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Signal Transduction by Tga3, a Novel G Protein α Subunit of *Trichoderma atroviride*

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Trichoderma **species are used commercially as biocontrol agents against a number of phytopathogenic fungi due to their mycoparasitic characterisitics. The mycoparasitic response is induced when *Trichoderma* specifically recognizes the presence of the host fungus and transduces the host-derived signals to their respective regulatory targets. We made deletion mutants of the *tga3* gene of *Trichoderma atroviride*, which encodes a novel G protein α subunit that belongs to subgroup III of fungal Gα proteins. Δ*tga3* mutants had changes in vegetative growth, conidiation, and conidial germination and reduced intracellular cyclic AMP levels. These mutants were avirulent in direct confrontation assays with *Rhizoctonia solani* or *Botrytis cinerea*, and mycoparasitism-related infection structures were not formed. When induced with colloidal chitin or *N*-acetylglucosamine in liquid culture, the mutants had reduced extracellular chitinase activity even though the chitinase-encoding genes *ech42* and *nag1* were transcribed at a significantly higher rate than they were in the wild type. Addition of exogenous cyclic AMP did not suppress the altered phenotype or restore mycoparasitic overgrowth, although it did restore the ability to produce the infection structures. Thus, *T. atroviride* Tga3 has a general role in vegetative growth and can alter mycoparasitism-related characteristics, such as infection structure formation and chitinase gene expression.**

Several fungi belonging to the genus *Trichoderma* can act as mycoparasites and are used commercially as biological control agents against plant-pathogenic fungi, such as *Rhizoctonia solani*, *Botrytis cinerea*, *Sclerotium rolfsii*, *Sclerotinia sclerotiorum*, and *Pythium* spp. (5, 6, 18). Mycoparasitic strains can penetrate and kill the host fungi. Mycoparasitism is accompanied by the secretion of antifungal metabolites, such as peptaibol antibiotics (32, 47), and morphological changes, such as coiling around the host and development of hooks and/or appressorium-like structures (11). Hydrolytic enzymes, such as chitinases, glucanases, and proteases, also are important for biocontrol activity, since they enable *Trichoderma* to degrade the host's cell wall and to utilize its cellular contents (20). Chitinase gene expression is induced by colloidal chitin, fungal cell walls, or the chitin monomer *N*-acetylglucosamine (29). In *Trichoderma atroviride* the *N*-acetylglucosaminidase (NAGase)-encoding gene, *nag1*, has a major impact on the induction by chitin of other chitinases (4). In mycoparasitic interactions between *T. atroviride* and *R. solani*, expression of *ech42*, which encodes endochitinase 42, is triggered by a low-molecular-weight diffusible factor released from the host prior to physical contact between the two fungi (10, 50). Lectins in the host's cell wall can induce coiling of the mycoparasite around the host (1, 24, 41).

Elucidation of the signaling pathways underlying mycoparasitism has only recently begun. Heterotrimeric G proteins are composed of α, β, and γ subunits. Gα subunits play pivotal roles in the recognition process, virulence, and virulence-dependent development in a number of plant-pathogenic fungi (3, 15, 26, 27, 31). Fungal Gα subunits are highly conserved and can be divided into three major subgroups (3). In *T. atroviride*, the subgroup I Gα protein Tga1 affects light-induced conidiation and mycoparasitism-related coiling (41). The corresponding protein in *Trichoderma virens* is involved in antagonism against *S. rolfsii* but not in antagonism against *R. solani*. Δ*tgaA* loss-of-function mutants sporulate and have coiling behavior similar to that of the wild type (37), suggesting that the subgroup I Gα subunits have different functions in these mycoparasites. The *T. virens* subgroup II G protein α subunit, TgaB, has no specific role in development or mycoparasitism (37). Fungal subgroup III Gα proteins also regulate morphological and developmental processes, such as germination, conidiation, and secondary metabolite production (28, 53), and they may increase intracellular cyclic AMP (cAMP) levels by stimulating adenylyl cyclase (28, 33, 39).

The objectives of this study were to isolate a subgroup III Gα subunit deletion mutant of *T. atroviride* and to determine its role in development and mycoparasitism. This report is the first report of the role of the Tga3-mediated G protein pathway in mycoparasitism-related properties, such as host recognition, chitinase expression, and secretion.

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MATERIALS AND METHODS

Strains and culture conditions. *T. atroviride* strain P1 (formerly *Trichoderma harzianum* ATCC 74058) was used for this study and was grown on potato dextrose agar (PDA) (Difco, Detroit, Mich.) at 28°C until sporulation. *B. cinerea*

and *R. solani* were used as pathogens and were obtained from the collection of the Institute of Plant Pathology, Università degli Studi di Napoli Federico II, Naples, Italy. *Escherichia coli* JM 109 was the host for plasmid amplification.

T. atroviride was grown in liquid synthetic medium (SM) as described previously (4). For induction experiments, the fungus was precultivated for 36 h in SM containing 1% (wt/vol) glycerol as the carbon source, harvested by filtration, washed with sterile tap water, and transferred to fresh SM containing 1% (wt/vol) colloidal chitin or 1% (wt/vol) *N*-acetyl-β-D-glucosamine. To test the influence of exogenous cAMP (Sigma, St. Louis, Mo.), cAMP was added to PDA cooled to 48°C after autoclaving.

Cloning of *tga3*. Two products (~1,000 and ~1,100 bp) were obtained by PCR amplification of genomic DNA from *T. atroviride* strain P1 with degenerate oligonucleotide primers based on conserved regions of Gα sequences (primers GαF [5′-GGNGCNGGNGARWSNGGNAA-3′] and GaR [5′-GTRTCNGTNG CNYDNGT-3′]). The products were subcloned into pGEM-T (Promega, Madison, Wis.) and sequenced. For screening of a genomic λ BlueStar library of *T. atroviride* P1, the *tga3*-containing PCR fragment was radioactively labeled with ^{32}P by random priming and used as a probe. Sequencing of isolated clones was performed by using the following internal primers: Tga3intF1 (5′-CTGCTTCG AGAACGTTACCTC-3′; bp 881 to 901), Tga3intF2 (5′-CGATGGCATTTAC GCGAAG-3′; bp 1632 to 1650), Tga3intF3 (5′-GTATTAACTTGCTGCGCTC ATTG-3′; bp 2476 to 2498), Tga3intR1 (5′-GTATTCGCTCAGCGCAACAC-3′; bp 913 to 932), Tga3intR2 (5′-GACAATGGTCGACTTGCCAC-3′; bp 220 to 239), Tga3intR3 (5′-CAGGGCTGGCAATGATGAAAG-3′; bp −486 to −466), and Tga3intR4 (5′-CTGTGCCAGCCTAAAAGTGTGC-3′; bp −1287 to −1266).

Fungal transformation. *Agrobacterium*-mediated transformation of *T. atroviride* was carried out as previously described (51). Briefly, after cocultivation of a conidial suspension (10^7 spores ml^{-1}) for 24 h with an *Agrobacterium tumefaciens* strain containing the disruption construct pTSZ-Δtga3, in which the entire *tga3* coding region was replaced by an *hph* (hygromycin B phosphotransferase-encoding) expression cassette (51), fungal transformants were selected on PDA containing 200 μg of hygromycin B per ml. Transformants were recovered and purified to mitotic stability by repeated transfer to selective medium and by two rounds of single-spore isolation.

DNA and RNA manipulations. Genomic DNA was isolated as previously described (16); Southern hybridizations were carried out as described by Sambrook et al. (43). RNA was isolated as described by Chomczynski and Sacchi (7). Northern blotting of total RNA was performed by using BiodyneB nylon membranes (Pall Europe, Portsmouth, United Kingdom) and applying 20 μg of RNA. For hybridization, the radioactively labeled PCR product containing most of the *tga3* structural gene described above was used. For analysis of chitinase gene transcription, a 1.9-kb SalI/XbaI fragment of *nag1*, a 1.0-kb PstI fragment of *ech42*, and a 1.9-kb KpnI fragment of *act1* were used as probes.

Real-time reverse transcriptase PCR. RNAs were incubated with DNase I (1 U/μg of RNA; Fermentas, St. Leon-Rot, Germany) according to the manufacturer's instructions to remove any remaining chromosomal DNA. First-strand cDNA synthesis was carried out with an oligo$(dT)_{18}$ primer (0.5 μg/μl), a random hexamer primer (0.2 μg/μl), 0.45 μg of total RNA, and RevertAid H Minus Moloney murine leukemia virus reverse transcriptase (200 U/μl; Fermentas) according to the manufacturer's instructions. For amplification of an ~60-bp fragment of the actin-encoding gene *act1*, real-time PCR was carried out with primers act1F (5′-TATGTGCAAGGCCGGTTTCG-3′) and act1R (5′–GGTC TTCCGACAATGGAGG-3′). A 370-bp *nag1* fragment was amplified with primers nag1intF (5′-GCAAATGCGCTGTGGCCCATC-3′) and nag1intR (5′–GC TTGAAGGTGGTCGCGGTATC-3′).

Real-time PCR amplification was carried out with the iCycler real-time detection system (Bio-Rad, Hercules, Calif.) in a 50-μl mixture containing iQ SYBR Green Supermix (Bio-Rad), each primer at a concentration of 100 nM, 5 μl of sample, 10 nM fluorescein, and 1× SYBR Green. The *act1* fragment was amplified with the following PCR program: initial denaturation for 90 s at 95°C, followed by 50 cycles consisting of 95°C for 15 s, 57°C for 20 s, and 72°C for 20 s. *nag1* was amplified by 50 cycles of 95°C for 15 s, 61°C for 20 s, and 72°C for 20 s. Successful amplification was verified by determination of the melting temperature and by agarose gel electrophoresis. A nonamplification control and a control with RNA without reverse transcription to exclude DNA contaminants were performed in parallel for every assay. All determinations were performed three times with two different sample dilutions. The results were displayed in the PCR base line subtracted mode, where the software (Bio-Rad) determined the cycle number at which the reaction reached the threshold.

The efficiency (E) of the real-time PCR was calculated by amplification of two different dilutions of the samples by using the equation $E = 10^{1/tc}$ (12), and the threshold cycles (tc) were amended for an optimum efficiency of 2. To compare different samples, the threshold cycle of the analysis with the primers for *nag1* was corrected with a factor for the *act1* amplification. The sample of wild-type *T. atroviride* P1 harvested 10 h after replacement with glucose (Gluc/10h sample) was assigned the factor 1 because it had the highest threshold cycle. As 3.32 cycles theoretically gives a 10-fold increase in the amount of PCR product, the difference between the threshold cycle of the wild-type Gluc/10h sample and the corrected threshold cycle of the other samples was divided by 3.32, yielding X. Inducibility (I) was calculated by using the equation $I = 10^X$.

Protoplasting and determination of growth inhibition by SDS. Protoplasts were prepared from mycelia grown for 15 h on PDA containing cellophane (Maildor, Gournay en Bray, France) as previously described (16) with lysing enzymes from *T. harzianum* (Glucanex; Sigma). The inhibitory effect of sodium dodecyl sulfate (SDS) was determined by measuring the radial growth rates on plates containing PDA supplemented with 0.01 or 0.02% SDS and comparing these growth rates to the growth rates on plates containing PDA without SDS. Hyphal elongation was measured twice a day, and the growth rate was determined.

Biocontrol assays. Tests of inhibition of *B. cinerea* spore germination were performed as described previously (4). Inhibition by culture filtrates was adjusted for the biomass present in the culture. Plate confrontation assays with *R. solani* or *B. cinerea* as the host were performed as previously described (32, 50).

Enzyme assays. Chitinase activity was determined in culture filtrates, in culture broth including mycelia, and in intracellular samples. Mycelium-bound activity was determined with medium containing mycelium, which was removed by centrifugation at 13,000 × *g* at 22°C for 5 min before spectrophotometric analysis. For determination of intracellular enzyme activities, harvested mycelium was ground in liquid N_2 and suspended in 1.5 volumes of protease inhibitor cocktail containing 0.5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 μM pepstatin A, and 0.6 μM leupeptin hemisulfate (all obtained from Sigma). Cells were broken by sonication for 1 min at 4°C (cycle 30 with 40% power; Sonopuls Sonicator [Bandelin, Berlin, Germany]), and the extract was centrifuged for 20 min at 20,000 × *g* at 4°C. The supernatant was collected and stored at −20°C after addition of 10% glycerol.

Enzyme assays were performed as previously described (17) with *p*-nitrophenyl *N*-acetyl-β-D-glucosaminide as the substrate for determination of *N*-acetylglucosaminidase activity and with *p*-nitrophenyl β-D-*N*′,*N*″-triacetylchitotriose as the substrate for determination of endochitinase activity (all chemicals were obtained from Sigma). Enzyme activity was related to biomass by determination of the mycelial dry weight.

Measurement of intracellular cAMP levels. Mycelia from strains grown for 72 h on PDA plates covered with a cellophane membrane were ground in liquid N_2 to a fine powder, weighed, and homogenized in 10 volumes of 0.1 M HCl. The samples were centrifuged at 600 × *g* at 22°C, and the cAMP content was measured with a Direct cAMP enzyme immunoassay kit (Sigma) used according to the manufacturer's instructions. cAMP levels were related to the protein concentrations (as measured by the Bio-Rad protein assay [Bio-Rad]) of the samples and were expressed as means ± standard deviations for three independent experiments.

Determination of cell wall chitin and glucan contents. Triplicate cultures of each strain were grown for 36 h in potato dextrose broth (PDB) (Difco). Mycelia were harvested, pressed dry between sheets of filter paper, weighed, ground in liquid N_2, and suspended in distilled water. Cells were broken by sonication for 1 min at 4°C (cycle 50 and 70% power; Sonopuls Sonicator), and the extract was centrifuged for 10 min at 13,000 × *g* at 4°C, washed four times with distilled water, and lyophilized.

Chitin content was determined as previously described (48) after the samples were hydrolyzed by boiling for 4 h with 4 M HCl. For glucan determination, the samples were hydrolyzed as described by Nemcovic and Farkas (38), and the amount of glucan was determined as described previously (35).

Nucleotide sequence accession number. The nucleotide sequence of *tga3* has been deposited in the GenBank database under accession number AF452097.

RESULTS

Cloning and characterization of *tga3*. PCR amplification resulted in two products that were approximately 1,000 and 1,100 bp long, both of which correspond to Gα subunits based on amino acid sequences deduced from the sequenced PCR fragments. A 958-bp fragment encoded Tga1 (GenBank accession number AY036905) (41). A 1,189-bp fragment, designated *tga3*, encoded a protein that is 88% identical (9) to

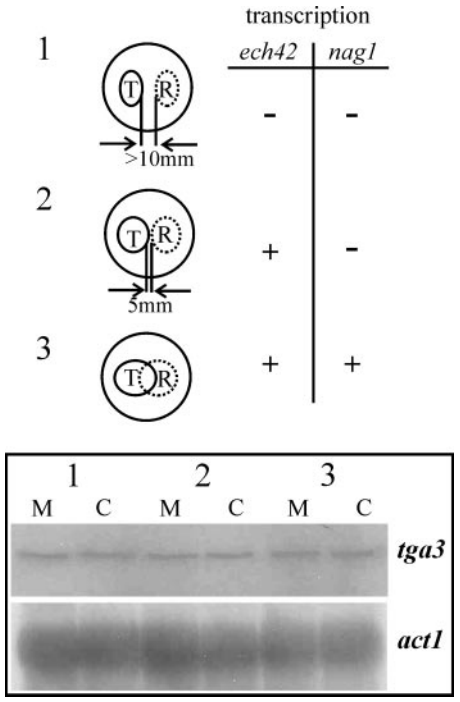

FIG. 1. Transcription of *tga3* during different stages of a direct confrontation of *T. atroviride* P1 with *R. solani*. A schematic representation of the different confrontation stages (stages 1, 2, and 3) of the mycoparasitic interaction is shown, and expression of the chitinase-encoding genes *ech42* and *nag1* is indicated by a plus sign or a minus sign. T, *T. atroviride*; R, *R. solani*. For Northern analysis of *tga3* gene transcription, 20 μg of total RNA was hybridized with a ~1.1-kb PCR fragment of the *tga3* structural gene or a 1.9-kb KpnI fragment of the actin-encoding gene *act1* as a probe. Lanes M, mycoparasitic interaction between *Trichoderma* and *Rhizoctonia*; lanes C, control, in which *Trichoderma* was confronted with itself.

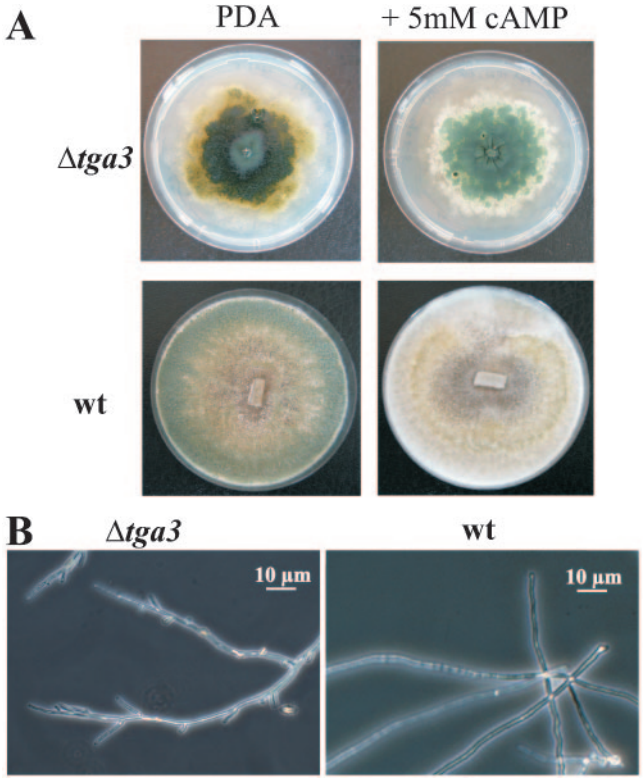

FIG. 2. Morphology of strain Δ*tga3-3* compared to the morphology of wild-type *T. atroviride* P1 (wt). (A) Colony morphology after growth on solid medium (PDA) for 7 days and effect of addition of 5 mM exogenous cAMP on the phenotype. (B) Microscopic examination of the *T. atroviride* wild type and Δ*tga3-3* mutant after growth in liquid culture (PDB) for 36 h at 28°C.

Neurospora crassa GNA-3, which belongs to subgroup III of fungal Gα subunits as classified by Bölker (3). The 1,189-bp PCR product was used to screen a genomic λ BlueStar library and to identify multiple clones carrying 10- to 15-kb inserts. Based on sequencing with internal primers Tga3intF1-F3 and Tga3intR1-R4, *tga3* (GenBank accession number AF452097) consists of a predicted 1,470-bp open reading frame interrupted by five putative introns at positions conserved in other fungal Gα homologues. The amino acid sequence was 90% identical to the MAGA sequence (GenBank accession number AF011340) from *Magnaporthe grisea* and 88% identical to the GNA-3 sequence (GenBank accession number AF281862) from *N. crassa*. Most of the differences occurred in the N-terminal one-half of the protein. Tga3 was only 49% identical to *T. atroviride* Tga1. Based on Southern analyses performed with genomic DNA of *T. atroviride* digested with several restriction enzymes and with the 1,189-bp PCR product as the probe, *tga3* is present in a single copy in the genome of *T. atroviride* (data not shown).

***tga3* expression during confrontation between *T. atroviride* and *R. solani*.** *tga3* gene transcription was examined during confrontation of *T. atroviride* with *R. solani* by isolating total RNA from different stages during a plate confrontation assay (Fig. 1). Samples from stage 1 (the distance between *Trichoderma* and the host was >10 mm; neither *ech42* nor *nag1* was expressed), stage 2 (precontact; *ech42* was expressed, but *nag1* was not expressed), and stage 3 (direct contact; both *nag1* and *ech42* were expressed) (50), including controls in which *T. atroviride* was confronted with itself, were subjected to Northern analysis. *tga3* was transcribed constitutively whether the host was present or not (Fig. 1), although detection of the *tga3* transcript required a long exposure of the Northern blot, suggesting that *tga3* was not highly expressed.

Phenotype of *tga3* disruptants. Sixty-two percent of the transformants had single-copy integration of the disruption cassette at the homologous *tga3* gene locus. Three arbitrarily chosen disruptants, Δ*tga3-1*, Δ*tga3-3*, and Δ*tga3-5*, were analyzed. The disruptants grew at ~45% the rate of the wild type on PDA and formed little aerial mycelium. The mutants formed flat, glossy colonies that continuously produced dark green conidia (Fig. 2A) that were the normal size and shape. The Δ*tga3* strains also sporulated in the dark, suggesting that the Tga3 Gα subunit has a role in light-induced conidiation. The intracellular cAMP levels in the mutants (6.5 ± 1.3 pmol of cAMP/mg of protein) were less than one-half those found in the wild-type strain (15.9 ± 4.2 pmol/mg) or a transformant with an ectopic integration of the disruption cassette (13.5 ± 2.9 pmol/mg). Addition of exogenous cAMP (2, 5, and 10 mM) did not alter the slowly growing, hypersporulating phenotype of the mutants (Fig. 2A), although inclusion of 5 mM cAMP in

the growth medium more than doubled the intracellular cAMP levels (35.5 ± 5.4 pmol of cAMP/mg of protein) in all three strains tested compared to the wild-type strain grown on PDA without cAMP.

In liquid cultures in PDB or SM with 1% glycerol for 36 h, no conidiation occurred. The germination of conidia from the Δ*tga3* mutants was both reduced and delayed compared to the germination of conidia from wild-type strain P1. After 9 h of incubation at 28°C, ~30% of the wild-type conidia, but only 10% of the Δ*tga3* conidia, had formed germ tubes, and after 11 h, 55% of the wild-type conidia and 33% of the Δ*tga3* conidia had germinated. After incubation for 36 h in liquid cultures, the Δ*tga3* mutants produced ~30% less biomass than the comparable wild-type cultures produced. The mycelia of Δ*tga3* strains were more highly branched than those of the wild-type strain (Fig. 2B).

Mycoparasitic ability of Δ*tga3* mutants. After 3 to 4 days of cocultivation of wild-type strain P1 with *R. solani*, P1 began to overgrow and lyse the host fungus (Fig. 3A), while the Δ*tga3* mutants were avirulent in similar cultures (Fig. 3B). Even when the mutants were grown until their mycelia covered about one-half of the plate before *R. solani* was inoculated on the opposite side of the petri dish, the Δ*tga3* strains were unable to parasitize the *R. solani* colonies (Fig. 3B). Thus, the Tga3 Gα subunit could have an important role in signal transduction during host recognition and/or the mycoparasitic response of *T. atroviride*. Similar results were obtained for the Δ*tga3* mutants when *B. cinerea* (Fig. 3C) or *S. sclerotiorum* (data not shown) was used in the confrontation assays. Typical morphological changes associated with mycoparasitism (34) (e.g., growth of the mycoparasite alongside the host's hyphae and attachment to these hyphae) were not observed in confrontations with the Δ*tga3* mutants (Fig. 3D). Addition of exogenous cAMP to the plate confrontation assays (PDA supplemented with 2, 5, or 10 mM cAMP) did not restore the mycoparasitic behavior (i.e., overgrowth and host lysis) of the Δ*tga3* strains, although cultures supplemented with 5 mM cAMP regained the ability to form mycoparasitism-related infection structures (data not shown). Culture filtrates of the Δ*tga3* mutants from cultures grown on *N*-acetylglucosamine for 10 h and on colloidal chitin for 48 h as sole carbon sources had 25 to 75% of the antifungal activity (inhibition of *Botrytis* spore germination) of the wild-type culture filtrates.

Extracellular chitinase activities in Δ*tga3* strains. In the wild-type strain *T. atroviride* P1, no chitinase production was observed during incubation on glucose or glycerol for 5 and 10 h (data not shown). When induced with *N*-acetylglucosamine, the wild type produced up to 300 U of NAGase per g (dry weight) for up to 10 h; after 12 h the NAGase activity began to decline again (Table 1). Upon incubation on colloidal chitin for 48 h, both NAGase activity (up to 1,900 U/g [dry weight]) and endochitinase activity (up to 205 U/g [dry weight]) also were induced in *T. atroviride* P1.

As with the wild type, chitinase production was not observed in the Δ*tga3* mutants when they were cultured on glucose or glycerol. The extracellular NAGase activities of the *N*-acetylglucosamine-induced cultures of the Δ*tga3* strains were 7 to 21% that of the wild-type strain (Table 1). In cultures induced with colloidal chitin, both the NAGase and endochitinase activities of the Δ*tga3* mutants were no more than 22 to 60% of those of the wild type. These results suggest that Tga3 influences the production of extracellular endo- and exochitinases.

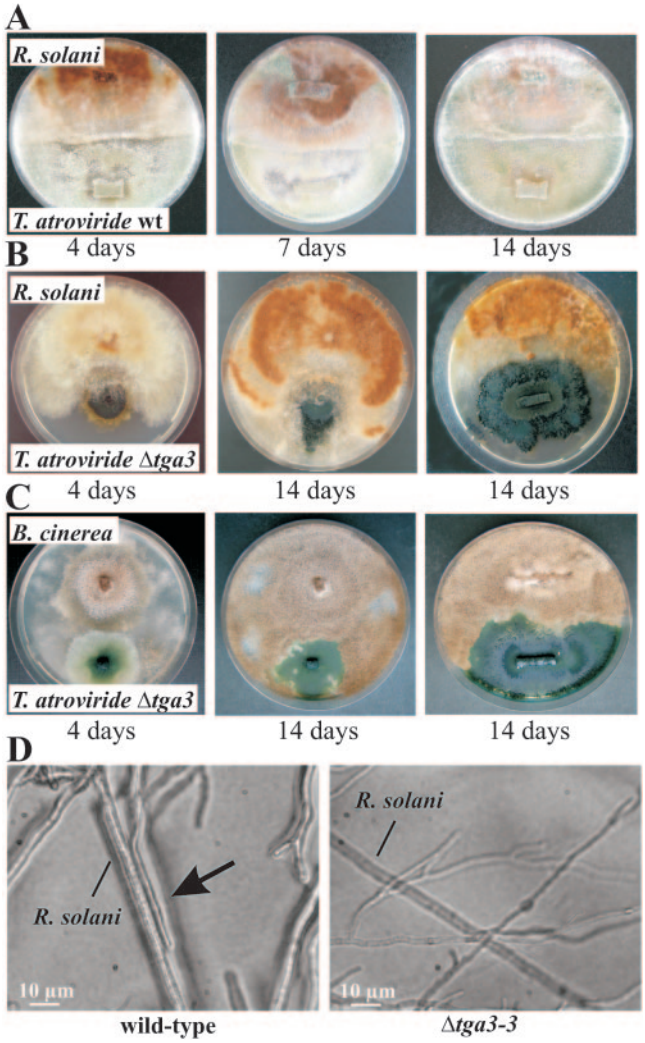

FIG. 3. Biocontrol assays for analyzing mycoparasitic ability. (A to C) Plate confrontation assays of *T. atroviride* wild-type strain P1 (wt) (A) and the Δ*tga3-3* mutant with *R. solani* (B) and *B. cinerea* (C). In the plates on the right in panels B and C, the *tga3*-negative mutant was grown until it covered about one-half of the plate, and then the host fungi were inoculated. Pictures were taken 4, 7, and 14 days (A) or 4 and 14 days (B and C) after inoculation. (D) Microscopic examination of the mycoparasitic interaction between the *T. atroviride* wild type or the Δ*tga3-3* mutant and *R. solani*. Attachment of the wild type to the host's hyphae is indicated by the arrow.

***nag1* and *ech42* transcription in Δ*tga3* mutants.** In wild-type strain P1, *nag1* transcript formation was greatest 5 h after transfer to a medium containing *N*-acetylglucosamine, lower after 10 h, and undetectable by 12 h. Following transfer to colloidal chitin, *nag1* and *ech42* transcripts could be detected at both 36 and 48 h. At all times tested, the Δ*tga3* mutant produced significantly more *nag1* transcript than the wild type produced when the organisms were induced with *N*-acetylglucosamine, and it produced both *nag1* and *ech42* transcripts when the organisms were induced with colloidal chitin (Fig. 4).

The *nag1* mRNA levels of selected samples were quantified

TABLE 1. Extracellular, mycelium-bound, and intracellular NAGase activities of *N*-acetylglucosamine-induced cultures of *T. atroviride* wild-type strain P1 and the Δ*tga3* mutant

Sample	Induction time (h)	NAGase activity (U/g [dry wt])[a]	
		Wild type	Δ*tga3*
Extracellular	5	75 ± 7	16 ± 9
	10	300 ± 70	21 ± 3
	12	270 ± 50	37 ± 8
Myceliumbound	5	900 ± 400 A	1,100 ± 500 C
	10	1,500 ± 400 A	700 ± 300 C
	12	700 ± 200 A	700 ± 100 C
Intracellular	5	10,500 ± 300 B	12,400 ± 300 D
	10	10,600 ± 100 B	12,500 ± 300 D
	12	11,400 ± 300 B	12,800 ± 400 D

[a] The values are means ± standard deviations of three independent experiments. The values followed by the same letter were pooled, and the data for the Δ*tga3* mutant were compared to the data for the wild type by using the Mann-Whitney U test. For the mycelium-bound samples two replicates of nine samples were examined, and the *P* value was >0.5. For the intracellular samples two replicates of nine samples were examined, and the *P* value was ≤0.001.

by real-time reverse transcriptase PCR. *nag1* mRNA was barely detectable in both the wild type and the Δ*tga3-3* mutant 10 h after transfer to glucose or glycerol. After induction with *N*-acetylglucosamine for 10 h, the *nag1* transcript levels were at their highest values in both strains; the wild type showed ~2,700-fold induction, and Δ*tga3* showed ~3,500-fold induction. After 12 h, the *nag1* transcript level for the wild-type strain had decreased to ~600-fold inducibility, reflecting the results of the Northern blot analysis, while the *nag1* transcript level for the Δ*tga3* strain had dropped only slightly to ~2,650-fold inducibility. When organisms were induced with colloidal chitin, a similar pattern was observed; the Δ*tga3* strain again had elevated *nag1* transcript levels at both 36 h (1.9-fold higher than the wild type) and 48 h (1.5-fold higher than the wild type) after induction.

Localization of *N*-acetylglucosaminidase activity in Δ*tga3* mutants. Although the Δ*tga3* mutant had elevated *nag1* and *ech42* transcript levels, its extracellular chitinase activities in induced cultures were reduced. Similar levels of mycelium-bound NAGase activity were found in the wild type and the mutant, but intracellular NAGase activity was significantly higher in the Δ*tga3* mutant than in the wild-type control (Table 1).

Cell wall of the Δ*tga3* mutants. We could not produce protoplasts from the Δ*tga3* strains even if we incubated them for up to 20 h (normal incubation time, 3 to 4 h) and added twice as much of the lysing enzymes. Resistance to protoplasting by cell wall lytic enzymes may be due to a change in cell wall polymer content, an increase in cross-linking, or a change in the type of cross-linking occurring in the cell wall. Any of these changes could affect cell wall porosity (21). Nevertheless, neither the cell wall chitin nor the glucan content of the Δ*tga3* mutants was significantly different from the content of the wild type or the transformant with ectopic integration of the disruption construct (data not shown). Growth inhibition by SDS often is used as an indicator of cell wall integrity (8, 21), but the reduction in hyphal growth by the Δ*tga3* mutants in the presence of 0.01 or 0.02% SDS was comparable to that of the control strains (data not shown).

DISCUSSION

In filamentous fungi, α subunits of heterotrimeric G proteins, which are activated upon ligand binding to a corresponding receptor, play crucial roles in regulating functions such as asexual development, sexual reproduction, and pathogenicity, in which fungi sense the presence of mating partners by pheromones and pathogenic fungi respond to signals from their hosts (22, 25, 26, 28, 31, 39, 44, 52). We identified and characterized a novel G protein α subunit-encoding gene, *tga3*, of the mycoparasitic fungus *T. atroviride*, which groups with subgroup III of fungal Gα proteins. Thus, *Trichoderma* spp. have members of each Gα protein subgroup, as previously shown for, e.g., *N. crassa* and *M. grisea* (28, 31, 45). The *tga3* gene is transcribed constitutively at a low level during different growth stages and irrespective of the presence of a host fungus. Deleting the *T. atroviride* subgroup I Gα protein-encoding gene *tga1* did not alter *tga3* transcript levels (Zeilinger, unpublished data), which is consistent with results obtained for *N. crassa*, where deleting any of the three Gα subunit-encoding genes did not affect the expression of the other G proteins (28).

For generating Δ*tga3* knockout mutants, the *hph* (hygromycin B phosphotransferase-encoding) gene was employed as a selection marker. This marker gene is frequently used for transformation of *T. atroviride* (34, 41, 50, 51), and its gene product so far has not been reported to interfere with growth

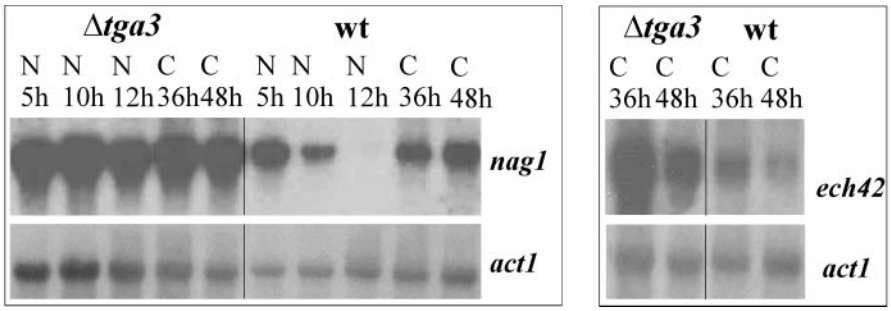

FIG. 4. Analysis of transcription of the chitinase-encoding genes *nag1* and *ech42* in the *T. atroviride* wild type (wt) and the Δ*tga3-3* mutant strain following induction with *N*-acetylglucosamine or colloidal chitin: Northern analysis of *nag1* gene transcription 5, 10, and 12 h after transfer to 1% *N*-acetylglucosamine (lanes N) and 36 and 48 h after transfer to 1% colloidal chitin (lanes C). A 1.9-kb SalI/XbaI fragment of *nag1* was used as the probe. *ech42* gene transcription was analyzed 36 and 48 h after transfer to colloidal chitin by using a 1.0-kb PstI fragment of *ech42* as the probe. Hybridization with a 1.9-kb KpnI fragment of the actin-encoding gene *act1* was used as a loading control.

or the mycoparasitic ability of the fungus. This is consistent with our results for an ectopic transformant used as a control in selected experiments, in which it behaved like the wild type.

Δ*tga3* mutants grow slowly with nearly no aerial hyphae, and they continuously produce conidia when they are cultured on solid media, a phenotype similar to that reported for an *N. crassa* Δ*gna-3* mutant (28). However the *Neurospora* mutant can form conidia in submerged culture. Conidia from Δ*tga3* mutants also have reduced and delayed germination, a characteristic similar to a characteristic observed for the *gasC* mutants of *Penicillium marneffei* (53).

Mutants of some subgroup III fungal Gα proteins, including *Cryphonectria parasitica* CPG-2 (14), *N. crassa* GNA-3 (28), *Ustilago maydis* Gpa3 (39), and *Podospora anserina* MOD-D (33), can be phenotypically suppressed by exogenous cAMP. The finding that the Δ*tga3* mutant phenotype was not altered by the addition of exogenous cAMP suggests that these properties are mediated through a cAMP-independent pathway. Nevertheless, intracellular cAMP levels were significantly reduced in Δ*tga3* mutants, suggesting that Tga3 can increase the internal cAMP level.

In *Trichoderma*, as in other filamentous fungi, asexual sporulation is activated by light (2, 13, 42, 49), and no conidia are produced when the fungus is grown in the dark. When grown in the dark, Δ*tga3* mutants sporulated in the same manner as when they were cultured in the light, like *tga1* antisense mutants (41). Thus, both Gα proteins (Tga1 and Tga3) act as negative regulators of conidiation, perhaps by sharing downstream signaling components. Overlapping roles for Gα subunit proteins also are known in *N. crassa*, in which both GNA-1 and GNA-3 regulate conidiation (25, 28), and in *P. marneffei*, in which GasA- and GasC-mediated signaling blocks conidiation and regulates production of a red pigment (52, 53).

Tga3 also has a major impact on mycoparasitism, as Δ*tga3* mutants were avirulent in plate confrontation assays with *R. solani*, *B. cinerea* (Fig. 3), and *S. sclerotiorum* (data not shown). The antifungal activity of chitinolytic enzymes and their role during mycoparasitism are well established (29, 40, 46). While Δ*tga3* strains had reduced extracellular endochitinase and *N*-acetylglucosaminidase levels, *nag1* and *ech42* gene transcription and intracellular chitinase levels were increased in the mutants. Compared to *Aspergillus nidulans*, in which the subgroup I Gα subunit FadA and the Gβ subunit SfaD regulate cell wall porosity and chitin content (8), cell wall composition was not altered in Δ*tga3* mutants. Thus, the observed accumulation of chitinolytic enzymes inside the cell and the retention of these enzymes in the cell wall may be due to Tga3-mediated posttranscriptional regulation of chitinase gene expression and its influence on chitinase secretion.

When chitinase-induced culture supernatants were used in in vitro *Botrytis* spore germination assays, the antifungal activity of the Δ*tga3* mutants was reduced compared to the activity of the wild type and roughly reflected the extracellular chitinase activity. These experiments showed that the Δ*tga3* mutants can still produce extracellular chitinases and suggested that the complete loss of mycoparasitism in direct confrontation assays is caused by other defects. One hypothesis is that the Δ*tga3* strains no longer recognize the host. This hypothesis is strongly supported by the lack of mycoparasitism-related infection structures (e.g., growth alongside, attaching to, or coiling around the host's hyphae) (11, 34) in dual cultures of the Δ*tga3* mutants with a host fungus. In *U. maydis*, deleting the subgroup III Gα protein Gpa-3 rendered the fungus avirulent. The resulting mutants also were mating deficient, as they could not respond to the pheromone signal (39). Another hypothesis for the loss of mycoparasitism by the Δ*tga3* mutants is that hyphal polarity could be changed due to the altered, hyperbranching phenotype, resulting in an inability to form the typical mycoparasitism-accompanying morphological changes.

Recognition of the host fungus by *Trichoderma* seems to play an important role in triggering the mycoparasitic response (1, 36, 37). The host-derived signals identified so far are diffusible, low-molecular-weight compounds released from the host's cell wall by the action of *Trichoderma*-secreted chitinases, which function as precontact inducers of antagonistic genes in the mycoparasite and as elicitors of plant defense mechanisms (19, 30, 50). Since most of the chitinase activity is retained inside the cells of Δ*tga3* strains, one hypothesis for the loss of host recognition could be a reduction in or a complete lack of this inducer, resulting in the failure of precontact induction of mycoparasitism-relevant genes. In addition, lectins present on the host's cell wall may be involved in host recognition, as they induce mycoparasitic coiling upon direct contact (23, 24). When different lectins were used to coat nylon fibers for biomimetic experiments, the lectins promoted attachment to and coiling around the fibers, regardless of their sugar moiety, suggesting that lectins were not the specificity-determining factor in host recognition (41). Tga1 antisense transformants could not overgrow *R. solani* and exhibited decreased coiling around nylon fibers when they were tested in biomimetic assays (41), similar to Δ*tga3* mutants which showed a loss of mycoparasitism-related infection structure formation upon direct contact with a living host fungus.

In summary, the data strongly support the hypothesis that the loss of mycoparasitism of Δ*tga3* mutants is a combination of indirect effects (e.g., reduced growth, a defect in chitinase secretion) and direct effects (e.g., loss of infection structure formation). Furthermore, Tga3 directly influences the regulation of mycoparasitism-related chitinase gene transcription, as the mutants had enhanced *nag1* and *ech42* mRNA levels.

The two G protein α subunits of *T. atroviride* have overlapping roles in conidiation and mycoparasitism, so it is necessary to identify the specific receptors and the signaling pathways used to transmit the host-derived signals to downstream effectors in order to understand the complex biochemical processes that result in fungal biocontrol.

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