Glyceraldehyde-3-phosphate dehydrogenase expression in *Trichoderma harzianum* is repressed during conidiation and mycoparasitism

Mariela Puyesky, Patricia Ponce-Noyola, Benjamin A. Horwitz and Alfredo Herrera-Estrella

Author for correspondence: Alfredo Herrera-Estrella. Tel: +52 462 39600. Fax: +52 462 45849/45846. e-mail: aherrera@irapuato.ira.cinvestav.mx

A glyceraldehyde-3-phosphate dehydrogenase (gpd) cDNA was isolated from the filamentous fungus *Trichoderma harzianum* in the course of a search for light-regulated genes in this organism. There is apparently only one copy of gpd in the *T. harzianum* genome, and its sequence is most similar to that of other filamentous ascomycetes. *Trichoderma* grows in the soil as a saprophyte or mycoparasite. A brief pulse of blue light, or nutrient depletion, induces sporulation, which is accompanied by altered patterns of abundance of specific polypeptides. Mycoparasitic development is also accompanied by changes in gene expression. The abundance of gpd mRNA decreased strongly during sporulation, and was lowest in samples consisting of mature conidiophores and conidia. When *T. harzianum* was grown in the presence of cell walls of the phytopathogen *Rhizoctonia solani*, the gpd mRNA level was much lower than in similar cultures grown on glucose. The repression of gpd, which is usually considered a constitutively expressed gene, may be part of the switch to sporulation or to the simulated mycoparasitic state. The implications of these findings for the use of gpd promoters to confer high constitutive expression are discussed.

Keywords: glyceraldehyde-3-phosphate dehydrogenase, biocontrol, sporulation, fungi, *Trichoderma harzianum*

INTRODUCTION

The common soil fungus *Trichoderma harzianum* is well known for its potential as a biological control agent of phytopathogenic fungi. Another important characteristic of the genus is its response to blue light. In total darkness, *T. harzianum* (some of the isolates widely used in photobiology are referred to as *Trichoderma viride* in work published before the early 1980s) grows indefinitely as a mycelium, provided that nutrients are not limiting. A brief pulse of blue light, as well as nutrient limitation, induce sporulation. In a symmetrically expanding colony, conidiophores are produced in a well-defined ring located where the colony perimeter was at the moment of photoinduction (Gressel & Rau, 1983; Betina, 1984). The inductive pulse can be as short as 10 ns and morphogenesis then proceeds in the dark with mature spores produced by 24 h (Horwitz et al., 1990).

Mycoparasitism is thought to be the main mechanism involved in biological control mediated by species of *Trichoderma* (Chet & Baker, 1981). During mycoparasitism *Trichoderma* coils around the hyphae of its host, forms appressoria-like structures, and finally penetrates it. Hydrolytic enzymes such as 1,3-β-glucanases, proteases and chitinases are important for mycoparasitism, and it is becoming clear that the expression of these genes is regulated during the mycoparasitic interaction (Flores et al., 1997; Carsolio et al., 1994; Inbar & Chet, 1995). Additionally, the expression of some of the mycoparasitism-related genes seems to be regulated.
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during development and affected by stress (Flores et al., 1997; Carsolio et al., 1994). At least some of these changes in gene expression can be induced in shake culture when the mycoparasitic state is simulated by growth on cell walls of the pathogen Rhizoctonia solani (Goldman et al., 1992; Geremia et al., 1993). A catabolite-repressed protein which is a likely component of the cell wall is also induced by growth on chitin (Lora et al., 1994).

The changes in gene expression that accompany conidiation of filamentous ascomycetes have been studied in detail, beginning with studies of enzyme activity (Urey, 1971; Weiss & Turian, 1966), and more recently at the mRNA level (Roberts & Yanofsky, 1989). In particular, three regulatory genes of Aspergillus nidulans can be placed on a linear pathway; the first of these, brlA, can direct conidiophore development when expressed in mycelia (Adams et al., 1988; Timberlake, 1993). For the most part, though, the functions, and polypeptides, corresponding to conidiation-specific genes are unknown. An exception is the Neurospora hydroporphin gene known as cccg-2, eas or bli-7, and its Aspergillus homologue rodA (Stringer et al., 1991). The various names that appeared in the literature for the Neurospora hydroporphin gene reflect its multiple regulation: by the circadian clock, blue light, and nitrogen limitation (Lauter et al., 1992; Arpaia et al., 1993). Regulation by light and by the sporulation process itself are not necessarily the same. A zinc finger DNA-binding protein of Neurospora, encoded by wc-1, is specific for light regulation, and is autoregulated by blue light (Ballario et al., 1996).

Little information has been available on the molecular correlation of photoinduction and sporulation in Trichoderma, though biochemical changes can be detected soon after induction (Farkas et al., 1990). The earliest stages of conidiation, in which aerial hyphae are stabilized, are not accompanied by changes in abundant polypeptides. Major differences, though, were detected when the synchronous, photoinduced ring of developing conidiophores was harvested separately from the mycelial mat (Baum & Horwitz, 1991). Together with the initiation of conidiophore development, an abundant polypeptide appears in conidiophore-enriched membrane fractions; several other proteins also decrease in abundance. One of the most prominent down-regulated species is a soluble polypeptide of about 39 kDa. In this study we used polyclonal antibodies to obtain the corresponding cDNA clones from an expression library and identified the gene as gpd, and followed the regulation of the mRNA during conidiation and simulated mycoparasitism.

METHODS

Growth, photoinduction and simulated mycoparasitism. T. harzianum, ATCC 32173, was grown at 25–26 °C on complete medium (PDYC: 24 g potato dextrose broth 1-3, 2 g yeast extract 1-3, 1.2 g casein hydrolysate 1-3; all from Difco). After 36 h growth in total darkness, colonies were photoinduced by a 4 min exposure to a standard blue source consisting of light from a cool-white fluorescent tube filtered through a blue acrylic filter (fluence rate 20 μmol m⁻² s⁻¹). Entire colonies were harvested by vacuum filtration after growth in stationary liquid culture: the inoculum was fixed to the bottom of the Petri dish with molten agar, and 12 ml PDYC was added. To obtain conidiophore-enriched samples, the colonies were grown on filter paper soaked with PDYC medium: an 8 cm disk of Whatman 50 overlaying a 7 cm disk of Whatman 1, in a 9 cm plastic Petri dish. The conidiophore ring was scraped from the surface of the Whatman 50 filter paper with a spatula. Samples for RNA extraction were immediately frozen in liquid nitrogen. To obtain the growth curve for conidiophores, samples from three colonies each were dried overnight at 70 °C on pre-weighed Whatman GF/C filters, and the dry weight was divided by three to obtain each point in Fig. 9.

For simulated mycoparasitism, cultures were grown for 48 h in minimal medium with 2% glucose with shaking, under laboratory light. At zero time (Fig. 8), the mycelium was collected by suction onto filter paper and washed with sterile distilled water. The sample was divided in two, and one was transferred to minimal medium with 2% glucose, and the other to minimal medium with 0.2% R. solani cell walls as carbon source. The cultures were then grown with shaking for a further 48 h, and samples harvested by filtration at the times indicated in Fig. 9.

Protein extraction and antibody production. Samples were ground for 1 min at medium speed with a Polytron homogenizer (Brinkmann) in 30 mM Tris/HCl pH 7.4, 20 mM NaCl, 0.3 M sucrose, 5 mM MgCl₂, 1 mM PMSF, and centrifuged 10 min at 12,000 g. The supernatant was centrifuged 30 min at 100,000 g in a Ti50 rotor (Beckman) to obtain a soluble protein fraction. Polypeptides were separated by SDS-PAGE, stained with Coomassie Blue, and the 39 kDa band was cut from the gel. Combined excised bands from 60 lanes were equilibrated with Laemmli sample buffer and subjected to electrophoresis on a second SDS-PAGE gel, blotted to nitrocellulose (BA4.5 Schleicher & Schuell) and detected by staining with Ponceau Red. The nitrocellulose strips with the 39 kDa band were vacuum-dried and dissolved in DMSO, mixed at 1:1 with complete Freund's adjuvant, and used to immunize rabbits; about 10 μg protein were used for the first injection and for a booster 3 weeks later. Serum obtained by bleeding the marginal ear vein at 10–14 d after the booster was used at 1:1000 dilution for Western blot analysis (Harlow & Lane, 1988). Detection of the primary antibody was either with Protein A–peroxidase and H₂O₂/3,3' diamobenzidine, or by goat anti-rabbit IgG–alkaline phosphatase and 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium. Equal loading of lanes on the Western blots was confirmed by Ponceau Red staining after transfer.

Nucleic acid extraction and analysis. RNA was isolated by a modification of the phenol/SDS method (Ausubel et al., 1987). Samples (2 g) were ground to a fine powder in liquid nitrogen, and then suspended by shaking for 10 min in 4 ml NTE'S (0.1 M NaCl, 10 mM Tris/HCl pH 7.5, 1 mM EDTA, 1% SDS) and 3 ml phenol/chloroform/isoamyl alcohol (25:24:1, by vol.). After centrifugation for 10 min at 7650 g, nucleic acids were ethanol-precipitated from the aqueous phase; the pellet was redissolved and RNA was precipitated overnight on ice addition of lithium acetate to a final concentration of 2 M, followed by a second ethanol precipitation. Northern analysis was by separation in formaldehyde agarose gels (Ausubel et al., 1987), blotting to Zeta-Probe GT membranes (Bio-Rad), and hybridization at 65 °C in 7% SDS, 0.25 M Na₂HPO₄, pH 7.2. The blots were washed twice for 30 min at
65 °C in 10 mM NaHPO₄, pH 7.2, 5% SDS, and if background was still present, for an additional 15 min in the same buffer with 1% SDS at 65 °C. Quantitative data in Fig. 9 were obtained by densitometric scanning of non-saturated autoradiograms. Southern blotting was to Hybond-N+ (Amer sham) according to the manufacturer’s recommendations, or by alkaline blotting (transfer in 0.5 M NaOH, 1.5 M NaCl). Fragments for use as probes were purified from agarose gels using glass milk, and labelled by random priming (Dupont-NEN). Poly(A)+ RNA was obtained using the PolyAttract mRNA isolation system (Promega). Briefly, with total RNA as the starting material, the poly(A)+ RNA fraction was isolated using a biotinylated oligo(dT) primer to hybridize in solution to the 3' poly(A)+ region. The hybrids were captured and washed at high stringency using streptavidin coupled to paramagnetic particles and a magnetic separation stand. The poly(A)+ RNA was eluted from the solid phase by addition of DEPC-treated water.

**Library construction, screening and sequencing.** Directional cDNA libraries were constructed in zZAP (Stratagene) from poly(A)+ RNA of colonies 0, 2 and 16 h after photoinduction. First strand cDNA synthesis was made with SuperScript RT (Gibco-BRL) instead of the reverse transcriptase supplied with Stratagene’s zZAP-cDNA synthesis kit. Second strand synthesis, ligation and digestion of adaptors, size selection and ligation to the vector arms were according to the supplier. For antibody screening, nitrocellulose filters were soaked with 10 mM IPTG, dried and incubated 6–14 h on plates of about 3 x 10⁴ plaques of the 2 h library (total 2 x 10⁴) and blocked by incubation for 2 h in a mixture of four parts non-fat milk to one part Tris-buffered saline, then washed with Tris-buffered saline and incubated overnight in the pre-adsorbed 39 kDa antiserum at 1:200 dilution. After two rounds of plaque purification, positive phage clones were rescued to plasmid form in pBluescript by in vivo excision using helper phage R408 (Stratagene). Sequencing was performed with double-stranded DNA of pGPDA1 as template, on an automated sequencer (Applied Biosystems; sequencing unit at the Weizmann Institute of Science, Rehovot, Israel), several oligonucleotides were synthesized (DNA synthesis unit, Department of Plant Genetic Engineering, CINVESTAV, Irapuato, Mexico) and used to sequence both strands.

**RESULTS**

**Isolation of Trichoderma gpd cDNA**

A prominent polypeptide band migrating at about 39 kDa is down-regulated during conidiation (Fig. 1). Antibody raised against this band detected an abundant polypeptide that was strongly down-regulated in conidiophores (Fig. 2). At 8 h, the earliest time at which conidiophores can be harvested, the signal is comparable to that in extracts of the whole colony. By 16 h, when conidial differentiation has begun, a strong decrease is apparent, and by 24 h, when mature conidia are present, the signal is very low, often near the limit of detection (Fig. 2). No decrease is noticeable in samples extracted from the entire colony (Figs 1 and 2). A zZAP cDNA library constructed from poly(A)+ RNA of colonies 2 h after photoinduction was screened with the antibody. Thirteen positive clones were isolated by screening approximately 2 x 10⁴ plaques. Of these, six were converted to plasmids by in vivo excision, and found to contain inserts of apparently identical size, 1–3 kb, and having the same restriction patterns upon digestion with KpnI, EcoRI, PstI and XhoI. In all six, a fusion protein about 6 kDa larger than the polypeptide from dark-grown mycelia was detected by the antibody. The difference corresponds well, within the resolution of the gel, with the additional 45 kDa peptide expected from the fusion.

The recognition of the fusion protein by the antibody, as well as the regulation of the corresponding mRNA
Fig. 3. Homology of *T. harzianum* gpd amino acid sequence with those encoded by both gpd genes from *T. koningii* (Watanabe et al., 1993), aligned using GeneWorks (IntelliGenetics). Boxes indicate regions of identity.

Fig. 4. Dendrogram indicating the relationship of *T. harzianum* to other known GPD protein sequences (EMBL accession nos: M33539; X52674; X63516; X58718; X39996; X62824; X07879; D88426; X99652; X99732; M81724; M81754; M93427; M81727; M11259; D14518; D14519; M11254; SWISS-PROT accession nos: 1730161; 729551; 706953; 462136; 462135; 120659; 120675; P00354). Diagram was generated using the program LASER GENE (DNASTAR); the scale indicates distance.

described below, indicates that the cDNA clone corresponds to the abundant 39 kDa polypeptide against which the antibody was raised. The antibody recognized a single spot in Western blots of two-dimensional gels (data not shown). It is still possible that other down-regulated protein(s), less abundant than GAPDH, are present in the original antigen, but no clones other than gpd have been isolated so far.
Expression of the Trichoderma harzianum gpd gene

Characterization of gpd sequences

The cDNA clone pGPD1A has extensive homology to glyceraldehyde-phosphate dehydrogenase (gpd) sequences from other organisms, as discussed in detail below. It contains the entire coding sequence, as well as the 3' untranslated region; there is a possible polyadenylation signal at 1328 bp, ATAAA, 36 bp upstream from the poly(A). The ATG at 10 bp from the start of the cDNA is likely to encode the initial methionine residue, based on homology to other gpd sequences; the sequence surrounding this codon, GCCATCATGTC, is quite consistent with the consensus for translation start sites in Neurospora crassa (Bruchez, 1993). Most of the 5' untranslated region appears to be missing from the cDNA clone, based on the size of the mRNA (1.8 kb). The molecular mass calculated from the sequence is 36 kDa, somewhat smaller than originally estimated from migration on SDS-PAGE (39 kDa, Figs 1 and 2).

The deduced amino acid sequence shows strong homology, 78% identity (Fig. 3) to a gpd sequence (GAPDH I) from Trichoderma koningii, another member of the genus. The second gpd gene of T. koningii, encoding GAPDH II, shows lower homology (62%) to T. harzianum, and also appears distant from many other fungal sequences (Fig. 4, and Jungehulsing et al., 1994). GAPDH II is an unusual GPD, which is resistant to the mycotoxin koningic acid produced by T. koningii (Watanabe et al., 1993). Among sequences tested (Fig. 4), T. harzianum is, as expected, closest to other ascomycetes, with the exception of T. koningii GAPDH II. T. harzianum fits into the pattern reported by Smith (1989), in which the gpd sequences of filamentous ascomycetes are closer to other filamentous fungi and to animals than to ascomycete yeasts.

Southern analysis of total DNA of both strains used in this study showed an identical pattern (Fig. 5). The hybridizing band in the SalI digests probably represents two fragments, consistent with the SalI site at 568 bp in the cDNA sequence; BamHI does not cut within the cDNA sequence, again consistent with the genomic Southern blot. There is a HindIII site at 931 bp in the cDNA; two additional sites are needed to explain the restriction pattern on the genomic Southern. The small (about 0.4 kb) band was detected in two independent experiments. It did not increase in intensity upon hybridization at lower stringency so it seems unlikely that this band indicates another gpd copy, though this cannot be completely excluded. The additional HindIII sites might be located just outside the available cDNA sequence, or in introns; the gpd gene of A. nidulans contains seven introns, five in or before the translational start site, and two near the 3' end (Punt et al., 1988).

To localize the gpd gene on the molecular karyotype, chromosomes of both strains were separated by contour-clamped homogeneous field electrophoresis, and probed with the gpd cDNA. The hybridization signal corresponds to the location of chromosomes V or VI (IMI 206040) and their homologues IVa or IVb in ATCC 32173 (Fig. 6). The pgk gene was also localized to chromosome V or VI of T. harzianum IMI 206040 (Herrera-Estrella et al., 1993); whether this means that glycolysis genes in Trichoderma are clustered remains to be investigated.

Developmental regulation of the mRNA hybridizing to
Fig. 7. Regulation of gpd mRNA levels during photoinduced conidiation. Colonies were grown in total darkness and photoinduced as for Figs 1 and 2. Upper panel, Northern blot of total RNA (15 μg per lane), probed with the XhoI fragment from pGPDAl. Lower panel, hybridization of a replica blot with a fragment of the human 28S rRNA gene (see Methods) as a control for equal loading of the lanes. Experiments were carried out twice for all time-points, except for 0 and 24 h which were done four times. The pGPD1A cDNA is shown in Fig. 7. The transcript, of about 1.8 kb, is very abundant in dark-grown mycelia, and is strongly repressed in developing conidiophores. In contrast to the polypeptide abundance (Fig. 2), the decrease in mRNA level was also found for RNA samples from the entire colony, but the regulation is much more striking in the conidiophore samples. After 48 h growth on minimal medium with 2% glucose, the gpd transcript is abundant (time zero of the simulated mycoparasitism experiment, Fig. 8). From transfer to 2 h, the transcript level increased on both glucose and cell walls. At 4–8 h, the gpd mRNA was markedly repressed in cultures growing on cell walls as compared to the glucose controls. By 24 and 48 h after transfer, the transcript level decreased in cultures growing on either glucose or cell walls, and at 48 h remained somewhat higher in the cell-wall-grown cultures than on glucose (Fig. 8). Glucose is totally exhausted after about 14 h of growth under these conditions, perhaps explaining the decrease in both transcripts. In the time window at 4–8 h when the mRNA is down-regulated, the cultures are still growing.

Fig. 8. Regulation of gpd mRNA levels during simulated mycoparasitism. Samples were harvested at the indicated times of growth on minimal medium with glucose (G) or on Rhizoctonia cell walls (C). Upper panel, Northern blot of total RNA (20 μg per lane), probed with the gpd cDNA as in Fig. 7. Lower panel, second hybridization of the same blot with a fragment of the human 28S rRNA gene, as a control for equal loading of the lanes. The experiment was repeated three times.

Temporal comparison of gpd down-regulation and conidiophore development

Both simulated mycoparasitism and conidiation represent a shift to less favourable conditions, and in both cases, growth eventually slows down or stops. The synchronous development of conidiophores provided an opportunity to determine which occurs first, down-regulation of gpd expression, or the decrease in growth rate. The growth curve for the conidiophore ring induced by blue light is shown in Fig. 9. The decrease in mRNA level occurs together with morphogenesis, and is very clear by 16 h after photoinduction, at a time when the growth rate of the conidiophore ring is maximal (Fig. 9).

DISCUSSION

Although gpd is considered to be a constitutively expressed gene in filamentous fungi, there is precedent for regulation of gpd genes in other organisms, for example in vertebrates (Dobson et al., 1987), Drosophila (Sun et al., 1988) and Caenorhabditis elegans (Huang et al., 1989). The nuclear genes encoding chloroplast GPD in Arabidopsis thaliana are light-regulated, albeit upward rather than downward (Kwon et al., 1994). Furthermore, phosphoglycerate kinase (PGK) mRNA is...
also down-regulated in *T. harzianum* during conidiation and simulated mycoparasitism (Goldman *et al.*, 1992). It has been reported that constitutively expressed proteins, such as GPD or the plasma membrane ATPase, are regulated by glucose in yeast (Capieaux *et al.*, 1989; Rao *et al.*, 1993). In yeast cells entering the stationary phase, however, GPD was one of the few enzymes that continued to be expressed at high levels (Boucherie, 1985). Adams & Timberlake (1990) showed that constitutive expression of *brlA* in *Aspergillus* growing in favourable conditions induces sporulation, and at the same time represses two genes which are normally highly expressed, *alcA* and *aldA*. They proposed that the induction of sporulation leads to a generalized metabolic shutdown. *Trichoderma*, when exposed to blue light, behaves in a similar way: the colony sporulates, even though the nutrient conditions are favourable. The view that blue light causes a transient metabolic stress (Gressel & Rau, 1983) fits well with this picture. Either blue light or nitrogen stress can regulate many of the same genes in *Neurospora* (Baker, 1981), though this does not yet imply that stress acts downstream from light in the transduction chain. In mycoparasitism, like conidiation, the organism must contend with less favourable conditions. One could argue that the repression of *gpd* may simply result from a slow-down in growth. That this is not the case is clear from Fig. 9; down-regulation begins early and continues while the conidiophores are growing most rapidly. By 24 h after induction, growth is slow, conidia are maturing, and the *gpd* mRNA level is very low (Figs 7 and 9). Developing conidiophores have rapid growth and metabolism, as shown by rapid incorporation of radioactive metabolites such as methionine (B. A. Horwitz, unpublished). Though we do not have unequivocal proof that the protein recognized by the antibody (Fig. 2) consists entirely of GPD, there is much circumstantial evidence (cross-reactivity with the fusion protein from the *gpd* clone; single spot on 2 d gels; the fact that GPD is an abundant protein; down-regulation of mRNA and of the polypeptide recognized by the antibody). If so, the amount of enzyme remaining in conidiophores (Fig. 2) would need to be enough to support spore maturation, and germ tube emergence upon germination. There is no evidence from Southern hybridizations and library screening for more than one *gpd* copy in *Trichoderma*.

The down-regulation of *gpd* may have consequences for the use of *gpd* expression signals to drive dominant selectable marker genes such as hygromycin phosphotransferase (Punt *et al.*, 1987). It is often observed that hygromycin inhibits sporulation of otherwise resistant transformants. Sporulation is inhibited under some selectable marker genes such as hygromycin phosphotransferase, while at the same time the *Trichoderma gpd* mRNA levels sharply decreased (data not shown). Thus, the down-regulation should not create any difficulty in using the *gpd* promoter to express heterologous genes in conidiophores and conidia, although further work is needed to pursue this question.

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