Plant-Derived Sucrose Is a Key Element in the Symbiotic Association between *Trichoderma virens* and Maize Plants[1[C][W]]

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Fungal species belonging to the genus *Trichoderma* colonize the rhizosphere of many plants, resulting in beneficial effects such as increased resistance to pathogens and greater yield and productivity. However, the molecular mechanisms that govern the recognition and association between *Trichoderma* and their hosts are still largely unknown. In this report, we demonstrate that plant-derived sucrose (Suc) is an important resource provided to *Trichoderma* cells and is also associated with the control of root colonization. We describe the identification and characterization of an intracellular invertase from *Trichoderma virens* (TvInv) important for the mechanisms that control the symbiotic association and fungal growth in the presence of Suc. Gene expression studies revealed that the hydrolysis of plant-derived Suc in *T. virens* is necessary for the up-regulation of Sm1, the *Trichoderma*-secreted elicitor that systemically activates the defense mechanisms in leaves. We determined that as a result of colonization of maize (*Zea mays*) roots by *T. virens*, photosynthetic rate increases in leaves and the functional expression of *tvinv* is crucial for such effect. In agreement, the steady-state levels of mRNA for Rubisco small subunit and the oxygen-evolving enhancer 3-1 were increased in leaves of plants colonized by wild-type *T. virens*. We conclude that during the symbiosis, the sucrolytic activity in the fungal cells affects the sink activity of roots, directing carbon partitioning toward roots and increasing the rate of photosynthesis in leaves. A discussion of the role of Suc in controlling the fungal proliferation on roots and its pivotal role in the coordination of plant-microbe associations is provided.

Animals and plants are exposed to a complex and dynamic consortia of beneficial (nonpathogenic) bacteria and fungi, such as in the oral cavity, the intestines, and the rhizosphere (Hooper et al., 1998; McFall-Ngai, 1998; Palmer et al., 2001; Hirsch et al., 2003; Dethlefsen et al., 2007). These beneficial interactions usually result in a heightened resistance to prevent subsequent pathogen attacks or establish a commensal association with the host. Therefore, the identification and understanding of the molecular mechanisms that regulate such nonpathogenic associations is warranted.

Among the mutualistic associations in the rhizosphere, the most studied systems are the symbioses between rhizobacteria and leguminous plants and the mycorrhizal associations of fungal species with numerous trees and crops (Hirsch et al., 2003; Sarma et al., 2007; Parniske, 2008). During these interactions, a molecular dialogue between both partners leads to metabolic modifications of host tissues, providing a favorable environment for the symbiont. In the case of mycorrhizal associations, plant-derived carbohydrates are exchanged for essential nutrients supplied by the fungus (Hahn and Mendgen, 2001; Nehls et al., 2001). It has been proposed that enzymes from the plant hydrolyze Suc, and the monosaccharides that are released are transported by the fungal cells (Blee and Anderson, 1998; Nehls et al., 2001). Importantly, the hexose concentration in the apoplast is speculated to control fungal metabolism during the symbiosis, with the monosaccharides acting as nutrients and, at the same time, as signals that regulate gene expression in the fungal cell (Nehls et al., 2001; Nehls, 2008).

Trichoderma species are nonpathogenic, soil-borne (free-living) fungi that also colonize roots of numerous plants (Harman et al., 2004). These fungi are recognized for their important benefits to agriculture, such as their ability to protect crops against diseases and increase crop yield under field conditions (Harman et al., 2004). *Trichoderma*-treated plants have demonstrated an increase in biomass production (Chang et al., 1986), induced systemic resistance (ISR) to diseases (Yedidia et al., 1999; Djonović et al., 2006, 2007a; Viterbo et al., 2007), increased nutrient uptake, as well as higher efficiency for fertilizer utilization and seed germination rates (Yedidia et al., 2001). The hyphae of *Trichoderma* grow mainly in the intercellular

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spaces of roots, where they are restricted to epidermal cell layers and a few cells beyond this level (Yedidia et al., 1999; Harman et al., 2004). This invasion pattern is suggestive of a colonization process strictly controlled by both partners; however, the biochemical pathways involved in the control of such an association are not well understood.

One of the most intriguing outcomes of plant-*Trichoderma* associations is the systemic control of gene expression in distant leaves. Proteomic and transcriptomic studies have demonstrated that when *Trichoderma* colonizes plant roots, it induces a systemic change in the expression of plant genes involved in the scavenging of reactive oxygen species, stress responses, isoprenoid oxylipins and ethylene biosynthesis, photosynthesis, photorespiration, and carbohydrate metabolism (Djonović et al., 2006, 2007a; Alfano et al., 2007; Segarra et al., 2007, Shoresh and Harman, 2008). The effect of peptaibols (peptide antibiotics) and the small protein Sm1, both produced by the fungal cells, has been shown to be responsible for the systemic activation of the defense responses in leaves (Djonović et al., 2006; Viterbo et al., 2007). In cucumber (*Cucumis sativus*) plants, a mitogen-activated protein kinase was also demonstrated to be involved in the signaling events that lead to the induction of resistance (Shoresh et al., 2006). However, the mechanisms that *Trichoderma* employs to detect the presence of plants in the surroundings to initiate the root invasion remain to be discovered.

By secreting compounds such as organic acids, amino acids, and sugars into the rhizosphere, plants provide carbon-rich resources and also establish a molecular communication with soil microbes that triggers root colonization (Bais et al., 2006). One of the main carbohydrates secreted by plant roots is Suc, which has been detected in high concentrations near root tips (Kraffczyk et al., 1984; Jaeger et al., 1999; Mahmood et al., 2002). However, the function of this exuded disaccharide has been barely explored, with studies mainly focused on its relationship to the uptake of minerals and in the association of plants with bacterial communities (Kraffczyk et al., 1984; Jaeger et al., 1999; Mahmood et al., 2002; Baudoin et al., 2003). On the other hand, it is well established that Suc plays vital roles in the control of plant developmental processes. As the main product of photosynthesis, Suc is produced in source leaves and exported to heterotrophic tissues. Suc controls carbohydrate distribution, acts as an important molecule in carbohydrate-mediated signaling in the plant, and also is degraded by plant cells to yield a carbon source for microbes during plant-microbe associations (Dennis and Blakeley, 2000; Koch, 2004). During the establishment of many plant-pathogen associations, Suc hydrolysis is stimulated in plant cells to enhance the flux of monosaccharides toward the infection site (Biemelt and Sonnewald, 2006). In addition, the presence of invertases (Inv; β-fructofuranosidases belonging to the glycoside hydrolase family GH32) that hydrolyze Suc has been described in some pathogenic fungal species such as *Thermomyces lanuginosus*, *Uromyces fabae*, and *Aspergillus niger* (Chaudhuri et al., 1999; Rubio and Navarro, 2006; Voegele et al., 2006). In the case of *U. fabae*, the induction of fungal Inv expression was detected upon penetration of the fungus into the leaf with high expression levels in haustoria. In contrast, with the exception of *Laccaria bicolor*, mycorrhizal fungi lack sucrolytic activities and rely on monosaccharides provided by the plant (Nehls et al., 2001). Most *Trichoderma* species are able to grow in vitro using Suc as a substrate, suggesting the presence of enzymes capable of hydrolyzing the disaccharide in fungal cells. Moreover, previous studies with *Trichoderma virens* demonstrated that the expression of *tex1* and *sm1*, involved in the induction of ISR responses, was specifically up-regulated in the presence of Suc or plant roots (Djonović et al., 2006; Viterbo et al., 2007). Based on these observations, we hypothesize plant-derived Suc (similarly to monosaccharides in mycorrhiza) as a nutrient for fungal cells and also as part of the molecular dialogue between *Trichoderma* and root cells.

In this study, we investigated the presence of sucrolytic activity in *T. virens* cells and its relevance to colonization of maize (*Zea mays*) roots by hyphae of *T. virens*. We describe the identification and functional characterization of *tvinv*, a gene encoding an intracellular Inv in *T. virens*. Reverse genetic experiments demonstrated that TvInv is responsible for Suc hydrolysis and the normal growth of *T. virens* in the presence of Suc. Our data suggest that plant-derived Suc represents not only a carbon source for the fungal cells but also that the extent of root colonization, production of fungal chlamydospores, and systemic induction of photosynthesis in leaves depend upon its metabolism. Although carbohydrate metabolism, mainly monosaccharides, has been shown to be involved in mycorrhizal associations, we are presenting to the best of our knowledge a novel scenario for microbe-host interaction in the rhizosphere. The ability of *Trichoderma* to transport and metabolize Suc released by plants may be a specific and fine-tuned strategy for root perception and subsequent colonization that makes these fungal species unique among the symbiotic root colonizers.

RESULTS

Identification and Characterization of an Inv from *T. virens*

The hypothesis that Suc is part of the molecular communication during *Trichoderma*-plant associations prompted us to investigate enzymes from *T. virens* that are involved in Suc metabolism. Using an acid Inv from *Saccharomyces cerevisiae* (accession no. YJM789) as a query, we identified its homolog (TvInv) in the *T. virens* Gv29-8 genome (www.genome.jgi-psf.org/Trive1/Trive1.home.html). The genomic sequence

contains a predicted 1,902-bp open reading frame coding for a putative 633-amino acid protein (Supplemental Fig. S1). The deduced amino acid sequence is 40% identical to the *S. cerevisiae* Inv and similar to SucB, the intracellular acid Inv from *A. niger* (Goosen et al., 2007). TvInv does not contain a transit peptide for secretion or glycosylation sites. Sequence analysis and phylogenetic reconstruction (including homologous sequences from plants, bacteria, and fungi) grouped TvInv with fungal β-fructofuranosidases, levanases, inulinases, and other members of the glycosyl hydrolase family 32 (Fig. 1A). The deduced amino acid sequence of the putative protein displayed the eight conserved motifs associated with catalytic activity in known Inv with acidic optimum pH (Goosen et al., 2007; Supplemental Fig. S1). Among these motifs are four amino acid residues (NDPXG) of the pentapeptide NDPNG, which has been shown to be essential for enzymatic activity in the *S. cerevisiae* homolog (Reddy and Maley, 1990; Sturm, 1999). Interestingly, we also identified a TvInv homolog sequence in the *Trichoderma atroviride* genome, but no homolog was detected in the *Trichoderma reesei* genome (Martinez et al., 2008). A previous report on the carbon utilization profile of *T. reesei* (*Hypocrea jecorina*) demonstrated that this fungal species grows very slowly in a medium containing Suc as the sole carbon source (Druzhinina et al., 2006). In good agreement with these observations, *T. reesei* presented poor hyphal development and sparse sporulation compared with *T. virens* and *T. atroviride* when cultured on Vogel's minimal medium supplemented with Suc (VMS; Fig. 1B). To better understand the relevance of TvInv for *T. virens* growth, we compared gene expression and Inv activity under different cultural conditions. The enzymatic activity was analyzed in protein extracts from fungal mycelia incubated in the presence of different carbon sources or collected from hydroponic systems containing maize seedlings. The enzymatic activity was only detected in fungal cells cultivated in the presence of Suc or in the hydroponic systems (Fig. 2A). Likewise, northern-blot assays demonstrated that *tvinv* was expressed only in Suc-containing medium or in the presence of plants (Fig. 2B). Additionally, the presence of alternative substrates for growth, such as chitin, fungal cell walls, and starch, failed to induce the expression of *tvinv* (Supplemental Fig. S2).

A time-course experiment demonstrated the presence of TvInv enzymatic activity and mRNA within 24 h of incubation of the fungal hyphae with Suc, increasing after 48 h of culture (Fig. 2, C and D). Inv activity was not detected in culture filtrates from any conditions tested. Specific changes were detected in the intracellular proteomes of *T. virens* mycelia incubated in the presence of Suc or maize plants in hydroponic systems (Fig. 2E). Compared with the other

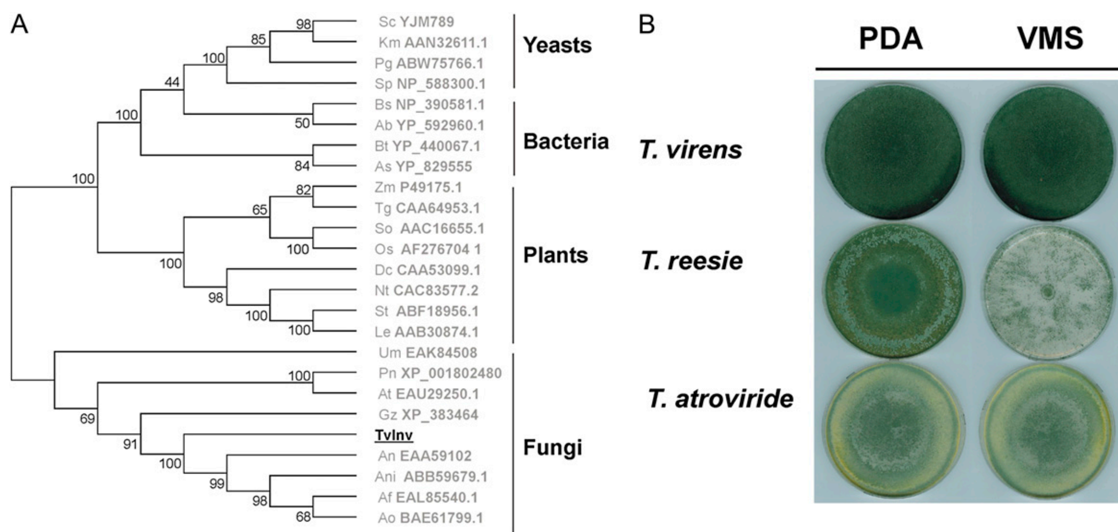

Figure 1. Presence of a putative acid Inv (TvInv) similar to β-fructofuranosidases in the *T. virens* genome sequence. A, Sequence comparison and phylogenetic reconstruction of β-fructofuranosidases from different organisms, including TvInv. TvInv is presented in boldface and underlined. The tree was constructed with MEGA 4.1 with a bootstrap trial of 1,000. The name of each sequence represents the initials of the organism followed by the GenBank accession number. Ab, *Acidobacteria bacterium* Ellin345; Af, *Aspergillus fumigatus* Af293; An, *Aspergillus nidulans*; Ani, *A. niger*; Ao, *Aspergillus oryzae*; As, *Arthrobacter* sp. FB24; At, *Aspergillus terreus*; Bs, *Bacillus subtilis* subsp. *subtilis* strain 168; Bt, *Burkholderia thailandensis* E264; Dc, *Daucus carota*; Gz, *Gibberella zeae*; Km, *Kluyveromyces marxianus*; Le, *Lycopersicon esculentum* (now *Solanum lycopersicum*); Nt, *N. tabacum*; Os, *Oryza sativa*; Pg, *Pichia guilliermondii*; Pn, *Phaeosphaeria nodorum*; Sc, *S. cerevisiae*; So, *Saccharum officinarum*; Sp, *Schizosaccharomyces pombe* 972h; St, *Solanum tuberosum*; Tg, *Tulipa gesneriana*; Um, *Ustilagus maydis* 521; Zm, *Z. mays*. B, In order to compare *T. virens*, *T. atroviride*, and *T. reesei* ability to grow using Suc, spores of each strain were cultured on VMS or PDA medium and photographed after 7 d. [See online article for color version of this figure.]

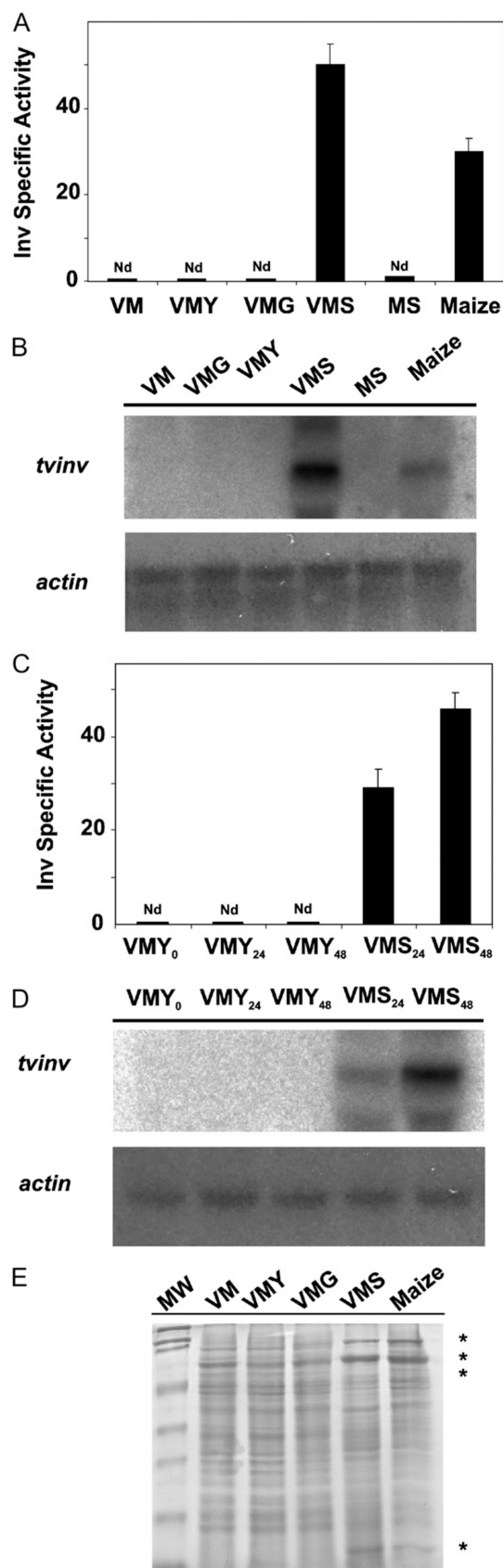

Figure 2. TvInv is expressed in the presence of Suc and maize plants. A and B, The levels of enzymatic activity (A) and gene expression (northern blot; B) for TvInv were determined in *T. virens* hyphae cultured for 4 d in VM or VM supplemented with 1.5% (v/v) glycerol (VMY), Glc (VMG), or Suc (VMS). Also, the expression was tested in fungal tissue that was coinoculated with maize plants in hydroponic systems (Maize) or cultivated in Murashige and Skoog medium (MS) as a control. C and D, The induction of *tvinv* in the presence of Suc was determined as enzymatic activity (C) or mRNA steady state (northern blot; D). *T. virens* spores were inoculated in VMY medium, and after 48 h the mycelia was harvested and transferred to fresh VMY or VMS; samples were collected after 0, 24, and 48 h of incubation. All of the experiments were independently repeated twice with similar results. The bars depict mean values ± SD. E, Protein profile in fungal cells incubated in the presence of VM, VMY, VMG, or VMS or when grown with maize plants. Proteins specifically accumulated in cells cultured in VMS or with maize plants are indicated by asterisks. Nd, Not detected.

conditions tested, several polypeptides (indicated by asterisks) differentially accumulated in VMS or in the hydroponic system. These results suggested that the presence of Suc in the medium elicits, in *T. virens*, very similar responses to those observed in the presence of plant roots, including the up-regulation of *tvinv*.

To provide a more complete understanding of TvInv and its biochemical properties, the enzyme was purified from mycelia collected after 48 h of incubation in VMS medium. Protein extracts were prepared, and the enzyme was purified through anion-exchange chromatography followed by gel-filtration chromatography (data not shown). The purified enzyme from *T. virens* displayed biochemical properties similar to those described for the intracellular Inv from *A. niger* (Goosen et al., 2007; Fig. 3). The enzyme presented an optimum pH of 5.0 for activity and a K_m of 3 ± 0.5 mM for Suc. The purified enzyme hydrolyzed stachyose and raffinose but not trehalose or melizitose, confirming its hydrolytic activity of β-fructofuranosidase. The enzymatic activity was inhibited by Fru and by $CuCl_2$. These results confirmed that *T. virens* does have an intracellular acid Inv with biochemical properties similar to those of SacB from *A. niger* and that its expression is only activated in the presence of Suc or plant roots.

Loss-of-Function Experiments Confirmed that *tvinv* Encodes a Functional Acid Inv

To confirm that *tvinv* encodes a functional Inv and to perform functional studies, we generated *T. virens* strains impaired in the expression of *tvinv*. A disruption cassette was constructed using the double-joint PCR strategy (Supplemental Fig. S3; Kuwayama et al., 2002). The final PCR amplification product was transformed into protoplasts of *T. virens* Gv29-8, and transformed protoplasts were selected for hygromycin resistance. The stable transformants (18 independent transformants) were evaluated for *tvinv* disruption by PCR using genomic DNA as a template. Two transformants, designated Δ*tvinv2* and Δ*tvinv5*, did not contain the wild-type allele (Supplemental Fig. S4A),

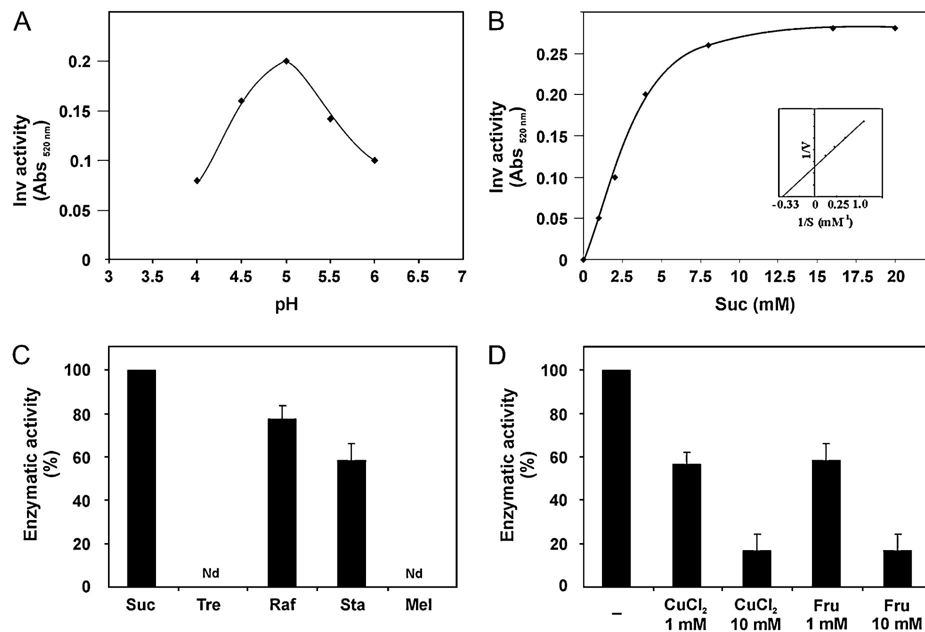

Figure 3. Biochemical characterization of TvInv. A, Optimum pH for activity was assayed in the presence of 50 mM Suc and 100 mM citrate-acetate buffer. This assay was repeated twice with similar results. B, TvInv activity (pH 5) under a gradient of Suc concentration (1–20 mM) was determined, and the K_m value for the enzyme was calculated from three different experiments with similar results. The values were calculated from Lineweaver-Burk plots (inset). We present means ± SD of three independent experiments. C, The activity of the purified enzyme was assayed in the presence of 50 mM Suc, trehalose (Tre), raffinose (Raf), stachyose (Sta), and melizitose (Mel) at pH 5. The products of the enzymatic activity were assayed by Somogyi-Nelson methodology. D, The enzymatic activity of TvInv was assayed at pH 5 in the presence of 50 mM Suc with the addition of 0, 1, or 10 mM $CuCl_2$ or Fru. Nd, Not detected.

and no mRNA for *tvinv* was detected in either of them (Supplemental Fig. S4B). These two null mutants were further analyzed by Southern blotting. The blots were probed with the downstream 1.1-kb DNA fragment used for double-joint PCR (Supplemental Fig. S3, A and B). A 4.1-kb hybridizing fragment was expected for the wild type, a 5.8-kb fragment was expected in the null mutant, and both bands were expected in ectopic integration events (Supplemental Fig. S3A). Both Δ*tvinv2* and Δ*tvinv5* presented only the expected hybridization pattern for the null allele (Fig. 4A). As expected, the Inv activity was not detected in protein extracts from the mutant strains cultured in the presence of Suc (Supplemental Fig. S4C) or when inoculated into hydroponic systems containing maize seedlings (Fig. 4B). The absence of Inv activity in protein extracts of the mutant strains confirmed that *tvinv* encodes the intracellular acid Inv up-regulated in the presence of Suc or maize seedlings.

The Loss of Functional *tvinv* Suppresses the Ability of *T. virens* to Grow Using Suc as a Carbon Source

In the rhizosphere, a plethora of natural compounds are delivered by plants and microorganisms. The ability of the soil community to perceive and respond to such stimuli controls its success as a mutualistic ecosystem. Therefore, we sought to determine the role of *tvinv* in the growth and development of *T. virens* in the presence of Suc and the extent to which mutant strains were affected in their ability to utilize Suc. Colonies of the null mutant strains, unlike the wild type (Gv29-8), were unable to respond to the addition of Suc (VMS) in the medium. In the presence of Suc, both mutant strains grew at a slower rate, resembling the wild-type strain incubated on minimal medium (Fig. 5A). However, the mutant strains grew at a rate similar to that of Gv29-8 on the other media tested (water-agar [WA], Vogel's minimal medium [VM], or potato dextrose agar [PDA]). When cultured on VMS, initial sporulation in the mutant strains was delayed compared with the wild type (occurring at day 4), but after 10 d in culture, the wild type and mutant strains were similar in their level of sporulation (Fig. 5B). Thus, sporulation initiation was delayed in the mutant strains, reaching similar levels after a prolonged incubation time. No difference in the sporulation process was detected for Gv29-8, Δ*tvinv2*, and Δ*tvinv5* in WA, VM, or PDA medium.

The strains impaired in the expression of *tvinv* were not affected in their rate of conidial germination with respect to the wild-type strain on WA, VM, VMS, or PDA. However, on VMS medium, a noticeable restriction in the development of the germlings was evident (Fig. 6A). A similar behavior was also observed for Δ*tvinv* chlamydospores, which presented similar ger-

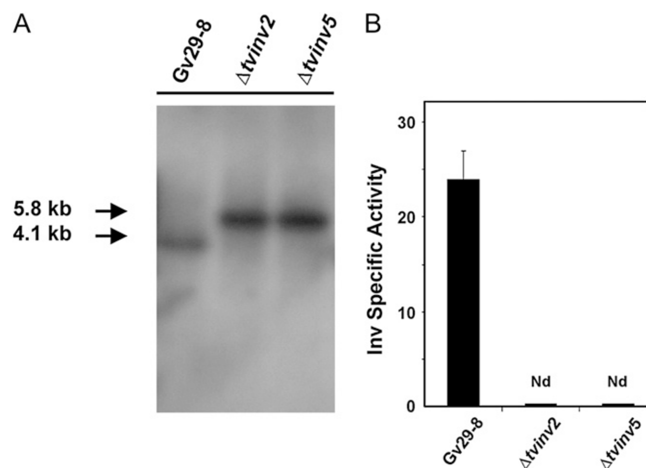

Figure 4. Confirmation of *tvinv* disruption. A, Southern-blot analysis of *T. virens* wild-type strain (Gv29-8) and *tvinv* deletion transformants (Δ*tvinv2* and Δ*tvinv5*). An autoradiograph of a DNA gel blot hybridized with [^{32}P]dCTP-labeled probe is shown in Supplemental Figure S2A. Fifteen micrograms of genomic DNA was digested with *Hin*dIII and loaded in each lane. The expected molecular sizes for native and deletion events are indicated on the left. B, TvInv activity in wild-type and mutant cells collected from hydroponic systems. Inv activity was determined in protein extracts from *T. virens* Gv29-8 and Δ*tvinv* strains coinoculated with maize plants in hydroponic systems. The bar depicts mean values ± SD of two independent experiments. Nd, Not detected.

mination in WA, VM, VMS, or PDA, but the further development of the germlings was impaired only in VMS (data not shown).

Colony morphology and development assays on sugar gradients demonstrated that after conidia germination, the mutant germlings preferably used Glc over Suc to support colony development (Fig. 6, B and C). In this experiment, an aliquot of the spore suspension was placed in the center of a VM plate between two paper discs (sugar 1 and sugar 2) saturated with either Suc or Glc to create a concentration gradient for each sugar after diffusion (Fig. 6B). After incubation for 24 h, the mutant strains preferably grew toward the paper disc saturated with Glc, generating an asymmetric colony (Fig. 6C, row Glc/Suc). The slight growth of the mutants toward the Suc discs is comparable to the slight growth detected in the presence of Suc (Fig. 6C, row Suc/Suc). These results suggested that TvInv is a central component for the utilization of Suc and that Suc may control *T. virens* development in the rhizosphere.

Disruption of *tvinv* Disturbs the Symbiotic Association between *T. virens* and Maize Roots

To further analyze the involvement of Suc during plant-*Trichoderma* associations, we tested the ability of maize plants to exude Suc into the rhizosphere and the ability of strains of *T. virens* to colonize the roots. By thin-layer chromatography, we detected the presence of Suc in root exudates of 7-d-old maize plants, including the inbred lines B73 and Mo940 as well as the hybrid Silver Queen (Supplemental Fig. S5). To determine the effects that plant-derived Suc may have on the fungal cells, we compared the ability of Gv29-8, Δ*tvinv2*, and Δ*tvinv5* to colonize roots of B73 (low levels of Suc exudation) and Mo940 (high levels of Suc exudation). The root systems of 2-week-old maize

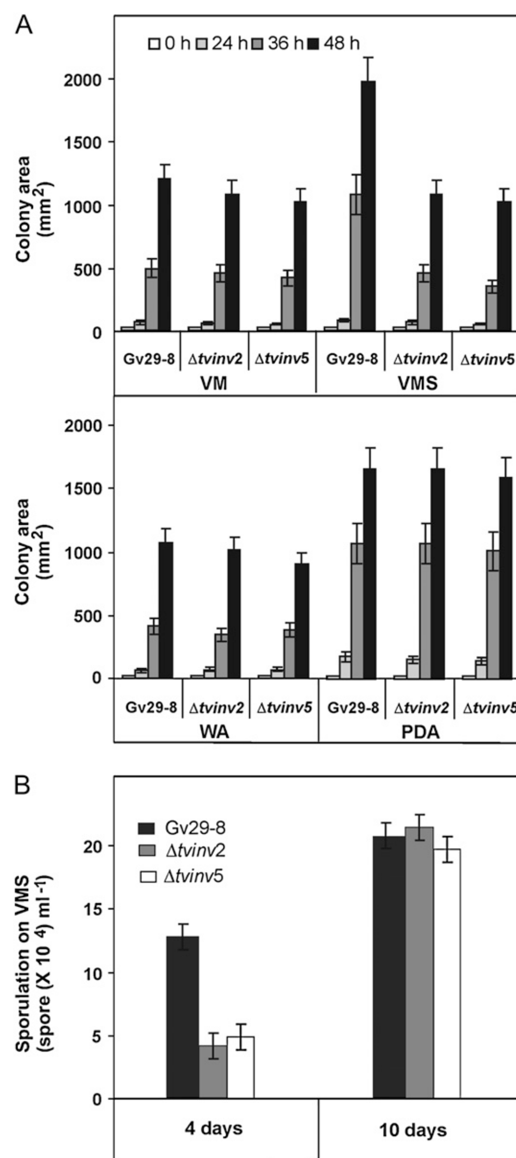

Figure 5. Comparison of growth and sporulation among Gv29-8, Δ*tvinv2*, and Δ*tvinv5*. A, The three strains were inoculated in the center of plates containing WA, VM, VMS, or PDA medium. The plates were incubated at 27°C, and growth was monitored at 0, 24, 36, and 48 h. For every strain, three plates with each medium were inoculated in each experiment. The bars depict mean values ± SD of three independent experiments. B, For each strain, a suspension of 10^3 spores mL^{-1} was prepared, and 200 μL was spread on each of three VMS plates. After 4 and 10 d, spores were determined from three random plugs (3 mm diameter) per plate. The bars depict mean values ± SD from two independent experiments.

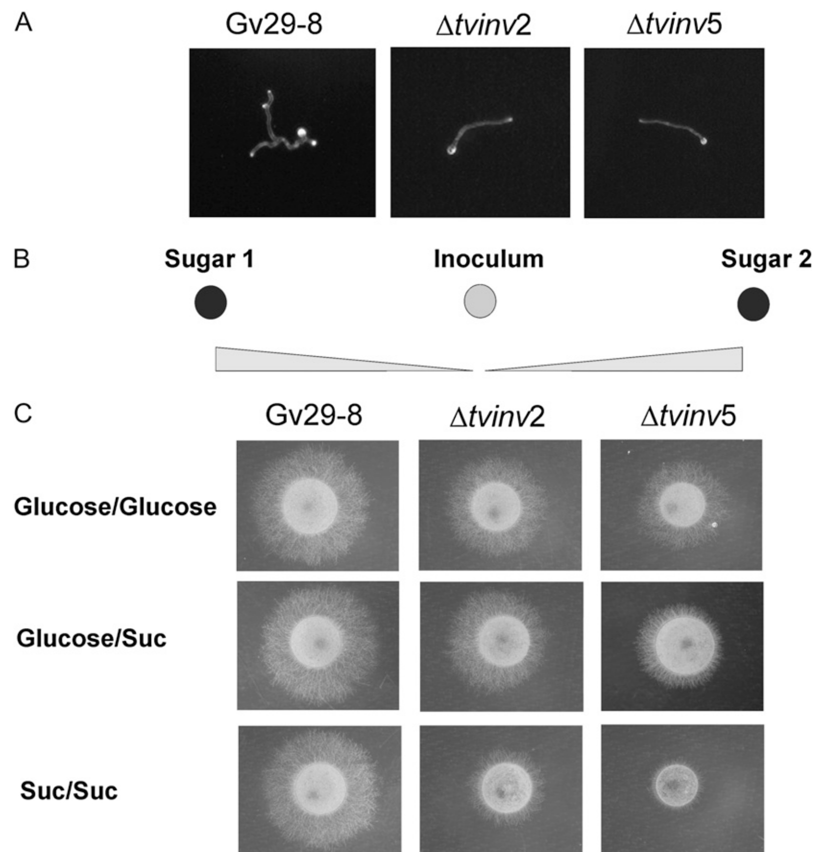

Figure 6. Germling development and Suc perception by Δ*tvinv* mutants and wild-type strains. A, Germling development of Gv29-8, Δ*tvinv2*, and Δ*tvinv5* strains after 12 h on VMS medium incubated in moist dark chambers at 27°C. B, Schematic depicting the experimental designs to assess the differential use of sugars by each strain. Spore suspensions for each strain were placed in the center of the plates. Different combinations of two paper discs (sugar 1 and sugar 2) imbibed with 3% (w/v) Suc or 1.5% (w/v) Glc were placed 3 cm away from the center. C, The sugars are expected to diffuse and generate a concentration gradient on both sides of the initial inoculum. Fungal growth was evaluated after 24 h of incubation at 27°C.

plants grown from seed inoculated with each of the three strains were harvested, weighed, and surface disinfested to eliminate fungal cells attached to the roots (Viterbo et al., 2007). After treatment, the roots were ground and plated on *Gliocladium virens* specific medium (GVSM; Park et al., 1992). The wild-type strain colonized line B73 at a significantly higher level than Mo949 (Fig. 7A). However, inoculation with either mutant strain resulted in a 4-fold increase in colony-forming units (per gram) with respect to the wild-type strain, and both mutants equally colonized the roots of B73 or Mo940 (Fig. 7A).

To understand the effect of plant-derived Suc on root colonization, we examined whether the mutant strains failed to induce plant defense responses that would restrict fungal growth. Maize plants were simultaneously inoculated with the wild type and each of the mutant strains; the number of colony-forming units corresponding to each strain was determined after 2 weeks. By coinoculating with the wild type and a mutant strain, colonization by the wild-type strain was expected to induce any defense response the plant uses to restrict the growth of hyphae. In this case, the colonization extent of the mutant strains would be reduced. The results indicated that the presence of the wild-type strain in the rhizosphere did not have an effect on the colonization extent determined for the mutant strains (Fig. 7B). The difference of root colonization was also observed microscopically after staining the roots for hyphae using a method to detect fungal succinate dehydrogenase activity as described by MacDonald and Lewis (1978). A greater amount of hyphae was detected on roots inoculated with Δ*tvinv2* or Δ*tvinv5*, and importantly, both mutants formed a greater number of chlamydospores per square millimeter of root than the wild type (4 ± 1.5 and 32.9 ± 4.5 in the wild type and mutants strains, respectively; Fig. 7C).

Similar autofluorescence patterns inside root cells (Fig. 7D) suggest no differential responses of the roots toward the mutant and wild-type strains.

Hydrolytic enzymes play important roles in the root colonization process (Yedidia et al., 1999). In agreement, Δ*tvinv2* and Δ*tvinv5* produced higher levels of cell wall-degrading enzymes, which may explain their increased ability to colonize maize roots (Fig. 8). We compared the activity of secreted glucanases, proteinase, and chitinase in both mutants (Δ*tvinv2* and Δ*tvinv5*) and the wild type when incubated in VMS medium or hydroponic systems with maize plants. In the presence of VMS, both mutants yielded a larger increase (about eight times) in the amount of secreted proteins, β-1,6-glucanase, and proteinase activities in the culture filtrates compared with the wild type (Fig. 8A). This drastic change was also accompanied by a significant increase of the levels of β-1,3-glucanase and

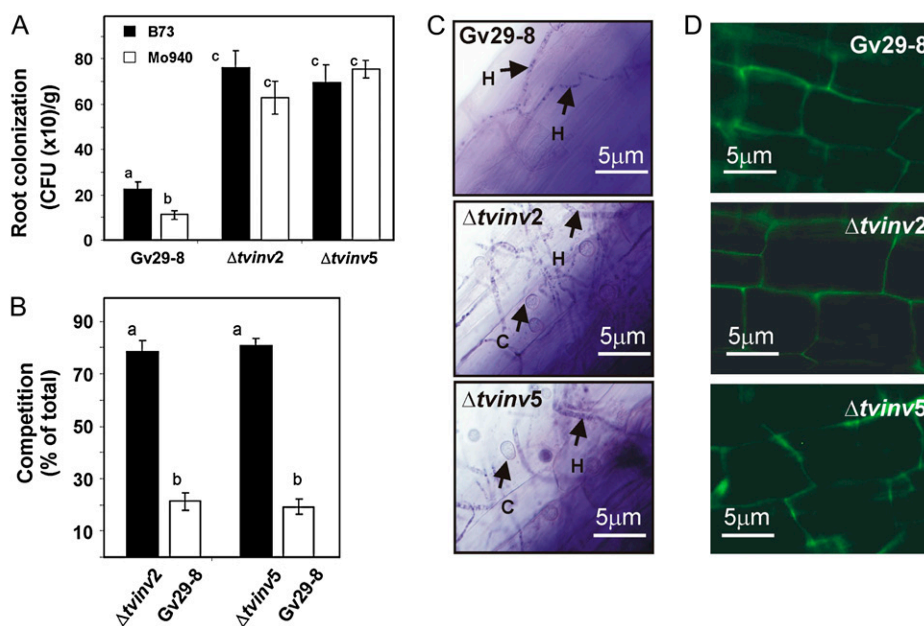

Figure 7. Suc metabolism in *T. virens* is involved in the coordination of root colonization and fungal development. A, Colonization of maize inbred lines B73 and Mo940 by the wild type (Gv29-8) and mutant strain Δ*tvinv2* or Δ*tvinv5*. The experiment was performed two times, and colony-forming units (CFUs) were determined in roots of five different plants for each treatment in each experiment. The bars depict mean values ± SD of independent experiments. Bars with different letters differ significantly according to Tukey's HSD test at a significance level of 5%. B, Coinoculation of Gv29-8 and Δ*tvinv2* or Δ*tvinv5* on maize roots. The wild-type strain and each of the mutants were equally coinoculated on maize seeds. After 7 d, the root systems were collected and surface sterilized for CFU determination. The ratio of mutant to wild-type colonies was compared for each root system. CFUs were determined in roots of five different plants for each treatment, and the experiment was repeated three times. The bars depict mean values ± SD determined in three independent experiments, and columns with different letters differ significantly according to Tukey's HSD test at a significance level of 5%. C, Bright-field micrographs of root systems of plants grown in hydroponic systems and inoculated with Gv29-8, Δ*tvinv2*, or Δ*tvinv5*. Fungal spores were germinated in VM medium supplemented with 1.5% (v/v) glycerol, and 1 g of fungal hyphae was inoculated into the hydroponic system containing 4-d-old maize plants. Two days after inoculation, roots were collected and photographed. D, Autofluorescence micrographs of maize roots inoculated with Gv29-8, Δ*tvinv2*, or Δ*tvinv5*. Fungal spores were germinated in VM medium supplemented with 1.5% (v/v) glycerol, and 1 g of fungal hyphae was inoculated into the hydroponic system containing 4-d-old maize plants. Two days after inoculation, roots were collected and photographed with a fluorescence microscope.

chitinase in both mutant strains. A similar effect was also determined when the strains were cultivated in the presence of maize plants. Both Δ*tvinv2* and Δ*tvinv5* strains exhibited significant increases in the production of hydrolytic enzymes and secreted proteins compared with Gv29-8 (Fig. 8B). However, such an effect was not observed when the fungal cells were cultured in VM medium supplemented with 1.5% glycerol (data not shown). In good agreement, immunoassays demonstrated higher levels of fungal proteinase polypeptides in protein extracts from maize roots inoculated with Δ*tvinv2* or Δ*tvinv5* (Supplemental Fig. S6).

To further investigate the involvement of Suc metabolism in fungus-plant interactions, the expression of *sm1* was compared among the mutant and wild-type strains inoculated into hydroponic systems containing maize plants. It was recently described that *sm1* is up-regulated in fungal tissues during root colonization. This small protein is part of the molecular dialogue associated with the activation of ISR in maize plants (Djonović et al., 2006, 2007a; Vargas et al., 2008). Reverse transcription followed by real-time PCR experiments showed that the up-regulation of *sm1* in the presence of plant roots requires the functional expression of *tvinv* (Fig. 9A; Supplemental Fig. S7A). However, the mutant strains still were able to induce systemic disease protection in leaves (Fig. 9B; Supplemental Fig. S7B).

The Photosynthetic Rate in Leaves of Maize Plants Is Affected by the Ability of *T. virens* to Use Suc within Roots

As photosynthesis is the primary energy and carbon supplier supporting the plant, we analyzed the effect on photosynthetic CO_2 fixation in leaves when maize roots are colonized by *T. virens*. Carbon assimilation was measured as an estimate of the rate of CO_2 uptake in the distal tip of the third leaf of 14-d-old plants. The results demonstrated that colonization of the maize rhizosphere with *T. virens* Gv29-8 causes a systemic

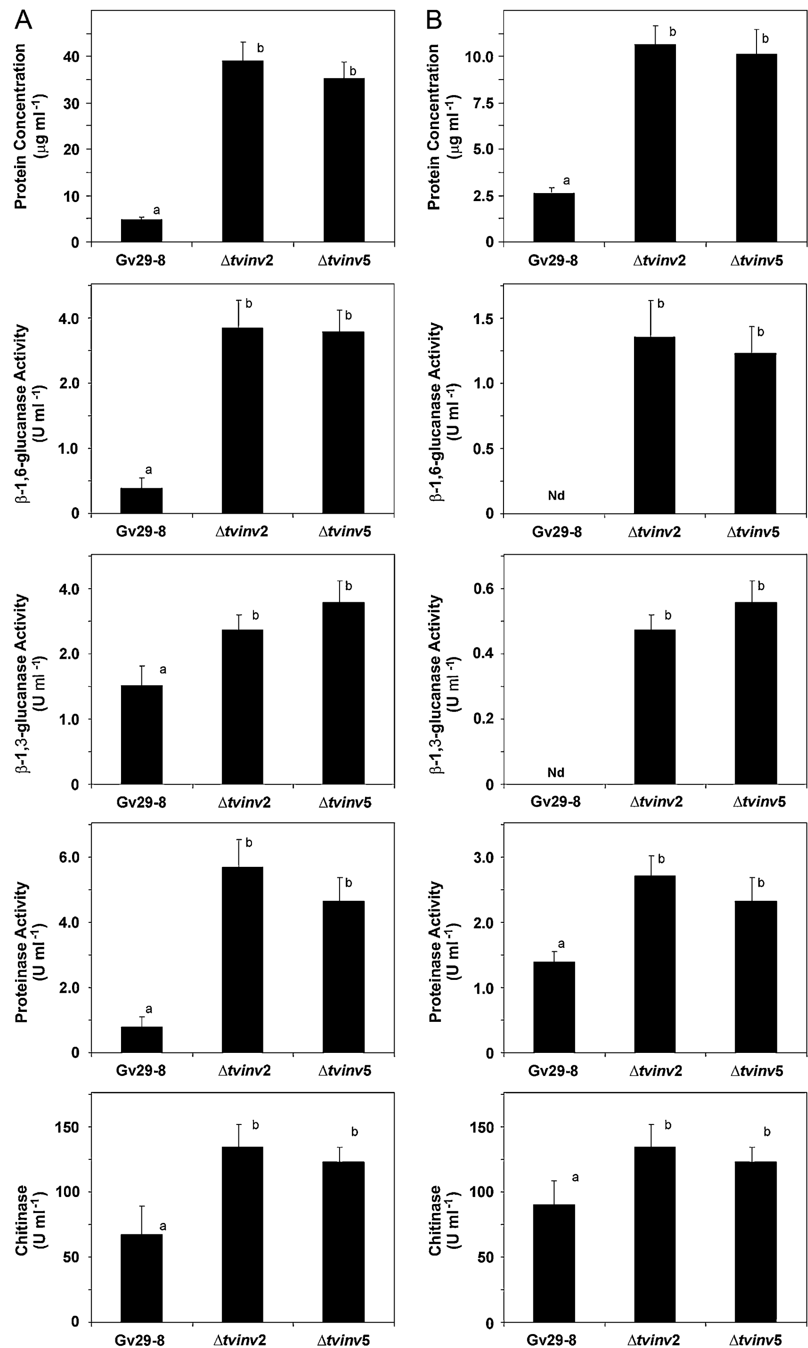

Figure 8. The activity of hydrolytic enzymes secreted by *T. virens* is increased in the Δ*tvinv2* and Δ*tvinv5* mutant strains. A, Determination of enzymatic activities produced in VMS medium after incubation for 4 d. Spores (10^6 spores mL^{-1}) from the three strains were germinated on VM supplemented with glycerol for 48 h, and harvested mycelia were inoculated into fresh VMS medium and cultured for an additional 4 d at 25°C. The enzymatic activities were determined in the culture filtrates. The bars depict mean values ± SD of two independent experiments. Bars with different letters differ significantly according to Tukey's HSD test at a significance level of 5%. B, Determination of enzymatic activities produced in hydroponic systems in the presence of maize plants after 4 d of incubation. Spores (10^6 spores mL^{-1}) from the three strains were germinated on VM medium supplemented with glycerol for 48 h, and mycelia were collected and transferred to the hydroponic systems and then incubated for 4 d at 25°C. The enzymatic activities were determined in the culture filtrates. The bars depict mean values ± SD of two independent experiments. Bars with different letters differ significantly according to Tukey's HSD test at a significance level of 5%.

increase in the uptake of CO_2 in leaves (Fig. 10A). Although the mutant strains displayed a greater colonization ability (four times higher) than the wild type (Fig. 7C), neither Δ*tvinv2* nor Δ*tvinv5* affected CO_2 uptake in leaves compared with nontreated plants (Fig. 10A).

To further confirm the systemic effects of *T. virens* on photosynthesis in maize plants, we analyzed the expression of photosynthesis-related genes in plants inoculated with wild-type *T. virens* or mutants impaired in the expression of *tvinv.* Northern-blot assays demonstrated that the accumulation of transcripts of Rubisco small subunit (*rbcS*) and the oxygen-evolving enhancer 3-1 (*oee3-1*; from the water-splitting complex) was systemically up-regulated after *T. virens* Gv29-8 colonized the roots of maize plants (Fig. 10, B and C). The up-regulation of these photosynthetic genes was dependent on Suc degradation in *Trichoderma* cells, as the up-regulation of these two genes was not detected in plants inoculated with either Δ*tvinv2* or Δ*tvinv5*. These findings indicate that during the symbiotic association, the degradation of

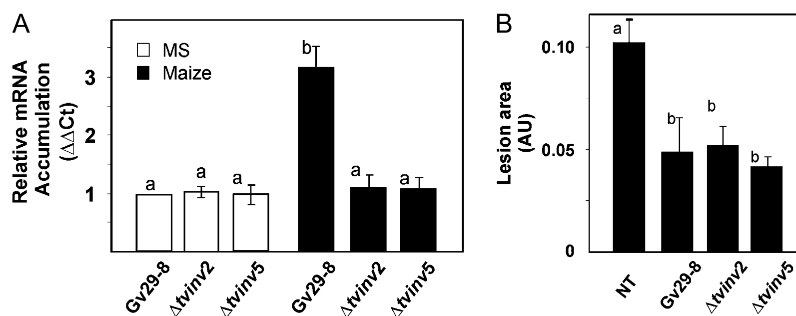

Figure 9. The ability of *T. virens* to hydrolyze Suc is necessary for the up-regulation of *sm1*. A, Comparison of the expression of *sm1* in Gv29-8, Δ*tvinv2*, or Δ*tvinv5*. Fungal spores were germinated in VM medium supplemented with 1.5% (v/v) glycerol. One gram of fungal hyphae was inoculated into the hydroponic systems containing 4-d-old maize plants or Murashige and Skoog medium (MS) as control. Two days after inoculation, fungal material was collected and *sm1* expression was assayed by quantitative real-time RT-PCR. Expression of *actin* was used as a quantitative internal control. The gene expression data were normalized to the reference control. The data depicted are mean values ± SD of two independent experiments. Bars with different letters differ significantly according to Tukey's HSD test at a significance level of 5%. B, Quantitative analysis of the foliar lesions caused by *Colletotrichum graminicola* on leaves of maize plants inoculated with Gv29-8, Δ*tvinv2*, or Δ*tvinv5*. The bars depict the average area of lesions (in arbitrary units [AU]) on maize leaves 4 d post inoculation with the pathogen. The data represent mean areas ± SD of five lesions produced on five different plants from two independent experiments. Bars with letters in common did not differ significantly according to Tukey's HSD test at a significance level of 5%. NT, Nontreated.

plant-derived Suc in the fungal cells systemically affects gene expression and photosynthetic rate in leaves.

DISCUSSION

Carbohydrates are associated with the most important metabolic processes, such as protein, lipid, and nucleic acid biosynthesis. In animals and plants, sugars have also been described to have important functions as signal molecules regulating gene expression and carbohydrate distribution (Towle, 1995; Kaylor et al., 1997; Pego et al., 2000; Rolland et al., 2001; León and Sheen, 2003; Koch, 2004). In this report, we present evidence that suggests a novel role for Suc metabolism in the coordination of plant interactions with microorganisms in the rhizosphere. The ability of the nonpathogenic fungus *T. virens* to metabolize Suc is a critical aspect in the control of root colonization of maize plants and for a systemic up-regulation of photosynthesis in leaves. Our findings provide what is to our knowledge a novel scenario for Suc metabolism, photosynthesis, and the control of plant-microbe associations.

In mycorrhizal associations, Suc-derived monosaccharides from the plant support the growth of the fungal cells (Nehls et al., 2001). Mycorrhizal species are unable to directly use Suc and must rely on enzymes from the plant to hydrolyze Suc to deliver monosaccharides for fungal metabolism (Blee and Anderson, 1998; Hahn and Mendgen, 2001). Previous evidence has shown that monosaccharides also act as signals, and their concentration in the apoplast con-

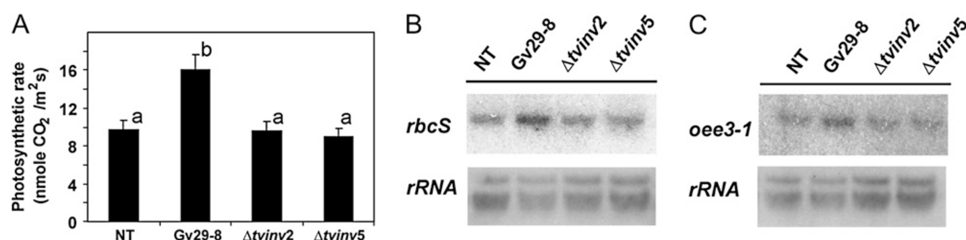

Figure 10. The expression of *tvinv* is required for the systemic induction of photosynthesis in maize plants. A, Effect of *T. virens* strains on photosynthesis. The photosynthetic CO_2 uptake was determined in mature leaves of 14-d-old maize plants as an estimation of the photosynthetic rate in maize leaves. The mean ± SD value of two independent experiments is presented. In each experiment, the photosynthetic rate was determined from the third leaf of five different plants and each measurement was repeated twice. Bars with different letters differ significantly according to Tukey's HSD test at a significance level of 5%. B and C, mRNA steady state of *rbcS* (accession no. X06535) and *oee3-1* (accession no. MN_001111878), respectively. Total RNA was extracted from the leaf area where photosynthetic rate was determined. Fifteen micrograms of total RNA was loaded in each treatment and separated on 1% (w/v) agarose gels. The probes were generated using the primers rbcSF/rbcSR and oee31F/oee31R (Supplemental Table S1), and both strands of each probe were sequenced. NT, Nontreated.

trols the extent of the root invasion (Nehls et al., 2001; Biemelt and Sonnewald, 2006). Both mycorrhiza- and *T. virens*-plant associations share in common the metabolism of plant-derived carbohydrates; however, different strategies have evolved to obtain these plant resources. Our data suggest that some *Trichoderma* species have incorporated an intracellular metabolic pathway that facilitates the use of Suc synthesized by the plant. As only oxygenic photosynthetic organisms are able to produce Suc (Salerno and Curatti, 2003), plants are the sole source of Suc in the rhizosphere. The ability of *Trichoderma* species to use plant-derived Suc in the cytosol may have emerged through coevolution of both organisms, resulting in a commensal relationship.

We demonstrate that the nonpathogenic symbiont *T. virens* produces TvInv, an intracellular Inv expressed in the presence of Suc or plant roots (Fig. 2). This finding provides novel insights into carbohydrate metabolism and the role of the fungal Inv during the association between *T. virens* and plants. TvInv is an intracellular Inv with an acidic optimum pH for Suc hydrolysis, and the enzyme displays β-fructofuranosidase activity with biochemical properties similar to those described for an intracellular Inv (SacB) recently characterized from *A. niger* (Fig. 3; Goosen et al., 2007). BLAST searches in the *T. virens* genome sequence, using *S. cerevisiae* Ac-Inv and SacB as queries, retrieved only one homologous sequence (*tvinv*) displaying the eight motifs conserved in characterized β-fructofuranosidases (Fig. 1A; Supplemental Fig. S1). In addition, two putative membrane-associated Suc transporters, similar to those characterized in plants (Lemoine, 2000), are present in the *T. virens* genome and complete the pathway for Suc metabolism in *Trichoderma* cells (W.A. Vargas and C.M. Kenerley, unpublished data). Analysis of strains impaired for *tvinv* expression showed that TvInv is necessary for the normal growth and development of *T. virens* in the presence of Suc (Figs. 5 and 6). Moreover, secondary metabolism appears to be also regulated by Suc metabolism in the fungal cells. Thin-layer chromatography experiments demonstrated alteration in the production of secondary metabolites (data not shown) that was also evident by the pigmentation of the culture filtrates (Supplemental Fig. S8). We are pursuing the identification of these metabolites and the metabolic pathways involved in their biosynthesis as possible components of the chemical interaction between *T. virens* and plants.

Secreted chemical signals from both plants and microbes mediate a complex dialogue that will determine whether an interaction will be malevolent or benign (Bais et al., 2006). Plant roots initiate chemical communication with soil microbes by producing signals that are recognized by the microbes, which in turn produce signals that trigger colonization (Hirsch et al., 2003; Akiyama and Hayashi, 2006; Bais et al., 2006; Bouwmeester et al., 2007; Parniske, 2008; Oldroyd et al., 2009). Only a few chemical signals have been characterized from plants, being mostly restricted to strigolactons and flavonoids exuded by the roots (Hirsch et al., 2003; Akiyama and Hayashi, 2006). Plants can also exude sugars through their roots, but the role of root-exuded sugars is not fully understood (Kraffczyk et al., 1984; Jaeger et al., 1999; Mahmood et al., 2002; Baudoin et al., 2003; Bais et al., 2006). In our studies, we demonstrated that plant-*Trichoderma* associations are affected by plant-derived Suc, with TvInv activity being crucial for the control of root colonization (Fig. 7). Root colonization by *Trichoderma* appears to be tightly controlled, with the hyphae restricted to the superficial cell layers of the roots, including the epidermis and some cell layers below (Yedidia et al., 1999). Control of fungal proliferation in mycorrhizal associations has been ascribed to the activation of different responses, including defense mechanisms from the plant cells such as phenyl propanoid biosynthesis, oxidative stress-induced enzymes, and pathogenesis-related genes, as well as the availability of monosaccharides (Gianinazzi-Pearson, 1996; Garcia-Garrido and Ocampo, 2002).

The mechanisms controlling the extent of root colonization by *Trichoderma* is not fully understood. It was suggested that for penetration of the epidermis and further ingress into the outer cortex, small quantities of cell wall hydrolytic enzymes are produced by the fungus to locally weaken or loosen epidermal cell walls to facilitate the fungal spread into the roots (Yedidia et al., 1999). By ultrastructural studies, Yedidia et al. (1999) showed that the thickening of plant cell walls and callose deposition act as physical barriers that *Trichoderma asperellum* has to overcome to spread within cucumber roots. In addition, the evidence presented in that report indicated that during root colonization, fungal metabolic status would play an important role in controlling the invasion. *T. virens* mutant strains impaired in the expression of *tvinv*, unable to metabolize Suc, presented higher levels of root colonization (Fig. 7). Moreover, we observed that when Gv29-8 and Δ*tvinv2* or Δ*tvinv5* were coinoculated on maize roots, the mutant strains still displayed increased colonization ability (Fig. 7B). With the presence of the wild-type strain in the coinoculation experiment, it was expected that any plant defense response to restrict the growth of *T. virens* (wild type) inside the roots, such as callose deposition or cell wall thickening (Yedidia et al., 1999), would be activated. In this case, root colonization would be reduced for both strains. However, according to the results obtained, the increased root colonization displayed by the Δ*tvinv* mutant strains is independent from such defense responses activated in the roots.

The increased root colonization by Δ*tvinv2* and Δ*tvinv5* seems to be controlled by the metabolic status of the fungal cells, with a strong correlation between the production of cell wall-degrading enzymes and the greater ability of the mutant strains to colonize maize roots (Fig. 8). Moreover, the higher levels of fungal

proteinase in roots inoculated with Δ*tvinv2* or Δ*tvinv5* mutants (Supplemental Fig. S6) suggest that these strains are producing higher levels of hydrolytic enzymes also within the roots. Interestingly, the regulation of the expression of hydrolytic enzymes in different *Trichoderma* species was reported as being subjected to carbon catabolic repression (Mach et al., 1996; Noronha et al., 2000; Seidl et al., 2008). Carbon catabolic repression of gene expression is a process present in many organisms where Glc and related sugars represses gene expression (Gancedo, 1998). In *T. virens*, the hydrolysis of Suc provides Glc and Fru, sugars that are involved in the repression of fungal hydrolytic enzymes (Gancedo, 1998; Noronha et al., 2000; Seidl et al., 2008). The control of the expression of enzymes that affect hyphal plasticity (Adams, 2004) ultimately will influence the ability of the fungal cells to spread into roots. When the ability of *T. virens* to hydrolyze Suc is impaired, catabolic repression mediated by the products of Suc hydrolysis is relieved. The hydrolytic enzymes become derepressed, increasing the level of such enzymatic activities facilitating the fungal dispersion within roots to probe for alternative sources of carbon and energy.

Chlamydospores are produced by many fungi and represent enlarged, thick-walled vegetative cells of varied forms containing condensed cytoplasm (Lin and Heitman, 2005). In some plant pathogenic fungi, chlamydospores have been described as long-term survival structures and important propagules as primary inoculum for plant infection (Abou-Gabal and Fagerland, 1981; Couteaudier and Alabouvette, 1990). The environmental signals for chlamydospore formation in fungi may be species specific and include nutrients, osmolarity, light, temperature, and plant stimulants (Barran et al., 1977; Regulez et al., 1980; Kertesz-Chaloupkova et al., 1998; Kües et al., 1998; Khan et al., 2004). However, the molecular mechanisms of formation and developmental control remain enigmatic (Lin and Heitman, 2005). Species of the genus *Trichoderma* are also able to form chlamydospores in culture conditions or in the presence of plants (Papavizas, 1985; Chacón et al., 2007), but the environmental conditions that induce this cellular differentiation are unknown. The ability of *T. virens* to metabolize plant-derived Suc seems to greatly influence chlamydospore differentiation. Even though greater levels of hyphal tissue were present inside roots colonized by the Δ*tvinv2* and Δ*tvinv5* mutants, these strains also differentiated a massive number of chlamydospores on the surface of maize roots (Fig. 7C). It is likely that the inability of Δ*tvinv2* or Δ*tvinv5* to metabolize root-exuded Suc results in a nutritional stress to the fungal cells growing outside the roots that triggers chlamydospore formation. However, the proliferation of fungal cells inside the roots may be supported by any alternative source of carbon that the fungal cells can withdraw directly from the plant cells. Based on our observations, we speculate that Suc availability during the mutualistic association supplies the nutritional demands of the fungus and regulates the morphological development of *T. virens* in natural environments.

The beneficial effects of plant-*Trichoderma* interactions are well described, including protection from plant pathogens and enhancing plant growth and crop yield (Harman et al., 2004). *T. virens* is known to systematically induce the up-regulation of defense-related genes in plants (Djonović et al., 2006, 2007a; Viterbo et al., 2007; Shoresh and Harman, 2008). Djonović et al. (2006) described the isolation and characterization of a small protein named Sm1, which has the ability to activate ISR in plants. Sm1 was classified as a proteinaceous elicitor that is part of the chemical dialogue and molecular signals that activate plant defenses during the symbiotic association between *T. virens* and roots. Further experiments demonstrated that the expression of *sm1* in the fungal tissue was up-regulated in the presence of plant roots or when cultured in the presence of Suc (Djonović et al., 2006, 2007a; Vargas et al., 2008). In this report, we present further insights into the regulation of *sm1* expression as related to the metabolism of plant-derived Suc in *Trichoderma* cells. Our results suggest that the hydrolysis of Suc is involved in the control of the expression of *sm1* in the presence of maize roots (Fig. 9A; Supplemental Fig. S7A). Mechanisms for sugar signaling, involving the phosphorylation of monosaccharides and gene expression regulation, are conserved among different organisms including unicellular eukaryotes such as yeasts (Stulke and Hillen, 1999; Rolland et al., 2001). Moreover, it is known that in plants one of the mechanisms for sugar signaling involves Suc hydrolysis and further phosphorylation of the monosaccharides (Koch, 2004). We speculate that those pathways for sugar signaling might be conserved in *T. virens* and regulate the intimate association between *T. virens* and roots. In addition, we tested the ability of Δ*tvinv2* and Δ*tvinv5* to activate ISR; even thought both mutant strains were unable to increase the expression of *sm1* in the presence of maize roots, the plants were still able to activate ISR in leaves (Fig. 9B; Supplemental Fig. S7B). This phenomenon may be attributed either to basal levels of expression of *sm1* or to the presence of other ISR elicitors produced by the fungal cells (Viterbo et al., 2007).

Proteomic experiments demonstrated that in cucumber plants, *T. asperellum* systemically activates the expression of genes involved in energy and carbohydrate metabolism, including some photosynthetic genes (Segarra et al., 2007; Shoresh and Harman, 2008). In plants, photosynthesis and carbohydrate metabolism establish the basis for the control of plant growth and productivity. Among carbohydrates, Suc is the main photosynthetic product that is transported from source to sink organs (Dennis and Blakeley, 2000). Two of the many functions attributed to Suc in plant cells are as a signal molecule and a controller of carbon partitioning and distribution throughout the

plant (Koch, 2004). The expression of photosynthetic genes, such as *rbcS*, is controlled by Suc and carbohydrate demands from sink tissues, and Ac-Inv activity is known to control the strength of the sink and the sink activity in roots (Urwin and Jenkins, 1997; Sturm, 1999; Pego et al., 2000; Koch, 2004). Increased sink activity in roots represents an enhanced demand for photoassimilates, a higher rate of Suc transport from source leaves, and a release of photosynthesis from the feedback inhibition exerted by sugars (Roitsch, 1999; Pego et al., 2000; Paul and Foyer, 2001). This phenomenon includes the control of the expression of many genes, including the up-regulation of genes involved in photosynthesis (Pego et al., 2000; Paul and Foyer, 2001). In this report, we describe the transcriptional up-regulation of two photosynthetic genes, *rbcS* and *oee31* (Fig. 10, B and C), which is in agreement with a higher photosynthetic rate detected in leaves of maize plants inoculated with *T. virens* Gv29-8 (Fig. 10A). Recently, the up-regulation of some photosynthesis-related genes was described in plants after treatment with *Trichoderma* (Segarra et al., 2007; Shoresh and Harman, 2008). We demonstrate that *Trichoderma* systemically induces the photosynthetic process at different levels, including CO_2 fixation and the photochemical production of energy. The up-regulation of *oee3-1* (Fig. 10C) suggests that the electron transport in PSII is probably enhanced to support a higher photoassimilation rate in the photosynthetic cells. We found the *Trichoderma*-mediated up-regulation of photosynthesis is influenced by the expression of *tvinv* in the fungal cells during the mutualistic association (Fig. 10). We speculate that when *Trichoderma* colonizes the roots, the up-regulation of *tvinv* increases the sink activity in roots and consequently the demand for carbon. This increased demand of photoassimilates in roots may release the feedback inhibition of photosynthesis in leaves, stimulate CO_2 fixation, and enhance carbon transport toward roots.

Suc degradation in the fungal cells may be considered an extension of plant carbohydrate metabolism that increases the demand for sugars and systemically alters plant metabolism. In mycorrhizal associations, the fungal cells within the root obtain monosaccharides from the plant, affecting carbon metabolism in the whole plant and photosynthesis in leaves (Loewe et al., 2000; Miller et al., 2002; Nehls, 2008). Carbohydrate flow to the symbiotic partner is controlled by the plant through the hydrolysis of Suc. For instance, the importance of plant apoplastic Inv in controlling carbon delivery and root colonization by arbuscular mycorrhiza fungi has been demonstrated in transgenic *Nicotiana tabacum* plants (Schaarschmidt et al., 2007a, 2007b). Transgenic plants with strongly enhanced Inv activity in leaves resulted in an accumulation of hexoses in source leaves, a decrease of Suc availability for roots, and a reduction of mycorrhization. However, the mechanisms controlling carbon distribution in the plant and arbuscular mycorrhiza colonization are not fully understood. For instance, contrary to the effect observed in plants with a strong increase in Inv activity, mycorrhization was stimulated in plants with a slight increase in leaf Inv activity (Schaarschmidt et al., 2007b). Unexpectedly, arbuscular mycorrhization was not improved in transgenic plants with increased Inv activity in roots that produced high levels of monosaccharides for the fungal cells (Schaarschmidt et al., 2007a). These observations suggest a very tight and complex process that regulates carbohydrate accumulation, distribution, and root colonization by arbuscular mycorrhizae that we do not fully understand. On the other hand, compared with arbuscular mycorrhizae, the metabolic scenario is different for the soil-borne fungus *T. virens*. In this report, we clearly show the importance of the hydrolysis of plant-derived Suc inside *T. virens* cells for the symbiotic association with maize roots. In this interaction, *T. virens* is distinct from mycorrhizal partners, as *T. virens* has acquired the ability to metabolize Suc without the dependence upon plant Inv. The precise mechanisms associated with Suc metabolism, chemical communication, and plant growth promotion by *Trichoderma* species are still unclear and will require additional studies to provide a better understanding of such complex processes. Based on the results presented in this and in previously published reports, we introduce Suc as a novel component in the symbiotic association between fungal and root cells as a plant-derived source of carbon and energy with a direct effect on *Trichoderma* development and growth in the rhizosphere.

MATERIALS AND METHODS

Fungal Strains and Plant Culture

Trichoderma virens Gv29-8 (wild type), *T. virens* Δ*tvsp1* (Pozo et al., 2004), *Trichoderma atroviride* IMI206040, and *Trichoderma reesei* Q6 were routinely maintained on potato dextrose agar (Difco) unless otherwise indicated. Maize (*Zea mays* Silver Queen and inbred lines B73 and Mo940) seedlings used in this study were grown in a hydroponic system containing Murashige and Skoog medium (Djonović et al., 2006), in pots containing soil-less mix (Metromix 366; Scotts) or sand, and incubated in a growth chamber at 25°C with a 14-h photoperiod and 60% humidity. Plants grown in pots containing Metromix 366 were inoculated with *Trichoderma* as reported previously (Djonović et al., 2007a).

Protein Extracts and Enzymatic Assays

Fungal tissue was ground with a mortar and pestle in the presence of liquid nitrogen. The proteins were extracted by treating the ground tissue for 10 min in 50 mM HEPES-NaOH (pH 7.5), 1 mM EDTA, 20% (v/v) glycerol, 20 mM β-mercaptoethanol, and 0.05% (v/v) protease inhibitor cocktail (Sigma). The protein extracts were used for enzymatic assays or enzyme purification. TvInv activity was routinely assayed in reaction mixtures containing 50 mM Suc, 100 mM acetic acid/sodium acetate buffer (pH 5.0), and an aliquot of the protein fraction to be tested. The mixture was incubated at 37°C for different times. The released monosaccharides were quantified with Somogyi-Nelson reagent (Ashwell, 1957).

Purification of TvInv

Crude extracts from fungal tissue grown in liquid Vogel's minimal medium (Vogel, 1956) supplemented with 1.5% (w/v) Suc (VMS) for 48 h were

loaded onto High Q support columns (0.5 × 20 cm; Bio-Rad) preequilibrated with 50 mM HEPES-NaOH (pH 6.5), 1 mM EDTA, 5 mM β-mercaptoethanol, and 20% (v/v) glycerol. Proteins were eluted with a 0 to 0.5 M NaCl linear gradient in the equilibration buffer. Fractions containing Inv activity were pooled, concentrated in an Amicon ultrafiltration cell, and further purified by gel filtration through Sephacryl S100 (1.0 × 100 cm; Amersham-Pharmacia) preequilibrated with 50 mM potassium phosphate buffer (pH 6.5), 1 mM EDTA, 10% (v/v) glycerol, 5 mM β-mercaptoethanol, and 150 mM KCl. This column had been previously calibrated using phosphorylase *b* (97.4 kD), bovine serum albumin (66 kD), carbonic anhydrase (29 kD), and cytochrome *c* (12 kD) as standards. Since TvInv activity was labile or susceptible to inactivation, fractions from gel-filtration chromatography were collected in tubes containing bovine serum albumin in a final concentration of 5 mg mL^{-1}. The protein was purified approximately 400-fold, and the concentrated fraction was termed the purified enzyme.

Enzyme Characterization

For pH optimum determination, 100 mM citrate-acetate buffer in the pH range 4.0 to 6.0 was added to the reaction medium. Apparent K_m determination was performed in the presence of 100 mM acetic acid/sodium acetate buffer (pH 5.0), Suc (1, 2, 4, 8, 16, and 20 mM), and an aliquot of the purified protein. The inhibition of TvInv was assayed in the presence of 1 or 10 mM $CuCl_2$ or Fru plus 5 mM Suc. The substrate specificity was assayed at pH 5.0 in the presence of 50 mM Suc, trehalose, raffinose, stachyose, or melizitose. The products of TvInv activity were quantified using Somogyi-Nelson reagent. In the presence of Fru, the enzymatic activity was determined by Glc oxidase assay as the amount of released Glc (Lloyd and Whelan, 1969).

DNA and RNA Manipulations

Genomic DNA from *T. virens* was obtained as described previously (Djonović et al., 2006). Total RNA from maize or fungal tissue was prepared using the TRIZOL reagent (Gibco-BRL). RNA quality was analyzed by electrophoresis on agarose gels. Southern- and northern-blot analyses were performed using Hybond-N$^+$ membranes (Amersham Biosciences) according to the manufacturer's suggestions. For northern-blot assays, the probes were PCR-amplified fragments from fungal or maize genomic DNA and radioactively labeled using [^{32}P]dCTP. The fragments amplified corresponded to exons of each gene, and the correct amplification product was confirmed after sequencing. The primers used for the PCR are listed in Supplemental Table S1.

Real-Time PCR Assays

For *sm1* expression assays, quantitative real-time reverse transcription (RT)-PCR experiments were conducted as described previously (Vargas et al., 2008) using *actin* as an internal reference. The experiments were conducted with the QuantiTect SYBR Green RT-PCR kit (Qiagen). The reactions were performed in a 20 μL reaction containing 1× QuantiTect SYBR Green Master Mix, 1× RT QuantiTect Mix, 200 nM primers, and 100 ng of total RNA. The reactions were performed with a 7500 Fast Real-Time PCR System (Applied Biosystems) following the protocols suggested by the manufacturer.

Construction of the *tvinv* Allele Replacement Cassette by Double-Joint PCR

A DNA fragment consisting of a hygromycin resistance gene (*HygB*) flanked by DNA regions from the 5′ and 3′ ends of the *tvinv* gene was amplified by double-joint PCR (Kuwayama et al., 2002). The 5′ (1,354 bp) and 3′ (1,101 bp) fragments of *tvinv* were amplified using the primer pairs InvUpF/InvUpR and InvDwnF/InvDwnR, respectively (Supplemental Table S1). A 1,430-bp fragment containing the *trpC* promoter and terminator was amplified from the vector pSCN44 (Fungal Genomic Stock Center) with the primers HygF/HygR (Supplemental Table S1). The three purified fragments were mixed at a 1:3:1 molar ratio and joined by PCR (Kuwayama et al., 2002). The PCR product was used as a template for a final amplification step using the primer pair NestInvF/NestInvR (Supplemental Table S1), which are nested in the 5′ and 3′ fragments of *tvinv*, respectively. The final PCR product expected was a 3,885-bp DNA fragment with the *HygB* cassette fused to the 5′ and 3′ regions of *tvinv*. The two strands of all the DNA fragments generated during the double-joint PCR process were sequenced to assess fidelity.

Fungal Transformation

T. virens protoplasts were prepared and transformed using the lineal DNA fragments according to the method described by Baek and Kenerley (1998). Prototrophic transformants were selected in PDA medium supplemented with hygromycin (100 μg mL^{-1}). Disruption of the *tvinv* gene in transformants was confirmed by PCR and Southern-blot analysis.

Growth Rate Comparison

Cultures of Gv29-8, Δ*tvinv2*, and Δ*tvinv5* were compared for colony morphology and radial growth. Spore suspensions (10^7 spores mL^{-1}) of each strain were prepared, and 3 μL of that suspension was inoculated in the center of WA, VM, VMS, or PDA plates. Plates were visually inspected for production of aerial hyphae, color, and morphology of the colony. The diameter of each colony was recorded at 24, 36, and 48 h of growth at 27°C. Each treatment contained four replicates, and each experiment was repeated three times.

Sugar perception was tested by placing a 3-μL droplet of a spore suspension (10^7 spores mL^{-1}) in the center of a plate containing VM medium and between two paper discs (sugar 1 and sugar 2). The papers discs were saturated with either 30% (w/v) Suc or 15% (w/v) Glc and placed 3 cm from the inoculation site, establishing a concentration gradient for each sugar after diffusion. The radial symmetry of the colony was evaluated as a measure of the ability of the strains to detect and equally use either Suc or Glc.

Protein Extraction from Roots

Roots of maize seedlings from hydroponic systems were first washed to remove the superficial hyphae of *T. virens* from the surface. Washed roots were frozen with liquid nitrogen and then ground with mortar and pestle under liquid nitrogen. The total proteins were extracted as described by Hendriks et al. (2003) using 16% (w/v) TCA in diethyl ether and resuspended in Laemmli sample buffer (Laemmli, 1970).

Enzymatic Activity Assays and Protein Quantification in Culture Filtrates

The activity of β-1,3-glucanase, β-1,6-glucanase, endochitinase, and proteinase secreted by the *Trichoderma* strains used in this study was determined. Enzymatic activities were assayed in the strains when grown in VM or VMS or cultured in Murashige and Skoog medium in the presence or absence (control) of maize seedlings. The activity of β-1,3-glucanase and β-1,6-glucanase was determined in the presence of pustulan and laminarin, respectively, by detecting the reducing sugars released after incubation (Djonović et al., 2007b). Protease and endochitinase activities were determined in the presence of Suc-Ala-Ala-Pro-Phe-pNA (Sigma) and 4-methylumbelliferyl-β-D-*N*,*N*′,*N*′′-triacetylchitotriose (Sigma), respectively, as recently described (Djonović et al., 2007b). Protein concentration was determined in a microplate assay using the Protein Reagent (Bio-Rad) according to the manufacturer's instructions or by detecting A_{280} using bovine serum albumin as a standard.

SDS-PAGE of Proteins and Western Blotting

Protein extracts prepared from fungal tissue and from maize roots or leaves were separated on 12% (w/v) polyacrylamide denaturing gels (SDS-PAGE; Laemmli, 1970). The polypeptides were stained with Coomassie Brilliant Blue or blotted onto a nitrocellulose membrane (Hybond C; Amersham) for immunoassays (Renart and Sandoval, 1984). Protein blots were probed with specific antibodies raised against TvSP1 (Pozo et al., 2004).

Infection of *Trichoderma*-Treated Maize Plants with *Colletotrichum graminicola*

The inoculation of maize leaves was performed as described previously (Djonović et al., 2007a). Control (nontreated) and treated seeds (coated with chlamydospore preparations of Gv29-8, Δ*tvinv2*, or Δ*tvinv5*) were grown in plastic cylinders containing a soil-less mix (Metromix 366) and incubated in a growth chamber at 25°C. The third leaf of 14-d-old plants was inoculated with 5 μL of a suspension of 6.5 × 10^4 spores mL^{-1} *C. graminicola*. After incubation

for 4 d, the leaves were scanned and the infection area was determined and compared among the treatments (Djonović et al., 2007a).

Microscopy and Imaging

Plants in hydroponic systems were inoculated with fungal hyphae, and after 2 d of culture the roots were collected for microscopic visualization and to compare root cell autofluorescence. The fungal hyphae growing on the root surface were stained by the succinate dehydrogenase method as reported by MacDonald and Lewis (1978). This method was originally developed to stain metabolically active arbuscular mycorrhiza inside roots and is based on the reaction catalyzed by succinate dehydrogenase with nitroblue tetrazolium chloride, resulting in the formation of insoluble formazan. Chlamydospores on the root surface were counted on micrographs from five plants per treatment in each of three independent experiments; the results are presented as number of chlamydospores per square millimeter of root ± SD. For autofluorescence, the roots were visualized with an Olympus BX51 fluorescence microscope. Images were recorded using an Olympus DP70 camera and processed with DPController 1.1.165 software.

Bioinformatics and Statistics

Sequence comparisons were performed using deduced amino acid sequences for β-fructofuranosidases retrieved from the databases at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/). Sequence alignments and graphical representations of phylogenetic trees were performed with MEGA (version 4) software (Takamura et al., 2007). The presence of putative subcellular signals or posttranslational sites was calculated using the resources available at ExPASy proteomics tools (http://www.expasy.ch/tools/). Statistical analysis was performed by one-way ANOVA followed by Tukey's honestly significant difference (HSD) test using VassarStat (http://faculty.vassar.edu/lowry/VassarStats.html).

Germination and Conidiation Assays

Spore germination assay was performed as described previously (Baek and Kenerley, 1998). Spore suspensions of each strain were spread on WA-, VM-, VMS-, or PDA-coated slides and incubated for 12 h at 27°C in the dark in moist chambers. Germinated conidia were counted along random transects across the slide. Conidial formation was assayed as described previously (Djonović et al., 2007a). Spore suspensions (10^3 spores mL^{-1}) of each strain were spread on WA, VM, VMS, or PDA plates, and after 4 or 10 d three plugs were removed from each plate and resuspended in 10 mL of water. Number of spores in the suspension was determined with a hemacytometer.

Root Colonization Assays

The roots of 2-week-old plants inoculated with either the wild type or the mutant strains and grown in Metromix were surface sterilized and ground in the presence of 100 mM phosphate buffer, pH 7, 20 mM $MgCl_2$, and Silwett 77 (root-grinding buffer), and serial dilutions were plated on GVSM medium (Park et al., 1992). Competition between Gv29-8 and either Δ*tvinv2* or Δ*tvinv5* for their ability to colonize maize roots was determined by infesting Metromix with equal amounts of *T. virens* chlamydospore preparation. After 7 d of incubation, roots were processed as above and dilutions were plated on GVSM medium. Colonies that appeared after 3 d were individually transferred to GVSM supplemented with hygromycin. The colonies that were able to grow in the presence of hygromycin corresponded to the mutant strains, and those unable to grow in these conditions corresponded to Gv29-8.

Photosynthetic Uptake of CO_2

The uptake of CO_2 was determined using the LI-COR LI-6400 portable photosynthesis system. The measurement parameters were set as follows: CO_2 flux, 500 μm s^{-1}; sample cell CO_2, 360 μmol CO_2 mol^{-1}; block temperature, 25°C; photosynthetically active radiation, 600 μmol; area, 6; stomata, 0.50. The measurements (nmol CO_2 m^{-2} s^{-1}) were performed in triplicate on the distal tip of the third leaf of five different plants. The experiment was independently repeated twice.

Collection of Root Exudates and Thin-Layer Chromatography

Maize seeds were surface sterilized using hydrogen peroxide as described previously (Djonović et al., 2007a) and planted in pots containing sterile sand. After 7 d, the plants were carefully removed from the pots and the sand was washed from the roots. Plants with intact root systems were used for exudate collection. Four plants of each line were placed in individual flasks (wrapped with aluminum foil) containing 10 mL of distilled water and incubated under constant light for 4 h with orbital shaking. The liquid suspension was then filter sterilized and lyophilized, and concentrated solids were dissolved in 50 μL of water. The concentrated root exudates were separated by thin-layer chromatography using Silca Gel GF 2000 Uniplate (Analtech) by developing three times with 1-butanol:isopropanol:water (3:12:4). Suc, raffinose, and stachyose were chromatographed in parallel as standards. The positions of the sugars were ascertained with urea-phosphoric acid reagent (Wise et al., 1955).

The sequence data of TvInv have been submitted to the EMBL sequence database under the accession number FM206347.

Supplemental Data

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

Supplemental Figure S1. Deduced amino acid sequence for TvInv.

Supplemental Figure S2. Expression of *tvinv* in *T. virens* cells cultured in alternative sources of carbon.

Supplemental Figure S3. Gene-deletion strategy.

Supplemental Figure S4. Screening of *T. virens* transformants.

Supplemental Figure S5. Detection of Suc in root exudates of maize plants.

Supplemental Figure S6. Immunodetection of TvSP1 in protein extracts from maize roots inoculated with *T. virens* wild type (Gv29-8), Δ*tvsp1*, Δ*tvinv2*, and Δ*tvinv5*.

Supplemental Figure S7. Sm1 expression and ISR induction.

Supplemental Figure S8. Growth of *T. virens* Gv29-8, Δ*tvinv2*, and Δ*tvinv5* in VMS medium.

Supplemental Table S1. Primers used in PCR

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