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Dimerization Controls the Activity of Fungal Elicitors That Trigger Systemic Resistance in Plants*[S]

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The soilborne fungus *Trichoderma virens* secretes a small protein (Sm1) that induces local and systemic defenses in plants. This protein belongs to the ceratoplatanin protein family and is mainly present as a monomer in culture filtrates. However, *Hypocrea atroviride* (the telomorph form of *Trichoderma atroviride*) secretes an Sm1-homologous protein, Epl1, with high levels of dimerization. Nonetheless, the molecular mechanisms involved in recognition and the signaling pathways involved in the induction of systemic resistance in plants are still unclear. In this report, we demonstrate that Sm1 and Epl1 are mainly produced as monomer and a dimer, respectively, in the presence of maize seedlings. The results presented show that the ability to induce plant defenses reside only in the monomeric form of both Sm1 and Epl1, and we demonstrate for the first time that the monomeric form of Epl1, likewise Sm1, induces defenses in maize plants. Biochemical analyses indicate that monomeric Sm1 is produced as a glycoprotein, but the glycosyl moiety is missing from its dimeric form, and Epl1 is produced as a nonglycosylated protein. Moreover, for Sm1 homologues in various fungal strains, there is a negative correlation between the presence of the glycosylation site and their ability to aggregate. We propose a subdivision in the ceratoplatanin protein family according to the presence of the glycosylation site and the ability of the proteins to aggregate. The data presented suggest that the elicitor's aggregation may control the *Trichoderma*-plant molecular dialogue and block the activation of induced systemic resistance in plants.

Rhizosphere colonization by certain bacterial strains results in a state of heightened resistance in plants, locally and systemically, to subsequent pathogen attack (1, 2). During the colonization process, a complex molecular dialogue is established between plants and the microorganisms in which signaling molecules (elicitors) play an essential role (3–6). Various molecules associated with the basic metabolism of microbes, such as cell wall glucans, chitin oligomers, and glycopeptides, have been described with elicitor activity (7–9). In addition, certain structural proteins and enzymes encoded in the invader genome (such as Sm1, chitinase, and flagellin) have also been shown to act as signal molecules (10–15).

One important outcome of some plant-microbe interactions is the induction of plant defenses mediated by the activation of induced systemic resistance (ISR).[3] ISR confers systemic protection in plants by mechanisms different from those of the well known and well studied systemic acquired resistance (reviewed by Vallard and Goodman (2)). However, the mechanisms that underlie the molecular cross-talk between plants and the microbes that initiate ISR are not fully understood. This type of induced resistance has primarily been linked to the colonization of plant roots by plant growth-promoting rhizobacteria and recently to some species of the filamentous fungus *Trichoderma* (13, 14, 16–18). The characterization of two ISR elicitors secreted by *T. virens* Gv29-8 was recently described. Peptaibols (peptides with antimicrobial activity) produced by *T. virens* were demonstrated to have ISR elicitor effects, and they systemically induce defenses in cucumber leaves (18). The second ISR elicitor produced by *T. virens* is the extracellular small protein Sm1, whose gene expression was demonstrated to be up-regulated in the presence of cotton plants. This elicitor is able to locally and systemically induce defense responses in plants (13). Further *in vivo* studies, using reverse genetic analyses, demonstrated that expression of *Sm1* is essential for triggering ISR in maize plants and providing protection against the foliar pathogen *Colletotrichum graminicola* (14). In maize, the metabolic pathways that lead to the establishment of Sm1-mediated ISR involve the signaling networks associated with salicylic acid, green leafy volatiles, and jasmonic acid metabolism and seem to be independent of PR proteins (14). However, the molecular mechanisms relevant to Sm1 recognition and signal transduction have not yet been described.

Sm1 was the first and the only (to our knowledge) proteinaceous elicitor, with no enzymatic activity, involved in ISR responses described to date. Sm1 is a secreted hydrophobin-

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S5.

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[3] The abbreviations used are: ISR, induced systemic resistance; CP, ceratoplatanin; CF, culture filtrate; GFC, gel filtration chromatography; GFP, green fluorescent protein; MS, mass spectrometry; VM, Vogel's minimal medium; VMS, VM supplemented with 1.5% sucrose; MALDI, matrix-assisted laser desorption ionization; RT, reverse transcription; HSD, honestly significant difference.

like protein, with a predicted molecular mass of 12,545.8 Da. According to structural and sequence analyses, Sm1 belongs to the cerato-platanin (CP) protein family (13, 19), which consists mainly of proteins produced by plant and human fungal pathogens, and is associated with toxicity and infection processes (20–24). Paradoxically, characterization of Sm1 demonstrated a lack of phytotoxicity toward various plants, including cotton, rice, tobacco, and peanut (13). Sm1 was previously purified from *T. virens* Gv29-8 culture filtrates as a monomeric protein (13, 14). However, in some growth conditions, a faint band corresponding to the SDS-resistant dimeric form was detected in Western blots.[4] The existence of Sm1 monomer and dimer forms could be important for its biological activity. For example, there are reports of key regulators of defense responses whose activity *in vivo* is regulated by redox changes (25, 26). Recently, Epl1, the Sm1 homologue produced by *Hypocrea atroviride*, was also detected in two forms (monomer and dimer) in fungal culture filtrates (27). To gain novel insights into ISR and the molecular mechanisms underlying the elicitor recognition and response induction, we have investigated the relevance of the oligomerization states of Sm1 and Epl1 for ISR. In this report, we demonstrate that Epl1 indeed elicits the activation of the plant defense mechanism, but only the monomeric forms of Sm1 and Epl1 are able to induce such responses, whereas both dimers fail to protect maize plants against a leaf pathogen. Further biochemical characterization of Sm1 and Epl1 demonstrates that the monomeric form of Sm1 is produced as a glycoprotein. However, glycosylation was not present in either dimer forms of Sm1 or Epl1. The occurrence of Sm1/Epl1 homologues was analyzed in different fungal strains by sequence analysis and immunodetection of the polypeptides. The results of this work led us to conclude that the glycosylation state may be controlling the protein oligomerization status and altering the recognition of the elicitor and the induction of defenses in plants. We discuss the importance of the glycosylation state of Sm1 in the molecular dialogue during plant-*Trichoderma* associations and in the stimulation of ISR in plants. A novel subdivision on the CP protein superfamily, based on to the protein's ability to aggregate and the presence of glycosylation sites, is also proposed.

EXPERIMENTAL PROCEDURES

Fungal and Plant Materials—The *Trichoderma* species used in this study were *Trichoderma virens* Gv29-8, *T. virens* G6, *T. virens* G9, *T. virens* Gv29-8-Δ*Sm1* (14), *Trichoderma atroviride* IMI206040, *Trichoderma reesei* 6, and *Trichoderma viride* 21. Also for this research, we used the filamentous fungi *Magnaporthe grisea* 70-15, *Aspergillus fumigatus* FGSC1152, and *Neurospora crassa* 74A. A GFP-tagged *Colletotrichum graminicola* M1.001-BH isolate (28) was used as a foliar pathogen for disease development studies in maize. The fungal strains were routinely maintained on potato dextrose agar (Difco). Maize (*Zea mays* inbred line B73) seedlings used in this study were grown in a hydroponic system (13) or planted in plastic containers (3.81 × 20.9 cm) containing a soilless mix (Metromix 366) and incubated in a growth chamber at 25 °C, with a 14-h photoperiod and 60% humidity.

Analysis of Fungal Secreted Proteins from the Hydroponic Growth System—A hydroponic system (13) was used to evaluate the plant defense response of maize seedlings when inoculated with *T. virens* or *T. atroviride* strains. Maize seeds were surface-sterilized using 10% hydrogen peroxide as previously described (13). The mycelial inoculum of the *Trichoderma* strains was aseptically added into the hydroponic systems containing 300 ml of fresh Murashige and Skoog (MS) medium and 4-day-old maize seedlings. Two days later, the medium from each treatment was collected and successively filtered through a 10-μm NITEX nylon cloth and a 0.45-μm filter. Culture filtrates (CFs) were then treated with a protease inhibitor mixture (0.05%, v/v; Sigma) and allowed to incubate at room temperature for 20 min, and then 300 ml of each treatment were concentrated by using 10 kDa cut-off Millipore filter devices (Bedford, MA). Protein extracts were used to determine enzymatic activity and also used for SDS-PAGE or Western blotting analyses.

Enzymatic Activity Assays and Protein Quantification—The activity of β-1,3-glucanase, β-1,6-glucanase, endochitinase, and proteinase secreted by *T. virens* and *T. atroviride* in various culture conditions was compared. Enzymatic activities of both strains when grown in liquid Vogel's minimal medium (VM) (29) or VM supplemented with 1.5% sucrose (VMS) or cultured in MS medium in the presence or absence (control) of maize seedlings were assayed. The activities of β-1,3- and β-1,6-glucanase were determined in the presence of pustulan and laminarin, respectively, by detecting the reducing sugars released after incubation (30). Protease and endochitinase activities were determined in the presence of Suc-Ala-Ala-Pro-Phe-*p*-nitroanalide (Sigma) and 4-methylumbelliferyl-β-D-*N,N′,N″*-triacetylchitotriose (Sigma), respectively, as recently described (30). Protein concentration was determined in a microplate assay using the protein reagent (Bio-Rad) according to the manufacturer's instructions or by detecting absorbance at 280 nm, using bovine serum albumin as a standard. The specific activities were calculated as units/mg protein.

SDS-PAGE and Western Blot Assays—Polypeptides were separated on SDS-PAGE (15% polyacrylamide) (31) and stained with Coomassie Blue or blotted onto a nitrocellulose membrane (HyBond C®; Amersham Biosciences) for immunoassays (32). Protein blots were probed with specific antibodies raised against Sm1 (13).

Purification of Sm1 and Epl1 Isoforms—For Sm1 and Epl1 purification, *T. virens* Gv29-8 and *T. atroviride* IMI206040 were grown for 7 days in VMS medium, and CFs were collected by filtration through a 10-μm NITEX nylon cloth. Proteins were precipitated with 80% ammonium sulfate, collected by centrifugation, dissolved in 20 mM NH_4HCO_3, and dialyzed against the same solution. Total protein was loaded onto High Q support columns (0.5 × 20 cm) (Bio-Rad) preequilibrated with 20 mN Tris-NaOH (pH 7.5) (anion exchange chromatography). Proteins were eluted with a 0–0.5 M NaCl linear gradient in the equilibration buffer. The elution of Sm1 and Epl1 was followed by SDS-PAGE and protein immunodetection. The fractions containing the protein of interest were pooled, dia-

[4] C. M. Kenerley, unpublished observations.

TABLE 1
List of primers used for gene expression assays

Gene name	Primer sequence	Product size	Accession number
		bp	
AOS	5′-AAACCGACGAATTTGAGCAA-3′	74	AY488135.1
	5′-GGAGGCTCGCAACAAGTTG-3′		
HPL	5′-GAAGGACGCCATCAACAACA-3′	80	AY540745.1
	5′-ACCAGGAAGGGCAGGAACAC-3′		
OPR7	5′-AGAGGCGGAGTATTGTTTGTATGT-3′	64	AY921644.1
	5′-CGCGGCATTACCCAGATG-3′		
PAL	5′-CCCATCGGCGTGTCCAT-3′	110	L77912.1
	5′-GGTGAGCCCGTTGTTGTAGAA-3′		
GAPc	5′-TCCTGATCTGAATGGCAAGCT-3′	63	X07156.1
	5′-AACCGAGACATCCACAGTAGGAA-3′		
TvSm1	5′-ACGCTGCTTCTGGCTTCAACATT-3′	125	DQ121133
	5′-CTTTAGAGACCGCAGTTCTTAACA-3′		
TaEpl1	5′-ATCCCTCGCTTCCCATACATTG-3′	221	AJ901879
	5′-ACTTGAGAGGCAGTGGCAGAAAC-3′		
Actin	5′-GTATCATGATCGGTATGGGTCAGA-3′	156	X75421
	5′-TAGAAGGTGTGGTGCCAGATCTT-3′		

lyzed against 20 mM NH_4HCO_3, and vacuum-dried. The dried protein was dissolved in 50 mM Tris-NaOH (pH 7.5), 150 mM NaCl and further purified by gel filtration chromatography (GFC) through Bio-Gel P30 columns (1.0 × 100 cm) (Bio-Rad) preequilibrated with the same solution. The elution of Sm1 and Epl1 was followed by SDS-PAGE and Western blotting. Although the monomeric form of Epl1 was not detected in the column eluant, the monomeric form of Sm1 eluted pure after this purification step. The fractions containing pure Sm1 were pooled, dialyzed (20 mM NH_4HCO_3), and vacuum-dried for further experiments. For purification of the dimeric forms of Sm1 and Epl1, an immunoaffinity purification assay was required. The fractions containing the dimers were pooled, dialyzed and concentrated to continue their purification. Both dimers were purified through an Affi-Gel column, where anti-Sm1 antibodies (13) had been immobilized according to the manufacturer's directions (Affi-Gel® Hz Immunoaffinity Kit; Bio-Rad). In order to obtain the dissociated form of Sm1 and Epl1, the purified dimers were sonicated in trifluoroacetic acid at 0 °C (33). The dissociated fraction was then dialyzed, dried under vacuum, and dissolved in water.

Sm1 Tryptic Digestion and MS Fingerprint Analysis—The bands corresponding to both isoforms of Sm1 after separation on SDS-PAGE were excised manually and subjected to in-gel trypsin digestion. The digestion products were extracted and prepared for MALDI-MS. The peptide mass fingerprint was analyzed in a Shimadzu/Kratos MALDI-time-of-flight mass spectrometer at the Protein Chemistry Laboratory (Texas A&M University).

Protein Glycosylation Assays—Glycosylation state was assayed using a glycoprotein detection reagent (Pierce) according to the manufacturer's suggestions. Different amounts of purified Epl1 (dimer) and both isoforms of Sm1 were oxidized with sodium *meta*-periodate and subjected to a reaction with a freshly made 0.5% solution of glycoprotein detection reagent in 1 N NaOH. A negative (bovine serum albumin) and a positive control (ovalbumin) were also included in the assay. In-gel glycoprotein staining was performed after SDS-PAGE protein separation, using the GelCode glycoprotein staining kit (Pierce) as suggested by the manufacturer.

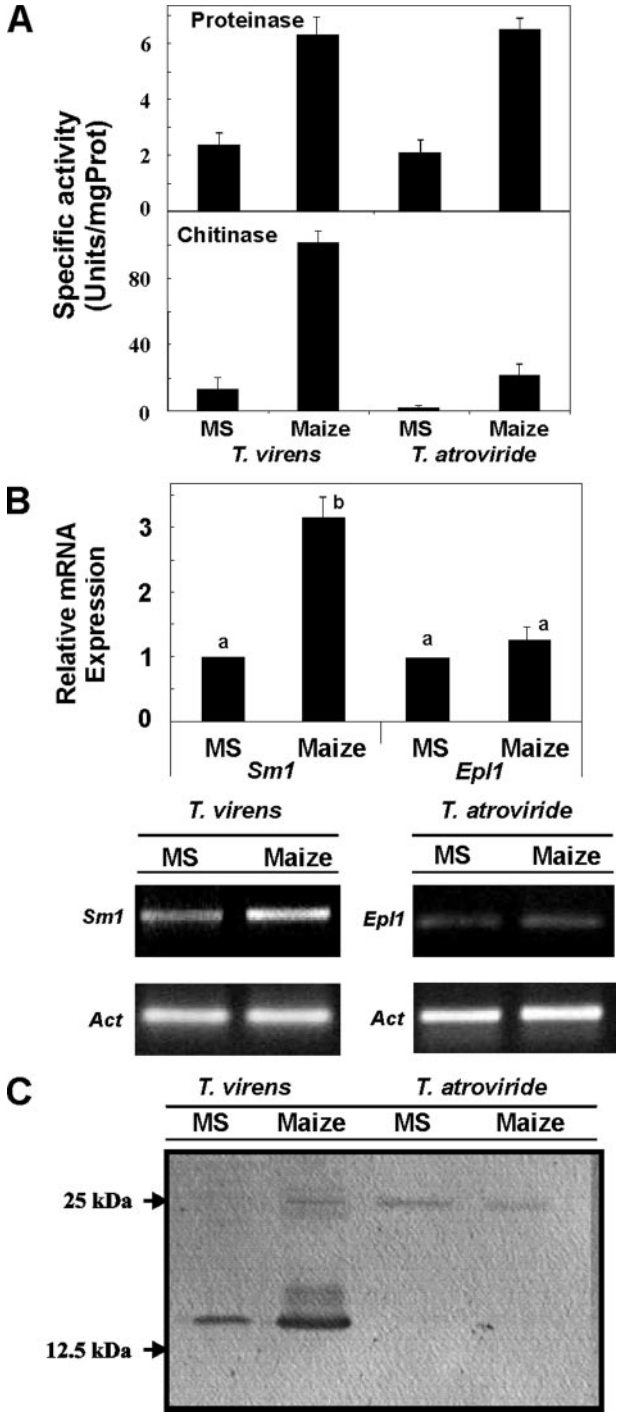

FIGURE 1. **Comparison of secreted proteins from *T. virens* and *T. atroviride* incubated in the presence of maize plants in a hydroponic system.** As a control, both strains were cultured in MS medium supplemented with 0.05% sucrose (13). After a 48-h incubation, mycelia were collected for gene expression, and CFs were used to assay enzymatic activities. *A*, specific activity of chitinase and proteinase in CF. The values are the mean value ± S.E. of two independent experiments. *B* (*top*), quantitative RT-PCR analysis of *Sm1* and *Epl1* from total RNA of *T. virens* Gv29-8 and *T. atroviride* IMI206040, respectively, cultured in MS or with maize plants. The gene expression data presented were normalized to a reference control and depict the mean value ± S.E. of two independent experiments. *Columns* with *different letters* differ significantly according to Tukey's HSD test at a significance level of 1%. *Bottom*, PCR products visualized after agarose gel electrophoresis and ethidium bromide staining. Amplification of the actin gene was used as an internal loading control. *C*, Sm1 and Epl1 immunodetection in CF from hydroponic systems. Proteins were separated by SDS-PAGE on 15% polyacrylamide gels and blotted onto nitrocellulose membranes. Blots were probed with anti-Sm1 antibodies.

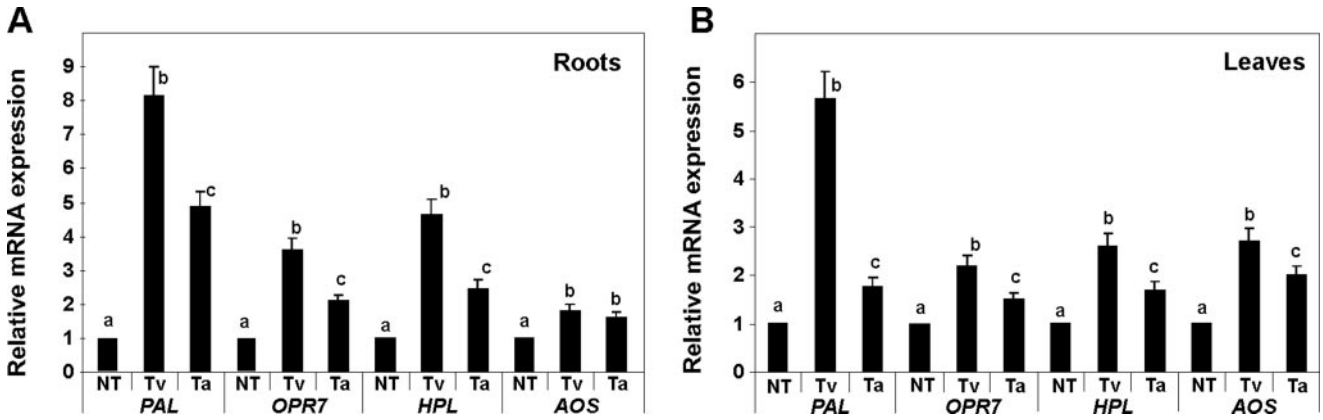

FIGURE 2. **Quantitative RT-PCR analysis of defense-related genes in roots and leaves of *T. virens*- or *T. atroviride*-treated maize plants.** Plants grown without *Trichoderma* were included as controls. Two days after inoculation, quantitative RT-PCR assays were performed with 100 ng of total RNA extracted from roots (*A*) or leaves (*B*). The expression of *AOS*, *HPL*, *OPR7*, and *PAL* was analyzed using the primers described in Table 1. *GAPc* was used as the reference internal control, and the expression results were normalized to its abundance. The values shown are the average of two independent experiments ± S.E. *Columns* with *different letters* (for each gene) differ significantly according to Tukey's HSD test at a significance level of 1%. *NT*, nontreated; *Tv*, plants inoculated with *T. virens* GV29-8; *Ta*, plants inoculated with *T. atroviride* IMI206040.

RNA Extraction and Gene Expression Analyses—Total RNA from maize or fungal tissue was prepared using the TRIZOL® reagent (Invitrogen). Previous to the extraction, plant tissues were ground with a mortar and pestle in the presence of liquid nitrogen; in the case of the fungal tissue, it was lyophilized and crushed prior to the extraction process. RNA quality and PCR products were analyzed by electrophoresis on agarose gels. In all cases, total RNA was DNase-treated before expression assays using a DNA-free kit (Ambion). For *Sm1* and *Epl1* expression assays, quantitative real time RT-PCR experiments were performed. The constitutively expressed gene for *T. reesei* actin (X75421) was used to design primers to amplify its homologue in *T. virens* and *T. atroviride* as a control reference (13). In maize, the expression of *AOS* (allene oxide synthase), *HPL* (hydroperoxide lyase), *OPR7* (12-oxo-phytodienoic acid reductase 7), and *PAL* (phenylalanine ammonia-lyase) was evaluated by quantitative real time RT-PCR using *GAPc* (glyceraldehyde-3-phosphate-dehydrogenase) as the control reference. The experiments were performed using the QuantiTect® SYBR® green RT-PCR kit (Qiagen). The reactions were carried out in a 20-μl reaction with 1× QuantiTect SYBR Green Master Mix, 1× RT QuantiTect Mix, 200 nM primers (Table 1), and 100 ng of total RNA. The reactions were performed in a 7500 Fast real time PCR system (Applied Biosystems) with the conditions suggested by the manufacturer. The absence of primer dimers was confirmed in reactions without RNA. The experiments were independently repeated two times (with similar results), and each reaction was performed in triplicate using relative quantification analysis. The expression of each specific gene was normalized *versus* the control reference with the formula, $2^{-\Delta\Delta C_T}$, where $\Delta C_T = C_T$(specific gene) − C_T(*GAPc* gene); $\Delta\Delta C_T = \Delta C_T$(arbitrary constant) (the highest ΔC_T) (59). Statistical analysis was performed by one-way analysis of variance followed by Tukey's honestly significant difference (HSD) test using VassarStat (available on the World Wide Web).

Infection of Trichoderma-treated Maize Plants with C. graminicola—Cultures of *C. graminicola* strain M1.001-BH expressing the *GFP* gene were grown for 14 days on potato dextrose agar at room temperature under constant light. Spores were scraped from plates, filtered through Miracloth (Calbiochem), and washed three times with water by centrifugation for 1 min at 10,000 rpm. Spores were counted by using a hemacytometer, and a spore suspension of 6.5×10^4 spores/ml was prepared in a solution of 0.02% Tween 20 in water. Inoculation with *T. virens* Gv29-8, *T. virens* Δ*Sm1* and *T. atroviride* IMI206040 strains was performed by coating maize seeds with a latex sticker (Rohm and Haas, Philadelphia, PA) and then coating with fine granules of chlamydospore preparations (34). Control (nontreated) and coated seeds were planted in plastic containers (3.81 × 20.9 cm) containing a soilless mix (Metromix 366) and incubated in a growth chamber at 25 °C with a 14-h photoperiod and 60% humidity for 2 weeks. Fourteen-day-old plants (at the V4 developmental stage) were inoculated with *C. graminicola* by placing the plants in trays and proceeding as previously described (14). The third leaf from all plants was inoculated with six droplets (10 μl) of the spore suspension, placed on the adaxial side, away from the midvein of the leaf. The trays were sealed and incubated for 24 h at room temperature. After 48 h of incubation in growth chambers, inoculated leaves were used for microscopy or immediately frozen for gene expression assays. For microscopy assays, five plants were infected in each of three independent experiments. Forty-eight hours after infection, five infection sites were excised form each leaf and analyzed with an Olympus BX51 fluorescence microscope (Olympus America Inc., Melville, NY).

Infection of Maize Plants with C. graminicola after Elicitor Infiltration—The purified monomer of Sm1, dissociated Epl1, and dimers of both Sm1 and Epl1 (1 nmol), were syringe-infiltrated into the third leaf of 14-day-old plants (13). The infiltrated plants were incubated for 24 h in growth chamber, and then leaves were harvested for RNA extraction or infected with the GFP-tagged *C. graminicola* strain as described above. In the case of the infection with the GFP-tagged pathogen, three independent experiments were performed, infecting five plants in each case. Forty-eight hours after inoculation, five infection sites were excised from each leaf and analyzed with a fluorescence microscope as above.

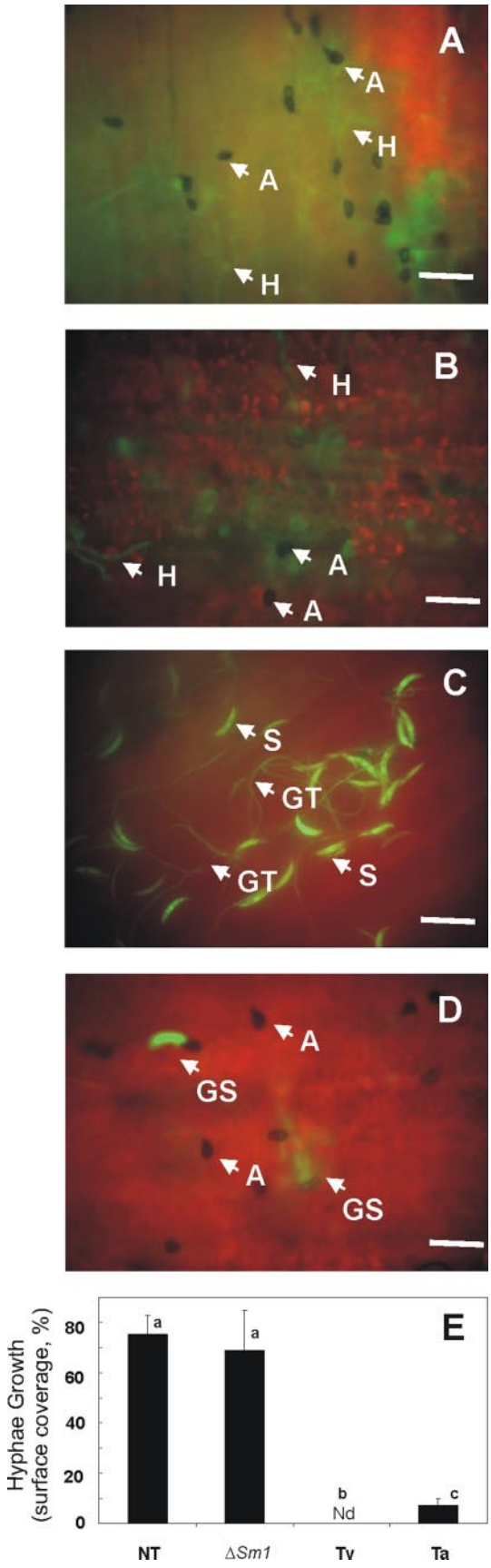

FIGURE 3. **Systemic disease protection mediated by *T. virens* Gv29-8 or *T. atroviride* IMI206040 in maize plants.** The development of the GFP-tagged *C. graminicola* was assayed on leaves of non-*Trichoderma*-treated maize plants (*A*) and plants that had been root-inoculated with *T. virens* Δ*Sm1* (*B*), *T. virens* Gv29-8 (*C*), or *T. atroviride* IMI206040 (*D*). Fourteen-day-old plants were challenged with the pathogen and analyzed 48 h after infection. *E*, quantification of *C. graminicola* growth on leaves. The data presented show the percentage of the inoculation area covered by the pathogen hyphae. The *bars* depict the mean value ± S.D. determined in pictures taken in three independent experiments. *Columns* with *different letters* differ significantly according to Tukey's HSD test at a significance level of 5%. Fluorescent micrographs of the pathogen were taken with an Olympus BX-51 fluorescent microscope with excitation from 470 to 490 nm and emission from 510 to 550 nm. The areas on the micrographs were determined using ImageJ software. *A*, appressoria; *S*, spore; *GS*, germinating spore; *GT*, germ tube; *H*, hypha; *Nd*, no detected. *Bars*, 50 μm.

Sequence Analyses—Sequence comparisons were performed using deduced amino acid sequences available in the data bases at the National Center for Biotechnology Information (NCBI; available on the World Wide Web) and those previously reported by Seidl *et al.* (27). Sequence alignments were generated with the ClustalX (version 1.8) software (35), and graphic representations of phylogenetic trees were performed using the TREEVIEW16 software. The presence of putative glycosylation sites was predicted using the resources available at ExPASy proteomics tools (available on the World Wide Web).

RESULTS

Proteins Secreted by T. virens Gv29-8 and T. atroviride IMI206040 in the Presence of Maize Seedlings—Fungal cell wall-degrading enzymes (*e.g.* chitinases and glucanases) are known to be important factors in plant-microbe interactions (36). We determined the level of activity of chitinase, proteinase, and β-1,3- and β-1,6-glucanase secreted by both strains in the presence of maize plants. Although no glucanase activity was detected in the CF of either strain, similar levels of proteinase activity were detected for both of them. Although *T. atroviride* presented lower chitinase activity than *T. virens*, both strains produced higher levels of the activity when cultured in the presence of maize plants (Fig. 1*A*). The activity of the hydrolytic enzymes was compared also from CFs collected after 4 days of culture in VM or VMS medium. In both conditions, the activity of all enzymes assayed was detected in *T. virens* and *T. atroviride* in either culture condition. In both strains, proteinase and chitinase were down-regulated in VMS medium, the activity of the β-1,6-glucanase was up-regulated in the presence of sucrose, and no differences were detected between the strains for the activity of β-1,3-glucanase (supplemental Fig. S1). The production of Sm1 and its homologue in *T. atroviride* (Epl1) was also compared. As previously described (13, 14), *Sm1* expression was up-regulated in the presence of maize plants grown hydroponically. However, no significant difference ($p < 0.01$) in the steady state for *Epl1* mRNA was evident when *T. atroviride* was cultured in the presence of plants or under control conditions (Fig. 1*B*). Polypeptide levels of both Sm1 and Epl1 reflected the differences detected in mRNA levels. In addition, in the presence of plants, Sm1 is mainly produced with a mass suggestive of a monomer, but no monomeric form was detected for Epl1 (Fig. 1*C*).

Defense Induction and Systemic Protection in Maize Seedlings Mediated by T. virens Gv29-8 and T. atroviride IMI206040—Previously, we demonstrated that the presence of Sm1 systemically up-regulates genes for defense mechanisms

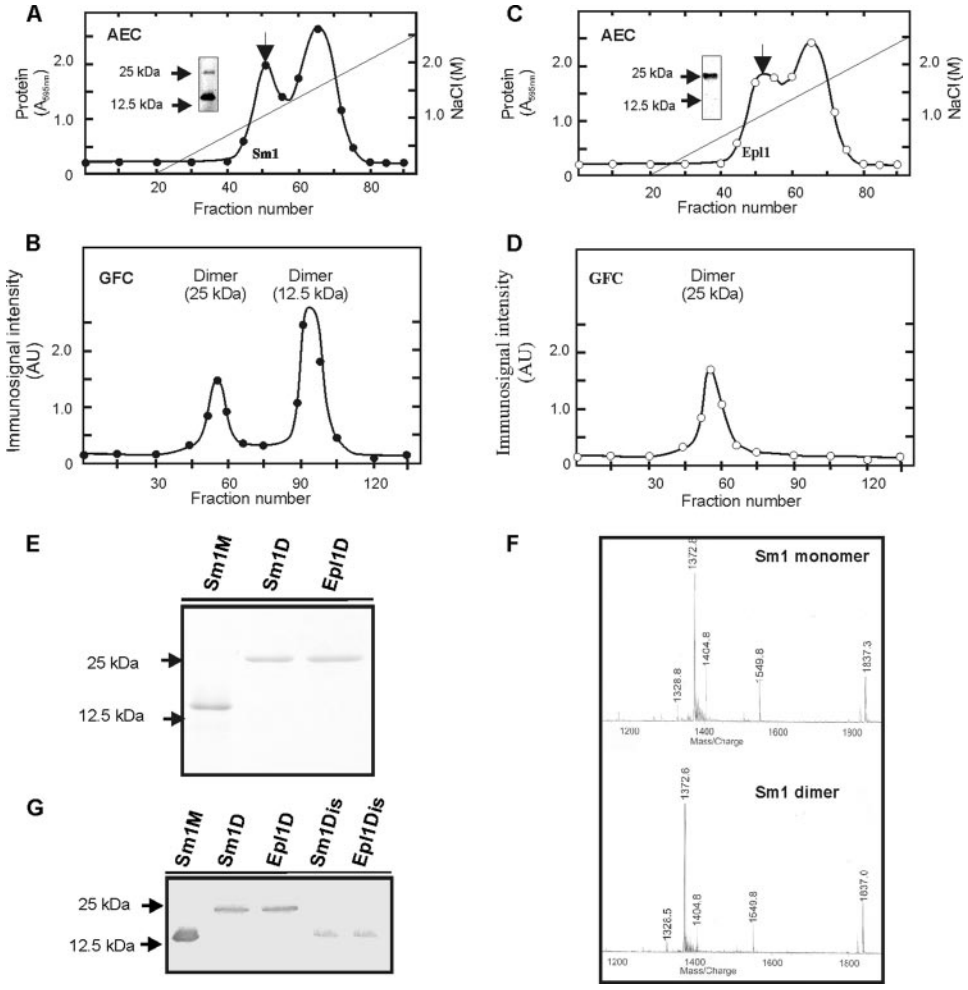

FIGURE 4. **Purification and molecular studies of Sm1 and Epl1.** Sm1 and Epl1 were isolated from CF of *T. virens* Gv29-8 or *T. atroviride* IMI602040 cultured in VMS liquid medium for 7 days. *A* and *B*, purification of Sm1 through anion exchange and gel filtration chromatography, respectively. *C* and *D*, purification of Epl1 through anion exchange and gel filtration chromatography, respectively. The protein elution profile was assayed by determining protein concentration, and the elution of Sm1 or Epl1 was followed by Western blot. The *insets* in *A* and *C* show the immunodetection of both proteins after concentration of the peak from anion exchange chromatography. *E*, Sm1 and Epl1 dimeric isoforms were purified to homogeneity by immunoaffinity chromatography, and the purified proteins were analyzed on SDS-PAGE. *F*, comparison of the MS polypeptide fingerprint of the Sm1 monomer and dimer after tryptic digestion. Both isoforms were digested in gel, and the polypeptides were analyzed by MS-MALDI. *G*, comparison of dissociated Epl1 and Sm1 isoforms by immunoassay. Proteins were electroblotted onto nitrocellulose membranes, and protein blots were probed with antibodies to Sm1. *AEC*, anion exchange chromatography; *Sm1M*, monomer form of Sm1; *Sm1D*, dimer form of Sm1; *Sm1Dis*, dissociated form of dimer Sm1; *Epl1D*, dimer form of Epl1; *Epl1Dis*, dissociated form of Epl1.

(*AOS*, *HPL*, *OPR7*, and *PAL*) in maize plants inoculated with *T. virens* (14). To compare the effects of *T. virens* and *T. atroviride* on the induction of resistance, we tested (locally and systemically) the expression of those genes in maize plants grown in the hydroponic systems and inoculated with fungal tissue. After 48 h of inoculation, expression levels of *HPL*, *OPR7*, and *PAL* were assayed in roots and leaves by quantitative real time RT-PCR. Significant differences in the modulation of gene expression *in planta*, mediated by *T. virens* and *T. atroviride*, were detected ($p < 0.01$). In roots, *PAL*, *HPL*, and *OPR7* were up-regulated by both strains, with higher levels of expression detected in plants inoculated with *T. virens* (Fig. 2*A*). A similar effect of the strains was detected in leaves. The expression of all genes was systemically up-regulated by both strains, with higher levels of expression induced by the presence of *T. virens* (Fig. 2*B*). The PCR products were also analyzed on agarose gel electrophoresis after ethidium bromide staining (Fig. S2). To assess the *in vivo* relevance of *T. virens* Gv29-8 and *T. atroviride* IMI206040 to induce disease resistance, the systemic protection of 2-week-old maize seedlings against the foliar pathogen *C. graminicola* was assayed (as a reference, *C. graminicola* development, on maize leaves, is presented in Fig. S3). We also included a *T. virens* Gv29-8 strain impaired in the expression of *Sm1* (Δ*Sm1*) (14). Fluorescent micrographs of the leaf areas inoculated with *C. graminicola* M1.001-BH-GFP are presented in Fig. 3. The pathogen applied to non-*Trichoderma*-treated plants (control plants) and in plants treated with the Δ*Sm1* strain showed normal development. The germinated spores produced melanized appressoria, and the invading hyphae were colonizing the leaf epidermis and mesophyl (Fig. 3, *A* and *B*). However, on plants treated with *T. virens* or *T. atroviride*, the infection cycle was delayed (Fig. 3, *C* and *D*), but a distinct difference was observed in the developmental stage of *C. graminicola*. On plants treated with *T. atroviride*, the pathogen had already developed melanized appressoria, and some primary hyphae had already developed (developmental stage 3), but on plants treated with *T. virens*, appressoria had not developed yet (developmental stage 1), and the presence of elongated germ tubes was noticeable (Fig. 3, *C* and *D*). The quantitative comparison of the pathogen hyphal growth is presented in Fig. 3*E*, indicating a significant difference ($p < 0.05$) between *T. virens* and *T. atroviride.*

Purification and Biochemical Characterization of Sm1 and Epl1 Isoforms—Since *T. virens* Gv29-8 and *T. atroviride* IMI206040 presented differences in the resistance response in maize, we purified and further characterized both isoforms. Both isoforms of Sm1 and Epll eluted from anion exchange chromatography in a single peak at an NaCl concentration of 100 mM (Fig. 4, *A* and *C*, indicated by an *arrow*). With the second chromatographic procedure (GFC), the monomer of Sm1 was purified to homogeneity, but the monomer of Epl1 was not detected (Fig. 4, *B* and *D*). After GFC, the dimer of Sm1 and Epl1 co-eluted with other proteins of similar molecular weight (not shown). To further purify the dimers of both proteins, an immunoaffinity chromatography using anti-Sm1 antibodies was implemented. The fractions containing the dimers

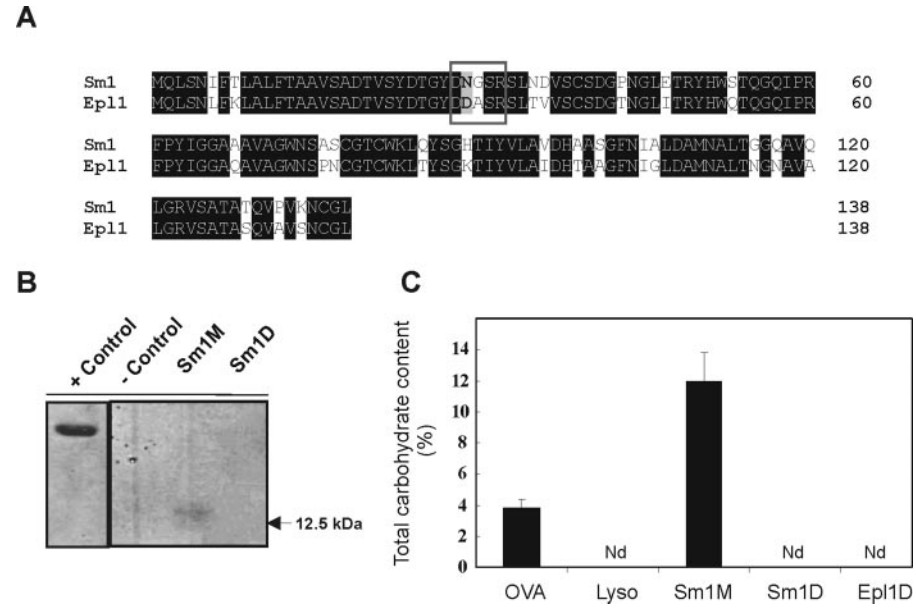

FIGURE 5. **Protein analysis and post-translational modifications on Sm1 and Epl1.** *A*, alignment of deduced amino acid sequences of Sm1 and Epl1. Immature protein sequences were aligned using ClustalX software. *Black shading*, residues conserved in both sequences. The putative motif for glycosylation is denoted by the *gray frame*, and the residue predicted to be glycosylated is *gray-shaded*. *B*, in-gel glycoprotein staining. The purified monomer and dimer of Sm1 were separated in a 15% SDS-polyacrylamide gel, and carbohydrate-containing proteins were stained. *C*, carbohydrate detection and quantification on Sm1 and Epl1 isoforms. The assay was performed in triplicate and using two batches of proteins purified independently. The data are the mean value ± S.E. *Sm1M*, monomer form of Sm1; *Sm1D*, dimer form of Sm1; *Epl1D*, dimer form of Epl1; *OVA*, ovalbumin; *Lys*, lysozyme; *Nd*, not detected.

TABLE 2

Comparison of the glycosylation motif identified in Sm1 homologues

Glycosylation prediction was performed using the NetNGlyc 1.0 server. The putative glycosylation motif is shaded in gray, and the residue target for *N*-glycosylation is boldface type.

Name[a]	Sequence	Accession Number
Gv29-8 Sm1	SYDTGYD**N**GSRSLND	AAZ80388
Hv Snodprot1	SYDTGYD**N**GSRSLND	Q1KHY4
HvT59 Epl1	SYDTGYD**N**GSRSLND	AJ907781
TlT52 Epl1	SYDTGYD**N**GSRSLND	AJ905125
TaIMI Epl1	SYDTGYDDASRSLTV	ABDG01000181
TaT53 Epl1	SYDTGYDDASRSLTV	AJ902344
HaB11 Epl1	SYDTGYDDASRSLTV	AJ901879
HaP1 Epl1	SYDTGYDDASRSLTV	CAL80754
TvT78 Epl1	SYDTGYDDGSRSLNV	AJ908086
Tre Epl1	SYDTGYDDGSRSLTA	AAIL01001181
HaP1 Epl2	SFDPGYDDTSRSLRD	AJ912903
TaIMI Epl2	SFDPGYDDASRSLRD	ABDG01000104
Gv29-8 Sm2	SFDTGYDDPSRSMTQ	ABDF01000320
Hv Snodprot2	TFDTIYDDPSRSLSE	Q1KHY3
Tre Epl2	TFNSLYDDPSRSLSE	AAIL01000958
Nc Snodprot1	SYDTGYDDP**N**RSLTV	Q9C2Q5
Mg UP1	SYDTGYDDGSRSLTA	UPI000021A10F
Af13	SYDPRYDNAGTSMND	XP_750502.1

[a] Gv29-8, *T. virens* Gv29-8; Hv, *H. virens;* HvT59, *H. virens* T59; TlT52, *T. longibrachiatum* T52; HaP1, *H. atroviride* P1; TaT53, *T. asperellum* T53; HaB11, *H. atroviride* B11; TvT78, *T. viride* T78; Tre, *T. reesei* Q56; TaIMI, *T. atroviride* IMI206040; Nc, *N. crassa;* Mg, *M. grisea;* AI293, *A. fumigatus* AF293. Accession numbers are according to Seidl *et al.* (27). For Gv29-8Sm2 and *T. reesei* and *T. atroviride* IMI296949 sequences, the accession numbers were retrieved from the whole genome sequence available at the NCBI site on the World Wide Web.

of Sm1 and Epl1 after GFC were collected, concentrated, and loaded onto the immunospecific column. This purification step allowed the isolation of both dimers to homogeneity, as determined by SDS-PAGE (Fig. 4*E*). To confirm that Sm1 can self-associate to assemble a homodimer, as was demonstrated for Epl1 (27), the purified monomer and dimer were subjected to in-gel tryptic digestion. The peptide mass fingerprint was assayed, and both isoforms presented similar patterns (Fig. 4*F*). We also succeeded in dissociating the purified dimeric isoforms of the Sm1 and Epl1 proteins. Dissociation was achieved by sonicating the protein in trifluoroacetic acid as described by Peña *et al.* (33). The dissociation of both dimers was determined by SDS-PAGE and Western blotting. The immunoassay revealed that the molecular masses of the dissociation products were in the range of the monomeric forms, and no dimer was detected by Western blotting (Fig. 4*G*). Dissociation of both Sm1 and Epl1 dimers was also attempted in the presence of a variety of detergents and reducing agents (SDS, mercaptoethanol, DTT, Nonidet P-40, and Triton X-100) with various heating times and combinations of them. However, no dissociation was achieved in any of these conditions (not shown).

Glycosylation Status of Sm1 and Epl1—Deduced amino acid sequences of Sm1 and Epl1 were compared after sequence alignment demonstrating 82% sequence identity (Fig. 5*A*). Previously, the presence of putative post-translational sites on Sm1 has been described (13), and we compared the presence of these putative sites on Sm1 and Epl1. The Asn-29 residue in Sm1 defines a putative glycosylation site that was replaced by Asp-29 in Epl1. The Asn-29 residue is in the motif DNGSR, which is the putative motif recognized by the glyscosylation machinery. Because of this modification in the putative glycosylation motif (Asn-29 → Asp), no glycosylation site was predicted for Epl1 (Table 2). We performed in-gel glycoprotein staining and carbohydrate content quantification using purified Sm1 (monomer and dimer) and Epl1 (dimer). Only the monomeric form of Sm1 was detected in gel as a glycoprotein (Fig. 5*B*), and the quantitative assay revealed that the monomeric form of Sm1 displays a carbohydrate content of about 14% on a weight basis. On the other hand, no reaction was detected for the dimeric forms of Sm1 or Epl1 in either of the tests (Fig. 5, *B* and *C*).

To compare the occurrence of the *N*-glycosylation site in members of the Epl1, Epl2, and Epl3 clusters reported by Seidl *et al.* (27) (Table 2), we performed a neighbor-joining analysis, including Sm1 from *T. virens* Gv29-8 and homologous sequences belonging to those clusters. All of the sequences that presented a putative *N*-glycosylation site belonged to strains of *Trichoderma*/*Hypocrea virens* and grouped together in a cluster including a sequence from *Trichoderma longibrachiatum* (TlT52 Epl1) (Fig. 6*A*). We compared the oligomerization state

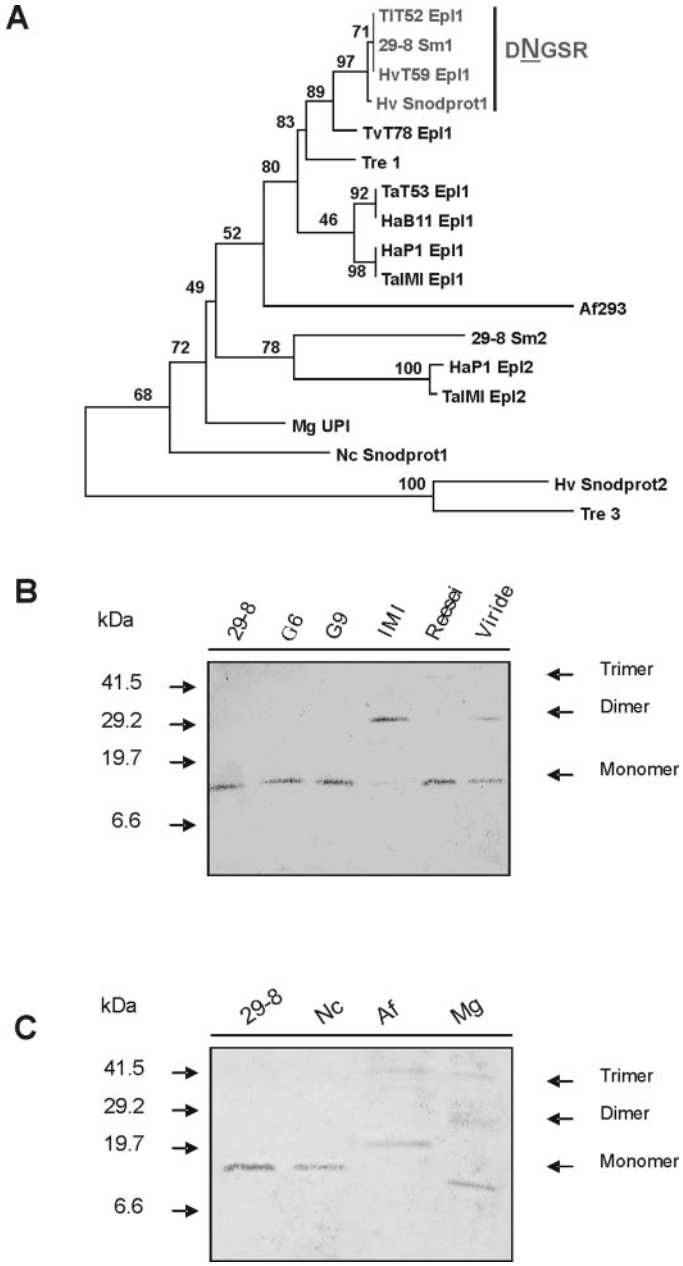

FIGURE 6. **Analysis of the occurrence of Sm1 and Epl1 in different fungal strains.** *A*, phylogenetic reconstruction of Sm1/Epl1 homologue proteins. Protein sequences were selected according to the study performed by Seidl *et al.* (27). Sequence designation is presented in Tables 2 and 3. The tree was constructed with MEGA 3.1 after sequence alignment using ClustalX with a BLOSSUM matrix and a bootstrap trial of 1000. *B*, immunodetection of Sm1/Epl1 homologues in different *Trichoderma* strains. Polypeptides were separated by SDS-PAGE and blotted onto nitrocellulose membranes. Membranes were probed with anti-Sm1 antibodies. *C*, immunodetection of Sm1/Epl1 homologues in non-*Trichodema* species. Polypeptides in CF of *A. fumigatus* (*Af*), *N. crassa* (*Nc*), and *M. grisea* (*Mg*) were electrophoresed on denaturing 15% acrylamide gels, transferred to nitrocellulose membranes, and probed with anti-Sm1 antibodies. *29-8*, *T. virens* Gv29-8; *G6*, *T. virens* G6; *G9*, *T. virens* G9; *IMI*, *T. atroviride* IMI206040; *Reesei*, *T. reesei*; *Viride*, *T. viride*.

of Sm1/Epl1 proteins in the CFs of some strains of *Trichoderma* spp. by Western blotting assays. In the CF of *T. virens* Gv29-8, G6, and G9, Sm1 was immunodetected as a single band with a molecular weight similar to that previously described for the monomeric isoform (13). On the other hand, in CF of *T. viride* and *T. atroviride*, the antibodies detected bands corresponding

TABLE 3

Comparison of the oxidized peptide of Epl1 with homologue sequences from various *Trichoderma* species

The Trp residue reported to be oxidized in the dimeric form of Epl1 is shown in boldface type, and the Gln → Ser substitution in *T. virens* strains is underlined. Identical residues are gray-shaded.

Name[a]	Sequence
Gv29-8 Sm1	YH**W**STQGQIPR
Hv Snodprot1	YH**W**STQGQIPR
HvT59 Epl1	YH**W**STQGQIPR
TlT52 Epl1	YH**W**STQGQIPR
TaIMI Epl1	YH**W**QTQGQIPR
TaT53 Epl1	YH**W**QTQGQIPR
HaB11 Epl1	YH**W**QTQGQIPR
HaP1 Epl1	YH**W**QTQGQIPR
TvT78 Epl1	YH**W**STQGQIPR
Tre Epl1	YH**W**QTQGQIPK

[a] Gv29-8, *T. virens* Gv29-8; Hv, *H. virens;* HvT59, *H. virens* T59; TlT52, *T. longibrachiatum* T52; HaP1, *H. atroviride* P1; TaT53, *T. asperellum* T53; HaB11, *H. atroviride* B11; TvT78, *T. viride* T78; Tre, *T. reesei* Q56; TaIMI, *T. atroviride* IMI206040. For accession numbers, see Table 2.

to the monomer and the dimer, and in the case of *T. reesei*, the presence of bands with M_r corresponding to the monomer and a trimer was detected (Fig. 6*B*). Table 2 lists homologous sequences from *N. crassa* (Snodprot1), *A. fumigatus* (XP_750502.1), and *M. grisea* (UniParc accession number UPI000021A10F). Among those sequences, the presence of a putative glycosylation site was predicted only in *N. crassa* Snodprot1, and Western blotting revealed a single band with M_r corresponding to a monomer (Fig. 6*C*, *Nc*). Seidl *et al.* (27) reported that the dimeric form of Epl1 from *T. atroviride* presented an oxidized Trp residue in the motif YHWQTQGQIPR. The oxidation of Trp residues is involved in the aggregation and inactivation of some proteins (37, 38). We compared this motif in Sm1/Epl1 sequences from the different *Trichoderma* strains analyzed previously (Table 3). This motif is highly conserved in all *Trichoderma* species, and only *Trichoderma*/*Hypocrea virens* sequences presented a point difference (Gln → Ser) next to the Trp residue to be oxidized. This motif is less conserved in *N. crassa*, *A. fumigatus*, and *M. grisea*; however, the Trp residue is still conserved in this region of the protein among these fungi (data not shown).

Differential Effect of Monomer and Dimer Forms of Sm1 and Epl1 on Defenses in Maize Leaves—We have previously demonstrated that Sm1 induces plant defense reactions when infiltrated into leaves (13). In the present report, we adopted this strategy as a rapid and reliable method to compare the responses that Sm1 and Epl1 induced in maize leaves. To test the effect of the oligomerization state of Sm1 and Epl1 on the expression of plant defense-related genes, the purified forms of the Sm1 monomer and dimer, the Epl1 dimer, and its dissociated form were syringe-infiltrated (1 nmol/leaf) into leaves of 14-day-old maize plants.

Quantitative real time PCR experiments indicated that the expression of *AOS*, *HPL*, *OPR7*, and *PAL* was significantly ($p < 0.01$) up-regulated 24 h after the monomer of either Sm1 or

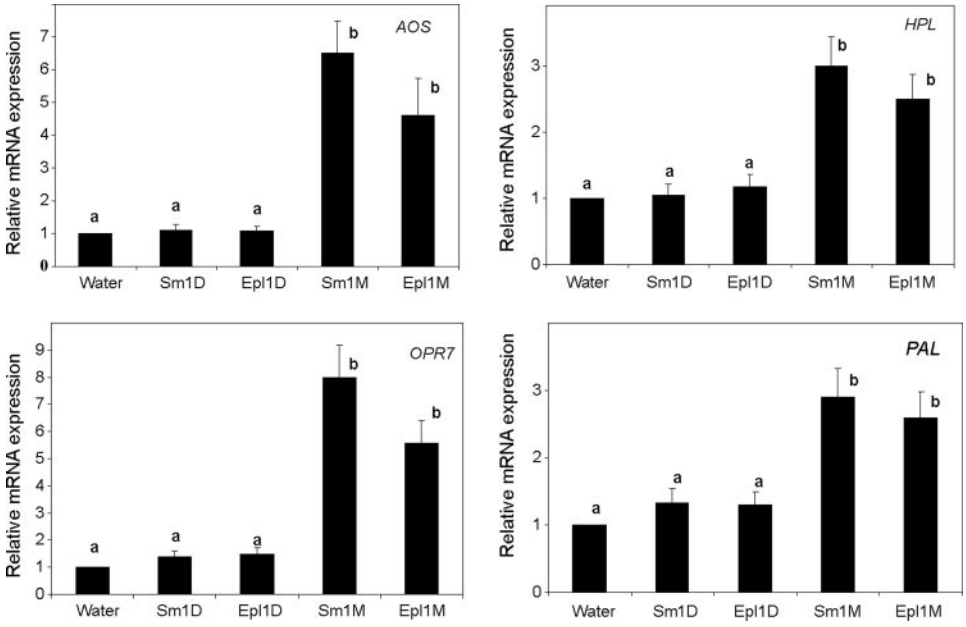

FIGURE 7. **Analysis of the expression of defense-related genes (*AOS*, *HPL*, *OPR7*, and *PAL*) in maize leaves after Sm1 and Epl1 infiltration.** The third leaves of 14-day-old maize plants were infiltrated with 1 nmol/leaf of Sm1 monomer (*Sm1M*), Sm1 dimer (*Sm1D*), dissociated Epl1 (*Epl1M*), Epl1 dimer (*Epl1D*), or water as a control (*Water*). After 24 h of treatment, total RNA was extracted and used for quantitative real time RT-PCR assays. Expression of *GAPc* was used as quantitative internal control. The gene expression data presented were normalized to the reference control. The data depicted are the mean values ± S.E. of two independent experiments. *Columns* with *different letters* differ significantly according to Tukey's HSD test at a significance level of 1%.

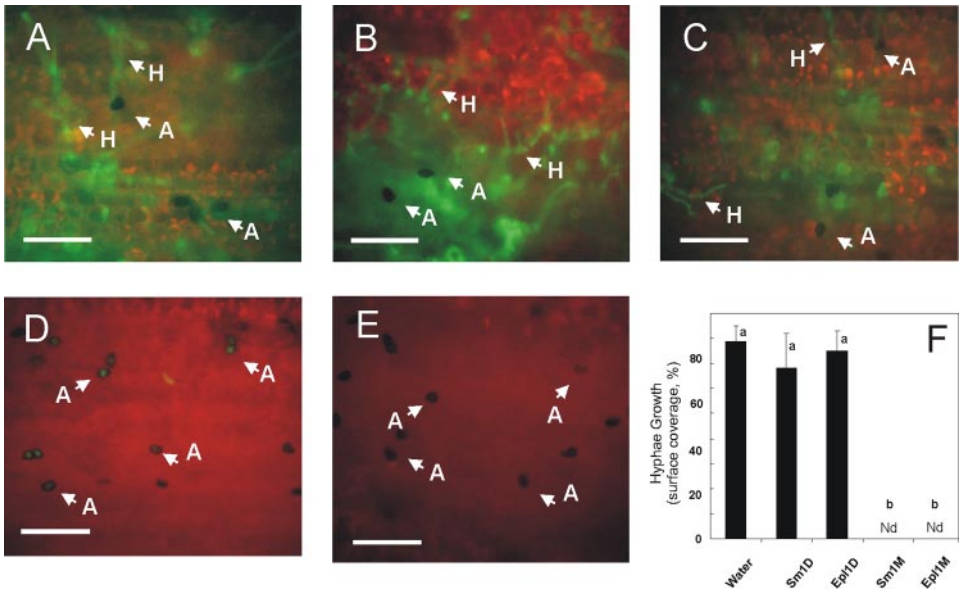

FIGURE 8. **Effect of Sm1 and Epl1 isoforms on the development of the foliar pathogenic fungus *C. graminicola*.** Leaves were infiltrated with water (*A*), dimeric Sm1 (*B*), dimeric Epl1 (*C*), monomeric Sm1 (*D*), or dissociated Epl1 (*E*) and inoculated with a GFP-tagged *C. graminicola* M1.001-BH strain 24 h postinfiltration. *F*, quantification of *C. graminicola* growth on leaves. The data presented show the percentage of the inoculation area covered by the pathogen hyphae. The *bars* depict the mean value ± S.D. determined in pictures taken in three independent experiments. *Columns* with *different letters* differ significantly according to Tukey's HSD test at a significance level of 1%. Pathogen development was compared 48 h after inoculation with an Olympus BX-51 fluorescent microscope with excitation from 470 to 490 nm and emission from 510 to 550 nm. The areas on the micrographs were determined using ImageJ software. *Bars*, 50 μm. *A*, appressoria; *H*, hypha; *Nd*, not detected. *Bars*, 50 μm.

Epl1 was infiltrated (Fig. 7). These experiments indicated that only the monomeric form of either protein up-regulates the expression of the defense-related genes. These data were confirmed by analyzing the PCR amplification products after separation on agarose gels (Fig. S4). As previously described for other plant species (13), none of the treatments presented phytotoxicity toward maize, since no visible lesions on leaves were observed (not shown). The effect of the infiltrated proteins on disease protection was evaluated by inoculating plants with an isolate of the foliar maize pathogen *C. graminicola* expressing *GFP* as a reporter (28). Forty-eight hours after inoculation, the leaves that had been infiltrated with water or the dimeric forms of Sm1 or Epl1 (Fig. 8, *A–C*) displayed an extensive hyphal development of the pathogen (similar to that of the non-*Trichoderma*-treated plants; Fig. 3*A*). In contrast, the leaves that had been infiltrated with the monomeric form of either elicitor displayed a more delayed pathogenic development. The spores had only developed mature appressoria, and no hyphal colonization was detected (Fig. 8, *D* and *E*). The quantitative comparison of the pathogen hyphal growth is presented in Fig. 8*F*.

DISCUSSION

Protein aggregation or self-association is a widespread phenomenon that occurs in different organisms (39–41). Moreover, recent findings show that protein dimerization or oligomerization is a key factor in the regulation of proteins such as enzymes, ion channels, receptors, and transcription factors (reviewed by Marianayagam *et al.* (39)). In this report, we demonstrate that the dimerization of an elicitor protein (Sm1) involved in plant-microbe interactions results in the loss of its eliciting properties, blocking the signaling transduction pathways that lead to the activation of resistance mechanisms in its host plant (maize).

Recently, Sm1 was described as essential for the induction of ISR in plants (13, 14), but only the monomeric form of the elicitor was examined. The characterization of Epl1, a homologue of Sm1, was limited to gene expression assays and to the description of structural traits of the protein

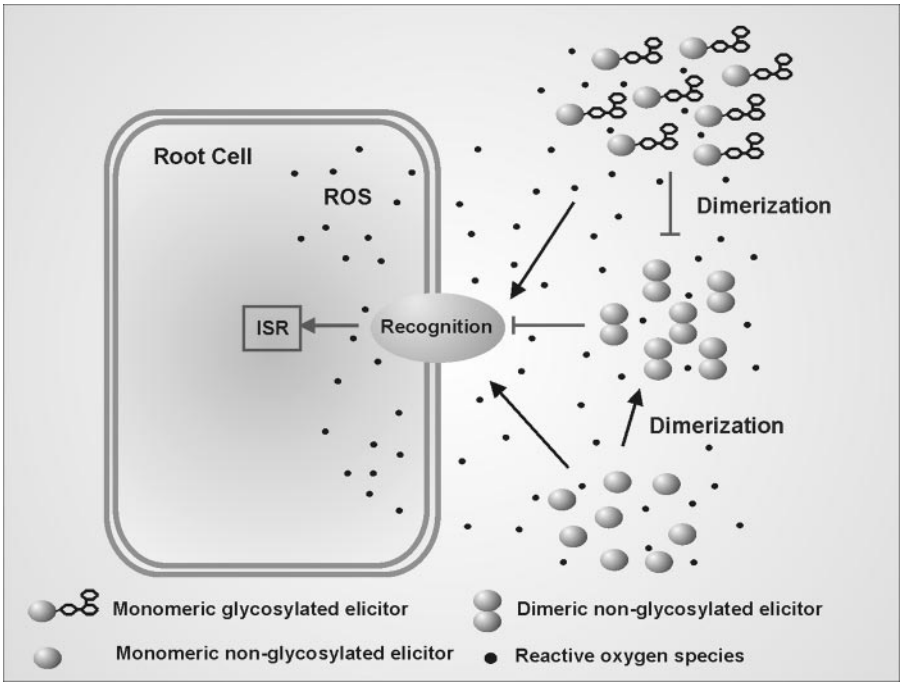

FIGURE 9. **Proposed biochemical processes involved in the control of ISR induction mediated by proteinaceous elicitors.** When the elicitors were produced as a monomer they were recognized in the cell interface, the defense responses were activated, and reactive oxygen species (*ROS*) reaction was elicited. The monomeric form of the elicitors in their nonglycosylated state would be susceptible to oxidative-driven dimerization and no longer active to induce ISR. However, when the monomeric elicitor is produced in its glycosylated form, the presence of the glycosyl moiety will not allow the monomers to dimerize, and they will remain in their active form for activating the defense responses in plants.

(27), but elicitor assays were not performed. Here, the ability Epl1 to induce plant defenses is demonstrated for the first time. The functional comparison of both isoforms from Sm1 and Epl1 highlighted that only the monomeric isoform of these small proteins was able to elicit the resistance mechanisms in maize leaves, and no activity was detected for the dimer (Figs. 7, 8, and S4). Since *T. virens* Gv29-8 and *T. atroviride* IMI206040 displayed different effects on systemic protection in maize, this result was attributed to variations in the production of the elicitor. Both strains secreted similar levels of hydrolytic enzymes important for *Trichoderma*-plant interactions, but *T. virens* produced higher levels of the monomeric form of Sm1. The production of the monomeric form of Epl1 by *T. atroviride* was at such low levels as not to be immunodetected. However, it was described that various *Trichoderma* species also produce other elicitors that induce resistance mechanisms in plants (18, 42). Thus, the fact that *T. atroviride* still displays ISR might be ascribed to the presence of additional elicitors other than monomeric Epl1.

The dimeric form of Sm1 was only detected in the presence of maize seedlings, and Epl1 was only immunorevealed as a dimer in the hydroponic experiment (Fig. 1*C*). However, when *T. virens* was grown in VM supplemented with sucrose, glucose, or glycerol, as well as in GYEC medium (Figs. 6*B* and S5), the presence of only the monomer was immunodetected. These data suggest that aggregation of the elicitors may be influenced by the presence of the plant. These observations lead us to hypothesize that as part of the *Trichoderma*-plant interaction, the plant may alter the aggregation state of Sm1/Epl1 and ultimately affect its ability to induce defenses.

The glycosylation state of proteins from the cell surface of pathogenic bacteria, such as flagellin and pilin, has been recognized as an important factor in host-pathogen interactions (43, 44). Recent reports describe the importance of the glycosylation state of flagellin from *Pseudomonas syringae* during plant-pathogen associations (45–47). Glycosylation of flagellin was demonstrated to be strongly related to the pathogen's ability to cause a hypersensitive reaction leading to cell death and could be the specific determinant of compatibility between phytopathogenic bacteria and plant species (45). For Sm1/Epl1 proteins, glycosylation appears to be important in controlling the elicitor's activity during the plant-microbe interaction. We conclude that the glycosyl moieties might be associated with structural functions, where they contribute to controlling the monomer-dimer dynamic, offering a steric impediment to subunit associations. Our results illustrate molecular and biochemical mechanisms that control the manner in which ISR, activated by proteinaceous elicitors, is triggered in plants. We speculate the existence of a very specific mechanism for the recognition of elicitors by the plant. This mechanism could either enable the plant to discriminate between monomeric and dimeric forms or detect special features of the monomeric form that are no longer exposed when the subunits aggregate.

The intermolecular interaction between monomers of Sm1 or Epl1 is resistant to SDS/β-mercaptoethanol treatment (and other detergents at various temperatures), suggesting an intersubunit covalent interaction other than disulfide bridges. In the case of hydrophobins secreted by *Trichoderma* spp. and other fungi, different aggregation states with strong interactions (similar to Sm1 and Epl1) have been described (33, 48–50). However, the molecular mechanisms involved in such strong interactions were not further studied. In humans, plants, and fungi, it has been reported that oxidative cross-linking can control activity and aggregation states of proteins, generating post-translational covalent linkages resistant to SDS/β-mercaptoethanol treatment (37, 38, 51–57). In *T. reesei* and *Agaricus bisporus*, an enzymatically driven protein cross-linking in which tyrosines are involved has been characterized (57). An oxide reduction mechanism controlling the activity and the aggregation state of human superoxide dismutase 1 (hSOD1) has also been described (37). This mechanism involves the oxidation of a tryptophan residue to its peroxyl radical to form kynurenine, a highly reactive species that drives the reaction

between both monomers and generates a covalent bond between the molecules (37). Peptide mass fingerprinting and peptide analysis of the dimer form of Epl1 demonstrated the presence of one peptide (YHWQTQGQIPR) with two different oxidation states. It was proposed that the oxidations occurred on the tryptophan residue to form *N*-formylkynurenine (27). Likewise, in the amino acid sequence of Sm1, we identified a segment of the sequence (YHWSTQGQIPR) very similar to that for Epl1 presenting only one substitution (Gln → Ser) (Table 3). This motif was 100% conserved in all Sm1 homologues from all *Trichoderma*/*Hypocrea virens* sequences analyzed and in the homologous sequence identified in *T. viride* T78 (Table 3). When Sm1 is in contact with plant cells, an oxidative burst is elicited (13). This accumulation of reactive oxygen species in the plant cells probably stimulates an oxidative-driven dimerization of the monomeric proteins, altering the concentration of the active elicitor in the environment. However, in the case of *T. virens* strains, the presence of a glycosyl moiety on the elicitor may prevent that dimerization, keeping in solution a protein with high elicitor activity (Fig. 9).

We also analyzed the sequences of Sm1 homologues in various fungal strains to identify differences in the glycosylation motif in protein from different species. The glycosylation site was only present among members of a closely related cluster containing sequences from *Trichoderma*/*Hypocrea virens* strains and an Sm1 homologue from *T. longibrachiatum* T52 (Fig. 6 and Table 2). According to phylogenetic reconstruction of the genus *Trichoderma* using parsimony analysis of ITS1, ITS2, *mitSSU* DNA, *tef1*, and *ech42* sequences, it was demonstrated that *T. longibrachiatum* is distantly related to *T. virens* strains (58). This phylogenetic difference coupled with a high similarity between *T. virens* Sm1 and *T. longibrachiatum* Epl1 sequences might suggest functional and molecular characteristics conserved throughout the evolution of both species. Remarkably, we also found the presence of the putative glycosylation site in some other sequences of CP proteins from various fungal species (Table 2) (not shown). The aggregation and oligomerization patterns of Sm1 homologues in different filamentous fungus strains (Fig. 6, *B* and *C*) correlate with the prediction that oligomerization may be prevented by the glycosylation of the polypeptides. According to these results, we suggest that the CP protein family be divided into two subfamilies according to the presence or absence of the glycosylation site and their ability to self-aggregate.

Our future research is focused on characterizing modified proteins lacking the glycosylation site and/or the tryptophan residue putatively involved in an oxidative cross-linking of the monomers. Further structural analyses, such as three-dimensional structure determination and carbohydrate moiety characterization, will contribute to our understanding of the processes involved in the recognition of elicitors and the initiation of ISR in plants.

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