Applied and Environmental Microbiology, May 2011, p. 3009–3016 Vol. 77, No. 9
0099-2240/11/$12.00 doi:10.1128/AEM.02486-10

Functional Analysis of the *Trichoderma harzianum nox1* Gene, Encoding an NADPH Oxidase, Relates Production of Reactive Oxygen Species to Specific Biocontrol Activity against *Pythium ultimum*▽†

M. Montero-Barrientos,[1] R. Hermosa,[1] R. E. Cardoza,[2] S. Gutiérrez,[2] and E. Monte[1]*

Spanish-Portuguese Center for Agricultural Research (CIALE), Department of Microbiology and Genetics, University of Salamanca, Campus of Villamayor, Río Duero 12, 37185 Salamanca, Spain,[1] and Area de Microbiología, Escuela Universitaria de Ciencias de la Salud, Universidad de León, Campus de Ponferrada, Avda. Astorga s/n, 24400 Ponferrada, Spain[2]

Received 21 October 2010/Accepted 4 March 2011

The synthesis of reactive oxygen species (ROS) is one of the first events following pathogenic interactions in eukaryotic cells, and NADPH oxidases are involved in the formation of such ROS. The *nox1* gene of *Trichoderma harzianum* was cloned, and its role in antagonism against phytopathogens was analyzed in *nox1*-overexpressed transformants. The increased levels of *nox1* expression in these transformants were accompanied by an increase in ROS production during their direct confrontation with *Pythium ultimum*. The transformants displayed an increased hydrolytic pattern, as determined by comparing protease, cellulase, and chitinase activities with those for the wild type. In confrontation assays against *P. ultimum* the *nox1*-overexpressed transformants were more effective than the wild type, but not in assays against *Botrytis cinerea* or *Rhizoctonia solani*. A transcriptomic analysis using a *Trichoderma* high-density oligonucleotide (HDO) microarray also showed that, compared to gene expression for the interaction of wild-type *T. harzianum* and *P. ultimum*, genes related to protease, cellulase, and chitinase activities were differentially upregulated in the interaction of a *nox1*-overexpressed transformant with this pathogen. Our results show that *nox1* is involved in *T. harzianum* ROS production and antagonism against *P. ultimum*.

Trichoderma is a fungal genus that includes species in current use as biological control agents due to their ability to antagonize other fungi (28). The ability of *Trichoderma* species to suppress plant diseases caused by phytopathogenic fungi has long been known (19). The antagonistic properties of *Trichoderma* have been related to mechanisms of action such as the production of antibiotics (48, 52) and/or hydrolytic enzymes (6) and competition for nutrients (11). It is also known that these fungi have the ability to interact with plants, inducing resistance to biotic and abiotic stresses and promoting plant growth (17, 21). These characteristics have encouraged extensive research into the use of *Trichoderma* strains as biocontrol agents (BCAs) to combat fungal and oomycetous diseases (28, 35), *Trichoderma harzianum* being the most cited species as an active agent in a variety of commercial biopesticides and biofertilizers (62).

One of the earliest manifestations of defense responses in animals and plants is the production of reactive oxygen species (ROS) by specific NADPH oxidases (Nox) (22, 59). Three different Nox subfamilies have been found in the kingdom *Fungi* (2): NoxA, which possesses domains for the catalytic core but no additional motifs and which is very similar in structure to human gp91^{phox} (23, 55); NoxB, which has an additional N-terminal extension with no evident functional motifs (30, 56); and NoxC, which has an even longer N-terminal region that contains a putative calcium-binding EF-hand motif, similar to the respiratory burst oxidase homolog (Rboh) enzymes from plants (24). The most widely studied member of this group of enzymes is the mammalian gp91^{phox}, which is responsible for the phagocytic oxidative burst in response to microbial pathogens. In activated macrophages, Nox enzymes produce superoxide, and its dismutation product, hydrogen peroxide, can kill pathogens directly or through the activation of proteases (42). Plant cells are also capable of an oxidative burst in response to pathogen recognition (14). *Arabidopsis thaliana* possesses 10 Rboh genes, and their enzymes are involved in plant defense signaling, programmed cell death, and root hair growth (12, 58).

Functional analyses of gp91^{phox} homologs from filamentous fungi have shown that these enzymes play a key role in fungal development and defense. A *noxA* disruption in *Aspergillus nidulans* blocks fungal sexual development since fruiting bodies cannot be formed (23). Similar phenotypes have been observed in *Podospora anserina* and *Neurospora crassa* after *nox* gene deletions, demonstrating that Nox enzymes are critical for the development of sexual structures in filamentous fungi (2, 30). Additional studies have reported that the *Magnaporthe grisea nox1* gene is also involved in the development of appressoria, which are important structures during plant infection processes (10). Nox-mediated fungal ROS production has been also related to mechanisms for defense against other fungi (15, 51).

This paper reports the isolation and characterization of the *nox1* gene in *T. harzianum* T34 and its functional analysis through a gene overexpression strategy. Transformants with higher *nox1* expression levels showed more antagonistic activ-

* Corresponding author. Mailing address: Spanish-Portuguese Center for Agricultural Research (CIALE), Campus of Villamayor, c/Río Duero no. 12, 37185 Salamanca, Spain. Phone: 34 923294500, ext. 5119. Fax: 34 923294399. E-mail: emv@usal.es.

† Supplemental material for this article may be found at http://aem.asm.org/.

▽ Published ahead of print on 18 March 2011.

ity than the wild-type strain against the phytopathogen *Pythium ultimum*, but not against *Rhizoctonia solani* or *Botrytis cinerea*. Increased ROS production correlated with increased induction of protease, cellulase, and chitinase activities, which were the main overproduced enzymes in the *nox1* transformants in *P. ultimum* interaction conditions.

MATERIALS AND METHODS

Microorganisms. *Trichoderma harzianum* T34 (CECT 2413; Spanish Type Culture Collection, Burjassot, Spain) was used in this study. The fungal phytopathogens *Rhizoctonia solani* CECT 2815 and *Botrytis cinerea* 98, isolated by us from diseased strawberry plants, and the oomycete *Pythium ultimum* 8, obtained from the ArBoPaVe collection of the University of Naples (Italy), were used as targets in dual confrontation assays. Strains were cultured on potato dextrose agar (PDA; Difco-Becton Dickinson, Sparks, MD).

Source of the *nox1* gene: EST database and cDNA library. The L03T34P075R07015 clone with accession number AJ897753 (hereafter referred to as EST 7015) was generated from the *T. harzianum* T34 cDNA library L03, one of the 26 cDNA libraries that were constructed for the TrichoEST project, using different mRNA populations from 10 strains belonging to eight *Trichoderma* species (including *T. harzianum* T34) expressed under conditions that simulate biocontrol processes (45). The growth conditions for the T34 strain used for preparing the L03 cDNA library have been described previously (66). 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 (66, 67).

Sequence analyses. Sequences were analyzed using the DNAstar package (Lasergene, Madison, WI). Transmembrane domains were identified using the TopPred II program (9). The NCBI database was used for protein domain identification (31).

PCR procedures. *nox1* cDNA was amplified by PCR with the primers nadph8 (5′-ATGGCTGGCCAACTCACATAT-3′) and nadph9 (5′-TTAAAAGTGCTC TTTCCAGAAGC-3′) and with phagemid DNA from the cDNA library L03 as the template using the *Taq* polymerase system (Biotools, Edmonton, Canada), according to the manufacturer's instructions. PCR was carried out for 35 cycles of 1 min at 94°C, 1 min 30 s at 59°C, and 1 min 30 s at 72°C. To construct the pJL7015 plasmid, the *nox1* cDNA was amplified by PCR using the oligonucleotides X5 (5′-TCTAGAATGGCTGGCCAACTC-3′) and X3 (5′-TCTAGATTA AAAGTGCTCTTTCC-3′), which contain XbaI recognition sites, and the conditions described above. Screening of *T. harzianum* T34 *nox1*-overexpressed transformants was performed by PCR with the primers Cyc-F (5′-ACAACCTG AAGTCTAGGTCCC-3′) and nadph11 (5′-ATCCTCTCACTGAGCTCAAG-3′) to amplify a 1,431-bp fragment from the pJL7015 plasmid. The oligonucleotide pair ble (5′-CCTTTCAGTTCGAGCTTTCCC-3′) and ble-r (5′-GGGACC TAGACTTCAGGTTGT-3′) served to amplify the phleomycin resistance gene (*ble*), which was used as a probe in a Southern analysis under the reaction conditions described above.

Construction of the pJL7015 vector and *Trichoderma* transformation. *nox1* cDNA was amplified by PCR with the primer pair X5 and X3 and cloned to the pGEM-T Easy vector. After digestion with XbaI, the 1.8-kb fragment corresponding to the *nox1* cDNA was ligated to the pLMG vector (36), previously digested with XbaI. The *nox1* expression cassette of 3.6 kb, which contains the *gpdA* (glyceraldehyde 3-phosphate dehydrogenase) gene promoter from *A. nidulans* and the *cbh2* (cellobiohydrolase II) termination region from *Trichoderma reesei*, was isolated by digestion with HindIII and cloned into plasmid pJL43b1. The resulting 8.11-kb vector, pJL7015, contained the *ble* gene from *Streptoalloteichus hindustanus* under the control of the *gpdA* gene promoter. Plasmid pJL7015 was used to transform protoplasts of *T. harzianum* T34 as previously described (8), and the transformants were selected for phleomycin resistance.

DNA manipulations. Fungal DNA was extracted by following the method of Raeder and Broda (41). Mycelia were recovered by filtration from potato dextrose broth (PDB) cultures, washed twice with water, frozen in liquid nitrogen, lyophilized, and ground. Bacterial DNAs were obtained using a routine miniprep procedure. For Southern analysis, 10 μg of genomic DNA was digested with EcoRI and restriction fragments were separated in 0.7% (wt/vol) agarose gels and transferred to a Hybond-N+ membrane (Amersham, Piscataway, NJ). The *ble* gene was labeled with the PCR DIG labeling mix kit (Roche, Penzberg, Germany) by following the supplier's protocols and used as a probe. Hybridizations were carried out for 16 h at 65°C. Membranes were washed under high-stringency conditions. Immunological detection was performed using the DIG nucleic acid detection kit (Roche), according to the manufacturer's instructions.

Confrontation assays *in vitro*. Confrontations between the *Trichoderma* strains and the phytopathogens *B. cinerea*, *R. solani*, and *P. ultimum* were carried out. Agar plugs cut from the growing edge of a 4-day colony of each phytopathogen were placed 2 cm from the borders of petri dishes containing PDA covered with sterile cellophane sheets. *B. cinerea* was allowed to grow at 25°C for 1 day before the sowing of *Trichoderma* strains at 2 cm from the borders on the opposite sides of the same petri dishes where this target pathogen was grown. In the same way, *R. solani*, *P. ultimum*, and *T. harzianum* strains were sown at the same time. *Trichoderma-Trichoderma* confrontations were used as a control condition. Mycelia were collected from the 5-mm interaction zone between the microorganisms confronted in dual cultures and used for both RNA and protein extractions, which were included in analyses of gene expression and hydrolytic activity, respectively. *In vitro* confrontations using PDA plates that were not covered with cellophane sheets were also conducted in triplicate as described above in order to analyze the antagonism of *Trichoderma* against these three phytopathogens. Dual cultures were photographed after 5 days.

Superoxide production *in situ* assay. Plates containing *Trichoderma*-pathogen confrontations, conducted as described above, were incubated for 5 days, after which 1 ml of an NBT solution (0.05% Nitro Blue Tetrazolium in 50 mM phosphate [pH 7.5 buffer]) (Sigma-Aldrich Química S.A., Madrid, Spain) was added to the interaction zone and then plates were photographed.

Activity assays. Mycelia collected from interaction zones between microorganisms were homogenized in 100 mM Tris (pH 7.5 buffer) at 4°C for 1 h using a Thermomixer (Eppendorf, Hamburg, Germany), and then the supernatants were recovered by centrifugation at 12,000 × *g* at 4°C for 20 min. Quantitative protein determination was performed with the Bradford assay (7), with bovine serum albumin as a protein standard. Ten micrograms of total proteins was included in each enzymatic assay. Tests were performed in triplicate, and the data represent mean values with standard deviations.

NADPH oxidase activity was determined in a colorimetric assay by measuring the reduction of the tetrazolium dye 3′-(1-[phenylamino-carbonyl]-3,4-tetrazolium)-bis(4-methoxy-6-nitro) benzenesulfonic acid hydrate (XTT) by O_2^- radicals at 470 nm (1). In this assay, XTT is reduced by HO_2^-/O_2^- to a soluble formazan that can be readily quantified in solution. The reaction mixture (0.25 ml) contained 0.3 mM XTT (Sigma-Aldrich Química), 100 μM NADPH in 100 mM Tris (pH 7.5 buffer), and 10 μg of proteins from protein extract. The reaction was initiated by the addition of NADPH and XTT, and the subsequent reduction was determined after incubation at room temperature for 10 min. Total activity corresponds to nmol of O_2^- produced in 1 min, and specific activity corresponds to nmol of O_2^- produced in 1 min per mg of protein.

Protease activity was determined in a colorimetric assay by measuring the hydrolysis of azocasein at 366 nm, as described previously (18). Azocasein is a nonspecific protease substrate that, when it is hydrolyzed, releases the azo dye into the media, where it is detected by measuring absorbance. The reaction mixture (0.325 ml), containing 1% (wt/vol) azocasein (Sigma-Aldrich Química) in 50 mM sodium acetate (pH 5.5 buffer) and 10 μg of proteins from protein extracts, was incubated at 30°C for 1 h. Total activity corresponds to nmol of azocasein hydrolyzed in 1 min, and specific activity corresponds to nmol of azocasein hydrolyzed in 1 min per mg of protein.

Cellulase activity was determined in a colorimetric assay by measuring the release of reducing groups during the hydrolysis of carboxymethylcellulose (CMC) at 520 nm (37, 53). The reaction mixture (0.25 ml), containing 1% (wt/vol) CMC (Sigma-Aldrich Química) in 50 mM sodium acetate (pH 5.5 buffer) and 10 μg of proteins from protein extracts, was incubated at 37°C for 1 h. Total activity corresponds to nmol of glucose released in 1 min, and specific activity corresponds to nmol of glucose released in 1 min per mg of protein.

Chitinase activity was determined in a colorimetric assay by measuring the release of *N*-acetylglucosamine during the hydrolysis of chitin at 585 nm. The reaction mixture (0.25 ml), containing 0.1% (wt/vol) colloidal chitin (Sigma-Aldrich Química) in 50 mM sodium acetate (pH 5.0 buffer) and 10 μg of protein from protein extracts, was incubated at 37°C in a water bath with constant shaking for 2 h. The *N*-acetylglucosamine released into the reaction mixture was estimated as previously described (44). Total activity corresponds to nmol of *N*-acetylglucosamine released in 1 min, and specific activity corresponds to nmol of *N*-acetylglucosamine released in 1 min per mg of protein.

Protease and cellulase activities were also measured, in triplicate, in mycelia from T34, Tnox2, and Tnox5 strains after 48 h growth in PDB medium supplemented with 1% CMC.

Quantitative real-time PCR analyses. *Trichoderma* mycelia collected from confrontation assay interaction zones were frozen, lyophilized, ground, and used for RNA extraction with Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. cDNAs were synthesized from 1 μg of total RNA using the AffinityScript quantitative PCR (QPCR) cDNA synthesis kit

(Stratagene, La Jolla, CA) with an oligo(dT) primer. Then, 0.5 μl of the cDNA was used in the subsequent PCR. Quantitative real-time PCR was performed using an ABI Prism 7000 sequence detection system with Brilliant SYBR green QPCR master mix (Stratagene). All PCRs were performed in triplicate in a total volume of 12 μl for 40 cycles under the following conditions: denaturation, 95°C, 30 s; annealing, 60°C, 1 min; extension, 72°C, 1 min. Threshold cycles (C_T) were determined using the 7000 SDS system software, and C_T values were calculated using the β-*tubulin* gene as an endogenous control. Data are expressed as $\log_{10}$ of $2^{-\Delta\Delta CT}$ (26), and three biological replicates were used for statistical analysis. The following specific primer pairs were used (GenBank accession numbers of encoded proteins are in parentheses): Nox1 (HM565864), 5′-CACCACCTGTTCATCCC-3′ and 5′-GTCAAATGGCGAGAATCC-3′; catalase (XP_002480230), 5′-ACTGCA TTGTCCGTTTCT-3′ and 5′-AGTTGCCCTCCTCTGTG-3′; cellulase signaling protein OOC1 (AAY25948), 5′-CAATGCTCCGACAATTACAG-3′ and 5′-CACCGATCACAGCACAGCA-3′; trypsin-like protease (CAC80694), 5′-C TGCCATCACTCCTCGT-3′ and 5′-AGAAGTGCGAACCACCA-3′; and β-tubulin, 5′-TTCTTGCATTGGTACACTAGCG-3′ and 5′-ATCGTTCATG TTGGACTCAGCC-3′.

Northern blot analysis. Four-month-old olive plants (*Olea europaea* L., var. Picual) were cultured in a 250-ml Erlenmeyer flask containing 100 ml of liquid Murashige and Skoog (MS) medium (Duchefa Biochemie B.V., Haarlem, Netherlands) inoculated with 10^5 conidial germlings ml^{-1} of *T. harzianum* T34, which was incubated in an orbital shaker at 80 rpm and 25°C. After 4, 8, or 24 h of plant-*Trichoderma* interaction, mycelia were collected, washed with distilled water, frozen, lyophilized, and kept at −80°C until total RNA extraction. In parallel, fungal mycelia from cultures without olive plants were also obtained (control). Conidial germlings were obtained from 15-h-old cultures of strain T34 in 200 ml of minimal medium (MM) (40) shaken at 200 rpm and incubated at 25°C. *nox1* gene expression was analyzed by Northern blot hybridization, and 20 μg of total RNA was separated on a 1.2% formaldehyde-agarose gel and transferred to a Hybond-N+ membrane. Blots were hybridized with ^{32}P-labeled *nox1* or 18S ribosomal DNA (rDNA) probes. Hybridization conditions and membrane washes were as described above for Southern analysis.

Microarray assay. Mycelia collected from the interaction zone in confrontations between *P. ultimum* and the *T. harzianum* T34 or Tnox5 strain were used for RNA extraction as described above and then purified using the RNeasy MinElute cleanup kit (Qiagen, Hilden, Germany). The quality and quantity of the RNA were determined using a Nanodrop spectrophotometer. High-quality purified RNAs, 40 μg per set of three microarrays, were submitted to Roche-NimbleGen (Madison, WI), where cDNAs were synthesized, amplified, labeled, and then used for subsequent hybridizations.

A self-designed *Trichoderma* high-density oligonucleotide (HDO) microarray (Roche-NimbleGen) was constructed by a method similar to that for a previous *Trichoderma* HDO microarray (49). The microarray was composed of 392,779 60-mer probes designed against 14,081 EST-derived transcripts and 11,100 genes of *Trichoderma atroviride* and 11,643 of *Trichoderma virens*.

Digitalization of the fluorescent signals emitted after the hybridization was performed with the Gene Spring GX program (Agilent Technologies, Santa Clara, CA). This program allows the statistical analysis and identification of induced and repressed genes in each experimental condition. Background correction, normalization, and expression analysis from the data were performed using the RMA algorithm (20). The significance of the differential expression under the two conditions compared was determined by statistical analysis performed with the *t* test algorithm (5).

Statistical analyses. The mean values of the assays carried out in triplicate were recorded in the StatView 4.01 program and analyzed using a Fisher exact test.

Nucleotide sequence accession number. The DNA sequence of *nox1* was determined and deposited as GenBank accession number HM565864.

RESULTS

The *T. harzianum* T34 *nox1* gene. A 690-bp fragment derived from the *T. harzianum* T34 EST 7015 was used as the probe to screen a lambda genomic DNA library (27). A total of 2,613 bp containing 547 bp of the promoter region and 267 bp corresponding to the terminator region was sequenced from one positive phage. The *nox1* cDNA was amplified from L03 library phagemids and cloned into pGEM-T Easy. The *nox1* coding region contains 1,799 bp with two introns. The open reading

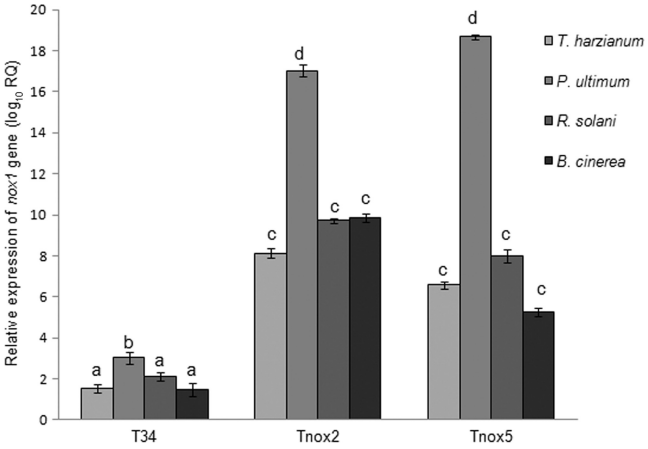

FIG. 1. Quantitative RT-PCR analysis of the *nox1* transcript of *T. harzianum* T34 (wild type) and the homologous *nox1*-overexpressed transformants Tnox2 and Tnox5. The experiments were carried out with mycelia obtained from the interaction zone of *Trichoderma* confrontations with *T. harzianum* itself, *P. ultimum*, *R. solani*, or *B. cinerea*. *T. harzianum* T34 β-*tubulin* was used as an internal reference gene. Data are expressed as $\log_{10}$ relative quantification (RQ) ($2^{-\Delta\Delta CT}$). Bars represent the standard deviations of the mean values of two biological replicates. Histograms with different letters are significantly different ($P < 0.0001$).

frame (ORF) contains 1,674 bp and encodes a protein of 557 amino acids with a theoretical molecular mass of 64.1 kDa and an isoelectric point of 8.8. The highest degree of similarity (87% amino acid sequence identity) was found with the NoxA protein from *Epichloe festucae* (GenBank accession number BAE72680). Additionally, one homologous gene was detected in the publicly available genomes of *T. reesei* (http://genome.jgi-psf.org/Trire2/Trire2.home.html) (95% identity of encoded protein with ID_79489 protein), *T. atroviride* (http://genome.jgi-psf.org/Triat2/Triat2.info.html) (94% identity of encoded protein with ID_302802 protein), and *T. virens* (http://genome.jgi-psf.org/TriviGv29_8_2/TriviGv29_8_2.info.html) (99% identity of encoded protein with ID_32702 protein). Bioinformatic analysis of the promoter region showed protein-binding motifs that may give hints as to its regulation, such as HAP1 (central regulator of oxygen metabolism genes), STUAP (involved in sexual development in fungi), and a region binding to the WRKY transcription factor, which regulates defense pathogenesis-related (PR) genes in plants.

Analysis of the 557 amino acids of the predicted *T. harzianum* T34 Nox1 protein revealed the presence of six transmembrane domains and one flavin adenine dinucleotide (FAD)/NADPH binding cytosolic domain as described previously for $gp91^{phox}$. The Nox1 structure was similar to that described for fungal NoxA enzymes.

***nox1* expression analysis.** Mycelia from the area of interaction between *T. harzianum* T34 and phytopathogen *B. cinerea*, *R. solani*, or *P. ultimum* were recovered and used to analyze *nox1* expression by quantitative reverse transcription-PCR (RT-PCR). As shown in Fig. 1, the highest transcript levels were detected in mycelia from T34-*P. ultimum* interactions, followed by those detected in T34-*R. solani* interactions. Among the four interactions considered, statistically significant higher *nox1* transcript levels were observed only in mycelia

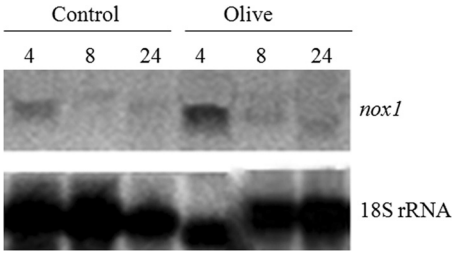

FIG. 2. Northern analysis of the *nox1* gene of *T. harzianum* T34 in the presence of olive plants. The experiments were carried out with mycelia obtained after growing the fungus in MS medium for 4, 8, or 24 h without plants (control) or in the presence of olive plants. The *nox1* ORF and 18S rRNA gene were used as probes.

TABLE 1. NADPH oxidase activity measured in intracellular protein extracts from interaction zones in dual cultures of *T. harzianum* T34 or *nox1*-overexpressed transformants and itself, *P. ultimum*, or *R. solani*

Strain	Mean NADPH activity[a] (nmol min^{-1}/mg protein) ± SD for:		
	T-T[b]	*T. harzianum*-*P. ultimum*	*T. harzianum*-*R. solani*
T34	38.44 ± 1.85	33.07 ± 1.57	27.67 ± 0.97
Tnox1	27.30 ± 1.08*	35.76 ± 0.94	32.07 ± 3.46*
Tnox2	65.19 ± 4.17*	68.08 ± 2.97*	32.28 ± 0.56*
Tnox5	54.57 ± 0.32*	60.08 ± 5.71*	44.23 ± 4.37*

[a] *, significant difference with the T34 wild type ($P < 0.0001$).
[b] T-T, interaction of *T. harzianum* with itself.

from T34-*P. ultimum* interactions compared to those observed in mycelia from T34-T34 interactions. Moreover, high transcript levels were also detected when the fungus was cultured for 4 h in the presence of olive plants (Fig. 2).

Overexpression of *nox1* in *T. harzianum* T34. In order to characterize the *nox1* gene functionally, the pJL7015 plasmid was constructed, linearized with ApaI to facilitate its integration, and used to transform *T. harzianum* T34. Ten transformants showing phleomycin resistance were checked by PCR. A 1.4-kb PCR product was amplified in all transformants analyzed using the primer pair Cyc-F and nadph11. Four PCR-positive *nox1*-overexpressed transformants were checked by Southern blotting to determine the additional *nox1* copies due to the insertion of the transformation cassette in the *T. harzianum* genome using EcoRI-digested genomic DNA and a fragment of the *ble* gene (with one EcoRI cut inside) as a probe (Fig. 3). DNA from the wild type was included as a control. Several blotted bands corresponding to the *ble* gene were observed only in DNAs from all transformant strains, indicating that the transformation cassette had been inserted several times into the four overexpressed transformant genomes. At this stage, a Nox activity assay of three transformants, Tnox1, Tnox2, and Tnox5, which showed different patterns of integration of the transformation cassette, was performed. Protein extracts obtained from dual confrontation experiments of wild-type or transformant strains against *Pythium*, *Rhizoctonia*, or *Trichoderma* (control condition) were assayed for superoxide production. As shown in Table 1, higher activity levels were detected in *nox1*-overexpressed transformant strains than in the wild type in *Pythium* confrontations. Except for the Tnox1 transformant, all *Trichoderma* strains showed lower activity levels in *Rhizoctonia* confrontations than those observed in control confrontations. Tnox2 and Tnox5 strains that produced the highest quantities of superoxide were selected and used in further characterization studies. In addition, a higher superoxide production in Tnox2- or Tnox5-*P. ultimum* interactions than in wild-type or *R. solani* interactions was observed in a staining assay with NBT (see Fig. S1 in the supplementary material).

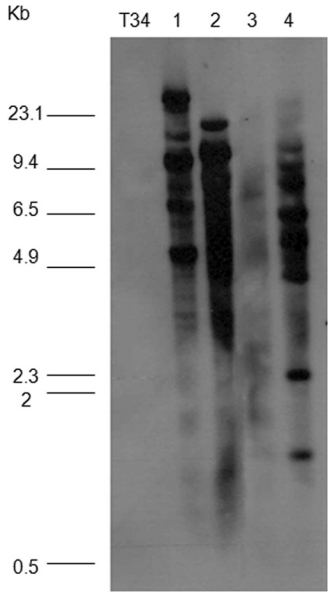

FIG. 3. Southern blots of EcoRI-digested genomic DNA of *Trichoderma* strains hybridized with the *ble* gene as a probe. DNA was obtained from wild-type *T. harzianum* (T34) and the *nox1*-overexpressed transformant strains Tnox1 (lane 1), Tnox2 (lane 2), Tnox4 (lane 3), and Tnox5 (lane 4). Molecular size standards of HindIII-digested λ DNA are at the left.

We analyzed the expression of the *nox1* gene in the wild type and the two previously selected transformant strains in the following interaction zones (Fig. 1): *Trichoderma-Pythium*, *Trichoderma-Rhizoctonia*, *Trichoderma-Botrytis*, and *Trichoderma-Trichoderma*. Under identical growth conditions, the Tnox2 and Tnox5 transformants showed *nox1* transcript levels higher than that of the endogenous *nox1* gene in the wild-type strain. Figure 1 shows that the greatest differences in *nox1* expression between the transformant strains and the wild type were observed in mycelia obtained from *P. ultimum* confrontations.

Confrontation assays. Plate confrontation experiments between T34 or *nox1*-overexpressed transformants Tnox2 and Tnox5 and the pathogens *B. cinerea*, *R. solani*, and *P. ultimum* were also carried out for 5 days at 25°C to investigate the effect of *nox1* overexpression on *T. harzianum* T34 antifungal activity. Plates were photographed at the end of the confrontation assay. In all cases, after this incubation time the three pathogens completely covered the surfaces of 90-mm-diameter PDA plates used as controls (data not shown). As shown in Fig. 4, *Trichoderma* strains overgrew the colonies of *R. solani* and *P. ultimum* and surrounded the colonies of *B. cinerea* in the dual cultures. No differences between the wild type and *nox1*-over-

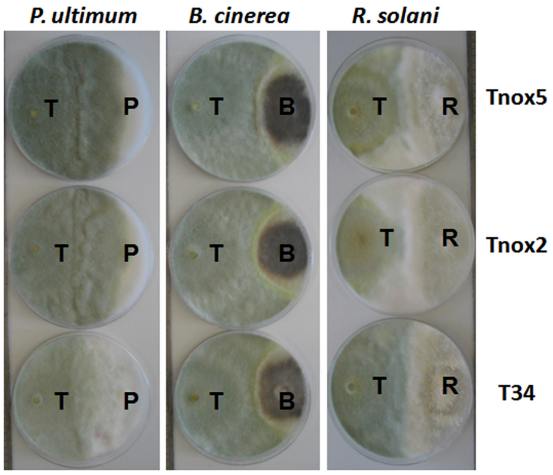

FIG. 4. Dual cultures of strains T34 and the *nox1*-overexpressed transformants Tnox2 and Tnox5 of *T. harzianum* (T) and the pathogens *P. ultimum* (P), *B. cinerea* (B), and *R. solani* (R) on PDA medium. *T. harzianum*, *P. ultimum*, and *R. solani* were sown at the same time. *B. cinerea* was allowed to grow for 1 day before *Trichoderma* sowing. Plates were incubated at 25°C for 5 days.

expressed transformant strains in inhibition of the pathogens *B. cinerea* and *R. solani* were observed. However, differences in growth inhibition and sporulation between T34 and the *nox1*-overexpressed transformants were observed in the *Pythium* confrontation plates. Smaller *P. ultimum* colony diameters were observed in *nox1* transformant confrontations than in those corresponding to the wild type. Sporulation in the pathogen overgrowing area was also more abundant in the *nox1* transformant confrontations.

Transcriptomic analysis of *nox1* during the *T. harzianum-P. ultimum* interaction. The role of *nox1* in the *Trichoderma-Pythium* interaction was analyzed using a *Trichoderma* HDO microarray (GEO submission: GSE24630). Full microarray data are shown in Table S1 in the supplemental material. After statistical analysis of normalized hybridization data, comparison of the T34-*P. ultimum* and Tnox5-*P. ultimum* transcriptomes showed that 108 *Trichoderma* genes, with fold changes (FC) ≥ 4 and P values ≤ 0.05, were differentially expressed in the Tnox5 *nox1*-overexpressed transformant. One hundred of them were upregulated, and eight were downregulated. The differentially regulated genes were classified into several biological processes of the Gene Ontology Consortium (GO). Proteolysis and binding and cellular transport processes were highly represented in Tnox5 during the interaction with *P. ultimum*.

To check the level of reliability of the array-based data, we selected two upregulated genes and one downregulated gene in the Tnox5-*Pythium* interaction and analyzed their expression levels by quantitative real-time PCR. In addition, the expression of these three genes in the Tnox2-*Pythium* interaction was analyzed. As expected, the catalase gene downregulated in the microarray data was also downregulated in the two transformant strains (Fig. 5A). Also, higher transcript levels of genes encoding the cellulose signaling protein and the trypsin-like protease (Fig. 5B and C, respectively) were detected in Tnox2 and Tnox5 transformant strains than in the wild-type T34 during the interaction with *Pythium*.

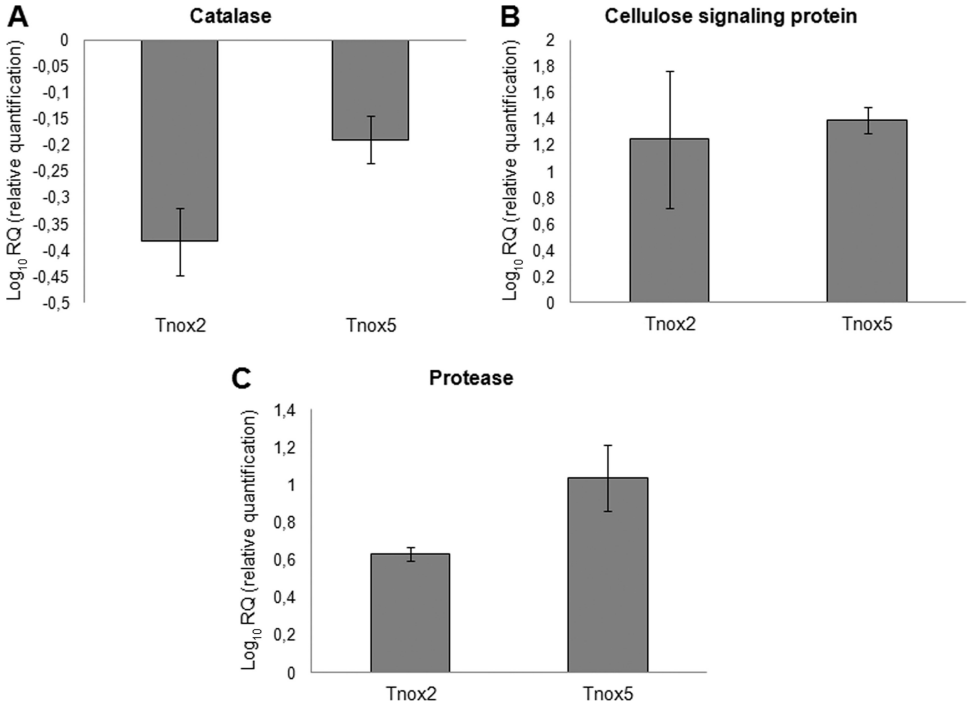

FIG. 5. Quantitative RT-PCR analysis of catalase (A), cellulose signaling (B), and trypsin-like protease (C) genes in the *nox1*-overexpressed transformants Tnox2 and Tnox5. Values ($2^{-\Delta\Delta CT}$) correspond to relative measurements against the *nox1* transcript in the wild-type *T. harzianum* T34 ($2^{-\Delta\Delta CT} = 1$). Mycelia were obtained from the interaction area of *T. harzianum-P. ultimum* dual cultures. *T. harzianum* T34 β-*tubulin* was used as an internal reference gene. Bars represent standard deviations of the mean values of three biological replicates.

TABLE 2. Protease, cellulase, and chitinase activities[a]

Enzyme and strain	Mean activity (nmol min^{-1}/mg protein) ± SD for:			
	T-T	T-P	T-R	CMC
Protease				
T34	0.1 ± 0.2^{A}	2.7 ± 0.8^{C}	3.0 ± 1.1^{C}	3.1 ± 0.35^{C}
Tnox2	1.6 ± 0.1^{B}	6.8 ± 0.8^{D}	4.2 ± 0.8^{C}	6.8 ± 0.34^{D}
Tnox5	1.1 ± 0.6^{B}	5.5 ± 0.6^{D}	4.2 ± 0.1^{C}	6.6 ± 0.33^{D}
Cellulase				
T34	77.7 ± 11.1^{A}	83.3 ± 5.5^{A}	94.4 ± 27.7^{A}	91.89 ± 1.6^{A}
Tnox2	216.6 ± 2.7^{B}	244.4 ± 5.5^{C}	127.7 ± 11.1^{A}	222.7 ± 1.54^{C}
Tnox5	172.2 ± 5.5^{C}	227.7 ± 5.5^{C}	116.6 ± 11.1^{A}	232.5 ± 1.57^{C}
Chitinase				
T34	18.1 ± 1.6^{A}	21.8 ± 1.4^{A}	16.6 ± 0.2^{A}	ND[b]
Tnox2	23.7 ± 1.8^{B}	38.0 ± 1.5^{C}	13.3 ± 1.6^{A}	ND
Tnox5	23.2 ± 1.4^{B}	32.2 ± 1.8^{C}	14.7 ± 1.9^{A}	ND

[a] Activities were measured in intracellular protein extracts from interaction zones in dual cultures of *T. harzianum* T34 or each *nox1*-overexpressed transformant and itself (T-T), *P. ultimum* (T-P), or *R. solani* (T-R), and protease and cellulase activities in T34 and transformant intracellular protein extracts obtained from 48-h liquid cultures in PDB supplemented with 1% CMC were also measured. For each activity, values followed by different superscript letters are significantly different ($P < 0.001$).

[b] ND, no data.

Most upregulated genes represented, with FC values between 4.1 and 19.3, were related to genes encoding (i) proteases (19%), including aspartic, subtilisin serine, and trypsin-like proteases; (ii) hydrophobins (7%); (iii) hypothetical proteins with domains related to fungal pathogenesis (5%); (iv) oligopeptide transporters (4%); (v) proteins involved in cell wall (CW) integrity (4%); (vi) cellulose signaling proteins (2%); and (vii) chitinases (2%). Most downregulated genes represented were those related to catalases (2%).

Protease, cellulose, and chitinase activities. Taking into account that an enhanced antagonistic activity of *nox1* transformants against *P. ultimum*, an oomycete with cellulose and minor amounts of chitin in its CW, was observed and that the highest number of upregulated genes were protease genes in the microarray assay, we compared the protease, cellulase, and chitinase activities in the wild-type and *nox1*-overexpressed transformant strains during their interaction with *T. harzianum* itself, *P. ultimum*, or *R. solani*. As shown in Table 2, higher levels of cellulase activity were detected in the two transformants than in the wild-type strain; these differences were statistically significant for both *Trichoderma-Trichoderma* and *Trichoderma-Pythium* confrontations but not for *Trichoderma-Rhizoctonia* confrontations. A similar relationship between the transformants and the wild-type strain was observed when protease and chitinase activities were analyzed under the different interaction conditions considered. Protease and cellulase activity levels detected in wild-type or transformant strains after 48 h of growth in liquid medium with 1% CMC were similar to those measured in *Trichoderma-Pythium* interactions.

DISCUSSION

The present study provides a contribution to understanding the role of a *T. harzianum* protein related to ROS production in the interaction between a biocontrol agent and three phytopathogenic preys. The isolation and characterization of a novel *Trichoderma* gene coding for a Nox protein are reported.

ROS production by specific Nox proteins has been related to defense responses in animals and plants (22, 59), and three Nox subfamilies have been described in the kingdom *Fungi* (2). EST 7015, showing high identity with ESTs for Nox proteins in a BlastX analysis, was identified in a *T. harzianum* T34 cDNA library constructed under antagonistic conditions (66). Southern blot analysis showed that *nox1* was present as a single copy in the genome of *T. harzianum* T34 (data not shown), and this result is in agreement with the presence of a single homologous gene in the three *Trichoderma* genomes available on line.

It has previously been demonstrated that *nox* genes are induced by the presence of pathogens in mammals (46) and plants (57, 60). We observed motifs for binding the regulators of oxygen metabolism, sexual development, and defense PR proteins in the *nox1* promoter region. These data are in agreement with the role of Nox proteins as critical enzymes for sexual development (2, 24, 30) and sclerotial differentiation (39) in filamentous fungi, plant infection processes (10), and antimicrobial activity due to their involvement in ROS production (15, 51).

Bearing in mind that some fungi are endowed with the ability to recognize potential contestants and have built up a response that involves cell death as a result of the generation of ROS through NADPH oxidase activity at the contact zone between fungi (51), that this gene was also induced by plant material (Fig. 2), and that cellulose is a major component of *Pythium* and plant CWs, it seems clear that this polysaccharide must be related to *nox1* induction. In contrast, *B. cinerea* and *R. solani* are ascomycete and basidiomycete fungi, respectively, without cellulose in their CWs, and no increase in *nox1* expression was detected in the zones of interaction between *T. harzianum* T34 and these two phytopathogens.

Since the frequency of homologous recombination in *T. harzianum* is very low (29) and generation of null mutants of this species has been achieved only twice (47, 48), the function of the *nox1* gene was studied following a homologous overexpression approach.

Because Southern analysis revealed that several copies of the transformation cassette were inserted into the genomes of transformant strains, superoxide production served to select two *nox1*-overexpressed transformants. The Tnox2 and Tnox5 transformants produced higher quantities of superoxide than the wild-type strain under both control and *Pythium* confrontation conditions. Although an increased *nox1* expression level had been detected only in *Pythium* interactions, similar Nox1 activity values were measured in transformant-*Pythium* confrontations and their controls (Table 1). According to *nox1* expression results (Fig. 1), we have observed higher superoxide *in situ* production in the zones of interaction between the transformants and *Pythium* than those from *Rhizoctonia* or *Trichoderma* confrontations, where stain differences were not observed (see Fig. S1 in the supplementary material). Dual-culture experiments showed that the wild-type and transformant strains displayed the same behavior against *R. solani* or *B. cinerea*. However, against *P. ultimum*, *nox1*-overexpressed transformants displayed more biocontrol efficiency than the wild type. This suggests that *nox1* is involved in *T. harzianum* antagonistic activity against the oomycete *P. ultimum*, but not

against *R. solani* or *B. cinerea*. It is well documented that ROS can induce sclerotial metamorphosis in *R. solani* (39), but this pathogen can also repress ROS formation through the production of the scavenger ascorbic acid in certain concentration gradients and proportions in response to oxidative stress caused by ROS during mycelial and sclerotial differentiation (13). On the other hand, *B. cinerea* seems to be better adapted to oxidative stress than other phytopathogens since it has been observed that ROS function generated by *Nicotiana benthamiana* has a positive role in expansion of disease lesions during *B. cinerea*-plant interaction (3).

The transcriptomic response of *T. harzianum* T34 to *nox1* overexpression was analyzed in interactions with *P. ultimum* using HDO microarrays. Compared to results for T34, the largest number of upregulated genes (19%) of the Tnox5 transformant in contact with *P. ultimum* were genes encoding proteases, including aspartic, subtilisin serine, and trypsin-like proteases, all of which were related to a biocontrol function and previously described in *Trichoderma* spp. (25, 54, 63, 68). Other highly represented differential genes were genes encoding hydrophobins (7%), also identified in proteomic studies as overexpressed *T. atroviride* proteins in interactions with bean roots and *R. solani* (32) or related to plant root colonization in *Trichoderma asperellum* (64). Hydrophobins have also been found to be a part of the antioxidant protective system of *Neurospora crassa* (16). Other differentially upregulated genes identified in Tnox5-*P. ultimum* interaction were those encoding hypothetical proteins with PR domains (5%), oligopeptide transporters (4%) induced by chitin in *Trichoderma* spp. (65), proteins involved in the maintenance of CW integrity (4%) (61), and cellulose signaling proteins (2%), induced only by cellulose in *T. reesei* (50).

That ROS promote the modification of cellular proteins and that intracellular proteolytic enzymes selectively degrade oxidized proteins is well documented (38). It has also been proposed that the proteasome is the major player involved in the removal of these oxidized proteins (43). However, proteolytic genes related to the proteasome, the ubiquitin pathway, and ROS detoxification were not differentially expressed in the *T. harzianum-P. ultimum* microarray assay. The induction of several proteases, an amine oxidase, and proteins related to cellulose signaling and pathogenesis, as well as the repression of catalase genes, suggests that Nox1 would be involved in a mechanism of defense against pathogens in a way similar to plant defense responses. In plants, the suppression of catalase or ascorbate peroxidase activities during attack by pathogens results in an overaccumulation of ROS and the activation of defense responses (34).

ROS production by NADPH oxidases is a universal signaling system among multicellular organisms, and the battery of ROS scavenging systems present in cells, including ascorbate peroxidases, glutathione, superoxide dismutases, and catalases, ensures rapid turnover of the ROS to maintain ROS homeostasis (55, 59). Then, it is generally accepted that a high catalase activity is related to high ROS levels as a mechanism for protection against oxidative damage. However, the repression of catalase genes observed in overexpressed *Trichoderma* transformants during the contact with *P. ultimum* indicates that high ROS levels downregulate catalase genes. This is in agreement with a recent study that describes how high ROS levels produce a hypermethylation of catalase promoters that reduce the expression of catalase genes in human tumoral cell lines (33).

A direct correlation between hydrolase production and biocontrol activity has been reported in transcriptomic studies of *Trichoderma* spp. (28, 66, 67). The detection of higher protease, cellulose, and chitinase activities in *nox1*-overexpressed transformants than in T34 in *P. ultimum* interactions is in agreement with the biocontrol efficiency observed in dual cultures and the microarray transcriptomic results. The fact that protease and cellulase activity levels detected in *Trichoderma* strains in the presence of CMC were similar to those observed in *Trichoderma-Pythium* interactions indicates that cellulose alone is the triggering factor of these *Trichoderma* hydrolytic activities. These results are in agreement with the *nox1* expression studies indicating that cellulose from *Pythium* CWs is the inducing factor for the *nox1* gene. The *nox1*-overexpressed transformants also displayed more chitinase activity in the interaction with *P. ultimum* than in that with *R. solani*. In this case, the higher chitinase activity could be due to the residual chitin present in *Pythium* CWs (4). The increased ROS levels measured in *nox1* transformants in interactions with *P. ultimum* seem be involved in their increased hydrolytic activities and therefore in the biocontrol efficiency of *nox1* transformants against this pathogen.

Taking our results as a whole, they indicate that the *T. harzianum nox1* gene is involved in control of the oomycete *P. ultimum* by maintaining high ROS levels, which are accompanied by the upregulation of protease-, cellulase-, and chitinase-encoding genes, leading to an increase in the corresponding enzymatic activities.

ACKNOWLEDGMENTS

Research project funding was from the Junta de Castilla y León (GR67) and the Spanish Ministry of Science and Innovation (AGL2008-0512/AGR and AGL2009-13431).

Thanks are due to Ricardo López Pérez for microarray data analysis.

REFERENCES

1. **Able, A. J., D. I. Guest, and M. W. Sutherland.** 1998. Use of a new tetrazolium-based assay to study the production of superoxide radicals by tobacco cell cultures challenged with avirulent zoospores of *Phytophthora parasitica* var. *nicotianae.* Plant Physiol. **117:**491–499.
2. **Aguirre, J., M. Rios-Momberg, D. Hewitt, and W. Hansberg.** 2005. Reactive oxygen species and development in microbial eukaryotes. Trends Microbiol. **13:**111–118.
3. **Asai, S., and H. Yoshioka.** 2009. Nitric oxide as a partner of reactive oxygen species participates in disease resistance to nectrotophic pathogen *Botryis cinerea* in *Nicotiana benthamiana*. Mol. Plant Microbe Interact. **22:**619–629.
4. **Asiegbu, F. O., A. M. Lönneborg, and M. Johansson.** 1996. Chitin and glucans detected in the cell walls of *Pythium dimorphum*—an oomycetous fungus. Eur. J. Forest Pathol. **26:**315–321.
5. **Baldi, P., and A. D. Long.** 2001. A Bayesian framework for the analysis of microarray expression data: regularized t-test and statistical inferences of gene changes. Bioinformatics **17:**509–519.
6. **Benítez, T., A. M. Rincón, M. C. Limón, and A. C. Codón.** 2004. Biocontrol mechanisms of *Trichoderma* strains. Int. Microbiol. **7:**249–260.
7. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
8. **Cardoza, R. E., J. A. Vizcaíno, M. R. Hermosa, E. Monte, and S. Gutiérrez.** 2006. A comparison of the phenotypic and genetic stability of recombinant *Trichoderma* spp. generated by protoplast- and *Agrobacterium*-mediated transformation. J. Microbiol. **44:**383–395.
9. **Claros, M. G., and G. von Heijne.** 1994. TopPred II: an improved software for membrane protein structure predictions. Comput. Appl. Biosci. **10:**685–686.
10. **Egan, M. J., Z. Wang, M. A. Jones, N. Smirnoff, and N. J. Talbot.** 2007.

Generation of reactive oxygen species by fungal NADPH oxidases is required for rice blast disease. Proc. Natl. Acad. Sci. U. S. A. **104:**11772–11777.
11. **Elad, Y.** 2000. Biological control of foliar pathogens by means of *Trichoderma harzianum* and potential modes of action. Crop Prot. **19:**709–714.
12. **Foreman, J., et al.** 2003. Reactive oxygen species produced by NADPH oxidase regulate plant cell growth. Nature **422:**442–446.
13. **Georgiou, C. D., and K. P. Petropoulou.** 2001. Effect of the antioxidant ascorbic acid on sclerotial differentiation in *Rhizoctonia solani*. Plant Pathol. **50:**594–600.
14. **Grant, M., et al.** 2000. The *RPMI* plant disease resistance gene facilitates a rapid and sustained increase in cytosolic calcium that is necessary for the oxidative burst and hypersensitive cell death. Plant J. **23:**441–450.
15. **Haedens, V., F. Malagnac, and P. Silar.** 2005. Genetic control of an epigenetic cell degeneration syndrome in *Podospora anserine*. Fungal Genet. Biol. **42:**564–577.
16. **Hansberg, W., and J. Aguirre.** 1990. Hyperoxidant states cause microbial cell differentiation by cell isolation from dioxygen. J. Theor. Biol. **142:**201–221.
17. **Harman, G. E., C. R. Howell, A. Viterbo, I. Chet, and M. Lorito.** 2004. *Trichoderma* species—opportunistic, avirulent plant symbionts. Nat. Rev. Microbiol. **2:**43–56.
18. **Holwerda, B. C., and J. C. Rogers.** 1992. Purification and characterization of aleurain: a plant thiol protease functionally homologous to mammalian cathepsin H. Plant Physiol. **99:**848–855.
19. **Howell, C. R.** 2003. Mechanisms employed by *Trichoderma* species in the biological control of plant diseases: the history and evolution of current concepts. Plant Dis. **87:**4–10.
20. **Irizarry, R. A., et al.** 2003. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. Biostatistics **4:**249–264.
21. **Kuc, J.** 2001. Concepts and direction of induced systemic resistance in plants and its application. Eur. J. Plant Pathol. **107:**7–12.
22. **Lambeth, J. D.** 2004. Nox enzymes and the biology of reactive oxygen. Nat. Rev. Immunol. **4:**181–189.
23. **Lara-Ortiz, T., H. Riveros-Rosas, and J. Aguirre.** 2003. Reactive oxygen species generated by microbial NADPH oxidase NoxA regulate sexual development in *Aspergillus nidulans*. Mol. Microbiol. **50:**1241–1255.
24. **Lewit-Bentley, A., and S. Rety.** 2000. EF-hand calcium-binding proteins. Curr. Opin. Struct. Biol. **10:**637–643.
25. **Liu, Y., and Q. Yang.** 2007. Cloning and heterologous expression of aspartic protease SA76 related to biocontrol in *Trichoderma harzianum*. FEMS Microbiol. Lett. **227:**173–181.
26. **Livak, K. J., and T. D. Schmittgen.** 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2(-\Delta\Delta CT)$ method. Methods **25:**402–408.
27. **Lora, J. M., J. De la Cruz, A. Llobell, T. Benítez, and J. A. Pintor-Toro.** 1995. Molecular characterization and heterologous expression of an endo-beta-1,6-glucanase gene from the mycoparasitic fungus *Trichoderma harzianum*. Mol. Gen. Genet. **247:**639–645.
28. **Lorito, M., S. L. Woo, G. E. Harman, and E. Monte.** 2010. Translational research on *Trichoderma*: from 'omics to the field. Annu. Rev. Phytopathol. **48:**395–417.
29. **Mach, R. L., and S. Zeilinger.** 1998. Genetic transformation of *Trichoderma* and *Gliocladium*, p. 225–242. *In* C. P. Kubicek and G. E. Harman (ed.), *Trichoderma* and *Gliocladium*. Taylor & Francis, Ltd., London, United Kingdom.
30. **Malagnac, F., H. Lalucque, G. Lepere, and P. Silar.** 2004. Two NADPH oxidase isoforms are required for sexual reproduction and ascospore germination in the filamentous fungus *Podospora anserine*. Fungal Genet. Biol. **41:**982–997.
31. **Marchler-Bauer, A., et al.** 2003. CDD: a curated Entrez database of conserved domain alignments. Nucleic Acids Res. **31:**383–387.
32. **Marra, R., et al.** 2006. Study of the three-way interaction between *Trichoderma atroviride*, plant and fungal pathogens by using a proteomic approach. Curr. Genet. **50:**307–321.
33. **Min, J. Y., S.-O. Lin, and G. Jung.** 2010. Downregulation of catalase by reactive oxygen species via hypermethylation of CpG island II on the catalase promoter. FEBS Lett. **584:**2427–2432.
34. **Mittler, R.** 2002. Oxidative stress, antioxidants and stress tolerance. Trends Plant Sci. **7:**405–410.
35. **Monte, E.** 2001. Understanding *Trichoderma:* between biotechnology and microbial ecology. Int. Microbiol. **4:**1–4.
36. **Montero-Barrientos, M., et al.** 2008. Overexpression of a *Trichoderma* HSP70 gene increases fungal resistance to heat and other abiotic stresses. Fungal Genet. Biol. **45:**1506–1513.
37. **Nelson, N. J.** 1944. A photometric adaptation of the Somogyi method for the determination of glucose. J. Biol. Chem. **153:**375–380.
38. **Okada, K., et al.** 1999. 4-Hydroxy-2-nonenal-mediated impairment of intracellular proteolysis during oxidative stress. J. Biol. Chem. **274:**23787–23793.
39. **Papapostolou, I., and C. D. Georgiou.** 2010. Superoxide radical induces sclerotial differentiation in filamentous phytopathogenic fungi: a superoxide dismutase mimetics study. Microbiology **156:**960–966.
40. **Penttilä, M., H. Nevalainen, M. Ratto, E. Salminen, and J. Knowles.** 1987. A versatile transformation system for the cellulolytic filamentous fungus *Trichoderma reesei*. Gene **61:**155–164.
41. **Raeder, U., and P. Broda.** 1985. Rapid preparation of DNA from filamentous fungi. Lett. Appl. Microbiol. **1:**17–20.
42. **Reeves, E. P., et al.** 2002. Killing activity of neutrophils is mediated through activation of proteases by K^+ flux. Nature **416:**291–297.
43. **Reinheckel, T., et al.** 1998. Comparative resistance of the 20S and 26S proteasome to oxidative stress. Biochem. J. **335:**637–642.
44. **Reissig, J. L., J. L. Strominger, and L. F. Leloir.** 1955. A modified colorimetric method for the stomation of N-acetylamino sugars. J. Biol. Chem. **217:**959–966.
45. **Rey, M., A. Llobell, E. Monte, F. Scala, and M. Lorito.** 2004. Genomics of *Trichoderma*, p. 225–248. *In* G. G. Khachatourians (ed.), Fungal genomics, vol. 4. Elsevier Science, Amsterdam, Netherlands.
46. **Roos, D., et al.** 1996. Mutations in the X-linked and autosomal recessive forms of chronic granulomatous disease. Blood **87:**1663–1681.
47. **Rosado, I. V., et al.** 2007. QID74 cell wall protein of *Trichoderma harzianum* is involved in cell protection and adherence to hydrophobic surfaces. Fungal Genet. Biol. **44:**950–964.
48. **Rubio, M. B., R. Hermosa, J. L. Reino, I. G. Collado, and E. Monte.** 2009. *Thctf1* transcription factor of *Trichoderma harzianum* is involved in 6-pentyl-2H-pyran-2-one production and antifungal activity. Fungal Genet. Biol. **46:**17–27.
49. **Samolski, I., A. de Luis, J. A. Vizcaíno, E. Monte, and M. B. Suárez.** 2009. Gene expression analysis of the biocontrol fungus *Trichoderma harzianum* in the presence of tomato plants, chitin, or glucose using a high-density oligonucleotide microarray. BMC Microbiol. **9:**217.
50. **Schmoll, M., and C. P. Kubicek.** 2005. *ooc1*, a unique gene expressed only during growth of *Hypocrea jecorina* (anamorph: *Trichoderma reesei*) on cellulose. Curr. Genet. **48:**126–133.
51. **Silar, P.** 2005. Peroxide accumulation and cell death in filamentous fungi induced by contact with a contestant. Mycol. Res. **109:**137–149.
52. **Sivasithamparam, K., and E. L. Ghisalberti.** 1998. Secondary metabolism in *Trichoderma* and *Gliocladium*, p. 139–191. *In* C. P. Kubicek and G. E. Harman (ed.), *Trichoderma* and *Gliocladium*, Taylor & Francis, London, United Kingdom.
53. **Somogyi, M.** 1952. Notes on sugar determination. J. Biol. Chem. **195:**19–23.
54. **Suárez, M. B., et al.** 2005. Proteomic analysis of secreted proteins from *Trichoderma harzianum*. Identification of a fungal cell wall-induced aspartic protease. Fungal Genet. Biol. **42:**924–934.
55. **Takemoto, D., A. Tanaka, and B. Scott.** 2007. NADPH oxidases in fungi: diverse roles of reactive oxygen species in fungal cellular differentiation. Fungal Genet. Biol. **44:**1065–1076.
56. **Tanaka, A., M. J. Christensen, D. Takemoto, P. Park, and B. Scott.** 2006. Reactive oxygen species play a role in regulating a fungus-perennial ryegrass mutualistic association. Plant Cell **18:**1052–1066.
57. **Torres, M. A., J. L. Dangl, and J. D. G. Jones.** 2002. *Arabidopsis gp91phox* homologues *AtrbohD* and *AtrbohF* are required for accumulation of reactive oxygen intermediates in the plant defense response. Proc. Natl. Acad. Sci. U. S. A. **99:**517–522.
58. **Torres, M. A., J. D. G. Jones, and J. L. Dangl.** 2005. Pathogen-induced, NADPH oxidase-derived reactive oxygen intermediates suppress spread of cell death in *Arabidopsis thaliana*. Nat. Genet. **37:**1130–1134.
59. **Torres, M. A., and J. L. Dangl.** 2005. Functions of the respiratory burst oxidase in biotic interactions, abiotic stress and development. Curr. Opin. Plant Biol. **8:**397–403.
60. **Torres, M. A., et al.** 1998. Six *Arabidopsis thaliana* homologues of the human respiratory burst oxidase ($gp91^{phox}$). Plant J. **14:**365–370.
61. **Verna, J., A. Lodder, K. Lee, A. Vagts, and R. Ballester.** 1997. A family of genes required for maintenance of cell wall integrity and for the stress response in *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. U. S. A. **94:**13804–13809.
62. **Vinale, F., et al.** 2006. Major secondary metabolites produced by two commercial *Trichoderma* strains active against different phytopathogens. Lett. Appl. Microbiol. **43:**143–148.
63. **Viterbo, A., and I. Chet.** 2006. *TasHyd1*, a new hydrophobin gene from the biocontrol agent *Trichoderma asperellum*, is involved in plant root colonization. Mol. Plant Pathol. **7:**249–258.
64. **Viterbo, A., M. Harel, and I. Chet.** 2004. Isolation of two aspartyl proteases from *Trichoderma asperellum* expressed during colonization of cucumber roots. FEMS Microbiol. Lett. **238:**151–158.
65. **Vizcaíno, J. A., et al.** 2006. *ThPTR2*, a di/tri-peptide transporter gene from *Trichoderma harzianum*. Fungal Genet. Biol. **43:**234–246.
66. **Vizcaíno, J. A., et al.** 2006. Generation, annotation and analysis of ESTs from *Trichoderma harzianum* CECT 2413. BMC Genomics **7:**193.
67. **Vizcaíno, J. A., et al.** 2007. Generation, annotation, and analysis of ESTs from four different *Trichoderma* strains grown under conditions related to biocontrol. Appl. Microbiol. Biotechnol. **75:**853–862.
68. **Yang, J. K., et al.** 2005. Isolation and characterization of a serine protease from the nematophagous fungus, Lecanicillium psalliotae, displaying nematicidal activity. Biotechnol. Lett. **27:**1123–1128.