A Proteinaceous Elicitor Sm1 from the Beneficial Fungus *Trichoderma virens* Is Required for Induced Systemic Resistance in Maize[1][W]

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We have previously shown that the beneficial filamentous fungus *Trichoderma virens* secretes the highly effective hydrophobin-like elicitor Sm1 that induces systemic disease resistance in the dicot cotton (*Gossypium hirsutum*). In this study we tested whether colonization of roots by *T. virens* can induce systemic protection against a foliar pathogen in the monocot maize (*Zea mays*), and we further demonstrated the importance of Sm1 during maize-fungal interactions using a functional genomics approach. Maize seedlings were inoculated with *T. virens* Gv29-8 wild type and transformants in which *SM1* was disrupted or constitutively overexpressed in a hydroponic system or in soil-grown maize seedlings challenged with the pathogen *Colletotrichum graminicola*. We show that similar to dicot plants, colonization of maize roots by *T. virens* induces systemic protection of the leaves inoculated with *C. graminicola*. This protection was associated with notable induction of jasmonic acid- and green leaf volatile-biosynthetic genes. Neither deletion nor overexpression of *SM1* affected normal growth or development of *T. virens*, conidial germination, production of gliotoxin, hyphal coiling, hydrophobicity, or the ability to colonize maize roots. Plant bioassays showed that maize grown with *SM1*-deletion strains exhibited the same levels of systemic protection as non-*Trichoderma*-treated plants. Moreover, deletion and overexpression of *SM1* resulted in significantly reduced and enhanced levels of disease protection, respectively, compared to the wild type. These data together indicate that *T. virens* is able to effectively activate systemic disease protection in maize and that the functional Sm1 elicitor is required for this activity.

Plants in their natural settings are surrounded by a range of beneficial or deleterious microorganisms. By evolving mechanisms that enable recognition of the invader followed by production of an arsenal of antimicrobial and/or antiherbivory compounds, plants have the ability to localize and reduce the impact of the pathogen invasion (Paul et al., 2000; Veronese et al., 2003). One of the most effective resistance mechanisms, an induced defense response, is activated only upon pathogen invasion and involves multiple signal transduction pathways. This strategy appears more cost effective than constitutive defenses as these pathways are only activated upon invasion (Heil and Bostock, 2002). Deployment of the plant defenses occurs both locally, at the site of infection, and/or systemically, e.g. in tissues distant from the point of pathogen entry (Gozzo, 2003). A number of plant species have been shown to develop systemic acquired resistance (SAR) in response to local infection with necrotizing pathogens conferring a broad-range resistance (Durrant and Dong, 2004; Glazebrook, 2005). SAR is characterized by an early increase in endogenous salicylic acid (SA) and accumulation of pathogenesis-related (PR) proteins (Van Loon and Van Strien, 1999; Durrant and Dong, 2004). Interestingly, induced systemic responses are not just initiated by pathogens, but also may result from interactions with avirulent microbes. The colonization of the rhizosphere by certain strains of plant growth-promoting rhizobacteria (PGPR) results in a state of heightened resistance to subsequent pathogen attack, a phenomenon generally known as induced systemic resistance (ISR; van Loon et al., 1998; Pieterse et al., 2003). Rhizobacteria-mediated ISR can occur in many plant species and was also demonstrated to be effective against a broad range of pathogens (van Loon et al., 1998). Unlike SA-dependent SAR, extensive work with Arabidopsis (*Arabidopsis thaliana*) has demonstrated that ISR depends primarily on signal transduction pathways involving jasmonic acid (JA) and ethylene (ET; Ton et al., 2002). Recent findings indicate that there is cross talk and a certain degree of overlap between SA and JA signaling pathways (Schenk et al., 2000; Dong, 2001; Glazebrook et al., 2003).

In addition to plant growth-promoting rhizobacteria, there is another group of root-colonizing beneficial microorganisms that have been found to induce plant resistance to pathogens. They represent the anamorphic

[1] This work was supported by grants from the U.S. Department of Agriculture National Research Initiative (2003–35316–13861) and the National Science Foundation (IOB0445650) to C.M.K., and a fellowship to S.D. from the Storkan-Hanes-McCaslin Foundation.

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[W] The online version of this article contains Web-only data.
www.plantphysiol.org/cgi/doi/10.1104/pp.107.103689

stages of several fungi, including *Trichoderma* spp., *Fusarium* spp., binucleate *Rhizoctonia*, and *Pythium oligandrum*, and are commonly found in most soils throughout the world (Hwang and Benson, 2003; Harman et al., 2004a; Le Floch et al., 2005). Members of the genus *Trichoderma* have long been recognized as agents for the biocontrol of plant diseases. It is now widely accepted that the biocontrol properties of *Trichoderma* spp. are also based on their ability to induce both local and systemic resistance responses. To date, however, the induction mechanisms as well as the fungal elicitors and the plant signals involved are largely undefined. Previous studies have reported that the root colonization by *Trichoderma* spp. resulted in the accumulation of antimicrobial compounds both locally in the roots (Howell et al., 2000) and systemically in the leaves (Yedidia et al., 2003). More recently, a JA/ET signaling pathway and a mitogen-activated protein kinase signaling pathway of both the plant and the fungus were identified to be important for the *Trichoderma* spp.-mediated ISR in cucumber (*Cucumis sativus*) plants (Shoresh et al., 2005, 2006; Viterbo et al., 2005). However, fungal elicitors and/or the plant signaling compounds related to these pathways still await discovery.

The highly coordinated molecular dialogue that occurs between plants and microbes during the early stages of their association, in which signaling molecules play an essential role, determines the final outcome of the relationship, which ranges from parasitism to mutualism (Bais et al., 2004; Pozo et al., 2005). A large array of microbial elicitors that initiate plant defense responses has been characterized (for review, see Nimchuk et al., 2003). Plant cells exposed to elicitors (i.e. crude fungal cell wall fragments or defined molecules such as purified proteins and avirulence gene products) respond with a battery of cellular changes, including rapid ion fluxes and the generation of reactive oxygen species, accumulation of phytoalexins, and synthesis of PR proteins (Nicholson and Hammerschmidt, 1992; Van Loon and Van Strien, 1999; Mittler et al., 2004). However, a clear understanding of the *Trichoderma*-plant recognition and communication process is lacking. Only proteins with enzymatic activity (i.e. cellulase and xylanase) have been described as proteinaceous elicitors in *Trichoderma* spp. (Bailey et al., 1992; Calderon et al., 1993). It should be noted that in case of xylanase, it was demonstrated that its enzymatic activity is unrelated to the elicitation processes (Furman-Matarasso et al., 1999; Rotblat et al., 2002). Although evidence exists indicating that other metabolites capable of elicitation of plant defense are produced by *Trichoderma* (Hanson and Howell, 2004; Harman et al., 2004a), these proposed elicitors have not been characterized.

To obtain new insights into the events underlining the processes of plant-*Trichoderma* interactions, we previously identified and characterized an elicitor produced by *Trichoderma virens* named Sm1, a novel proteinaceous nonenzymatic elicitor from this group of rhizosphere-competent fungi (Djonovic et al., 2006a). Sm1 is induced and secreted by the fungus at the early stages of plant-*Trichoderma* interaction, suggesting a signaling role for this protein. Indeed, the purified Sm1 efficiently elicited plant defense responses and systemic resistance against a foliar pathogen of cotton (*Gossypium hirsutum*; Djonovic et al., 2006a). The protective activity of Sm1 was associated with the accumulation of reactive oxygen species and phenolic compounds, and increased levels of transcription of the defense genes regulated by SA and JA/ET as well as genes involved in the biosynthesis of sesquiterpenoid phytoalexins (Djonovic et al., 2006a).

Most of the molecular mechanisms that underlie the *Trichoderma*-mediated systemic induced resistance have been studied in dicot plants (Yedidia et al., 2003; Shoresh et al., 2005; Viterbo et al., 2005; Djonovic et al., 2006a). In contrast, the current knowledge of SAR/ISR mechanisms in monocots is limited. The only available data suggesting that monocots may undergo induced resistance responses similar to those found in dicots have been generated from the exogenous application of chemical inducers of SAR (Kogel et al., 1994; Schweizer et al., 1999). However, conclusive evidence of the biological relevance of this chemically induced SAR is lacking. Moreover, unlike multiple reports on dicot species, the monocot "SAR genes" that are inducible by pathogens were not inducible by chemicals (Schaffrath et al., 1997; Schweizer et al., 1999). It is worth noting, however, that despite these differences, many lines of evidence point to a similarity in various aspects of induced resistance between monocots and dicots (Morris et al., 1998; Dong, 2004; Chern et al., 2005).

In this study we aimed (1) to determine whether colonization of roots by *T. virens* can induce systemic protection against a foliar pathogen in the monocot maize (*Zea mays*); (2) to examine the requirement of a functional Sm1 for induction of systemic resistance in maize; (3) to elucidate the potential involvement of signaling pathways mediated by SA, JA, and other oxygenated fatty acids in the induced resistance of maize triggered by *T. virens*; and (4) to assess a number of phenotypic traits to address a potential role of Sm1 in the physiology of *T. virens*. This study provides compelling evidence that, similar to dicot plants, colonization of maize roots by *T. virens* induces ISR-like systemic protection of the leaves inoculated with *Colletotrichum graminicola*. Furthermore, our results demonstrate that Sm1 is required for activation of ISR in maize, since plants grown with *SM1*-deletion strains exhibited the same levels of systemic protection as the control (non-*Trichoderma*-treated) plants and, conversely, plants grown with *SM1*-overexpression strains displayed increased protection compared to the wild type.

RESULTS

Identification of *SM1*-Deletion and -Overexpression Transformants

Previously, we showed that a *T. virens* hydrophobin-like elicitor, Sm1, induced plant defense responses and

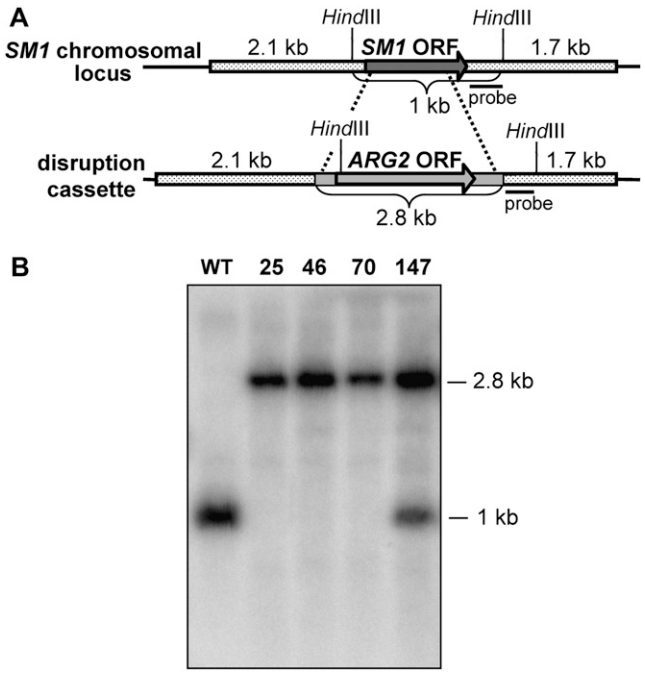

Figure 1. Southern analysis and confirmation of *SM1* disruptants. A, Scheme of the gene-deletion strategy. The expected sizes in native (1 kb) and deletion (2.8 kb) events are indicated. The dimensions are not drawn to scale. B, Southern analysis of *T. virens* wild-type (WT) strain and *SM1*-deletion transformants (KO25, KO46, and KO70). Autoradiograph of DNA gel blot hybridized with ^{32}P-dCTP-labeled probe indicated in A. Fifteen micrograms of genomic DNA was digested with *Hin*dIII and loaded per lane. Numbers on the right indicate expected size in native and deletion events. Strain 147 contains the native and overexpression bands indicating ectopic integration.

provided high levels of systemic resistance against the foliar pathogen *Colletotrichum* sp. in cotton (Djonovic et al., 2006a). To confirm the biological relevance of Sm1 in the *T. virens* Gv29-8 (wild type) systemic induced resistance in maize, we generated *SM1*-deletion (KO) and -overexpression (OE) strains. The vector for gene replacement, pSZD25, was constructed to replace the entire *SM1* open reading frame (ORF) with a selectable marker (Fig. 1A). A total of 147 stable transformants were tested for a gene disruption by PCR (data not shown). Nine PCR-selected candidates were further analyzed by Southern hybridization to verify the gene disruption. After digestion of the genomic DNA with *Hin*dIII and hybridization with a 460-bp *Bam*HI/*Hin*dIII fragment immediately downstream of *SM1*, a positive disruption event yielded a 2.8-kb band versus a 1-kb band in the wild type (Fig. 1A). Three strains, KO25, KO46, and KO70, were clearly disrupted in the gene, and strain 147 contained both 1-kb and 2.8-kb bands, indicating ectopic integration of pSZD25 (Fig. 1B). After probing with a 710-bp *Xho*I fragment from the *ARG2* gene, a single integration event was detected in disruption transformants (data not shown).

The overexpression vector pSZD26 was constructed for constitutive overproduction of the *SM1* gene (Fig. 2A). From 14 randomly selected putative overexpression transformants, 10 demonstrated the presence of the overexpression cassette based on PCR analysis (data not shown). These 10 candidates were further verified by Southern-blotting analysis. As Figure 2 demonstrates, the *SM1* probe hybridized to the native gene, yielding a 4-kb and a 1.5-kb band in overexpression strains, as expected (Fig. 2B). The intensity of hybridization of the 1.5-kb band in some of the transformants, such as OE38 and OE39, suggests integration of multiple copies of the construct at one site, resulting in tandem repeats.

Expression of *SM1* in Transformants

Gene expression analysis was performed to test whether disruption or overexpression of the *SM1* gene resulted in the expected alteration of transcript and protein accumulation. Northern-blot experiments showed no transcripts of *SM1* in any of the deletion transformants (KO25, KO46, or KO70; Fig. 3A). These results were further verified by western-blot analysis (Fig. 3B), confirming the successful replacement of the gene. Additionally, there was no obvious difference in the extracellular protein profile of the wild-type and deletion strains except for the absence of 12.6-kD Sm1 band in deletion strains (data not shown). Examination of the seven overexpression strains (OE8, OE9, OE24, OE38, OE39, OE126, and OE150) revealed higher *SM1* mRNA levels than for the wild type after 3 d of growth in a complete medium (GYEC; Fig. 3C). Polypeptide levels for the overexpression strains (OE38 and OE39) with the highest *SM1* mRNA levels were

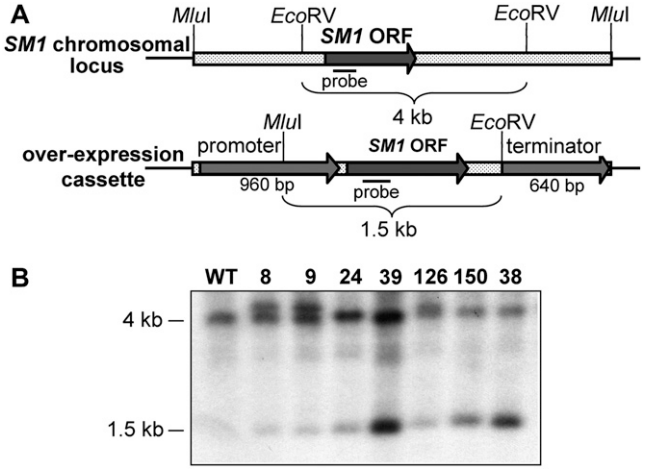

Figure 2. Confirmation of *SM1*-overexpression transformants. A, Gene-overexpression strategy. The expected sizes in native (4 kb) and overexpression (1.5 kb) events are indicated. The dimensions are not drawn to scale. B, Southern analysis of *T. virens* wild-type (WT) strain and *SM1*-overexpression transformants (OE8, OE9, OE24, OE39, OE126, OE150, and OE38). Fifteen micrograms of genomic DNA was double digested with *Mlu*I/*Eco*RV, blotted on a membrane, and probed with the ^{32}P-dCTP-labeled 264-bp PCR product amplified from *SM1* ORF. Numbers on the left indicate expected size in overexpression and wild-type sequences.

compared to the wild type by western-blot analysis. Both overexpression stains produced greater amounts of Sm1 than the wild type (Fig. 3D).

Phenotypic Analysis of Transformants

A number of phenotypic assays were conducted with the gene-disruption, gene-overexpression, and wild-type strains to assess if the morphology and physiology of strains were affected by genetic manipulations of *SM1*.

There were no evident changes in the cultures of the deletion or overexpression transformants when compared to the wild type with respect to colony appearance and pigmentation during sporulation. We further assessed the possible involvement of *SM1* in fungal growth under different nutritional conditions. Three disruptants, KO25, KO46, and KO70, and two overexpression strains, OE38 and OE39, were selected for growth analysis. Growth area was compared after 1 and 2 d growth on minimal (Vogel's minimal medium [Vogel, 1956] supplemented with 1.5% Suc [VMS], water agar [WA]) or complex (potato dextrose agar [PDA]) medium. An ANOVA ($P < 0.05$) indicated there was no significant differences in growth area among the strains on any of the media tested (Supplemental Table S1). Additionally, no differences among the strains were found in conidial germination. The percentage of germination ranged from 92.3% to 93.7% and was not significantly different. The strains were also assessed for their ability to coil around their target fungal hosts as a component of the mycoparasitic process (Benitez et al., 2004). The ability to recognize and attach and coil around the fungal host *Rhizopus oryzae* was not affected by either *SM1* deletion or overexpression (Supplemental Fig. S1). Zones of inhibition on antibacterial indicator plates resulting from the presence of gliotoxin in culture filtrates (CFs) were similar among all strains tested.

Since Sm1 is a hydrophobin-like protein, we further assessed the hydrophobicity of transformants by performing two different assays. In the first experiment, the hydrophobicity of the mycelia was visualized by placing water droplets on the *T. virens* strains growing on a solid agar medium (Fig. 4A). The greater the contact angle of a droplet, the greater the hydrophobicity and vice versa (van der Mei et al., 1991). Figure 4A shows that the hydrophobicity of the wild-type strain and the *SM1*-deletion or -overexpression strains did not visibly differ. The second assay involved separation of conidia into aqueous and organic phases to assess hydrophobicity of conidia (Whiteford and Spanu, 2001). Titrating conidia from this interface with a detergent (e.g. Triton X-100) can release conidia and may reveal quantifiable differences in conidium hydrophobicity between strains (Fig. 4B). From the Figure 4B we can see that the amount of detergent needed to release the conidia of wild-type, *SM1*-deletion, and *SM1*-overexpression strains into the aqueous phase was very similar. The absence of any visible alteration of the fungal growth provided confidence that subsequent results from the biocontrol assays were a function of genetic manipulation in *SM1* rather than the transformation procedure.

Genetic Manipulation of *SM1* Results in Altered Local and Systemic Expression of a Defense-Related *PAL* Gene in Hydroponically Grown Maize

We have previously shown that colonization of cotton roots by *T. virens* resulted in an increased expression of *PR* genes (Djonovic et al., 2006a). To investigate plant

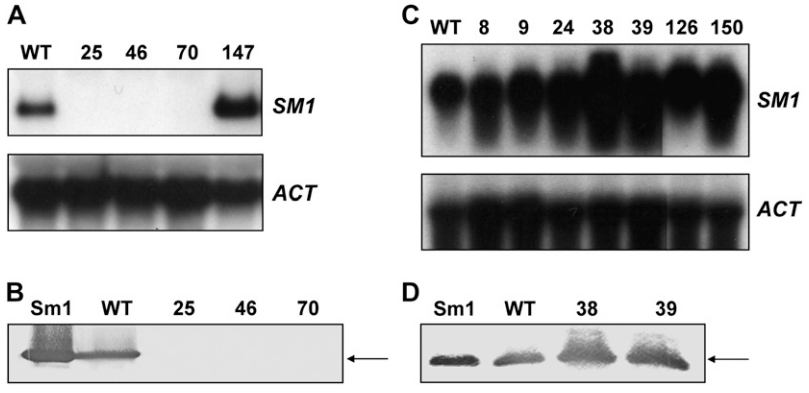

Figure 3. *SM1* expression in transformants. A and C, Northern analysis of transformants. Total RNA was extracted from wild type (WT), deletion strains (SKO25, SKO46, SKO70), and strain with ectopic integration of the construct (147) cultured in VMS media for 5 d (A), and overexpression strains (OE8, OE9, OE24, OE38, OE39, OE126, and OE150) cultured in GYEC medium for 3 d (C). Fifteen micrograms in A and 10 μg in C of total RNA were separated on formaldehyde-agarose gel, transferred to a Hydrobond-N^+ nylon membrane, and hybridized with the 264-bp PCR product of the *SM1* gene (A and C, top). The same membrane was hybridized with a 550-bp actin fragment PCR amplified from *T. virens* wild-type cDNA as a control for even loading (A and C, bottom). B and D, Western analysis of transformants. Immunoblot of the protein profile of 5-d VMS CFs from *T. virens* wild-type and deletion strains (B) and 3-d CFs of overexpression and wild-type strains cultured in GYEC medium (D). Pure Sm1 was included as positive control (Sm1). Six micrograms of total protein of each strain was loaded per lane. The 12.6-kD Sm1 is indicated by arrows.

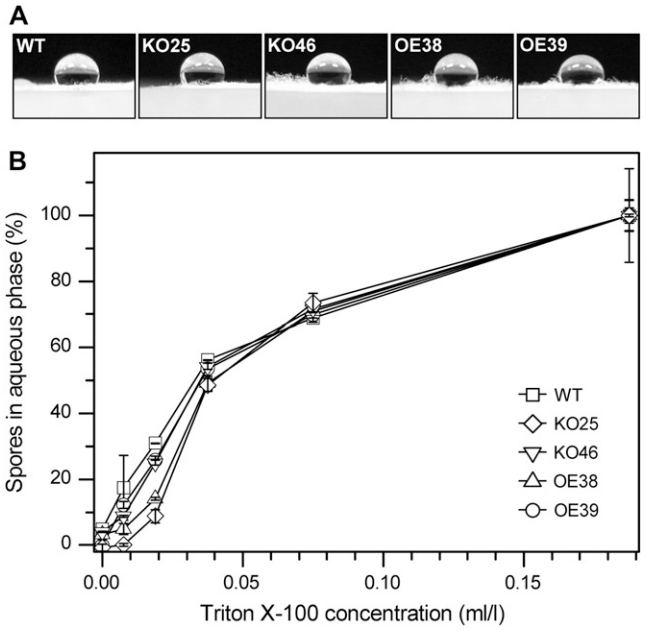

Figure 4. Hydrophobicity of wild-type, *SM1*-deletion, and *SM1*-overexpression strains. A, Hydrophobicity of wild-type (WT), *SM1*-deletion (KO25 and KO46), and *SM1*-overexpression strains (OE38 and OE39). Water droplet (20 μL) was placed on the surface of the colony of each strain after 2 d growth on PDA (nonsporulating mycelium) and photographed. B, Hydrophobicity of conidia using MATH assay (Whiteford and Spanu, 2001). The graph shows the amount of conidia in the aqueous phase in the presence of increasing concentrations of the surfactant Triton X-100 (each point is the mean of two replicates).

defense responses to treatment of roots with *T. virens* in a monocot species, maize (inbred line B73) was selected as a model plant. An aseptic hydroponic growth system was used (Djonovic et al., 2006a) to spatially separate the root-inducing agent from the distal cotyledon tissue. To verify that the fungal strains did not colonize the entire seedling, maize aboveground organs were plated onto a *Trichoderma* selective medium (GVSM; Park et al., 1992). The presence of *T. virens* was not detected in any of the maize aboveground organs. Expression of maize defense genes was tested in root and leaf tissues at 48 h after inoculation of the root system with the wild type or transformants. Figure 5A illustrates that defense-related *PAL* (Phe ammonia-lyase) gene was up-regulated locally, in roots, and systemically, in leaves, when maize seedlings were grown with the wild-type and overexpression strains. In contrast, no significant changes in the levels of expression of *PAL* were detected in either roots or leaves of seedlings cocultured with the deletion strains (Fig. 5A). These results indicated that the functional Sm1 elicitor is required for induction of the plant *PAL* gene.

Detection of Sm1 during Fungal-Maize Interaction in the Hydroponic System

As we have previously shown that Sm1 was abundantly secreted outside the cell in the presence of cotton seedlings (Djonovic et al., 2006a), we performed western analysis to examine the expression of Sm1 in wild-type and overexpression strains in the hydroponically cocultured *Trichoderma*-maize seedlings (Fig. 5B). The analysis of the secreted proteins in the plant growth medium showed that Sm1 was more abundant in the filtrate from the plant-overexpression strain coculture than in the filtrate from the plant-wild type coculture (Fig. 5B). As expected, no Sm1 was detected in the plant-deletion strain coculture (Fig. 5B).

Sm1 Is Required for ISR in Maize against *C. graminicola*

To assess the in vivo biological relevance of *T. virens* to induce disease resistance in maize through Sm1 expression, wild-type strain, two deletion strains, and two overexpression strains were compared in their ability to systemically protect 2-week-old maize seedlings

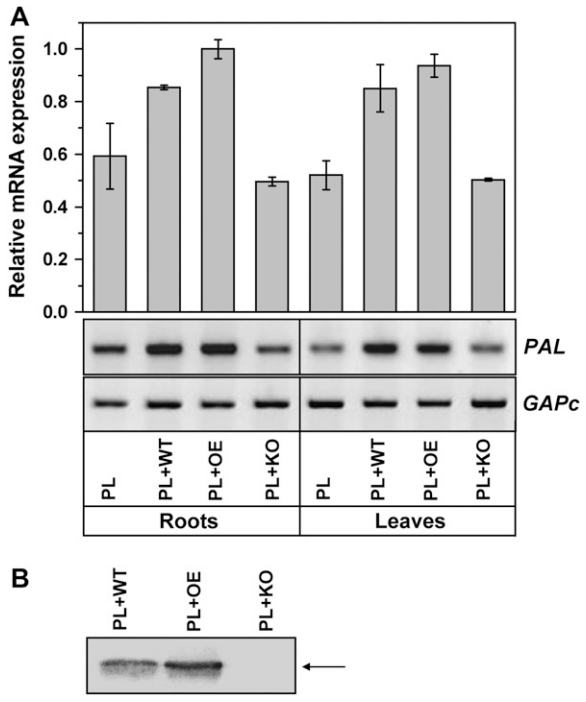

Figure 5. sqRT-PCR analysis of *PAL* expression in maize hydroponically grown with *T. virens* strains and Sm1 expression. Shown is *PAL* expression in maize root and leaf tissue 48 h after root inoculation with *T. virens* wild type (PL + WT), *SM1*-overexpression strain OE38 (PL + OE), or *SM1*-deletion strain KO25 (PL + KO). Plants grown without *Trichoderma* were included as control (PL). Maize *GAPc* was used to as control for equal amounts of cDNA. Intensities of bands were quantified using Scion Image software and the data expressed as arbitrary units of *PAL* expression normalized to *GAPc*. The values are shown relative to the highest value (bars) and are representative of two independent experiments with SE bars. B, Immunoblot analysis of Sm1 secreted into the growth medium in the hydroponically cocultured *Trichoderma*-maize seedlings. Equal volumes of concentrated samples equivalent to 300 mL growth medium from the hydroponic system (PL + WT, PL + OE, PL + KO) were loaded on a 15% SDS-PAGE and electroblotted to a nitrocellulose membrane. The 12.6-kD Sm1 is indicated by an arrow.

challenged with the foliar pathogen *C. graminicola*. Maize seedlings from *T. virens*-treated seed were inoculated with a spore suspension of the pathogen with disease assessment conducted 4 d following inoculation. In contrast to the typical large lesions that appeared earlier and had began to coalesce in the plants not treated with *T. virens* or plants treated with *SM1*-deletion strains, the lesions were significantly smaller in plants treated with either wild-type strain or *SM1*-overexpression strains. Disease protection due to treatment with overexpression strains was even more evident than with the wild type, as the lesions were much smaller, and development of some lesions was delayed or arrested (Fig. 6A). The mean lesion area for the plants treated with the disruptant strains was significantly greater than wild-type or overexpression strains, but was the same as the nontreated control. Both overexpression strains increased disease protection as compared to the wild-type strain with OE38 significantly reducing lesion area ($P < 0.05$; Fig. 6B).

Colonization of Roots by *T. virens* Strains in Hydroponics and in Soil Is Not Affected by *SM1* Deletion or Overexpression

As Sm1 is a hydrophobin-like protein and hydrophobins are known to be involved in several plant-fungal symbiotic interactions (Tagu et al., 2002; Duplessis et al., 2005; Viterbo and Chet, 2006), we hypothesized that the inability of *T. virens* to produce Sm1 may have an affect on the ability of these strains to colonize plant roots. However, our data show that neither deletion nor overexpression of *SM1* affected the ability of the wild-type strain to colonize seedlings grown hydroponically or in soil (Table I).

To further examine the colonization of maize seedlings roots by the wild-type and *SM1*-deletion strains, we generated *T. virens* wild-type and deletion strains expressing *GFP*. Southern analysis of genomic DNAs of transformants confirmed integration of the *GFP* and *HPH* (hygromycin B phosphotransferase gene) vectors, pTEFEGFP and pCSN44, respectively (data not shown). The growth rate and colony morphology of selected cotransformants were similar to the original strains as evaluated by growth comparison on various nutritional media (PDA, VMS, and WA plates). Six-day-old maize seedlings grown in the hydroponic system were examined for the pattern of colonization by these strains. The extent of colonization (fungal hyphae along the main root axis as well as encircling the roots) as well terminal and intercalary formation of chlamydospores appeared similar for both strains (Fig. 7).

Expression of Maize Defense Genes during *T. virens*-Induced Resistance to *C. graminicola*

To elucidate the potential involvement of signaling pathways mediated by SA, JA, and other octadecanoids in the induced resistance of maize triggered by *T. virens*, we examined the expression of nine well-characterized maize defense genes. The housekeeping gene, the cytosolic form of glycerol phosphate dehydrogenase (*GAPc*; Farag et al., 2005), was used to ensure equal amount of template cDNA. Because many of the genes tested belong to large gene families and share high sequence identity throughout their entire cDNA, gene-specific primers were generated and semiquantitative reverse transcription (sqRT)-PCR analyses were performed. A range of PCR reactions was conducted to determine the optimal number of PCR cycles for linear amplification of the genes. Fourteen days postinoculation with *T. virens* wild-type or transformant strains, maize seedlings were

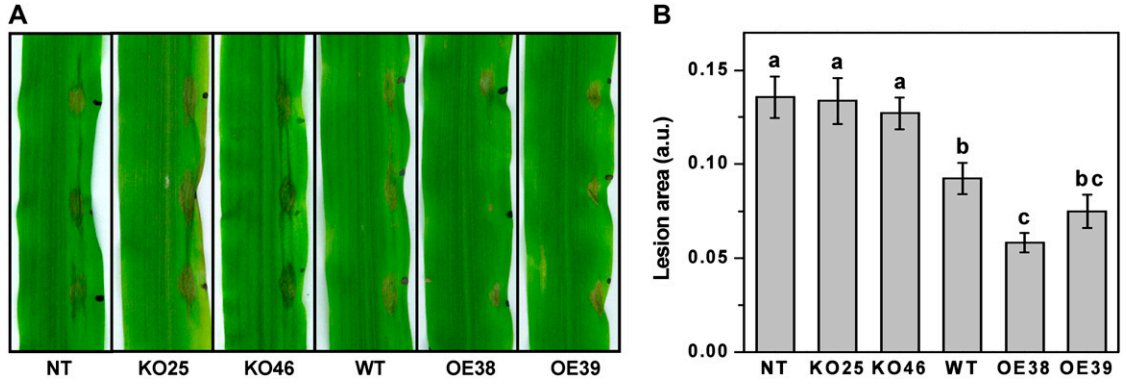

Figure 6. Effect of *T. virens* wild-type, *SM1*-deletion, and *SM1*-overexpression strains on ISR in maize seedlings to *C. graminicola*. A, Lesion development in leaves of *T. virens*-induced maize plants 4 d postchallenge with the pathogen *C. graminicola*. From left to right: control seedlings non-*Trichoderma* inoculated (NT), and seedlings inoculated with *SM1*-deletion (KO25 and KO46), *T. virens* wild-type (WT), and *SM1*-overexpression (OE38 and OE39) strains. B, The graph illustrates the levels of systemic disease protection observed in each treatment. The mean lesion area (given as the arbitrary units) was evaluated 4 d postinoculation as described in ''Materials and Methods.'' Each bar represents mean lesion area of six replicates with four plants each from three independent experiments with SE bars. Columns with letter in common did not differ significantly according to Fisher's PLSD test at a significance level of 5%.

Table I. *Colonization by wild-type (WT), SM1-deletion (KO), or SM1-overexpression (OE) strains of maize seedlings grown in hydroponic system or in soil*

Strain	Hydroponics[a]	Soil[b]
WT	94.40 ± 1.88	1.92 ± 0.21
KO25	97.25 ± 0.64	2.57 ± 0.21
KO46	96.15 ± 1.29	1.96 ± 0.15
OE38	95.02 ± 0.91	1.98 ± 0.38
OE39	94.83 ± 1.46	2.12 ± 0.32

[a]The percentage of the total length of excised root fragments from the hydroponic system colonized by the strains of *T. virens* given as a mean of six replicates with the SE. [b]Total number of colonies of the strains per centimeter of root harvested from the maize seedling grown in soil given as a mean of five replicates with the SE. These experiments were replicated at least once with similar results. Based on ANOVA ($P < 0.05$), there was no significant differences in root colonization in any of the growth systems.

inoculated with *C. graminicola*. Systemic induced resistance was evaluated before pathogen inoculation (T = 0 or equivalent of 14 d growth) or 1 d after pathogen inoculation (T = 1). Total RNA was extracted from the leaf tissue at these time points and sqRT-PCR performed. As the expression of all of the genes studied after pathogen attack, in general, did not differ significantly among *Trichoderma*-treated or nontreated plants but was inducible by *C. graminicola* infection (M.V. Kolomiets, unpublished data), we have shown the expression of the maize defense genes activated by the presence of the *T. virens* strains only (T = 0; Fig. 8).

The maize genes of the SA-responsive pathway examined were *PR1* and *PR5* (Morris et al., 1998), oxophytodienoate reductase *OPR2* (Zhang et al., 2005), and *PAL* (Farag et al., 2005). *PR1* and *PR5* in the treated plants were expressed at similar levels as the nontreated (control) plants. *OPR2* transcripts were not detected at this time point. For *PAL*, there was a notable up-regulation by wild-type and overexpression strains, whereas expression was similar in nontreated and deletion strain-treated plants (Fig. 8A).

Since lipoxygenase (LOX) products such as jasmonates and other oxygenated fatty acids called oxylipins were implicated as signals in ISR (Conrath et al., 2006), the expression of the LOX pathway (Fig. 8B) was examined. Currently, the best studied branches of this pathway are those initiated by the oxygenation of linolenic acid by 13-LOXs: the allene oxide synthase (AOS) branch and the hydroperoxide lyase (HPL) branch (Feussner and Wasternack, 2002). The AOS branch involves a series of enzymes including allene oxide cyclase and 12-oxo-phytodienoic acid reductase (OPR) and produces the diverse group of jasmonates (Feussner and Wasternack, 2002). The HPL branch final products include a number of volatile C-6 aldehydes and alcohols, or so-called green leaf volatiles (GLVs) that function as signaling molecules in defense responses to pathogens and pests (Matsui, 2006). *AOS* and *OPR7* and/or *OPR8* are implicated in JA biosynthesis (Zhang et al., 2005; Mei et al., 2006). Similar to the *PAL* gene, the expression of *AOS* and *OPR7* was notably up-regulated by wild-type and overexpression strains, whereas a basal level of expression was detected in either nontreated or deletion strain-treated plants. Interestingly, *OPR8* was not expressed at any of the stages or treatments tested, suggesting a differential regulation of this other putative JA-producing OPR isoform (Fig. 8B).

LOX10 and *HPL* are most likely GLV-producing enzymes in maize (Nemchenko et al., 2006). Moreover, *LOX10* is the closest maize homolog of a bean (*Phaseolus vulgaris* 'Prelude') *LOX* gene associated with ISR by a nonpathogenic *Pseudomonas* strain (Ongena et al., 2004). In our study, *LOX10* was expressed at very low levels throughout the time course, and did not differ among nontreated and *Trichoderma*-treated plants (Fig. 8C). In contrast, the expression of *HPL* was significantly up-regulated by wild-type and overexpression strains, and expressed at similar levels in deletion strain-treated and nontreated plants. Interestingly, *HPL* transcript levels were notably higher in overexpression strain treatment compared to the wild type (Fig. 8C).

SM1 Is Expressed in Planta Before and After Pathogen Attack

It has been shown recently that several proteins that are involved in *Trichoderma*-plant early interaction are up-regulated during the interaction with plants but also expressed in planta (Viterbo et al., 2004; Viterbo and Chet, 2006). We have also shown that *SM1* was

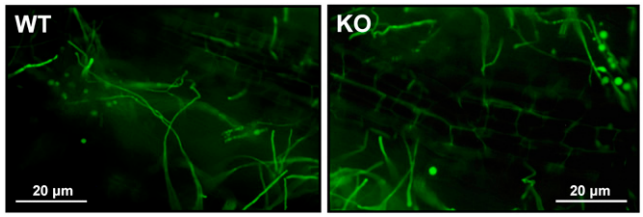

Figure 7. Fluorescent micrographs of 6-d-old maize seedlings grown in the hydroponic system and colonized with *T. virens* wild-type or deletion strain (KO25) expressing *GFP*. Microscopy was performed using Olympus BX-51 fluorescent microscope with excitation from 470 to 490 nm, emission from 510 to 550 nm, and 200× magnification.

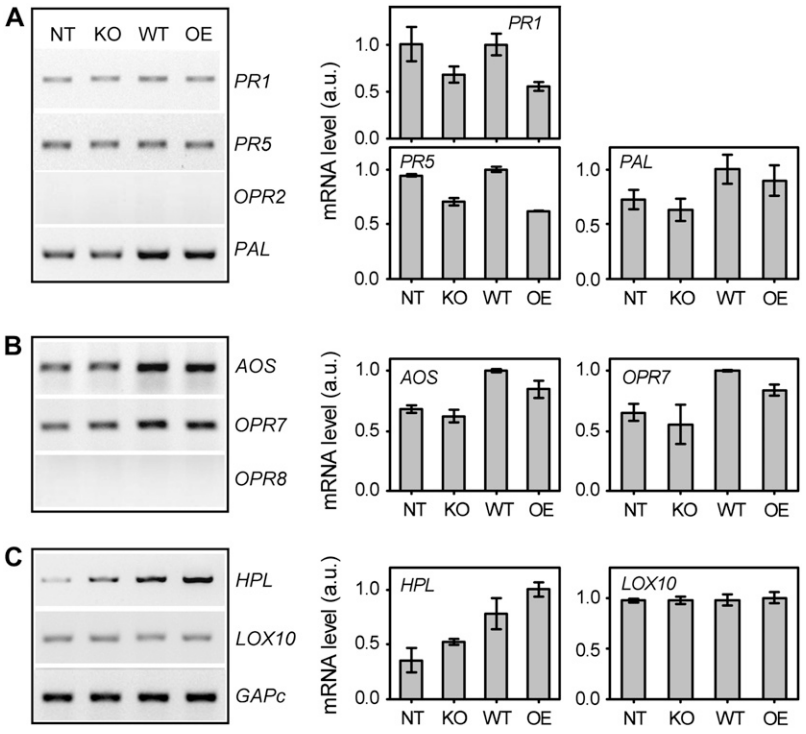

Figure 8. Expression (sqRT-PCR) of defense-related genes in leaves of *T. virens* wild type-, deletion strain-, and overexpression strain-induced maize plants challenged with *C. graminicola*. A, SA-responsive genes: *PR1* and *PR5*, *OPR2*, and *PAL*. B, JA-biosynthetic or -inductive genes: AOS, *OPR7*, and *OPR8*. C, GLV-biosynthetic or -inductive genes: *HPL* and *LOX10*. Expression of *GAPc* was used as a quantitative standard. The negative control for sqRT-PCR was DNase-treated and cleaned RNA reactions without reverse transcription (not shown). Treatments are: maize seedlings not inoculated with *T. virens* (NT), or preinoculated with KO25 (KO), Gv29.8 (WT), or OE38 (OE) for 14 d (T = 0, before pathogen challenge). Intensities of bands were quantified using Scion Image software (right panels) and the data expressed as arbitrary units of expression of the indicated defense genes normalized to *GAPc*. The values shown are the average of two independent experiments with SE bars (note: the images of gene expression on the left are from those of the second experiment).

up-regulated in the presence of the cotton plants in the early interaction (Djonovic et al., 2006a). In this study, after observing the protection of maize seedlings pretreated with *T. virens* wild-type and overexpression strains against a foliar pathogen, we wanted to test if *SM1* was indeed expressed in planta after the root colonization by *T. virens* strains. Figure 9 shows that *SM1* was expressed both after 14 d of growth with *Trichoderma* (Fig. 9, left) as well as after pathogen attack (Fig. 9, right). As expected, no transcripts of *SM1* were detected in seedlings grown with *SM1*-deletion strain or non-*Trichoderma*-treated plants (Fig. 9). However, the technique used for this experiment was not quantitative (RT-PCR performed with 40 cycles as the level of expression was low), but was employed to confirm that *SM1* was expressed in planta in both wild-type and overexpression strains during *T. virens* colonization of healthy maize seedlings as well as during the infection process.

DISCUSSION

T. virens Induces Systemic Disease Resistance in Maize

Resistance responses triggered by defense-related signal compounds such as SA or its chemical analogs have been described in maize (Morris et al., 1998). There is no evidence, however, that these chemically induced responses result in systemic protection of plants against pathogens. Additionally, there are only a few reports demonstrating that the colonization of roots of any monocot species by beneficial microorganisms induces systemic disease resistance to leaf pathogens (Harman et al., 2004b; Waller et al., 2005). This study provides evidence that *T. virens* induces systemic resistance to the fungal foliar pathogen *C. graminicola* in maize as demonstrated by significant reduction in disease symptoms in plants grown with *T. virens* Gv29-8 compared to nontreated plants. A previous report indicated that *T. virens* strains G6, G11, and G6-5 were able to induce local but not systemic resistance in cotton (Howell et al., 2000). We have now demonstrated that *T. virens* Gv29-8 as well as an exogenous application of Sm1 elicitor produced by Gv29-8 (Djonovic et al., 2006a) are able to effectively activate ISR (Fig. 6).

The important component of local and systemic resistance induced by *Trichoderma asperellum* in cucumber

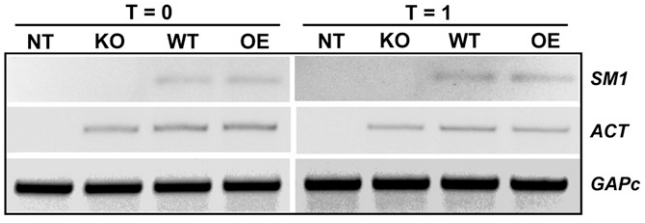

Figure 9. Detection of *SM1* expression in maize roots (RT-PCR). A, Total RNA was extracted from tergitol-metaphosphate-washed roots of maize seedlings not inoculated with *T. virens* (NT), or preinoculated with KO25, wild type, or OE38 for 14 d (T = 0, before pathogen challenge) or 1 d after *C. graminicola* challenge (T = 1). Gene-specific primers for *T. virens SM1* and *ACT* are indicated on the right. *GAPc* gene was amplified as a reference for equal amounts of maize cDNA.

plants (Yedidia et al., 2000, 2003) was shown to be mediated by the ability of *Trichoderma* hyphae to penetrate several epidermal layers and colonize the intercellular spaces (Yedidia et al., 1999). This colonization was accompanied by increased levels of plant defense transcripts and phytoalexin production. We have previously demonstrated that *T. virens* Gv29-8 is rhizosphere-competent on cotton (Park et al., 1992) and that colonization of cotton roots by *T. virens* Gv29-8 resulted in increased expression of *PR* genes (Djonovic et al., 2006a). Here, we report that *T. virens* Gv29-8 is able to successfully colonize maize root systems produced in either an aseptic hydroponic system or soil, resulting in a systemic disease resistance accompanied with increased levels of expression of defense-related genes.

Sm1 Is Required for the Ability of *T. virens* to Induce Systemic Resistance in Maize

As we have shown that *T. virens* induces systemic resistance in maize, we further tested the hypothesis that deletion of *SM1* in *T. virens* results in attenuated defense responses reducing the levels of protection against pathogen attack. Results from biological assays performed in this study support this hypothesis. We show that *SM1*-disruptant strains appear not to trigger ISR as plants grown with deletion strains exhibited similar levels of symptoms as control plants (non-*Trichoderma*-treated plants). Moreover, this effect was further supported by our results showing that *SM1*-overexpression strains induced even higher levels of protection than wild-type strain-treated plants (Fig. 6). The enhanced levels of protection with overexpression strain-treated plants may have resulted from the higher levels of production of Sm1 in overexpression strains compared to the wild type. This observation is in agreement with our previous finding that Sm1 secreted by the wild type was up-regulated in the presence of the plants compared to the growth in culture medium (Murashige and Skoog + 0.05% Suc) without the plants (Djonovic et al., 2006a). The same observation was made in this study, when maize seedlings were hydroponically cocultured with *T. virens* in Murashige and Skoog medium (data not shown).

All together, these data indicate that Sm1 is a key player for *T. virens*-induced systemic disease resistance in maize. To date, most studies report the effect of exogenous application of purified microbial elicitor on plant resistance. This study provides genetic evidence that an elicitor of defense response is required for ISR.

T. virens ISR in Maize Is Modulated through JA- and GLV- Rather Than SA-Regulated Defense Pathways

Since we demonstrated that root colonization of maize plants by *T. virens* Gv29-8 triggered systemic disease resistance, we sought to determine which defense-related pathways were associated with the observed protection. Considerable progress has been made recently to elucidate the molecular mechanisms involved in *Trichoderma*-induced systemic resistance; however, most of this work has been performed with dicot plants (Yedidia et al., 2003; Shoresh et al., 2005; Viterbo et al., 2005; Djonovic et al., 2006a). The signal transduction pathways and the defense genes involved in the resistance mechanisms in dicot plants may differ from those in monocot plants. For instance, in dicot plants, JA was suggested to mediate induced resistance through a distinct, SA-independent signaling pathway, and the most common markers of SA-mediated signal transduction pathways are *PR* genes (Pieterse and van Loon, 1999). In monocots, accumulating evidence suggests that both signal pathways contribute simultaneously to resistance mechanisms. In maize, *PR1* and *PR5* genes can be induced by pathogen infection and functional mimics of SA (Morris et al., 1998), whereas in rice (*Oryza sativa*) exogenous application of JA induces the expression of a number of *PR* genes (Agrawal et al., 2000; Rakwal and Komatsu, 2000). Moreover, a recent report provided evidence of the importance of JA in mediating rice *PR* gene expression and disease resistance (Mei et al., 2006). In our study, *PR1* and *PR5* were expressed at similar levels in the control (noninoculated) and *T. virens* wild-type strain-inoculated plants, suggesting that these two genes are not involved in *T. virens*-mediated systemic resistance in maize. This is in agreement with a recent report (Waller et al., 2005), in which systemic disease protection of barley (*Hordeum vulgare*) infected with the endophytic fungus *Piriformospora indica* was not accompanied by increased levels of *PR5*. The potential involvement of the SA-mediated pathway by *T. virens* was further examined by evaluating the expression of the *OPR2* gene, previously shown to be inducible by SA and pathogens but not by JA (Zhang et al., 2005). In this study, *OPR2* was only responsive to pathogen attack at the later time point when disease was already established (data not shown). Collectively, the described expression of *PR* genes as well as *OPR2* suggests that SA, a major signal in SAR responses triggered by pathogen infection, is most likely not a major player in *T. virens*-mediated systemic resistance in maize.

We further tested the expression of the JA/ET-responsive and -biosynthetic genes in *T. virens*-mediated resistance responses. Expression of *PAL* has been reported to be activated by the JA/ET signaling pathway (Diallinas and Kanellis, 1994; Kato et al., 2000; Shoresh et al., 2005). PAL is the first enzyme in the phenylpropanoid biosynthetic pathway, which provides precursors for the formation of an array of antimicrobial compounds (Dixon et al., 2002). We have previously shown that treatment of cotton cotyledons with Sm1 elicitor resulted in increased levels of autofluorescence (Djonovic et al., 2006a), which is usually associated with accumulation and oxidation of phenolic compounds such as phytoalexins and lignin (Nicholson and Hammerschmidt, 1992; Heath, 2000). Howell et al. (2000) found that cotton radicles treated with *T. virens*

or *T. virens* protein fractions exhibited elevated levels of the biosynthesis of sesquiterpenoid phytoalexins. The consistent pattern of up-regulated *PAL* expression in *T. virens* wild type- and overexpression strain-inoculated maize (both locally and systemically) and the similar basal expression in nontreated and deletion strain-treated plants illustrate the importance of this gene and the corresponding pathway for *T. virens*-Sm1-mediated defense responses in maize.

Existing evidence has implicated 13-LOX-derived oxylipins, including jasmonates and GLVs, in plant resistance mechanisms against diverse pathogens and pests. Both jasmonates produced by the AOS branch of the LOX pathway, and GLVs, the derivatives of the HPL branch, have potent signaling activities that regulate expression of numerous defense-related and developmental genes (Bate and Rothstein, 1998; Birkett et al., 2000; Turner et al., 2002). Our study shows that *AOS* may be of relevance to *T. virens*-Sm1-mediated defense responses in maize because *AOS* transcripts were up-regulated by wild-type and overexpression strains of *T. virens* but remained low in nontreated and deletion strain-treated plants. Recently, it was shown that overexpression of the *AOS* gene in rice increased endogenous JA levels, followed by enhanced resistance to fungal infection (Mei et al., 2006). Another gene from the JA biosynthetic pathway, *OPR7*, which was previously shown to be highly induced in maize by JA/ET treatments but not by SA (Zhang et al., 2005), was also induced by *T. virens* wild-type and overexpression strains. Interestingly, a gene encoding the putative GLV-producing enzyme HPL exhibited the highest level of expression in plants induced by a *SM1*-overexpression strain. Similar to this finding, the *T. asperellum*-mediated systemic disease protection of cucumber plants from a bacterial leaf pathogen was also associated with increased levels of *HPL* transcripts (Yedidia et al., 2003). Priming of bean plants with nonpathogenic *Pseudomonas putida* reduced the symptoms caused by the pathogen *Botrytis cinerea* and was accompanied by stimulation of the LOX pathway that may have resulted in higher levels of endogenous GLVs (Ongena et al., 2004). Indeed, overexpression of the *HPL* gene in Arabidopsis resulted in enhanced resistance against *B. cinerea* (Shiojiri et al., 2006). Additionally, very recently, GLVs were shown to play an important ''within-plant'' role in defense signaling (Heil and Silva Bueno, 2007).

Taken together, the gene expression data suggest that *T. virens* induces systemic resistance in maize by an ISR-like mechanism involving JA/ET/GLV-mediated pathways rather than a SAR-like mechanism that requires SA.

Sm1 Is Expressed in Maize Roots during Interaction with *T. virens* But Does Not Affect Root Colonization

The role of hydrophobins in several mutualistic symbioses has been reported previously (Honegger, 1991; Tagu et al., 1996; Scherrer et al., 2000). Recently, a hydrophobin gene, *TasHyd1*, was detected during cucumber root colonization by *T. asperellum*. Transformants disrupted in this gene were severely impaired in root attachment and colonization (Viterbo and Chet, 2006). Thus, we examined strains disrupted in *SM1* to test their ability to colonize maize roots. We did not detect any difference among wild-type and transformant strains as all colonized maize roots at very high efficiency. For example, in the hydroponic system, after 2 d of growth, more than 94% root fragments were colonized by all strains of *T. virens* (Table I). Additionally, *GFP*-transformed wild type and deletion transformants showed a similar pattern of colonization on maize roots (Fig. 7).

While the colonization of roots by *T. virens* does not yield the elaborate hyphal structures seen with ectomycorrhizal fungi or the level of penetration as with arbuscular mycorrhizal fungi, an intimate association is formed that involves a signaling process (Yedidia et al., 2003). Here, we have shown that several maize defense genes were affected by the presence of Sm1. Maize seedlings grown with transformants that fail to produce Sm1 express these same defense genes at levels similar to the non-*Trichoderma*-treated plants. The involvement of Sm1 in the induction of defense responses was further supported by demonstrating the expression of *SM1* in roots colonized by *Trichoderma* before and after pathogen challenge. This finding indicated again that besides being an elicitor, this protein may have some further signaling role in plant-fungal interaction as proposed earlier (Djonovic et al., 2006a). For example, the hydrophobins HydPt and HydPt-1, produced during the early stages of *Pisolithus-Eucalyptus* ectomycorrhizae formation, have been proposed to be involved in the adhesion of the mycelia to the root surface (Tagu et al., 1996). In *T. asperellum*, hydrophobin TasHyd1 was proposed to provide a protection to the hyphae from plant defense compounds during the first steps of colonization of the intercellular spaces of the plant roots (Viterbo and Chet, 2006). We are currently examining other potential roles of Sm1 by identifying plant proteins that interact with Sm1.

Possible Role of Sm1 in *T. virens* Physiology

Sm1 is a member of a new cerato-platanin (Pazzagli et al., 1999) family of hydrophobin-like, small (approximately 150 amino acids) secreted proteins, mainly associated with toxicity and infection processes, and produced by plant and human fungal pathogens. In contrast to these reported functions of cerato-platanin members, we have demonstrated that Sm1, produced by a beneficial microorganism *T. virens*, does not have toxic activity and instead is an effective elicitor of systemic resistance (Djonovic et al., 2006a). Very little is known about the role of these proteins in fungal development and physiology (Boddi et al., 2004). When a *SM1* homolog from *Leptosphaeria maculans*, *SP1*, was disrupted, it was shown not to be crucial for

pathogenicity; however, the effect of the *SP1* deletion for fungal development has not been reported (Wilson et al., 2002). Here, we show that neither deletion nor overexpression of *SM1* altered the fungal phenotypic traits (i.e. colony appearance and pigmentation during sporulation, germination, growth, hydrophobicity of mycelia or conidia, and mycoparasitic characteristics such as coiling and production of gliotoxin) we examined. Other roles in the development of *T. virens* may yet be realized for Sm1 as cerato-platanin, a homolog of Sm1, has been shown to be located in the cell walls of ascospores, hyphae, and conidia, and the authors have proposed that this protein may have some new structural functions for *Ceratocystis fimbriata* (Boddi et al., 2004). Further studies are in progress to address the role of Sm1 in fungal development using *SM1*::*GFP* fusion strains (C.M. Kenerley, unpublished data).

In summary, this study provides evidence that, similar to dicotyledonous plants, colonization of maize roots by *T. virens* induces ISR-like systemic protection associated with notable induction of JA- and GLV-biosynthetic genes. We also demonstrated that the activity of a functional Sm1 elicitor was required for *T. virens*-mediated ISR. The fact that overexpression of this gene led to enhanced levels of systemic protection suggests that genetic manipulation of fungal biocontrol agents or plants to express this elicitor may lead to improved crop resistance against pests.

MATERIALS AND METHODS

Fungal and Plant Materials

Two strains of *Trichoderma virens* were used in this study, a wild-type strain, Gv29-8, and an Arg auxotrophic strain, Tv10.4, as the recipient for fungal transformation (Baek and Kenerley, 1998). An isolate of the foliar pathogen of maize (*Zea mays*), *Colletotrichum graminicola*, was kindly provided by Dr. S. Sukno (Texas A&M University). The isolate of *Rhizopus oryzae* were kindly provided by Dr. C. Howell (Southern Plains Agricultural Research Center, U.S. Department of Agriculture Agricultural Research Service, College Station, TX). The strains were routinely maintained on PDA (Difco) with *C. graminicola* grown under constant light. For screening of transformants, VMS and PDA were used.

Maize (inbred line B73) seedlings used in this study were grown in a hydroponic system (Djonovic et al., 2006a) or in the soil-less growth medium Metro-Mix 366 (Scotts). The disease resistance bioassay was performed with maize plants grown in a growth chamber at 25°C and with a 14-h photoperiod and 60% humidity.

Construction of *SM1*-Disruption and -Overexpression Vectors

To obtain the flanking regions of the *SM1* ORF for construction of deletion strains, a clone with a high-molecular-mass insert was isolated from *T. virens* BAC library as described previously (Djonovic et al., 2006a). This BAC clone was further digested with several restriction enzymes and the fragments subcloned into pBluescript II SK (±) to obtain the following vectors: pSZD14 (a 5.6-kb *Sac*I subclone), pSZD15 (a 1-kb *Hin*dIII subclone), and pSZD21 (a 7.5-kb *Cla*I subclone) that were used for sequencing and further analysis. Nucleotide sequencing was performed by a primer-walking strategy. All sequencing reactions were performed at the Gene Technologies Laboratory (Texas A&M University). DNA sequences were analyzed by DNA Strider 1.2 and Sequencher 4.1 (Gene Code Corporation). The QIAprep spin miniprep kit (Qiagen) was used for plasmid DNA purification.

The *SM1*-disruption vector (pSZD25) was constructed by replacing a 483-bp ORF with a 3.0-kb *Sma*I/*Eco*RV fragment of the *T. virens ARG2* gene (Baek and Kenerley, 1998), which served as a selectable marker for transformation. The resulting vector contained 2.1-kb and 1.7-kb noncoding upstream and downstream *SM1* sequences from pSZD21 flanking the selectable *ARG2* gene. The 5.6-kb *Pst*I/*Xba*I deletion cassette was isolated from pSZD25 and used for fungal transformation. An overexpression vector (pSZD26) was constructed for constitutive overproduction of the *SM1* gene by placing a 1-kb *Hin*dIII insert from pSDZ15 subclone containing the *SM1* ORF between the promoter and the terminator regions of the *T. virens GPD* (glyceraldehyde-3-P dehydrogenase) gene (Xu et al., 1996). To obtain overexpression strains, Tv10.4 was cotransformed with pSZD26 and pJMB4, which contain *ARG2* gene as a selectable marker.

Transformation and Screening of Transformants

Stable prototrophic transformants were selected by consecutive transfer of single colonies to VMS, PDA, and VMS (Baek and Kenerley, 1998). Screening of potential deletion and overexpression transformants was first performed by PCR. For deletion transformants, *SM1*-specific primers SmF and SmR (Djonovic et al., 2006a) were used to amplify the 264-bp PCR product from the wild-type genomic DNA. PCR amplification of the *SM1* fragment comprised 28 cycles (each cycle: 30 s at 94°C, 20 s at 55°C, and 20 s at 72°C). Strains that yielded no PCR product were further screened by Southern analysis. For overexpression strains, forward (SmF) and reverse (R50) 5′-TACAGACAATGATTCATG-3′ primers were designed to amplify a 1-kb fragment from the *SM1* ORF and a *GPD* terminator region from the overexpression cassette. Strains that yielded the 1-kb PCR product were selected for further analysis by Southern blotting. PCR amplification comprised 28 cycles (each cycle: 30 s at 94°C, 20 s at 40°C, and 30 s at 72°C). PCR was performed according to manufacturer's instructions using the Invitrogen *Taq* DNA polymerase kit.

Northern Analysis of Transformants

Conidia of 7-d-old *T. virens* wild type or transformants cultured on PDA were used to inoculate VMS (for deletion strains) or GYEC (15 g of Glc, 3 g of yeast extract, 5 g of casein hydrolysate per liter; for overexpression strains) liquid medium (Thomas and Kenerley, 1989) to a final concentration of 10^6 spores/mL. All cultures were incubated on an orbital shaker (130 rpm) at room temperature. After 5 and 3 d of growth for deletion and overexpression strains, respectively, the mycelia were harvested and rinsed thoroughly with sterile water. Total RNA was extracted from the harvested mycelia as described previously (Djonovic et al., 2006a). Fifteen micrograms for deletion stains and 10 μg for overexpression strains of total RNA per sample was denaturated, resolved in 1.5% agarose formaldehyde gel, transferred to a Hydrobond-N^+ nylon membrane (Amersham Biosciences), and hybridized overnight at 42°C using Ultrahyb (Ambion). The 264-bp (SmF-SmR) PCR product was used as *SM1* probe. The blots were reprobed with a 550-bp PCR product from the actin (*ACT*) gene (Djonovic et al., 2006b) as positive control for even loading.

SDS-PAGE and Western Analysis of Transformants

Fungal CFs were obtained by inoculating 200 mL of VMS with a conidial suspension of the appropriate fungal strain to a final concentration of 10^6/mL conidia. Following incubation on a rotary shaker at 130 rpm for 5 d at 23°C, CFs were successively filtered through a 10-μm NITEX nylon cloth (TETKO) and a 0.45-μm filter (Fisher Scientific). Proteins in the CFs were precipitated by 80% ammonium sulfate (Fisher Scientific). Pellets were resuspended in small amounts of 10 mM Tris, pH 7.8, and dialyzed against the same buffer (10-kD mwco; Pierce). Protein concentrations were determined by Bio-Rad Bradford microassay using bovine serum albumin as a standard. Protein extracts were subjected to SDS-PAGE following silver or Coomassie Brilliant Blue R-250 staining for protein visualization. Prestained SDS-PAGE broad-range molecular mass standards (Bio-Rad) or Kaleidoscope polypeptide molecular mass standards (Bio-Rad) were used for molecular mass determination.

Protein extracts (obtained as described above) were electrophoresed on SDS-PAGE gels and electroblotted to a nitrocellulose membrane (Osmonics). Sm1 protein was detected using Sm1 polyclonal antibodies (dilution 1:1,000) in a standard western-blot procedure (Sambrook et al., 1989). SDS-PAGE and

western analysis for comparison of Sm1 expression in wild-type, overexpression, and deletion strains hydroponically grown with maize seedlings was performed as we have reported previously (Djonovic et al., 2006a).

Phenotypic Analysis of Transformants

Cultures of selected transformants were compared with the wild-type strain for colony morphology and radial growth. Agar plugs from actively growing colonies were inoculated in the center of VMS, PDA, or WA plates. Plates were visually inspected for production of aerial hyphae, and color and morphology of the colony. Hyphal extension was recorded at 24 and 48 h of growth at 27°C. The border of the hyphal extension was marked each day, and each plate was photographed and the surface area of growth for each day determined using ImageJ software (http://rsb.info.nih.gov/ij/). Each treatment contained four repetitions and each experiment was repeated at least twice. Data were analyzed by ANOVA and Fisher's PLSD test ($P < 0.05$; Statview Version 5.0.1; SAS Institute).

For germination study, conidial suspensions (1×10^6 /mL) of each strain were plated onto PDA and incubated for 10 d at 27°C. Sterile glass slides were coated with approximately 1 mL of PDA. Conidia were gently removed from the plates and spread onto the coated slides at a concentration of 10^3 per slide. The slides were incubated for 12 h at 27°C in the dark in moist chambers. The number of germinated conidia was determined by observing 100 conidia along random transects on the slides. Each strain was replicated three times and the experiment repeated at least twice.

Two methods were used to assay hydrophobicity of the mutants and the wild type. In the first assay, each strain was assayed on PDA solid medium at 2 d prior to sporulation. Water drops (20 μL) were placed on the surface and digitally photographed immediately. The shape of the drop and the angle of contact of the drop with the colony provide an indication of the hydrophobicity of the surface (van der Mei et al., 1991). The second method involves separation of conidia into aqueous and organic phases to assess hydrophobicity (Whiteford and Spanu, 2001). Briefly, the conidia of each strain are diluted in phosphate buffered saline to obtain an optical density (OD) of about 0.7 at 420 nm. A 750-μL aliquot of this dilution is added to 250 μL of hexadecane. This solution is vortexed for 1 min, allowed to settle for 5 min, 200 μL is added to a 96-well plate, and the optical density recorded with a microwell plate reader. Increasing amounts of triton were added to the conidia prior to the addition of the hexadecane to lower the affinity of the conidia for the aqueous-organic interphase.

For experiment to detect the coiling phase of mycoparasitism, glass slides were coated with VMS by dipping the slides into the molten agar. An agar plug (3 mm in diameter) of the selected strain of *T. virens* was added to one side of the coated slide and incubated at 27°C for 36 h. The isolate of *R. oryzae* was then placed (agar plugs) opposite the hyphae of *T. virens* (1–1.5 cm) and the slides further incubated at 27°C. After 24 to 48 h, the slides were microscopically examined (see section "Microscopy and Imaging") for evidence of mycoparasitism (coiling) at the zones of interaction.

Infection of *Trichoderma*-Treated Maize Plants with *C. graminicola*

Cultures of *C. graminicola* for inoculation were grown for 14 d on PDA at room temperature under constant light. Conidia were scraped from plates, filtered through Miracloth (Calbiochem), and washed three times in distilled water, followed by centrifugation for 1 min at 10,000 rpm. Conidia were counted by using a hemacytometer, spore suspension was adjusted to 6.5×10^4 conidia/mL, and Tween 20 was added to a final concentration of 0.005%.

Inoculation of maize seeds with the wild type, KO26, KO46, OE38, or OE39 was performed by first coating the seeds with a latex sticker (Phoplex AC-33; Rohm and Haas), followed by the addition of fine powder of chlamydospore preparations (Weaver and Kenerley, 2005). Control (nontreated seeds) and fungus-coated seeds were planted in containers (3.81 × 20.9 cm; Stuewe & Sons) containing a soil-less mix (Metro-Mix 366) and incubated in a growth chamber (EGC) 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 (78.7 × 63.5 × 6.9 cm; Molded Fiber Glass Tray Company) and taping the leaves onto moist paper towels in the bottom of the trays. The third leaf from all plants was inoculated with six droplets (10 μL), each containing 650 conidia, placed on the adaxial side, away from the midvein of the leaf. The trays were sealed with plastic wrap to maintain the moisture and incubated for 24 h. After incubation, the plants were left for 3 to 4 h for droplets to dry, and the location of each droplet was marked to allow for later identification. Then, the plants were gently untaped, returned to controlled growth conditions, and monitored for the appearance of symptoms. Following a 4-d incubation period, inoculated leaves were scanned and percentage of the leaf area with symptomatic lesions was determined using ImageJ software. Each treatment, consisting of six spots per leaf per one plant, was replicated four times, and the experiment was repeated three times. Data were analyzed by ANOVA and Fisher's PLSD test ($P < 0.05$; Statview Version 5.0.1; SAS Institute).

T. virens-Root Colonization Studies

Plants were removed from the hydroponic system and the roots were excised from the plant. The entire root system was placed in 100 mL of 1% sodium hexametaphosphate and 0.1% Tergitol P10 (Sigma Aldrich) and shaken for 45 min at 100 rpm on an orbital shaker. The roots were then removed, rinsed twice in sterile water, and then shaken for 45 min in 100 mL of sterile water. Root systems were then spread apart in a petri dish (150 mm diameter) containing sterile water and harvested (excised with a scalpel) for plating on GVSM (Park et al., 1992). Excised root fragments were blotted dry on sterile filter paper before placement in GVSM. After 48 h of incubation at 27°C, the total length and length colonized by *T. virens* were measured. As these roots were extensively colonized, the length of the root from which numerous coalescing colonies emerged was used as a measure of colonization.

After 14 d of growth in soil, plants were removed from containers, shoots excised from the root systems, root systems extensively washed under tap water and collected on a sieve. The same washing protocol was followed as cited above. Roots were excised and plated on GVSM. Over a 48 h period of incubation, colonies of *T. virens* growing from the root fragments onto GVSM were assessed. Root fragments were measured and the data are expressed as colonies/cm of root length.

GFP-Tagged *T. virens* Strain Construction

T. virens wild-type and KO25 strains expressing *GFP* were constructed by cotransformation (Djonovic et al., 2007) using the plasmids pTEFEGFP and pCSN44. pTEFEGFP contains the *GFP* coding sequence under the action of the *Aureobasidium pullulans* translation elongation factor promoter and the *Aspergillus awamori* glucoamylase terminator (Vanden Wymelenberg et al., 1997). Plasmid pCSN44 containing *HPH* (Staben et al., 1989) was used as a selectable marker for cotransformation procedure.

Stable cotransformants were selected on PDA plates supplemented with hygromycin (Dave et al., 1994). Resistant colonies were screened for the presence of green fluorescence and the insertion of pTEFEGFP was further analyzed by Southern-blot analysis. Genomic DNA was prepared and digested using *Bam*HI or *Hin*dIII restriction endonucleases. DNA digests were electrophoresed in 1% agarose gels, blotted onto nylon membranes, and probed with *GFP* or *HPH* gene-specific probes. The growth rate and colony morphology of selected cotransformants were similar to the original strains as judged by growth comparison on PDA, VMS, and WA plates.

Expression Analysis of Maize Defense-Related Genes

Expression of defense-related genes was analyzed in maize seedlings (roots and leaves) grown hydroponically without or with wild-type, *SM1*-deletion, and *SM1*-overexpression strains (as described above). In the experiment with maize seeds coated with wild-type strain, two deletion strains, and two overexpression strains (described in section above) and challenged with *C. graminicola*, the seedlings were grown in soil. Plants were harvested after 14 d of growth with *Trichoderma*, 1 d after incubation with the pathogen, and 4 d after inoculation when the symptoms were evaluated. All harvested samples were immediately frozen in liquid nitrogen. Total RNA from maize was extracted using TRI reagent (Molecular Research Center) according to the manufacturer's protocols.

Expression of plant defense-related genes in this study was assessed by sqRT-PCR. Sequences for gene-specific primer pairs were obtained from published studies (*ZmOPR2*, *ZmOPR7*, and *ZmOPR8* [Zhang et al., 2005]; *ZmPAL* and *ZmHPL* [Farag et al., 2005]; *AOS* [He et al., 2005]; and *ZmLOX10* [Nemchenko et al., 2006]), whereas primer pairs for *PR* genes were designed based on sequences available in the GenBank database, namely, *ZmPR1* (U82200) and *ZmPR5* (U82201; Morris et al., 1998; Gao et al., 2007). The forward and reverse primers were: 5′-AAC AAT GGC ACC GAG GCT AGC

GT-3′and 5′-GTA TGC ATG ACA GTC TAG TAG GG-3′, respectively, for *ZmPR1*, and 5′-TAG CTC TAT AGC TCG AGT ATT GCT-3′ and 5′-TCA CTA GCC CAT GCA TGC AGA GC-3′ for *ZmPR5*. Maize *GAPc* (Farag et al., 2005) was used as an internal control. Extracted RNA was DNase treated and cleaned using a DNA-free kit (Ambion). Total RNA (2.5 μg) was reverse transcribed with a first-strand cDNA synthesis kit (G.E. Healthcare) using the random hexamer pd$(N)_6$ as a primer. To define the optimal number of PCR cycles for linear amplification of each gene, a range of PCR amplifications was performed. Subsequently, PCR products were electrophoresed, stained with ethidium bromide, and band signals quantified by Scion image software (http://www.scioncorp.com/). PCR amplification of *PAL* and *GAPc* gene fragments in both hydroponic and soil-grown maize seedlings experiments comprised 25 cycles (each cycle: 30 s at 94°C, 30 s at 58°C, and 30 s at 72°C) for leaf RNA and 23 cycles for *PAL* root RNA. To detect the expression of genes that were expressed at very low levels (i.e. *PR1*, *PR5*, *OPR2*, and *LOX10*), the number of PCR cycles for those genes was increased by an additional one to three cycles. PCR amplification for *PR1*, *PR5*, *OPR7*, *OPR8*, and *HPL* gene fragments comprised 28 cycles (each cycle: 30 s at 94°C, 30 s at 58°C, and 30 s at 72°C); *OPR2*: 28 cycles (each cycle: 30 s at 94°C, 30 s at 53°C, and 1.3 min at 72°C); *AOS*: 28 cycles (each cycle: 30 s at 94°C, 30 s at 56°C, and 28 s at 72°C); and *LOX10*: 29 cycles (each cycle: 30 s at 94°C, 30 s at 50°C, and 15 s at 72°C). The negative control for RT-PCR was DNase-treated and cleaned RNA reactions without reverse transcription. PCR was performed according to the manufacturer's instructions using the Invitrogen *Taq* DNA polymerase kit. PCR products were electrophoresed on 2% agarose gels, and band intensities compared within each experiment after ethidium bromide staining and intensities of bands were quantified using Scion Image software.

In Planta *SM1* mRNA Detection

To detect *SM1* mRNA in roots of maize plants inoculated with *T. virens* wild-type or mutant strains, total RNA from roots was extracted and used for RT-PCR experiments. After harvesting, the roots were washed with tergitol-metaphosphate solution as described above and total RNA was extracted. Reverse transcription reactions were performed using 2.5 μg of total RNA. The reactions for *SM1* and *ACT* genes from *T. virens* were carried out for 40 cycles of 94°C (30 s), 58°C (30 s), and 72°C (30 s), plus a single final step at 72°C for 5 min, and using the primers 5′GACACTGGTGAGACAAGCAC3′ (forward) and 5′TTAGAGACCGCAGTTCTTAACAG3′ (reverse) for the *SM1* gene or 5′GTATCATGATCGGTATGGGTCAGAA3′ (forward) and 5′TAGAAGGTGTGGTGCCAGATCTT3′ (reverse) for the *ACT* gene. PCR reactions for *GAPc* were conducted on the conditions mentioned above. PCR products were separated on 2% agarose gels, ethidium bromide stained, and visualized under UV light. Maize defense gene expression was profiled from two independent experiments. As the results from the two experiments were similar, the data from one trial are presented here.

Microscopy and Imaging

Microscopy of coiling experiments was carried out with an Olympus BX60 microscope and 100× magnification. The images were captured using the Q-Free Olympus camera and processed using Adobe Photoshop imaging software. Fluorescence microscopy was performed with an Olympus BX51 fluorescence microscope. Excitation and emission wavelengths of 470 to 490 and 510 to 550 nm, respectively, and 200× magnification were used. Images were recorded using an Olympus DP70 camera and processed with DPController 1.1.165 software.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Coiling capability of wild-type, *SM1*-deletion, and *SM1*-overexpression strains.

Supplemental Table S1. Growth analysis of agar plates inoculated with wild-type, *SM1*-deletion, or *SM1*-overexpression strains.

ACKNOWLEDGMENTS

We thank Dr. Serenella Sukno for sharing the protocol for *Colletotrichum* inoculation of maize; Dr. Brain Shaw for expert assistance in fluorescence microscopy; Dr. Daniel Cullen for providing the GFP vector and Dr. Deborah Bell-Pedersen for providing the hygromycin vector; Dr. Xiquan Gao and Dr. Andriy Nemchenko for providing the primers for maize gene expression; and Gloria Vittone for assistance with plant bioassays and hydrophobicity assay.

Received June 10, 2007; accepted September 17, 2007; published September 20, 2007.

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