Secretome of Trichoderma Interacting With Maize Roots: Role in Induced Systemic Resistance*‡

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Trichoderma virens is a biocontrol agent used in agriculture to antagonize pathogens of crop plants. In addition to direct mycoparasitism of soil-borne fungal pathogens, T. virens interacts with roots. This interaction induces systemic resistance (ISR), which reduces disease in aboveground parts of the plant. In the molecular dialog between fungus and plant leading to ISR, proteins secreted by T. virens provide signals. Only a few such proteins have been characterized previously. To study the secretome, proteins were characterized from hydroponic culture systems with T. virens alone or with maize seedlings, and combined with a bioassay for ISR in maize leaves infected by the pathogen Cochliobolus heterostrophus. The secreted protein fraction from coculture of maize roots and T. virens (Tv+M) was found to have a higher ISR activity than from T. virens grown alone (Tv). A total of 280 fungal proteins were identified, 66 showing significant differences in abundance between the two conditions: 32 were higher in Tv+M and 34 were higher in Tv. Among the 34 found in higher abundance in Tv and negatively regulated by roots were 13 SSCPs (small, secreted, cysteine rich proteins), known to be important in the molecular dialog between plants and fungi. The role of four SSCPs in ISR was studied by gene knockout. All four knockout lines showed better ISR activity than WT without affecting colonization of maize roots. Furthermore, the secreted protein fraction from each of the mutant lines showed improved ISR activity compared with WT. These SSCPs, apparently, act as negative effectors reducing the defense levels in the plant and may be important for the fine tuning of ISR by Trichoderma. The down-regulation of SSCPs in interaction with plant roots implies a revision of the current model for the Trichoderma-plant symbiosis and its induction of resistance to pathogens. Molecular & Cellular Proteomics 14: 10.1074/mcp.M114.046607, 1054–1063, 2015.

Fungi belonging to the genus Trichoderma are used as biocontrol agents in agriculture. In addition to direct antagonism of soil-borne pathogens, these fungi intimately interact with plant roots, and are thus considered rhizosphere-competent (1). The interaction is, in general, a beneficial one, promoting plant growth as well as inducing systemic resistance (ISR)

1 to pathogens (2–6). The elicitation of defense response in the leaves of plants whose roots are colonized with Trichoderma enhances the plant’s resistance to foliar pathogens. This clear potential for application in agriculture is already beginning to be realized (7–9).

Secreted proteins are central to the molecular dialog between fungi and their plant hosts. Recent studies addressed, for example, the molecular basis for mutualistic interactions between soil fungi and plants in mycorrhizae, a fungus-root symbiosis of widespread importance for nutrient acquisition. Specific secreted proteins were found to have targets in the plant (10, 11). The Trichoderma-root mutualism is distinct from these well-studied mycorrhizal symbioses, but some of the principles may be shared. Proteomic studies on several Trichoderma species have been reviewed (12). These studies employed total protein extracts from Trichoderma interacting with plants, or the three-way Trichoderma-plant-pathogen interaction (13, 14), and led to the identification of some secreted proteins expressed during the interaction with plant and fungal hosts. Indeed, the first studies of secreted proteins demonstrated an abundant Trichoderma secreted protein, belonging to the ceratoplatanins, which are a fungal family of secreted elicitors and toxins. This protein, Sm1 (in T. virens)/Ep1 (in T. atroviride (15–20) was shown to elicit ISR. The ceratoplatin Sm1/Ep1 also belongs to a larger class of fungal proteins defined as SSCPs or SSPs: small, secreted (cysteine rich) proteins (21–23). There are no sequence motifs

1 The abbreviations used are: ISR, inducing systemic resistance; Tv, Trichoderma virens; Tv+M, T. virens plus maize; LC-MS/MS, liquid chromatography tandem MS; SSCP, small, secreted, cysteine rich protein; WT, wild type.

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or domains common to the members of the entire SSCP class. Within the wide definition, though, there are subfamilies of proteins that do share sequence homology, for example the ceratoplatanin family to which Sm1 belongs.

A bioinformatic survey of the SSeps encoded in the genomes of three *Trichoderma* species, *T. virens* (Tv), *T. atroviride* (Ta), and *T. reesei* (Tr) revealed several hundred candidate SSeps in each species (24). Approximately half of the SSeps from each species have homologs in the same and/or in the other two species, whereas the other half are unique and do not share homology in or between the species. This diversity between the three species suggests that SSeps are evolving rapidly.

Given the known importance of Sm1, and the wide host range of *Trichoderma* species, it seemed likely that many SSeps might be involved in the *Trichoderma*-root interaction. Secreted proteins (with emphasis on SSeps), whose abundance changes in response to association with plant roots, may function in the fungal-plant molecular dialog. To test the hypothesis that the abundance of specific SSeps and other secreted proteins is regulated by the interaction with plant roots, we compared the secretome of *Trichoderma* alone to the secretome of *Trichoderma* cocultured with the roots of maize seedlings. Functional experiments using knock out mutants in the genes encoding some of the regulated SSeps were carried out in order to shed light on their role in the molecular interaction between the plant and the fungus.

**EXPERIMENTAL PROCEDURES**

Plant Inoculation with Trichoderma and Pathogen Challenge—The hydroponic system was adapted from those of (20, 25). Glass beakers (600 ml) were filled with 200 ml of plant nutrient solution [half-strength Murashige and Skoog (MS) basal medium (Duchefa, Haarlem, The Netherlands) with 2.5 mM MES buffer, pH 5.7]]. Perforated plastic stands for supporting the seeds were cut from 200 μl micropipette tips holders. Maize seeds (Royalty, local hybrid, purchased from Ben Shachar, Tel Aviv, Israel) were surface sterilized by soaking them in 10% H2O2 for three hours, followed by three washes with sterile water. Treated seeds were dried on sterile filter paper (Whatman 1), placed in sterile Petri dishes containing half-strength MS agar, and incubated in the dark for 3 days at 30 °C to allow germination. Twelve germinated seeds with similar-sized roots and shoots were placed on the stands in each aseptic beaker. The plants were maintained in a controlled environment at 23 °C and a 16-h photoperiod with moderate shaking on an orbital shaker (100 rpm). After 4 days of incubation, the growth medium was replaced by fresh growth medium supplemented with 0.05% sucrose and plants were inoculated with a suspension of *T. virens* Gv 29–8 (26) conidia (asexually produced spores) to yield a final concentration of 10⁵ spores/ml. Roots and fungus were allowed to interact for 4 days before pathogen challenge. For pathogen challenge, plants—with their roots—were taken out of the beakers and the second leaf of each plant was attached to a tray from the edges of the leaf. The roots of each treatment were wrapped separately in wet paper towels.

The maize pathogen *Cochliobolus heterostrophus* (strain C4) was grown for 7 days on complete xylene medium (27) in the same controlled environment as the plants. The second leaf was inoculated with 7 μl droplets of 0.02% Tween 20 in deionized water containing 1000 spores. Trays were closed in clear plastic bags to keep the plants moist and maintained in the controlled environment. Pictures of the challenged leaves were taken after 48 h and lesions were measured using ImageJ software (http://image.nih.gov/ij/index.html).

**Concentration of the *T. virens* Secreted Protein (SP) Fractions—** Hydroponics medium from beakers containing maize roots grown with *T. virens* was collected after 4 days of co-incubation. Hydroponic medium from *T. virens* without plants grown in the same conditions served as control. Media were filtered through monofilament nylon mesh followed by a 0.22 μm filter. Twenty microliters of each filtered secretome were concentrated to a final volume of 200 μl using VIVASPIN 20-ml concentrator tubes (cutoff 3000 D; GE Healthcare) in a benchtop centrifuge at 4 °C. Protease inhibitor mixture (Sigma) was added to the concentrated samples to a final dilution of 1:100.

**Evaluation of the ISR Activity of Secreted Protein Fractions (SP)—** To evaluate the ISR activity of both secretomes, 12 day-old maize-roots were supplemented with the secreted protein fraction diluted to the original volume (100 μl of the concentrated secretome in 10 ml medium supplemented with 0.05% sucrose for four plants). After 24 h of interaction, the plants were challenged with *C. heterostrophus*, and the lesion size was measured as described above. Graphs and statistical analyses were performed with the GraphPad Prism software version 5.00 (GraphPad Software, San Diego, CA). Unless otherwise indicated, the threshold level chosen for comparison of means was p < 0.05 by Student’s t test (one-tailed, non-paired, equal variance).

**Preparation of Samples, Mass Spectrometry and Data Analysis—** Preparation of samples: Samples representing equal volumes of the secretome were prepared as described in section (2) above. The samples at final concentrations of 8 mM urea and 100 mM ammonium bicarbonate were reduced with 2.8 mM dithiotreitol for 30 min at 60 °C, modified with 8.8 mM iodoacetamide in 100 mM ammonium bicarbonate in the dark for 30 min at room temperature, and digested in 2 mM urea, 25 mM ammonium bicarbonate with modified trypsin (Promega, Madison, WI) at a 1:50 enzyme-to-substrate ratio overnight at 37 °C. An additional second trypsinization was performed for an additional 4 hours. The tryptic peptides were desalted using C18 tips, (Harvard Apparatus, Cambridge, MA) dried, and resuspended in 0.1% formic acid.

Mass spectrometry: The resulting tryptic peptides from the supernatant were analyzed by liquid chromatography-(LC)-MS/MS using a Q exactive mass spectrometer (Thermo) fitted with a capillary HPLC (easy nLC 1000, Thermo). The peptides were loaded onto a C18 trap column (0.3 × 5 mm, LC-Packings) connected on-line to a homemade capillary column (20 cm, 75 micron ID) packed with Reprosil C18-Aqua (Dr Maisch GmbH, Germany) in solvent A (0.1% formic acid in water). The peptide mixtures from each treatment were resolved with a linear gradient (5 to 28%) of solvent B (95% acetonitrile with 0.1% formic acid) for 180 min followed by a gradient of 5 min (20% B to 25% B at 37 °C). An additional second trypsinization was performed for an additional 4 hours. The tryptic peptides were desalted using C18 tips, (Harvard Apparatus, Cambridge, MA) dried, and resuspended in 0.1% formic acid.

Data Analysis: The mass spectrometry data from three biological repeats were analyzed using the MaxQuant software 1.4.0.8 (www.maxquant.org) for peak picking identification and quantitation using the Andromeda search engine, searching against the *T. virens* proteome set of the UniProt database (with 12,389 entries, Feb 2014) using default parameters. The search was performed versus full tryptic peptides with mass tolerance of 20 ppm for the precursor masses.
and 20 ppm for the fragment ions. Methionine oxidation, and protein N terminus acetylation were accepted as variable modifications, and carbamidomethyl on cysteine was accepted as a static modification. Minimal peptide length was set to six amino acids and a maximum of two miscleavages was allowed. Peptide- and protein-level false discovery rates (FDRs) were filtered to 1% using the target-decoy strategy. Proteins listed in tables were filtered to eliminate the identifications from the reverse database, and common contaminants. Moreover proteins with PEP score >0.002 and single peptide identifications were filtered out. Identification parameters also included minimum PEP score of one and at least two unique peptides for identification. The data was quantified by label free analysis using the same software. Based on extracted ion currents of peptides, quantitation of peptides identified from each LC/MS run in any of experiments was achieved (28). The analysis was performed after a first recalibration of the retention times. The data was also presented as iBAQ, the sum of intensities of all tryptic peptides for each protein divided by the number of theoretically observable peptides. The resulting iBAQ intensities provide an accurate determination of the relative abundance of all proteins identified in a sample. The missing intensity values were replaced with 1020, which is below the background intensity. All intensities were log2-transformed. Both side t test was performed using Perseus 1.4.0.20 with 0.05 FDR and 250 randomizations.

All the proteins that were identified with at least two peptides in the project were listed in supplemental Table S1. Each single row in the table contains the group of proteins that could be reconstructed from a set of peptides including homologous proteins that do not have an additional unique peptide. They are sorted by number of identified peptides in descending order. Pearson column correlation followed by correlation clustering tool in the Perseus software (1.4.0.20) were used in order to evaluate the level of correlation between biological repeats and treatments.

Quantification of T. virens by qPCR—In order to compare the biomass of T. virens between the two treatments (Tv and Tv+M) qPCR was carried out, using genomic DNA extracted from the samples as a template. T. virens mycelium was collected from the Tv treatment medium and the combined T. virens mycelium and root tissue from the Tv+M treatment medium. Excess hydroporics medium was removed by blotting and samples were weighed and ground in liquid nitrogen. Two hundred milligrams of each sample were used for genomic DNA extraction using a standard phenol-chloroform procedure. Primers for genomic T. virens tubulin were used (tubF: 5’-TCTGACCATGCTACACTCGC-3’; tubR: 5’-ATCGTCTGATGGTGGTCAAGC-3’) (13). These primers did not amplify any detectable product from control maize genomic DNA extracted from noninoculated root samples from this study. A calibration curve relating tubulin qPCR signal to the amount of fungal genomic DNA was prepared using serial dilutions from a pool of the T. virens samples. Samples were brought to a concentration of 20 ng/μl, diluted 1:8, and used as template in duplicate qPCR reactions, along with the calibration curve. After determining the DNA concentration from the calibration curve for the tubulin qPCR signal as a function of T. virens genomic DNA in the Tv samples, the total quantity of DNA for each sample was calculated by dividing the tubulin signal by the fraction of the total sample fresh weight that was extracted.

Knockout Strain Generation: Construction, Transformation, and Screening of Transformants—Transformation techniques followed established methods (26, 27). The split-marker method (29, 30) was used for the construction of a linear DNA fragment, which upon integration into the genome of the wild type strain (Gv 29–8), replaces the gene of interest with a hygromycin selectable marker (27). The list of primer pairs for construction of each mutant is provided in supplemental Table S2. Selection was by overlay of the transformation plates with 600 μg/ml of hygromycin B (Calbiochem, EMD Millipore, Billerica, MA) in 1% agar. Colonies that grew from single spores, obtained by serial dilution, were isolated on PDA with 100 μg/ml of hygromycin B and screened by PCR for the absence of the ORF of the gene of interest, and the insertion of the hygromycin selectable marker in the correct position (supplemental Fig. S2).

Colonization Assay—To determine the extent of root colonization by Trichoderma, maize roots were detached 96 h postinoculation (105 spores/ml, in the hydroponic system) and extensively washed in water. After sterilization in 1% NaOCl (a threefold dilution of commercial bleach) for 1 min, the roots of two plants per replicate were washed three times with sterile distilled water, weighed and homogenized using a Polytron homogenizer (Kinematica GmbH) in 15 ml water for 30 s. One ml of the homogenate was plated on PDA supplemented with 100 μg/ml ampicillin and 0.1% Triton X-100 in 145 mm Petri dishes to determine colony forming units. The addition of Triton X-100 is important for limiting the spread of the colony and promoting formation of denser colonies, allowing colony counts even after spore formation. Colonies were counted after 48 h incubation at room temperature.

RESULTS

Assay for Improved Disease Resistance—Coculture of T. virens with maize roots in the hydroponic system (Fig. 1A) reduced the severity of disease following inoculation of the leaves with C. heterostrophus, (Fig. 1B, 1C). For simplicity, this reduction in disease will be referred to as ISR and to the ability of Trichoderma or its secreted protein fractions to promote resistance as “ISR activity,” even though the pathways and regulated genes in the maize plant are not yet fully.
characterized. On *Trichoderma*-treated seedlings, lesion sizes were reduced by almost 40% and the lesions tended to be darker compared with controls grown without *Trichoderma* (Fig. 1B).

**Tv+M SP Have Greater ISR Activity Than Tv SP**—To test the hypothesis that secreted proteins are involved, we compared the ISR activity of the protein fraction obtained from hydroponic culture of *Trichoderma* alone (Tv) or cocultured with the roots of maize seedlings (Tv+M). The protein fractions were isolated from the two cultures and applied separately to maize roots for 24 h, followed by the pathogen challenge. Both fractions had ISR activity compared with those from the mock treatment (plants alone), but the secreted proteins of Tv cultures alone reduced the lesion size by 13%, whereas the secreted proteins of Tv+M reduced the lesion size by 43% (Fig. 2). These results indicate that the two fractions are different in their ISR abilities, probably as a result of different protein composition.

**Proteomic Analysis**—To obtain a better understanding of the different composition of proteins that are responsible for the different ISR activity of the secreted protein fractions (Tv+M SP or Tv SP), the two secretomes were compared. The concentrated fractions of the secreted proteins were separated by one-dimensional SDS-PAGE, with three biological repeats from each treatment (Fig. 3). The overall patterns were similar, but several differences were observed, including bands visible only in Tv, as well as bands visible only in Tv+M. The fractions were tested for ISR activity on a per-volume basis. The average protein concentration obtained from Tv+M was higher than Tv (355 μg/ml and 239 μg/ml, respectively). This trend toward higher protein concentration in the Tv+M samples (albeit not statistically significant) could be the result of enhanced secretion, or the contribution of maize proteins in the Tv+M samples. There were no visible differences in the mass of the fungus in the harvested samples. The amount of fungal genomic DNA was measured by qPCR with primers specific for fungal tubulin (see “Methods”), and was 1426 ± 479 ng (mean and S.E. for four experiments, of which three were those from the proteomic study) for Tv alone, and 1639 ± 305 ng for the corresponding four Tv+M samples. The calculated fresh weight of fungal material in the Tv+M samples was 1865 ± 347 mg, or about 35% of the total fresh weight of these samples. The difference in estimated fungal biomass between Tv and Tv+M samples was not statistically significant (p = 0.72, two-tailed t test). The original concentration ratio was therefore retained and the samples were not normalized by protein concentration. The samples were further analyzed by quantitative label free proteomics analysis. The protein samples were trypsinized, separated by capillary liquid chromatography and analyzed by tandem mass spectrometry (LC/MSMS). Identification and quantification were done by MaxQuant software.

Correlation analysis of the signal intensities showed that the biological repeats of each treatment were clustered together, with an average correlation of 0.77 for Tv and 0.72 for Tv+M, whereas the two treatments were clustered separately with much lower correlation of 0.41, highlighting the differences between the two treatments (supplemental Fig. S1).

By this approach a total of 280 fungal proteins were identified, of which 86% contained a predicted signal peptide in their sequence [see the genome website (http://genome.jgi.doe.gov/programs/fungi/index.jsf) for further details]. Out of the 280 identified proteins, 66 proteins were significantly different at p < 0.05. Examining other proteins that were
highly differential, 78 more proteins were different in their intensities in the secreted protein fractions from Tv or Tv+M, based upon analysis of a t test difference cutoff of three, but were not t test significant. Out of the 66 significantly different proteins, almost half (32 proteins) were found in higher abundance in Tv+M, whereas 34 proteins were found in higher abundance in Tv (Fig. 4).

Of the 280 soluble proteins detected, the largest annotated group of proteins identified was the glycoside hydrolases (GH) with total of 107 proteins from various GH families. Most of them (80 proteins) were not significantly different between Tv and Tv+M. Of the 27 GH that were significantly different, 17 proteins were found in higher abundance in Tv+M and ten GH were found in higher abundance in Tv. Interestingly, most of the GH that were found in higher abundance in Tv (six out of ten) belong to GH families which are specific fungal cell degradative enzymes [GH families (following the classification used by (31)]. On the other hand, most of the GH that were found in higher abundance in Tv+M (10 out of 17) are predicted to be specific for plant cell wall degradation.

Another large group of annotated proteins comprises enzymes with proteolytic activity (total of 40 proteins), from several families, primarily aspartic-type endopeptidases and peptidases belonging to the subtilisin-like superfamily. However, the abundance of most of the proteases did not differ significantly between the two secretomes. Lipases and phospholipases, in contrast, were mainly more abundant with the maize roots, and of a total of six identified lipases/phospholipases, four are found in significantly higher amounts with the plant roots.

The Tv ortholog of the expansin-domain protein swollenin, protein ID 49838, (32, 33), was below detection in Tv samples, but increased strongly in Tv+M (supplemental Table S1). Two carbohydrate esterases were strongly secreted in Tv+M, but undetectable in Tv. One of these, protein ID 31131, is a pectinesterase, catalyzing the de-esterification of pectin to pectate and methanol; the other, protein ID 47222, is an acetyl xylan esterase that catalyzes the hydrolysis of acetyl groups from polymeric xylan and xylo-oligosaccharides. In addition, a secreted protein whose abundance was significantly higher in Tv was identified, containing four LysM domains, protein ID 201746. LysM domains bind chitin; LysM effectors are widespread in fungi and may interfere with detection of chitin by the host (34).

We identified a total of 29 SSCPs including Sm1 (supplemental Table S1, Fig. 5). When examining the abundance of all secreted proteins, Sm1 was the most abundant protein in both secretomes, and its expression level was not different between the two secretomes (Fig. 5). This is consistent with previous findings that Sm1 is highly abundant (15, 20). In interaction with cotton, Sm1 is more abundant in T. virens – root interactions compared with axenic culture (20). Lack of up-regulation in this study could result from our choice of a different plant host, other environmental or cultural conditions or the time course of the interaction, but it is important when making this comparison to note that the previously reported up-regulation was only around two to threefold. There were two main trends in the abundance of the SSCPs between the two treatments; SSCPs that did not change in their abundance (as Sm1) and SSCPs that were found in higher abundance in Tv+M (Fig. 5). Only one SSCP, protein ID 215947, showed an increased abundance in Tv+M. The abundance of SSCP 215947 increased strongly in all three replicate experiments, indicating a promising candidate for further study even though in one Tv replicate its level was below detection (10 units, supplemental Table S1), resulting in a large S.D. and lack of significant difference from Tv+M. The abundance of 13 SSCPs showed significant down-regulation in Tv+M (Figs. 4, 5). Some of these were below the limit of detection in Tv+M samples, whereas others were present at
decreased abundance (supplemental Table S1). Ten of these 13 are conserved among the three sequenced *Trichoderma* species (24). Two are unique to *T. virens*, and one shared only with *T. reesei* at the homology cutoff level used by (24). Three belong to a small gene family denoted cluster 1 and contain CFEM domains, which are unique to fungal cell surface and secreted proteins. Another two (MRSP1, MRSP3) are expansin-like proteins.

There is little if any overlap between the regulated proteins found here and those identified by Marra et al. (14). In addition to using a different plant host and a different *Trichoderma* species, the latter study did not focus on the secretome, but rather was designed to identify proteins whose abundance changes in response to the three-way fungal-plant-pathogen interaction. Thus, it is not surprising that different classes of proteins were found in these studies.

**Mutants – Generation and Functional Analysis**—Taking together the importance of SSCPs in the interactions between fungi and plants, and the down-regulation of a large group of SSCPs in the *Tv+M* treatment prompted us to test the involvement of some of those SSCPs in the molecular dialog between the fungi and the plant by generation of knock-out strains. We chose four candidate SSCPs whose abundance was significantly higher in *Tv* than in *Tv+M*. Two of them were below the limit of detection in the *Tv+M* secreted protein fraction (protein IDs 111486 and 77560, Fig. 5), whereas the other two were found in significantly lower abundance in *Tv+M* (protein IDs 71692 and 92810, Fig. 5). We generated knock-out strains for each gene, purifying three to four independent colonies for each mutant (supplemental Fig. S2). All the mutants were similar to wild type (WT) regarding physiological parameters including germination, growth and sporulation on PDA (data not shown). All of the mutants assayed, however, had greater ISR ability than WT, as maize plants interacting with the mutants were more resistant to *C. heterostrophus* than plants interacting with WT *T. virens* (Fig. 6A). The average lesion size on leaves of maize with roots colonized by SSCPs mutants was reduced by 8% to 35% from that of plants colonized by WT. For each mutant, three independently isolated transformants, confirmed for the double-crossover integration event, were tested with similar results (data not shown). The colonization abilities of these mutants were compared with WT, as better colonization may be the reason for better ISR ability. There were no significant differ-
ences between the extent of colonization by the SSCPs mutants and WT (Fig. 6B). If colonization does not play any major role in the improved ISR abilities of the SSCPs mutants, perhaps these SSCPs have a direct role in the inhibition of ISR. If so, deletion of the gene would lead to an improvement of ISR. Thus, the ISR abilities of the secreted proteins obtained from each of the SSCPs knockouts were compared with WT. Secreted proteins were obtained from each line and applied to maize roots for 24 h, followed by pathogen challenge. All four groups of plants treated with the secreted proteins obtained from the SSCPs knock-outs showed much smaller lesion size compared with the group treated with WT secreted proteins, a decrease of 56–65% (Fig. 6C).

**DISCUSSION**

*Trichoderma* ssp. and other beneficial microorganisms do not cause disease, but result in a stronger immune response upon subsequent challenge by a pathogen (35–37). The working hypothesis of this study was that secreted proteins are likely to make up at least part of the molecular dialog between the host and the symbiont, much as they do in plant-pathogen interactions, or in the better-characterized ecto- and endomycorrhizal symbioses (38). A hydroponic system was used to establish the interaction between the plant host, maize, and the symbiont *T. virens*. The plant resistance induced by the secreted protein fraction collected from *Trichoderma* alone was compared with that collected from the *Trichoderma*-maize root interaction. By this approach, the specific inducer activity was shown to reside in the secreted fraction and that this activity is increased by the presence of the maize roots. Previous work demonstrated that the ceratoplatanin family protein Sm1 is an inducer of systemic resistance in maize (18). Here, the abundance of Sm1 was demonstrated to be similar in *Tv* and *Tv/H11001* secreted protein fractions, yet these samples had different ISR activity, implying that soluble factors in addition to Sm1 participate in ISR.

The maize–*C. heterostrophus* pathosystem was used as an assay for plant disease (Fig. 1). The darker lesions in *Trichoderma*-inoculated plants suggest that the decreased development of lesions is accompanied by increased oxidative activity. Resistance may result from colonization of the roots, soluble factors secreted by the fungus, or a combination of these or other mechanisms. The interaction with maize roots may induce the secretion of elicitors of the plant defense pathways as shown for the small induction of Sm1 transcript and protein upon interaction with cotton roots (20). Another interpretation is that this interaction suppresses the secretion of negative regulators (effectors), which inhibit, early in the interaction, the plant defense pathways to promote colonization.

A large number of proteins were identified in the secretome (supplemental Table S1). Because there are on the order of 1000 proteins with predicted signal peptides for secretion (JGI website), around one fourth of the bioinformatically predicted...
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secretome was identified in this study. Among the most prominent classes of secreted proteins whose abundance is affected by the interaction with roots are GH and SSCPs, whereas several other classes are also noticeable. Regarding the GH composition, it is interesting to note that in the Tv+M treatment there is an enrichment of GH specific to plant cell wall degradation. These enzymes may play a role in the penetration of the Trichoderma into the roots. In contrast, GH specific to fungal cell degradative enzymes are negatively regulated perhaps as an adaptation to obtaining nutrition from the plant host in the rhizosphere, rather than from a fungal host in the soil. Induction of lipases may, likewise, reflect partial degradation of living or dead root cells during the interaction of the fungus with the outer root layers. Up-regulation of an expansin-like protein also fits with the endophytic lifestyle. Endogenous plant expansins regulate cell wall extensibility; there is recent strong support for the hypothesis that microbial pathogens of plants may exploit expansin-like proteins in order to penetrate into plant tissue (39). The expansin-like protein swollenin (SWO) is a colonization factor in the T. asperellum-cucumber interaction, where it was also up-regulated in interaction with roots (33). Up-regulation of this protein here suggests that the mechanism is a general one in Trichoderma. Furthermore, SWO orthologs may act as elicitors of ISR, as shown in the T. asperellum-cucumber interaction (33). Both pectin and xylo-oligosaccharides are found in maize root cell walls (40) and the secretion of enzymes that degrade those oligosaccharides upon interaction with maize roots can promote the close interaction between the fungus and plant. LysM domains bind chitin and can play a role in preventing hyphal lysis by plant chitinases. Another known role of some LysM proteins is interference with chitin detection by the plant through sequestration of cell wall-derived chitin fragments that would otherwise be perceived by the plant immune receptors (34). Down-regulation of the LysM protein is curious, as one might expect this protein to allow the fungus to elude the plant’s PAMP/MAMP (pathogen/microbe associated molecular pattern)—triggered defenses upon recognition of chitin, as studied in plant pathogens (41, 42). Perhaps the presentation of chitin to the plant is indeed one of the triggers of ISR. The mutual advantage of ISR would explain suppression of LysM-domain protein(s) to allow presentation of chitin, yet attenuate more aggressive, pathogenic attack of the plant tissues by permitting a plant immune response to chitin.

For SSCPs, perhaps the most surprising finding was that most of the regulated members of this (loosely-defined) protein class were decreased in interaction with maize (Fig. 5). This down-regulation observed in the protein abundance detected in the culture medium could result from different scenarios. Maize roots may affect the secretion or stability of the proteins. Proteases secreted by the fungus, roots, or both may increase the turnover rate of the SSCPs after their secretion. Another possibility is that the roots sequester these SSCPs, so that despite their abundance and secretion, they are not detected in the culture medium.

The primary functional approach in fungal genetics is to construct knock-mutants. These loss-of-function alleles are created by homologous recombination (double crossover) between an introduced construct and the fungal genome. Knock-out mutants were constructed for four of the SSCP-encoding genes identified in the secretome of T. virens. Maize plants treated with two of the mutants, Δ111486 and Δ77560, had average lesion sizes of 71 and 65%, respectively, compared with WT (set at 100%) treated plants. Coculture with the other two mutants, Δ92810 and Δ71692, also conferred reduced lesion sizes compared with the WT, but not as reduced as Δ111486 or Δ77560. Of importance to note is that 111486 and 77560 totally “disappeared” from the secretome of Tv+M, whereas 71692 and 92810 were merely down-regulated (Fig. 5). A more dramatic decrease in abundance may be correlated with a stronger phenotype in the corresponding knock-out mutant. The activity of these mutants is the first report of a knock-out mutant in Trichoderma that has better ISR abilities than WT, as all the proteins from Trichoderma were found to be involved in ISR promote ISR (reviewed by (43)).

The improved ISR abilities by the SSCPs knock-outs is not related to the colonization abilities of those mutants, as the level of colonization in maize roots by T. virens was not different among mutants and WT (Fig. 6B). This result suggests that the lack of the SSCP in the knock-out mutant directly affect the ISR abilities of the symbiont and not indirectly by colonization. These four SSCPs might act as negative effectors of ISR, reducing the defense level in the plant. Indeed, the significantly higher ISR abilities of the secreted protein fraction obtained from the SSCPs mutants compare with WT (Fig. 6C) suggests that those proteins actually operate as negative effectors, and when not present, increase the ISR abilities of this soluble fraction. This is in agreement with the higher ISR capacity of the soluble protein fraction obtained from Tv+M cocultures, which also have lower levels of these SSCPs (Fig. 2). Also of interest is that whereas examining the ISR abilities of the mutants, diverse levels of improved abilities were obtained (Fig. 6A), for example Δ92810 provides a modest but significant decrease in lesion size. When testing the ISR capacity of the cell-free secreted protein fractions, all were similar (Fig. 6C). The reason for the difference between application of the fungal strains and of their secreted protein fractions could be some redundancy and compensation mechanisms during the interaction with the roots. The protein fraction, on the other hand, has a static composition, with the exception perhaps of breakdown of proteins or other soluble active molecules.

In this model, other proteins (for example, Sm1) would then be the primary signals resulting in resistance. ISR requires the participation of multiple plant signaling pathways (44), and ISR may not be the only mechanism responsible for Trichoderma-induced resistance in maize. Another question
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raised is how loss of different SSCP genes results in the same phenotype, suggesting that Trichoderma has evolved to echo the phases of the plant’s response. Down-regulation of a negative effector at the appropriate phase in the interaction could thus promote ISR, to higher levels than WT. These new hypotheses can be addressed in further genetic studies on both Trichoderma and the plant host.

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[This article contains supplemental Figs. S1 and S2 and Tables S1 and S2.]

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