Role of Swollenin, an Expansin-Like Protein from *Trichoderma*, in Plant Root Colonization¹[W]

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Swollenin, a protein first characterized in the saprophytic fungus *Trichoderma reesei*, contains an N-terminal carbohydrate-binding module family 1 domain (CBD) with cellulose-binding function and a C-terminal expansin-like domain. This protein was identified by liquid chromatography-mass spectrometry among many other cellulolytic proteins secreted in the coculture hydroponics medium of cucumber (*Cucumis sativus*) seedlings and *Trichoderma asperellum*, a well-known biocontrol agent and inducer of plant defense responses. The swollenin gene was isolated and its coding region was overexpressed in the same strain under the control of the constitutive *pki1* promoter. *Trichoderma* transformants showed a remarkably increased ability to colonize cucumber roots within 6 h after inoculation. On the other hand, overexpressors of a truncated swollenin sequence bearing a 36-amino acid deletion of the CBD did not differ from the wild type, showing in vivo that this domain is necessary for full protein activity. Root colonization rates were reduced in transformants silenced in swollenin gene expression. A synthetic 36-mer swollenin CBD peptide was shown to be capable of stimulating local defense responses in cucumber roots and leaves and to afford local protection toward *Botrytis cinerea* and *Pseudomonas syringae pv lachrymans* infection. This indicates that the CBD domain might be recognized by the plant as a microbe-associated molecular pattern in the *Trichoderma*-plant interaction.

*Trichoderma* spp. are well-known plant biocontrol agents mainly due to their mycoparasitic activity against soil-borne plant pathogens (Benitez et al., 2004). Some *Trichoderma* rhizosphere-competent strains can also colonize entire root surfaces with morphological features reminiscent of those seen during mycoparasitism (Yedidia et al., 1999) and have been shown to have dramatic direct effects on plants. The effects noted include increased growth and yields, increased nutrient uptake, as well as increased fertilizer efficiency utilization, percentage and rate of seed germination, and induced systemic resistance (ISR) to diseases (Harman et al., 2004). *Trichoderma* strains capable of establishing such interactions induce metabolic changes in plants that increase resistance to a wide range of plant-pathogenic microorganisms and viruses (Harman et al., 2004). This response seems to be broadly effective for many plants, which indicates that there is little or no plant specificity. The systemic response in plants occurs through the jasmonic acid/ethylene signaling pathway in a way similar to the rhizobacteria-ISR (Shoresh et al., 2005; van Loon, 2007). At least three classes of substances that elicit plant defense responses have been identified. These elicitors (XE elicitors) include proteins, peptides, and low-M, compounds (Harman et al., 2004; Djonovic et al., 2007; Viterbo et al., 2007).

The *Trichoderma asperellum*-cucumber (*Cucumis sativus*) root interaction has been the subject of many detailed studies. Penetration of the root tissue is usually limited to the first or second layers of cells and is restricted to the intercellular spaces. Attachment of the fungus to the root by appressoria-like structures is mediated by a class I hydrophobin, *TasHyd1* (Yedidia et al., 1999; Viterbo and Chet, 2006) and root penetration is achieved by secretion of cellulolytic and proteolytic enzymes (Viterbo et al., 2004). In this study, we performed a more accurate analysis of the proteins secreted to the plant-*Trichoderma* coculture medium by liquid chromatography-mass spectrometry (LC-MS), which allowed the identification of several additional proteins. Among them is swollenin, a protein first isolated and characterized from the saprophytic cellulolytic fungus *Trichoderma reesei*. The protein was named swollenin due to its ability to swell cotton (*Gossypium hirsutum*) fibers without producing detectable amounts of reducing sugars (Saloheimo et al., 2002). The protein has an N-terminal fungal-type carbohydrate-binding module family 1 domain (CBD) with cellulose-binding function, connected by a linker region to an expansin-like domain with homology to the group β-expansins (EXPBs). EXPB and EXPB appear to act on different cell wall components, but their native targets have not been well defined yet (Cosgrove, 2000). A subset of EXPBs is known in the immunological

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RESULTS

Identification of Proteins Secreted to the Plant-Trichoderma Coculture Medium

Concentrated growth medium (see “Materials and Methods”) recovered from cultures of cucumber seedlings grown in the presence or absence of *Trichoderma* in the root compartment were subjected to LC-MS. Table I shows the partial sequences obtained from several proteins differentially secreted to the *Trichoderma*-plant coculture medium. This protein pattern was reproducible in two separate experiments. The majority of the proteins could be classified as plant cell wall-degrading enzymes: cellulases (cellulbiohydrolase, endoglucanase), hemicellulases (glucan 1,3-β-glucosidase and arabinofuranosidases), and an aspartyl protease. A glucoamylase, a starch-degrading enzyme, was also detected and swollenin, a protein first isolated from *T. reesei* (Saloheimo et al., 2002), related to the fungus cellulolytic activity during its saprophytic growth. In addition, the medium contained three plant proteins (chitinase 1, chitinase 3, peroxidase) that could be classified as plant defense proteins.

Isolation of the Swollenin Gene *TasSwo* from *T. asperellum*

Degenerate primers (see “Materials and Methods”) were designed according to the partial sequences obtained from identified swollenin peptides (Table I) and according to conserved regions between swollenin from *T. reesei* and the homologous sequence from *A. fumigatus* (GI70984483). A 360-bp genomic amplification product was obtained. A larger 1,762-bp genomic sequence (*TasSwo*) was cloned by nested PCR amplification with swollenin-specific primers, according to the GenomeWalker procedure (Viterbo et al., 2002). A *TasSwo* cDNA (1,488 bp) was obtained by PCR amplification of the full coding sequence using cDNA generated from *T. asperellum* mycelium grown with 2% Glc for 48 h and subsequent induction by 2% cellulose for another 48 h (Saloheimo et al., 2002).

The *TasSWO* protein contains a 19-amino acid secretion signal peptide with a predicted cleavage site (SignalP 3.0) between amino acids 19 and 20, yielding a mature protein of 477 amino acids. Smart-architecture domain analysis predicts a CBD between amino acids 24 and 57 (1.27e-15), and an expansin domain (pollen allergen 1 domain) at amino acids 381 to 471 (5.40e-04). BLAST search with two-thirds (amino acids 170–477) of the *TasSWO* C-terminal sequence showed low homology to an EXPB-like protein precursor from tomato (*Solanum lycopersicum*; ABB71677.1; 6e-09) and EXB10 from maize (*Zea mays*; ABF81662.1; 1e-08).

The *TasSWO* open reading frame (ORF) shows 89% similarity both to SWO from *T. reesei* and to swollenin from *Trichoderma virens* (gw_1.22.344.1; Trivel:49838), 88% to swollenin from *Hypocrea pseudokoningii* (ABV57767.1), 75% to the fungal cellulose-binding domain protein from *N. fischeri* (EAW15624.1), and 73% to the putative swollenin from *A. fumigatus* (EAL85710.1; Fig. 1). The genomic *TasSwo* sequence presents five short introns in the ORF whose positions are conserved in the swollenin genes of *T. reesei* and *T. virens*. The last intron seems not to be spliced in the *A. fumigatus* sequence (Fig. 1).
Table 1. Peptide sequences of differentially secreted proteins in plant-Trichoderma growth medium

<table>
<thead>
<tr>
<th>Peptide Sequence</th>
<th>Best Database Match</th>
<th>Accession No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPSFESLSNQGIFR, DVGLENDTDLVALSGAHTFGR</td>
<td>Peroxidase (C. sativus)</td>
<td>GI167533</td>
</tr>
<tr>
<td>LGGRQSDRPLGAAVL</td>
<td>Chitinase 1 (Cucumis melo)</td>
<td>GI7595839</td>
</tr>
<tr>
<td>LGGRSSRPGLDAVGLDVG</td>
<td>Chitinase 3-like protein precursor (Trichosanthes kirilowii)</td>
<td>GI5553476</td>
</tr>
<tr>
<td>IGTGFFGIPSAANTGDSL, LTNNAPSLFL</td>
<td>Cellulohydrolase II (Trichoderma viride)</td>
<td>GI34582636</td>
</tr>
<tr>
<td>YGGTCDPDCDWNPRY, YGTGCDSQCPY, YPTNLAGAK, YVQONGVK, VVLANDWR, AALVVEDLANKR, TWFSNASKK</td>
<td>Cellulohydrolase I (Trichoderma harzianum, T. asperellum)</td>
<td>GI1073767, GI42741023</td>
</tr>
<tr>
<td>VSSEFSDTNSSLGLGLGSLINTVSPK, TWFSNASIK, AIALIGYSK, FEILTSQFTGNGWRQOR, DSAVLFK</td>
<td>Aspartyl protease (T. asperellum)</td>
<td>GI47027997</td>
</tr>
<tr>
<td>ANSGATWDNDSHGDLIR, SFTATLGGSWTPQATSESQFAGK</td>
<td>Glucoamylase</td>
<td>GI61657886</td>
</tr>
<tr>
<td>IVGEIWPIMVGGSK, GILLESTK</td>
<td>Arabinoxylanase 2 (T. asperellum)</td>
<td>GI45269104</td>
</tr>
<tr>
<td>LAVDPANIVYFGAR</td>
<td>Glucan 1,3-β-glucosidase (Trichoderma atroviride)</td>
<td>GI8886891</td>
</tr>
<tr>
<td>LKVQGILLATLP</td>
<td>Endoglucanase, Cell74a (Hypocrea jecorina)</td>
<td>GI31747160</td>
</tr>
<tr>
<td>GYPNTALNIPNKR</td>
<td>Hypothetical amino acid transporter (Neurospora crassa)</td>
<td>GI85113614</td>
</tr>
<tr>
<td>WCCGPGADHCGEIDFKLTSPTQEVNQAIK</td>
<td>Endoglucanase 1 (T. viride)</td>
<td>GI42521642</td>
</tr>
<tr>
<td>FALTAVNTPGSGTVKIE</td>
<td>Swollenin (H. jecorina)</td>
<td>GI8052455</td>
</tr>
</tbody>
</table>

TasSwo Is Induced by Cellulose and during Trichoderma Plant-Root Interaction

Real-time reverse transcription (RT)-PCR was used to study the expression of TasSwo. The transcript is subjected to catabolic repression by Glc and is remarkably induced (110-fold induction) after 48-h growth in minimal medium supplemented with 2% cellulose (Fig. 2A), similar to the report by Saloheimo et al. (2002) for swollenin from T. reesei. TasSwo expression is also induced (3-fold induction) in Trichoderma mycelium recovered from roots 12 h after inoculation (Fig. 2B), supporting the LC-MS data on swollenin secretion into the Trichoderma-plant coculture medium.

CBD Is Necessary for Improved Root Colonization Ability Conferred by Swollenin Overexpression in Trichoderma

Trichoderma transformants overexpressing swollenin, or swollenin deleted in the CBD domain, driven by the constitutive pki1 promoter, were obtained following particle bombardment and hygromycin selection. Transformants were further identified by PCR screening of genomic DNA, the forward primer located on the pki1 constitutive promoter and the reverse primer within the swollenin coding region (Fig. 3A). Constitutive expression of the transgenes in mycelia grown on repressive medium (1.5% Glc) was verified by RT-PCR with specific primers (Fig. 3B). Four independent mutants, T1 and T2 overexpressing the complete swollenin ORF, and ΔT3 and ΔT4 overexpressing a swollenin clone deleted in the CBD domain, were selected for further analysis. In the in vitro growth rates of all the transformants were comparable to that of the wild type, although mutants T2 and ΔT4 showed somewhat reduced sporulation. Lines T1 and T2 overexpressing the full-length swollenin showed remarkably increased ability to colonize cucumber roots already 6 h postinoculation (Fig. 4). On the other hand, lines ΔT3 and ΔT4, expressing swollenin deleted in the CBD, did not differ from the wild type. The same trend was apparent also after 12 h (Fig. 4).

We also tested whether the swollenin overexpressors can trigger enhanced ISR when compared to Trichoderma wild type. We could not detect any significant difference when cucumber seedlings were infected with Pseudomonas syringae pv lachrimans (Psl), 12 or 24 h post Trichoderma inoculation (Supplemental Fig. S1).

TasSwo RNA Interference Silencing Reduces Root Colonization by Trichoderma

Saloheimo et al. (2002) reported that, according to Southern-blot analysis, more than one copy of the swel1 gene might be present in the Trichoderma genome. Southern hybridization of T. asperellum genomic DNA with a TasSwo gene fragment encoding the expansin-like domain as a probe showed one single band under both high- and low-stringency conditions (Supplemental Fig. S2). These data, together with the information obtained from the genome sequence of T. reesei (http://genome.jgi-psf.org/Trire2/Trire2.home.html) and T. viride (Department of Energy Joint Genome Institute assembly; draft version), suggest that the swollenin gene is a single-copy gene in these strains.

Several TasSwo RNA interference (RNAi) transformants were obtained and subcultured to mitotic stability by repeated transfer on selective medium. Inhibition of TasSwo expression was followed by real-time RT-PCR on mRNA extracted from cultures grown in 2% cellulose induction medium for 2 d. Two transform-
ants (PS1 and PS2), which exhibited 95% reduction in mRNA expression (Fig. 5A), were selected and evaluated for root colonization ability. These two lines presented growth rates similar to the wild type. *Trichoderma* mycelia were recovered from root tissues 12 h postinoculation. Lines silenced in *TasSwo* expression exhibited reduced root colonization ability (40%) compared to the wild type (Fig. 5B).

**Local Induction of Defense-Related Gene Expression by a Synthetic 36-mer CBD in Cucumber Leaves and Roots**

We tested whether selected cucumber defense genes were induced by the CBD of swollenin. Local induction of $\beta$-glucanase ($\beta$-gluc), chitinase 1 (*chit*), hydroxyperoxide lyase (*hpl*), Phe ammonia lyase (*pal1*), and peroxidase (*prx*) genes by synthetic CBD was followed.
in cucumber leaves that were directly injected with a 5 μM CBD peptide solution or mock injected with 0.002% Glc. No phytotoxic symptoms were detected in infiltrated cucumber leaves at this concentration. Real-time RT-PCR analysis revealed a 100-fold up-regulation for the β-glucanase and chitinase 1 genes (Fig. 6A), compared to 3- and 9-fold induction for prx and pal1, respectively. No induction was detected for hpl (Fig. 6B). A 4- and 9-fold local induction of the β-glucanase and chitinase 1 genes, respectively, was observed in seedling roots that were incubated in a 10 μM CBD solution (Fig. 6C). No detectable induction was observed in the roots for the other genes.

CBD Provides Local Resistance against Botrytis cinerea and Psl

The involvement of the CBD peptide in ISR was evaluated by incubating cucumber seedlings in a 25 μM CBD solution for 48 h and subsequently infecting the cotyledons with the bacterial pathogen Psl. No significant difference could be detected compared to injection of a mock solution in the induction of the selected defense genes by real-time PCR (data not shown).

Finally, we wished to determine whether local induction of defense genes by the CBD could confer disease resistance to the elicited plant. Detached cucumber leaves or leaves on whole plants were infected with the gray mold B. cinerea 24 h after CBD injection or mock injection of 0.002% Glc. Fungal infection development was monitored for 3 to 4 d after infection. Expanded yellow necrotic lesions were observed in the control untreated leaves that were not pretreated with CBD. In contrast, lesion expansion was totally inhibited or significantly reduced in infected leaves that were treated with CBD (Fig. 7A; Supplemental Fig. S3). Disease severity was assessed by scoring the symptoms on a 0 (no symptoms) to 3 (severe lesions) scale (Fig. 7B).

Cucumber cotyledons were treated by infiltrating the CBD peptide 24 h before inoculation with Psl. Multiplication of the bacteria in infected cucumber cotyledons (Fig. 7C) was 50% lower in the plants pretreated with CBD compared to control plants infiltrated with mock solution (0.002% Glc).

DISCUSSION

In a previous study (Viterbo et al., 2004), we were able to show that penetration of the epidermis by T. asperellum T-203 and subsequent ingress into the outer cortex of cucumber seedlings requires secretion of cell wall lytic enzymes. Two arabinofuranosidases (Abf1 and Abf2) and an aspartyl protease (PapA) were identified by sequencing of proteins, differentially secreted in Trichoderma–plant coculture medium, after separation by one-dimensional gel electrophoresis. In this article, we essentially repeated the same experiment, but we analyzed the concentrated extracellular growth medium by LC-MS, a more powerful method that allows a more comprehensive analysis. This afforded the identification of additional cellulytic enzymes and a glucoamylase (Table I). The occurrence of a starch-degrading enzyme during a plant-fungal interaction may be significant to provide energy to the mold via plant starch degradation during the infection and for pathogenicity potency (Brown et al., 2001; Martel et al., 2002). Swollenin was also identified among the differentially secreted proteins. The presence of this protein in T. reesei has been related to the fungal cellulytic activity during its saprophytic growth (Salohimeo et al., 2002). In T. reesei, an industrially important cellulytic filamentous fungus, but not a common biocontrol-competent species nor as far as is known a plant opportunistic symbiont, it was shown that swollenin can disrupt the structure of cotton fibers without detectable formation of reducing sugars. Here, we show by overexpression and silencing of the TasSwo gene that this protein is involved in plant root colonization by a rhizocompetent Trichoderma strain.

Similarly, the plant-parasitic roundworm, G. rostochiensis, can produce a functional expansin (Gr-EXPB1) used to loosen cell walls when invading its host plant (Qin et al., 2004). Gr-EXPB1 is a member of a multigene family, present in other nematode species as well (Roze et al., 2008). It consists of a bacterial type II carbohydrate-binding module and an expansin domain, with significant similarity to EXPB-like proteins from tobacco (Nicotiana tabacum) and Arabidopsis (Arabidopsis thaliana). It is even more similar to two hypothetical proteins from the aerial mycelium-forming soil bacteria Amycolatus mediterranei and Streptomyces lavendulae, suggesting a prokaryotic origin and its probable acquisition by horizontal gene transfer (Kudla et al., 2005).

Figure 2. Modulation of TasSwo expression in T. asperellum mycelium by different growth conditions. Gene induction was quantified by real-time RT-PCR, normalized versus the endogenous β-tubulin gene (see “Materials and Methods”). A, Total RNA was extracted from Trichoderma mycelium grown for 48 h in liquid SM medium with different carbon sources (1.5% Glc or 2% cellulose). B, Total RNA was extracted from Trichoderma mycelium wrapped around cucumber roots 12 h after inoculation. RNA extracted from mycelium of Trichoderma grown in hydroponic medium supplemented with 0.05% Glc was used as a control.
Unlike plant expansins, and similar to the roundworm protein, swollenin has a bimodular structure, composed of an N-terminal CBD connected by a linker region to the plant expansin homologous domain, with significant similarity to an EXPB-like protein precursor from tomato and maize.

A modular structure is typical of fungal cellulases and some hemicellulases, which contain one or several CBDs to target the catalytic module close to the substrate. The CBDs of fungal cellulases interact with crystalline cellulose through their hydrophobic flat surface, formed by three conserved aromatic amino acid residues (Takashima et al., 2007). The observation that improved root colonization of the overexpressors is abolished by deletion of the swollenin CBD underlines the importance of the latter for full protein activity. Previous studies have also shown that removal of CBD from the cellulase or from the scaffolding in cellulosomes dramatically decreases the enzymatic activity (van Tilbeurgh et al., 1986; Goldstein et al., 1993). Moreover, fusion of carbohydrate-binding modules to enzymatic partners has been reported to improve the efficiency of the enzyme partner, helping to target the enzyme to the substrate and increasing the local concentration of the enzyme (Boraston et al., 2004). Levasseur et al. (2006) demonstrated that fusion of swollenin to feruloyl esterase A (FAEA) is more efficient than applying the free modules, SWOI and FAEA, for ferulic acid release. They speculate that the CBD of SWOI may increase the local concentration of the enzyme close to the substrate and increase the final hydrolysis yields. In addition, the efficiency of the chimeric protein may be improved by the particular mobility of the SWOI expansin module (Cosgrove, 2000), which may facilitate the lateral diffusion of the FAEA along the surface of the cellulose microfibrils.

Along the same lines, we can speculate that cell wall disruption and subsequent root colonization by Trichoderma are more efficient due to swollenin, which could facilitate the access of other cellulolytic enzymes to less accessible areas of the substrate.

Trichoderma has a wide host range, including dicots and monocots. In this article, we tested root colonization of cucumber seedling, a dicot plant. The function of EXPBs in dicotyledons is not clear. The predominance of EXPB genes in grass genomes could be explained if we assume that EXPB and EXPB proceed on different polysaccharide matrices in the cell wall (Li et al., 2002); matrix composition in grasses differs significantly from that of dicotyledonous species (Carpita, 1996). Trichoderma, like the potato cyst nematodes, produces an expansin with an apparently limited impact on dicot cell walls but, like the nematode, it seems to efficiently loosen the dicot root tissue.

Because Trichoderma is able to induce defense responses in plants (Harman et al., 2004), we also wanted to check whether swollenin overexpressors can trigger enhanced resistance in addition to increasing root colonization. We could not detect any significant difference when cucumber seedlings were infected with PsI, 12 or 24 h post Trichoderma inoculation (Supplemental Fig. S1). At these time points, although root colonization by the swollenin overexpressor is higher, apparently the amount of Trichoderma wild type is sufficient to trigger the ISR responses.

Moreover, we studied the involvement of the fungal CBD domain in elicitation of plant defense mecha-
nisms. As part of their innate immune system, plants recognize invaders by virtue of pathogen-associated molecular patterns/MAMPs that trigger intracellular metabolic changes in plant cells to cope with pathogen attacks (Nürnberger et al., 2004). Such patterns correspond to motifs or domains with conserved structural traits found in widely occurring compounds of microbes, but not present in their hosts. The CBEL from *P. parasitica nicotianae* contains two CBDs belonging to the carbohydrate-binding module family 1. Infiltration of tobacco and Arabidopsis leaves using synthetic peptides showed that the CBDs of CBEL are essential and sufficient to stimulate defense responses (Gaulin et al., 2006).

We could not detect involvement of the CBD peptide in ISR by incubating cucumber seedlings at concentrations up to 25 μM CBD solution for 48 h and subsequent infection of the cotyledons with the bacterial pathogen *Psl*. However, we could detect an increased expression of chitinase and β-glucanase genes in the root tissue after 24-h incubation with 10 μM CBD, indicating that this domain indeed elicits local defense responses in roots, although the induced expression is lower than in infiltrated leaves.

It is well established that, during the first 72 h of *Trichoderma* root colonization, there is a rise in plant root local defense responses (Yedidia et al., 1999; Shoresh et al., 2005), which limits *Trichoderma* root colonization to the intercellular spaces. In the communication zone between plant and *Trichoderma*, there are many molecules that can act as MAMPs/elicitors that can affect different signaling pathways, in turn affecting different modes of reaction and classes of pathogens. From our present data, it seems that the fungal CBD domain is not involved in the ISR response, but more in local sensing of the invading fungus by the root.

Our present results, demonstrating local elicitation of defense genes in cucumber and protection against a fungal and a bacterial pathogen, confirm and extend the data of Gaulin et al. (2006), showing that CBDs are a novel class of molecular patterns that might function by interaction with the cell wall. The carbohydrate-binding module family 1 is found almost exclusively...
in fungi. It is noteworthy that chitinase and glucanase genes, major enzymes used by plants for defense against fungal pathogens, showed remarkably higher induction both in leaves and roots, compared to prx and pal genes that are related to secondary metabolite synthesis and cell wall strengthening.

The fact that swollenin homologs are found (by current homology-search algorithms) only in Trichoderma species, A. fumigatus, or its close relative N. fischeri, and not in other fungal phytopathogens, is quite intriguing. A. fumigatus, like Trichoderma, is not a plant pathogen, although a survey of the A. fumigatus genome (Tekaia and Latgé, 2005) shows that it encodes a wide range of glycosylhydrolases that have the capacity to degrade the major plant cell wall polymers. Like in Trichoderma, no global differences have been demonstrated between the enzymes produced by true phytopathogens and saprophytic Aspergillus strains.

These findings raise questions on the evolution of such protein architecture in saprophytic fungi like Trichoderma and Aspergillus. Although sequence data and knowledge of molecular evolution suggest gene transfer among eukaryotes, the mechanism by which this transfer occurs remains elusive (Friesen et al., 2006). A cross-kingdom horizontal gene transfer of a fungal mitochondrial intron was suggested to have occurred in a sponge, facilitated by the symbiotic relationship between the fungus and sponge (Rot et al., 2006). Bacterial expansin-like sequences, as well as animal and fungal expansin-like sequences, appear to be restricted to a very small group of organisms, mainly involved in plant pathogenesis or plant cell wall digestion, suggesting horizontal transfer rather than preservation of an ancestral form (Li et al., 2002).

In conclusion, we show that the swollenin protein remarkably increases plant root colonization efficiency in Trichoderma. The CBD is indispensable for full protein activity; it stimulates local defense responses in the plant and affords plant protection, indicating that this domain might be recognized by the plant as a MAMP in the Trichoderma-plant interaction.

**MATERIALS AND METHODS**

**Plant Material**

Cucumber (Cucumis sativus ‘Kfir’) seedlings (Gedera Seeds Co.) were grown as described by Yedidia et al. (1999). Briefly surface-sterilized seeds (25 per box) were placed on a sterile gauze sheet in an axenic hydroponics growth system. Plants were grown in a controlled environment: 26°C, 80% relative humidity, and a circadian cycle of 14 h light/10 h darkness.

**Plant Inoculation with Trichoderma and Isolation of Proteins Secreted to the Growth Medium**

*Trichoderma asperellum* T-203 was grown on potato dextrose agar (PDA; Difco) for 10 d. Spores (10⁷) were harvested and grown overnight in 100 mL of synthetic medium (SM; Yedidia et al., 1999) to allow germination. The inoculum was added to plant growth medium (300 mL) of 7-d-old seedlings, resulting in a concentration of 10³ germinated spores mL⁻¹. At inoculation time, the plant growth medium was changed to avoid high plant extracellular protein background. Coculture medium was collected after 48 h. Hydroponics medium from plants grown without *Trichoderma* was collected as control. Hydroponics medium in which the mycelium grew with 0.05% Glc without plants with continuous shaking served as an additional control. Complete protease inhibitor cocktail (Roche) was added to the filtrated medium (300 mL) prior to concentration to a final volume of 100 μL using VIVASPIN 20-mL concentrator tubes (cutoff 10,000 D; Vivascience).

**Proteolysis and MS Analysis**

Proteins were analyzed at the Smoler Proteomics Center. The proteins were reduced with 10 mM dithiothreitol (60°C for 30 min), modified with 100 mM iodoacetamide in 10 mM ammonium bicarbonate at room temperature for 30 min, and trypsinized in 10 mM ammonium bicarbonate containing trypsin (modified trypsin [Promega] at 1:100 enzyme-to-substrate ratio), overnight at 37°C.

The tryptic peptides were resolved by reverse-phase chromatography on 0.1-× 200-mm fused silica capillaries (J&W; 100-μm i.d.) packed with Everest reversed-phase material (Grace Vydac). The peptides were eluted with linear 80-min gradients of 5% to 95% of acetonitrile with 0.1% formic acid in water at flow rates of 0.4 μL/min. MS was performed by an LC-MS ion-trap mass spectrometer (LCQ-DecaXP; Finnigan) in a positive mode using repetitively full MS scan followed by collision-induced dissociation of the three most dominant ions selected from the first MS scan. The MS data were clustered and analyzed using Sequest software (J. Eng and J. Yates, unpublished data; Finnigan) or Pep-Miner (Beer et al., 2004) searching against the National Center for Biotechnology Information nonredundant database.

**Cloning of Swollenin from *T. asperellum***

Degenerate primers were designed according to the amino acid sequences aa-VDVNCVC, 5′-GGNCRCTNGTRTNACNGC-3′; and aa-KPDGTDY, 5′-AARCNGGNGNCNGAY-3′. The full-length TasSw gene was obtained using the Universal GenomeWalker kit (CLONTECH) as described by...
Vector Construction, Transformation Procedure, and Mutant Selection

The pAN7 vector containing the Esherichia coli hygromycin phospho-transferase (hph) gene was purchased from Stratagene. The vector pRLMex was kindly provided by Professor R.L. Mach (Mach et al., 1994). The coding region was amplified from cDNA by PCR with primers Swoxil (5′-CGCTGCGCGCTGCCAG-3′), 5′-GGAGAGTACAATGCCCCAAG-3′, and 5′-CAAGTTTCTGATGCTAAGTTCTG-3′. GenBank accession number for the full isolated genomic sequence of TasSw is EU307698.

RNA Extraction and Gene Expression Analysis by Real-Time RT-PCR

Total RNA from fungal mycelium or plant leaves and roots was extracted using the GenElute Plant/RNA kit (Sigma-Aldrich). The total RNA was treated with DNase I (Promega) and treated with RNAase-free DNase (Fermentas). Total RNA was quantified by spectrophotometry and its integrity was assessed by gel electrophoresis. A 185-bp fragment was amplified with the primers QTF (5′-GGATTTGC-3′) and SwoGen (5′-GGCCCTACTGG-3′) using SuperScript II reverse transcriptase (Invitrogen) and oligo(dT) as a primer. As a control, 1 μl of cDNA was used for first-strand synthesis using SuperScript II reverse transcriptase (Invitrogen) according to the manufacturer’s procedure, using oligo(dT) as a primer. As a negative control, the reactions were performed in the absence of the enzyme.

Real-Time RT-PCR experiments with cucumber genes were as in Shoresh et al. (2005) for pki1 and SwoGen (5′-CGCTGCGCGCTGCCAG-3′) and ΔCBD-R (5′-CCACGGTGTTGCCCTCTC-3′) flanking the CBD region were used in two separate PCR reactions with primers SwoGen and SwoGen, and 1 μl aliquot from each reaction was then combined for a second PCR reaction with 2 μl of each of the resulting product into the pRLMex vector.

CBD Infiltration to Cucumber Leaves and Infection with Botrytis cinerea

The 36-amino acid CBD synthetic peptide (ALYQGCCGGGWGATCCVS-GAQNALNDYQQCVL; BioSight Ltd.) was diluted in a 0.2% Glc solution (Wassenberg et al., 1997) to give a 500 μM stock solution. Cucumber seedlings (Kfir variety) were grown in soil in 250-mL pots in a controlled environment. One hundred microliters of a 5 μM water-diluted solution, or mock solution (0.002% Glc), was infiltrated in cucumber leaves of 10-d-old plants or detached leaves, using a syringe without needle 24 h postinfiltration. Leaves were inoculated with 5-mm-diameter mycelial agar discs of B. cinerea strain BC03 (vine pathovar from Prof. Paul, Dijon, France) taken from 10- to 7-d-old cultures maintained on PDA. The disc (approximately 2 × 106 spores) was placed in the middle of each leaflet (Elad, 1990). After 24 h, the discs were removed and disease development was assessed 3 to 4 d after inoculation. Whole plants were wrapped in plastic bags with wet paper and detached leaves were placed in closed plastic containers on wet filter paper to maintain high humidity. The containers were placed at room temperature during the entire experiment.

CBD Treatment of Cucumber Seedlings and Cotyledons and Infection with Pseudomonas syringae pv lachrymans

Cucumber seedlings were grown in axenic hydroponic growth systems as described in the previous section. Fully expanded cotyledons were infiltrated with a 5 μM CBD solution or mock solution (0.002% Glc). After 24 h, the seedlings were inoculated with 10 μL of bacterial suspension and colony proliferation was assayed after 48 h as described in Yedidia et al. (2003). Six-day-old seedlings with fully expanded cotyledons were transferred to small vials containing 400 μL of a solution of 10 or 25 μM of CBD solution or mock (0.002% Glc). The vials were then placed in a sterile polycarbonate culture box (Djonovic et al., 2006). After 48 h, the seedlings were inoculated with 10 μL of bacterial suspension.

Southern Hybridization

T. asperellum chromosomal DNA was isolated according to Raeder and Broda (1985). The hybridization was performed as described in Sambrook et al. (1989) at stringent (65°C) and nonstringent (55°C) conditions. The blot was probed with a TasSw fragment located between bases 1,744 and 2,151 of the genomic sequence.

Statistical Analysis

All experimental data were subjected to Student’s t test or ANOVA with the Benjamini-Hochberg correction for multiple testing when necessary containing 1× SYBR Green PCR Master Mix (PE Applied Biosystems), 500 nM primers (for each forward and reverse primer), and one-twenty-fifth of the RT reaction. Quantitative analysis was performed using the GeneAmp 7300 sequence detection system (PE Applied Biosystems) with PCR conditions of 95°C for 15 s and 60°C for 1 min for 40 cycles. The absence of primer-dimer formation was examined in no-template controls. Three independent experiments were carried out. Each sample was examined in triplicate using relative quantitation analysis. This method normalizes the expression of the specific gene versus the control reference with the formula ΔCt = Ct-specific gene − Ct reference gene, and ΔΔCt = ΔCt − arbitrary constant (the highest ΔCt; for further elaboration, see PE Applied Biosystem Sequence Detection User Bulletin No. 2). The threshold cycle value is defined as the PCR cycle number that crosses an arbitrarily placed threshold line.

Root Colonization Assay

Root colonization assays were performed according to Viterbo et al. (2005). Briefly, roots were detached 48 h postinoculation and extensively washed with water. After sterilization in 1% NaOCl for 2 min, the roots were washed with sterile distilled water, weighed, and homogenized using an Ultra Turrax apparatus (Janke & Kunkel) in 20 mL of water for 1 min. Serial dilutions were plated for colony forming unit counts on Trichoderma selective medium (Vargas Gil et al., 2008) at 28°C.

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