	—
wt2001-e04	No hits found	Unknown	—
wt2001-e06	No hits found	Unknown	—
wt2001-f11	No hits found	Unknown	—
wt2001-g09	No hits found	Unknown	—
wt2001-h01	No hits found	Unknown	—
wt6003-f11	No hits found	Unknown	—
wt2001-a07	No hits found	Unknown	—
wt6004-a11	No hits found	Unknown	—
wt6004-f10	No hits found	Unknown	—
wt6004-f12	No hits found	Unknown	—
wt2001-b05	No hits found	Unknown	—
wt6004-g11	No hits found	Unknown	—
wt6004-f08	No hits found	Unknown	—
wt6004-h07	No hits found	Unknown	—

identical to HFBII from *T. reesei* and 58 % identical to Srh1 from *Hypocrea lixii*. Tv-cfth1 showed high similarity with the three domains of the tri-hydrophobin Cfth1 from *Claviceps fusiformis* (57 %), whereas Tv-qid3 showed high identity (66.0 %) to QID3 from *H. lixii*. On the other hand, Tv-hfb3 had high identity (87 %) to TasHyd1 from *Trichoderma asperellum*, 56 % identity to a hypothetical protein from *Gibberella zeae* (gil42545447) and 41 % identity to hydrophobin Pbhyd1 from *Paracoccidioides brasiliensis*.

In addition to the genes encoding hydrophobin-like proteins we identified one additional gene encoding a cell

wall protein (*Tv-qid74*) and one which encodes a small secreted protein (*Tv-ccg14/Sm1*). The latter corresponds to the recently reported gene *Sm1* from *T. virens* (Djonović *et al.*, 2006), which encodes a protein that displayed strong identity to a probable SnodProt1 precursor from *N. crassa* (50 % identity), which has been reported also as a clock-controlled gene (*ccg-14*) in *N. crassa* (Zhu *et al.*, 2001). A comparative analysis of Tv-snodprot1 and its homologues in other fungi showed high identity among them (Djonović *et al.*, 2006). Tv-qid74 had a high identity to the repetitive domains of Qid74, a cell wall protein reported in *H. lixii* (Rey *et al.*, 1998).

Fig. 1. Morphological differences between aerial and submerged conidia of wild-type and $\Delta tvk1$ strains of *T. vires*. (A) SEM images of wild-type (Gv 29.8), and $\Delta tvk1$ conidia produced on solid and liquid medium. (B) TEM images of the same samples as in (A). (C) Magnification of (B). W1, inner wall layer; W2, outer wall layer. Scale bars: (A) and (B), 1 μ m; (C), 100 nm.

Genes encoding cell wall proteins are differentially expressed during the conidiation process

Expression of the genes encoding cell wall proteins mentioned above was investigated and compared between the wild-type and $\Delta tvk1$ null mutant strains. Total RNA from mycelium of wild-type and $\Delta tvk1$ strains grown in liquid shaken cultures in VMS medium was used for this analysis (Fig. 3). *Tv-hfb1*, *Tv-srh1*, *Tv-cfth1* and *Tv-ccg14/Sm1* were expressed in both strains; however, higher expression was found in the $\Delta tvk1$ strain (Fig. 3). The expression pattern of these genes was similar, most of them showing maximum expression 52 h post-inoculation, a few hours before noticeable morphological changes occurred, and thereafter their transcript levels started to decrease. Interestingly, *Tv-qid74* and *Tv-hfb3* were expressed at higher levels in the wild-type strain than in the $\Delta tvk1$ null mutant, in which their expression level was scarcely detectable (Fig. 3). *Tv-qid74* reached its highest level of expression 60 h post-inoculation; after this its expression decreased sharply (Fig. 3). On the other hand, *Tv-hfb3* showed the highest expression level between 60 and 64 h (Fig. 3). The highest level of expression of *Tv-qid74* and

Tv-hfb3 coincided with the start of the production of phialides and conidia (Fig. 2b–c).

Conidia from the wild-type and $\Delta tvk1$ strains were inoculated in VMS and incubated for 24 h at 28 °C. The mycelium was then transferred to Vogel's minimal medium (VMM) without carbon (VM–C) or nitrogen sources (VM–N). In these experiments the $\Delta tvk1$ null mutant always showed higher expression levels of *Tv-hfb1*, *Tv-srh1*, *Tv-cfth1* and *Tv-ccg14/Sm1* than the wild-type strain. Unexpectedly, no differences were observed between the samples obtained from VMM and VM–C; however, a discrete increase in *Tv-hfb1*, *Tv-srh1*, *Tv-cfth1* and *Tv-ccg14/Sm1* expression in VM–N with respect the VMM was observed (data not shown).

Conidia produced in submerged culture show higher hydrophobicity than those generated on solid media

The hydrophobic character of the reproductive structures generated in liquid and on solid media was determined according to the phase distribution method (Mozes & Rouxhet, 1987). The hydrophobicity indices obtained

Fig. 2. (A–E) Submerged conidiation in the $\Delta tvk1$ strain of *T. virens*. The arrows in (B–D) indicate conidia (c) and phialides (ph). The arrow in (E) indicates the release of a phialide together with the conidium. (F–H) Micrographs of the wild-type strain serving as a control. Scale bars, 10 μ m.

indicated that aerial conidia produced by the wild-type strain were highly hydrophobic whereas those obtained from the $\Delta tvk1$ mutant had notably reduced hydrophobicity (Table 3). Surprisingly, conidia of the $\Delta tvk1$ mutant generated in submerged cultures were the most hydrophobic and this characteristic was prevalent at the different pH values analysed. In contrast, hydrophobicity indices from aerial conidia decreased at high pH.

Breakage of the liquid–air interface is altered in the $\Delta tvk1$ strain

The ability of the $\Delta tvk1$ and wild-type strains to escape from a hydrophilic to a hydrophobic environment was evaluated. Wild-type and $\Delta tvk1$ spores were grown in PDB or VMS medium placed on Parafilm. Depending on the medium used, two interesting differences, opposite to each other, in the $\Delta tvk1$ strain were observed. (1) In VMS medium, the $\Delta tvk1$ strain formed the most prominent and strong filaments; these filaments were not strongly attached to the hydrophobic surface, since they easily came off when the drop was collected with a micropipette tip. Together with this prominent filament formation, a collapse in the middle of the drop was observed (Fig. 4a). (2) In contrast, in PDB the mutant was unable to break the liquid–air interface (Fig. 4a). This characteristic was not due to growth alterations in the $\Delta tvk1$ strain, which appeared to form normal filaments in the drops.

The ability to escape from a hydrophilic to a hydrophobic environment involves changes in the pattern of hydrophobin gene expression. We tested the relationship between the ability to break the liquid–air interface and the patterns of gene expression (Fig. 4b). *Tv-srh1* mRNA levels decreased with time and those of *Tv-qid3* increased in both strains. However, the highest expression level of both genes was observed in the $\Delta tvk1$ strain. In the wild-type strain *Tv-hfb3* was not expressed until 40 h post-inoculation, when it reached its maximum level, with a clear reduction after this point. *Tv-qid74* was expressed at all times examined; it increased slightly by 40 h after drop deposition and then started to decrease. The pattern of expression of *Tv-cfth1* in the wild-type strain was similar to that of *Tv-qid74*, except that it reached an apparent maximum by 35 h, with a significant reduction at 40 h and increased again by 45 h. In contrast, in the $\Delta tvk1$ mutant the expression of *Tv-cfth1* reached a maximum by 35 h, with a pronounced drop at 45 h. Interestingly, *Tv-hfb3* and *Tv-qid74* were expressed exclusively in the wild-type strain. In contrast, *Tv-ccg14/Sm1* showed increasing levels of expression over time and was detected only in the $\Delta tvk1$ mutant. *Tv-hfb1* was expressed weakly in the $\Delta tvk1$ strain in VMS and was completely absent in PDB (Fig. 4b and data not shown). A good correlation between hydrophobin gene expression and hyphal emergence under the conditions tested was shown (Fig. 4). Taken together these data suggest a complex regulation of hydrophobin gene

Fig. 3. Gene expression comparison in submerged culture of the wild-type and $\Delta tvk1$ null mutant. Samples of the two strains grown in submerged culture were collected at the indicated time points and total RNA was extracted for Northern analysis. Twenty micrograms of total RNA were loaded in each lane. The probes used were as follows: *Tv-hfb1* was obtained from wt6004-d07; *Tv-srh1* from wt2001-h09, *Tv-cfth1* from wt2001-d08, *Tv-ccg14* from wt6003-g11, *Tv-qid74* from k2001-h09, *Tv-hfb3* from k6001-c01; and *Tv-qid3* from wt2001-d03 (see Tables 1 and 2). The lowest panel shows the ethidium bromide staining of the gel, as a loading control.

expression and cell wall components during changes in environment.

DISCUSSION

Ultrastructural analyses of the cell wall of aerial conidia from the wild-type and $\Delta tvk1$ strains of *T. virens* showed morphological differences in the first cell wall layer (W2). The W2 layer has been considered to be spore specific and it could be related to pigmentation (Muñoz *et al.*, 1995).

Table 3. Hydrophobicity indices for aerial and submerged *T. virens* conidia, calculated by the phase distribution method (Mozes & Rouxhet, 1987)

Conidia type	pH		
	3.0	6.0	9.0
Wild-type aerial	93.7	92.4	81.7
$\Delta tvk1$ aerial	86.6	83.3	45.8
$\Delta tvk1$ submerged	100.0	100.0	100.0

Because $\Delta tvk1$ aerial conidia are pale green, in contrast to the intense dark green colour from the wild-type strain, the idea of thickness and green colour being related would be supported by our observations. However, this correlation is not valid for submerged conidia, which contain a very thin outer layer (W2) and have an intense green colour. The developmental programme of *T. virens* was completed in 72 h, when the conidia were released from the conidiophore, probably through enzymic activities. *Tv-nag1*, a gene found among those expressed only in $\Delta tvk1$, encodes an *N*-acetyl- β -D-glucosaminidase and its homologue in *Trichoderma atroviride* is secreted and partially bound to the cell wall (Brunner *et al.*, 2003). Another interesting finding was that of a G β -subunit-encoding gene identified in the subtractive library of the genes absent in $\Delta tvk1$. G β subunit proteins are considered negative regulators of conidiation in submerged culture in *Aspergillus nidulans* and *N. crassa* (Rosen *et al.*, 1999; Yang *et al.*, 2002).

Five hydrophobin-like proteins have been reported in *Trichoderma*, and four belong to class II (Linder *et al.*, 2005; Viterbo & Chet, 2006). We identified two additional hydrophobin-like proteins in *T. virens*. *Tv-hfb3* encodes a novel protein related to class I hydrophobins. Although *Tv-hfb3* almost contains the 'classical' cysteine distribution, it has an unusual spacing between the fourth and fifth cysteine. A similar difference was also observed in Hyd3, a hydrophobin from *Fusarium verticillioides*, but in this case the discrepancy was located between the third and fourth cysteine (Fuchs *et al.*, 2004). *Tv-cfth1* encodes the second novel hydrophobin-like protein in *T. virens*. The open reading frame deduced from the genomic clone of the gene showed high identity to each domain of the tri-hydrophobin Cfth1 from *C. fusiformis* (de Vries *et al.*, 1999), and allowed us to identify eight cysteines in the deduced protein (data not shown). However, one of the cysteines is located in an unusual position, between the fourth and fifth cysteine found in typical hydrophobins. Another class II hydrophobin with an additional cysteine in an equivalent location is HFBIII from *T. reesei*. Furthermore, when the structure of *Tv-cfth1* was compared to the structure of hydrophobin HFBII (PDB code 1R2M), the cysteine was found on the protein surface and in the region of the α -helix in HFBII. Therefore, *Tv-cfth1* is very likely to adopt the typical structure of hydrophobins. On the other hand, *Tv-qid74* is highly homologous to Qid74, an extracellular protein which has been detected in several species of *Trichoderma*; however, the physiological role of this protein is still unknown (Rey *et al.*, 1998).

Our results indicate that *Tvk1* is involved in the negative regulation of the genes encoding type II hydrophobins, *Tv-hfb1*, *Tv-srh1* and *Tv-cfth1*, in submerged culture. Furthermore, the high expression of *Tv-hfb1*, *Tv-srh1* and *Tv-cfth1* during conidiation in submerged culture in $\Delta tvk1$ suggests a role for the corresponding proteins in this developmental programme. The expression of *Tv-hfb3* and *Tv-qid74* was abolished in the absence of *Tvk1*, suggesting a positive role for *Tvk1* in the expression of these genes. The

Fig. 4. Breaking water–air interface and expression of cell wall protein and hydrophobin genes. (A) Breaching of PDB or VMS drops at different time points. Fifty-microlitre drops containing 1×10^6 conidia suspended in PDB or VMS medium were deposited on Parafilm and incubated for the times indicated at 28 °C. (B) Expression of cell wall protein genes in *T. vires* during emergence from VMS medium. Twenty micrograms of total RNA were loaded in each lane and probes used were as described in Fig. 3. Hybridization with the 28S rRNA gene was used as a loading control.

fact that neither gene was expressed during conidiation in submerged culture indicates that they are dispensable for this process. Viterbo & Chet (2006) showed that Tashyd1, the homologue of *Tv-hfb3*, is needed for hydrophobicity of young hyphae and during plant root colonization. The class I hydrophobin Mpg1 of *Magnaporthe grisea* is required for appressoria function (Talbot *et al.*, 1996) and regulated by the homologue of *tvk1* (Soanes *et al.*, 2002). These data suggest conserved signalling in the regulation of this class of hydrophobins through Pmk1 homologues. Conidia of $\Delta tvk1$ generated in submerged cultures were more hydrophobic than those generated on plates. Although these results were the opposite of those reported for other systems (Muñoz *et al.*, 1995; Pascual *et al.*, 2000), they clearly correlated with the high levels of expression of the hydrophobin-encoding genes in submerged culture.

Hydrophobins seem to serve a dual role by first lowering the water surface tension, which allows the fungus to penetrate physical barriers, and then forming a protective coating on aerial structures and spores (Wösten *et al.*, 1994b, 1999). In *T. reesei* HFBII is involved in conidiation and HFBI is involved in breaking hydrophilic–hydrophobic interfaces (Askolin *et al.*, 2005). Hyperconidiation and the high level of expression of *Tv-srh1* in submerged cultures could be explained if we consider that *Tv-srh1* is also needed for conidiation as in *T. reesei* (Askolin *et al.*, 2005). Nevertheless, expression of *hfb2* from *T. reesei* in both conidiation and normal vegetative growth suggests that the protein has other functions in addition to its presumed role in protecting the spores (Nakari-Setälä *et al.*, 1997). Deletion of *hfb2* in *T. reesei* promoted intense conidiation when lactose medium was used as the carbon source, but

when glucose was used the mutant produced fewer conidia than the wild-type (Askolin *et al.*, 2005; Bailey *et al.*, 2002). These phenotypes resemble those of $\Delta tvk1$, which shows reduced conidiation on solid medium and hyperconidiates in submerged culture (Mendoza-Mendoza *et al.*, 2003).

In contrast to HFBII, which is secreted, most HFBI is attached to the cell walls in *T. reesei* (Askolin *et al.*, 2001). Deletion of *hfb1* affects sporulation, probably due to the reduction in aerial hyphae formation. The role of HFBI in hyphal development and during the breaking of the liquid–air interface by filaments was shown in glucose-containing medium (Askolin *et al.*, 2005). Our results indicate that during breakage of the liquid–air interface *Tv-hfb1* was expressed at very low levels, whereas the expression of *Tv-srh1* and *Tv-qid3* was very high, and they may perform the function of *Tv-hfb1* in this process. The differences in expression and behaviour of the fungi in the same situation depend on the environment, as reflected by the ability of $\Delta tvk1$ to break the hydrophilic–hydrophobic interface in VMS and its inability to do so in PDB. However, the breaking of the interface in PDB in the wild-type could be explained by the participation of other hydrophobins expressed in this medium. *Tv-qid3* expression was reduced in submerged cultures of the $\Delta tvk1$ strain until it became undetectable. In contrast, it was highly expressed during breakage of the hydrophilic–hydrophobic interface in the $\Delta tvk1$ strain, whereas the expression in the wild-type was lower. These data suggest a role of *Tv-qid3* in filamentous growth in hydrophobic environments but not in liquid media or during submerged conidiation.

All things considered, the MAP kinase *Tvk1* appears to play a major role in the control of the production of conidia

through its action as a molecular switch for the expression of a set of genes involved in the corresponding developmental programme.

ACKNOWLEDGEMENTS

The authors wish to thank Dr Octavio Martínez for assistance with the BLAST analysis, and Beatriz Jimenez and Guillermo Corona for their invaluable help in sequencing. We are also grateful to Dr Johanna Kallio and Bernadette Heinze for stimulating discussion and suggestions. This research was supported by grant GTO-2002-C01-5965 from CONCyTEG to A.H.-E.; A.M.M and T.R.-S. each received a fellowship for doctoral studies from Conacyt México.

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Edited by: H. A. B. Wösten