RESEARCH ARTICLE

TvDim1 of Trichoderma virens is involved in redox-processes and confers resistance to oxidative stresses

M. Eugenia Morán-Diez · Rosa E. Cardoza · Santiago Gutiérrez · Enrique Monte · Rosa Hermosa

Abstract The evolutionarily conserved Dim1 proteins belong to the TRX fold superfamily. An EST showing high identity values with genes coding for Dim1 proteins was selected from an EST library collection of *Trichoderma virens* T59. Here, we report the cloning, characterization, and functional analysis of a *T. virens* T59 *TvDim1* gene. The *TvDim1* gene, with a sequence size of 614 bp, was PCR-amplified and found to contain three introns. The *TvDim1* gene was present as a single copy in the *T. virens* genome and was also present in another five *Trichoderma* strains investigated. Increased levels of expression and redox-activity were detected when the fungus was grown in the presence of H_2O_2. The overexpression and silencing of *TvDim1* in *T. harzianum* T34 gave rise to transformants, with higher and lower growth, redox-activity, and quantities of biomass, respectively, than the wild-type strain after culture under oxidative stress.

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Introduction

Most species of *Trichoderma* have been linked to biocontrol and biotechnological applications. The antagonistic properties of *Trichoderma* species are well known and have been related to mechanisms of action, such as mycoparasitism (Benítez et al. 2004; Lorito 1998), antibiosis (Vinale et al. 2006), and competition for nutrients (Sivan and Chet 1989), and their capacity to promote plant growth (Harman et al. 2004) and induce plant defense (Alfano et al. 2007; Djonovic et al. 2006, 2007; Shoresh and Harman 2008; Viterbo and Chet 2006). Nevertheless, little is known about aspects related to the development and cell cycle of this fungus (Brunner et al. 2008; Esquivel-Naranjo and Herrera-Estrella 2007; Rocha-Ramírez et al. 2002).

The thioredoxin (TRX)-like fold superfamily is a group of proteins that has been related to a large number of functions in the cell, such as the regulation of redox homeostasis (Grant 2001), impaired cell cycles and sexual development (Berry and Gould 1997; Malagnac et al. 2007), pre-mRNA splicing (Simeoni and Divita 2007), and protein folding (Ferrari et al. 1998). These proteins show similar three-dimensional structures, the so-called TRX fold (Martin 1995), which is present in several classes of proteins, such as TRX, glutaredoxin, the Dsb protein from *Escherichia coli*, glutathione peroxidase, glutathione *S*-transferase, peroxiredoxin, disulfide isomerase, cytochrome *c* oxidase, iodothyronine selenodeiodinases, and the evolutionarily conserved Dim1 protein (Stefankova et al. 2005). In these proteins the TRX fold can be modified by additional amino acid residues at the N and/or C terminus of the sequence and some insertion in the specific

regions of this fold (Stefankova et al. 2005). In this sense, although Dim1 proteins structurally belong to the TRX superfamily, the most important feature of TRXs—the canonical CxxC motif—is replaced by a DxxC motif (Reuter et al. 1999). The importance of the two cysteines in the reduction of protein disulfide bonds, and hence in protecting the cell from oxidative stress, is not clear (Fomenko and Gladyshev 2003).

A TRX study including several Dim1 proteins revealed high homology and similarity of protein secondary structure, although such proteins also showed a highly conserved C-terminal extension, important for its biological function (Zhang et al. 1999). These proteins have been identified as part of the spliceosome, a complex machine comprising several elements involved in the removal of introns from nuclear mRNA precursors. Dim-encoding genes have been identified in *Homo sapiens*, *hDim1*; in *Saccharomyces cerevisiae*, *Dib1p/Snu16p* (Reuter et al. 1999); and in *Schizosaccharomyces pombe*, *Dim1p* (Berry and Gould 1997). Their role in pre-mRNA splicing is not clear, but this protein appears to bind other U5-specific proteins, such as Prp1p/Prp6p (Carnahan et al. 2005).

In work with mutants, other functions have also been assigned to Dim proteins. Thus, *Dim1p* has been shown to be essential for G2/M progression and for chromosome segregation during mitosis in *S. pombe* (Berry and Gould 1997). Gene deletions—of *Dim1p* in *S. pombe* and of *Dib1p* in *S. cerevisiae*—produce a lethal G2 arrest (Carnahan et al. 2005), and *Dim1* deletion also induces lethality during embryogenesis in metazoans, such as *Caenorhabditis elegans* (Zhang et al. 2000).

Highly conserved Dim sequences are present in all genomes but to date no Dim1 proteins have been described in filamentous fungi. In the present work, we explored the presence of Dim1-encoding genes in our collection of *Trichoderma* expressed sequence tags (ESTs), generated in a functional genomics project carried out on eight *Trichoderma* species (Rey et al. 2004). An EST showing high identity with Dim1 proteins was identified in a *Trichoderma virens* T59 cDNA library. Here, we report the cloning and characterization of the *TvDim1* gene in the biocontrol fungus *T. virens* T59. Using a heterologous expression approach, the *TvDim1* gene from *T. virens* T59 was overexpressed and silenced in *T. harzianum* T34, a strain commonly used in basic research studies, to investigate the relationship of this gene with the regulation of redox homeostasis.

Materials and methods

Bacterial and fungal strains

Escherichia coli DH5α (Invitrogen Life Technologies, Carlsbad, CA, USA) was used as host for plasmid construction and propagation. This bacterial strain was grown in Luria–Bertani (LB) broth or on LB plates, which were supplemented with ampicillin (0.1 mg/ml), X-gal (40 μg/ml), and IPTG (10 μg/ml) when required.

Six *Trichoderma* strains were used in this study: *T. atroviride* T11, *T. asperellum* T25, *T. longibrachiatum* T52, and *T. virens* T59 from the NBT collection (Newbiotechnic S.A., Sevilla, Spain), *T. harzianum* T22 (ATCC 20847, American Type Culture Collection, Manassas, USA), and *T. harzianum* T34 (CECT 2413, Spanish Type Culture Colletion, Burjasot, Spain). Four pathogenic fungal strains were used in the antifungal assays: *Botrytis cinerea* 98, isolated from diseased strawberry plants; *Fusarium oxysporum* f. sp. *lycopersici* (CECT 2866); *Rhizoctonia solani* (CECT 2815), and *Colletotrichum acutatum* 73 (IMI 364856, CABI Bioscience, Egham, UK), isolated from diseased oranges. All fungal strains were grown on Potato Dextrose Agar (PDA, Difco Becton Dickinson, Sparks, MD, USA) and were maintained at −80°C in a 20% glycerol solution, with the exception of the *R. solani* strain, which was maintained at 25°C in sterilized wheat grains and *B. cinerea* strain, which was grown on Malt Extract Agar (MEA).

EST database

As much as 26 cDNA libraries were previously constructed for the TrichoEST project using different mRNA populations from 10 strains belonging to eight *Trichoderma* species expressed under mycoparasitic and nutrient stress conditions (Rey et al. 2004). Expressed sequence tags (ESTs) were generated by sequencing cDNA clones from the 5′ end, and an EST database was compiled with 13,814 unique ESTs (Vizcaíno et al. 2006, 2007).

cDNA library

L20, a cDNA library constructed with RNA from *T. virens* T59 for the TrichoEST project, was used (Vizcaíno et al. 2007). Biomass was obtained following a two-step liquid culture procedure. First, the fungus was grown in minimal medium (MM) (Penttilä et al. 1987) containing 2% glucose at 28°C for 36 h. Mycelia were harvested and transferred to MM without glucose under the following substrates/conditions in separate cultures: 1.5% crab chitin for 8 h; nitrogen starvation for 8 h; 1.5% crab chitin and pH 3.5 for 4 h, and 2% plant cell wall polymers for 96 h. Nitrogen starvation conditions included a 100-fold decrease in the concentration of ammonium sulfate in the medium (50 mg/l). RNA was extracted using the TRIZOL® reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. After total RNA extraction, equal amounts of RNA from the four different growth

conditions were mixed and used for mRNA purification, employing Dynabeads (Dynal, Oslo, Norway). The cDNA library was constructed using the Uni-ZAP XR Vector System (Stratagene, La Jolla, CA, USA), following the manufacturer's instructions.

DNA and RNA manipulations

Standard molecular techniques were performed throughout these studies (Sambrook and Russel 2001). Mycelia for DNA extractions were obtained from Potato Dextrose Broth (PDB, Difco) cultures incubated at 28°C and 200 rpm for 2 days. Then, they were collected by filtration, washed with distilled water, frozen, and lyophilized. Fungal genomic DNA was isolated according to previously described protocols (Raeder and Broda 1985).

For Southern analysis, 10 μg of genomic DNA was digested with restriction enzymes, electrophoresed on 0.7% agarose gels, and transferred to a Hybond-N+ membrane (Amersham Biosciences AB, Uppsala, Sweden). The probe was labeled with ^{32}P using Ready-To-Go DNA labeling beads (32P-dCTP) kit (Amersham). Hybridizations were carried out at 65°C for 16 h. Membranes were washed under high-stringency conditions.

Mycelia for RNA extraction and qRT-PCR expression analyses were obtained following a two-step liquid culture procedure, as described above, except that the following substrates/conditions were used in separate cultures: glucose starvation (0% glucose), 1 mM H_2O_2, 0.5 mM Paraquat® (Afrasa S.A., Valencia, Spain), and 10% NaCl. Cultures were maintained at 25°C on a rotary shaker at 150 rpm for 4, 8, or 24 h. Mycelia were collected by filtration, thoroughly washed with sterile water, lyophilized, and kept at −80°C until RNA extraction. Fungal RNA was obtained as described above.

PCR procedures

The *TvDim1* gene was isolated by PCR from strain T59 genomic DNA using the degenerated primer 614-4 (5′-ATGGG$^T/_C$TC$^C/_T$GTCGTTCTCCC-3′), designed on the 5′ end from an alignment of *Dim1* genes from databases, and the primer 614-3 (5′-GTAATCCTTGGGGCTGACC-3′), designed on the 3′ end from the EST 614. The *TvDim1* cDNA was PCR-amplified using phagemid DNA from the cDNA library L20 as template and the TRX-1 (5′-ATGG GCTCTGTCGTTCTC-3′) and TRX-3 (5′-CTA GTATCT GTGTCTCGT-3′) primers.

The primer pair TRX-1 and 614-3 was used to amplify the *TvDim1* gene, using the genomic DNA from *T. virens* T59 as template. This PCR product was used as probe in Southern blot experiment.

Screening of the *T. harzianum* T34 overexpressed transformants was carried out by PCR with the primers pKi-614 (5′-GGAGCTTGACGAAGACCTG-3′) and 614-3, described above. The screening of *T. harzianum* T34 silenced transformants was also carried out by PCR with the primer pair pta-p (5′-GATGCACTGCAGTCCAC-ATTG-3′) and 614-3.

PCR amplifications were carried out for 35 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min 30 s at 72°C, with the *Pfu* or *Taq* (for the screening of transformants) polymerase system (Biotools, Edmonton, Canada), following the manufacturer's instructions.

qRT-PCR analysis

For the expression analysis of *TvDim1* gene in *T. virens* T59, total RNA was extracted using the TRIZOL® reagent (Invitrogen) and 1 μg of total RNA was used for a RT reaction using reverse transcription system (Promega, Madison, WI, USA) following the manufacturer's instructions. The real-time PCR was performed using the Stratagene's Brilliant SYBR® Green QPCR master mix (Stratagene, La Jolla, CA, USA). The reaction mix was prepared according the manufacturer's instructions. The primer pair TRX-5 (5′-GAAGAGGATCGTCTCGTCGTC-3′) and TRX-3 (5′-TCAGGAACCTCGTCAATGTCG-3′) was used to amplify a 143-bp fragment of *TvDim1* gene and the *β-tubulin* gene was used as an internal reference. Amplifications were carried out in the ABI PRISM® 7000 (Applied Biosystems, Foster City, CA, USA) and the thermal cycler was programmed as follows: one cycle of 10 min at 95°C, 40 two-step cycles of 15 s at 95°C, and 1 min at 60°C.

In a similar way, the relative expression of *TvDim1* gene in the overexpressed transformants *T. harzianum* TvDim1-1, TvDim1-2, and TvDim1-3 respecting to *ThDim1* (the endogenous gene) was analyzed by qRT-PCR using the primer pair TRX-5 and TRX-3.

Plasmid constructions and *Trichoderma* transformation procedure

For gene overexpression, plasmid pLMRS3, which contained the *pki1* (pyruvate kinase I) gene promoter and the *cbh2* (cellobiohydrolase II) terminator region, both from *T. reesei* (Mach et al. 1994), was digested with *Xba*I, treated with a Klenow fragment, and dephosphorylated with calf intestine alkaline phosphatase (CIAP). Then, it was ligated to the *TvDim1* ORF. An *Nsi*I–Klenow–*Eco*RI fragment, containing the *pki* promoter and the *TvDim1* gene, was ligated into plasmid pJL43b (Gutiérrez et al. 1997), to construct the plasmid designated pSPDim1-1 (4.43 kb), which contained the expression cassette

(1465 bp). This cassette, containing the *pki* promoter, the *TvDim1* gene, and the *CYC1* terminator from *Saccharomyces cerevisiae*, was isolated by digestion with *Xho*I and ligated to plasmid pJL43b1 linearized with *Xho*I. This vector, designated pJLTvDim1 (5.95 kb), contained the phleomycin resistance gene (*ble*) from *Streptoalloteichus hindustanus* under the control of the *Aspergillus nidulans gpdA* gene promoter. The pJLTvDim1 plasmid was used to transform *T. harzianum* T34 using the previously described protoplast method (Cardoza et al. 2006). Transformants were selected for phleomycin resistance.

For gene silencing, the plasmid pSIL (Sousa 2004) was linearized with *Bam*HI, treated with Klenow fragment, and dephosphorylated with CIAP. Then, it was ligated to a dephosphorylated 432 bp fragment, corresponding to the *TvDim1* ORF. This fragment was "in sense" relative to the *tss1* promoter. This construction (pSILDim-0) was later linearized with *Eco*RV and treated with CIAP, after which the ORF was ligated in "anti-sense" with respect to the *tss1* promoter. An intron of 150 bp was introduced between both the "sense" and "anti-sense" ORF of the *TvDim1* gene. This plasmid also contained the terminator region of the *cbh2* gene from *T. reesei* and was designated pSILTvDim1 (6,114 bp). Plasmids pSILTvDim1 and pJL43b1, which contained phleomycin resistance gene (Gutiérrez and Martín unpublished), were used at a proportion of 9:1 to co-transform *T. harzianum* T34 using a previously described protoplast method (Cardoza et al. 2006). Transformants were selected as described above.

TRX activity assays

TRX activity was tested in a 1,000-µl reaction volume using the insulin-disulfide reduction assay according to the method of Holmgren (1979). Briefly, the assay was monitored by the addition of a master mix of the following reaction components at the indicated end concentration— 63 mM sodium phosphate buffer, pH 7.0, 2 mM EDTA, 0.1% (w/v) insulin solution, and 1 mM DTT—to intracellular protein extracts from *Trichoderma* cultures. The reactions were incubated at 25°C for 20 min. Then, measurements were performed at OD_{650} nm every 5 min over a total of 20 min on a spectrophotometer. TRX from *Escherichia coli* (Sigma-Aldrich, San Luis, MO, USA) at an end concentration of 1 µg/ml was used as a positive control, and the master mix without TRX was used as a negative control. Total activity corresponds to µmol formed in 1 min, and specific activity corresponds to µmol formed in 1 min per mg of protein. Quantitative protein determination was carried out with the Bradford assay (Bradford 1976). Tests were performed in triplicate and data represent mean values with standard deviations.

Oxidative stress resistance assays

The wild-type, three *TvDim1*-overexpressing transformant strains and three *TvDim1*-silenced transformant strains were tested for resistance to oxidative stress by measuring colony diameters after incubation under different growth conditions. As much as 200 conidia from each strain were inoculated onto plates containing MM supplemented or not (control) with 1 mM or 3 mM H_2O_2 and incubated at 28°C for 1 week.

The growth of strain T34, one overexpressing transformant and two silenced transformants was also tested in liquid media. A 250-Erlenmeyer flask containing 100 ml of MM 1% glucose supplemented or not (control) with 1 mM H_2O_2 was inoculated with 10^6 conidia and maintained at 28°C for 48 h. Fungal growth was determined by measuring the accumulation of biomass dry weight according to Montero-Barrientos et al. (2007). Both growth assays— in liquid medium and on plates—were carried out in triplicate.

Direct confrontation assays

In vitro confrontation assays between *Trichoderma* strains and the pathogens *F. oxysporum* f. sp. *lycopersici*, *C. acutatum*, and *R. solani* (on PDA plates), and *B. cinerea* (on MEA plates) were carried out at 25°C as previously described (Rubio et al. 2009). These assays were performed in triplicate, and single cultures of *Trichoderma* strains and pathogens were used as controls.

Sequence analyses

Sequences were analyzed using the DNAstar package (Lasergene). For phylogenetic analysis, gene sequences were aligned using the CLUSTAL X algorithm (Thompson et al. 1997). The NJ tree was constructed with the MEGA software (Kumar et al. 2001) and a bootstrap analysis of 1,000 replicates was performed. The nucleotide sequence of *TvDim1* was deposited in the GenBank database under accession number FJ788527.

Results

The *TvDim1* gene of *T. virens* T59

The 421-bp EST 614 was selected for further characterization, based on its high degree of similarity with Dim1 proteins, using the BLASTX algorithm. EST 614 was obtained from the *T. virens* T59 cDNA library L20, which was constructed after growing the T59 strain under simulated mycoparasitic and plant interaction conditions. The

TvDim1 gene was obtained by PCR with the primers 614-4 and 614-3 and T59 genomic DNA as template, and was found to be 614-bp long. The *TvDim1* gene contained three introns of 52, 53, and 77 bp. To obtain the ORF of the *TvDim1* gene by PCR, the TRX-1 and 614-3 primer pair was designed using the *TvDim1* genomic sequence and a cDNA of T59 strain as template. The ORF of the *TvDim1* gene was 432-bp long and encoded a protein of 143 amino acids, with a theoretical molecular mass of 16.78 kDa and a deduced isoelectric point of 5.56 units.

The highest degree of similarity (97% amino acid sequence identity) was found with a conserved hypothetical protein from *Chaetomium globosum* (accession number XP_001229514). The analysis of the 143 amino acids of the predicted sequence revealed the presence of a conserved DXXC domain located at positions 36 to 39, and the carboxy-terminal extension located beyond the TRX homology region, from amino acid 130 to end of the protein. As a result of the TvDim1 structural analysis using the SMART database (http://www.smart.embl.de), a Dim1 domain (pfam 02966) extending from amino acid 5 to 137 was found.

A NJ tree (Fig. 1) was constructed after an alignment of *TvDim1* gene with other 13 GenBank-retrieved sequences, representing genes encoding Dim1 proteins from ascomycetes, yeasts, animals and plants, as well as other encoding thioredoxin and peroxiredoxin proteins. Three independent clades containing Dim1, thioredoxin, and peroxiredoxin genes could be identified, supported by bootstrap values of 100%. *Dim1* genes were separated according to their evolutionary relationships. The *TvDim1* gene from *T. virens*

T59 was located with the other three fungi sequences and particularly, it formed a subclade with a hypothetical *Dim1* gene from *T. atroviride*, separated from animal, plant, and yeast sequences, supported by a bootstrap value of 99%.

A Southern blot analysis was performed to determine the copy number of the *TvDim1* gene in the *T. virens* T59 genome and the presence of homologs of this gene in other *Trichoderma* species (Fig. 2). A single band was detected in the T59 genomic DNA digested with the endonuclease *Xho*I, which does not cut inside the probe, indicating that the *TvDim1* gene exists as a single copy in the genome of *T. virens* T59. In addition, when the *TvDim1* probe was used to hybridize *Xho*I-digested genomic DNA from another five strains belonging to four different *Trichoderma* species, hybridization signals were also observed: a single signal in the DNA from *T. harzianum* T34, *T. atroviride* T11, and *T. asperellum* T25 strains, and two signals in the DNA from *T. harzianum* T22 and *T. longibrachiatum* T52, indicating that a homologous gene was also present in these strains.

ESTs showing a high degree of identity with Dim1 proteins were only observed in another two cDNA libraries, obtained under simulated conditions of mycoparasitism and stress (Rey et al. 2004): one from *T. stromaticum* (92% identity) and one from *T. aggressivum* (91%). We also detected a single homologous gene in the available on-line genomes of *T. virens* strain Gv29-8 (an identity percentage of 99.3% with the *TvDim1* gene), *Hypocrea jecorina* (anamorph *T. reesei*) (89%), and *T. atroviride* (91%) (http://www.genome.jgi-psf.org/).
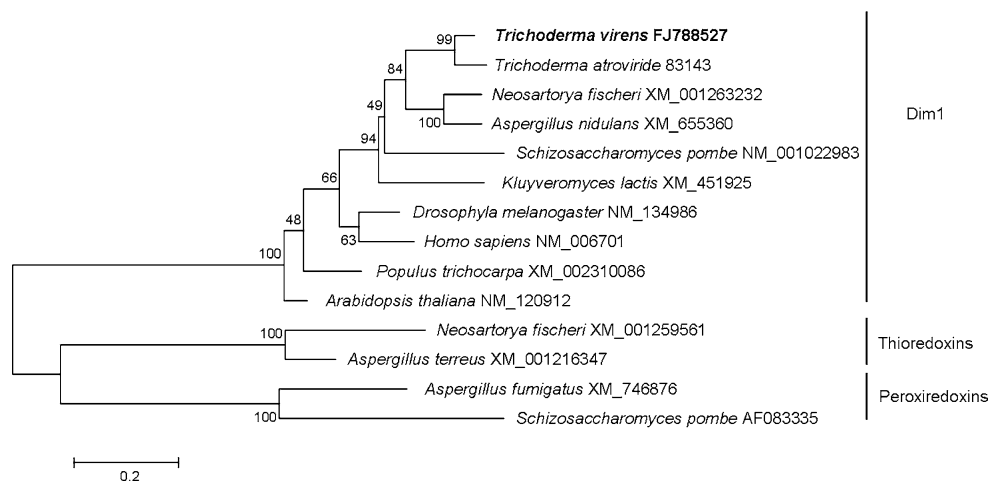

Fig. 1 Neighbor-joining (NJ) tree of *TvDim1* gene from *T. virens* T59 and other 13 GenBank-retrieved sequences, representing genes encoding Dim1, thioredoxin, and peroxiredoxin proteins. Nucleotide sequences were obtained from *T. atroviride* (protein id: 83143, http://www.genome.jgi-psf.org/), *Neosartorya fischeri*, *Aspergillus nidulans*, *Schizosaccharomyces pombe*, *Kluyveromyces lactis*, *Drosophyla melanogaster*, *Homo sapiens*, *Populus trichocarpa*, *Arabidopsis thaliana*, *Aspergillus terreus*, and *Aspergillus fumigatus*. Sequences were aligned using the CLUSTAL X algorithm (Thompson et al. 1997) and the NJ tree was constructed with the MEGA software (Kumar et al. 2001). Sequences are indicated using their GenBank accession numbers. *Bar* represents one substitution per 10 nucleotides
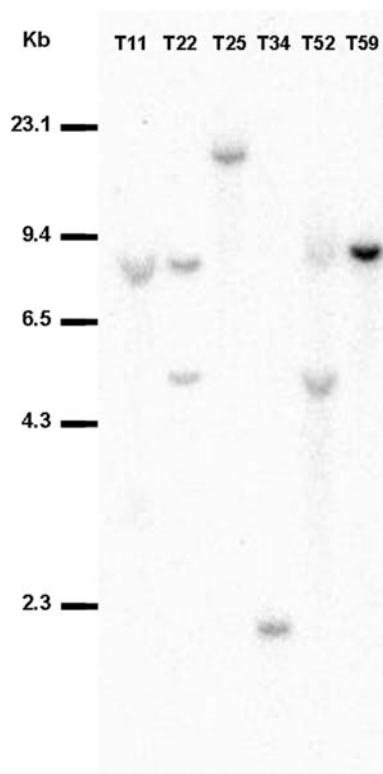
Fig. 2 Southern blot analysis of the *TvDim1* gene in different *Trichoderma* species. Genomic DNA from *T. atroviride* T11, *T. harzianum* T22, *T. asperellum* T25, *T. harzianum* T34, *T. longibrachiatum* T52, and *T. virens* T59 *Xba*I-digested. Hybridization was carried out at 65°C using the *TvDim1* gene as probe. Molecular sizes (Kb) are indicated

Expression and TRX activity analysis in *T. virens* T59

Expression analysis was carried out after culturing *T. virens* T59 under conditions related to abiotic stresses, using glucose starvation (0% glucose) as control condition (Fig. 3). *TvDim1* gene expression was only detected for 1 mM H_2O_2 at 8 and 24 h and for 0.5 mM Paraquat 8 h, being observed the highest transcript levels in 24 h cultures of 1 mM H_2O_2. No *TvDim1* gene expression was detected in the rest of conditions analyzed: 10% NaCl at 4, 8, and 24 h, 1 mM H_2O_2 at 4 h, and for 0.5 mM Paraquat at 4 and 24 h.

Total TRX activity was quantified after the T59 strain had been grown in the presence of 1 mM H_2O_2 for 8 h, obtaining a value of 6.12 μmol/min mg^{-1}.

Heterologous expression of *TvDim1* in *T. harzianum* T34

To characterize the *T. virens* T59 *TvDim1* gene functionally, pJLTvDim1 and pSILTvDim1 plasmids were constructed and transformed and cotransformed, respectively, in *T. harzianum* T34. Ten overexpressing and six silenced
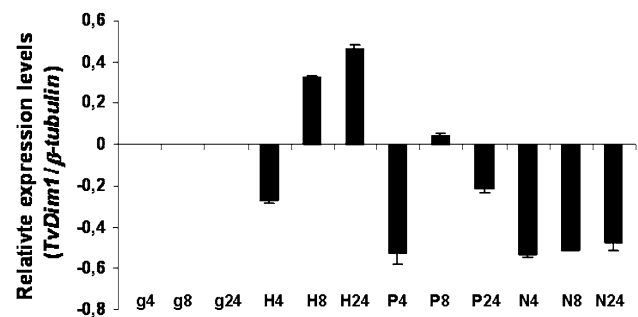

Fig. 3 qRT-PCR analysis of the *TvDim1* gene. The experiment was carried out from mycelia of *T. virens* T59 grown in MM (Penttilä et al. 1987), without glucose, in the presence of 1 mM H_2O_2 (H), 0.5 mM Paraquat® (Afrasa S.A., Valencia, Spain) (P), 10% NaCl (N), and glucose starvation (g). Cultures were maintained at 25°C on a rotary shaker at 150 rpm for 4, 8, or 24 h. *T. harzianum* T34 *β-tubulin* was used as an internal reference gene

transformants showing phleomycin resistance were checked by PCR. A 527-bp fragment, corresponding to the *TvDim1* expression cassette, was amplified in nine overexpressing transformants, using the primer pair pKi-614 and 614-3. A 596-bp fragment was amplified with the primers pta-p and 614-3 in the six silenced transformants analyzed. Four *TvDim1*-overexpressing and four *TvDim1*-silenced transformants were checked by Southern blot to determine the copy number of the cassette in the *T. harzianum* T34 genome, using *Xho*I-digested genomic DNA and the *TvDim1* gene as a probe (Fig. 4a, b). Three *TvDim1*-overexpressing transformants, TvDim1-1, TvDim1-2, and TvDim1-3, and three *TvDim1*-silenced transformants, pSILT-42, pSILT-45, and pSILT-62, representing different integration patterns for both types of transformants, were selected for further characterization.

The *TvDim1* expression level was investigated in mycelia from *T. harzianum* T34 and previously selected transformants, three overexpressing and three silenced strains, by qRT-PCR (Fig. 5) and Northern blot analysis (data not shown), respectively. *Trichoderma* RNAs were obtained from mycelia grown in MM, without glucose, supplemented with 1 mM H_2O_2 for 8 h. The transformants TvDim1-1, TvDim1-2, and TvDim1-3 showed at least five times higher *TvDim1* transcript level than that from the endogenous *ThDim1* gene in the wild-type strain *T. harzianum* T34 under identical growth conditions. Lower *TvDim1* transcript levels were observed in the RNA from strain pSIL62, but several hybridization signals were observed in RNAs from the transformants pSIL42 and pSIL45.

Transformant characterization assays

Since the highest *TvDim1* transcript levels in *T. virens* T59 were observed after growth under oxidative stress, TRX

Fig. 4 Southern blot analysis of wild-type (T34) and transformant strains. Genomic DNAs were *Xho*I-digested and the *TvDim1* ORF was used as probe. *Eco*RI–*Hin*dIII-digested λ DNA (Roche) was used as a marker and molecular sizes (bp) are indicated (line M). **a** *T. harzianum* T34 (line 1), TvDim1-1 (line 2), TvDim1-2 (line 3), TvDim1-3 (line 4), and TvDim1-4 (line 5). **b** *T. harzianum* T34 (line 1), pSILT-22 (line 2), pSILT-42 (line 3), pSILT-45 (line 4), and pSILT-62 (line 5)
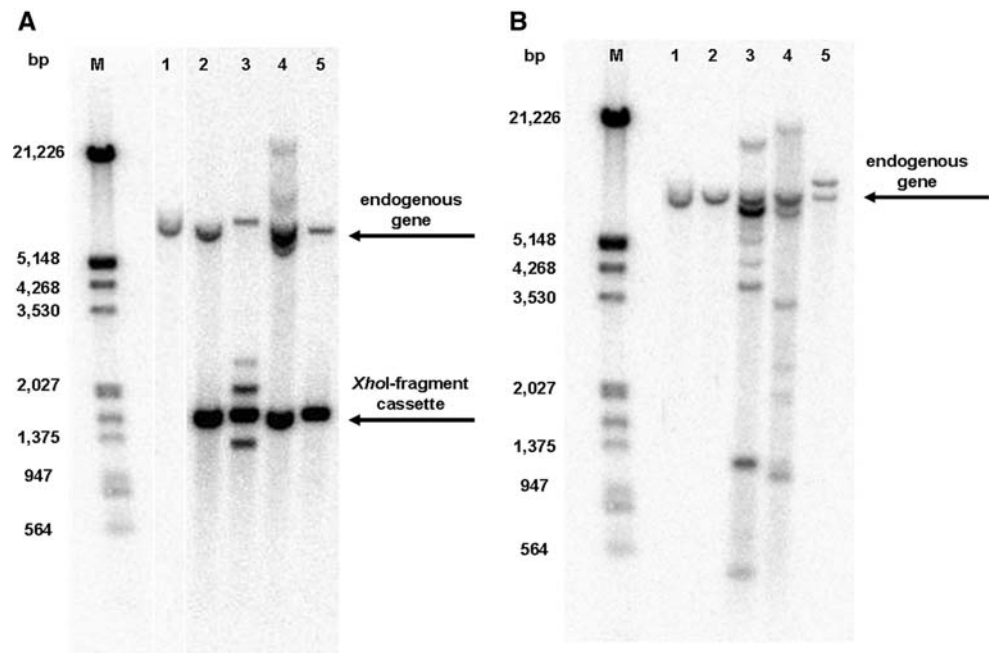
activity was determined in extracts from *T. harzianum* T34 and the six selected transformant strains after growth in MM supplemented with 1 mM H_2O_2 for 8 h (Table 1). Higher specific activity levels were observed in the three *TvDim1*-overexpressing transformants than in T34, with statistically significant differences (P value < 0.05) between the T34 and TvDim1-2, and between the T34 and TvDim1-3 strains. Lower thioredoxin activity levels were observed in the three *TvDim1*-silenced transformants than in the T34 strain, the three cases showing statistically significant differences ($P < 0.05$).

Next we investigated the growth of *T. harzianum* T34 and of the six selected transformants under oxidative conditions, using MM without glucose (control), or MM without glucose but supplemented with 1 or 3 mM H_2O_2
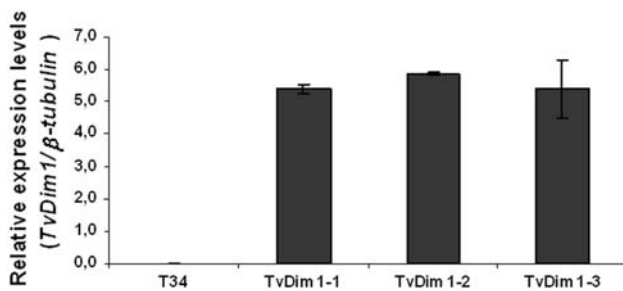
(Fig. 6a, b). Higher colony diameters were observed only for overexpressing transformant strains than for T34 after growth in the presence of 1 or 3 mM H_2O_2. As shown in Fig. 6a, these significant solid-medium growth differences were higher in the transformants TvDim1-2 and TvDim1-3 when they were grown on MM with 3 mM H_2O_2. As shown in Fig. 6b, growth diameters were slightly higher in the wild-type strain than in the silenced transformants on MM plates (control). However, the differences observed in the mycelial diameters of the silenced transformants were only statistically significant ($P < 0.05$) with respect to the T34 strain after growth in the presence of 1 or 3 mM H_2O_2 and they were higher on 3 mM plates, where no growth of silenced transformants was observed.

Similar results were obtained in a liquid growth assay with strain T34 and the TvDim1-1, pSILT-42, and pSILT-62

Fig. 5 qRT-PCR quantification of *TvDim1* transcript in the heterologous overexpressed transformants *T. harzianum* TvDim1-1, TvDim1-2, and TvDim1-3. Values correspond to relative measures against the *ThDim1* transcript in *T. harzianum* T34 wild type (endogenous gene). The experiment was carried out with mycelia grown for 8 h in MM (Penttilä et al. 1987), without glucose, and supplemented with 1 mM H_2O_2. *T. harzianum* T34 *β-tubulin* was used as an internal reference gene

Table 1 Thioredoxin activity measured in intracellular protein extracts from *T. harzianum* T34, *TvDim1*-overexpressing (TvDim1-1, TvDim1-2, and TvDim1-3) and *TvDim1*-silenced (pSILT-42, pSILT45, and pSILT62) strains after 8 h growth in MM supplemented with 1 mM H_2O_2

Strain	DA_{650}/min	Relative specific activity[a]
T34	0.088	5.42 ± 0.05
TvDim1-1	0.107	6.48 ± 0.12
TvDim1-2	0.124	8.63 ± 0.24
TvDim1-3	0.100	6.45 ± 0.20
pSILT-42	0.049	3.64 ± 0.03
pSILT-45	0.065	3.98 ± 0.08
pSILT-62	0.047	2.83 ± 0.08

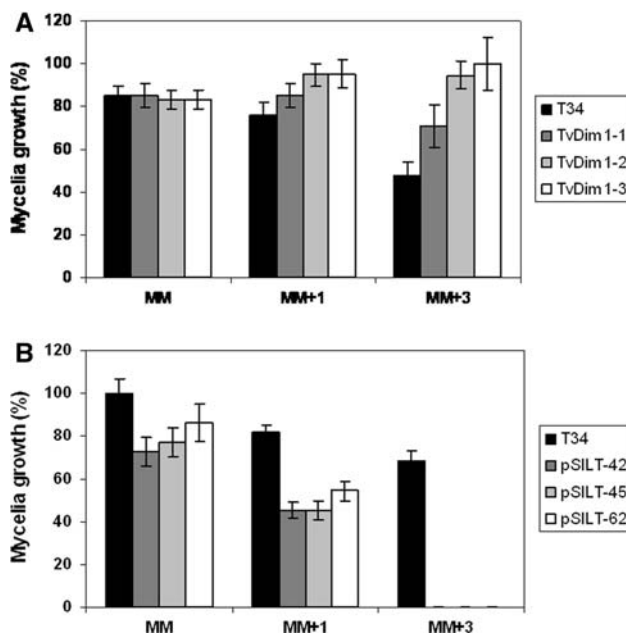[a] μmole min^{-1} per mg of protein

Fig. 6 Mycelial growth diameters of *T. harzianum* T34 and over-expressing and silenced transformant strains measured on MM, without glucose, (MM) (control) or MM, without glucose, supplemented with 1 (MM + 1) or 3 mM H_2O_2 (MM + 3), after incubation at 28°C for 1 week. **a** Mycelial growth diameters of *T. harzianum* T34, TvDim1-1, TvDim1-2, and TvDim1-3. **b** Mycelial growth diameters of *T. harzianum* T34, pSILT-42, pSILT-45, and pSILT-62

Table 2 Accumulation of biomass dry weight in *T. harzianum* T34, TvDim1-1, pSILT-42, and pSILT62 strains after growth in MM (control) or MM supplemented with 1 mM H_2O_2

Biomass dry weight (g)		
Strain	MM	MM + 1 mM H_2O_2
T34	2.47 ± 0.08	0.88 ± 0.03
TvDim1-1	2.88 ± 0.06	1.26 ± 0.19
pSILT-42	2.03 ± 0.18	0.44 ± 0.03
pSILT-62	0.54 ± 0.01	0.28 ± 0.01

transformants. A higher quantity of biomass (dry weight) was measured in the overexpressing transformants than in T34, while both silenced transformants showed a lower amount of biomass than T34 after growth for 48 h in MM, with 1% glucose, supplemented with 1 mM H_2O_2 (Table 2).

No growth differences were observed between the *T. harzianum* T34 and the TF2 strain, which was transformed with empty pJL43b1 plasmid and was used as a transformation control in all experiments (data not shown).

Plate confrontation experiments between T34, TvDim1-1 or pSILT-62, and the pathogens *F. oxysporum* f. sp. *lycopersici*, *R. solani*, *C. acutatum*, and *B. cinerea* were carried out at 25°C, and the plates were observed after 8 days of incubation (data not shown). In all cases, after this incubation time the four pathogens completely covered

the surface of 90 mm-diameter PDA or MEA plates used as control. In the dual cultures, *Trichoderma* strains overgrew the colonies of *R. solani* and *B. cinerea*, and they surrounded the colonies of *F. oxysporum* and *C. acutatum*. All *Trichoderma* strains were able to inhibit the growth of the four pathogens tested, reducing the colony diameter of the pathogens to not more than 25 mm, and no significant differences were observed between the inhibition effect of T34 and that of the transformants.

Discussion

Trichoderma is a fungus of high biotechnological value (Monte 2001). Within the framework of the EU-funded TrichoEST project (Rey et al. 2004), a functional genomics project carried out on 10 strains belonging to eight *Trichoderma* species, only three ESTs showing high identity with Dim1 proteins were identified out of a total of 13,814 unigenes (Vizcaíno et al. 2006, 2007). To date, these proteins have not been reported in filamentous fungi. Here, we explored the role of the *TvDim1* gene in *Trichoderma*.

An EST showing high identity with the evolutionarily conserved Dim1 proteins, belonging to the TRX fold superfamily (Zhang et al. 1999), was identified in library L20 from *T. virens* T59, a biocontrol strain. EST 614, containing the partial sequence of the *TvDim1* gene, was aligned with other *Dim1* genes, and the pair of primers designed in that alignment was used to isolate the full-length genomic DNA clone encoding the TvDim1 protein. Southern analysis revealed that the *TvDim1* gene was present as a single copy in *T. virens* T59, and a homologous gene was also present in the other five strains investigated, belonging to four different species. A single *TvDim1* homologous gene was also identified in the available online genomes of *T. atroviride*, *T. reesei*, and *T. virens* strain Gv29-8, the latter showing 99.3% of similarity with the *TvDim1* gene. In light of these data, it seems reasonable to assume that one *Dim1* gene would be present in all *Trichoderma* genomes. This result is in agreement with the single *Dim1* gene detected after exploring genomes from other organisms, such as *S. cerevisiae* (http://www.yeastgenome.org/), *C. elegans* (http://www.sanger.ac.uk/Projects/C_elegans/) or *A. thaliana* (http://www.arabidopsis.org/).

The ORF of *TvDim1* encodes a protein of 143 amino acids, and analysis of the sequence revealed a conserved domain with a typical TRX-fold from amino acids 5–137. Upon comparing the TvDim1 sequence with that of Dim1 or hypothetical Dim1 proteins, we observed high homology throughout the entire length of 143 amino acids, Dim1 shows an identity higher than 85% in all cases. This is according to the closer clades observed for *Dim1* genes in the NJ tree (Fig. 1). Evolutionarily conserved Dim1

proteins have been described throughout eukaryotic organisms in analyses of mammals, plants, yeasts, and nematode species (Simeoni and Divita 2007).

The highest levels of *TvDim1* expression in *T. virens* T59 were observed after growth under oxidative stress, such as in the presence of H_2O_2 (Fig. 3). We also observed that *TvDim1* transcript levels were increased in the presence of heavy metals—i.e., 100 μM Cd^{++}—in the medium (data not shown). These expression patterns seem to be related to TRX genes. In this sense, H_2O_2 treatment increased the transcription of the *KlTRR1* gene, coding for a TRX reductase, but not of the *KlGLR1* gene, encoding a glutathione reductase, in the yeast *Kluyveromyces lactis* (Tarrío et al. 2004). An enhanced *TrxR* mRNA level in cells from the yeast *S. pombe* was detected in the presence of mercuric chloride (Hong et al. 2004). These results suggest that the *T. virens* T59 *TvDim1* gene is one of the stress response-related genes, and they are in agreement with previous work suggesting the involvement of TRXs in defense mechanisms against heavy metals in algae (Lemaire et al. 1999).

We analyzed *TvDim1* expression after growing T59 under abiotic stress conditions and gene expression was detected only in the presence of H_2O_2 and the herbicide Paraquat, indicating its relation with oxidative stress.

The biological function of Dim1 proteins has been related to both pre-mRNA splicing (Reuter et al. 1999) and to the control of cell cycle progression (Berry and Gould 1997), but its biochemical function is not clear. First, TvDim1 showed the TRX-fold but did not display the classic redox active CxxC motif. However, TRX activity was detected in intracellular protein extracts from 8 h-cultures grown in the presence of 1 mM H_2O_2, in which the highest expression levels were also detected. In view of these results, TvDim1 could act as a single active cysteine protein in redox processes; alternatively, the cysteine from the DxxC motif could form a disulfide bond with other C-terminal cysteines. Regarding this, previous results are contradictory. Thus, in some cases the CxxC motif seemed to be essential for redox-related activity (Holmgren 1979; Martin 1995; Stefankova et al. 2005) while other works have reported a redox function in proteins that lacked one of the two cysteines in this motif (Fomenko and Gladyshev 2003; Jung et al. 2005; Lyles and Gilbert 1994; Reuter et al. 1999).

Trichoderma harzianum T34 is a strain commonly used in basic research studies. To investigate the function of *TvDim1* in vivo, heterologous expressions in the strain T34 were performed and *TvDim1*-overexpressing and *TvDim1*-silenced transformants were obtained. Since Southern analysis revealed that *T. harzianum* T34 had a homologous gene to *TvDim1*, but no ESTs with Dim1 homology were detected in the 3,478 unigenes of strain T34 identified, we used the TRX-1 and 614-3 primer pair to isolate the gene from *T. harzianum* T34 genomic DNA. The *ThDim1* gene

showed 94% similarity with *TvDim1*, and ThDim1 shared 100% identity with the TvDim1 protein. However, H_2O_2 treatment increased the transcription of *ThDim1* less than that of *TvDim1*. This result indicates that both genes were subjected to different forms of regulation. We have also previously reported that the *hsp23* gene, encoding a small heat-shock protein, showed 83% identity between strains T34 and T59 but displayed different expression patterns in both strains (Montero-Barrientos et al. 2007). A different expression pattern could explain why no ESTs with Dim1 homology were present out of the total of 3,478 unigenes obtained from *T. harzianum* T34 (Vizcaíno et al. 2006).

To characterize the transformants, we analyzed *TvDim1* gene expression and TRX activity in strain T34 as well as in three overexpressing and three silenced strains after culturing them in the presence of 1 mM H_2O_2 (Fig. 5, Table 1). These transformants were chosen because in Southern analyses they showed different integration patterns of cassette transformation (Fig. 4a, b). In a relative quantification by qRT-PCR under identical growth conditions, the overexpressing transformants showed at least 5 times higher *TvDim1* transcript level referred to that from the *ThDim1* in the wild-type strain *T. harzianum* T34 (endogenous gene). These transformants also showed higher TRX activity than strain T34. The highest expression and activity levels were observed in strain TvDim1-2, an overexpressing transformant with more insertions of the transformation cassette. Similar *TvDim1* expression levels were detected in strains TvDim1-1 and TvDim1-3 although the latter exhibited a higher TRX activity level even though both transformant strains had a similar integration pattern of pJLTvDim1, the plasmid used for transformation. In this sense, it has also been reported that no correlation exists between the number of inserted copies of the *chit33*, *hsp23*, and *hsp70* genes and the expression levels observed in *Trichoderma* transformant strains (Limón et al. 1999; Montero-Barrientos et al. 2007, 2008). The silenced transformant strains showed lower TRX activity than that observed in *T. harzianum* T34 corresponding the lowest level to strain pSILT-62, transformant analyzed with the least inserted copies of plasmid pSILTvDim1 (Table 1). Considering the hybridization profiles obtained from the three silenced transformants analyzed, it is difficult to propose a direct correlation between TRX activity and expression levels. Since the method used to silence the *TvDim1* gene expression in *T. harzianum* T34 was based on the generation of an intron containing self-complementary "hairpin" RNAs (ihpRNA) or perfect duplex hairpin RNAs (Dykxhoorn et al. 2003), the strong and abundant Northern signals could be due to degraded transcripts.

To analyze the contribution of the *TvDim1* gene to abiotic stress resistance in *T. harzianum* T34, we compared

the growth of strain T34 and of three overexpressing and three silenced transformant strains with or without H_2O_2 in the medium. The higher growth observed in the *TvDim1*-overexpressing transformants and the lower growth pattern seen in the *TvDim1*-silenced transformants than in the wild-type strain only in presence of this stress, together with the enhanced differences when the amount of H_2O_2 added to the medium was increased, are in agreement with the redox activity observed in the insulin assays. Statistically significant biomass quantities, higher in the TvDim1-1 transformant and lower in the silenced pSILT-42 and pSILT-62 transformants than in strain T34, were obtained after growing the strains in liquid medium containing 1 mM H_2O_2 for 48 h. These results point to the ability of *TvDim1* to confer resistance to oxidative stress and are in agreement with others previously reported for the Prx6 human peroxiredoxin protein, which has only one active cysteine but maintains its capacity to reduce H_2O_2 (Pedrajas et al. 2000).

To date, Dim1 proteins have been related to the control of cell cycle progression, which seems to be a consequence of a fault in the pre-mRNA splicing of the proteins responsible for entry into mitosis (Simeoni and Divita 2007). In this sense, we observed a lower growth of *TvDim1*-silenced transformants than that of strain T34 in MM (a basal medium). Dim1 proteins are related to the cell cycle, since *Dim1*-deficient mutants in *S. pombe* (Berry and Gould 1997) and in *C. elegans* (Zhang et al. 2000) are lethal, and this could account for the reduced growth of *TvDim1*-silenced transformants under basal conditions. In contrast, no growth differences were detected between T34 and the overexpressing transformants in the basal medium. Considering that the Dim1 protein acts as part of a complex, in these transformants only the level of Dim1, but not that of other associated proteins, was increased and hence it could be expected that their growth would not be affected after growth in basal conditions.

Since *T. harzianum* is considered as a good biocontrol agent, we also analyzed the *TvDim1* overexpression and silencing effect in two *T. harzianum* T34 transformants (TvDim1-1 and pSILT-62) in a direct confrontation assay against four phytopathogenic fungi. The behavior of both transformant strains was similar to that of strain T34 against the four pathogens assayed. In light of these data, it may be proposed that the *TvDim1* gene would not be related to biocontrol activity.

Our results indicate that *TvDim1* is a stress-related TRX gene and confirm the involvement of TRXs in defense mechanisms against oxidative stresses.

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