Dimerization Controls the Activity of Fungal Elicitors That Trigger Systemic Resistance in Plants*§

Walter A. Vargas1, Slavica Djonović1, Christy A. Kemerly1, and Charles M. Kenerley1,2

From the 1Department of Plant Pathology and Microbiology, Texas A&M University, College Station, Texas 77843 and 2Centro Hispano-Luso de Investigaciones Agrarias, Departamento de Microbiologia y Genética, Universidad de Salamanca, 37007 Salamanca, Spain

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 teleomorph form of Trichoderma atroviride) secretes an Sm1-homologous protein, Ep11, 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 Ep11 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 Ep11, and we demonstrate for the first time that the monomeric form of Ep11, 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 Ep11 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 hydrophin-
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. 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-ΔSm1 (14), *Trichoderma atroviride* IMI206040, *Trichoderma reesei* 6, and *Trichoderma viride* 21. Also for this research, we used the filamentous fungus *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.

**Modifications of Elicitors That Activate Disease Resistance**

*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-nitroanilide (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 immunoblotting (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 NH4HCO3, and dialyzed against the same solution. Total protein was loaded onto High Q support columns (0.5 × 20 cm) (Bio-Rad) equilibrated with 20 mM 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.
Modifications of Elicitors That Activate Disease Resistance

TABLE 1
List of primers used for gene expression assays

| Gene name | Primer sequence | Product size (bp) | Accession number |
|-----------|----------------|------------------|-----------------|
| AOS       | 5’-AAACCGACATTTTGACGCA-3’ | 74 |AY68135.1 |
| HPL       | 5’-TGAGCGGCTCCACGAGAAATGG-3’ | 80 |AY540745.1 |
| OPR7      | 5’-ACACAGAAAGGCGAGAAC-3’ | 64 |AY921644.1 |
| PAL       | 5’-CCCTAGGCCTGTCATCAAT-3’ | 110 |L37912.1 |
| GAPc      | 5’-GGCCGTTCAAGCAGTCCAT-3’ | 63 |X07156.1 |
| TvsSm1    | 5’-ACGCTGTCTGCTGTCGACACAT-3’ | 125 |DQ121133 |
| TaEpl1    | 5’-CTAACTGCTTGGCACTTGCAAAC-3’ | 221 |AI901879 |
| Actin     | 5’-TGAATGATGTCGATGGCAGAAGAC-3’ | 156 |X75421 |

**PROTEIN GLYOSYLATION 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 metaperiodate and subjected to a reaction with a freshly made 0.5% solution of glycoprotein detection reagent in 1 M 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.

**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 metaperiodate and subjected to a reaction with a freshly made 0.5% solution of glycoprotein detection reagent in 1 M 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.

**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 Gt29-8 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 1.**
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), OP7 (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 \( \Delta C_T \)) (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 \( 6.5 \times 10^4 \) spores/ml was prepared in a solution of 0.02% Tween 20 in water. Inoculation with T. virens Gv29-8, T. virens ΔSm1 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 (Metro-mix 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.
Sequence Analyses—Sequence comparisons were performed using deduced amino acid sequences available in the databases 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. 1A). 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. 1B). 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. 1C).

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
Modifications of Elicitors That Activate Disease Resistance

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 (ΔSm1) (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 ΔSm1 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. 3E, 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 Epl1 eluted from anion exchange chromatography in a single peak at an NaCl concentration of 100 mM (Fig. 4, A and C). Dimeric isoforms were detected (ΔSm1) (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 ΔSm1 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. 3E, indicating a significant difference (p < 0.05) between *T. virens* and *T. atroviride*.

*Scopalia moniliformis* (Sm1) and *Epidermophyton juncosum* (Epl1) 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 system 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. 2A). 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. 2B). The PCR products were also analyzed on agarose gel electrophoresis after ethidium bromide staining (Fig. S2).
Modifications of Elicitors That Activate Disease Resistance

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 | Sequence | Accession Number |
|------|----------|-----------------|
| Gv29-8 Sm1 | SYDGTG1DBSSLDQD | AA199008 |
| Hv Stdox1 | SYDGTG1DBSSLDQD | QI196814 |
| Hv TTS9 Eppl | SYDGTG1DBSSLDQD | A907781 |
| TTS9 Eppl | SYDGTG1DBSSLDQD | A905325 |
| TelM Eppl | SYDGTG1DBSSLDQD | ABG0000101 |
| Tfs75 Eppl | SYDGTG1DBSSLDQD | A902744 |
| HaHl1 Eppl | SYDGTG1DBSSLDQD | A9301670 |
| HaHl1 Eppl | SYDGTG1DBSSLDQD | A900754 |
| Tps79 Eppl | SYDGTG1DBSSLDQD | A900866 |
| Tep Epl | SYDGTG1DBSSLDQD | A9050901 |
| Hfl1 Eppl | SYDGTG1DBSSLDQD | A9301670 |
| Nc Stdox2 | SYDGTG1DBSSLDQD | ABG0000101 |
| Nc Stdox2 | SYDGTG1DBSSLDQD | A9050901 |
| Mg UPl | SYDGTG1DBSSLDQD | UP199002 |
| Apf | SYDGTG1DBSSLDQD | XP400702 |

a Gv29-8, T. virens; Gv29-8, H. virens; HvTS9, H. virens T59; TTS9, T. longibrachiatum T52; HaP1, H. atrochoroides P1; Taf53, T. asperellum T53; HaB11, H. atrochoroides B11; HvT78, T. viride T78; Tre, T. reesei Q56; TelM, T. atroviride IMI12006040; Nc, N. crassa; Mg, M. grisea; Apf, A. fumigatus AF293. Accession numbers are according to Seidl et al. (33). For Gv29-8 Sm1 and T. reesei and T. atroviride IMI12006040, sequences 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 deter-
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 the CF of *T. viride* and *T. atroviride*, the antibodies detected bands corresponding to the monomer and the dimer, and in the case of *T. reesei*, the presence of bands with *M* corresponding to the monomer and a trimer was detected (Fig. 6B). 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* corresponding to a monomer (Fig. 6C, 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/Hypocrean* strains 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
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 (similar to that of the non-*Trichoderma*-treated plants; Fig. 3A). 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. 8F.

**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.
Modifications of Elicitors That Activate Disease Resistance

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.

The dimeric form of Sm1 was only detected in the presence of maize seedlings, and Ep1 was only immunorevealed as a dimer in the hydroponic experiment (Fig. 1C). However, when T. virens was grown in VM supplemented with sucrose, glucose, or glycerol, as well as in GYEC medium (Figs. 6B 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 Ep1 is resistant to SDS/β-mercaptoethanol treatment (and other detergents at various temperatures), suggesting an inter-subunit 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 Ep11) 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 oxidation 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 (YHWQYQGQIPR) 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.

Acknowledgments—We thank E. Monte (Universidad de Salamanca, Spain), A. Herrera-Estrella (CINVESTAV, Mexico), C. Howell (United States Department of Agriculture, College Station, TX), and H. Wilkinson (Texas A&M University, College Station, TX) for providing fungal isolates for this study.

REFERENCES
1. Cook, R. J., Thomashow, L. S., Weller, D. M., Fujimoto, D., Mazzola, M., Bangera, G., and Kim, D. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 4197–4201
2. Vallard, G. E., and Goodman, R. M. (2004) Crop Sci. 44, 1920–1934
3. Nimchuk, Z., Elgjem, T., Holt, B. F., III, and Dangl, J. L. (2003) Annu. Rev. Genet. 37, 579–609
4. Nürnberger, T., Brunner, F., Kemmerling, B., and Pieterse, C. M. I. (2004) Immuno- mol. Rev. 198, 249–266
5. Bais, H. P., Park, S. W., Weir, T. L., Callaway, R. M., and Vivanco, J. M. (2004) Trends Plant Sci. 9, 26–32
6. Pozo, M. J., Van Loon, L. C., and Pieterse, C. M. I. (2005) J. Plant Growth Regul. 24, 211–222
7. Reymond, P., Grünberger, S., Paul, K., Müller, M., and Farmer, E. E. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 4145–4149
8. Ramonnelli, K. M., Zhang, B., Ewing, R. M., Chen, Y., Xu, D., Stacey, G., and Somervell, S. (2002) Mol. Plant Pathol. 3, 301–311
9. Kaku, H., Nishizawa, I., Ishii-Minami, N., Akimoto-Tomiyama, C., Dohmae, N., Takio, K., Minam, E., and Shibuya, N. (2006) Proc. Natl. Acad. Sci. U. S. A. 103, 11086–11091
10. Felix, G., Duran, J. D., Volkso, S., and Boller, T. (1999) Plant J. 18, 265–276
11. Brunner, F., Rosahl, S., Lee, J., Ridd, J., J., Geiler, C., Kauppinen, S., Rasmussen, G., Scheel, D., and Nürnberger, T. (2002) EMBO J. 21, 6681–6688
12. Kunze, G., Zipfel, C., Robatzeck, S., Niehaus, K., Boller, T., and Felix, G. (2004) Plant Cell 16, 3496–3507
13. Djonovic, S., Pozo, M. J., Dangott, L. J., Howell, C. R., and Kenerley, C. M. (2006) Mol. Plant-Microbe Interact. 19, 838–853
14. Djonovic, S., Vargas, W. A., Kolomietz, M. V., Horneskjold, M., Wiest, A., and Kenerley, C. M. (2007) Plant Physiol. 145, 875–889
15. Ferrari, S., Galletti, R., Denoux, C., De Lorenzo, G., Ausubel, F. M., and Dewdney, J. (2007) Plant Physiol. 144, 367–379
16. Yedidia, I., Shores, M., Kerem, K., Benhamou, N., Kapulnik, Y., and Chet, I. (2003) Appl. Environ. Microbiol. 69, 7343–7353
17. Shores, M., Yedidia, I., and Chet, I. (2005) Phytopathology 95, 76–84
18. Viterbo, A., Wiest, A., Brotman, Y., Chet, I., and Kenerley, C. (2007) Mol. Plant Pathol. 8, 737–746
19. Wosten, H. A. B. (2001) Annu. Rev. Microbiol. 55, 625–646
20. Pan, S. C., and Cole, G. T. (1995) Infect. Immun. 63, 3994–4002
21. Hemmann, S., Blaser, K., and Cramer, R. (1997) Ann. J. Respir. Crit. Care Med. 156, 1956–1962
22. Hall, N., Keon, J. P. R., and Hargreaves, J. A. (1999) Physiol. Mol. Plant Pathol. 55, 69–73
23. Pazzagli, L., Cappugi, G., Manao, G., Camici, G., Santini, A., and Scala, A. (1999) J. Biol. Chem. 274, 24959–24964
24. Wilson, L. M., Idnurm, A., and Howlett, B. J. (2002) Mol. Plant Pathol. 3, 487–493
25. Mou, Z., Fan, W., and Dong, X. (2003) Cell 113, 935–944
26. Durrant, W. E., and Dong, X. (2004) Annu. Rev. Phytopathol. 42, 185–209
27. Seidl, V., Marchetti, M., Schandl, R., Allmaier, G., and Kubicke, C. P. (2006) FEBS J. 273, 4346–4359
28. Sukno, S. A., Garcia, V. M., Shaw, B. D., and Thon, M. R. (2008) Appl. Environ. Microbiol. 74, 823–832
29. Vogel, H. J. (1956) Microbiol. Genet. Bull. 13, 42–43
30. Djonovic, S., Vittone, G., Mendoza-Herrera, A., and Kenerley, C. M. (2007) Mol. Plant Pathol. 8, 469–480
31. Laemmli, U. K. (1970) Nature 227, 680–685
32. Renart, J., and Sandoval, I. V. (1984) Methods Enzymol. 104, 455–460
33. Pitas, M. A., Åsgeirsdóttir, S. A., Lasa, I., Ciliañez-Maciá, F. A., Pi-sabarro, A. G., Wessels, J. G. H., and Ramírez, L. (1998) Appl. Environ. Microbiol. 64, 4028–4034
34. Weaver, M., and Kenerley, C. (2005) Biocontrol. Sci. Technol. 15, 847–857
35. Harman, G. E. (2006) Phytopathology 96, 190–194
36. Thompson, J. D., Gibson, T. J., Plewniak, F. M., Jeanmougin, F., and Higgins, D. G. (1997) Nucleic Acids Res. 15, 4876–4882
37. Zhang, H., Andrekopoulou, C., Joseph, J., Chandran, K., Karoui, H., Crow, J. P., and Kalyanaraman, B. (2003) J. Biol. Chem. 278, 24078–24089
38. Fu, X., Kao, J. L. F., Bergt, C., Kassim, S. Y., Huq, N. P., d’Avignon, A., Parks,
W. C., Mecham, R. P., and Heinecke, J. W. (2004) J. Biol. Chem. 279, 6209–6212
39. Marianayagam, N. J., Sunde, M., and Matthews, J. M. (2004) Trends Biochem. Sci. 29, 618–625
40. Paul, R., Abel, S., Wassmann, P., Beck, A., Heerklotz, H., and Jenal, U. (2007) J. Biol. Chem. 282, 29170–29177
41. Wayne, N., and Bolon, D. N. (2007) J. Biol. Chem. 282, 35386–35395
42. Brotman, Y., Briff, E., Viterbo, A., and Chet, I. (2008) Plant Physiol. 147, 779–789
43. Benz, I., and Schmidt, M. A. (2002) Mol. Microbiol. 45, 267–276
44. Logan, S. M. (2006) Microbiology 152, 1249–1262
45. Takeuchi, K., Taguchi, F., Inagaki, Y., Toyoda, K., Shiraishi, T., and Ichnose, Y. (2003) Plant Cell Physiol. 44, 342–349
46. Takeuchi, K., Taguchi, F., Inagaki, Y., Toyoda, K., Shiraishi, T., and Ichinose, Y. (2003) J. Bacteriol. 185, 6658–6665
47. Takeuchi, K., Ono, H., Yoshida, M., Ishii, T., Katoh, E., Taguchi, F., Miki, R., Murata, K., Kaku, H., and Ichinose, Y. (2007) J. Bacteriol. 189, 6945–6956
48. Wessels, J. G. H. (1994) Annu. Rev. Phytopathol. 32, 413–437
49. Torkkeli, M., Serimaa, R., Ikkala, O., and Linder, M. (2002) Biophys. J. 83, 2240–2247
50. Linder, M. B., Szilvay, G. R., Nakari-Setälä, T., and Penttilä, M. E. (2005) FEMS Microbiol. Rev. 29, 877–896
51. Bradley, D. J., Kjellborn, P., and Lamb, C. J. (1992) Cell 70, 21–30
52. Heinecke, J. W., Li, W., Francis, G. A., and Goldstein, J. A. (1993) J. Clin. Invest. 91, 2866–2872
53. Brisson, L. F., Tenhaken, R., and Lamb, C. (1994) Plant Cell 6, 1703–1712
54. Brown, I., Trehowan, J., Kerry, M., Mansfield, J., and Bolwell, G. P. (1998) Plant J. 15, 333–343
55. Ziouzenkova, O., Asatryan, L., Akmal, M., Tetta, C., Wratten, M. L., Wich, G. L., Jürgens, G., Heinecke, J., and Sevanian, A. (1999) J. Biol. Chem. 274, 18916–18924
56. Larios, J. M., Budhiraja, R., Fanburg, B. L., and Thannickal, V. J. (2001) J. Biol. Chem. 276, 17437–17441
57. Mattien, M.-L., Lantto, R., Selinheimo, E., Kruus, K., and Buchert, J. (2008) J. Biotechnol. 133, 395–402
58. Kullnig-Gradinger, C. M., Szakacs, G., and Kubicek, C. P. (2002) Mycol. Res. 106, 757–767
59. PE Applied Biosystems (2001) Sequence Detector User Bulletin 2, PE Applied Biosystems, 1–36