The Mycoparasite *Ampelomyces quisqualis* Expresses *exgA* Encoding an *exo*- β -1,3-Glucanase in Culture and During Mycoparasitism

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ABSTRACT

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Ampelomyces quisqualis, a mycoparasite of fungi causing powdery mildews, exhibited high levels of extracellular *exo*- β -1,3-glucanase activity in culture compared with *Neurospora crassa* and *Gliocladium roseum*. *A. quisqualis* culture filtrates affected powdery mildew caused by *Sphaerotheca fusca* in a manner indicative of cell wall degradation, as determined by microscopic examination. A gene encoding an *exo*- β -1,3-glucanase in *A. quisqualis*, designated *exgA*, was isolated and sequenced. The predicted polypeptide deduced from *exgA* had 46, 42, and 30%

identity with amino acid sequences of *Trichoderma harzianum* *exo*- β -1,3-glucanase and *Cochliobolus carbonum* *EXG1* (both encoding *exo*- β -1,3-glucanase) and *T. harzianum* *bng13.1* (encoding an *endo*- β -1,3-glucanase), respectively. The *exgA* gene had a predicted molecular mass of 84 kDa and a pI of 4.79. The gene was expressed during the late stages of growth in culture, and transcription was induced by fungal cell wall components. Transcript levels for *exgA* were present during late stages of hyperparasitism and were abundant along *A. quisqualis* mycelium and were slightly less abundant in *A. quisqualis* pycnidia.

Additional keywords: lytic enzymes.

Ampelomyces quisqualis Ces. is a mycoparasite of many fungal species that cause powdery mildews (17). The mycoparasite infects and forms pycnidia inside fungal hyphae, conidiophores, conidia, and cleistothecia (8). Cells of the mycoparasite grow normally inside host cells, in spite of gradual degeneration of host cells (9).

β -1,3-Glucanases have been identified and studied in fungi, bacteria, actinomycetes, algae, mollusks, and higher plants (13). In fungi, β -glucanase activity has been found in most isolates examined (13). Various putative functions have been assigned to fungal β -glucanases, including metabolic, morphogenetic, and ecological functions (13). One example of a possible ecological role played by these enzymes is their involvement in the interaction between species of the mycoparasite *Trichoderma* and its host, *Sclerotium rolfsii*; high amounts of β -1,3-glucanase and chitinase have been detected in dual cultures of *T. harzianum* and *Sclerotium rolfsii* (7). *Penicillium purpurogenum* is another antagonist that produces β -1,3-glucanase. The enzymatic complex of *P. purpurogenum* attacks the mycelium of its host, *Monilinia laxa*, causing lysis of hyphae and spores (10). Studies on *Schizophyllum commune* mycoparasitism in a dual culture with its host, *Rhizoctonia solani*, revealed that the main enzyme detected in cultures was *endo*- β -1,3(4)-glucanase; the activity of this enzyme was enhanced markedly compared with cultures of the mycoparasite alone (5). β -1,3-Glucanase of *Tilletiopsis* spp. has been suggested as a potential component of the biocontrol action of this yeast against powdery mildew (19).

Some fungal β -1,3-glucanases are produced constitutively, whereas production of others is regulated. Regulation mechanisms include induction and catabolite repression (13). Little is known about extracellular enzymes of *A. quisqualis*. However, it has been established that in vitro *A. quisqualis* constitutively produces several extracellular enzymes, among them a β -1,3-glucanase (12).

Genes encoding β -1,3-glucanases from various organisms, including plants, bacteria, and yeasts have been isolated and characterized (6). However, to date such genes have been isolated only from two filamentous fungi. The *EXG1* gene of the filamentous plant-pathogenic fungus *Cochliobolus carbonum*, encoding *exo*- β -1,3-glucanase, has been isolated, following the determination of the partial amino acid sequence of the purified enzyme (15). A gene encoding an *endo*- β -1,3-glucanase of the mycoparasite *T. harzianum* has been cloned and sequenced. Its structural analysis suggests that its *endo*- β -1,3-glucanase contains a hydrophobic leader peptide that may be cleaved by an endoprotease (6).

In this paper, we report on some of the characteristics of *A. quisqualis* *exo*- β -1,3-glucanases and the isolation and expression of the *A. quisqualis* *exgA* gene encoding an *exo*- β -1,3-glucanase.

MATERIALS AND METHODS

Fungal isolates and growth conditions. Isolate 10 of *A. quisqualis* was used. The isolate was maintained on bran extract agar medium (water-boiled, cheesecloth-filtered extract of 100 g of bran, 20 g of malt extract [Difco, Detroit, MI], 2 g of DL-asparagine, 0.25 g of chloramphenicol, and 1 liter of deionized water) (17).

Strain 74-OR23-1A(FGSC987) of *Neurospora crassa* Shear & Dodge and isolate 2479 of *Gliocladium roseum* (Link) Bainier used in this study were from the fungal collection of the Department of Plant Pathology and Microbiology, The Hebrew University, Rehovot, Israel.

The liquid medium used to study glucanase production was potato broth (PB) containing water-boiled, cheesecloth-filtered extract of 200 g of PB per liter of deionized water. A pycnidiospore inoculum of *A. quisqualis* was prepared by flooding 3-week-old fungus cultures grown on solid bran extract medium with sterile water and collecting the spore suspension. For glucanase production, *A. quisqualis* was grown in a 2-liter fermentor (MultiGen, model F-2000, New Brunswick Scientific Co., Edison, NJ) inoculated with 2×10^5 spores per ml of medium. During growth, the aeration rate

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was 1 liter of air per min per liter of medium, agitation speed was 180 rpm, and temperature was maintained at 25°C. Samples were taken daily and assayed for enzyme activity. PB medium, amended with glucose at 20 g/liter (potato dextrose broth, PDB) was used to obtain the fungal biomass used for DNA extraction.

To compare the β -1,3-glucanase activity in culture filtrates of *A. quisqualis* to the activity in cultures of *N. crassa* and *G. roseum*, the fungi were grown on nutrient yeast dextrose broth medium (8 g of nutrient broth [Difco], 5 g of yeast extract [Difco], 10 g of D-glucose, and 1 liter of deionized water). Inoculum consisted of 1×10^5 conidia per ml. The fungus was grown in 100-ml flasks containing 30 ml of medium at 120 rpm on an orbital shaker (New Brunswick Scientific) at 25°C. For experiments in which dry fungal mycelium was added to the medium, *A. quisqualis* was grown in 100-ml flasks containing 30 ml of Vogel's medium (20) containing 1.5% sucrose and 0.2 mg of lyophilized *N. crassa* mycelium per ml.

To examine the effect of *A. quisqualis* culture filtrate on powdery mildew caused by *Sphaerotheca fusca* (Fr.) Blumer, *A. quisqualis* was grown in 100-ml flasks containing 30 ml of PB medium. Inoculum consisted of 3×10^5 conidia per ml, and the fungus was grown for 6 days on an orbital shaker (120 rpm) at 25°C. Mycelium was removed by filtration through Whatman No. 1 filter paper (Whatman International, Maidstone, England), followed by filtration through a 0.22- μ m-pore microbial filter (Millipore, Bedford, MA). Culture filtrate was concentrated ($\times 10$) with a Speed-Vac device (Heto, Allerød, Denmark). As a control, sterile PB medium was concentrated by the same method.

β -1,3-Glucanase activity assays. Qualitative assays were performed on plates containing 0.5 M 4-methylumbelliferyl β -D-glucoside (MUG), phosphate-citrate buffer (50 mM phosphate per 12 mM citrate, pH 5.0; PCB), and 1.5% agar (MUG plates) by spotting 8- μ l samples on MUG plates, incubating at 37°C for 10 min, and visualizing with UV illumination (Spectronics, Westbury, NY) at 302 nm.

Quantitative assays were performed with a substrate mixture containing 0.08% (wt/vol) soluble laminarin dissolved in PCB. Enzyme solution (10 μ l) was mixed with substrate (40 μ l), and the reaction mixture was incubated at 37°C for 60 min. The reaction was terminated by boiling, and the amount of glucose liberated during the reaction was determined with glucose hexokinase reagent (Sigma Chemical Co., St. Louis) according to the manufacturer's instructions. Both qualitative and quantitative analyses were repeated at least twice.

Isolation and analysis of nucleic acids. *A. quisqualis* genomic DNA was extracted and purified as described by Beth Din and Yarden (2). *A. quisqualis* was grown on PDB at 120 rpm on an orbital shaker (New Brunswick Scientific) for 4 days at 25°C. Mycelium was harvested by filtration through Whatman No. 1 filter paper, frozen in liquid nitrogen, and lyophilized.

DNA manipulation (digestion with restriction enzymes, Southern blot analyses) was performed as described by Sambrook et al. (14). A 2.6-kb *EcoRI-XhoI* fragment derived from a cDNA clone that encoded an *exo*- β -1,3-glucanase of *C. carbonum* was separated electrophoretically in agarose gel and excised. The fragment was labeled with [α - 32 P]dCTP (Amersham International, Little Chalfont, England) with a Prime-a-Gene system, random-priming labeling kit (Promega, Madison, WI) in a total volume of 50 μ l. Unincorporated nucleotides were removed by chromatography on a Sephadex G-50 column (Pharmacia Biotech, Uppsala, Sweden). After digestion of *A. quisqualis* genomic DNA with various restriction endonucleases, DNA samples were resolved on 1% agarose gels and blotted on nylon membranes (Magnacharge NT, Micron Separations Inc., Westborough, MA), followed by Southern blot analysis. Hybridization was performed at 42°C in the presence of 35% formamide, $6\times$ SSC ($1\times$ SSC is 0.15 M sodium chloride plus 0.015 M sodium citrate, pH 7.0), $5\times$ Denhardt's solution, 0.5% sodium dodecyl sulfate (SDS), and 100 μ g of denatured salmon sperm

DNA per ml. Final membrane washes were at 42°C with a solution containing $1\times$ SSC and 0.25% SDS. The radioactive signal on membranes was visualized either by autoradiography or scanning with a bioimaging analyzer (BAS1000, Fuji, Tokyo).

DNA was sequenced with *Taq* FS DNA polymerase and fluorescent-dideoxy terminators by a cycle-sequencing method. The resultant DNA fragments were electrophoresed and analyzed with an automated DNA sequencer (Model 373A, Applied Biosystems, Foster City, CA). Sequencing was performed at the W. M. Keck facility, Yale University (New Haven, CT).

To extract RNA, mycelium from a liquid culture of *A. quisqualis* was harvested by filtration through filter paper (Whatman No. 1) and frozen in liquid nitrogen. Total RNA was isolated by LiCl precipitation (16). Poly(A)⁺ RNA was purified by PolyATract mRNA Isolation System IV (Promega).

RNA separation and blotting were performed according to Beth Din and Yarden (2). Total RNA (3 to 5 μ l) was resolved on formamide gels and subsequently blotted on nylon membranes (Magnacharge NT). Prehybridization and hybridization were performed at 42°C in $5\times$ SSC, 50% formamide, $2\times$ Denhardt's solution, 1% SDS, 50 mM sodium phosphate buffer (pH 6.5), 100 μ g of denatured salmon sperm DNA per ml, and 100 μ g of yeast tRNA per ml. A 2.8-kb *EcoRI-XhoI* restriction fragment of the *A. quisqualis* *exgA* gene, which was isolated from an *A. quisqualis* cDNA library constructed during this study and labeled by the same procedure described for *EXG1*, was used as a DNA probe. Final membrane washes were at 65°C with a solution containing $2\times$ SSC and 1% SDS.

cDNA library construction and screening. cDNA was synthesized with a cDNA synthesis kit (ZAP Express, Stratagene, La Jolla, CA) from poly(A)⁺ RNA extracted from an 8-day-old culture of *A. quisqualis* in which significant extracellular β -1,3-glucanase activity was detected (discussed below). cDNA was ligated into the *EcoRI-XhoI* arms of the ZAP Express vector. The library was screened by a plaque hybridization procedure (14), using *C. carbonum* *EXG1* as a probe.

Host, *S. fusca* inoculation, and treatment with *A. quisqualis*. Cucumber plants (*Cucumis sativus* L. 'Dalila') were grown in a peat/vermiculite (1:2) mixture and inoculated with the powdery mildew pathogen *S. fusca* as described previously (1). *A. quisqualis* inoculum was prepared from a commercial formulation (AQ10, Ecogen Inc., Langhorne, PA) in a suspension of 5×10^6 spores per ml. Powdery mildew-infected cucumbers (5 days after inoculation) were sprayed with the hyperparasite spore suspension and maintained at 100% relative humidity by placing pots in closed polyethylene bags. Bags were removed 24 h after application of *A. quisqualis*.

Scanning electron microscopy. Five days after inoculation with *S. fusca*, infected cucumber cotyledons were removed from plants and placed on 0.8% agar plates containing 0.4% benzimidazole (Sigma). *A. quisqualis* culture filtrate made from a 5-day-old PDB-grown culture was concentrated ($\times 10$) with a Speed-Vac device (Heto), and 20- μ l drops of concentrated filtrate were placed on the upper side of infected cotyledons. As a control, concentrated PDB medium ($\times 10$) was used. After placement of drops, plates were kept under light at 25°C. After 24 h, culture filtrate and control medium drops were removed with filter paper. Three-millimeter disks were cut from the cotyledons, immersed in glutaraldehyde (3%, vol/vol) for 1 h at room temperature, rinsed with 0.2 M sodium phosphate buffer (pH 7.2), and dehydrated in a graded series of ethanol solutions (30 to 100%). After dehydration, specimens were dried with CO₂ (critical-point drying) and coated with gold, using a Polaron E-500 (Watford, England) device. Specimens were examined with a scanning electron microscope (JSM 35C, Jeol, Tokyo). The experiment was repeated twice.

In situ hybridization. Disks 1 cm in diameter were cut from powdery mildew-infected cucumber leaves treated with *A. quisqualis*. Infected leaves not treated with the mycoparasite were used as controls. Disks were placed in a 24-well Nunclon multidish (Nalge Nunc, Roskilde, Denmark), and the entire procedure (fixation, hy-

bridization, and washing) was performed during gentle shaking. Disks were washed with 0.6 M KCl, 7 mM Na₂HPO₄, and 3 mM NaH₂PO₄ (PKB) for 20 min at room temperature, fixed in glutaraldehyde (2%, vol/vol, in PKB) for 2 h at room temperature, washed with PKB three times for 5 min each at room temperature, treated with Novozyme 234 (Novo Industries, Bagsvaerd, Denmark) at 10 mg/ml in PKB for 30 min at 30°C, washed with PKB three times for 5 min each at room temperature, treated with proteinase K at 100 mg/ml in PKB for 20 min at 37°C, washed with PKB three times for 5 min each at room temperature, fixed in glutaraldehyde (2%, vol/vol, in PKB) for 20 min at room temperature, and washed again with PKB three times for 5 min each at room temperature.

Disks then were subjected to prehybridization (4× SSC, 60% formamide, 1× Denhart's solution, 0.5 mg salmon sperm DNA per ml, and 0.25 mg of yeast tRNA per ml) at 42°C for 4 h, followed by hybridization with a probe labeled by the random-priming method with a 2.8-kb *EcoRI-XhoI* restriction fragment of the *A. quisqualis* *exgA* gene with fluororedd rhodamine-4-dUTP (Amersham), using a Prime-a-Gene Kit (Promega). Hybridization was performed for 20 h at 42°C. After hybridization, disks were washed in a series of SSC solutions (2×, 1×, 0.5×, and 0.2×) at 42°C for 30 min at each solution strength. Samples of *S. fusca* mycelium (with or without *A. quisqualis*) were removed from disks for light and fluorescence microscope (Axioscop, Zeiss, Oberkochen, Germany) examination. The experiment was repeated three times.

RESULTS

***exo*-β-1,3-Glucanase activity in *A. quisqualis*, *G. roseum*, and *N. crassa* culture filtrates.** As a first step in analyzing the extracellular *exo*-β-1,3-glucanase activity in *A. quisqualis*, enzyme activity was determined in the filtrate of a culture growing exponentially. Glucanase activity in culture filtrate was compared with activity observed in filtrates of *N. crassa*, which is a saprophyte, and *G. roseum*, which is a mycoparasite. After 4 days, β-1,3-glucanase activity in *A. quisqualis* culture filtrate was 516 μmol glucose liberated per h per mg dry weight, while β-1,3-glucanase activity in *N. crassa* culture filtrate was 2.77 μmol glucose liberated per h per mg dry weight and in *G. roseum* was 1.74 μmol glucose liberated per h per mg dry weight. The levels of glucanase activity in the three tests were significantly different ($P = 0.05$) according to Duncan's multiple range test. β-1,3-Glucanase activity in *A. quisqualis* was at least two orders of magnitude higher than in *N. crassa* and *G. roseum*.

Scanning electron microscopy. The effect of *A. quisqualis* culture filtrate on *S. fusca* mycelium was investigated by scanning electron microscopy (SEM). Examination of nontreated cucumber powdery mildew samples showed undamaged mycelium, conidiophores, and conidia (Fig. 1A). Examination of powdery mildew samples exposed to concentrated sterile medium revealed undamaged mycelium with unidentified sediments (Fig. 1B). It is likely, that the sediments were components of the growth medium. In contrast, examination of powdery mildew exposed to concentrated mycoparasite culture filtrate revealed mycelium that, in many instances, exhibited nonuniform external hyphal cell structures (Fig. 1C). It is possible that the observed changes in *S. fusca* hyphal structure resulted from the presence of enzymatic activity (such as cell wall-degrading enzymes) originating from the mycoparasite culture filtrate.

Isolation of the *A. quisqualis* *exgA* gene. A genetic approach was used to isolate a gene encoding an *exo*-β-1,3-glucanase from *A. quisqualis*. A 2.6-kbp cDNA fragment of *EXG1* from *C. carbonum*, a gene encoding an *exo*-β-1,3-glucanase (15), was used to probe for similar genes in *A. quisqualis*. Analysis of an *A. quisqualis* genomic DNA Southern blot revealed a single band in each restriction endonuclease-digested sample of genomic DNA resolved in the gel (Fig. 2), suggesting the presence of a single-copy DNA fragment resembling *EXG1* in *A. quisqualis*.

Fig. 1. Scanning electron micrographs of cucumber cotyledons infected with *Sphaerotheca fusca* 5 days after inoculation: **A**, without treatment; **B**, 24 h after exposure to a droplet of concentrated (×10) sterile potato broth (PB) medium; **C**, 24 h after exposure to a droplet of a concentrated (×10) culture filtrate of *Ampelomyces quisqualis* (grown for 6 days on PB medium). H = *S. fusca* hypha; CP = conidiophore; and C = conidium.

To isolate an expressed fragment corresponding to the DNA fragment detected by Southern blot analysis, a cDNA library of *A. quisqualis* was constructed from poly(A)⁺ RNA isolated from a 6-day-old culture of *A. quisqualis*. The library contained $\approx 1 \times 10^6$ recombinants per ml. A screen of $\approx 30,000$ plaques was performed, using the *EXG1* cDNA fragment from *C. carbonum* as a probe. Five positive clones were isolated. Southern blot analysis showed that all clones had inserts of identical size, as detected by the *EXG1* probe. One of the clones (designated pYR-1) was used for further analysis.

DNA and deduced polypeptide sequence analysis of a putative β -1,3-glucanase-encoding gene of *A. quisqualis*. The 2,746-bp insert of pYR-1 was sequenced completely on both strands. The comparison of the pYR-1 nucleotide sequence (GenBank accession no. AF029354) to that of *C. carbonum EXG1* (provided by J. S. Craig and J. Walton, Michigan State University, East Lansing) revealed a very high degree of similarity: 61.5% identity in nucleotide sequences throughout the entire lengths of the clones. At the correct pYR-1 reading frame (determined by comparison with *EXG1*), the translation yielded a predicted polypeptide composed of 777 amino acids (aa). Comparison of the deduced polypeptide sequence to *EXG1* showed 42.6% identity (Fig. 3). *T. harzianum* *exo*- β -1,3-glucanase (GenBank accession no. AJ002397) also had an amino acid sequence similar to pYR-1: 46.3% identical to the predicted *A. quisqualis* polypeptide (Fig. 3). Two other genes that encoded nonexoglucanase sugar-degrading polypeptides also were identified in the database search: *T. harzianum* *endo*- β -1,3-glucanase (6), which was 29.6% identical to the predicted *A. quisqualis* polypeptide (Fig. 3), and *Nicotiana* sp. polygalacturonase (EMBL accession no. 322767, deposited by Tebbutt and Lonsdale [1995]), which was 24.5% identical to *A. quisqualis* pYR-1 (data not shown).

The high level of similarity between a single open reading frame encoded by the pYR-1 *A. quisqualis* cDNA clone and the deduced amino acid sequence of two fungal *exo*- β -1,3-glucanases suggests that the isolated *A. quisqualis* cDNA is an *exo*- β -1,3-glucanase-encoding gene. We designated this gene *exgA*.

The presumed *exgA* start codon segment is CCATCATGCT, which is different than the putative start codon segment (CCGCTATGGC)

of *A. quisqualis chsA*, the only other gene analyzed so far in *A. quisqualis* (21). Both of the presumed start codon segments show limited similarity to the translation initiation consensus found in other fungi (3). The closest match to a polyadenylation signal is AATA, at nucleotides (nt) 2728-2731 (15 nt upstream of the poly(A) tail). In comparison, the common polyadenylation signal in *N. crassa* is AATAAA, which is located 3 to 23 nt upstream of the poly(A) tail (3).

Calculations based on the deduced amino acid sequence of *A. quisqualis EXGA* suggest the protein has a molecular mass of 84 kDa and a pI of 4.79. Hydrophobicity analysis of the deduced polypeptide suggests the first 40 aa create a hydrophobic region at the N terminus of EXGA. Similarly, the *endo*- β -1,3-glucanase of *T. harzianum* has a hydrophobic region (33 aa) at the N terminus, terminating with lysine and arginine. It has been suggested that this hydrophobic region may be a leader peptide that is cleaved by a KEX2-like protease at the Lys-Arg site (6). In EXGA of *A. quisqualis*, aa 47 and 48 are lysine and arginine, respectively. Thus, it is conceivable, based on these findings, that EXGA has a 48-aa hydrophobic leader sequence that is cleaved by a KEX2-like endoproteinase.

Comparison of a polypeptide fragment (NVAGEWSAA) of the *C. albicans* *exo*- β -1,3-glucanase activity site (11) with the EXGA amino acid sequence revealed a similar segment (NVAGETLLA) in the *A. quisqualis* polypeptide (aa 346-354; Fig. 3), including highly conserved (11) glutamic acid (Glu-350). Assuming functional similarity between the two glucanases exists, it is possible that the catalytic nucleophile of the enzyme may be in EXGA Glu-350.

Expression of *exgA* in vitro. *A. quisqualis* culture samples from various growth stages (as well as dormant spores) were harvested. Total RNA was extracted from samples, and 4 μ g of RNA from each was loaded on a formaldehyde gel. Ethidium bromide staining of ribosomal RNA confirmed that total RNA quantities of all samples were similar. Northern blot results showed there was no expression of the gene in spores, and there was low transcript abundance after 3 days of growth and significant expression after 6 days of growth, followed by a reduction in transcript levels after 10 days of growth (Fig. 4A). The size of the *exgA* transcript was calculated as ≈ 3 kb, based on comparison with the known sizes of *N. crassa* and *A. quisqualis* ribosomal RNA. The calculated size agrees with the length of the isolated *exgA* cDNA fragment (pYR-1).

The effect of dried fungal mycelium in growth medium on expression of *exgA* was analyzed. Dried *N. crassa* mycelium was added to liquid cultures of *A. quisqualis* 24 h prior to harvesting. Total RNA was extracted, and glucanase activity in culture filtrates was measured. Analysis of Northern blots indicated no expression of *exgA* at 3 days of growth without dried mycelium. However, at the same stage in cultures amended with dried mycelium, a distinct band that indicated gene expression was observed (Fig. 4B). Even after 6 days of growth, *exgA* expression in control medium was much lower than that measured after 3 days in dried mycelium-amended culture. After 6 days of growth in cultures amended with dried fungal mycelium, *exgA* expression had markedly increased compared with levels found in unamended culture. We concluded that *exgA* is expressed in vitro mainly in growing mycelium of *A. quisqualis* prior to pycnidia formation and that *exgA* expression is enhanced by the presence of fungal cell wall components.

Expression of *exgA* in vivo. To analyze *exgA* expression in vivo, *S. fusca* mycelium and conidia, with or without *A. quisqualis*, were collected from infected cucumber leaves. Total RNA was extracted from samples of powdery mildew 2, 5, and 7 days after inoculation with the hyperparasite and from a sample of powdery mildew without the hyperparasite. Each sample consisted of material collected from 60 cucumber leaves. The experiment was repeated three times.

Northern blot analysis indicated the presence of *exgA* transcript in samples of powdery mildew treated with *A. quisqualis* 5 and

Fig. 2. Southern blot analysis of *Ampelomyces quisqualis* genomic DNA digested with various restriction endonucleases and probed with cDNA of *Cochliobolus carbonum EXG1*. Hybridization and wash conditions are described in text.

Ccexogluc	1	MRFSSLLACLGAV-GIQAAAI PFQRRVDNTTDSGSLD	AAQAAAAIYDGYWLN	DL	LSG
Thexogluc	1	-----MGFIRS	AVLSALTFAAACRGLATPGS-EAEPSVEKRA	SS	WYENIAH
Aqexogluc	1	-----MLAFSAGAFLLTLRVFLTATPSAAAPVAQ	AVEVPQAG	SG	YWFN
Thendogluc	1	-----MLKLTALVALLLGAASATPTPSPPASDEGITKRA	TSFYYP	NMDH	
Ccexogluc	56	----KGRAPFNSNP-NYKVF	RNVKDYGAKGDGVTDDSDAFNRAIS	DGSR	CGP
Thexogluc	47	----QGIAPF-APS-NYTV	RNVKDYGAKGDGVTDDTA	AINNA	ILSGRCGR-LCT
Aqexogluc	49	----QGIAPYNENPAAYKVF	RNVKLLGAKGDGVTDDTA	AINNA	IA
Thendogluc	45	VNA PRGFAPDL DGD FNYPI	YQTVN----AGDG-----NALQNA	ITTDGKGS	SRHPQ
Ccexogluc	107	SSTDS	PAVVYVPSGTYLIN	KPIIF	YMTALIGNPRELPVLKAA
Thexogluc	96	SSTLT	PAVVYFPAGTYVI	STPIIDQYYTNI	IGDPTNLP
Aqexogluc	100	STTTS	PAIIYFPAGTYLISEPI	IQQYYTQFVGD	ATNPPTL
Thendogluc	92	WFASQ	PRVVYIPPGTYTIS	SKTLRFNTDTILMGDPTN	PIIKAAAGFS
Ccexogluc	163	Y--SNQNGEP	GWISTNLF	FLRQIRNLI	IDGTAVAPTS-GFQA
Thexogluc	152	YYGD	NNPNDPNWISTNV	FYRQV	RNFKLDMTS IPTSAPKIYGIHWP
Aqexogluc	156	YIPGD	-GANWYTNN	FYRQIRNF	VIDIKDTKAAA---GIHWQVS
Thendogluc	148	DPSTNEK	GEELS-----FAVA	IKNVVLD	TTAI-PGGNSFTALWVGVAQA
Ccexogluc	216	IRMTQA-SNSV	HAGIFVENGSG	GHMADLDIT	GGLYGMNI-GNQ
Thexogluc	208	ITMSTA-SGNS	QVGLFIENG	SAGFLTDMTFNGGLI	GAAI-GNQ
Aqexogluc	207	FEMATGEAGAN	QKGFQDNGSG	GFMSDLVFNGGA	IGAFL-GSQ
Thendogluc	197	ITMSSSSG	NGHTGIRMG	RGSTLGLAD	VVRVERGQNGIWI
Ccexogluc	270	VGISQ	IWNWGWLYSGLQIS	DCGTAF	SMVNGGSAGKQEVGSAV
Thexogluc	262	QPLSA	ASIGSGFTRAIS	IINNCGLGIDMT	-----AAESITL
Aqexogluc	262	TAIFM	NWNWLWTLK	SIFINDCK	LGLDMAN--SPDNQTVGSV
Thendogluc	253	IGMLI	SSGNTFSIFS	SSTFDT	CGTAFPTLAGSPW-----IAL
Ccexogluc	326	SAWSQTSN	PTGSGQLV	IENIKLT	NVPAAV-VSNGATV
Thexogluc	310	TSFRNQSP	PATSN	SLIVENLS	SLNVPVAIQSSSG
Aqexogluc	316	SSFTQDS	VPH	TGGTLID	NVDFEGSNVAVQNVAGETL
Thendogluc	299	-TE	TNQFP	-----SFM	IENLT
Ccexogluc	381	NASGPSKF	QGA	I-SGAT	TRPTGLLQNGK
Thexogluc	366	N--GPTTF	QGS	I-TPNS	RPPSLLSGSNYYTR
Aqexogluc	371	GQAQAGR	VQGDVNN	PTKPPQ	SLLG
Thendogluc	348	NPT	-----YGD	VTS	SNTRPSALA
Ccexogluc	431	-ATG	DGV	TDDTRAV	QAAVTQAASQNKVLF
Thexogluc	414	-ATG	NAV	TDDTAALQSV	INSATAAGQIVYFDAGIYRIT
Aqexogluc	422	-AVG	DGV	TDDTAMI	QKAI-DGLQDQILHADHGAYLIT
Thendogluc	399	QVK	G	DN	TINEADTLNAILEL
Ccexogluc	484	SAIMGS	SGSTFG	DQANPVPI	IQIG-KPGESGS
Thexogluc	467	PIIMSS	SGSFFNDQSN	PKPVVQVG-T	PGQTGGV
Aqexogluc	476	TMEFI	TGKFFGN	MDDPQPG	FVRVGGKSGDKGT
Thendogluc	453	ATITG	NGNPFKN	ENSPPQ	PVSVG-RAGDV
Ccexogluc	538	NTA-LGS	GLWDVH	TRIGGAKG	TNLQVAQC
Thexogluc	521	ATSGTPS	GMWDVH	TRIGGAKG	TNLQVAQC
Aqexogluc	531	NAEAGKA	GLWDVH	FRVGG	FAGTNLQSSNCKKNPDTEHPPNE
Thendogluc	508	GNNPGD	VALWNS	SLVTVGSTR	GAQALANACTNN-----SNECK
Ccexogluc	590	NGAYF	ENNW-E	WTADH	LDLDDADSTRIN
Thexogluc	576	SNLYM	ENNW-L	WTADH	DI
Aqexogluc	587	SG-Y	FENVW-L	WTADH	ELDQPDHAQ
Thendogluc	557	S-PY	I	QNVWEL	LGLRDHIAENFSGGTSHRRERW
Ccexogluc	644	YQFN	AAQDI	IFAGY	IQTETPYFQ--PTPIA
Thexogluc	631	YQFANT	QNIYAG	VIQTETPYFQ--PNP	DAPT
Aqexogluc	641	YQEQ	AKDITWY	GALQ	DETETPYFQ--ENPKAN
Thendogluc	612	LNLHNA	ANVVVSL	LQAE	TNYHOGANTQQIP
Ccexogluc	691	--SAW	GLRL	LD	AKNVLIIYGGGLYSFF
Thexogluc	683	CAEAW	GLR	IVSSQ	NILIIYAAGLYSFF
Aqexogluc	686	-TSAW	AVRI	IDSS	IWNYGAGTYSFF
Thendogluc	667	CRMGPAN	F	INGGS	NIYTYASA
Ccexogluc	744	TSVQA	F	GESEVGV	EWMTAAAGQDKANWKD
Thexogluc	738	TNIN	VYNL	GT	VGVVNQITQNGNVLATSSSNVNAFADVIAL
Aqexogluc	734	RNVN	I	FGL	STKASVNMIS
Thendogluc	716	SNLQA	F	GL	CSKDSVNTLR

Fig. 3. Comparison of deduced amino acid sequences of *Cochliobolus carbonum* EXG1 (Ccexogluc), *Trichoderma harzianum* α -1,3-glucanase (Thexogluc), *Ampelomyces quisqualis* putative α -1,3-glucanase (Aqexogluc), and *T. harzianum* α -1,3-glucanase BNG13.1 (Thendogluc). Dashes indicate gaps inserted for optimal alignment. *T. harzianum* α -1,3-glucanase has a longer amino acid sequence than the other sequences presented (1,032 aa). Thus, only the section that aligns with the other glucanases is shown.

7 days after inoculation (Fig. 4C). There was no indication of the presence of a structurally related transcript in nontreated powdery mildew and powdery mildew 2 days after treatment with *A. quisqualis*. The size of the transcript identified by the *exgA* probe was identical to the size of the transcript detected in pure *A. quisqualis* cultures (Fig. 4A and B).

In situ hybridization was conducted with samples obtained from a late stage of mycoparasitism (6 days after treatment with *A. quisqualis*). Prior to hybridization, samples of the fungi (both *S. fusca* with and without the mycoparasite from cucumber leaves and of *A. quisqualis* from culture) that were not subjected to hybridization with the *exgA* fluorescent probe were examined by fluorescence microscopy. None of the control samples emitted any significant signal (data not shown).

Examination of powdery mildew not treated with *A. quisqualis* after hybridization with the probe showed undamaged conidial chains, conidiophores, and mycelia (Fig. 5A). The signal produced by rhodamine, which indicates hybridization to the *exgA* probe, was very weak in the nontreated *S. fusca* conidia chain (Fig. 5B). Moreover, a relatively long image integration time ($1/250$ s) was required to capture the weak signal seen in this picture.

In contrast, an examination of *A. quisqualis*-treated *S. fusca* (after hybridization with the probe) showed collapsed conidia chains and conidiophores of *S. fusca* colonized by *A. quisqualis* (Fig. 5C and D). The rhodamine signal was intense inside the mycoparasite mycelium. A signal inside *A. quisqualis* pycnidia was observed that was somewhat weaker than the signal observed in the mycelia (Fig. 5E and F). A signal also was detected in pycnidiospores (Fig. 5F). In these cases (Fig. 5F), a much shorter integration time ($1/4,000$ sec) was required to obtain the signal. The difference between the integration time required to obtain the different images, in addition to the difference in rhodamine signal, suggests hybridization of the *exgA* probe is specific to the mycoparasite and that such a gene is not expressed by *S. fusca*. The *exgA* gene is expressed along *A. quisqualis* mycelia (at a late stage of mycoparasitism) and, based on signal intensity, is probably expressed less in pycnidia.

DISCUSSION

The anatomy of the mycoparasitic relationship between *A. quisqualis* and its hosts, the Erysiphales, has been investigated and described in detail (9). However, very little is known about cell-wall lytic enzymes that are produced by *A. quisqualis*. Our results,

Fig. 4. Northern blot analysis of *Ampelomyces quisqualis* *exgA* expression. Blots were probed with random-primed (α^{32} P)dCTP-labeled *exgA*. **A**, Samples taken from either mycelial cultures at different stages of growth (numbers indicate age, in days, of culture grown in liquid medium) or from pycnidiospores. **B**, Mycelial samples taken from 3- and 6-day-old liquid medium-grown cultures, with (N) or without added dried *Neurospora crassa* mycelium (0.2 mg/ml) 24 h prior to harvesting. **C**, Samples extracted from *Sphaerotheca fusca* infected with *A. quisqualis*. Numbers indicate days after inoculation of *S. fusca* with *A. quisqualis*. Sf = nontreated *S. fusca*. Hybridization and wash conditions are described in text. Bottom panels: ethidium bromide-stained rRNA, as indication of RNA loaded.

with respect to the ability of *A. quisqualis* to produce extracellular *exo*- β -1,3-glucanase in culture compared with other fungi, suggest that these enzymes play an important role in the mechanism of mycoparasitism.

The ability of extracellular enzymes produced by mycoparasitic fungi to cause lysis of host cell walls has been demonstrated in several studies. Larena and Melgarejo (10) found that *P. purpureogenum* produces a complex of extracellular enzymes, including β -

Fig. 5. In situ hybridization of *Sphaerotheca fusca* taken from cucumber leaves 11 days after inoculation (treated or nontreated with *Ampelomyces quisqualis*), probed with an *exgA* fluorescent DNA probe. **A**, Nontreated *S. fusca*, bright-field illumination. **B**, Nontreated *S. fusca*, epifluorescent illumination. **C** and **D**, *A. quisqualis*-treated *S. fusca*, bright-field illumination. **E** and **F**, *A. quisqualis*-treated *S. fusca*, epifluorescent illumination. C = *S. fusca* conidium, P = *A. quisqualis* pycnidium, and H = hypha. Bars = 10 μ m.

1,3-glucanase and β -1,6-glucanase, that is capable of degrading cell walls of *M. laxa* spores and mycelia. Purified β -1,3-glucanase of *Stachybotrys elegans* causes swelling and bursting of *R. solani* hyphal tips, cytoplasm leakage, and formation of septa in attacked hyphae (18). We found that a concentrated culture filtrate of *A. quisqualis* can cause lysis of *S. fusca* cell walls, indicating that extracellular cell wall-degrading enzymes (most likely glucanases and chitinases) of *A. quisqualis* are capable of degrading the entire host cell wall.

Reports concerning the extent of and differential roles of β -1,3-glucanase genes and protein families are still very limited. Thus, although the presence of multiple β -1,3-glucanases in different fungi has been established (6,13), their functions have yet to be determined.

It is likely that mycoparasites require additional β -1,3-glucanase activities for the mycoparasitic phase of their life cycle, in addition to the metabolic and morphogenetic β -1,3-glucanase activities that are common in other fungi. *T. harzianum* produces at least three extracellular β -1,3-glucanases (6). In contrast to *Trichoderma* spp., which are considered necrotrophic mycoparasites, *A. quisqualis* is a biotrophic mycoparasite (4). Because biotrophic mycoparasites tend to have different relationships with their hosts than those between necrotrophic mycoparasites and their hosts and probably a different pattern of glucanase activities, it is interesting to compare *A. quisqualis* and *Trichoderma* spp. with respect to their glucanase activities. For *A. quisqualis*, preliminary results of isozyme starch gel electrophoresis indicate there are at least five β -1,3-glucanases present, and at least two are extracellular (Y. Rotem, O. Yarden, and A. Szejnberg, unpublished data).

The *exgA* gene isolated from the *A. quisqualis* cDNA library is highly similar to an *exo*- β -1,3-glucanase gene of *T. harzianum* (46.3% identity of deduced polypeptide sequences) and to *EXG1* of *C. carbonum* (42.6% identity of deduced polypeptide sequences). To a lesser extent, *exgA* also is similar to the *T. harzianum bng13.1* gene (6) that encodes an *endo*- β -1,3-glucanase (29.6% identity of amino acid sequences; Fig. 3). Our analysis clearly indicates that the *exgA* gene encodes an *exo*- β -1,3-glucanase. It is also reasonable to assume that a gene encoding a β -1,3-glucanase would have some similarity to a gene encoding a polygalacturonase, which is another polysaccharide-degrading enzyme. Based on structural analysis, the calculated molecular mass of EXGA is 84 kDa, and its pI is 4.79. According to Pitson et al. (13), fungal β -glucanases have a broad range of molecular masses, commonly ranging between 20,000 and 80,000 Da, but some fungal glucanases have molecular masses of up to 230,000 Da. In view of the broad range, a molecular mass of 84,000 Da is not surprising. The common pI range of filamentous fungal β -glucanases is between 4.0 and 8.0, and the optimal activity of fungal β -glucanases usually ranges between pH 4.0 and 6.0. (13). Based on these glucanase enzyme properties, the *A. quisqualis* β -1,3-glucanase analyzed in our study has the properties of a common fungal glucanase.

Another similarity between EXGA of *A. quisqualis* and *T. harzianum* glucanases (both *endo*- and *exo*- β -1,3-glucanases) is that all have a putative hydrophobic leader sequence that ends with the amino acids Lys-Arg (6). This may indicate the presence of a leader peptide (33 aa long in *T. harzianum endo*- β -1,3-glucanase; 35 aa long in *T. harzianum exo*- β -1,3-glucanase; and 48 aa long in *A. quisqualis* glucanase) that could be cleaved by a KEX2-like endoprotease. Several secreted enzymes in *Trichoderma*, CHIT42 chitinase, BNG16.2 β -1,6-glucanase and PRB1 protease of *T. harzianum*, and XYN2 of *T. reesei* contain similar leader peptides (6).

Mackenzie et al. (11) identified Glu-330 as the catalytic nucleophile of *C. albicans exo*- β -1,3-glucanase. Their conclusion was based on site-directed mutagenesis. They found that glutamic acid in this position was completely conserved in this family of enzymes. A comparison of EXGA to the active site of an *exo*- β -1,3-glucanase from *C. albicans* revealed a close match in EXGA amino acid sequence, which includes the glutamic acid at position 350 (Fig.

10 in Mackenzie et al. [11]). This comparison suggests that Glu-350 is the catalytic nucleophile of *A. quisqualis exo*- β -1,3-glucanase. In contrast to the findings of Mackenzie et al. (11), one notices that glutamic acid is not completely conserved in all *exo*- β -1,3-glucanases: *EXG1* of *C. carbonum* has no glutamic acid in the same position. This is true for *T. harzianum* BNG13.1 as well, but BNG13.1 is an *endo*-glucanase and, therefore, may have a different active site.

Studies on the expression of the putative β -1,3-glucanase gene in liquid culture show that the gene is expressed mainly at the stage prior to pycnidia maturation. Expression tends to be minor at the young mycelium (3 days old) and mature pycnidia (10 days old) stages. There is no sign of *exgA* expression in mature pycnidiospores (Fig. 4A). The results of expression studies on *S. fusca* treated with *A. quisqualis* cannot be used as an indication of the relative abundance of glucanase mRNA within the total mRNA of *A. quisqualis*, because we cannot distinguish between the RNA of the host and mycoparasite. Thus, it is possible that the increased abundance in *exgA* signal may be due to the proportional increase of *A. quisqualis* RNA in the mycoparasite/host RNA extract ratio, even though such a gene does not seem to be expressed in *S. fusca*. Nonetheless, our in vitro experiments support the observed appearance of *exgA* gene expression during late phases of mycoparasite development in the host.

In situ hybridization did confirm that an *exgA* homolog is not expressed in *S. fusca*. Our procedure also clearly demonstrated that the expression of *exgA* was located along the mycelium of *A. quisqualis*, within *S. fusca* mycelium, and during late phases of mycoparasitism when the mycoparasite forms pycnidia.

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