

Trichoderma atroviride G-Protein α -Subunit Gene *tga1* Is Involved in Mycoparasitic Coiling and Conidiation

Víctor Rocha-Ramírez,¹ Carmi Omero,² Ilan Chet,² Benjamin A. Horwitz,³ and Alfredo Herrera-Estrella^{1*}

Department of Plant Genetic Engineering, Centro de Investigación y Estudios Avanzados, Unidad Irapuato, 36500 Irapuato, Guanajuato, México,¹ and Otto Warburg Center for Agricultural Biotechnology, Faculty of Agriculture, The Hebrew University of Jerusalem, Rehovot 76100,² and Department of Biology, Technion—Israel Institute of Technology, Haifa 32000,³ Israel

Received 23 January 2002/Accepted 15 May 2002

The soil fungus *Trichoderma atroviride*, a mycoparasite, responds to a number of external stimuli. In the presence of a fungal host, *T. atroviride* produces hydrolytic enzymes and coils around the host hyphae. In response to light or nutrient depletion, asexual sporulation is induced. In a biomimetic assay, different lectins induce coiling around nylon fibers; coiling in the absence of lectins can be induced by applying cyclic AMP (cAMP) or the heterotrimeric G-protein activator mastoparan. We isolated a *T. atroviride* G-protein α -subunit ($G\alpha$) gene (*tga1*) belonging to the fungal subfamily with the highest similarity to the $G\alpha_i$ class. Generated transgenic lines that overexpress $G\alpha$ show very delayed sporulation and coil at a higher frequency. Furthermore, transgenic lines that express an activated mutant protein with no GTPase activity do not sporulate and coil at a higher frequency. Lines that express an antisense version of the gene are hypersporulating and coil at a much lower frequency in the biomimetic assay. The loss of Tga1 in these mutants correlates with the loss of GTPase activity stimulated by the peptide toxin Mas-7. The application of Mas-7 to growing mycelial colonies raises intracellular cAMP levels, suggesting that Tga1 can activate adenylyl cyclase. In contrast, cAMP levels and cAMP-dependent protein kinase activity drop when diffusible host signals are encountered and the mycoparasitism-related genes *ech42* and *prb1* are highly expressed. Mycoparasitic signaling is unlikely to be a linear pathway from host signals to increased cAMP levels. Our results demonstrate that the product of the *tga1* gene is involved in both coiling and conidiation.

Members of the genus *Trichoderma* belong to an aggregate of *Deuteromycetes* species whose teleomorphs, where identified, belong to the *Ascomycetes* (*Hypocrea*) (35). A number of soil isolates are being studied because of their ability to antagonize soilborne plant pathogens, and these *Trichoderma* species are often referred to as biocontrol fungi (9). During the initial stages of this interfungal interaction, *Trichoderma atroviride* responds to the presence of the host by coiling around the host hyphae. At later stages, the mycoparasite produces hydrolytic enzymes, such as β -1,3-glucanases, β -1,6-glucanases, chitinases, and proteases, to penetrate the host and use its cellular content as a source of nutrients. Coiling occurs in response to signals that presumably originate from the host, its surface structure, or the by-products of its initial degradation by enzymes secreted by *Trichoderma* (36).

Recognition between a parasite and its host appears to be an essential step for successful continuation of the parasitic process (46). Surface molecules such as lectins are good candidates for the recognition process in *Trichoderma*. Evidence for this supposition comes from experiments on the proposed specific recognition of *Rhizoctonia* mediated by lectins present on its cell wall (3, 4). Further support for this hypothesis was generated by use of a biomimetic system based on the use of nylon fibers coated with lectins developed to detect attachment

and coiling similar to those observed during the *Rhizoctonia-Trichoderma* interaction (23, 24, 25). In addition, Inbar and Chet (25) showed an increase in the activity of an intracellular 102-kDa glucosaminidase by using the biomimetic system, suggesting that the recognition event mediated by lectins serves as a signal that triggers a general antifungal response in *Trichoderma*. In this sense, the expression of genes for at least some cell wall-degrading enzymes is induced by a soluble signal from the host with no coiling (11). Thus, the coiling response appears to follow signaling pathways different from those involved in the induction of the expression of these genes; alternatively, the coiling response involves multiple signals. However, the intracellular signaling pathways downstream of surface recognition and the detection of the soluble molecule are still unclear.

Another interesting developmental process in this fungus is asexual sporulation, which is induced by light or nutrient depletion. Asexually produced spores (conidia) are useful as inocula in the field or the greenhouse; however, sporulation is not conducive to efficient biocontrol because the resources of the culture are diverted to spore production rather than attack of the host. An understanding of the switch determining the relative priorities of conidiation and mycoparasitic coiling has intrinsic interest, as a rather unique example of fungal development. Furthermore, such an understanding may help in the selection of improved biocontrol strains.

Loss-of-function and gain-of-function experiments with G-protein α -subunit ($G\alpha$) and G-protein β -subunit ($G\beta$) genes have indicated that heterotrimeric G proteins are involved in

* Corresponding author. Mailing address: Centro de Investigación y Estudios Avanzados, Unidad Irapuato, A.P. 629, 36500 Irapuato, Guanajuato, Mexico. Phone: 52/462/6239600. Fax: 52/462/6245849. E-mail: aherrera@ira.cinvestav.mx.

sporulation, mating, pathogenicity, secondary metabolite production, and vegetative incompatibility in a variety of ascomycete (5, 22, 28, 29, 42, 51,) and basidiomycete (50) fungi.

In plant pathogens, loss-of-function mutations introduced into genes encoding heterotrimeric G proteins (14, 28, 38, 50) prevent the mutants from efficiently attacking their hosts. Mycoparasitism may well depend on similar, conserved eukaryotic signaling pathways. In *Trichoderma*, there is biochemical evidence for the participation of $G\alpha$ in coiling. Omero et al. have reported that the application of exogenous activators of G-protein-mediated signaling, the peptide toxin mastoparan and fluoroaluminate (AlF_4^-), results in an increase in coiling around nylon fibers of more than twofold compared to the results obtained for controls (47). Furthermore, the application of exogenous dibutylryl-cyclic AMP (cAMP) also increases coiling. In addition, cAMP affects another developmental process in *Trichoderma*; it bypasses the requirement for light for sporulation, while atropine, a compound known to block cAMP production in fungi, prevents sporulation even after photoinduction.

We have isolated from *T. atroviride* a $G\alpha$ gene belonging to the well-conserved fungal subfamily with the highest similarity to $G\alpha_i$. Sense and antisense expression in transgenic lines shows that the product of this gene is involved in at least two developmental pathways.

MATERIALS AND METHODS

Strains and culture conditions. *T. atroviride* (formerly *T. harzianum*) IMI 206040 was used in this work. *Escherichia coli* strains JM103, MC1061, and TOP10 (53) (Invitrogen) were used for DNA manipulations. The plasmids used were pBluescript (Stratagene), pCR2.1 (Invitrogen), pSPORT1 (Gibco-BRL, Madison, Wis.), pTZ18 (Pharmacia), pHat α (20), and pCB1004 (Fungal Genetics Stock Center). Plasmids pHat α and pCB1004 carry the *E. coli* hygromycin phosphotransferase gene under the control of the *Aspergillus nidulans* *gpdA* and *trpC* promoters, respectively.

Trichoderma was grown on potato dextrose agar (PDA; Difco) unless otherwise indicated. For transformation, soft agarose selection medium (1% agarose, 200 μ g of hygromycin/ml) was used as an overlay, and PDA with 200 μ g of hygromycin/ml was used as a base selection medium. For photoinduction experiments, *T. atroviride* was grown on complete medium (24 g of potato dextrose broth/liter, 2 g of yeast extract/liter, 1.2 g of casein hydrolysate/liter; PDYC; Difco).

DNA and RNA manipulations. Fungal chromosomal DNA was isolated as previously described (49). All other DNA manipulations were performed by standard techniques (53). Fungal RNA was isolated according to the protocol described by Jones et al. (27). Southern blotting and Northern blotting were performed by standard procedures (53).

Cloning of *iga1*. A DNA fragment of about 165 bp was amplified by PCR with *T. atroviride* genomic DNA as a template and degenerate primer oligonucleotides designed on the basis of conserved regions of known $G\alpha$ genes (56). The PCR cycles were as previously described (63). The products were subcloned into pTZ18 and sequenced by the dideoxynucleotide chain termination method of Sanger et al. (54) with a Sequenase kit (version 2.0; U.S. Biochemicals). The short fragment sequence showed high similarity to the sequences of many $G\alpha$ genes. The fragment was labeled with a random priming DNA labeling system and used to screen a cDNA library constructed from a *Trichoderma* culture grown in the presence of *Rhizoctonia solani* cell walls as a single carbon source in plasmid pSPORT1. Hybridization was carried out under high-stringency conditions as previously described (53). Several positive clones were identified, and their sequences were analyzed. The sequence data indicated that all clones corresponded to the same gene and that none was full length.

A full cDNA clone was obtained later by reverse transcription (RT)-PCR with a forward primer (5'-GGATGAGACTGCTAACCGAATGC-3') and a reverse primer (5'-GTCGTCTAAATGAGAGACCGC-3') designed based on the genomic sequence. The amplification product was cloned into pCR2.1 and later isolated as a 280-bp *AatII*-*BamHI* fragment, which was inserted into the cDNA

clone at the same sites. The resulting plasmid (pTGAC) contained a cDNA clone with the complete open reading frame. The cDNA clone was used to screen a λ EMBL3 genomic library. Four positive phage clones were identified, and DNAs were purified.

Southern analysis allowed the identification of 2.4-kb *BamHI* and 2.5-kb *XhoI* DNA-hybridizing fragments, which were subcloned, respectively, into *BamHI*- and *Sall*-digested pBluescript KS(+). Sequencing of the *BamHI* fragment indicated that it contained the sequence coding for the carboxy terminus of the protein (Tga1). The sequence of the *Sall* fragment contained the region of the gene coding for the amino terminus. A plasmid (pTGAG) containing the full-length *iga1* genomic sequence was constructed by ligating the 2.4-kb *BamHI* fragment to the *BamHI*-digested 2.5-kb clone. Correct orientation was determined by restriction mapping and sequencing across the *BamHI* site.

Constructs. Plasmids for antisense and sense expression were constructed as follows. pTGAC was digested with *EcoRI*; this fragment contained the complete cDNA and was ligated to the *EcoRI* site of plasmid pPR2, which carries the promoter of the *T. atroviride* *prb1* gene (15). The two resulting plasmids were pAsTGA, which is an antisense construct, and pSTGA, which is a sense construct; both are controlled by the *prb1* promoter.

A Q203L mutant gene was generated by using a PCR-based strategy. Three oligonucleotides were designed based on the genomic sequence to generate the desired mutation in a two-step procedure. A primer (5'-CGGTGCCAGCCA TC-3') containing a single base modification (marked in bold) which results in a single amino acid modification of Q to L and a primer (5'-GGACGCTTGAT GAACC-3') containing an *AatII* site were used to amplify a 187-bp fragment. The resulting mutant product was used as a reverse primer for a second round of PCR amplification with a forward primer having the sequence 5'-CGCGTCAT TCTCGA-3'. The PCR product (344 bp), which includes an *NcoI* site next to the reverse primer, was ligated to plasmid pCRII and used for the transformation of *E. coli*. Plasmids containing the expected insert were confirmed by digestion with *BalI* (*MseI*) (this site is lost due to the introduced mutation) and sequenced. Finally, the *AatII*-*NcoI* fragment (325 bp) cloned into pCRII was used to replace the same region in plasmid pTGAG, which carries the complete genomic clone, resulting in a modified *iga1* carrying the desired point mutation.

Transformation. Biolistic transformation of *Trichoderma* was carried out essentially as described by Lorito et al. (41), with minor modifications. Briefly, particle preparation was done according to the protocol described by Tomes et al. (59). Conidia (10^8) were plated on PDA supplemented with 0.4 M sorbitol, and plates were allowed to dry for 4 h. Projectiles carrying the DNA were delivered at a helium pressure of 800 lb/in². The fungus was allowed to recover for 7 h and was overlaid with soft agarose selection medium. Approximately 48 h later, single colonies reaching the surface were cut out and transferred to selection medium. For purification of transformants, selected colonies were allowed to sporulate, conidia were collected, and serial dilutions were plated on PDA containing 100 μ g of hygromycin/ml and 0.5% Triton X-100.

Photoinduction. Colonies were grown on filter paper soaked with 3 ml of PDYC: an 8-cm disk of Whatman 50 overlaying a 7-cm disk of Whatman 1 in a 9-cm plastic petri dish. Experiments were begun after 36 h of growth in total darkness, when colony diameter had reached 40 to 50 mm. The cultures were photoinduced by exposure to a standard blue source consisting of light from a cool-white fluorescent tube filtered through a blue acrylic filter (maximum transmission, 442 nm; fluence rate, 2 μ mol m⁻² s⁻¹) or two cool-white fluorescent tubes filtered with LEE filter no. 183 (fluence rate, 3 μ mol m⁻² s⁻¹). At various times, the mycelia or conidiophore rings were scraped from the surface of the filter paper under weak yellow or red safelight (0.1 μ mol m⁻² s⁻¹). Samples for RNA extraction were immediately frozen in liquid nitrogen.

Protein analysis. Cultures grown on PDYC in the dark at 28°C for 48 h were harvested, frozen, and ground in liquid nitrogen. The powder was suspended in homogenization buffer A (3 ml/g), which contained 50 mM HEPES (pH 7.5), 330 mM sucrose, 5 mM EDTA, 0.2% bovine serum albumin (BSA), 1 mM phenylmethylsulfonyl fluoride (PMSF), and 5 mM dithiothreitol (DTT). The suspension was then sonicated for 1 min at 4°C with 50% pulses by using a Sonicator model W-375 microtip (Heat Systems Ultrasonics Inc., Farmingdale, N.Y.). Samples were centrifuged for 10 min at 4°C and 10,000 \times g. The supernatant was collected and centrifuged for 1 h at 4°C and 100,000 \times g. Finally, the pellet obtained was suspended in homogenization buffer. The protein concentration was determined by the Bradford method with assay dye reagent from Bio-Rad Laboratories, Richmond, Calif., and BSA as a protein standard. Western blotting was performed according to standard procedures (53). Equal loading of the lanes was confirmed before probing by staining with Ponceau S. The anti-Gna1 and anti-Gna2 antibodies were kindly provided by K. Borkovich and were used at a 1:7,000 dilution. Goat peroxidase-coupled secondary antibody (Amersham) was used at a 1:12,000 dilution, followed by chemiluminescent signal detection in 1.25

mM luminol (Sigma)–400 μ M *p*-coumaric acid (Sigma)–2.7 mM H₂O₂ (Merck)–100 mM Tris base (pH 8.5).

Biomimetics. The culture conditions and the biomimetic system used were as described previously (23). For the biomimetic system, *T. atroviride* was grown on a synthetic medium (SM) with 0.2% (wt/vol) glucose (23) and covered with a cellophane (permeable cellulose) membrane. Nylon 66 fibers (approximate diameter, 14 μ m; kindly supplied by Nilit, Migdal-Haemek, Israel) were extended across grids (square polyester nets measuring 7 by 7 mm and having a pore size of 420 μ m), and the edges were fastened with cyanoacrylate glue (Loctite). The grids were washed several times with chloroform for 30 min and then were washed with 70% ethanol for 30 min. Finally, the grids were washed with sterile distilled water for a further 30 min.

Binding of lectins and BSA to the nylon fibers was brought about as described by Inbar and Chet (24). The various lectins bound to the nylon fibers were peanut (*Arachis hypogaea*), wheat germ (*Triticum vulgare*), gorse (*Ulex europaeus*), soybean (*Glycine max*), and concanavalin A (Sigma catalog no. L-0881, L-9640, L-5505, L-1395, and C-2631, respectively). Cultures were started by inoculation of an agar block with mycelium on top of the cellophane membrane. Sixteen hours later, the grids were placed 5 mm in front of the advancing colony. Experiments were performed 9 h after placement of the grids, when the front had reached one-third to one-half way across the grids. Incubation was done in total darkness at 28 to 30°C, and all manipulations of growing colonies were performed under a red safelight to avoid photoinduction of sporulation. The grids were removed aseptically and vapor fixed with 5% (wt/vol) OsO₄ and 25% glutaraldehyde (electron microscopy grade) for 48 h.

Specimens were dried for 24 h, coated with gold palladium in a model E-500 sputter coater (Polaron Equipment Ltd., Watford, United Kingdom), and observed under a scanning electron microscope (JEOL JSM 35C). For quantitative analysis of the results, the number of coils per milliliter of nylon fiber was counted in replicate scanning electron microscope images (23). Quantitative data represent the averages of data pooled from 9 to 11 replicate fields pooled from at least two independent sets of experiments. Statistical significance was assessed by Student's *t* test, and error bars in the figures indicate standard errors of the means.

GTPase activity assays. High-affinity membrane GTPase was measured by the assay of Cassel and Selinger (8). The reaction mixture contained 50 mM morpholinepropanesulfonic acid (MOPS) (pH 7.0), 1 mM MgCl₂, 0.2 mM EGTA, 0.5 μ M [γ -³²P]GTP (BLU/NEG/004; New England Nuclear) diluted with unlabeled GTP to a final activity of 5 to 20 Ci/mmol, 0.5 mM ATP, 2 mM DTT, 2 mM PMSF, 2 mM creatine phosphate, and creatine phosphokinase freshly added at 0.06 U/ μ l. The reaction was started by the addition of 50 μ l of the reaction mixture into 25 μ g of membrane protein in 50 μ l of homogenization buffer B, which contained 50 mM HEPES (pH 7.5), 330 mM sucrose, 5 mM EDTA, 5 mM EGTA, 0.2% (wt/vol) BSA, 0.2% (wt/vol) casein hydrolysate (acid), 5 mM DTT, and protease inhibitor cocktail (complete EDTA free; Roche). Incubation was done at room temperature and was terminated after 20 to 30 min by the addition of 500 μ l of ice-cold and well-stirred 6% activated charcoal (Sigma) in 50 mM phosphate buffer (pH 7.0). Samples were centrifuged for 5 min at 12,000 \times g, 440 μ l of the supernatant was added to 3 ml of a POP scintillation liquid containing 50% (vol/vol) toluene and 50% (vol/vol) Triton X-100, and counts were determined (1600 TR analyzer; Packard). Means and standard errors of the means are the combined results of at least two independent assays.

cAMP measurements. Mycelia were ground in liquid nitrogen to a fine powder and resuspended in ice-cold 6% trichloroacetic acid. Trichloroacetic acid extracts were incubated on ice for 20 min with frequent vortexing. A 1-ml aliquot of each sample was centrifuged for 25 min at 16,000 \times g in a microcentrifuge at 4°C. The pellet was solubilized in 0.1 N NaOH–5% sodium dodecyl sulfate at 60°C, and protein was quantitated with bicinchoninic acid reagent (Sigma) and BSA as a standard. This solution was extracted four times with 5 ml of ice-cold water-saturated ether and stored at –20°C. Radioimmunoassays were performed as described by Harper and Brooker (18). Results are representative of at least two independent experiments. Means and standard errors of the means are reported.

For induction with diffusible host signals, *T. atroviride* was pregrown for 3 days on SM plates covered with a cellophane membrane and then placed on an *R. solani* colony that had been pregrown for 2 days on the same medium. The pregrown *Trichoderma* colony was transferred to SM medium on which *Rhizoctonia* had been cultivated for 2 days and removed. In both instances, *Trichoderma* was incubated for a further 24 h after being transferred.

cAMP-dependent protein kinase activity. Approximately 50 mg of mycelium was frozen in liquid nitrogen, ground to a fine powder, and resuspended in 200 μ l of a buffer containing 20 mM Tris-HCl (pH 7.4), 1 mM EDTA (pH 8.0), and 0.5 mM PMSF. Samples were then vortexed three times for 10 s each time and kept at 4°C. The homogenate was centrifuged for 10 min at 12,000 rpm and 4°C

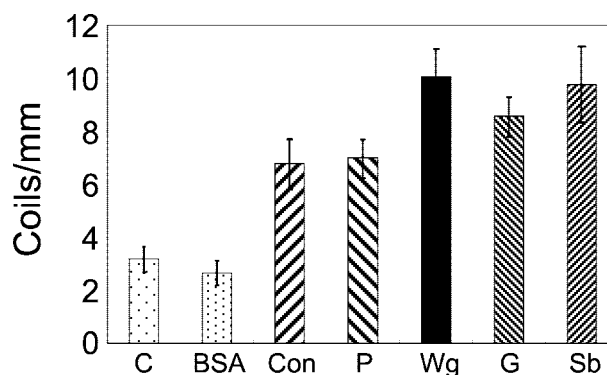


FIG. 1. Induction of coiling of *Trichoderma* IMI 206040 with different lectins. The commercial plant lectins used were peanut (P), wheat germ (Wg), gorse (G), soybean (Sb), and concanavalin A (Con). The lectins were coupled to nylon fibers, and the rate of coiling of *Trichoderma* around them was measured. Unmodified nylon fibers (C) or BSA-coupled nylon fibers (BSA) served as controls.

in an Eppendorf Microfuge. Supernatants were transferred to fresh Eppendorf tubes, and the protein content was determined by the Bradford method with a commercial product (Bio-Rad). Glycerol was added to each of the extracts to a final concentration of 20%. The protein concentration was then brought to 1 μ g/ μ l. The extracts were kept at –70°C until used.

Protein kinase A (PKA) activity was determined by the incorporation of ³²P into the specific substrate kemptide from [γ -³²P]ATP. The reaction was carried out in a 20- μ l mixture containing 50 mM Tris-HCl (pH 7.5), 1.5 mM EGTA, 5.8 mM MgSO₄, 50 μ M kemptide, 1 mM ATP, and 0.1 mM [γ -³²P]ATP (0.2 μ Ci) and with and without 10 μ M cAMP. The reaction was carried out with 10 μ g of protein, and the reaction mixture was incubated for 5 min at 30°C. After incubation, the reaction mixture was spotted on phosphocellulose filters (Whatman P-81), and the filters were washed three times with 75 mM phosphoric acid for 10 min with agitation and once with ethanol for 5 min. The phosphocellulose filters were allowed to dry, and radioactivity was measured with scintillation liquid in a 1600 TR liquid scintillation analyzer. The experiments were repeated three times.

RESULTS

Role of lectins in host recognition. To investigate whether lectins determine a specific recognition event between *Trichoderma* and its host, as previously proposed (25), a series of commercially available lectins with different sugar moieties covalently linked to nylon fibers were tested in biomimetic assays. Previous work (25) was done with a different *Trichoderma* strain and only two lectin types. As shown in Fig. 1, we confirmed and extended the results (25) with the strain used here. All tested lectins induced coiling of *Trichoderma* around the nylon fibers to different degrees. Wheat germ, gorse, and soybean lectins induced slightly higher coiling levels, reaching a maximum of 10 coils/mm, compared to concanavalin A and peanut lectins, which induced coiling levels of about 7 coils/mm. In contrast, the use of nylon fibers coated with BSA resulted in coiling levels (two or three coils per milliliter) similar to those obtained in control experiments with non-coated nylon fibers (three coils per milliliter). These results suggest that lectins play a role in host recognition.

Attachment and coiling are stimulated by activation of a G-protein signaling pathway. It has been suggested that a cAMP signaling pathway participates in a lectin-mediated host recognition process (47). Thus, we decided to examine the effect of the exogenous application of cAMP and the G-protein

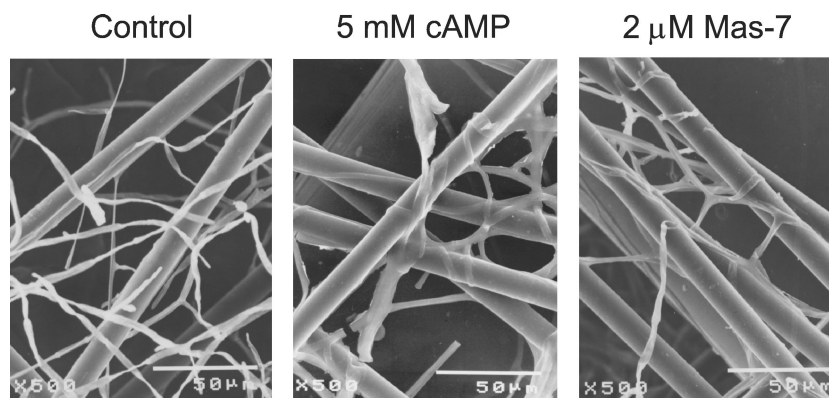


FIG. 2. Effect of the mastoparan analog Mas-7 and cAMP on coiling. The indicated concentration of Mas-7 or cAMP was applied under red safelight to the perimeter of growing colonies in the dark as described previously (47). After 6 h, the grids were removed and fixed for scanning electron microscopy.

activator Mas-7 in a biomimetic system with noncoated nylon fibers by scanning electron microscopy. As shown in Fig. 2, the application of 5 mM cAMP (Fig. 2B) and 2 μ M Mas-7 (Fig. 2C) resulted in *Trichoderma* attachment to and coiling around the nylon fibers. In contrast, *Trichoderma* did not attach to or coil around noncoated nylon fibers at a high frequency without the application of the mentioned compounds (Fig. 2A). These results suggest that a G-protein-mediated signaling pathway and cAMP are involved in both attachment and coiling.

Structure and expression of *tga1*, a *T. atroviride* $G\alpha$ gene. In order to determine whether heterotrimeric G proteins participate in coiling and conidiation, a $G\alpha$ gene was isolated. For this purpose, degenerate oligonucleotides were designed on the basis of conserved regions of other $G\alpha$ sequences in publically available data banks (see Materials and Methods). Using *T. atroviride* genomic DNA, we obtained a 165-bp PCR fragment. Sequence analysis indicated that this fragment corresponded to a portion of a $G\alpha$ gene. The cloned PCR fragment was used as a probe to screen a cDNA library. Several positive clones were obtained, and sequence analysis indicated that all clones corresponded to the same gene and that none of them was full length. A cDNA clone (1,332 bp) containing the complete open reading frame of 1,049 bp was later assembled (see Materials and Methods), resulting in plasmid pTGAC. The partial cDNA clone was used to screen a λ EMBL3 genomic library (see Materials and Methods). A 4.9-kb clone containing the full-length *tga1* genomic sequence was then also assembled (see Materials and Methods), resulting in plasmid pTGAG.

Comparison of the complete cDNA and genomic sequences of *tga1* (GenBank accession number AY036905) indicated the presence of four introns. The first intron is located 128 bp upstream of the ATG, and the other three introns are located 114, 873, and 1,065 bp downstream of the ATG, matching the intron positions of genes encoding proteins that belong to the $G\alpha_i$ superfamily (39). The deduced protein sequence of Tga1 corresponds to a peptide of 353 amino acids with a calculated molecular mass of 40.8 kDa, similar to that deduced from the *Neurospora crassa gna1* gene. *gna1* was the first gene cloned from a lower eukaryote encoding $G\alpha$ and was classified as part of the mammalian $G\alpha_i$ family (61). Both *tga1* and *gna1* belong to the same ascomycete group proposed by Horwitz et al. (22),

based on the three consensus sequences. It is noteworthy that all members of this group contain the consensus sequence for potential ADP ribosylation by pertussis toxin (62) and cholera toxin and have an N-myristoylation site (MGXXXS) in the amino-terminal region (6). The promoter region of *tga1* contains potential binding sites for several regulatory proteins, such as Stre, PacC, and StuAp (30, 58). Perhaps the most relevant elements are the 10 StuAp response elements detected in the *tga1* promoter. StuAp is an important regulator of multicellular development in *A. nidulans* (12). Response elements for this transcription factor have been found upstream of important developmental and cell cycle genes in *A. nidulans* (12). Southern analysis of *T. atroviride* genomic DNA under high-stringency hybridization conditions suggested the presence of a single copy of the gene. However, low-stringency hybridization showed one or two additional bands, suggesting the presence of at least one additional homologous gene in the *T. atroviride* genome (data not shown).

Generation of *Trichoderma* transgenic lines overexpressing *tga1* and expressing an antisense version. To elucidate the function of Tga1 in *Trichoderma*, we overexpressed the protein and expressed a mutant protein in transgenic lines. Two strategies were adopted with the objective of overexpressing Tga1. First, we generated transformants (MC1 and MC2) carrying multiple copies of vector pTGAG, which contains the complete genomic clone. With the same objective, we introduced multiple copies of plasmid pSTGA into transformants OE1 and OE2, in which the expression of the *tga1* coding sequence is driven by the derepressible promoter of the *prb1* gene from *T. atroviride*. This promoter is switched off when the fungus is grown in the presence of ammonium and is switched on when the fungus is grown on nitrate or deprived of nitrogen (unpublished data). All transformants obtained showed higher levels of Tga1 protein than did the wild type (Fig. 3A and B), had similar fluffy phenotypes, in contrast to the wild type, and showed no obvious alteration of their growth rates.

In order to gain further insight into the role of Tga1, a point mutation in the corresponding gene (*tga1*) was introduced (see Materials and Methods), resulting in the production of an activated mutant protein with a single amino acid change at position 203 (Q to L). This mutation was previously reported

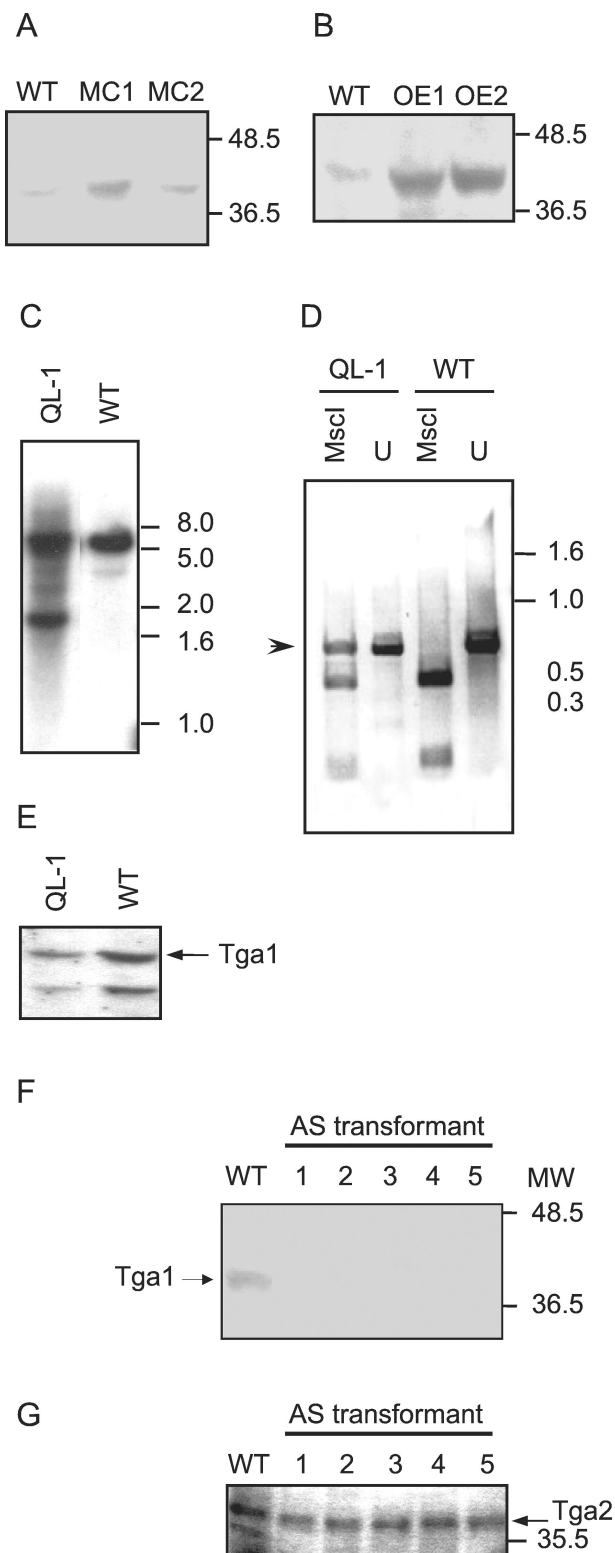


FIG. 3. Expression of Tga1 in wild-type and transgenic *Trichoderma* strains. (A and B) Western blots of membrane fractions from the indicated strains probed with antibody to Gna1. WT, wild type. (A) Lines MC1 and MC2, carrying multiple copies of *tga1*. (B) Lines OE1 and OE2, expressing *tga1* from the *prb1* promoter. (C) Southern analysis of wild-type and transgenic strains carrying a mutant allele of *tga1*. Genomic DNAs from the two strains were digested with *EcoRI*, and the blot was probed with a 1.2-kb *EcoRV* genomic fragment. The

to eliminate the intrinsic GTPase activity of $G\alpha$, switching on constitutive signaling in the absence of receptor activation in animal cells (19). This construct was used to transform *Trichoderma*. Southern blot (Fig. 3C) and RT-PCR (Fig. 3D) analyses of two lines from a total of about 10 different hygromycin-resistant colonies confirmed integration and expression of the introduced construct, respectively. The RT-PCR strategy used was designed to amplify a region which includes an intron, enabling us to distinguish between amplification products derived from DNA and RNA. Additionally, the mutation generated results in the loss of an *MscI* site, allowing us to distinguish between the products of the wild-type and mutant genes and at the same time to detect its expression. *MscI* digestion of the RT-PCR products obtained from the mutant showed three bands (Fig. 3D). The upper band corresponds to the expected cDNA product of the mutant gene, which cannot be digested with *MscI*; the two additional bands correspond to the product of the wild-type gene. Colonies of these types of transformants showed a fluffy phenotype similar to that of the overexpressing strains. The Tga1 protein, however, was detected at lower levels than in the wild type, so that the phenotype of these strains must be attributable to the mutant gene rather than to overexpression (Fig. 3E).

In a first attempt to elucidate the function of Tga1, we used a gene disruption strategy. For this purpose, we constructed a plasmid derived from pTGAG in which the central coding region of *tga1* was replaced by a hygromycin resistance cassette from plasmid pCB1004. However, even after multiple transformation experiments and the use of different purification strategies, all transformants obtained either carried the construct integrated ectopically or contained more than one nucleus with at least one carrying a wild-type copy of the gene. Thus, to gain further insight into the role of Tga1 in *T. atroviride*, a vector carrying an antisense *tga1* cDNA under the control of the derepressible promoter of the *prb1* gene was constructed. This plasmid was used in cotransformation experiments together with plasmid pHat α , which confers hygromycin resistance. Ten hygromycin-resistant transformants were purified through monoconidial cultures and used for further characterization. Southern analysis confirmed the integration of the construct in the *Trichoderma* genome, characterized by the presence of the expected 1.3-kb *EcoRI* band in addition to the 9-kb band corresponding to the wild-type copy of the gene (data not shown).

An antibody raised against *N. crassa* $G\alpha$ Gna1 (26) was used

positions of molecular size markers are indicated at the right in kilobases. (D) Detection of expression of the mutant *tga1* allele. RNA was extracted and used as a template for RT and PCR amplification as described in Materials and Methods. The products were digested to completion with *MscI* or left undigested (U). The arrowhead indicates the expected cDNA product of the mutant gene, which cannot be digested with *MscI*. DNA molecular size markers are indicated at the right in kilobases. (E) Immunoblot analysis of transformants carrying the mutant *tga1* allele. Membrane protein fractions from wild-type and transgenic strains were analyzed as described in panel A. The arrow indicates the signal corresponding to Tga1. (F and G) Immunoblot analyses of transformants expressing *tga1* in the antisense (AS) form. Shown are Western blots of membrane fractions from the indicated transformants probed with antibody to Gna1 (F) or Gna2 (G) of *N. crassa*. MW, molecular weight, in thousands.

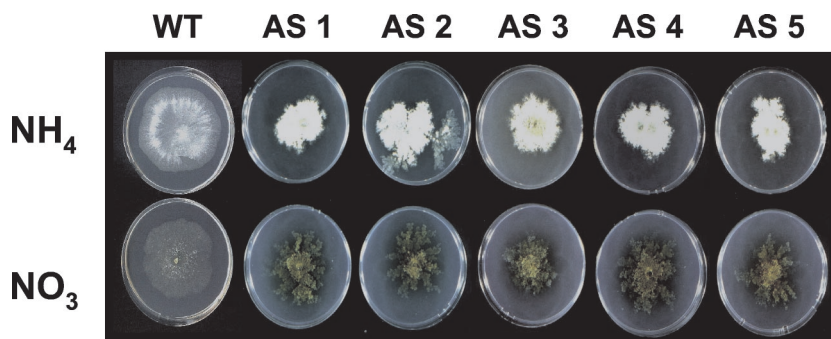


FIG. 4. Nitrogen source effects on phenotypes of *Trichoderma* transformants expressing *tga1* in the antisense (AS) form. *T. atroviride* and $G\alpha$ antisense transformants were grown on SM medium with 12.5 mM NH_4Cl or 12.5 mM KNO_3 as the sole nitrogen source and 0.5% glucose as the carbon source. Cultures were kept in light for 3 days at 28°C and photographed.

for immunoblot analysis of membrane fractions prepared from the different transformants (Fig. 3F). Antisense transformants (AS1, AS2, AS3, AS4, and AS5) showed no detectable Tga1 levels. There are only 17 amino acid substitutions between Gna1 and Tga1, so we expect the cross-reacting signal, which comigrates with the signal from *Neurospora* membranes at 40 kDa (data not shown), to correspond to Tga1. Probing of replicate blots with antibodies against Gna2, another *N. crassa* $G\alpha$ (1), gave similar signals in all the transformants (Fig. 3G). The signal obtained with anti-Gna2 indicates that *Trichoderma* has at least one more $G\alpha$ gene in addition to that for Tga1. Furthermore, this signal serves as an internal control for the amount of membrane protein loaded in each lane.

All 10 transformants showed a clearly aberrant phenotype, characterized by the formation of rather dense and small colonies, continuous sporulation, and the production of a yellowish pigment (data not shown). The phenotype of $G\alpha$ antisense transformants was most extreme on medium containing nitrate as a sole nitrogen source and was alleviated to some extent by growth on ammonium, as expected (Fig. 4).

Mas-7 activates Tga1 and raises cAMP levels. The activation of heterotrimeric G proteins can be monitored by measuring the intrinsic GTPase activity of $G\alpha$. By following the method used for animal cells (8), we developed an assay to detect high-affinity GTPase in fungal membrane fractions (C. Omero et al., unpublished data). In order to further characterize Tga1 and because mycoparasitic coiling is induced by the specific peptide toxin Mas-7, the membrane-associated GTPase activity in *Trichoderma* and its response to Mas-7 were analyzed. Figure 5A shows that GTPase activity increased in response to the application of Mas-7 in the wild-type strain, while no significant GTPase activity increase was detected for any of the antisense transgenic lines tested. Furthermore, basal GTPase activity was considerably lower (30 to 40%) in all antisense transformants than in the wild type. These experiments suggest that Tga1 is responsible for most of the Mas-7-inducible GTPase activity, since it was strongly decreased in antisense transformants (Fig. 5A). Mastoparan and Mas-7 are specific for the G_i and G_o classes of mammalian G-protein subunits, and the loss of Mas-7-stimulated GTPase activity in the antisense transformants is consistent with this fact, since Tga1 is most homologous to $G\alpha_i$.

Having obtained genetic evidence that Mas-7 is primarily a

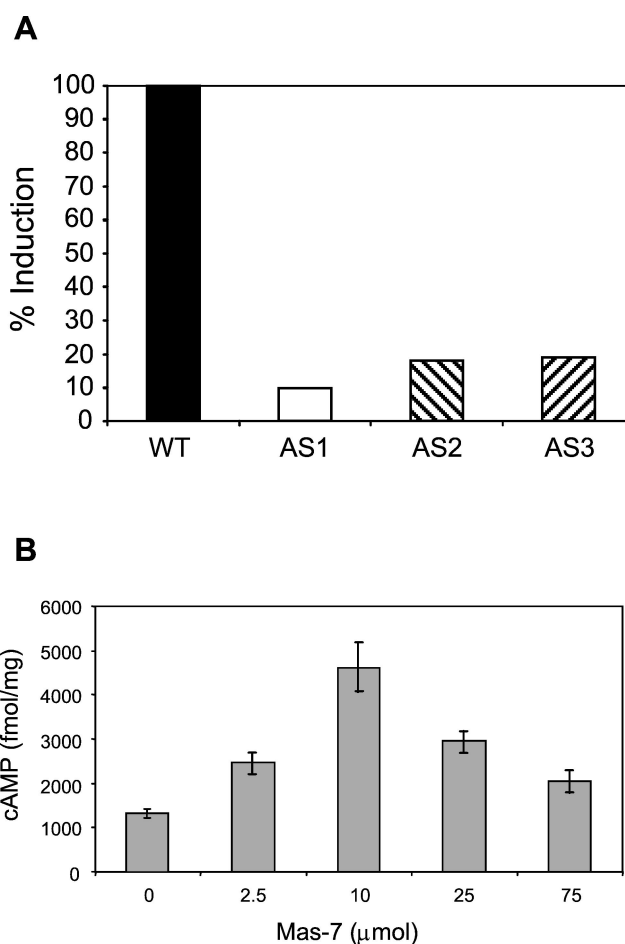


FIG. 5. Mas-7 activates Tga1 and raises cAMP levels. (A) Mas-7 activates GTPase activity. Membrane fractions from wild-type (WT) or Tga1 antisense (AS) lines (25 μg of protein) were assayed for high-affinity GTPase activity with or without the G-protein activator Mas-7. Radioactive phosphate released from $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ was assayed as described in Materials and Methods. Bars represent the difference between induced (with Mas-7) and noninduced (without Mas-7) GTPase activities relative to the wild type and indicate means of two or three independent experiments. (B) G-protein activators raise cAMP levels. The G-protein activator Mas-7 was applied to growing colonies under red safelight. After 2 h, the material was frozen and cAMP was assayed as described in Materials and Methods.

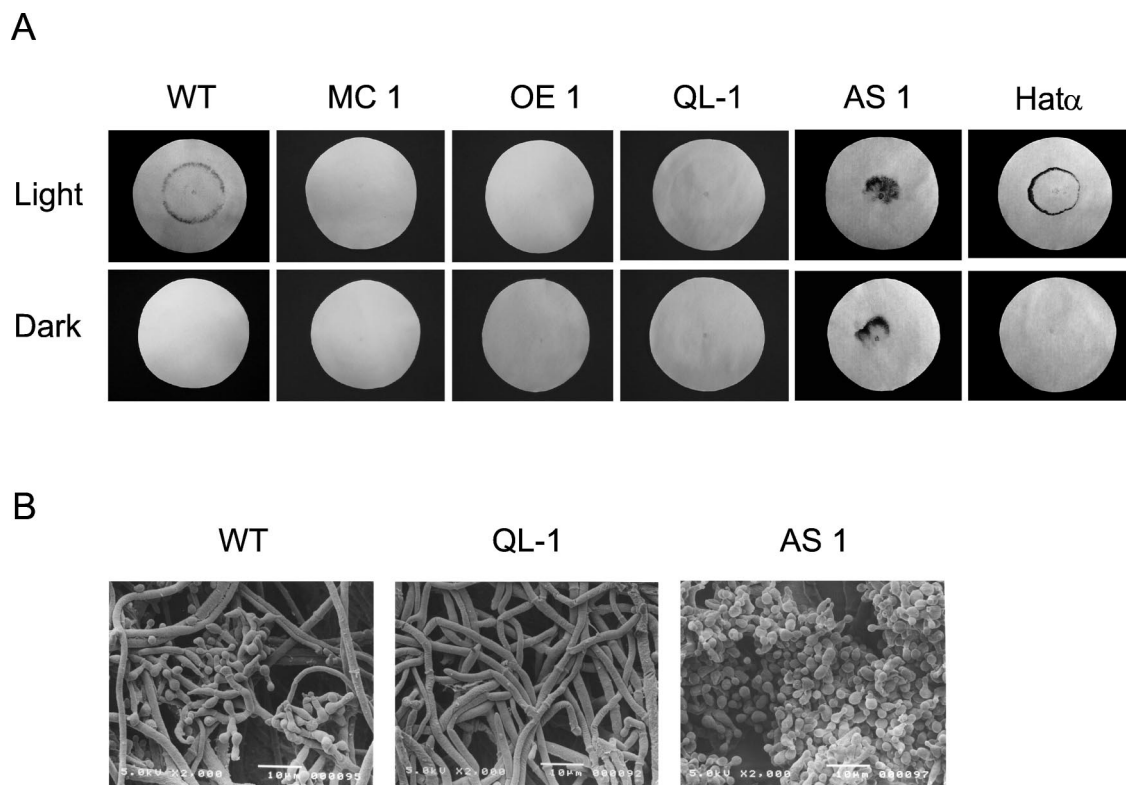


FIG. 6. Growth and developmental alterations in transgenic *Trichoderma* lines. (A) Photoinduction of conidiation in transgenic lines. Each sample was inoculated in the center of filter paper saturated with liquid medium (see Materials and Methods). After 36 h, the cultures were given a saturating pulse of blue light ($900 \mu\text{mol m}^{-2}$) (Light) or kept in total darkness (Dark); the cultures were then grown for a further 36 h in the dark, fixed in ethanol, and photographed. Shown are the wild type (WT); transgenic lines carrying multiple copies of *tga1* (MC 1), overexpressing *tga1* from the *prb1* promoter (OE 1), expressing a mutant Tga1 protein (QL-1), and expressing *tga1* in the antisense form (AS 1); and a control transgenic line transformed only with plasmid pHat α (Hata α). (B) Scanning electron microscopy analysis of transgenic lines expressing a mutant Tga1 protein (QL-1) and expressing *tga1* in the antisense form (AS 1) and the wild type (WT) as a control.

specific activator of Tga1, we tested the effect of Mas-7 on cAMP levels. The application of $7 \mu\text{M}$ to growing mycelial colonies resulted in more than a twofold increase in cAMP levels (Fig. 5B). By analogy with what is known for animal cells, this result suggests that the activation of Tga1 raises the activity of adenylyl cyclase, although other interpretations are possible (for example, activation of adenylyl cyclase by the G $\beta\gamma$ heterodimer released by the activation of Tga1 or inhibition of phosphodiesterase).

Transgenic *Trichoderma* lines with modified *tga1* expression levels or function show altered conidiation patterns. All transformants carrying multiple copies of *tga1* either under the control of its own promoter (MC1 and MC2) or under the control of the *prb1* promoter (OE1 and OE2) were used in photoinduced conidiation assays. In a typical assay, the wild-type strain produces a conidiation ring where the colony perimeter was located at the time of the light pulse. Conidia are mature by 36 h after the light pulse, while cultures kept in the dark do not produce conidia. None of the overexpressing lines produced a conidiation ring (Fig. 6A, MC1 and OE1). In contrast, the wild type and transformants carrying only the selectable vector (pHata α) formed conidia normally in response to light (Fig. 6A). Transgenic lines carrying the wild-type *tga1* gene and at least one copy of the mutant gene (QL-1) showed the same behavior as the overexpressing transformants in light-

induced conidiation experiments (Fig. 6A). These data suggest that Tga1 plays an important role in light-induced conidiation. A closer inspection of conidiation by scanning electron microscopy allowed us to determine that multicopy transformants produce a few conidiophores in response to the light stimulus, with a normal number of phalides per stalk and with a single conidium per phalide. Multicopy transformants eventually produced fully developed conidiophores and conidia. In contrast, transformants carrying the mutant *tga1* allele (QL-1) did not produce conidiophores at all (Fig. 6B).

Analyses of antisense transformants resulted in two distinct phenotypes. The most common phenotype was a constitutive sporulation pattern, with the presence of conidia over the whole colony, regardless of light stimulation (Fig. 6A, AS1) (data not shown for AS2, AS3, AS4, and AS5). The second phenotype was similar to that of the wild type, with a conidiation ring at the periphery of the colony (data not shown for AS6 and AS7). The transformants that produced a light-induced conidiation ring had Tga1 levels that appeared only slightly reduced compared to the wild-type level (data not shown). The phenotypes thus correlated well with the immunoblot analysis results and suggested variable levels of antisense expression and a consequent reduction of sense expression in different transformants. In addition, antisense transformants sporulated in both shaken and stationary cul-

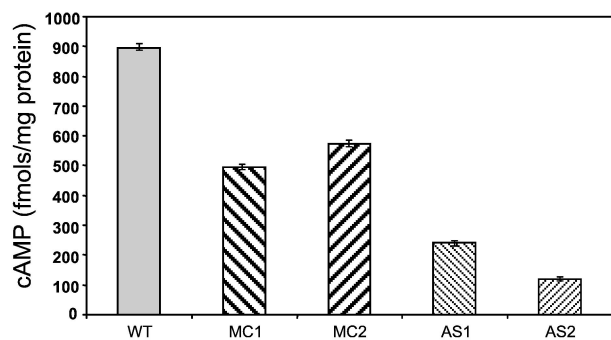


FIG. 7. Altered steady-state cAMP levels in transgenic lines. Bars indicate the cAMP levels detected in cultures of wild-type *Trichoderma* (WT) and *Trichoderma* transgenic lines carrying multiple copies of *tga1* (MC1 and MC2) or expressing *tga1* in the antisense form (AS1 and AS2).

tures, conditions under which the wild-type strain does not produce spores. In a closer analysis of conidiation by scanning electron microscopy, we were able to corroborate the hypersporulating phenotype of antisense transformants (Fig. 6B, AS1) and to determine that they produced many more phyalides per stalk (data not shown).

Altered cAMP levels in Tga1 transgenic lines. A likely effector of Tga1 is cAMP, since the application of cAMP stimulated coiling (47) (Fig. 2) and the activation of Tga1 with Mas-7 raised cAMP levels (Fig. 5). We therefore tested whether genetic manipulation of Tga1 affected cAMP levels. Both overexpressing and antisense lines had lower steady-state levels of cAMP than the wild type, but the overexpressing lines had moderately decreased cAMP levels while the antisense lines had much lower cAMP levels (Fig. 7). The reason for the moderately decreased cAMP levels in the overexpressing lines is not clear, but it is important to note that the overexpressed alpha subunit may titrate the beta-gamma heterodimer, which could also be an activator of adenylyl cyclase. The very low cAMP levels in the antisense lines may be due to a loss of adenylyl cyclase activation by Tga1 but may also be a secondary effect as well—a result of, rather than a cause of, the intense sporulation of these lines.

Role of Tga1 in mycoparasitism. We have shown that Mas-7 (or mastoparan) (47) induces the coiling of *Trichoderma* around nylon fibers (Fig. 2) and that Mas-7-induced GTPase activity is strongly decreased in antisense transformants (Fig. 4). Thus, we used the biomimetic system to test the possible participation of Tga1 in mycoparasitic coiling. Overexpressing transformants (MC1 and MC2) showed an increase of more than twofold in coiling, compared to the wild type (Fig. 8). Furthermore, coiling was also increased in transformants carrying the constitutively active Q203L allele (Fig. 6). In contrast, two antisense transformants that were typical of the extreme hypersporulation phenotype and whose Tga1 levels were below detection by immunoblotting (Fig. 3F) showed markedly decreased coiling around the nylon fibers (Fig. 8). Although their intense hypersporulation, even in the presence of ammonium in the medium, might have masked any response, these data strongly suggest that *tga1* plays an important role in mycoparasitic coiling.

A common test for the selection of *Trichoderma* strains with

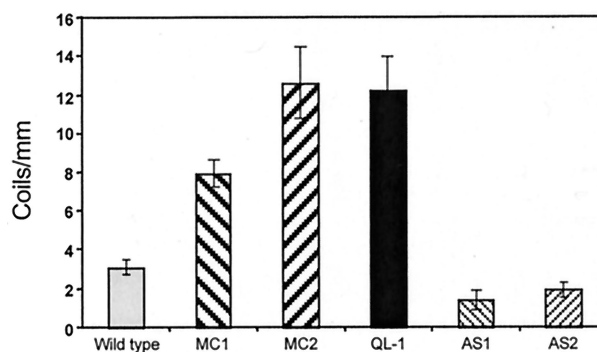


FIG. 8. Hyphal coiling around nylon fibers. The wild type and transformants overexpressing *tga1* (MC1 and MC2) or carrying the constitutively active Q203L allele (QL-1) or an antisense copy of *tga1* (AS1 and AS2) were grown on cellophane overlaying standard complete medium and allowed to overgrow grids with nylon fibers. The number of coils per millimeter of fiber was counted (see Materials and Methods) on scanning electron microscopy images of the cultures.

high biocontrol capacity is a direct confrontation with the host in dual cultures. To test the impact of the alteration of the expression of Tga1 in vivo, we established direct confrontation assays with *Trichoderma* and *R. solani*. Figure 9 shows that there was an impressive increase in the capacity of the parasite to overgrow its host when Tga1 was overexpressed (Fig. 9B), compared to the results for the nontransformed strain (Fig. 9A). In contrast, antisense transformants with no detectable levels of Tga1 protein (Fig. 3F) were unable to overgrow *R. solani* (Fig. 9C). These data suggest that Tga1 plays a major role in mycoparasitism.

Higher Tga1 levels are thus correlated with an increased capacity to overgrow the host. If Tga1 stimulates adenylyl cyclase, then one might predict that in the presence of the host, cAMP levels, PKA activity, and mycoparasitism-related gene expression all will be increased.

It has been shown that the genes *prb1* (encoding a basic protease) and *ech42* (encoding an endochitinase) play relevant roles in the biocontrol capacity of *Trichoderma* and that their expression is triggered during the interaction with the host (7, 13). Thus, we decided to test whether the activation of the expression of these genes correlated with the activation of a cAMP signaling pathway. Figure 10A shows the analysis of the expression of *prb1* and *ech42* during a direct confrontation assay with *R. solani*, where contact was avoided by the use of a cellophane membrane placed between the two fungi. As indicated in the autoradiographs, high levels of expression of both genes were obtained at the time of sampling, while their expression was nearly undetectable when *Trichoderma* was grown alone. Measurements of cAMP levels in the samples showed high cAMP levels when *Trichoderma* was grown in the absence of the host (Fig. 10B, T). Interestingly, cAMP levels dropped to nearly one-fourth when the parasite was grown in the presence of the host or just diffusible factors produced by it (Fig. 10B, TRt and TRd). Furthermore, cAMP-dependent protein kinase activity levels decreased in proportion to cAMP levels when *Trichoderma* was grown in the presence of the host, compared to the activity levels detected when the mycoparasite was grown alone (Fig. 10C). If Tga1 is responsible for the

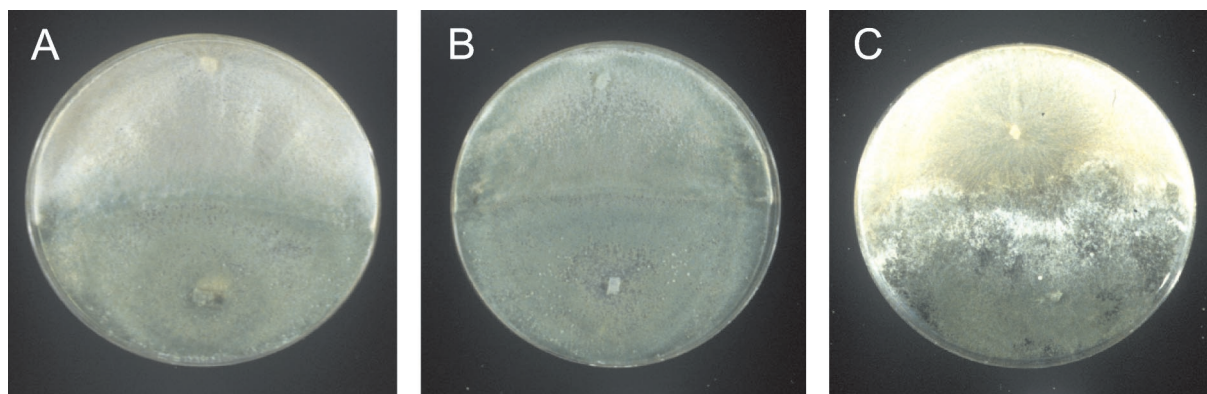


FIG. 9. Direct confrontation assays of wild-type and transgenic *Trichoderma* against *R. solani*. Overgrowth of *Rhizoctonia* by the different *Trichoderma* strains is evident from the production of green conidia. (A) Wild type. (B and C) Transgenic lines carrying multiple copies of *tga1* (MC1) or expressing *tga1* in the antisense form (AS1), respectively.

induction of *ech42* and *prb1* gene expression, it does not do so by increasing intracellular cAMP levels. Our data therefore suggest that these two responses to the host use distinct signaling pathways.

DISCUSSION

During the initial stages of mycoparasitism, *Trichoderma* attaches to and coils around the host. The signals for this developmental route are presumably cues immobilized on the host surface (23, 24), diffusible factors released from the host upon attack by *Trichoderma* (11), or both. While the receptors for these signals are likely to be very specific, eukaryotic signal transducers such as heterotrimeric G proteins are evolutionarily conserved from fungi to humans (5, 16, 44). In phytopathogenic fungi, G-protein and cAMP signaling pathways have been shown to play a major role in pathogenicity and, in particular, in the formation of invasive structures. *Trichoderma* hyphae coil around lectin-coated nylon fibers in the absence of a host (23). The G-protein activators fluoroaluminate, mastoparan, and MAS-7 trigger *Trichoderma* attachment to and coiling around noncoated nylon fibers (47) (Fig. 2). Since mastoparan is specific for the G_i and G_o classes of $G\alpha$ in animal cells, we postulated that a G protein of this type could transduce the signals for coiling. Furthermore, because it replaced the lectin signal, cAMP might be the second messenger.

We have cloned, characterized, and obtained loss- and gain-of-function mutations for the *tga1* gene encoding $G\alpha$ of *T. atroviride*. A sequence comparison shows that the *T. atroviride* Tga1 protein belongs to the $G\alpha_i$ superfamily. This group includes the corresponding $G\alpha$ of *Coprinus congregatus* (Gpa1) (32), *N. crassa* (Gna1) (61), *A. nidulans* (FadA) (64), *Cryphonectria parasitica* (Cpg-1) (10), *Magnaporthe grisea* (MagB) (38), *Ustilago maydis* (Gpa1) (50), *Cochliobolus heterostrophus* (Cga1) (22), and *Colletotrichum trifolii* (60). All of these proteins show a molecular mass (determined by immunoblotting and/or calculated from the sequence) of approximately 40 kDa, and all of the corresponding genes have at least two introns in identical positions which are conserved in mammalian $G\alpha_i$ genes (22). In addition, all of them contain putative N-myristoylation and ADP-ribosylation sites for pertussis and cholera

toxins. Southern analysis under high-stringency conditions indicated the presence of a single $G\alpha$ gene, but low-stringency hybridization suggested the presence of at least one more homologous gene (data not shown). Further evidence for another $G\alpha$ gene comes from immunoblot analysis, which revealed one protein similar to Gna1 of *N. crassa* and a second reacting with antiserum raised against Gna2 of *N. crassa*.

Since we have no viable deletion mutants, sense expression and antisense expression were used to manipulate the level of Tga1. Loss of Tga1 promoted intense sporulation, while overexpression or an activated allele inhibited sporulation and promoted coiling. Activation of the *A. nidulans* homolog of Tga1 or loss of its regulator, FlbA, likewise inhibits sporulation and promotes hyphal proliferation (55). In *A. nidulans*, activated $G\alpha$ (FadA) promotes active growth and inhibits sporulation (21, 55, 64). Our findings are consistent with the model proposed for *Aspergillus*. Loss of Tga1 would release the inhibition of sporulation normally mediated by the active GTP-bound form of $G\alpha_i$. The activated Q203L allele or overexpression of free Tga1 would have the opposite effect, promoting vegetative proliferation. Decreased sporulation and increased proliferation of hyphae may themselves be sufficient to account for the increase in coiling in both kinds of gain-of-function experiments. In contrast, loss of Tga1 due to antisense expression results in profusely sporulating and slowly growing colonies.

To reach a better understanding of how Tga1 acts to modulate sporulation and coiling, we adapted a high-affinity GTPase assay from the animal cell literature. This assay, combined with Western blot data, confirmed that Tga1 is reduced to very low levels in the antisense transformants, that other types of $G\alpha$ are probably unaffected by the antisense transgene, and that Mas-7 is specific to the $G\alpha_i$ class, as for mammalian G proteins. If Tga1 can activate adenylyl cyclase, then one would expect that the application of Mas-7 can raise intracellular cAMP levels, and this is indeed the case.

Steady-state cAMP levels have been used to infer whether fungal $G\alpha$ activates or inhibits adenylyl cyclase. The cAMP levels were moderately reduced in *Trichoderma* overexpressing lines and greatly reduced in antisense lines. Studies with other fungi, such as *M. grisea* (38), *N. crassa* (61), and *C. trifolii* (60), have suggested positive regulation of adenylyl cyclase by $G\alpha_i$.

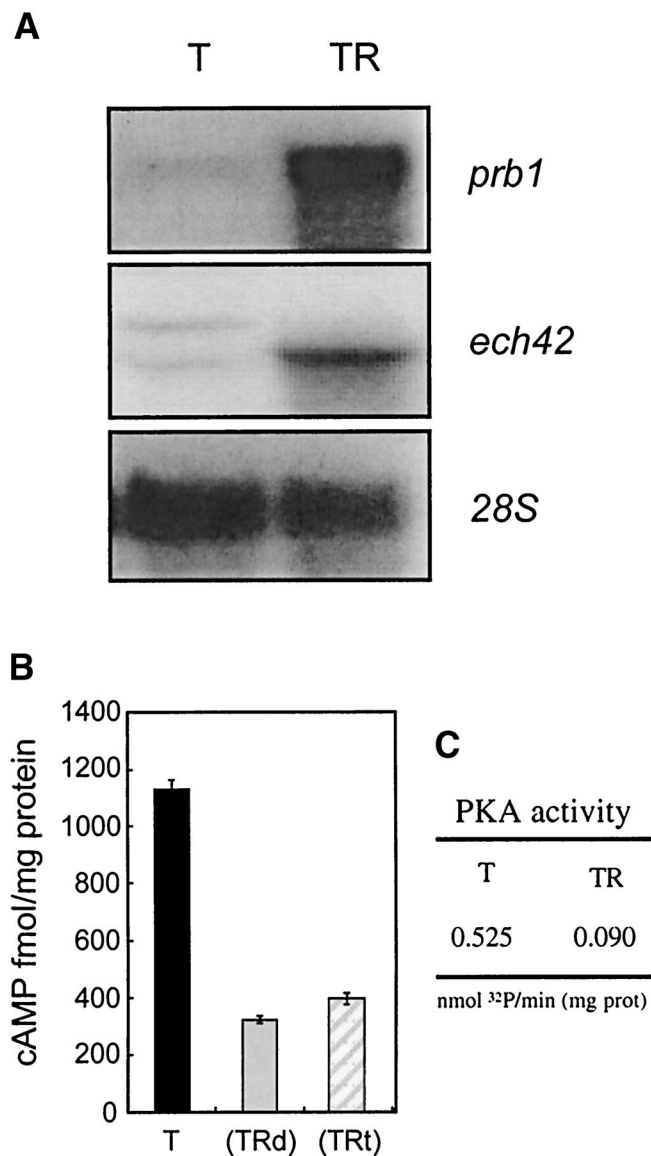


FIG. 10. Analysis of cAMP levels and cAMP-dependent protein kinase activity in confrontation experiments. (A) Northern analysis showing the levels of expression of two mycoparasitism-related genes (*prb1* and *ech42*) at the time when samples were collected for measurements of cAMP levels and cAMP-dependent protein kinase activity. T, *Trichoderma* growing alone; TR, *Trichoderma* growing on top of *Rhizoctonia*. (B) cAMP levels detected in cultures of *Trichoderma* growing alone (T), *Trichoderma* exposed to diffusible factors produced by *Rhizoctonia* (TRd), or *Trichoderma* growing on top of *Rhizoctonia* (TRt). (C) cAMP-dependent protein kinase activity measured in cultures of *Trichoderma* growing alone (T) or *Trichoderma* growing on top of *Rhizoctonia* (TR). PKA, protein kinase A; prot, protein. The results shown are those of a typical experiment out of three carried out.

homologs as well as by some members of the other fungal G α classes (33, 42). Gna3 of *N. crassa* appears to regulate post-transcriptionally the level of adenylyl cyclase (29). *C. parasitica* is an exception, in that CPGA1 null mutants display an increase in cAMP levels, suggesting that the G protein inhibits adenylyl cyclase or that this enzyme is stimulated by G $\beta\gamma$ (14). Mammalian adenylyl cyclase isoforms differ in their regulatory

sites and properties (57), so more information about the fungal adenylyl cyclases is needed before a general model is produced.

Although steady-state cAMP levels are likely to depend on cellular factors in addition to G α signaling, we can infer that the decreased sporulation and the increased coiling in overexpressing lines cannot be the result of increased steady-state levels of cAMP, because cAMP levels are somewhat lower in these strains than in wild-type controls. Thus, Tga1 has a cAMP-independent role in promoting coiling, consistent with the current model for *Aspergillus* (55). Precedent for this interpretation can be found in the animal cell literature; for example, the overexpression of stimulatory G α in transgenic mice enhanced L-type calcium channels through an adenylyl cyclase-independent route (37). Increased sporulation in our antisense lines could, on the other hand, result from the drastically decreased cAMP levels, consistent with hypersporulation in *Aspergillus* mutants lacking the catalytic PKA subunit gene *pkaA* (55). The hallmark of all the cases where the loss of a fungal G α gene decreases cAMP levels is that exogenous cAMP may rescue some or all of the G α mutant phenotypes. We found that exogenous cAMP did not rescue the phenotypes of the *Trichoderma* Tga1 antisense lines (data not shown). Again, this finding could have been the result of cAMP-independent signaling by Tga1. The corresponding experiments with *Aspergillus*, i.e., the application of cAMP or PKA overexpression in a G α_i (FadA) loss-of-function background, could shed light on this question in a genetically well-defined model system. A further element in the scheme is the release of G $\beta\gamma$ upon activation of the heterotrimer. Overexpression of G α would be expected to titrate G $\beta\gamma$, diminishing any G $\beta\gamma$ signal. Expression of the activated Q203L allele, in contrast, should not affect the availability of G $\beta\gamma$. The similarity in the phenotypes conferred by overexpression and by the activated allele suggests that G $\beta\gamma$ does not play a major role in sporulation and coiling.

In *Trichoderma*, light, which induces sporulation, triggers a transient biphasic oscillation in intracellular cAMP levels followed by the start of an upward trend (17). Exogenous cAMP promotes sporulation in the dark (45), and light results in the activation of adenylyl cyclase (31). This scenario appears difficult to reconcile with the decreased cAMP levels in the hypersporulating antisense lines. It is important to note, however, that transient activation and a steady-state low level are quite different effects. The induction and development of sporulation in *Trichoderma* are distinct events, separable by low temperature or nutrient limitation, and each may be under the control of different cAMP signals. It is development, rather than induction, that is studied in *Aspergillus*, as well as in our antisense lines.

All the data relevant to mycoparasitism might be arranged in a linear pathway: lectins or other signals, activation of Tga1, increased cAMP levels, activation of PKA, coiling, and gene expression. Some experiments were done as an initial test of this simple model. At the input of the postulated pathway, it was suggested that host lectins play a major role in attachment and that lectins may determine host specificity (24). Further research directed to investigating the role of host lectins in the mycoparasitic process showed that the expression of at least one chitinase is triggered by the detection of lectins in a bio-

mimetic assay (25). These data led to the suggestion that a lectin-carbohydrate recognition event could be the signal that triggers a general parasitic response. Using a variety of lectins to coat nylon fibers for use in biomimetic experiments, we have been able to show that lectins indeed promote attachment to and coiling around the fibers. The fact that all lectins tested induced coiling, regardless of their sugar moiety, suggests that they are not the specificity-determining factor in host recognition by *Trichoderma*. However, different lectins induced to different degrees (Fig. 1). Thus, it is still possible that saturating amounts of any lectin can produce similar effects but that, under nonsaturating conditions, the lectins may show higher degrees of specificity. Attachment requires the use of a solid support, and under these conditions, it is, unfortunately, not possible to control the amount of lectin linked to the support. Furthermore, *Trichoderma* may carry receptors capable of recognizing a wide spectrum of lectins; it has a wide host range and attacks fungi with disparate cell surface compositions.

Because one of our goals was to determine the role of *tga1* in mycoparasitism, we used our transgenic lines in direct confrontation assays with a host, a common procedure in the selection of strains with high biocontrol capacity. Transformed lines overexpressing Tga1 showed an impressive increase in the capacity of the fungus to overgrow the host, compared to the capacity of the wild type. In contrast, lines blocked in the production of Tga1 were unable to overgrow the host (Fig. 9). Taken together, our data suggest a major role for Tga1 in mycoparasitism.

Furthermore, we determined the levels of cAMP and cAMP-dependent protein kinase activity under conditions where mycoparasitism-related genes are expressed (Fig. 10A). Our results showed in both cases behavior exactly opposite that predicted from the simple linear model outlined above, with decreasing levels of cAMP (and, correspondingly, decreasing PKA activity) during the interaction with the host. Thus, either coiling uses a signaling pathway different from that used to trigger the expression of mycoparasitism-related genes or there is a sequential activation allowing gene expression only when cAMP levels and PKA activity are low again. We can conclude that, although several more signaling elements would need to be studied in *Trichoderma*, the signaling pathway to mycoparasitic development is not a single linear sequence. This conclusion is expected by analogy with genetic model systems such as *Aspergillus* and yeasts.

In budding yeast, a combination of cAMP-dependent (heterotrimeric G protein) and cAMP-independent (Ras-mitogen-activated protein kinase) pathways controls filamentous growth (34, 40, 43, 48, 52). Although a budding yeast cannot follow as many morphogenetic pathways as can a filamentous fungus, its signaling pathways can provide a basic biochemical model with which to understand the development of filamentous fungi, as proposed by Banuett (2). From an evolutionary point of view, invasive growth of yeasts and mycoparasitic coiling by filamentous fungi may be related. Both may be triggered by nutrient status and host signals.

ACKNOWLEDGMENTS

We are grateful to Edna Cukierman for some of the initial PCR experiments, to Katherine Borkovich for antisera to *Neurospora* G

proteins, and to June Simpson and Plinio Guzmán for critical reading of the manuscript.

This work was supported in part by grants from Conacyt (31790N) and ICGEB (CRP/MEX99-02) to A.H.-E., from the Israel Academy of Sciences (ISF) to B.A.H., and from BARD to I.C.

Víctor Rocha-Ramírez and Carmi Omero contributed equally to this work.

REFERENCES

1. Baasiri, R. A., X. Lu, P. S. Rowley, G. E. Turner, and K. A. Borkovich. 1997. Overlapping functions for two G protein α subunits in *Neurospora crassa*. *Genetics* **147**:137–145.
2. Banuett, F. 1998. Signalling in the yeasts: an informational cascade with links to the filamentous fungi. *Microbiol. Mol. Biol. Rev.* **62**:249–274.
3. Barak, R., and I. Chet. 1990. Lectin of *Sclerotium rolfsii*: its purification and possible function in fungal interaction. *J. Appl. Bacteriol.* **69**:101–112.
4. Barak, R., Y. Elad, D. Mirelman, and I. Chet. 1985. Lectins: a possible basis for specific recognition in the interaction of *Trichoderma* and *Sclerotium rolfsii*. *Phytopathology* **75**:458–462.
5. Boelker, M. 1998. Sex and crime: heterotrimeric G protein in fungal mating and pathogenesis. *Fungal Genet. Biol.* **25**:143–156.
6. Buss, J. E., S. E. Mumby, P. J. Casey, A. G. Gilman, and B. M. Sefton. 1987. Myristoylated alpha subunits of guanine nucleotide-binding regulatory proteins. *Proc. Natl. Acad. Sci. USA* **84**:7493–7497.
7. Carsolio, C., N. Benhamou, S. Haran, C. Cortés, A. Gutiérrez, I. Chet, and A. Herrera-Estrella. 1999. Role of the *Trichoderma harzianum* endochitinase gene, *ech42*, in mycoparasitism. *Appl. Environ. Microbiol.* **65**:929–935.
8. Cassel, D., and Z. Selinger. 1976. Catecholamine-stimulated GTPase activity in turkey erythrocyte membranes. *Biochim. Biophys. Acta* **452**:538–551.
9. Chet, I. 1987. *Trichoderma*—application, mode of action, and potential as a biocontrol agent of soilborn plant pathogenic fungi, p. 137–160. *In* I. Chet (ed.), *Innovative approaches to plant disease control*. John Wiley & Sons, Inc., New York, N.Y.
10. Chen, B., S. Gao, G. H. Choi, and D. L. Nuss. 1996. Extensive alteration of fungal gene transcript accumulation and elevation of G-protein-regulated cAMP levels by a virulence-attenuating hypovirus. *Proc. Natl. Acad. Sci. USA* **93**:7996–8000.
11. Cortés, C., A. Gutiérrez, V. Olmedo, J. Inbar, I. Chet, and A. Herrera-Estrella. 1998. The expression of genes involved in parasitism by *Trichoderma harzianum* is triggered by a diffusible factor. *Mol. Gen. Genet.* **260**: 218–225.
12. Dutton, J. R., S. Johns, and B. L. Miller. 1997. StuAp is a sequence-specific transcription factor that regulates developmental complexity in *Aspergillus nidulans*. *EMBO J.* **16**:5710–5721.
13. Flores, A., I. Chet, and A. Herrera-Estrella. 1997. Improved biocontrol activity of *Trichoderma harzianum* strains by overexpression of the proteinase encoding gene *prb1*. *Curr. Genet.* **31**:30–37.
14. Gao, S., and D. L. Nuss. 1996. Distinct roles for two G protein α subunits in fungal virulence, morphology, and reproduction revealed by targeted gene disruption. *Proc. Natl. Acad. Sci. USA* **93**:14122–14127.
15. Geremia, R. A., G. Goldman, D. Jacobs, W. Ardiles, M. Vila, M. Van Montagu, and A. Herrera-Estrella. 1993. Molecular characterization of a proteinase-encoding gene, *prb1*, related to mycoparasitism by *Trichoderma harzianum*. *Mol. Microbiol.* **6**:1231–1242.
16. Gilman, A. G. 1987. G proteins: transducers of receptor-generated signals. *Annu. Rev. Biochem.* **56**:615–649.
17. Gresik, M., N. Kolarova, and V. Farkas. 1988. Membrane potential, ATP, and cyclic AMP changes induced by light in *Trichoderma viride*. *Exp. Mycol.* **12**:295–301.
18. Harper, J. F., and G. Brooker. 1975. Femtomole sensitive radioimmunoassay for cyclic AMP and cyclic GMP after 2'-O-acetylation by acetic anhydride in aqueous solutions. *J. Cyclic Nucleotide Res.* **1**:207–218.
19. Hermouet, S., J. J. Merendino, J. S. Gutkind, and A. M. Spiegel. 1991. Activating and inactivating mutations of the α subunit of G_{12} protein have opposite effects on proliferation of NIH 3T3 cells. *Proc. Natl. Acad. Sci. USA* **88**:10455–10459.
20. Herrera-Estrella, A., G. H. Goldman, and M. Van Montagu. 1990. High-efficiency transformation system for the biocontrol agents, *Trichoderma* spp. *Mol. Microbiol.* **4**:839–843.
21. Hicks, J. K., J.-H. Yu, N. P. Keller, and T. H. Adams. 1997. *Aspergillus* sporulation and mycotoxin production both require inactivation of the FadA G protein-dependent signaling pathway. *EMBO J.* **16**:4916–4923.
22. Horwitz, B. A., A. Sharon, S. Lu, V. Ritter, T. M. Sandrock, O. C. Yoder, and B. G. Turgeon. 1999. A G protein alpha subunit from *Cochliobolus heterostrophus* involved in mating and appressorium formation. *Fungal Genet. Biol.* **26**:19–32.
23. Inbar, J., and I. Chet. 1992. Biomimics of fungal cell-cell recognition by use of lectin-coated nylon fibers. *J. Bacteriol.* **174**:1055–1059.
24. Inbar, J., and I. Chet. 1994. A newly isolated lectin from the plant pathogenic fungus *Sclerotium rolfsii*: purification, characterization and role in mycoparasitism. *Microbiology* **140**:651–657.

25. Inbar, J., and I. Chet. 1995. The role of recognition in the induction of specific chitinases during mycoparasitism by *Trichoderma harzianum*. *Microbiology* **141**:2823–2829.
26. Ivey, F. D., P. N. Hodge, G. E. Turner, and K. A. Borkovich. 1996. The G α_i homologue *gna-1* controls multiple differentiation pathways in *Neurospora crassa*. *Mol. Biol. Cell* **7**:1283–1289.
27. Jones, J. D. G. P. Dunsmuir, and J. Bedbrook. 1985. High level expression of induced chimeric genes in regenerated transformed plants. *EMBO J.* **4**:2411–2418.
28. Kasahara, S., and D. L. Nuss. 1997. Targeted disruption of a fungal G-protein α subunit gene results in increased vegetative growth but reduced virulence. *Mol. Plant-Microbe Interact.* **10**:984–993.
29. Kays, A. M., P. S. Rowlley, R. A. Baasiri, and K. A. Borkovich. 2000. Regulation of conidiation and adenyl cyclase levels by the G alpha protein GNA-3 in *Neurospora crassa*. *Mol. Cell. Biol.* **20**:7693–7705.
30. Kobayashi, N., and K. McEntee. 1993. Identification of *cis* and *trans* components of a novel heat shock stress regulatory pathway in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **13**:248–256.
31. Kolarova, N., J. Haplova, and M. Gresik. 1992. Light-activated adenyl cyclase from *Trichoderma viride*. *FEMS Microbiol. Lett.* **72**:275–278.
32. Kozak, K. R., M. F. Lyon, I. K. Ross, and K. R. Ian. 1995. Cloning and characterization of a G protein α -encoding gene from the basidiomycete, *Coprinus congregatus*. *Gene* **163**:133–137.
33. Kruger, J., G. Loubradou, E. Regenfelder, A. Hartmann, and R. Kahmann. 1998. Crosstalk between cAMP and pheromone signalling pathways in *Ustilago maydis*. *Mol. Gen. Genet.* **260**:193–198.
34. Kubler, E., H. U. Mosch, S. Rupp, and M. P. Lisanti. 1997. Gpa2p, a G-protein α -subunit, regulates growth and pseudohyphal development in *Saccharomyces cerevisiae* via a cAMP-dependent mechanism. *J. Biol. Chem.* **272**:20321–20323.
35. Kuhls, K., E. Lieckfeldt, G. J. Samuels, W. Kovacs, W. Meyer, O. Petrini, W. Gams, T. Borner, and C. P. Kubicek. 1996. Molecular evidence that the asexual industrial fungus *Trichoderma reesei* is a clonal derivative of the ascomycete *Hypocrea jecorina*. *Proc. Natl. Acad. Sci. USA* **93**:7755–7760.
36. Kullnig, C., R. L. Mach, M. Lorito, and C. P. Kubicek. 2000. Enzyme diffusion from *Trichoderma atroviride* (= *T. harzianum* P1) to *Rhizoctonia solani* is a prerequisite for triggering of *Trichoderma ech42* gene expression before mycoparasitic contact. *Appl. Environ. Microbiol.* **66**:2232–2234.
37. Lader, A. S., Y. F. Xiao, Y. Ishikawa, Y. Cui, D. E. Vatner, S. F. Vatner, C. J. Homcy, and H. F. Cantiello. 1998. Cardiac G α overexpression enhances L-type calcium channels through an adenyl cyclase independent pathway. *Proc. Natl. Acad. Sci. USA* **95**:9669–9674.
38. Liu, S., and R. A. Dean. 1997. G protein α subunit genes control growth: development, and pathogenicity of *Magnaporthe grisea*. *Mol. Plant-Microbe Interact.* **10**:1075–1086.
39. Lochrie, M. A., J. E. Mendel, P. W. Sterberg, and M. I. Simon. 1991. Homologous and unique G protein alpha subunits in the nematode *Caenorhabditis elegans*. *Cell Regul.* **2**:135–154.
40. Lorenz, M. C., and J. Heitman. 1997. Yeast pseudohyphal growth is regulated by GPA2, a G protein homolog. *EMBO J.* **16**:7008–7018.
41. Lorito, M., C. K. Hayes, A. Di Pietro, and G. E. Harman. 1993. Biolistic transformation of *Trichoderma harzianum* and *Gliocladium virens* using plasmid and genomic DNA. *Curr. Genet.* **24**:349–356.
42. Loubradou, G., J. Beguret, and B. Turcq. 1999. MOD-D, a G alpha subunit of fungus *Podospora anserina*, is involved in both regulation of development and vegetative incompatibility. *Genetics* **152**:519–528.
43. Mösch, H. U., R. L. Roberts, and G. R. Fink. 1996. Ras2 signals via the Cdc42/Ste20/mitogen-activated protein kinase module to induce filamentous growth in *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. USA* **93**:5352–5356.
44. Neer, E. J., and D. E. Clapham. 1988. Roles of G protein subunits in transmembrane signalling. *Nature* **333**:129–134.
45. Nemicovic, M., and V. Farkas. 1998. Stimulation of conidiation by derivatives of cAMP in *Trichoderma viride*. *Folia Microbiol.* **43**:399–402.
46. Ofek, I., I. Kahane, and N. Sharon. 1996. Toward anti-adhesion therapy for microbial diseases. *Trends Microbiol.* **4**:297–299.
47. Omero, C., J. Inbar, V. Rocha-Ramírez, A. Herrera-Estrella, I. Chet, and B. A. Horwitz. 1999. G protein activators and cAMP promote mycoparasitic behavior in *Trichoderma harzianum*. *Mycol. Res.* **103**:1637–1642.
48. Pan, X., and J. Heitman. 1999. Cyclic AMP-dependent protein kinase regulates pseudohyphal differentiation in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **19**:4874–4887.
49. Raeder, U., and P. Broda. 1989. Rapid preparation of DNA from filamentous fungi. *Lett. Appl. Microbiol.* **1**:17–20.
50. Regenfelder, E., T. Spellig, A. Hartmann, S. Lauenstein, M. Boelker, and R. Kahmann. 1997. G proteins in *Ustilago maydis*: transmission of multiple signals? *EMBO J.* **16**:1934–1942.
51. Rosen, S., J. H. Yu, and T. H. Adams. 1999. The *Aspergillus nidulans* *sfaD* gene encodes a G protein beta subunit that is required for normal growth and repression of sporulation. *EMBO J.* **18**:5592–5600.
52. Rupp, S., E. Summers, H. J. Lo, H. Madhani, and G. Fink. 1999. MAP kinase and cAMP filamentation signaling pathways converge on the unusually large promoter of the yeast FLO11 gene. *EMBO J.* **18**:1257–1269.
53. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: a laboratory manual*, 2nd ed., vol. 1. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
54. Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**:5463–5467.
55. Shimizu, K., and N. P. Keller. 2001. Genetic involvement of a cAMP-dependent protein kinase in a G protein signaling pathway regulating morphological and chemical transitions in *Aspergillus nidulans*. *Genetics* **157**:591–600.
56. Strathmann, M., T. M. Wilkie, and M. I. Simon. 1989. Diversity of the G-protein family: sequences from five additional α subunits in the mouse. *Proc. Natl. Acad. Sci. USA* **86**:7407–7409.
57. Taussig, R., W. J. Tang, J. R. Hepler, and A. G. Gilman. 1994. Distinct patterns of bidirectional regulation of mammalian adenyl cyclases. *J. Biol. Chem.* **269**:6093–6100.
58. Tilburn, J., S. Sarkar, D. A. Widdick, E. A. Espeso, M. Orejas, J. Mungroo, M. A. Peñalva, and H. N. Arst. 1995. The *Aspergillus* PacC zinc finger transcription factor mediates regulation of both acid- and alkaline-expressed genes by ambient pH. *EMBO J.* **14**:779–790.
59. Tomes, D. T., M. C. Ross, and D. D. Songstad. 1995. Direct DNA transfer into intact plant cells via microprojectile bombardment, p. 197–213. *In* O. L. Gamborg and G. C. Phillips (ed.), *Plant cell, tissue and organ culture: fundamental methods*. Springer-Verlag, Berlin, Germany.
60. Truesdell, G. M., Z. Yang, and M. B. Dickman. 2000. A G α subunit gene from the phytopathogenic fungus *Colletotrichum trifolii* is required for conidial germination. *Physiol. Mol. Plant Pathol.* **56**:131–140.
61. Turner, G. E., and K. A. Borkovich. 1993. Identification of a G protein α subunit from *Neurospora crassa* that is a member of the Gi family. *J. Biol. Chem.* **268**:14805–14811.
62. West, J. R., E. J. Moss, M. Vaughan, and T. Liu. 1985. Pertussis toxin-catalyzed ADP-ribosylation of transducin. *J. Biol. Chem.* **260**:14428–14430.
63. Wilkie, T. M., P. A. Scherle, M. P. Strathmann, V. Z. Slepak, and M. I. Simon. 1991. Characterization of G-protein alpha subunits in the Gq class: expression in murine tissues and in stromal and hematopoietic cell lines. *Proc. Natl. Acad. Sci. USA* **88**:10049–10053.
64. Yu, J. H., J. Wieser, and T. H. Adams. 1996. The *Aspergillus* FlbA RGS domain protein antagonizes G protein signaling to block proliferation and allow development. *EMBO J.* **15**:5184–5190.