Elicitors of Plant Defense Responses from Biocontrol Strains of *Trichoderma virens*

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ABSTRACT

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Effective biocontrol strains of *Trichoderma virens* can induce the production of defense-related compounds in the roots of cotton. Ineffective strains do not induce these compounds to significant levels. This elicitation was found to be heat stable, insoluble in chloroform, passed through a 5K molecular weight cut-off (MWCO) filter, but not a 3K MWCO filter, and was sensitive to treatment by proteinase K. When the active material was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, several bands were present in the material from bio-

control-active strains that were lacking in inactive strains. When eluted and tested for elicitation activity, with or without renaturation, four bands stimulated cotton terpenoid production. One band showed cross-reaction with an antibody to the ethylene-inducing xylanase from *T. viride*. Another band of approximately 18 kDa, gave significant stimulation of cotton terpenoid production and increased peroxidase activity in cotton radicles in all tests, with or without renaturation. The 18-kDa protein was subjected to amino-terminal sequence analysis, and the first 19 amino acids at the amino terminus were determined to be DTVSYDTGYDN-GSRSLNDV. A database homology search using the BLASTp algorithm showed the highest similarity to a serine proteinase from *Fusarium sporotrichioides*.

Trichoderma spp. have biocontrol activity against a number of different fungi on various hosts. On cotton, biocontrol activity has been reported against *Rhizoctonia solani* (12), *Fusarium oxysporum* f. sp. *vasinfectum* (26), and *Verticillium dahliae* (10). One proposed mechanism for biocontrol activity in *Trichoderma* sp. is stimulation of host defense responses (14). Induced resistance has been reported with *T. harzianum* on bean (5), an unidentified *Trichoderma* sp. on cucumber (18), and *T. virens* (*Gliocladium virens*) on cotton (10). Cotton seedlings treated with effective biocontrol strains of *T. virens* have higher levels of defense-related compounds such as terpenoids and higher peroxidase activity in the roots than seedlings treated with ineffective isolates (14).

Several fungi and oomycetes produce compounds that elicit the production of defense responses in plants (2,4,6-9,19,21). The majority of these fungi are plant pathogens, but metabolites from *Penicillium janczewskii* elicit resistance to stem rot in melon and cotton (19) and an ethylene-inducing xylanase (EIX) produced by *T. viride* (3) elicits the production of the phytoalexin resveratrol in grapevine cells (1). Little is known about the potential production of other elicitors by nonpathogenic plant-associated fungi.

Culture filtrates from effective biocontrol strains of *T. virens* were found to stimulate significantly greater terpenoid levels in cotton than filtrates from noneffective strains or media alone. The purpose of this research was to isolate and identify elicitors from such *T. virens* culture filtrate.

MATERIALS AND METHODS

Parent and mutant strains of *T. virens* were stored as conidia in 25% glycerol at -70°C until used. Prior to use, conidia were transferred to potato dextrose agar (PDA, Difco Laboratories, Detroit) plates containing 50 µg of rifampicin per ml, and colonies developing from them were used as a source of inoculum. Strains used in this experiment included: *T. virens* strain G6, an isolate from Texas cotton field soil with good biocontrol activity against *R. solani* (15) and two UV-induced mutants of G6 (G6-4 and G6-5) that were deficient for both mycoparasitism and gliotoxin production (provided by M. H. Wheeler, College Station, TX). Mutant G6-5 had good biocontrol activity against *R. solani*, but mutant G6-4 had no detectable biocontrol activity against *R. solani* (14).

To obtain culture filtrates (CFS), the *Trichoderma* isolates were grown in liquid shake cultures (G-10 gyratory shaker; New Brunswick Scientific Co., New Brunswick, NY) of 100 ml of 5% wheat bran and 1% peat moss (WBPM, pH 4.0) as described by Howell et al. (13). After 7 days incubation at 150 rpm and 27°C, the culture contents were filtered through Whatmann's No. 1 filter paper, and the CFS were collected. CFS were either used directly or lyophilized and brought to the desired concentration in sterile water. CFS were filter-sterilized by passage through 0.22-µm filters (Sterile Acrodisc; A. Daigger & Co., Lincolnshire, IL) and stored at 5°C until used. The hyphal material and peat moss particles were collected, air dried, and plated to compare growth in terms of colony-forming units for the different fungi.

Induction of terpenoids in cotton roots by culture filtrates. Cotton seeds (cv. DeltaPine 50) were surface-disinfested for 5 min in 2% sodium hypochlorite solution with 0.01% Triton X-100 on a rotary shaker (200 rpm). Seeds were rinsed twice for 4 h in sterile distilled water and placed on filter paper moistened with distilled water in glass petri dishes wrapped in sterile aluminum foil. After 4 to 6 days, CFS were applied to the radicles of germinated seeds by pipetting 3 ml of solution onto the radicles

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of 12 seeds per replicate, three replicates per treatment, when they were approximately 2 cm in length. The solution contacted the root and then saturated the sterile filter paper for continuous exposure. Controls included sterile water or sterile culture media applied to the same number of seeds and replicates. After 2 days of incubation at 26°C, the radicles were excised from the plants. The radicles from each of the three replicates were weighed, cut into approximately 5-mm sections, and soaked for 24 h in a volume of 90% acetone, 9.9% water, and 0.1% ascorbic acid (3 ml per g of tissue). The acetone extracts were drawn off by pipette and subjected to high-performance liquid chromatography (HPLC) analysis as described previously (27). The HPLC system consisted of a Model 1090 Liquid Chromatograph (Hewlett-Packard, Atlanta, GA) with a diode array detector set at 235 nm. The solvents, methanol (0.07% H₃PO₄) and H₂O (0.07% H₃PO₄), were delivered using a gradient starting with 80% H₂O, changing to 40% H₂O after 5 min, and ending at 13% H₂O after 22 min. The flow rate was 1.25 ml/min.

Stimulation of peroxidase activity. 'DeltaPine 50' seed were treated with CFS as described previously. Replicate 0.55-g samples of radicles were each ground separately with a glass tissue grinder (Ace Glass, Inc., Vineland, NJ) in 1-ml aliquants of 0.1 M phosphate buffer (pH 6.0) and centrifuged (Eppendorf 5414; Brinkmann Instruments, Inc., Westbury, NY) at 10,000 × *g* for 15 min. The supernatants were collected and stored on ice. Supernatants were assayed for peroxidase activity by the guaiacol assay as described by Smit and Dubery (24). Five microliters of supernatant was incubated for 5 min at 25°C with the reaction mix (24), and absorbance was determined at 470 nm in a spectrophotometer (DU-70; Beckman Coulter, Inc., Fullerton, CA). The reaction mix with 5 µl of heat-inactivated supernatant was used as a blank.

Alternate media for elicitor production. Cultures of *T. virens* strain G6 were prepared as described previously in 100 ml of each of the following media: potato dextrose broth (PDB, Difco Laboratories), Rawlin-Thom medium (RT) (22) and malt extract medium (ME) (25), and WBPM. After 7 days incubation, CFS were collected as described previously, and elicitation activity was determined. Sterile media were used as controls.

Characteristics of elicitation activity. CFS were concentrated by freeze-drying (DuraStop/DuraStop MP R114; FTS Systems Inc., Stone Ridge, NY). Lyophilized material was resuspended in sterile water for a final concentration of five times the concentration of the initial material (CFS5).

To test solubility of the eliciting materials, CFS5 were extracted five times with equal volumes of chloroform. The chloroform fractions were evaporated to dryness on a rotary evaporator (Buchi Rotoevaporator; Brinkman Instruments). The chloroform-soluble fraction was resuspended in a volume of sterile distilled water equal to that of the initial solution. The residual solvent was removed from the aqueous fraction with a rotary evaporator. The chloroform-soluble and aqueous fractions were assayed for terpenoid induction activity as described previously.

Ten-milliliter aliquants of CFS5 were placed into prewarmed 15-ml glass test tubes and incubated in an 80°C water bath (Thelco Model 82; Precision Scientific Co., Chicago) for 30 and 60 min. Unheated and heated materials were assayed for terpenoid induction activity.

CFS5 were passed through a series of molecular weight cut-off (MWCO) filters, 10,000 (10K), 5,000 (5K), and 3,000 (3K) (Pall Filtron Corp., Northborough, MA and Lida Mftg. Corp., Kenosha, WI). The material retained by the filter and the material that passed through the filter were brought to initial volume with sterile distilled water and assayed for terpenoid induction activity. According to company literature (Pall Filtron Corp.), for protein retention it is necessary to choose a device with an MWCO that is between three and six times smaller than the molecular weight of the protein to be retained, with optimum retention using a size six

times smaller than the protein molecular mass. Thus, for the filters used, the 10K MWCO filter would be expected to have optimal retention of proteins of 60 kDa or greater, the 5K filter would retain proteins of 30 kDa or greater, and the 3K filter would be optimum for retention of proteins of 18 kDa or greater.

CFS5 were treated with various enzymes to further characterize the elicitation activity. All enzymes were added to the CFS at 5 units of enzyme per ml of culture filtrate. Enzymes suspended in sterile distilled water were used to test for direct activity of the enzymes on seedlings. The enzymes used included β-glucosidase, proteinase K, protease, and trypsin (Sigma Chemical, St. Louis). Protease, proteinase K, and β-glucosidase were incubated at 37°C and trypsin was incubated at 25°C for 2 h in a shaking water bath (100 rpm). Materials were assayed for terpenoid induction activity.

The protein concentration in the preparations was tested with the Coomassie Plus Protein Assay Kit (Pierce, Rockford, IL). The concentration for two 50-µl replicates of each protein preparation was compared according to manufacturer's instructions. Absorbance was determined at 595 nm in a spectrophotometer and compared with bovine serum albumin standards.

Polyacrylamide gel electrophoresis and western blotting. CFS5 that had been passed through a 10K MWCO filter were fractionated with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using either a 15 or 18% resolving gel with 4% stacking gel (Bio-Rad, Hercules, CA). Size markers were broad range and polypeptide SDS-PAGE molecular weight standards (Bio-Rad). For western blotting, cellulysin (Calbiochem, La Jolla, CA) was suspended in water at 1% (wt/vol), mixed 1:2 with loading buffer, and included as a control. For western blots, G6-4 CFS5 was double loaded onto the gel. For direct observation, gels were stained with Coomassie blue (Sigma Chemical) or silver stain (Bio-Rad). Bands were transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad) for western blot analysis by electrophoretic transfer (Mini Trans-Blot; Bio-Rad) with Tris-glycine-SDS transfer buffer, pH 9.2 (48 mM Tris, 39 mM glycine, and 0.05% SDS). In western blots, membranes were blocked overnight with blocking buffer (Tris-buffered saline [TBS] with 0.1% Tween 20 and 5% powdered milk) at 4°C. Blots were probed with antibodies developed against the EIX from *T. viride*, obtained from J. D. Anderson (Beltsville, MD). Antibodies were diluted 1:10,000 in blocking buffer and incubated for 1 and 3 h for initial tests. Two sets of blots were incubated overnight to detect low levels of proteins. Blots were washed three times and developed with TBS-Tween for 10 min each. Washed blots were incubated for 1 h with goat anti-rabbit alkaline phosphatase conjugate (Bio-Rad) in blocking buffer at room temperature. Blots were washed three times, 10 min each, with TBS-Tween and developed according to manufacturer's instructions (Bio-Rad). When color developed, blots were washed twice for 5 min each in sterile nano-pure water and air dried.

Activity of fractions. Bands were cut from SDS-PAGE gels. Material in bands was collected by electroelution according to the manufacturer's instructions (422 Electro-Eluter; Bio-Rad), in which case the material was in a denatured form. Alternatively, gel slices (single bands) were macerated in a renaturation buffer (30 mM HEPES buffer, pH 7.0, 0.1% Tween 80, 10% glycerol, and 5 mM dithiothreitol), incubated at room temperature for 3 h, and centrifuged for 5 min at 12,000 × *g*. The supernatant was collected, with the materials in a renatured form. Eluted materials were assayed for activity on cotton seedlings.

Because one fraction described previously showed a cross-reaction with the EIX antibody from *T. viride*, cellulysin was assayed for elicitation activity on cotton radicles. Cellulysin was suspended in acetate buffer (pH 5.0) at a concentration of 0.5 mg/ml, assayed as described previously, and compared with water and acetate buffer controls.

The 18-kDa band was excised from a PVDF membrane and submitted to the Protein Chemistry Core Laboratory (Baylor College of Medicine, Houston, TX) for amino-terminal sequencing. The amino acid sequence was used in a database homology search using the BLASTp algorithm.

Statistics. All experiments were repeated at least two times. The data from the last trials are presented here. Statistics were performed using the general linear model (version 6; SAS Institute, Cary, NC) or the *t* test of correlation functions of EXCEL (Microsoft Corp., Bothell, WA).

RESULTS

CFS from the biocontrol-effective strains of *T. virens* significantly stimulated terpenoid levels in cotton radicles compared with the levels in the water and media-treated controls and the ineffective strain (Table 1). Peroxidase activity was similarly significantly stimulated ($P = 0.02$) in roots treated with extract from the biocontrol-effective strain G6 (11.77 ± 1.46 pkat) when compared with the ineffective strain G6-4 (8.72 ± 1.58 pkat). No significant differences ($\alpha = 0.05$) were found between the three different fungal strains in the number of colony-forming units produced in the media.

The levels of terpenoids detected in plants treated with the various media differed. For example, average desoxyhemigossypol (dHG) levels were 2.04, 4.36, 9.98, and 10.22 $\mu\text{g/g}$ of tissue for RT, WBPM, ME, and PDB, respectively. Levels of dHG for media in which G6 had grown were 5.31, 27.16, 21.69, and 12.36 $\mu\text{g/g}$ of tissue for RT, WBPM, ME, and PDB, respectively. The difference in terpenoid levels between the sterile medium control and the media in which G6 had been grown was significant ($\alpha = 0.05$) for filtrates of cultures grown in ME and RT as well as the WBPM, but not in PDB. The G6 culture grown in RT had significantly higher terpenoid levels than material exposed to sterile water (dHG of 2.02 $\mu\text{g/g}$ of tissue).

The elicitation activity for most terpenoids was not found in the chloroform extracts (Table 2) and was retained after heating at 80°C for up to 60 min. The elicitor(s) passed through a 5K MWCO filter, but not a 3K MWCO filter (Table 2), and were highly sensitive to proteinase K treatment, with less sensitivity to trypsin, β -glucosidase, and protease treatment (Table 3). Of the enzyme preparations, the protease, trypsin, and proteinase K had significantly ($\alpha = 0.05$) higher levels of hemigossypol in the treated seedlings than in the water control (Table 4), with the highest levels with the protease treatment. However, these levels were still lower than those found with any of the CFS treatments. Significantly higher levels of dHG and 5-methoxygossypol were

TABLE 1. Stimulation of cotton terpenoids in seedling radicles by treatment with culture filtrates from *Trichoderma virens* shake cultures

Treatment ^y	Terpenoid concentration (μg of compound per g of tissue) ^z					
	dHG	HG	dMHG	MHG	HGAL	G
Water	2.46 a	2.03 a	2.63 a	1.82 a	n.d.	94.68 a
WB+PM	4.36 a	14.89 b	2.38 a	3.08 a	3.88 a	133.19 a
G6-4	3.93 a	10.11 b	2.06 a	2.97 a	3.25 a	129.24 a
G6	27.16 b	90.58 c	10.26 b	6.76 b	39.96 b	250.79 b

^y Treatments include water control, filtrate from the wheat bran and peat moss medium (WB+PM) and culture filtrates from WB+PM medium in which an ineffective mutant (G6-4) of a biocontrol-effective strain (G6) of *T. virens* had been grown.

^z Terpenoids detected included dHG = desoxyhemigossypol, HG = hemigossypol, dMHG = desoxyhemigossypol 6-methyl ether, MHG = hemigossypol 6-methyl ether, HGAL = hemigossylic acid lactone, and G = gossypol. Numbers followed by the same letter are not significantly different ($\alpha = 0.05$) according to Fisher's least significant difference. n.d. = not detected; statistics were performed without this treatment when there was no detectable terpenoid production. Numbers are the mean of three replicates of 12 seedlings each.

TABLE 2. Characteristics of elicitation activity from *Trichoderma virens* culture filtrates^w

Treatment ^x	dHG	HG	dMHG	MHG	HGAL	G
Solubility						
G6-untreated	26.56 a	43.45 a	4.70 a	3.27 a	12.26 a	191.54 a
G6-aqueous	22.02 a	30.62 a	4.53 a	2.35 a	8.04 a	144.44 a
G6-chloroform	6.49 b	4.60 b	3.20 a	0.47 b	n.d.	101.36 a
Size exclusion						
G6	39.05 a	79.72 a	15.03 a	7.38 a	18.09 a	300.36 a
10K retained ^y	9.19 b	6.62 b	4.58 b	1.17 a	2.97 b	169.42 b
10K filtrate	48.89 a	88.82 a	18.94 a	9.81 a	21.97 a	343.11 a
5K retained	11.88 b	9.94 b	6.26 b	2.88 a	n.d.	168.72 b
5K filtrate	40.83 a	87.82 a	15.29 a	8.81 a	18.65 a	332.31 a
3K retained	26.26 a	53.10 a	8.19 ab	5.09 a	8.93 b	244.71 a
3K filtrate	14.13 b	12.78 b	7.86 b	4.52 a	4.97 b	166.55 b
Temperature ^z						
G6-28°C	34.43 a	46.34 a	4.25 a	2.95 a	8.29 a	230.46 a
G6-80°C	28.52 a	29.38 b	3.27 a	2.43 a	7.65 a	184.40 a

^w dHG = desoxyhemigossypol, HG = hemigossypol, dMHG = desoxyhemigossypol 6-methyl ether, MHG = hemigossypol 6-methyl ether, HGAL = hemigossylic acid lactone, and G = gossypol. All values are micrograms of compound per gram of tissue. Numbers followed by the same letter in the same experimental group are not significantly different ($\alpha = 0.05$) according to Fisher's least significant difference. n.d. = not detected; statistics were performed without these treatments for terpenoids for which there was no detectable production.

^x Treatments are all culture filtrate from wheat bran and peat moss media in which *T. virens* strain G6 had been grown, treated with different solvents, molecular weight cut-off filtration, or incubation at different temperatures.

^y Retained material remained on the membrane of the given size while the filtrate passed through and was collected following centrifugation. The expected protein molecular mass retained by the filters would be ≥ 60 kDa for the 10K filter, ≥ 30 kDa for the 5K filter, and ≥ 18 kDa for the 3K filter.

^z Culture filtrates were incubated at the given temperatures for 60 min. Results were similar for 30 min incubation (data not shown).

TABLE 3. Effect of enzyme treatment on elicitation activity of *Trichoderma virens* culture filtrate

Treatment	dHG	HG	dMHG	MHG	G	MG
G6	60.99	88.09	7.66	3.39	243.19	38.91
G6+ β -glucosidase	48.62	63.02	4.17**	2.27	152.10**	27.01
G6+protease	66.87	116.98	10.11	5.11	374.05***	73.99***
G6+trypsin	64.97	89.46	5.96	3.43	177.65*	27.45
G6+proteinase K	21.46**	19.20**	5.43*	1.25*	171.75*	25.57

^z Wheat bran and peat moss culture filtrate from *T. virens* strain G6 incubated with 5 units of enzyme per ml and assayed on cotton radicles for effect on stimulation of terpenoids, including desoxyhemigossypol (dHG) and hemigossypol (HG) and their respective 6-methyl ether forms (dMHG and MHG), gossypol (G), and 6-methoxygossypol (MG). All values are micrograms of compound per gram of tissue. The enzymes suspended in water served as controls (Table 4). * And ** indicate values significantly lower than untreated extract according to the *t* test ($-\alpha = 0.05$ and 0.01), respectively. *** Indicates values significantly higher than untreated extract according to the *t* test ($\alpha = 0.05$).

TABLE 4. Effect of water suspensions of enzymes on cotton terpenoids

Treatment	dHG	HG	dMHG	MHG	G	MG
Water	8.05	2.57	2.13	0.26	131.38	16.57
β -Glucosidase	8.45	4.05	2.25	0.33	138.75	20.35
Protease	14.58*	12.01**	2.65	0.78	153.72	27.08*
Trypsin	7.18	5.23*	2.46	0.86	164.30	12.41
Proteinase K	6.48	6.44*	1.67	0.25	104.53	21.09

^z Sterile distilled water alone or with 5 units of enzyme per ml assayed on cotton radicles for effect on stimulation of terpenoids, including desoxyhemigossypol (dHG) and hemigossypol (HG) and their respective 6-methyl ether forms (dMHG and MHG), gossypol (G), and 6-methoxygossypol (MG). All values are micrograms of compound per gram of tissue. * And ** indicate that values are significantly different than water according to the *t* test ($-\alpha = 0.05$ and 0.01), respectively.

detected in seedlings treated with protease suspensions, but not the other enzymes. No significant response to β -glucosidase was seen (Table 4).

When run on SDS-PAGE, several bands were visible in extracts from the biocontrol-effective strains, G6 and G6-5 (Fig. 1), that were not detectable in the ineffective strain, G6-4. Differences in protein levels were confirmed by a protein concentration assay. G6-4 had approximately 80 μ g of total protein per ml in the material pass through the 10K MWCO filter, whereas G6 and G6-5 had approximately 260 to 360 μ g/ml, respectively. Even when G6-4 CFS5 was double loaded onto a gel (Fig. 1), these bands were not detected.

Fig. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel of culture filtrates from effective and ineffective biocontrol strains of *Trichoderma virens*. The gel was stained with Coomassie blue. Several bands are detectable in the effective strains that are absent in ineffective strains. All odd lanes (lanes 1, 3, and 5) were loaded twice with culture filtrate. Lanes 1 and 2, culture filtrate from *T. virens* strain G6 (biocontrol-effective), lanes 3 and 4, culture filtrate from *T. virens* strain G6-5 (biocontrol-effective), lanes 5 and 6, culture filtrate from *T. virens* strain G6-4 (biocontrol-ineffective), and lane 7, size marker.

Fig. 2. Western blot with antiserum for the ethylene-inducing xylanase from *Trichoderma viride*. A single band of approximately 20 kDa in the biocontrol-active *T. virens* extract cross-reacted with the antibody for the *T. viride* xylanase (3). The second smaller band that showed cross-reaction in cellulysin was not detectable in *T. virens* extract.

Western blot analysis with the antibody for the EIX from *T. viride* showed the two bands reported from *T. viride* in the cellulysin (Fig. 2). A single band was detected in the biocontrol-effective *T. virens*, and no bands were detected in the ineffective *T. virens* strain when incubated for 1 or 3 h. When blots were incubated longer (12 h), a weak band was observed from the ineffective *T. virens* strain (data not shown).

When the material eluted from PAGE gels was tested for elicitation activity, an 18-kDa band was found to be active in both a denatured and a renatured form (Table 5). Three other bands showed activity in a renatured form. A band of approximately 20 kDa was the same size as the band that showed cross-reaction with the EIX antibody.

Cellulysin, a source for the EIX (3), was assayed for activity on cotton seedlings. Significant elicitation was observed for only hemigossypol (Table 6).

When the 18-kDa band was submitted for amino terminal sequencing, the first 19 amino acids at the amino terminus were determined to be DTVSYDTGYDNGSRSLNDV. A database homology search using the BLASTp algorithm was done to compare this sequence with known sequences in the protein database. The highest similarity (63% identity, 75% positives) was to a serine proteinase (GenBank pir|PN0172) from *Fusarium sporotrichioides*. This was followed by 58% identity, 68% positives to an allergen aspf13 precursor from *Aspergillus fumigatus* (GenBank sp|060022|AF13 ASPFU) and to the protein snodprot1 precursor from *Phaeosphaeria nodorum* (GenBank sp|074238|SNP1 PHANO) (Fig. 3).

DISCUSSION

Several bands were visible in CFS from biocontrol-effective strains that were not visible in CFS from the biocontrol-ineffective strain. There was no significant difference in the number

TABLE 5. Induction of cotton phytoalexins in seedling roots with different protein bands from polyacrylamide gel electrophoresis (PAGE)

Band ^y	Renatured ^z		Denatured ^z	
	dHG	HG	dHG	HG
Buffer	4.7 d	5.0 e	3.8 bc	2.8 b
6.5 kDa	10.8 ab	22.9 ab	4.1 b	2.6 bc
18 kDa	12.6 a	25.6 a	7.4 a	4.5 a
20 kDa	7.2 cd	13.9 cd	4.1 b	2.1 bc
26 kDa	12.2 a	24.6 a	4.5 b	4.0 ab
32 kDa	5.7 d	7.1 de	2.9 c	1.8 bc
42 kDa	10.0 ab	16.0 bc	2.9 c	1.4 c

^y Bands were extracted from PAGE gel and either used directly (denatured) or incubated in renaturation buffer before use. Buffer for treatment is the buffer in which the bands were applied to seedlings. Bands were used in either a denatured or a renatured state.

^z dHG = desoxyhemigossypol, HG = hemigossypol. Values are micrograms of compound per gram of tissue. Numbers followed by the same letter are not significantly different ($\alpha = 0.05$) according to Fisher's least significant difference.

TABLE 6. Effect of cellulysin treatment on cotton radicles^z

Treatment	Terpenoid concentration (μ g of compound per g of tissue)					
	dHG	HG	dMHG	MHG	HGAL	G
Water	n.d.	n.d.	n.d.	n.d.	n.d.	46.56 $\pm$ 23.60
Buffer	n.d.	n.d.	n.d.	n.d.	n.d.	117.47 $\pm$ 64.53
Cellulysin	n.d.	2.39 $\pm$ 0.37	n.d.	n.d.	n.d.	103.85 $\pm$ 14.67

^z Assayed water, acetate buffer, or a suspension of 0.5 mg/ml of cellulysin in acetate buffer on cotton radicles for effect on stimulation of terpenoids, including desoxyhemigossypol (dHG) and hemigossypol (HG) and their respective 6-methyl ether forms (dMHG and MHG), hemigossylic acid lactone (HGAL), and gossypol (G). n.d. = not detected by high-performance liquid chromatography. Numbers are averages followed by the standard deviation.

of colony-forming units produced by these strains in the culture medium, so this difference is unlikely to be due to differences in fungal growth.

Since the CFS activity was most reduced by treatment with proteinase K, the elicitors are most likely proteins or glycoproteins. The lower level of protein production by the ineffective biocontrol strain compared with the effective strains is further evidence for a role of these materials. The reduced detection of two of the induced compounds following treatment of the CFS with β -glucosidase may indicate that some of these proteins are glycosylated, and that this glycosylation has a role in activity. When a western blot was tested with antibodies for glycosylation, several bands above 20 kDa in size reacted with the antibody (data not shown).

The size of the bands observed on the SDS-PAGE gels is larger than what might be expected following passage through a 10K MWCO membrane. However, the results are consistent with the company's recommendations. The majority of the activity passed through the 5K filter but was retained by the 3K filter. This would be consistent with the optimal retention of proteins of a molecular mass of 30 kDa or more by the 5K filter and of 18 kDa or more by the 3K filter, because most of the bands excised from PAGE gels that showed activity were between 18 and 30 kDa. These results are consistent with work by Hosono et al. (11) in which material that passed through a 50-kDa cut-off membrane, but not through a 6-kDa cut-off membrane filter, was recorded with sizes between 60 and 2,460 kDa.

The base level of the terpenoids in the cotton radicles varied in different experiments (Tables 1 to 5; [14]). This may be due to differing levels in the seeds used for the tests, or to other factors. The buffers used in some of the experiments also may have affected these levels. For example, the terpenoid levels in radicles were consistently lower in the tests with materials extracted from PAGE gels (Table 4), which were in buffers, as opposed to those in which the materials were in water or media (Tables 1 to 4).

Glycoproteins and glycopeptides with elicitation activity have been reported from a number of fungi and oomycetes (review in literature citation 9). Some of these are from cell wall fractions (17), rather than culture filtrate like our elicitor fraction. Other known fungal or oomycetous elicitors include the elicitors from *Phytophthora* spp, which are holoproteins with molecular masses of about 10 kDa (16,20,21). Unlike the elicitor(s) reported here, the elicitors cause necrosis of tobacco and other plants within 24 h of application (21). No necrosis of cotton was observed within 24 h with our elicitors, but our elicitors were applied to roots rather than leaves. However, the cotyledons came into contact with the material saturating the filter paper, and no difference in necrosis was observed in cotyledons between those exposed to sterile water and those exposed to the CFS.

One band from *T. virens* cross-reacted with the EIX antibody, indicating that this band has antigenic similarity to the EIX in *T. viride*. However, this band migrated slightly farther on the SDS-PAGE gel than did the larger EIX fraction in *T. viride*, thus it may be smaller than the *T. viride* EIX. The crude CFS from *T. virens* had xylanase activity (data not shown). In a renatured form, this band (Table 4, band 20) elicited some terpenoid production in cotton roots, indicating that this may be an elicitor similar to the EIX in *T. viride*. However, cellulysin, which contains EIX (3 and

Fig. 2), produced significant elicitation of only one of the phytoalexins in cotton seedlings (Table 5). This weak induction indicates that this band is not the major elicitor in *T. virens*. There was a weak band observed after incubation overnight of the EIX antibody with the extract from the ineffective *T. virens* strain. This strain also had xylanase activity, but at significantly lower levels than did the effective strains ($P = 0.03$). Because there was no difference in the growth of these strains, as indicated by production of colony-forming units, these results indicate that the ineffective strain produced lower levels of the EIX-type material than did the biocontrol-effective strains.

An 18-kDa protein was isolated from the culture filtrate of *T. virens* and elicited defense responses in cotton seedling roots in either a denatured or renatured form. This gave the strongest and most consistent elicitation of any of the bands tested. This band did not cross-react with the EIX antibody, indicating that it is a different elicitor. The evidence above indicates that this may be a novel elicitor from *T. virens*. The amino-terminal end of this protein had highest similarity (63% identity) to a serine proteinase from *Fusarium sporotrichioides*. However, no putative conserved domains were detected, so the similarity to the serine proteinase is unlikely to be at an active site. The material was not tested for serine proteinase activity. Serine proteinases have been detected from other plant-associated fungi (23).

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Amino terminal from *T. virens* 18-kDa protein
 Serine proteinase from *Fusarium sporotrichioides*
 Allergen aspf13 precursor from *Aspergillus fumigatus*
 Snodprot1 precursor from *Phaeosphaeria nodorum*

DTVSYDTGYDNGSRSLNDV
 TVSYDTGYDDKSRPMTSV
 SVSYDPRYDNAGTSMNDV
 SVSYDQGYDDAGRSLTSV

Fig. 3. Results of BLAST search using the amino terminal sequence for the 18-kDa fraction from sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Amino acids common between the 18-kDa fraction and other proteins are highlighted in gray.

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