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Expression of Two Major Chitinase Genes of *Trichoderma atroviride* (*T. harzianum* P1) Is Triggered by Different Regulatory Signals

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Regulation of the expression of the two major chitinase genes, *ech42* (encoding the CHIT42 endochitinase) and *nag1* (encoding the CHIT73 *N*-acetyl-β-D-glucosaminidase), of the chitinolytic system of the mycoparasitic biocontrol fungus *Trichoderma atroviride* (= *Trichoderma harzianum* P1) was investigated by using a reporter system based on the *Aspergillus niger* glucose oxidase. Strains harboring fusions of the *ech42* or *nag1* 5′ upstream noncoding sequences with the *A. niger goxA* gene displayed a glucose oxidase activity pattern that was consistent under various conditions with expression of the native *ech42* and *nag1* genes, as assayed by Northern analysis. The expression product of *goxA* in the mutants was completely secreted into the medium, detectable on Western blots, and quantifiable by enzyme-linked immunosorbent assay. *nag1* gene expression was triggered during growth on fungal (*Botrytis cinerea*) cell walls and on the chitin degradation product *N*-acetylglucosamine. *N*-Acetylglucosamine, di-*N*-acetylchitobiose, or tri-*N*-acetylchitotriose also induced *nag1* gene expression when added to mycelia pregrown on different carbon sources. *ech42* expression was also observed during growth on fungal cell walls but, in contrast, was not triggered by addition of chitooligomers to pregrown mycelia. Significant *ech42* expression was observed after prolonged carbon starvation, independent of the use of glucose or glycerol as a carbon source, suggesting that relief of carbon catabolite repression was not involved in induction during starvation. In addition, *ech42* gene transcription was triggered by physiological stress, such as low temperature, high osmotic pressure, or the addition of ethanol. Four copies of a putative stress response element (CCCCT) were found in the *ech42* promoter.

Chitin, a β-1,4-linked polymer of *N*-acetylglucosamine (NAGa), is an abundant biopolymer whose degradation has a significant impact on the balance of natural ecosystems (15). Many prokaryotic and eukaryotic microorganisms degrade chitin by using enzyme systems such as endochitinases (EC 3.2.1.14), chitobiosidases (no EC number), and β-*N*-acetylhexosaminidases (*N*-acetyl-β-D-glucosaminidases) (EC 3.2.1.30) (4, 31).

Members of the fungal genus *Trichoderma* are known to produce chitinolytic enzymes that can degrade the cell wall of ascomycetes and basidiomycetes (6, 7, 10, 23). The chitinases of mycoparasitic species, e.g., *Trichoderma harzianum*, are also involved in the antagonistic ability of these fungi against plant pathogens and in biocontrol (39). Although a plethora of chitinolytic enzymes have been detected and purified from various *Trichoderma* spp. (23), only a limited number of chitinolytic genes have been cloned (*ech42*, *chit33*, and *nag1*) (5, 8, 9, 12, 14, 19, 22, 30).

Very little is known about the regulation of expression of these chitinolytic genes. Chitinase expression in fungi is thought to respond to degradation products that serve as inducers and to easily metabolizable carbon sources that serve as repressors (2, 31, 34, 35). Most studies of the regulation of chitinase formation in *Trichoderma* spp. have identified chitinases only by enzyme assays and have not addressed the possibility of differential regulation for the various isoenzymes. The small amount of data presently available indicate that *ech42*, *chit33*, and *nag1* are inducible by fungal cell walls and colloidal chitin (5, 12, 22, 30) or by carbon starvation (22, 28). In addition, *ech42* expression appears to be governed by carbon catabolite repression (5, 24) and to follow light-induced sporulation (5), while *nag1* transcription is triggered by NAGa (30). However, some confusion has occurred because a detailed investigation of the observed effects is lacking, and previous studies have used different species of *Trichoderma* under the name "*T. harzianum*" (21).

In this study, we have examined the regulation of expression of two major chitinase genes, *ech42* (*ThEn42*) and *nag1*, from *Trichoderma atroviride* (= *T. harzianum* P1 [21]), a strain that has been used in a number of theoretical and applied biocontrol studies (18).

MATERIALS AND METHODS

Strains. *T. atroviride* P1 ("*T. harzianum*" ATCC 74058 [21]) was used throughout this study and maintained on potato dextrose agar (Merck, Darmstadt, Germany). *Aspergillus niger* (ATCC 9029) (11) was the source of the glucose oxidase-encoding *goxA* gene, *Botrytis cinerea* 26 (25) was used to prepare fungal cell walls, and *Escherichia coli* JM109 (40) was the host for plasmid amplification (1, 32).

Cultivation conditions. *T. atroviride* and recombinant strains were grown in liquid synthetic medium (SM) containing the following (in grams per liter): $(NH_4)_2SO_4$, 2.8; urea, 0.6; KH_2PO_4, 4; $CaCl_2 \cdot 2H_2O$, 0.6; $MgSO_4 \cdot 7H_2O$, 0.2; $FeSO_4 \cdot 7H_2O$, 0.01; $ZnSO_4 \cdot 2H_2O$, 0.0028; $CoCl_2 \cdot 6H_2O$, 0.0032 (pH 5.4). SM was augmented with either glucose (10 g/liter, except as otherwise stated), glycerol (10 g/liter), colloidal chitin (2 g/liter, prepared according to reference 38), or *B. cinerea* cell walls (2 g/liter, prepared as described in reference 33). In experiments where the effect of oxygen transfer was studied, the volume of the medium in 1-liter shake flasks varied between 50 and 500 ml.

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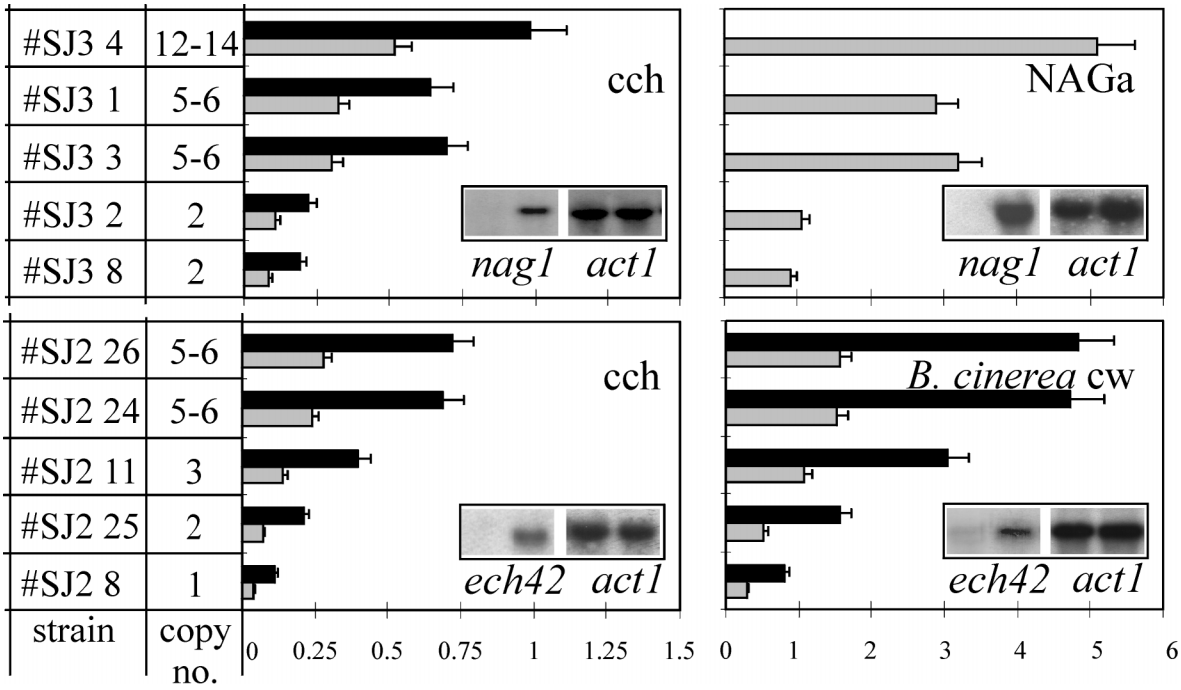

FIG. 1. Copy number and glucose oxidase (GOX) expression in different transformant strains. SJ3 and SJ2 define recombinant strains transformed with pSJ3 (*nag1*::*goxA*) and pSJ2 (*ech42*::*goxA*), respectively; strains were cultivated on SM with different C sources (colloidal chitin [cch], NAGa, and *B. cinerea* cell walls [cw]) for 48 h (grey bars) and 72 h (black bars). GOX enzyme activity (U/ml of culture filtrate) was determined in at least three independent experiments; error bars indicate standard deviations. Inserts show results from Northern analyses of *nag1* and *ech42* transcription of *T. atroviride* cultivated on the various C sources (right lanes) and glucose (left lanes), as a control, for 48 h (NAGa and glucose as C source) and 72 h (cch and *B. cinerea* cell walls as C source). A 1-kb *Pst*I fragment of the *ech42* gene, a 2-kb *Sal*I/*Xba*I fragment of the *nag1* gene of *T. atroviride*, and a 1.9-kb *Kpn*I fragment of the actin (*act1*) gene of *T. reesei* were used as probes, respectively.

For induction experiments in replacement cultures, *T. atroviride* was precultivated in SM containing glycerol as the carbon source for 36 h, harvested by filtration, washed with sterile tap water, and transferred to 25 ml of SM containing 1% glycerol and one of the chitooligomers (NAGa; *N*,*N*′-diacetylchitobiose [DACb]; *N*,*N*′-diacetylchitobiose hexaacetate [DACbHA]; or *N*,*N*′,*N*″-triacetylchitotriose [TACt] [Sigma, St. Louis, Mo.]) at a final concentration of 1 mM. Cultures without these putative inducers served as controls.

To examine carbon starvation, strains were grown on SM supplemented with glucose or glycerol as a carbon source at concentrations of 0.1 and 1% (wt/vol) and samples were taken after 24 and 48 h.

To elicit a stress response, *T. atroviride* was precultivated in SM containing 1% glycerol as the carbon source for 36 h, harvested by filtration, washed with sterile tap water, and transferred to 25 ml of SM containing 1% glycerol in 100-ml Erlenmeyer flasks. After 120 min of adaptation to the new conditions, the stress-inducing agent (final concentration) ethanol (2% [wt/vol], sorbitol (1 M), $CdSO_4$ (50 mg/liter), or H_2O_2 (2% [wt/vol] was added, and incubation was continued for 1 h. In the case of heat (40°C) or cold (4°C) shock, flasks were transferred to a water or ice bath previously adjusted to the indicated temperature, respectively, and further incubated with shaking for 1 h. For testing expression at low pH, SM was adjusted to pH 2 by addition of a predetermined amount of 1 M citric acid.

For isolation of genomic DNA, *A. niger* was grown in 1-liter shake flasks containing 250 ml of YEP medium (yeast extract, 10 g/liter; peptone, 20 g/liter; glucose, 30 g/liter). To obtain fungal cell walls, *B. cinerea* was cultivated as described by Schirmböck et al. (33).

Plasmids and plasmid constructions. Plasmid $pRLM_{ex}30$, containing the *E. coli hph* gene under control of the *Trichoderma reesei pki* (pyruvate kinase-encoding) promoter (26), was used for construction of vectors for transformation. The coding region and 367 bp of the 3′ noncoding region of the *A. niger goxA* gene (11) were amplified from genomic DNA with primers goxF (5′-CAT CTG C**TC TAG A**TG CAG ACT CTC C-3′) and goxR (5′-GCA TGT TGT TT**A AGC TT**A AAC ACC GCC-3′), designed according to the published sequence (11) and containing an *Xba*I site and a *Hin*dIII site, respectively (shown in boldface type); *Taq* polymerase (Promega, Madison, Wis.); and a Biometra TRIO thermocycler (Biometra, Göttingen, Germany). The amplification protocol consisted of an initial denaturation cycle of 1 min at 95°C, followed by 30 cycles of 1 min at 95°C, 1 min at 59°C, and 90 s at 74°C, followed by a final step of 7 min at 74°C. The amplified fragment was exchanged with the 2-kb *Xba*I/*Hin*dIII fragment from $pRLM_{ex}30$, resulting in vector $pRLM_{ex}60$. An 830-bp fragment of the *nag1* upstream regulatory sequence was amplified by PCR (as described above) with plasmid pCPN4 (30) used as template DNA and primers nagF (5′-GCT GAT ATG GCC G**CT CGA G**TA CCT AGA TC-3′) and nagR (5′-CCT TGG GCA GCA **TCT AGA** ACG ACC GAG G-3′), containing internal *Xho*I and *Xba*I restriction sites (in boldface type), respectively. The amplicon was exchanged with the *pki1* promoter fragment of $pRLM_{ex}60$, resulting in plasmid pSJ3. Analogously, an 810-bp fragment of the *ech42* upstream regulatory sequence (24) was amplified with primers echF (5′-ATG GTG AAG TG**C TCG AG**A GGA TAA CGG-3′) and echR (5′-CAG AAT TCG GCT TAT **GCT AGC** GTG TTT GAG ATT C-3′), containing the respective restriction sites *Xho*I and *Nhe*I (in boldface type), by the amplification protocol described above. The amplified fragment was exchanged with the *pki* promoter of $pRLM_{ex}60$, producing plasmid pSJ2.

All constructs were verified by means of automatic sequencing (LI-COR 4200 L-1; LI-COR Inc., Lincoln, Neb.) with both M13 primers.

Fungal transformation. *goxA*-bearing plasmids were introduced into *T. atroviride* by cotransformation with plasmid pHATα (20) by a protoplast-based protocol (24). The total amount of transforming DNA was 12 μg (8 μg of *goxA*-bearing plasmids and 4 μg of pHATα). Transformants were regenerated on potato dextrose agar supplemented with 100 μg of hygromycin B (Calbiochem, San Diego, Calif.) per ml. Mitotically stable transformants were obtained by at least three sequential transfers of conidia from nonselective to selective media.

DNA and RNA manipulations. Chromosomal DNA was isolated by the CTAB (cetyltrimethylammonium bromide) method (1), and plasmid DNA was isolated by using a midiprep kit (Qiagen Inc., Chatsworth, Calif.), as recommended by the manufacturer. RNA isolation and Northern analysis were carried out as described previously (30). Other molecular techniques were performed by standard protocols (1, 31).

Determination of fungal biomass. Fungal biomass was determined by extracting total protein from the 5,000 × *g* (10 min) pellet of 5 ml of culture broth with 0.1 N NaOH (3 h, room temperature) and determining the protein concentration in the supernatant (10,000 × *g*, 4°C, 20 min) by the dye binding procedure (3). Values are given as milligrams of extractable protein per liter of culture.

Glucose oxidase assay. Quantification of glucose oxidase activity in culture supernatants was done as described by Geisen (13); the assay mixture contained the following (final concentration in the assay): ABTS (2,2′-azino-di-[3-ethylbenzthiazoline sulfonate]) (Boehringer, Mannheim, Germany) (1 mM), horseradish peroxidase (Boehringer) (1 U/ml), and glucose (250 mM) in sodium phosphate buffer (100 mM) (pH 5.8). Blanks lacking glucose were always included. The increase in absorption at 420 nm was measured continuously in a photometer. One unit of activity was defined as the amount of enzyme required to oxidize 1 μmol of glucose per min at pH 5.8 and 25°C.

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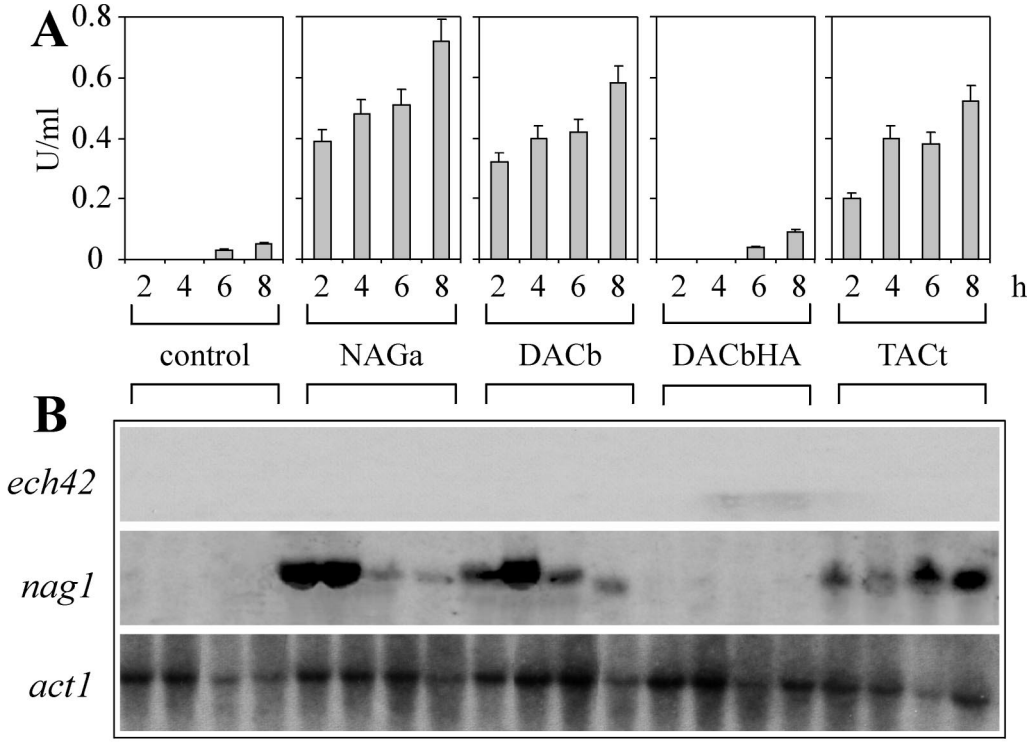

FIG. 2. Effects of soluble chitooligomers on expression of *nag1* and *ech42*. Shown are glucose oxidase activities of SJ3 strains after replacement on SM containing NAGa, DACb, DACbHA, TACt at a final concentration of 1 mM. Incubations without chitooligomers served as a control. (A) Results are means from an assay of five different transformant strains normalized to a single interpreted copy. Error bars indicate standard deviations ($n \geq 3$). (B) Northern analysis of *ech42* and *nag1* expression in the *T. atroviride* parent strain under the same conditions. Hybridization probes used are described in the legend to Fig. 1.

ELISA. Quantification of glucose oxidase protein in culture supernatants was carried out by standard enzyme-linked immunosorbent assay (ELISA) protocols (1). Two volumes of 96% (wt/vol) ethanol were added to 5 ml of culture supernatant, and the precipitate recovered by centrifugation at 10,000 × *g* (4°C, 30 min) was dissolved in 360 μl of phosphate-buffered saline (1). These samples were used for ELISA directly or after dilution in phosphate-buffered saline, as required, and measurements were taken only when they fell into the linear part of the relationship between glucose oxidase protein concentration and absorbance (0.08 to 0.15 μg/ml). Incubations with primary polyclonal antibody against *A. niger* glucose oxidase (Chemicon, Temecula, Calif.) and secondary anti-rabbit antibody (Promega, Madison, Wis.) were carried out for 1 h at 37°C. Glucose oxidase grade II (Boehringer) was used to calibrate the method and as a control in the experiments.

RESULTS

***A. niger goxA* as a monitor of *nag1* and *ech42* gene induction.** Glucose oxidase activity was not detected either in *T. atroviride* mycelia or cell walls or in extracellular culture broth when the fungus was grown under different conditions, including high (10%, wt/vol) glucose concentration, different volumes of medium (75 to 500 ml per 1-liter flask), and shaking speed (i.e., varying oxygen supply).

Twenty-one and 32 mitotically stable, hygromycin B-resistant transformants were selected after cotransformation of pHATα with pSJ2 and pSJ3, respectively. Among the transformants, 5 and 16 strains showed glucose oxidase activity when grown on *B. cinerea* cell walls or NAGa. Copy numbers of plasmids pSJ2 and pSJ3 in the transformants ranged from 1 to 14, and all were ectopically integrated in diverse genomic locations (data not shown).

To verify the utility of the reporter system, both the wild type and transformants were grown on media with colloidal chitin, NAGa, *B. cinerea* cell walls, or glucose as the sole carbon source. The presence of *ech42* and *nag1* transcripts was investigated by Northern analysis and compared to the glucose oxidase activity of the respective transformants (Fig. 1). *nag1* expression in the presence of NAGa and colloidal chitin and *ech42* expression in the presence of colloidal chitin and *B. cinerea* cell walls appeared to be similar with the two methods. Both enzyme activities and transcript levels of *ech42* and *nag1* were below the limit of detection during growth on glucose as the sole carbon source.

To study the relationship between copy number and glucose oxidase activity, we tested five transformants for each group (Fig. 1). Copy numbers below seven, but not higher, correlated linearly (R^2, 0.95 to 0.99 [depending on the inducer and the gene, respectively]) with glucose oxidase activity (Fig. 1). Therefore, all induction studies were performed with strains carrying one to six copies of the respective promoter-glucose oxidase constructs, and the glucose oxidase activities measured were always adjusted for copy number.

Induction of *nag1* and *ech42* expression by soluble chitooligomers. To determine whether *nag1* and *ech42* expression can be elicited by chitin degradation products such as soluble chitooligosaccharides, all transformants shown in Fig. 1 (with the exception of SJ3 4) were precultivated on glycerol as the carbon source and transferred to SM containing 1% glycerol and either NAGa, DACb, DACbHA, or TACt. The highest level of *nag1* expression, assayed as glucose oxidase formation, was observed with NAGa, followed by DACb and TACc (Fig. 2), while DACbHA and the controls induced only a very low level of expression of this gene. Results from Northern analyses were consistent with this pattern. Interestingly, NAGa and DACb produced the highest levels of transcripts within the first 4 h of incubation, whereas TACt-induced transcripts increased

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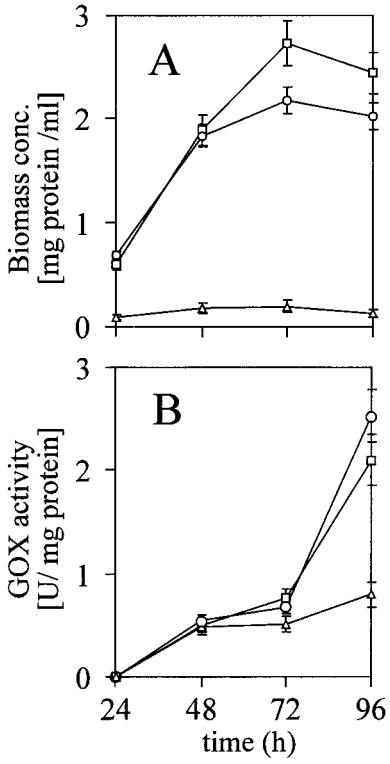

FIG. 3. Biomass formation (total extractable protein [A]) and *ech42* expression (glucose oxidase [GOX] activity [B]) of *T. atroviride* during cultivation on glucose (○), NAGa (□), and colloidal chitin (△). Values are means of at least three separate experiments carried out with five different transformant strains normalized by copy number; error bars indicate standard deviations. Enzyme activities are related to milligrams of biomass protein.

throughout the 8-h duration of the assay. This may be due to differences in the metabolic half-lives of these oligochitosides.

We also tested induction of *ech42* by the chitooligosaccharides. In contrast to *nag1*, no *ech42* transcription was observed by treatment with any of the soluble chitooligosaccharides used (Fig. 2B; glucose oxidase measurements not shown).

Triggering of *ech42* expression by carbon source starvation. We compared growth of *T. atroviride* and *ech42* gene expression on glucose, NAGa, and colloidal chitin (Fig. 3). Growth on chitin was very poor in comparison to that on glucose and NAGa. This poor growth was not due to the use of low (0.2%, wt/vol) concentrations of chitin, as an increase in chitin concentration (to 1%) did not increase biomass formation (data not shown). Microscopic examination showed that only about 1% of the spores (initially 5×10^7/liter) germinated on colloidal chitin, whereas the level of spores germinating on glucose or NAGa was close to 100% (data not shown). Under these conditions, *ech42* expression was first observed after 48 h and strongly increased after 96 h. When related to the amount of biomass, glucose oxidase activity (i.e., "induction" of *ech42*) was significantly higher on glucose and NAGa than on chitin.

This pattern of *ech42* expression during growth and with various carbon sources suggested that this gene is not induced by chitin but by carbon starvation. To test this hypothesis, the five transformants in the SJ2 (*ech42*::*goxA*) series were grown on SM supplemented with 0.1 and 1% (wt/vol) glucose or glycerol, respectively. Growth on the lower concentration of both carbon sources resulted in increased levels of *ech42* expression (i.e., glucose oxidase activity) after 48 h (i.e., when the carbon source was depleted from the medium), while only weak activities were detected at 24 h. Only very low activities were recorded at both 24 and 48 h when strains were grown at the higher concentration of these carbon sources (Table 1), which were not yet depleted at 48 h. We obtained essentially the same results when 0.5% colloidal chitin was added to either of these carbon sources, irrespective of their concentration (0.1 or 1% [wt/vol]). These data suggest that chitin does not increase the induction of *ech42* resulting from carbon source depletion.

***ech42* is stress inducible.** To test if *ech42* is stress inducible, the five transformants from the SJ2 series were precultivated on SM with 1% glycerol, transferred to fresh medium, incubated for 2 h to adapt to the new condition, and then exposed for 1 h to different stress conditions. *ech42* expression (quantified as glucose oxidase activities) was observed in the presence of 2% (wt/vol) ethanol (0.22 U/ml), at 4°C (0.11 U/ml), or after replacing the culture in 1 M sorbitol (0.095 U/ml). However, incubation at 40°C, at pH 2, in the presence of $CdSO_4$, or in the presence of H_2O_2 did not trigger expression of *ech42* (all glucose oxidase activities of <0.05 U/ml). All negative values were confirmed by ELISA, thus eliminating the possibility that stress-inducing substances or conditions had interfered with the glucose oxidase assay.

DISCUSSION

Secretion of extracellular enzymes as several isoenzymes is common and often coordinately regulated (e.g., cellulases) but, in many cases, is differentially controlled (17). Previous results on the formation (17) and gene expression of various chitinases during mycoparasitic interaction of *T. atroviride* with plant pathogenic fungi revealed a sequential order of appearance (41), but the mechanism of gene regulation remains unclear. The present work demonstrates that the mechanism of regulation of *nag1* and *ech42* expression is different. *nag1* was induced by low-molecular-weight chitooligosaccharides and its own catabolic products, while *ech42* expression appeared to be not directly induced by purified chitin or chitooligosaccharides but by carbon starvation and some stress conditions.

Induction of a hydrolase gene by products of its own activity, as shown here for *nag1* with NAGa in *T. atroviride*, has also been reported for *T. reesei* genes such as α-galactosidase, β-xylosidase, and α-arabinosidase (29), which are induced by galactose, xylose, and arabinose, respectively. Although we did

TABLE 1. Effects of different carbon sources and carbon source depletion on *ech42* expression in *T. atroviride*

Carbon source	Concn (% [wt/vol])	Mean *ech42* expression (SD)[a]	
		GOX formed (mg/mg [dry wt])	GOX activity (U/mg [dry wt])
Glucose	0.1	3.7 (0.7)	4.4 (0.48)
	1	<0.1	0.07 (0.04)
Glucose + chitin	0.1 + 0.5	4.3 (1.3)	3.9 (0.50)
	1 + 0.5	<0.1	0.05 (0.04)
Glycerol	0.1	1.8 (0.3)	3.1 (0.47)
	1	<0.1	0.14 (0.005)
Glycerol + chitin	0.1 + 0.5	2.1 (0.4)	2.0 (0.44)
	1 + 0.5	<0.1	0.09 (0.01)

[a] All values shown are for 48 h of cultivation and are from at least three separate experiments with all *ech42*::*gox* transformants described in Fig. 1 and normalized to a single gene copy. Values for 24 h were below the detection limit in all cases and are not given. GOX, glucose oxidase.

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not detect constitutive expression of *nag1*, Peterbauer et al. (30) claimed that a low level of *N*-acetyl-β-D-glucosaminidase activity, possibly due to a different isozyme, was bound to the cell walls of *T. atroviride*. It is possible that such low constitutive activities release small amounts of NAGa from chitooligomers originating from the host cell wall, which further induce *nag1* expression. DACb and TACt also were able to activate *nag1* expression, although their effects were slower than NAGa, probably because they need to be hydrolyzed to NAGa before acting as inducers. This result is consistent with the fact that CHIT73 was more active on DACb than on TACt and that TACt showed the lowest inducing effect on *nag1*. In addition, the DACb derivative DACbHA was not an effective inducer and was also a poor substrate for CHIT73.

Despite Northern blotting data indicating *ech42* expression during growth of *T. atroviride* on *B. cinerea* cell walls and colloidal chitin, *ech42* expression was induced neither by incubation of washed mycelia with colloidal chitin nor by any of the low-molecular-weight chitooligosaccharides that were effective inducers of *nag1* expression. One way to explain this apparent discrepancy is to assume that *ech42* expression is triggered by cell wall oligomers of higher molecular weight or higher complexity. The highly purified colloidal chitin used in our assays is significantly different from the polymer naturally occurring in fungal cell walls, as the latter is not amorphous but covalently linked to other cell wall polysaccharides; therefore, the induction potential could also be altered. With respect to *ech42* induction during "growth" on chitin, we doubt that the observed expression is due to induction by chitin, as biomass formation was less than a tenth of that on glucose or NAGa and *ech42* expression under these conditions could be due to carbon starvation. This speculation also is supported by the observation that even under these conditions *ech42* expression lags behind growth and was maximal at the time when autolysis was already apparent. In addition, a comparable level of *ech42* induction also was observed after exhaustion of glucose or glycerol from the cultures, and this expression was independent of the presence of chitin. Therefore, we suggest that carbon source depletion rather than direct induction by chitin induced *ech42* expression in *T. atroviride* under our assay conditions. These findings are consistent with those of Margolles-Clark et al. (28) and indicate that purified chitin may be a poor substrate for *T. atroviride* P1 and that other cell wall components are needed for the fungus to catabolize chitin in the host cell wall.

This work provides evidence, for the first time, of the triggering of *ech42* transcription by a stress response reaction, although it is limited to certain types of stress. Regulation of *ech42* transcription by stress is consistent with the finding of four stress response elements (27, 36) in its promoter. These elements in *Saccharomyces cerevisiae* bind the zinc finger transcription factors Msn2p and Msn4p and mediate various stress responses, including those tested in this study and that caused by nutrient depletion (27, 36). The fact that heat shock or the presence of heavy metal ions also elicits an Msn2p/Msn4p-mediated stress response in yeast, but not in *T. atroviride*, might be due to interference with the reporter gene system used here (e.g., a block in the secretion of glucose oxidase).

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