MPMI Vol. 22, No. 8, 2009, pp. 1021–1031. doi:10.1094/MPMI-22-8-1021. *e*-Xtra*

The ThPG1 Endopolygalacturonase Is Required for the *Trichoderma harzianum*–Plant Beneficial Interaction

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Submitted 14 November 2008. Accepted 14 April 2009.

Considering the complexity of the in vivo interactions established by a mycoparasitic biocontrol agent at the plant rhizosphere, proteomic, genomic, and transcriptomic approaches were used to study a novel *Trichoderma* gene coding for a plant cell wall (PCW)-degrading enzyme. A proteome analysis, using a three-component (*Trichoderma* spp.–tomato plantlets–pathogen) system, allowed us to identify a differentially expressed *Trichoderma harzianum* endopolygalacturonase (endoPG). Spot 0303 remarkably increased only in the presence of the soilborne pathogens *Rhizoctonia solani* and *Pythium ultimum*, and corresponded to an expressed sequence tag from a *T. harzianum* T34 cDNA library that was constructed in the presence of PCW polymers and used to isolate the *Thpg1* gene. Compared with the wild-type strain, *Thpg1*-silenced transformants showed lower PG activity, less growth on pectin medium, and reduced capability to colonize tomato roots. These results were combined with microarray comparative data from the transcriptome of *Arabidopsis* plants inoculated with the wild type or a *Thpg1*-silenced transformant (ePG5). The endoPG-encoding gene was found to be required for active root colonization and plant defense induction by *T. harzianum* T34. In vivo assays showed that *Botrytis cinerea* leaf necrotic lesions were slightly smaller in plants colonized by ePG5, although no statistically significant differences were observed.

Most species of *Trichoderma* have been linked to biocontrol and biotechnological applications. The versatility of *Trichoderma* strains to suppress diseases caused by pathogens has been further illustrated (Howell 2003; Papavizas 1985). Their antagonistic abilities are described as a combination of several mechanisms, including nutrient competition and direct mycoparasitism, which involves the production of antifungal metabolites and cell wall (CW)-degrading enzymes (Howell 1998; Kuc 2001). The capacity of this fungus to promote growth and induce resistance in plants has also been described (Yedidia et al. 1999). In this sense, *Trichoderma* spp. can generate a response in the plant similar to rhizobacteria-induced systemic resistance (ISR), as has been demonstrated by following different research approaches (Alfano et al. 2007; Djonovic et al. 2007; Shoresh and Harman 2008). Some CW oligomers may act as elicitor molecules released by plants following pathogen attack (Woo et al. 2006). These can include oligogalacturonides (OGA), biologically active homogalacturonan (HGA) fragments with a size between 2 and 20 residues of galacturonic acid. OGA released from pectins are related to defense responses and also modulate plant growth and development (Casasoli et al. 2008; Ridley et al. 2001).

Pectin is a major component of plant CW and pectinases are among the first enzymes produced during fungal attack on the plant (Annis and Goodwin 1997). These enzymes are involved in the cleavage of the three pectin domains: HGA, rhamnogalacturonan-I (RG-I), and rhamnogalacturonan-II (RG-II) (Willats et al. 2001). Endopolygalacturonases (endoPG) catalyze the hydrolysis of α-1,4-galacturonic acid linkages between two nonmethylated galacturonic acid residues from HGA. EndoPG are included in family 28 of the glycosyl hydrolase enzymes (E.C. 3.2.1.15) (Henrissat 1991) that, in general, use pectin as substrate (Wubben et al. 2000).

Several fungal endoPG have been purified and characterized (D'Ovidio et al. 2004a) and their role during plant pathogenesis has been investigated (Di Pietro and Roncero 1998; Roper et al. 2007; ten Have et al. 2001). To date, only two endoPG from *Trichoderma reesei* (Mohamed et al. 2003) and one from *T. harzianum* (Mohamed et al. 2006) have been purified in *Trichoderma* spp. but no gene coding for these proteins have been isolated yet. In fact, the genus *Trichoderma* is today considered an avirulent plant-symbiotic fungus (Harman et al. 2004) and studies focused on genes and proteins directly involved in plant–*Trichoderma* interaction are still relatively uncommon (Woo et al. 2006).

Using proteomic, genomic, and transcriptomic approaches, in the present work, we report for the first time the cloning and characterization of a gene, *Thpg1*, that encodes an endoPG protein from a *Trichoderma* sp. strain. We show that this gene is differentially upregulated during *Trichoderma* spp.–plant–pathogen interaction. Silencing of *Thpg1* resulted in a significant decrease of endoPG and root colonization activities in the biocontrol fungus and in a reduced expression of genes related to defense in the plant.

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Nucleotide sequence data is available from the GenBank database under accession numbers AM421521 (*Thpg1* gene) and AM489729 (promoter).

*The *e*-Xtra logo stands for "electronic extra" and indicates that four supplemental figures are published online.

RESULTS

Proteomic analysis.

A proteomic approach was used to investigate the cell protein profiles of *T. harzianum* T34 in order to identify differentially expressed proteins during *Trichoderma* spp.–plant–pathogen interactions. Two-dimensional electrophoresis (2-DE) maps of intracellular proteins were generated from mycelium obtained by growing *Trichoderma* spp. in a three-component system with tomato plantlets, *T. harzianum* T34, and a fungal or Oomycete plant pathogen (Fig. 1). The proteome of *T. harzianum* T34, obtained on water agar (WA) in the presence of the plant, was used as a control for comparisons. Differential spot numbers produced according to PD-Quest analysis are indicated in Table 1. The infection of the plantlets with the foliar pathogen *Botrytis cinerea* produced 132 differential spots whereas more than 220 spots were noted as differential when *Trichoderma* spp. were exposed to the plant and *Rhizoctonia solani* or *Pythium ultimum*, indicating that these soilborne phytopathogens induce major changes in the proteome of *T. harzianum* T34 interacting with the plant. The differential spots between the two-way (*Trichoderma* spp.–plant) and three-way (*Trichoderma* spp.–plant–pathogen) interaction condition were identified by mass spectroscopy (MS) and in silico analysis. Interesting homologies were found with the following type of proteins: i) hydrolases and other enzymes such as cellulases, chitin synthases, glucosidases, trehalases, and proteases; ii) stress-related proteins; iii) a hydrophobin; iv) an ABC transporter; v) transcription factors and regulators, such as IME4 and WD40; and vi) proteins involved in basic metabolism and various cellular processes, such as cyclophilins, kinases, phosphatases, a phosphoesterase, a 14.3.3 family protein, a glycosil hydrolase, a phosphocholine cytidyltransferase, a leucine-rich protein, a GTP cyclohydrolase, a cloacin-like protein, a septin, a glyceraldehyde-3-phosphate dehydrogenase, and so on.

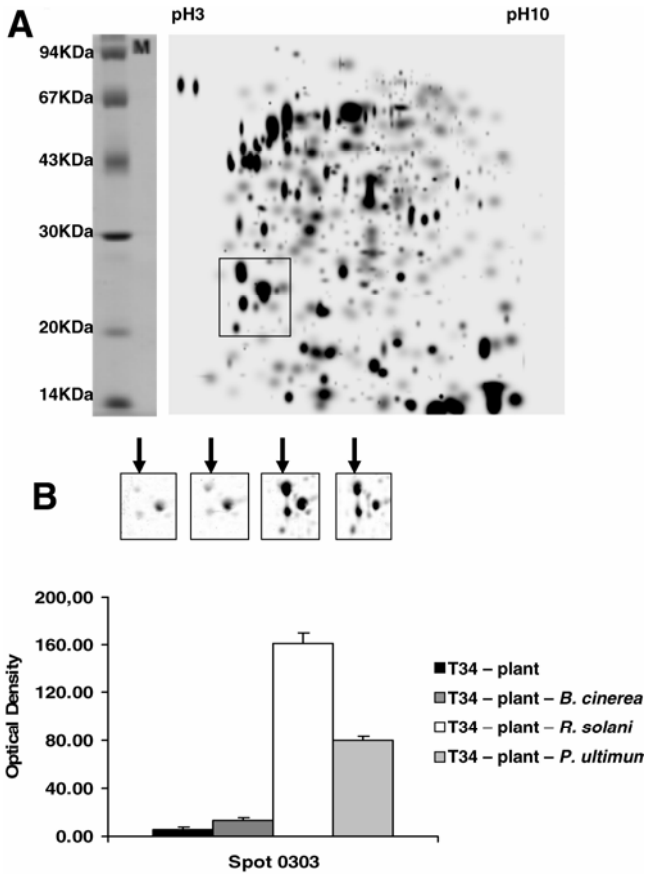

Fig. 1. Analysis of the intracellular proteome of *Trichoderma harzianum* T34 grown on water agar plates in the presence of plants (tomato plantlets) and three different phytopathogens (*Botrytis cinerea, Rhizoctonia solani*, or *Pythium ultimum).* **A,** Proteomic map. Two-dimensional gel of intracellular proteins extracted from *T. harzianum* T34 grown in the presence of tomato plantlets and *R. solani***. B,** Differential intensity levels of spot 0303. Spot intensity changes are shown by the enlarged gel regions (picture insets) placed over the corresponding relative intensity (histogram).

The intensity of the spot 0303 was strongly upregulated in the presence of *P. ultimum* (13-fold) and *R. solani* (27-fold) and less in the presence of *B. cinerea.* MS and in silico analysis of this spot showed a match with an endoPG B of *Aspergillus niger* (gi|6911545). The subsequent Mascot analysis related the spot 0303 to the expressed sequence tag (EST) L15T34P120R10663 (hereafter, EST 10663) from a *T. harzianum* T34 EST database (Vizcaíno et al. 2006) generated in the functional genomics TrichoEST project (Rey et al. 2004).

Isolation of *Thpg1*.

The spot 0303-related EST 10663 was used as a probe to screen a lambda genomic DNA library (L01). A positive phage was isolated, and a 2,199-bp fragment contained the complete sequence of the *Thpg1* gene: 1,387 bp, with an open reading frame (ORF) of 1,143 bp and four introns of 54 to 74 bp; plus 812 bp of the promoter region. Two oligonucleotides were designed using this genomic sequence for amplifying a 1,143-bp fragment corresponding to the full-length cDNA from library phages by polymerase chain reaction (PCR).

The ORF of *Thpg1* encodes a protein of 380 amino acids with a theoretical molecular mass of 38.3 kDa and an isoelectric point of 5.02. An analysis of the primary structure of ThPG1 revealed the presence of a putative signal peptide of 21 amino acids and the conserved glycosyl hydrolases family 28 domain related to its catalytic mechanism. The analysis of the *Thpg1* promoter region revealed up to six MCM1 motifs involved in the control of cell cycle, CW structure, and metabolism (Kuo and Grayhack 1994).

Southern analysis was carried out in order to analyze the number of *Thpg1* copies in the genome of *T. harzianum* T34, as well as to explore the presence of homologs of this gene in the other *Trichoderma* spp.: *T. asperellum*, *T. atroviride*, *T. harzianum*, *T. virens*, and *T. longibrachiatum* (Supplementary Fig. S1). Two and one hybridizing bands were detected with *Bam*HI-digested (one restriction site inside the probe) or *Xba*I-digested (no restriction sites) genomic DNA of *T. harzianum* T34, respectively, indicating that *Thpg1* is present as a single copy. Homologous genes were identified in the genomes of all the *Trichoderma* strains investigated.

Phylogenetic analysis.

A neighbor-joining (NJ) tree (Fig. 2) was obtained after an alignment of the *Thpg1* gene with 10 other GenBank-retrieved

Table 1. Changes occurring in the proteome of *Trichoderma harzianum* T34 during a three-way interaction: T34–tomato (Tom)–*Botrytis cinerea* (Bot), *Rhizoctonia solani* (Rhi), or *Pythium ultimum* (Pyt)[a]

Condition	Total[b]	On[c]	Off[c]	Up[d]	Down[d]
T34-Tom-Bot	132	28	32	46	26
T34-Tom-Rhi	227	81	24	105	17
T34-Tom-Pyt	222	82	29	101	10

[a] The combination T34–tomato was used as control.
[b] Total number of differential spots.
[c] Number of spots present in T34–tomato condition compared with T34-tomato-pathogen (On) or vice versa (Off).
[d] Number of spots whose intensity in T34-tomato condition increased (Up) or decreased (Down) at least twofold compared with T34–tomato-pathogen.

sequences, representing genes encoding endo- and exoPG from five Ascomycetes as well as from one Oomycete and one plant, and 11 sequences obtained from the three *Trichoderma* genomes available online. Two independent clades containing endo- or exoPG could be identified. Ascomycete endoPG formed a major subclade, separated from Oomycete and plant endoPG, as supported by a bootstrap value of 93%. Within the Ascomycete endoPG clade, several subclades, supported by high bootstrap values, were observed. The *Thpg1* sequence formed a 100%-supported subclade with hypothetical endoPG-encoding genes of *T. virens*, *T. atroviride*, and *T. reesei*.

Expression studies.

The expression of the *Thpg1* gene was analyzed by Northern blot under a simulated in vitro plant–*T. harzianum* interaction condition. Total RNA was isolated from strain T34 cultured in minimal medium (MM) liquid medium (control) or MM amended with different plant CW polymers or tomato plantlets and harvested at different time points (Fig. 3). A marked hybridization signal with a *Thpg1* probe was observed when the fungus was cultured for 24 h in the medium containing 0.5% pectin or 0.5% polygalacturonic acid (PGA). A less-marked signal was observed in 24-h-old mycelia grown together with tomato plantlets or in the presence of 0.1% pectin and 8-h-old mycelia in the presence of 0.5% pectin. No *Thpg1* gene expression was detected in the control condition (MM medium with glucose and nitrogen starvation).

In vivo *Thpg1* expression analysis was performed by real-time PCR using a three-component system, in which *T. harzianum* T34 was applied together with a phytopathogen in the rhizosphere of tomato plants. Total RNA from *T. harzianum* T34 grown for 24 h in plates hosting the three-component (T34–tomato–*P. ultimum*), two-component (T34–tomato), or T34 (control condition) system was used. Compared with the control, higher transcript levels of *Thpg1* were detected in the presence of *P. ultimum* (Fig. 4).

Thpg1 expression was investigated using real-time PCR after growing strain T34 for 24 or 48 h in concentrated (10- or 18-fold) tomato–*R. solani* co-culture filtrates. No gene expression was detected under the different conditions assayed.

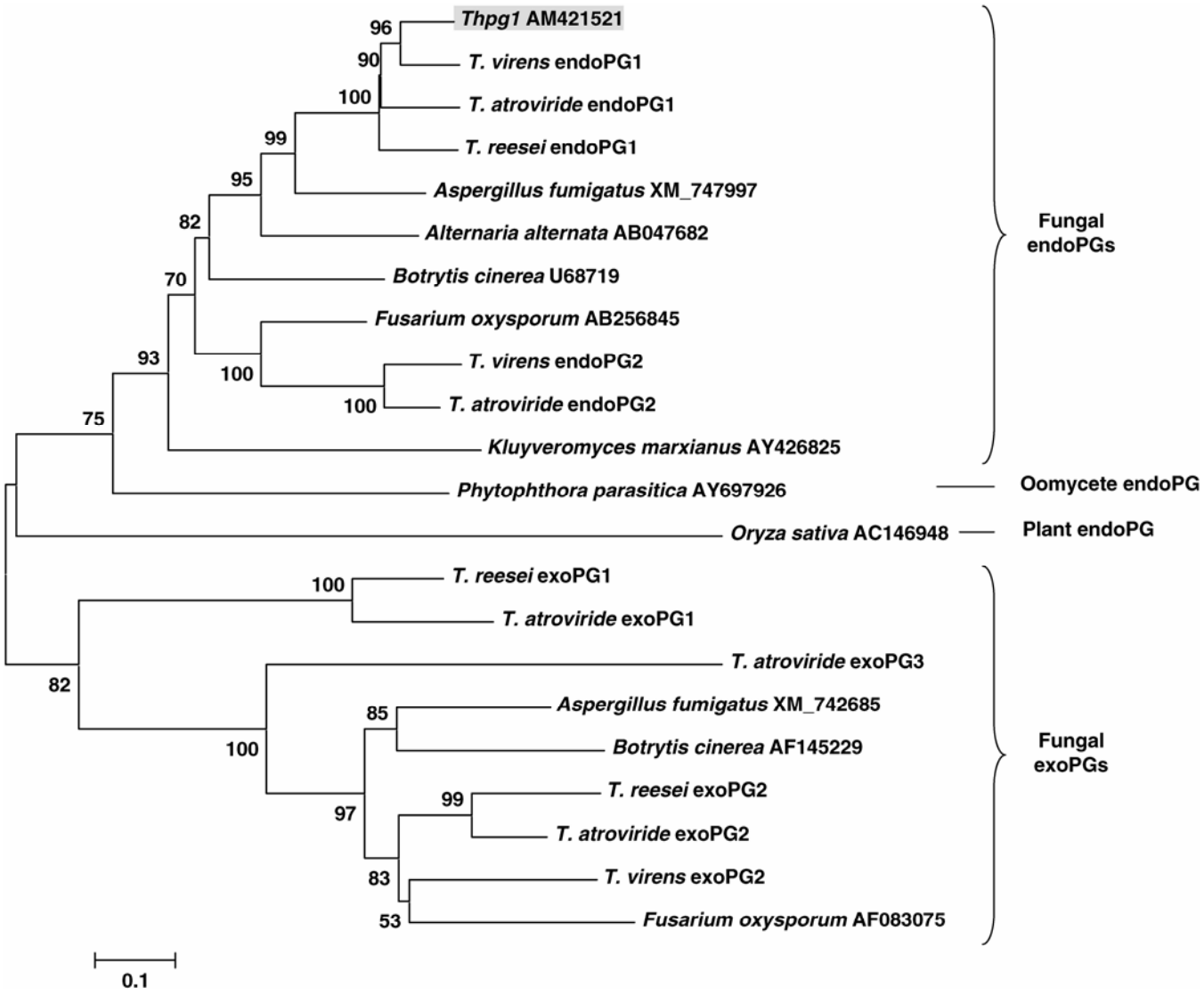

Fig. 2. Neighbor-joining (NJ) tree of *Thpg1* from *Trichoderma harzianum* T34 and another 21 polygalacturonase (PG) and hypothetical PG genes. Nucleotide sequences were obtained from *T. reesei* (protein ID: 103049 [endoPG1], 70186 [exoPG1], and 112140 [exoPG2]), *T. virens* (protein ID: 51095 [endoPG1], 58787 [endoPG2], and 48906 [exoPG2]), *T. atroviride* (protein ID: 83873 [endoPG1], 28947 [endoPG2], 86622 [exoPG1], 87581 [exoPG2], and 55045 [exoPG3]), *Aspergillus fumigatus*, *Alternaria alternata*, *Botrytis cinerea*, *Fusarium oxysporum*, *Kluyveromyces marxianus*, *Phytophthora parasitica*, and *Oryza sativa*. Sequences from *Trichoderma* spp. are available online from the DOE Joint Genome Institute. Sequences are indicated using their GenBank accession numbers. Bar represents ten substitutions per 100 nucleotides.

PG activity.

PG activity was determined in supernatants from *T. harzianum* T34 cultures after 4, 8, and 24 h of incubation in the *Thpg1* expression test conditions described above, as well as after 36 h in 0.5% pectin and with 0.5% PGA used as the substrate. A value of 7.40 enzyme units per milligram of protein was detected in the 24-h-old culture supernatant (0.5% pectin), decreasing to 3.39 U/mg of protein after 36 h of growth. In the 24-h-old culture supernatant obtained in the presence of 0.1% pectin, 0.5% PGA, or 1% tomato plant, where a lower transcript signal compared with the 0.5% pectin condition was observed (Fig. 3A), PG activity was detectable only if the sample was concentrated five- to ninefold, obtaining values of 1.37, 1.15, and 0.33 U/mg of protein, respectively. In the 8-h-old culture supernatant with 0.1% pectin, PG activity could be detected only after a 15-fold concentration.

PG inhibition by PGIP.

The sensitivity of ThPG1 to different PG inhibitor proteins (PGIP), two from *Arabidopsis thaliana* (AtPGIP1 and AtPGIP2) and two from *Phaseolus vulgaris* (PvPGIP1 and PvPGIP2), was assayed following the method described by Ferrari and associates (2003). *Arabidopsis* PGIP were unable to inhibit the ThPG1 activity. By contrast, an inhibitory effect of *Phaseolus* PGIP was observed: approximately 50 ng of PvPGIP2 and 130 ng of PvPGIP1 was necessary to inhibit 1 agarose plate unit of ThPG1.

Silencing of *Thpg1* gene and characterization of transformants.

The function of *Thpg1* in *T. harzianum* T34 was studied by gene silencing. The method used to silence this gene was based on the generation of an intron containing self-complementary "hairpin" RNAs (ihpRNA). *T. harzianum* T34 was transformed with the pSIL-PG1 construct (Supplementary Fig. S2). From the regenerated protoplasts, we selected 14 transformants growing on tryptone soy agar medium amended with phleomycin at 200 μg/ml. Single-spore cultures were isolated from transformants and they kept their resistance to phleomycin.

The integration of pSIL-PG1 was determined by PCR using the oligonucleotides IntF and Tcbh2 as the primers. The presence of a 1,218-bp amplification fragment was confirmed for all 14 transformants. Southern analysis was performed on T34 and four *Thpg1*-silenced strains (ePG4, ePG5, ePG6, and ePG8) by digesting gDNAs with *Eco*RI (no restriction site in *Thpg1)* and using *Thpg1* cDNA as a probe. The wild-type strain showed only one band of approximately 13 kb, whereas, for the transformants ePG4, ePG5, ePG6, and ePG8, additional insertions of the transformation cassette were detected (Supplementary Fig. S3). In particular, ePG4 and ePG8 contained four and three insertions, respectively.

To analyze the effect of *Thpg1* silencing on PG activity, supernatants for wild-type and transformant strains were obtained from 24-h-old cultures on MM plus 0.5% pectin. PG activity values for the different strains, obtained by using 0.2%

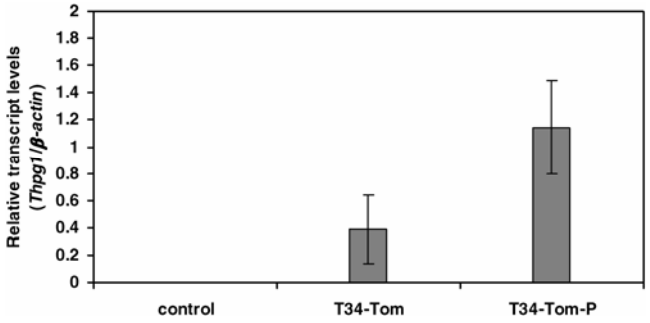

Fig. 4. Quantification of the *Thpg1* transcripts in *Trichoderma harzianum* T34 mycelia by real-time polymerase chain reaction. The fungus was grown on minimal medium (control) or in the presence of tomato (T34-Tom) or tomato and *Pythium ultimum* (T34-Tom-P), as described by Marra and associates (2006).

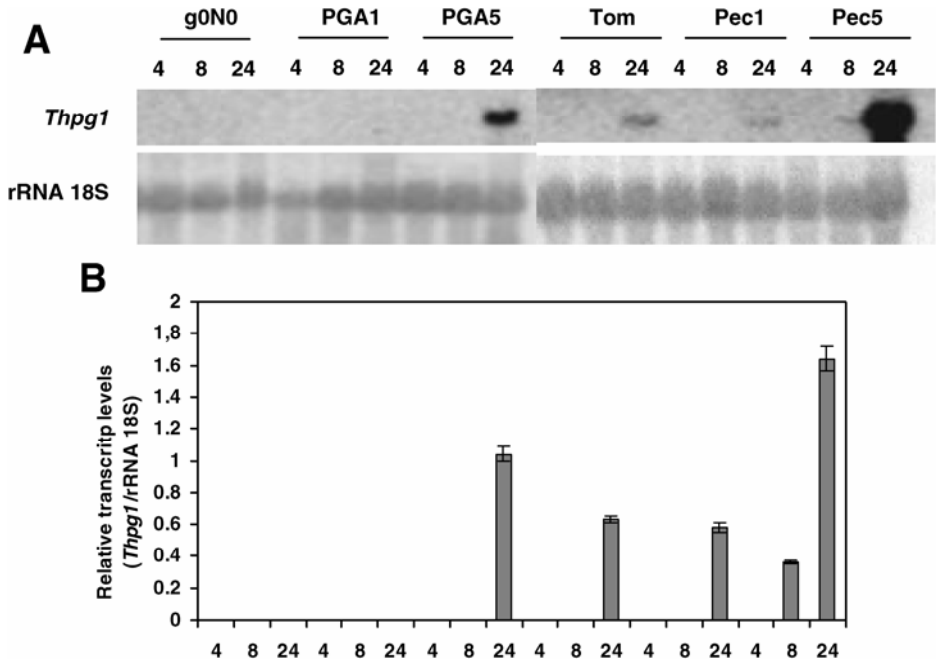

Fig. 3. In vitro expression analysis of *Thpg1* gene of *Trichoderma harzianum* T34. **A,** Northern blot experiments were carried out with total RNA (20 μg) extracted from mycelia of *T. harzianum* T34 grown on minimal medium (Penttilä et al. 1987) containing no glucose but one of the following carbon source: 0.1 or 0.5% polygalacturonic acid (PGA1 or PGA5), 0.1 or 0.5% pectin (Pec1 or Pec5), or 1% tomato plants (Tom). A glucose and nitrogen starvation condition (g0N0) served as the control. The *Thpg1* open reading frame and 18S rRNA gene from *T. harzianum* T34 were used as probes. **B,** Relative transcript levels of *Thpg1* gene versus rRNA 18S.

PGA as substrate, are shown in Figure 5A. All four *T. harzianum* transformants presented values of PG activity lower than the wild-type T34 strain. Particularly, ePG5 exhibited a decrease of 87.0% in the PG activity relative to T34. The PG activity of ePG4, ePG6, and ePG8 was reduced, compared with the wild type, 47, 83, and 68%, respectively.

We compared the expression level of *Thpg1* in three of the *Thpg1*-silenced transformants (ePG5, ePG6, and ePG8) and the wild-type strain by amplifying a fragment of 425 bp not used for the silencing using primers endop-1 and endop-5. Total RNA from *T. harzianum* T34 and transformants was extracted from 24-h-old mycelia grown on MM amended with 0.5% pectin. Compared with the wild-type strain (ratio 1), the ratio values were lower in ePG5, ePG6, and ePG8 (0.006, 0.010, and 0.071, respectively). Therefore, *Thpg1* expression was reduced by 167-, 100-, and 14-fold, respectively, for the three silenced transformants.

In order to investigate the role of *Thpg1* on pectin assimilation in *T. harzianum*, silenced mutant ePG5 and T34 wild types were grown on potato dextrose agar (PDA) and MM medium (control conditions) and MM medium supplemented with 0.5% pectin (Fig. 5B).

The two control treatments produced comparable colony sizes, and strain ePG5 showed a statistically significant reduced growth on the pectin-containing medium compared with the wild type.

The role of *Thpg1* in plant rhizosphere colonization was evaluated on tomato roots by real-time PCR using the silenced transformant ePG5 and *T. harzianum* T34. The amount of ePG5 DNA obtained from tomato roots was approximately eightfold lower than that of the wild type (Table 2).

The plant response to *Thpg1* was analyzed in the *T. harzianum–A. thaliana* interaction using *Arabidopsis* microarrays. The comparison of the plant T34-treated versus the plant ePG5-treated transcriptomes showed that just 10 *Arabidopsis* genes, with a false discovery rate (FDR) of 0.33, had a reduced expression level, between 11.9- and 1.8-fold, in the presence of the silenced transformant (Table 3). They are i) *GRP19* and *GRP20*, which encode CW structural proteins and may help to repair the CW (Mousavi and Hotta 2005); ii) *XTR7* and *XTH9*, which hydrolize plant xyloglucans and have been involved in *Medicago trunculata* root colonization by *Glomus versiforme* (Maldonado-Mendoza et al. 2005); iii) *GRP* and *XTH,* which were upregulated in *Pinus* microarrays after mycorrhizal colonization (Heller et al. 2008); iv) chalcone synthase (CHS), which was triggered by OGA and has been related to defense response (Shaw et al. 2006); v) a lipid transfer protein (LTP) gene belonging to a family involved in plant cell physiology and defense (Carvalho and Moreira 2007); and vi) a cysteine proteinase inhibitor gene elicited by CW oligomers and involved in defensive responses against insects and pathogens (Belenghi et al. 2003; Hermosa et al. 2006). The *GRP19*, *ATA20, XTR7*, and *CHS* gene expression was validated by Northern blot (data not shown). For all these *Arabidopsis* genes, a lower transcript level was observed 24 h after inocula-

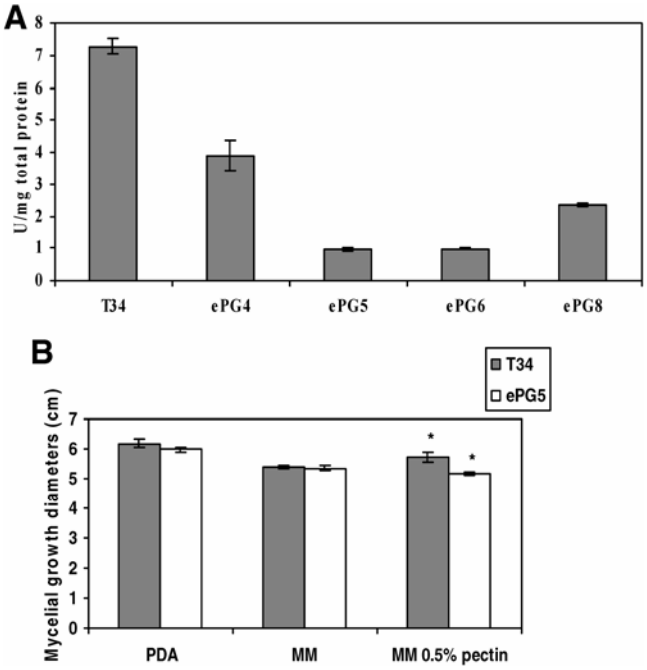

Fig. 5. Analysis of the *Trichoderma harzianum* T34 *Thpg1*-silenced transformants. **A,** Polygalacturonase (PG) activity measured in supernatants from *T. harzianum* T34 and silenced transformants ePG4, ePG5, ePG6, and ePG8 after 24 h of growth in minimal medium (MM) supplemented with 0.5% pectin. The release of reducing ends from polygalacturonic acid was measured, and results expressed as specific activity (U/mg of total protein). **B,** Colony diameter (cm) of *T. harzianum* T34 and the silenced strain ePG5 on potato dextrose agar (PDA), solid MM (Penttilä et al. 1987), and solid MM supplemented with 0.5% pectin. Values are means of three replicates with the corresponding standard deviations. Bars marked with asterisk differ significantly (Fisher's test).

Table 3. Differential expression of 10 *Arabidopsis* genes during the interaction with the wild-type *Trichoderma harzianum* strain T34 and the endopolygalacturonase-silenced mutant ePG5

Gene name and description	Probe set ID	Ratio[a]
Glycine-rich protein, *GRP19*	250610_at	11,929
Anther development protein, *ATA20*	258392_at	6,656
Lipid transfer protein, *LTP*	256381_at	8,400
Xyloglucosyl transferase, *XTR7*	245325_at	3,747
60S ribosomal protein, *RPP2C*	256597_at	2,877
Glycine-rich protein, *GRP20*	250639_at	2,386
Cysteine proteinase inhibitor B	265672_at	4,089
Chalcone synthase, *CHS*	250207_at	1,779
Xyloglucan endotransglucosylase, *XTH9*	255433_at	2,674
Extracellular lipase 6, *EXL6*	262675_at	2,892

[a] Indicated ratio value represents the fold change in gene expression during the interaction with T34 compared with ePG5 24 h after inoculation with fungi.

Table 2. Colonization of tomato roots by *Trichoderma harzianum* wild-type T34 and the *Thpg1*-silenced transformant ePG5[a]

	β-Tubulin				Chalcone synthase				
Condition	Ct	SD	Qty[b]	SD	Ct	SD	Qty[c]	SD	Ratio[d]
Tom-T34	22.39	0.164	9.71	1.119	25.79	0.174	10.73	1.274	0.905
Tom-ePG5	24.49	0.072	2.27	0.114	24.89	0.101	20.27	1.484	0.112

[a] Fungal DNA present on the tomato roots (Tom) 24 h after the inoculation was quantified by real-time polymerase chain reaction. Three replicates were made of each sample. Ct = threshold cycle and SD = standard deviation.
[b] Quantity of fungal DNA (ng) referred to *β-tubulin* gene in 1 µl of total DNA.
[c] Quantity of plant DNA (ng) referred to *chalcone synthase* gene in 1 µl of total DNA.
[d] Proportion of fungal DNA versus plant DNA per 1 µl of total DNA.

tion with the silenced strain ePG5 compared with strain T34, which was consistent with the microarray data.

Biocontrol assay in tomato.

Results obtained in *B. cinerea*-infected tomato assays with strains T34 and ePG5 showed that *Thpg1* silencing did not affect the biocontrol activity of *T. harzianum* (Fig. 6; Supplementary Fig. S4). Severity of leaf necrotic lesions (NSL) produced by *B. cinerea* B05.10 was measured in tomato plants previously seed-coated with an aqueous solution (control) or treated with conidia of T34 or ePG5. The highest sizes of necrotic lesions and tissue maceration were observed in the control and no statistically significant differences were observed among the necrotic lesion sizes observed in the T34 and ePG5 treatments. However, leaf necrotic lesions caused by *B. cinerea* were slightly smaller in plants pretreated with ePG5.

DISCUSSION

The present study provides a contribution to the understanding of the complex in vivo interaction occurring in the plant rhizosphere between a mycoparasitic biocontrol agent and its hosts. It reports the isolation and characterization, by proteomic, genomic, and transcriptomic methods, of a novel *Trichoderma* gene coding for a plant CW-degrading enzyme.

Trichoderma proteome changes during antagonist–plant–pathogen cross-talk were investigated. Several proteins differentially regulated by the presence of the pathogen in the three-way combination and that could be used by *T. harzianum* to colonize the roots—for instance, by partially degrading the plant CW (i.e., glucanases, proteases, cellulases, and so on)—were found (data not shown). Spot 0303, markedly increased in the presence of the soilborne pathogens *R. solani* and *Pythium ultimum*, was related to an EST clone (10663) as found by using a modified Mascot analysis (Suárez et al. 2005) and a database of *Trichoderma* EST constructed during the TrichoEST project (Rey et al. 2004), and showed a high identity with endoPG. Precisely, this clone was obtained from the unique L15 library constructed after exposing the fungus to plant CW polymers.

The endo- and exoPG NJ tree separation was in agreement with what was reported by other authors (Götesson et al. 2002). All other fungal endoPG genes, including those from *Aspergillus, Sclerotinia*, and *Fusarium* spp., have displayed high variability in intron number and sequence size (Annis and Goodwin 1997). Instead, the *Trichoderma* endoPG genes seemed to cluster in only two independent subclades made of members with relatively common features. The one that contained *Thpg1* included genes all having four introns and a size of approximately 1,300 bp while the other, which included endoPG of *T. virens* and *T. atroviride*, consisted of genes with one intron and of approximately 1,100 bp in size. These data suggest the presence of different endo- and exoPG families also in *Trichoderma* spp.

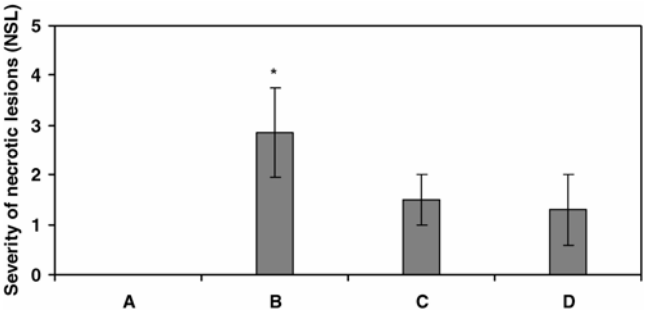

Fig. 6. Severity of necrotic lesions (NSL) observed in tomato leaves after *Trichoderma harzianum* seed treatment and *Botrytis cinerea* B05.10 conidia infection. Untreated and uninfected control (A), untreated seed and *B. cinerea*-infected leaves (B), *T. harzianum* T34-treated seed and *B. cinerea*-infected leaves (C), and *T. harzianum* ePG5-treated seed and *B. cinerea*-infected leaves (D). NSL data were registered 4 days after *B. cinerea* infection. The standard deviation bars were obtained from at least two different experiments, with eight replicates of infection spots. Asterisk represents statistically significant differences (Fisher's test).

An in silico analysis of 812 bp of the promoter region of *Thpg1* showed putative motifs related to development control (Adrianopoulos and Timberlake 1994), saprophytic growth (Herbert et al. 2002), pectinolytic gene activation (de Vries et al. 2002), carbon metabolism (Zeilinger et al. 2001), and mycoparasitism (Cortes et al. 1998). Results obtained after analyzing the *Thpg1* transcript levels in *T. harzianum* T34 under different in vitro growth conditions show that plant material and polymers trigger expression of this gene. Recently, D'Ovidio and coworkers (2004a) indicated that regulation of endoPG genes is generally conditioned by the available carbon source. We observed the same phenomenon in *T. harzianum*, where *Thpg1* expression could be increased by adding more pectin or PGA in the medium (from 0.1 to 0.5%) (Fig. 3A). Except for 0.5% pectin, expression data are not very consistent with PG activity. The delay between the timing of transcript expression and PG secretion as well as the contribution of others PG isoforms to total PG activity may also be taken into account. The possible involvement of *Thpg1* in mycoparasitism is unclear. A mycoparasitism-related motif (MYC) was observed in the promoter but no upregulation of the gene was detected by Northern analysis of mycelia grown in the presence of 1% *B. cinerea*, *P. ultimum*, or *R. solani* CW (data not shown).

It has been demonstrated that the presence or absence and location of some specific amino acids in the sequence of fungal endoPG is critical to PGIP binding (Raiola et al. 2008). Similarly to endoPG from *Fusarium moniliforme* and *Aspergillus niger* (De Lorenzo and Ferrari 2002), the *T. harzianum* ThPG1 was unaffected by PGIP of *Arabidopsis thaliana* and inhibited by PvPGIP1 and PvPGIP2 of *Phaseolus vulgaris*. However, a much higher amount of PvPGIP2 (130 ng) was needed to inhibit the activity of the biocontrol agent ThPG1 compared with endoPG from phytopathogenic fungi such as *Aspergillus niger*, *F. moniliforme*, *Colletotrichum acutatum*, or *B. cinerea* (D'Ovidio et al. 2004b). This is in agreement with the hypothesis that there is a relatively high degree of specificity in endoPG-PGIP interactions (De Lorenzo and Ferrari 2002; Kemp et al. 2004).

Northern and proteomic results showed that in vitro *Thpg1* expression and in vivo ThPG1 intracellular production were low during the two-player interaction (T34–tomato). However, a marked increase of ThPG1 amount was detected in the proteomes from the in vivo three-player interactions T34–tomato–*R. solani* and T34–tomato–*Pythium ultimum*. This result was subsequently confirmed by real-time PCR (Fig. 4), which demonstrated that the presence of a pathogen in the system triggers the expression of *Thpg1*. In order to analyze whether the presence of oligomers released by the pathogens could trigger a higher expression of *Thpg1* gene in a three-component system, strain T34 was grown in tomato–*R. solani* co-culture filtrates. Although different incubation times (24 and 48 h) and filtrates of different ages (48 h, 72 h, and 1 week) and concentrations (10- and 18-fold) were assayed, no *Thpg1* expression was detected. Probably, the amount of oligomers present in the filtrates tested was unable to trigger *Thpg1* gene expression. Eventually, we were not able to reproduce in vitro the many factors that regulate the multiplayer systems that we are investigating.

The gene-silencing strategy used in this work was successfully applied in *Trichoderma* spp. to analyze the function of

the terpene-related genes *erg1* and *hmgR* (Cardoza et al. 2006, 2007). We found no correlation between the number of pSILPG1 copies inserted and the level of PG activity in the best *Thpg1*-inducing conditions (24 h culturing on MM plus 0.5% pectin). In fact, the lowest PG activity reduction compared with the wild type occurred in those transformants, ePG4 and ePG8, with the highest number of transformation-cassette-inserted copies. The same lack of correlation was also reported when genes encoding chitinase and heat shock protein were overexpressed in *Trichoderma* spp. (Limón et al. 1999; Montero-Barrientos et al. 2007). Regardless, we confirmed by real-time PCR that the reduction of *Thpg1* expression and PG activity were related, and selected for the phenotype study a strongly silenced mutant (minus 167-fold *Thpg1* expression and a residual enzymatic activity of only 13%).

The mutant ePG5 generally showed a growth rate similar to that of the wild type on MM and PDA media, and was still able to use pectin as the only carbon source. This was probably due to the residual ThPG1 activity derived from the incomplete silencing of the gene or the presence of other endo- and exoPG in strain T34. A correlation between PG activity and growth reduction in pectin-based media was reported in double endo- and exoPG *Cochliobolus carbonum* mutants (Scott-Craig et al. 1998), and in an endo-PG mutant of *Alternaria citri* (Isshiki et al. 2001). On the contrary, an endo-PG mutant of *C. carbonum* with a 30% reduction in PG activity grew as well as the wild type in the presence of pectin as the sole carbon source (Scott-Craig et al. 1990).

A comparison of tomato root colonization by strains T34 and ePG5 showed that *Thpg1* is involved in this process. In fact, the amount of ePG5 DNA obtained from tomato roots was eightfold less than that of the wild type. This result, which is in agreement with the data from growth assays on pectin, indicates that ThPG1 supports rhizosphere colonization by the biocontrol fungus and pectin degradation may be an important step in the beneficial *Trichoderma*–plant interaction. Accordingly, endoPG disruptants of *A. citri* showed a reduced colonization ability of citrus fruit tissues (Isshiki et al. 2003).

Efficient root colonization is considered important for the well-known *Trichoderma* spp. beneficial effect on the plant (Brotman et al. 2008; Harman et al. 2004; Yedidia et al. 1999). Several authors have reported that OGA generated by PG enzyme activity can elicit plant defense responses (Casasoli et al. 2008; Poinssot et al. 2003). Using *Arabidopsis* microarrays, we have investigated the possibility that *Thpg1* may be involved in the often observed ability of *T. harzianum*, as well as other *Trichoderma* spp., to activate or prime plant defense mechanisms (Djonovic et al. 2007; Harman et al. 2004; van Wees et al. 2008). This method has permitted an in-depth investigation of rhizobacteria-ISR (Verhagen et al. 2004). We found only 10 plant genes related to CW metabolism and plant defense, which were downregulated in the presence of ePG5 relative to T34. Of these 10 genes, six (*GRP19*, *GRP20*, *ATA20*, *XTR7*, *XTH9*, and *EXL6*) encode proteins involved in CW construction or degradation and in plant defense responses. Others, such as *CHS*, *LTP*, or a protease inhibitor gene, are involved in plant defense. The downregulation of ISR-linked genes observed in *Arabidopsis* roots colonized with the *Thpg1*-silenced transformant could anticipate a possible reduction in the protection against plant pathogens. However, in vivo biocontrol assays showed that *B. cinerea* leaf necrotic lesions were slightly smaller in plants colonized by ePG5, although no statistically significant differences were observed between T34 and ePG5 treatments.

The data set presented in this article supports the hypothesis that activity of ThPG1 on the plant CW is required for an efficient root colonization by *T. harzianum*. However, further in vivo assays are needed to understand the *Thpg1* role in the biocontrol effect mediated by the activation of the plant ISR mechanism in other pathosystems.

MATERIALS AND METHODS

Fungal and plant material.

T. harzianum T34 (CECT 2413; Spanish Type Culture Collection, Valencia, Spain), *T. atroviride* T11 (NBT11; Newbiotechnic S.A., Seville, Spain), *T. harzianum* T22, *T. asperellum* T25 (NBT25), *T. longibrachiatum* T52 (NBT52), and *T. virens* T59 (NBT59) were used in this study. Strain 26 of *B. cinerea*, isolated from tobacco, and strain 19 of *R. solani* and strain 8 of *P. ultimum*, isolated from tomato, were used as sources of fungal CW. Strain B05.10 of *B. cinerea* was used as a pathogenic fungus in biocontrol assays in tomato. All microorganisms were maintained on PDA (Difco Becton Dickinson, Sparks, MD, U.S.A.).

Arabidopsis thaliana Col-0 ecotype and *Lycopersicon esculentum* cv. Marmande plants were used. Sterilized seed were sowed in tubes containing Murashige and Skoog medium (Murashige and Skoog 1962) supplemented with 1% (wt/vol) sucrose and 1% (wt/vol) agar, pH 5.7. They were placed in a growth chamber at 22°C and 40% humidity with a light-and-dark photoperiod of 16 and h, respectively, at 80 to 100 μE m^{-2} s^{-1}.

Culture conditions.

Trichoderma–plant–pathogen interaction was simulated on WA plates as previously described (Marra et al. 2006) by using 15-day-old tomato plants and separating the three components (plant, *Trichoderma* spp., and pathogen) with a sterile cellophane membrane.

For Southern analysis, *Trichoderma* strains were cultivated in shake flasks (150 rpm) in potato dextrose broth (PDB, Difco Becton Dickinson) at 25°C for 48 h.

For in vitro expression studies, *T. harzianum* T34 mycelia were obtained following a two-step liquid culture procedure (Vizcaíno et al. 2006). First, the fungus was grown in MM (Penttilä et al. 1987) containing 2% glucose at 28°C for 36 h. The fungal biomass was harvested and transferred to MM containing no glucose but one of the following carbon sources: 0.1 or 0.5% PGA, 0.1 or 0.5% pectin, or 1% tomato plants. A glucose and nitrogen starvation (g0N0) served as the control. Cultures were maintained at 25°C on a rotary shaker at 150 rpm for 4, 8, or 24 h. Nitrogen starvation conditions included a 100-fold decrease in the concentration of ammonium sulfate in the medium (50 mg/liter). Tomato plant material was obtained by removing the roots of 8-week-old plants and subsequently freezing and powdering the aerial part.

For T34 expression studies in tomato–*R. solani* co-culture filtrates, mycelium of T34 previously obtained from a liquid PDB culture was incubated for 24 and 48 h in *R. solani*–tomato co-culture filtrates. For this, MM supplemented with 2% of 15-day-old tomato plantlets was inoculated with 1% *R. solani* mycelium and cultured at 25°C and 120 rpm for 12 days. Supernatants were harvested by filtration and concentrated 10- and 18-fold using a speed-vacuum system.

For in vivo expression studies, *T. harzianum* T34 mycelia were obtained in the same growth conditions used to simulate the *Trichoderma*–plant–pathogen interaction described by Marra and associates (2006).

To perform tomato rhizosphere colonization tests, 20-day-old plants were cultured in a 10-ml Erlenmeyer flask containing 8 ml of liquid Murashige and Skoog medium inoculated with 10^5 conidial germlings ml^{-1} of *Trichoderma* strain (T34 or ePG5) or 100 μl of sterile water (control) in an orbital incubator at 80 rpm and 25°C. Conidia were obtained from 15-h-old

cultures of the strains T34 or ePG5 in PDB (Difco Becton Dickinson), and left to germinate at 25°C in 200 ml of PDB medium shaken at 200 rpm. After 24 h of plant–*Trichoderma* interaction, roots from six plants per treatment were collected, washed with distilled water, frozen, lyophilized, and kept at –80°C until total RNA extraction. Three independent tomato–*Trichoderma* co-culture experiments were carried out.

For the microarray-based experiments, 25-day-old *Arabidopsis* plants were cultured in the conditions described above for the colonization assays. After 24 h, samples from the aerial part of 10 *Trichoderma*-colonized plants per each treatment were collected, combined, treated as described above, and used for RNA extraction. Three technical replicates were performed per each treatment.

Cell proteins extraction.

The protein extraction protocol applied in this work was the one described by Jacobs and associates (2001) with the modifications indicated by Marra and associates (2006).

Isoelectric focusing and 2-DE.

Isoelectric focusing (IEF) was carried out in a PROTEAN IEF Cell system (Bio-Rad Laboratories Inc., Hercules, CA, U.S.A.) by using 11-cm immobilized-pH-gradient (IPG) strips (Bio-Rad Laboratories Inc.) with a pH of 3 to 10 (intracellular proteins). IPG, 2-DE, and staining procedure were performed as described by Marra and associates (2006).

Gel images were acquired by a GS-800 Imaging Densitometer (Bio-Rad Laboratories Inc.) and analyzed with the PD-Quest software. Image files were recorded by using a red filter (wavelength 595 to 750 nm) and a resolution of 36.3 by 36.3 microns. The signal intensity of each spot was determined in pixel units (optical density) from four different two-dimensional gels and normalized to the sum of the intensities of all the spots included in the standard gel. Protein spots were considered to be "differentially produced" if at least a twofold intensity variation was detected when responses to different interaction conditions were compared.

In-gel digestion, matrix-assisted laser desorption-ionization time-of-flight MS, and in silico analysis.

Protein spots were excised from gels and digested with bovine trypsin as described by Talamo and associates (2003). Each spot was indicated with the standard spot number assigned by PD-Quest software. Tryptic-digested peptides were resuspended in 10 µl of a 1% acetic acid solution and the samples were mixed (1:1) with a matrix of a saturated α-cyano-4-hydroxycinnaminic acid solution (acetonitrile at 10 mg/ml/0.2% trifluoroacetic acid, 70:30] (Sigma-Aldrich, St. Louis). Then, 1 µl of the mixture was matrix-assisted laser desorption-ionization time-of-flight analyzed in a mass spectrometer (Applied Biosystem, Foster City, CA, U.S.A.) according to the protocol described by Marra and associates (2006).

Peptide mass fingerprint data were matched to the National Centre of Biotechnology Information nonredundant database entries against proteins from fungal or all species, using the Mascot software (Matrix Science, London). A modified Mascot analysis was also performed by using a *Trichoderma* EST clones database, built as described by Vizcaíno and associates (2006, 2007), supported by the TrichoEST European Union project (Rey et al. 2004).

Libraries of *T. harzianum* T34.

L01 and L15 *T. harzianum* T34 libraries were used for cloning the *Thpg1* gene in this study. L01 is a lambda genomic library and was previously described (Lora et al. 1995). L15 is a cDNA library, constructed with RNA obtained after growing *T. harzianum* T34 in MM (Penttilä et al. 1987) without glucose but supplemented with 0.1% PGA, 0.1% CMC, 0.1% pectin, and 0.1% xylan (all from Sigma-Aldrich) for 36 h at 28°C and 160 rpm. The mycelium was harvested, total RNA was extracted, mRNA was purified, and the L15 library was constructed in Uni-ZP XR Vector System (Stratagene, La Jolla, CA, U.S.A.) (Vizcaíno et al. 2006).

Molecular cloning.

Standard molecular techniques were used throughout this work (Sambrook and Russel 2001). The L01 library was screened with a 510-bp PCR fragment, corresponding to the EST 10663, which was labeled by random primers (Roche Applied Science, Mannheim, Germany). Labeling, hybridization, and immunological detection were carried out using a nonradioactive labeling and immunological detection kit and CDP-Star as the chemiluminescent substrate (Roche Applied Science), as previously described (Hermosa et al. 2000). The DNA of a positive phage was isolated and sequenced to obtain the complete *Thpg1* gene.

PCR procedures.

The complete cDNA of *Thpg1* was obtained by PCR using the primers endop-1 (5′-CAT GAC CAA ACT ATC CCT TCT C-3′) and endop-2 (5′-CTT AAC AAG TAG CAA CAC TTG G-3′) and DNA from phages of L15 cDNA library as template. PCR was carried out for 35 cycles of 1 min at 94°C, 1.5 min at 55°C, and 2 min at 72°C, with a *Taq* polymerase system (Biotools Labs, Madrid) following the manufacturer's instructions. The screening of the silenced transformants was carried out with the primers IntF (5′-GTG CTA ATC GTG TTA TGC ACA G-3′) and Tcbh2 (5′-GAG CTC AAC CCA AAG GAG GG-3′). The PCR conditions were the same described above except that the annealing temperature was 57°C.

Southern and Northern blot analysis.

Total DNAs from fungi were extracted following the method of Raeder and Broda (1985). RNA extraction was carried out using the TRIZOL reagent (Invitrogen, Carlsbad, CA, U.S.A.) according to the manufacturer's instructions.

For Southern analysis, 7 µg of genomic DNA was digested with *Bam*HI, *Eco*RI, or *Xho*I restriction enzymes, electrophoresed on 0.7% agarose gels, and transferred to a Hybond-N^+ membrane (Amersham Biosciences AB, Uppsala, Sweden). For Northern blot analyses, 20 µg of total RNA was separated on a 1.2% formaldehyde-agarose gel and transferred to a Hybond-N^+ membrane. For both hybridizations, the *Thpg1* cDNA was labeled with ^{32}P and used as a probe. Hybridizations were carried out at 65°C for 16 h. Membranes were washed under high-stringency conditions.

Construction of pSIL-PG1.

Plasmid pSIL (Cardoza et al. 2006) was linearized with *Spe*I-*Bam*HI and then ligated to a 440-bp *Spe*I-*Bam*HI fragment of the *Thpg1* gene. This fragment was in "sense" relative to the orientation of the *tss1* promoter. This construct was later linearized with *Hin*dIII-*Xho*I and then ligated to the same 440-bp *Thpg1* fragment but in an "antisense" orientation relative to the *tss1* promoter. An intron of 159 bp was introduced between both sense and antisense fragments of the *Thpg1* gene. The resulting plasmid also contained the terminator region of the *cbh*2 gene from *T. reesei* and was designated pSIL-PG0. In order to transform this cassette in *T. harzianum* T34, pSIL-PG0 was digested with *Sac*I to isolate the SIL-*Thpg1* fragment, which was then ligated to the same restriction site of the plasmid pJL43b1 (S. Gutiérrez and Martín, *unpublished data*), giving rise to the final construct

pSIL-PG1 (7.46 kb). This was used to transform *T. harzianum* T34 by a protoplast-based method described elsewhere (Cardoza et al. 2006).

Enzyme assays.

Supernatants were dialyzed against milliQ water. PG activity was determined in a colorimetric assay by measuring the release of reducing groups during PG-catalyzed hydrolysis of PGA (Nelson 1957; Somogyi 1952). The reaction mixture (0.25 ml), containing 0.2% PGA in 50 mM acetic acid-acetate buffer, pH 5.5, and 10 μg of proteins from culture supernatant, was incubated at 37°C for 30 min. One unit of enzymatic activity (U) was defined as 1 μmol of galacturonic acid released per minute. Quantitative protein determination was carried out by using the Bradford assay (Bradford 1976). Tests were performed in triplicate and data represent mean values with standard deviation.

The inhibitor activity of different PGIP against the ThPG1 protein was evaluated in the Felice Cervone's laboratory at Universitá di Roma La Sapienza (Rome) following the method described by D'Ovidio and associates (2004b).

Real-time PCR analysis.

Highly purified, salt-free primers for *Thpg1* (endop-1, described above; endop-5, 5′-CTT TGG CTT CGT CTT GCC ACC-3′), β-actin (act-1, 5′-ATC GGT ATG GGT CAG AAG GA-3′; act2, 5′-ATG TCA ACA CGA GCA ATG G-3′), *CHS* (CS-f, 5′-TTC GGT TAA GCG GCT CAT GA-3′; CS-r, 5′-CTC GAG CAC CCT TGT TGT TCT C-3′), and β-tubulin (tub-f, 5′-TTC TTG CAT TGG TAC ACT AGC G-3′; tub-r, 5′-ATC GTT CAT GTT GGA CTC AGC C-3′) genes were used and they produced single amplicons of 425, 407, 100, and 71 bp, respectively. PCR conditions were as previously described (Cardoza et al. 2007), except that the annealing temperatures were as follows: *Thpg1*, 59°C; β-actin, 55°C; *CHS*, 60°C; and β-tubulin, 60°C.

Thpg1 expression was comparatively analyzed in the in vivo T34–tomato–*P. ultimum* and T34–tomato systems as well as in T34 and the silenced transformants by a semiquantitative real-time method described by Cardoza and associates (2007). The relative expression ratio of *Thpg1* was expressed in comparison with the reference gene (β-actin), according to the equation reported by Pfaffl (2001).

Quantification of wild-type T34 and the ePG5-silenced transformant DNA from the colonized tomato roots was performed using the Stratagene's Brilliant SYBR Green QPCR master mix (Stratagene). The mix was prepared in a 25-μl volume by using the following ingredients: 12.5 μl of 2× master mix, 0.25 μl of forward primer (100 nM), 0.25 μl of reverse primer (100 nM), 0.375 μl of diluted reference dye (300 nM), 50 ng of DNA, and nuclease-free PCR-grade water to adjust the final volume. DNA was extracted from roots 24 h after inoculation. Amplifications were carried out in the ABI PRISM 7700 (Applied Biosystems, Foster City, CA, U.S.A.) 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, followed by 1 min at 60°C. Cycle threshold values were calculated and the amount of fungal DNA was estimated using standard curves. Values were normalized to the amount of tomato DNA in the samples. Each sample was tested in triplicate.

Microarray analysis.

Total RNA from the aerial part of an *Arabidopsis* plant was obtained using the RNeasy plant mini kit (Qiagen, Hilden, Germany). The integrity and pureness of the RNA were determined using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, U.S.A.) and cDNA was synthetized with Superscript Choice System for cDNA (Invitrogen). Subsequently, labeled cRNA was obtained from cDNA with an IVT kit (Affymetrix, Santa Clara, CA, U.S.A.) and was used as a probe to hybridize an Affymetrix GeneChip *Arabidopsis* ATH1 Genome Array, which represents approximately 24,000 genes (Affymetrix). Hybridization and washing of the chips were performed with the procedure described in the GeneChip System Manual of Affymetrix in the Genomic and Proteomic Unit of the University of Salamanca (Salamanca, Spain). Three chips were used per each condition tested.

Biocontrol assays in vivo.

In vivo tests of the biocontrol ability of *T. harzianum* T34 or the ePG5-silenced transformant were carried out as previously described by Hermosa and associates (2006). *B. cinerea* B05.10 conidia were used to infect 4-week-old tomato leaves. Previously, sterilized tomato seed were coated with water (control) or an aqueous suspension containing of the T34 strain or ePG5-silenced transformant at 10^8 conidia/ml. In all, 1 ml of spore suspension served to coat 15 mg of seed that were air dried in an open petri dish overnight in a laminar flow hood.

Two leaves from each plant were inoculated in two points: one leaf with 5 μl of a germination solution (20 mM glucose and 20 mM potassium phosphate) containing *B. cinerea* at 2×10^8 conidia/ml and the other with germination solution alone. Eight plants, one leaf per plant and two inoculation points per leaf, were considered for every condition. Appearance of necrotic spots was assessed 4 days after inoculation. Values of 0 (absence of reaction) to 4 (high necrotic reaction more than 1 cm diameter) were used to indicate the NSL. Data are presented as mean values ± standard deviation of three experiments.

Bioinformatics programs and sequence analysis.

DNA-binding elements were found by looking for consensus sequences described elsewhere or by the MatInspector program on the Genomatix web server. Prediction of the signal peptide cleavage site was searched by the SignalIP 3.0 program, available online from the Swiss Institute of Bioinformatics. Homology analysis and consensus domain search were performed with the Blast program, available online from the National Center for Biotechnology Information. For phylogenetic analysis, 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.

For microarray analysis, the digitalization of the emitted fluorescent signals after the hybridization was performed with the Gene Array reader (Affymetrix) and the Desktop Mining Solution system (Micro DB 3.0, Data Mining Tool 3.0). This program allows statistical analysis and the identification of the induced and repressed genes in each experimental condition. Background correction, normalization, and expression analysis from the data were performed using the RMA algorithm (Irizarry et al. 2003). The significance of the differential expression in the two conditions compared was determined by statistical analysis performed with SAM (Tusher et al. 2001) and FDR (Benjamini and Hochberg 1995) algorithms, which indicated the proportion of false gene expression data. Microarray data processing was carried out in the Bioinformatic Unit of the University of Salamanca.

ACKNOWLEDGMENTS

This work has been funded by the Spanish Ministry of Education and Science (AGL2008-0512/AGR), Junta de Castilla y León (research group

GR67), and European Union (project QLK3-CT-2002-02032). We thank F. Cervone and G. De Lorenzo (Universitá di Roma La Sapienza, Rome) for their support in the PGIP evaluations.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

Joint Genome Institute genome *Trichoderma* genome portal: genome.jgi-psf.org/
Matrix Science Mascot software: www.matrix-science.com