Role of the 4-Phosphopantetheinyl Transferase of *Trichoderma virens* in Secondary Metabolism and Induction of Plant Defense Responses

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Trichoderma virens is a ubiquitous soil fungus successfully used in biological control due to its efficient colonization of plant roots. In fungi, 4-phosphopantetheinyl transferases (PPTases) activate enzymes involved in primary and secondary metabolism. Therefore, we cloned the PPTase gene *ppt1* from *T. virens* and generated PPTase-deficient ($\Delta ppt1$) and overexpressing strains to investigate the role of this enzyme in biocontrol and induction of plant defense responses. The $\Delta ppt1$ mutants were auxotrophic for lysine, produced nonpigmented conidia, and were unable to synthesize nonribosomal peptides. Although spore germination was severely compromised under both low and high iron availability, mycelial growth occurred faster than the wild type, and the mutants were able to efficiently colonize plant roots. The $\Delta ppt1$ mutants were unable of inhibiting growth of phytopathogenic fungi in vitro. *Arabidopsis thaliana* seedlings co-cultivated with wild-type *T. virens* showed increased expression of *pPr1a:uidA* and *pLox2:uidA* markers, which correlated with enhanced accumulation of salicylic acid (SA), jasmonic acid, camalexin, and resistance to *Botrytis cinerea*. Co-cultivation of *A. thaliana* seedlings with $\Delta ppt1$ mutants compromised the SA and camalexin responses, resulting in decreased protection against the pathogen. Our data reveal an important role of *T. virens* PPT1 in antibiosis and induction of SA and camalexin-dependent plant defense responses.

Phosphopantetheinyl transferases (PPTases) belong to a superfamily of enzymes found in prokaryotes and eukaryotes which are required for the synthesis of a wide range of compounds, including fatty acids, amino acids, polyketides, and nonribosomal peptides. PPTases activate carrier proteins in specific biosynthetic pathways by the transfer of a phosphopantetheinyl moiety to an invariant serine residue. PPTases catalyze the nucleophilic attack of the hydroxyl side chain of the conserved carrier protein serine residue on the 5'- β -pyro-

phosphate linkage of CoA. This causes the transfer of the phosphopantetheinyl moiety of CoA to the side chain of a conserved serine, converting the carrier protein from an inactive apo-form to an active holo-form (Lambalot et al. 1996; Walsh et al. 1997).

The PPT superfamily has been divided into two paralogous groups that correspond to substrate specificity. The first is the AcpS family that comprises homotrimers of 120 to 140 amino acids that are involved in fatty acid biosynthesis in bacteria and fungi (Allen et al. 2011; Hiltunen et al. 2010). Members of the second family such as Sfp are monomeric enzymes of 220 to 240 amino acids, which participate in the synthesis of secondary metabolites by activating nonribosomal peptide synthetases (NRPS) and polyketide synthetases (PKS) in both bacteria and eukaryotes (Lambalot et al. 1996). This family includes PPTases involved in cyanobacterial heterocyst differentiation, fungal lysine biosynthesis, β -alanine conjugation, hybrid peptide synthase/polyketide synthase complexes, and other enzymes with an as-yet-unidentified function (Copp and Neilan 2006). A third group of PPTases is characterized by being an integral part of fatty acid synthetases in eukaryotes (Mootz et al. 2001).

The importance of PPTases in the synthesis of antibiotics and siderophores has been widely demonstrated in prokaryotes such as *Escherichia coli* and *Pseudomonas syringae* (Flugel et al. 2000; Lambalot et al. 1996; Seidle et al. 2006). Many microorganisms possess multiple PPTases. The genome of *Bacillus subtilis* encodes Sfp for surfactin biosynthesis, and AcpS, involved in siderophore synthesis (Mootz et al. 2001; Ollinger et al. 2006). In comparison, the genome of *E. coli* encodes three PPTases: AcpS and EntD, for synthesis of fatty acids and the siderophore enterobactin; and YhhU, an uncharacterized PPTase (Flugel et al. 2000; Lambalot et al. 1996). These enzymes act in distinct pathways and display contrasting specificity for carrier proteins. The Sfp-like EntD is unable to complement an AcpS mutant of *E. coli*. In contrast, the *B. subtilis* Sfp displays a remarkable range of carrier protein substrates (Gehring et al. 1998; Keating and Walsh 1999; Marahiel et al. 1997). When an AcpS-like PPTase is not present in an organism, the Sfp-like PPT will act in both primary and secondary metabolic pathways, displaying a preference for the carrier proteins of fatty acid synthesis (FAS) (Finking et al. 2002). In the case of the fungal antagonistic bacteria *B. subtilis*, a PPTase

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was found to be involved in the synthesis of antibiotics such as surfactin, which promotes induced systemic resistance (ISR) in common bean and tomato (Ongena et al. 2007). Currently, PPTases of the Sfp family have been studied in bacteria pathogenic to humans or plants, or in model systems. In the case of microorganisms used in biological control in agriculture, one of the few cases in which their function has been studied is that of *P. luminescens* (Chiche et al. 2001). This bacterium is associated with an entomopathogenic nematode, and its corresponding PPTase was found to be essential for the synthesis of toxins that kill the insect.

In fungi, there is still limited information about the role of PPTases. A single multifunctional PPTase has been described in *Aspergillus nidulans* (Keszenman-Pereyra et al. 2003; Márquez-Fernández et al. 2007; Oberegger et al. 2003). Similar findings were reported in *A. fumigatus* (Neville et al. 2005), *Penicillium chrysogenum* (García-Estrada et al. 2008), and the plant pathogens *Colletotrichum graminicola* and *Magnaporthe oryzae* (Horbach et al. 2009). However, recently, a mitochondrial PPTase (PptB) of the AcpS type, specific for the mitochondrial acyl carrier protein AcpA, was reported in *A. fumigatus* (Allen et al. 2011). These studies also demonstrated that PPTases are necessary for synthesis of lysine and many secondary metabolites, including antibiotics, siderophores, and pigments.

The genus *Trichoderma* comprises a large number of filamentous fungi of wide distribution in agricultural ecosystems, which are well characterized in terms of production of polyketides and nonribosomal peptides (Reino et al. 2007). A single NRPS can produce up to three distinct peptaibols. Accordingly, Neuhoof and co-workers (2007) analyzed 34 phylogenetically related *Trichoderma* strains, identifying 58 different classes of peptaibols. Several species of the genus *Trichoderma* are necrotrophic mycoparasites of phytopathogenic fungi and are widely used in biological control of diseases in agriculturally important crops. Moreover, some *Trichoderma* isolates are capable of activating plant defense responses (Yedidia et al. 1999, 2003).

Plants possess various inducible defense mechanisms for protection against pathogen attack. An example of this is systemic acquired resistance (SAR), which is activated after infection by necrotizing pathogens (Ryals et al. 1996). Similarly, colonization of plant roots by certain nonpathogenic rhizobacteria can produce ISR in the host plant (van Loon et al. 1998). ISR is a plant-mediated mechanism similar to SAR which is initiated at the root and extends up to the shoot, conferring protection against different types of plant pathogens. Induction of ISR depends, in part, on phytohormone signaling mediated by jasmonic acid (JA) and ethylene (ET) (Glazebrook 2005). Accumulation of antimicrobial metabolites is integral to plant protection. In *Arabidopsis thaliana*, accumulation of the phytoalexin camalexin was found in tissue exposed to infection by either avirulent or virulent strains of the bacterium *Pseudomonas syringae* (Glazebrook and Ausubel 1994; Tsuji et al. 1992) and after inoculation with the fungus *Cochliobolus carbonum* (Glazebrook et al. 1997). In vitro studies demonstrated that camalexin inhibits bacterial and fungal growth (Ferrari et al. 2003; Jejelowo et al. 1991; Rogers et al. 1996; Tsuji et al. 1992).

There is a wide range of hormone-like factors that affect the responses of plants to *Trichoderma* spp. In cucumber (*Cucumis sativus*) and maize (*Zea mays*), the main signaling pathway by which *Trichoderma virens* induces systemic resistance involves JA and ET (Djonovi et al. 2007; Yedidia et al. 2003). Both salicylic acid (SA) and JA changes have been previously described in cucumber plants inoculated with *T. asperellum* T34 (Segarra et al. 2007), indicating that hormonal defense protection of plants is widespread among *Trichoderma* spp. By

using an *Arabidopsis*–*Trichoderma* co-cultivation system, we analyzed the response of pathogenesis-related reporter genes to *T. virens* or *T. atroviride*, which provided evidence that the defense signaling pathway activated by these fungi involves SA and JA (Salas-Marina et al. 2011). Interestingly, accumulating evidence suggests that the 18-mer peptaibols are critical for the chemical communication between *Trichoderma* spp. and the plant (Brotman et al. 2009; Viterbo et al. 2007). In fact, the peptaibol alameticin produced by *T. viride* sprayed on *Phaseolus lunatus* plants activates ISR, resulting in the production of defense compounds against herbivores (Engelberth et al. 2000). In a more recent study, cucumber plants co-cultivated with *T. virens* strains disrupted in the NRPS encoding gene *tex1* showed a significant reduction in systemic resistance against the leaf pathogen *Pseudomonas syringae* pv. *lachrymans*, and reduced production of phenolic compounds with inhibitory activity against this bacterium (Viterbo et al. 2007).

Here, we report the characterization of *T. virens* mutants defective in the *ppt1* gene and show that the corresponding protein is required for the synthesis of lysine, peptaibols, pigments, and siderophores. The *Δppt1* mutants showed a dramatically reduced capacity to inhibit the growth of phytopathogenic fungi in vitro. In addition, decreased activation of ISR in *A. thaliana* was observed when plants were grown in association with *T. virens* lacking PPTase PPT1, which correlated with a reduction in SA and camalexin levels in plants. Surprisingly, the production of JA and activation of JA-dependent *pLox2:uidA* were still observed in plants co-cultivated with *T. virens Δppt1* mutants, even though the mutants failed to produce polyketides and nonribosomal peptides. Our results reveal a key role of *T. virens* PPTase in antibiosis, and the specific induction of SA and camalexin-dependent plant defense responses.

RESULTS

***T. virens ppt1* mutants produce nonpigmented conidia, show enhanced growth, and require supplemental iron to germinate.**

Bioinformatics analysis of the sequenced genomes of members of the genus *Trichoderma* revealed that there are three sequences with similarity to PPTases in the genome of *T. virens* (protein ID 194983 of the Sfp type, protein ID 203026 of the AcpS type, and protein ID 48659 of the fatty acid synthesis type I), as well as those of *T. atroviride* (protein ID 52102 of the Sfp type, protein ID 286047 of the AcpS type, and protein ID 85662 of the fatty acid synthesis type I), whereas that of *T. reesei* has only two PPTases (protein ID 56081 of the AcpS type and protein ID 48788 of the fatty acid synthesis type I). Analysis of the deduced protein sequence of the *T. virens* gene (*ppt1*), corresponding to the PPTase of the Sfp type (ID 194983) indicated that it contains the conserved motifs FNVTHQ (102 to 107), VAIGTD (123 to 128), and WCLREAYVK (188 to 196) characteristic of PPTases. To determine the functional role of the *T. virens* phosphopantetheinyl transferase, the *ppt1* gene was replaced by the *T. virens arg2* gene in the arginine auxotrophic strain Tv10.4 of *T. virens* (Supplementary Fig. 1). Gene replacement was confirmed by polymerase chain reaction (PCR) and Southern blot. A null mutant was then retransformed with the wild-type *ppt1* gene to verify that all observed phenotypes were due to the replacement of the gene. Mutant complementation was confirmed by Southern blot, showing the integration of multiple copies of the gene in the complemented strains (data not shown), and the complemented strain included in all subsequent analyses. To determine if PPT1 is a limiting factor for the activation of PKS and NRPS in *T. virens*, overexpressing strains were generated by transformation of the wild-type strain using a plasmid containing the cod-

ing sequence of *ppt1* fused to the constitutive promoter of the *T. reesei* pyruvate kinase gene. Integration of the construct was confirmed in two transformants by Southern blot (Supplementary Fig. 2). Analysis of the expression of *ppt1* in these transformants (OE-4 and OE-6) revealed high levels of expression, whereas no mRNA could be detected in the wild-type strain (Supplementary Fig. 3). Expression of the gene in the wild-type strain (Tv29.8), the parental strain used to generate the mutant (Tv10.4), and the retransformed strain (TvC-24) used throughout our studies was confirmed by reverse-transcription PCR (data not shown).

The evaluation of growth and conidiation of the mutant (*Δppt1*-1) and complemented (TvC-24) strains in comparison with the wild-type (Tv29.8) and parental (Tv10.4) strains was carried out (Fig. 1A and B). A fluffy phenotype was observed in the gene-replacement mutant, which unexpectedly had a marked increase in radial growth (93%) compared with the wild type (Fig. 1A and C). In addition, the mutant produced white conidia, lacking the characteristic green pigment of the wild type (Fig. 1A), suggesting that the mutation likely affected the activation of the polyketide synthase responsible for the synthesis of this pigment. As expected, these phenotypes disappeared in the complemented strains, which showed no difference in growth or conidiation when compared with the wild-type strain (Fig. 1B and C). All *Δppt1* mutants obtained behaved similarly (data not shown).

Growth of the wild-type and mutant strains in minimal medium supplemented with iron revealed a clear reduction in conidial germination, which was even more drastically reduced in the absence of iron, suggesting an important role of siderophores in the iron capture mechanism necessary for germination of conidia (Fig. 2A and B). To determine whether the mutation also affected iron capture during active growth, the mutant was evaluated in minimal medium with or without iron and without the addition of siderophores. In contrast to germination of conidia, the mutant grew without any apparent problem without iron, although the mycelium was scarce and growth was arrested after 72 h (Fig. 2C). In medium supplemented with iron, the mutant grew normally and produced abundant mycelium (Fig. 2D).

The *ppt1* mutants show decreased antagonistic activity against phytopathogens.

To better understand the role played by secondary metabolites in the control of phytopathogenic fungi by *T. virens*, we evaluated the inhibitory activity of small molecules produced by the different *Trichoderma* strains generated on the growth of seven phytopathogenic fungi (*Alternaria solani*, *Fusarium* spp., *Fusarium oxysporum*, *Phytophthora capsici*, *Rhizoctonia solani*, *Sclerotium rolfsii*, and *S. cepivorum*). The growth of all pathogens was completely inhibited when grown in media where the wild type, overexpressing, or complemented *Trichoderma* strains had been pregrown, with the exception of *S. rolfsii*, which only showed a delay in growth. In contrast, the growth of pathogens in medium where the *Δppt1* strain had been pregrown was unaffected, except in the cases of *Fusarium* spp. and *F. oxysporum*, which grew but whose colonies clearly showed less dense mycelial mats (Fig. 3A). All seven phytopathogens grew normally in the medium without *Trichoderma* metabolites. These data suggested that the lack of activation of PKS and NRPS, responsible for the production of most secondary metabolites by *Trichoderma* spp., severely affects the capacity of *T. virens* to inhibit the growth of phytopathogenic fungi. To confirm that the loss of this capacity was due to the lack of antibiotic production, mycelial extracts of *Trichoderma* spp. grown in liquid medium were analyzed by mass spectrometry using electrospray ionization quantitative

time-of-flight (ESI-QTOF). In the cases of parental Tv10.4 (Fig. 3B) and wild-type Tv29.8 strains (data not shown), three classes of peptaibols were identified by mass spectrometry analysis, including those previously reported: 11, 14, and 18 mer (Viterbo et al. 2007). In contrast, the *Δppt1* mutant did not produce any of these metabolites (Fig. 3C), confirming that mutation of the corresponding gene severely affects the synthesis of peptaibols, thus decreasing the potential of *Trichoderma* spp. to inhibit the growth of other fungi.

T. virens Δppt1 mutants are capable of colonizing plant roots.

It has previously been demonstrated that *Trichoderma* spp. colonize plant roots. Following root penetration, the exchange of bioactive compounds controls the endophytic proliferation of the fungus (Chacón et al. 2007; Yedidia et al. 1999). Although it has been shown that some secondary metabolites may potentiate systemic induced response, it is not known if they play a role in the plant–*Trichoderma* communication. For this reason, the colonization of *Solanum lycopersicum* roots by wild-type *T. virens* and *Δppt1* mutants in vitro and their permanence in the root system were analyzed. Using vital staining of fungal hyphae and confocal microscopy, we detected the presence of all tested strains of *T. virens* Tv10.4, Tv29.8, and *Δppt1*-1 in the root epidermis after 48 h of plant-fungus interaction. At this time, all strains were present in the first cell layers of the root (Fig. 4B to D). Twenty-four hours later, the strains Tv10.4, Tv29.8, and TvC-24 colonized the root system without extending to the aerial part of the plant, whereas the *Δppt1* mutant colonized the root system but also invaded the stem of the plant (Supplementary Fig. 4). After 72 h of the interaction, plants were transferred to soil and grown for 15 additional days. At the end of this period, persistence of

Fig. 1. *Trichoderma virens* mutants in *ppt1* are altered in growth and conidiation. Representative photographs showing the aspect of the colonies of the strains photographed after **A**, 72 h of growth at 28°C in potato dextrose agar (PDA). Names on top of columns indicate the *T. virens* strain evaluated. **C**, Kinetics of growth of the indicated strains in PDA as determined by measuring colony diameter. Values shown represent the mean of three different plates $\pm$ standard deviation. Means with different letter in a column are statistically different ($P \leq 0.05$). The experiment was repeated twice with similar results.

Trichoderma spp. in the roots was evaluated by recovering them from surface-sterilized root fragments in selective media (Fig. 4E to H). Even though the gene replacement mutant ($\Delta ppt1$ -1) and the parental strain (Tv10.4) were auxotrophic for lysine and arginine, respectively, they could survive in the root system. The identity of the recovered strains was confirmed by PCR using oligonucleotides *ppt1-G* and *ppt1-H* that allowed the distinction between the wild type and the gene replacement mutant (data not shown).

Nonribosomal peptides and polyketides play a minor role in seed protection by *Trichoderma* spp.

The role of antibiotics in fungal antagonism by *Trichoderma* spp. has been well established in vitro, and synergism with cell wall-degrading enzymes has also been observed (Schirmböck et al. 1994). In spite of the efforts made to understand the role of antibiotics in the interaction with the plant (Tijerino et al. 2011; Vinale et al. 2006), their role in vivo remains poorly understood. Therefore, we next investigated the role of nonribosomal peptides and polyketides from *T. virens* in seed protection through the analysis of the protection conferred by wild-type *T. virens* and $\Delta ppt1$ mutants to *S. lycopersicum* seeds exposed to a substrate infested with *R. solani*. By measuring seed germination, we determined that, without *Trichoderma* spp., only 45% of *S. lycopersicum* seeds germinated in the presence of *R. solani*. Seed treated with *T. virens* Tv29.8 and Tv10.4 behaved similarly, showing 65% germination. A contrasting result was obtained when seeds were treated with the gene-replacement mutant, which apparently were more efficiently protected, showing 77% germination (Fig. 5A). Axenic seeds or *T. virens* wild-type and $\Delta ppt1$ -1-treated seeds showed a

roughly 80% germination in medium without *R. solani* (Fig. 5B). These data suggest that antibiotics of the NRP and polyketide type do not play a significant role in seed protection against *R. solani*.

T. virens $\Delta ppt1$ mutant is defective in induction of SA-dependent defense responses in *Arabidopsis thaliana*.

The defense signaling pathways that are activated in *A. thaliana* upon exposure to *Trichoderma* spp. involve both SA and JA (Salas-Marina et al. 2011; Segarra et al. 2007). To examine whether mutation in *ppt1* could affect the SA or JA responses, we monitored expression of selected marker genes that are upregulated by these hormones. We used *A. thaliana* transgenic lines expressing β -glucuronidase (GUS) (*uidA*) fusions to the promoters of *Pr1a*, a gene activated by SA (Shah et al. 1997) and *Lox2*, a gene activated by JA (Schommer et al. 2008). *A. thaliana* transgenic seedlings carrying each of these markers were co-cultivated with the different *T. virens* strains. Axenically grown seedlings did not express *pPr1a:uidA* (Fig. 6A, K, F, and O). *pPr1a:uidA* expression was activated in both shoots and roots of plants inoculated with the wild-type strain (Tv29.8), the parental strain (Tv10.4), and the complemented strain TvC-24 (Fig. 6B to D and G to I). In sharp contrast, co-cultivation of this reporter line with the *T. virens* $\Delta ppt1$ -1 mutant failed to activate GUS expression (Fig. 6E and J). Surprisingly, the JA-activated marker *pLox2:uidA* was expressed at a similar level in plants co-cultivated with wild-type *T. virens* or the $\Delta ppt1$ mutant strains (Fig. 6L to N and P to S). These data suggest that *T. virens* PPT1-dependent secondary metabolites are specifically involved in triggering a subset of *A. thaliana* responses mediated by SA.

Fig. 2. The $\Delta ppt1$ mutant of *Trichoderma virens* is affected in germination of conidia. Germination of conidia of the indicated *T. virens* strains in VMS medium (Viterbo et al. 2007) **A**, with or **B**, without iron. Mycelial growth of the indicated *T. virens* strains in Grimm-Allen medium **C**, with or **D**, without iron. **A** to **D**, Bars indicate standard deviation. Means with different letter in a column are statistically different ($P \leq 0.05$). The experiment was repeated twice with similar results.

Interaction between *A. thaliana* roots and *T. virens* Δ *ppt1* mutant fail to induce SA accumulation in leaves.

To determine whether the changes in the expression of *pPr-1a:uidA* and *pLox2:uidA* markers were associated with changes in endogenous SA or JA content in plants co-cultivated with *Trichoderma* spp., free SA and JA were measured in wild-type *A. thaliana* (Col-0) plants co-cultivated with wild-type *T. virens* or Δ *ppt1* mutants by gas chromatography–mass spectrometry (GC-MS). A fourfold increased accumulation in SA was observed in treatments with wild-type *Trichoderma* spp. when compared with axenically grown seedlings (Fig. 7A). The levels of SA dramatically decreased in plants co-cultivated with the Δ *ppt1*-1 mutant (Fig. 7A). In contrast, JA determinations clearly showed a three- to fourfold increase in JA in plants that were co-cultivated with either wild-type *T. virens* or Δ *ppt1*-1 mutant strains (Fig. 7B).

Trichoderma Δ *ppt1* mutants fail to induce camalexin accumulation in *A. thaliana*.

To investigate whether deletion of *ppt1* could affect camalexin production, axenically grown plants, or plants colonized with wild-type *T. virens* or the Δ *ppt1*-1 mutant were used for camalexin determinations. GC-MS analysis revealed that *A. thaliana*

seedlings interacting with wild-type *T. virens* increased camalexin levels by three- to fourfold when compared with axenically grown plants (Fig. 8). This effect was highly reduced in plants co-cultivated with Δ *ppt1* mutants (Fig. 8).

Deletion of *ppt1* compromises *T. virens* protection against the necrotizing pathogen *Botrytis cinerea*.

To determine whether the alterations in SA and camalexin-dependent responses observed in *A. thaliana* seedlings exposed to Δ *ppt1* mutants could affect pathogen resistance, we tested the responses of leaves from 12-day-old *Arabidopsis* plants whose roots had been colonized or not with wild-type *T. virens* or Δ *ppt1* mutant and inoculated with the necrotrophic pathogen *Botrytis cinerea*, which causes spreading necrotic lesions on leaves. In these experiments, *B. cinerea* spores were inoculated on the leaf surface and disease symptoms evaluated 3 and 5 days later. In control plants, *B. cinerea* was found to induce necrotic lesions in approximately 55% of inoculated leaves (Fig. 9A). In contrast, in plants colonized by the *T. virens* wild type, only 29% presented necrotic lesions caused by *B. cinerea* infection (Fig. 9A), whereas plants co-cultivated with the Δ *ppt1* mutant showed levels of necrotic lesions similar to the control plants (Fig. 9A). Furthermore, *B. cinerea* caused death

Fig. 3. Effect of antibiotics from *Trichoderma virens* on phytopathogenic fungi and production of peptaibols. **A**, Growth of phytopathogens on plates where *Trichoderma* had been pregrown photographed after 48 h of growth. Row 1, *Alternaria solani*; row 2, *Fusarium* spp.; row 3, *Fusarium oxysporum*; row 4, *Phytophthora capsici*; row 5, *Rhizoctonia solani*; row 6, *Sclerotium rolsii*; row 7, *S. cepivorum*. Names on top of each column indicate the *T. virens* strain evaluated. Detection of peptaibols by mass spectrometry in extracts from strains **B**, Tv 10.4 and **C**, Δ *ppt1*-1.

in approximately 90% of control plants, in contrast to only 25% death in plants colonized by the wild-type *Trichoderma* strain, whereas the *ppt1* mutant failed to confer protection (Fig. 9B).

DISCUSSION

Phosphopantetheinyl transferases compose a class of ubiquitous enzymes found in filamentous fungi, which are necessary for primary metabolism due to their role in activating the α -amino acid reductase and in secondary metabolism for the activation of polyketide synthases and NRPS. Here, we report the role of an Sfp-class phosphopantetheinyl transferase from *T. virens* in fungal physiology, biocontrol, and plant interaction.

We observed that $\Delta ppt1$ mutants of *T. virens* had an accelerated radial growth of vegetative hyphae and delayed production of conidia. Conidia were produced only when growth of aerial hyphae decreased. A similar alteration in vegetative growth and a sharp decrease in conidiation were reported in the *cfwA/ngpA* mutant of *Aspergillus nidulans*, which was attributed to a delay in hyphal branching (Márquez-Fernández et al. 2007). Increased growth was also observed in the conditional mutant *ngpA/cfwA* from *A. nidulans*, which produced more mycelial mass than the wild type in liquid culture (Keszenman-Pereyra et al. 2003). We hypothesize that the enhanced mycelium growth and decreased conidiation in the *T. virens* $\Delta ppt1$ mutant is due to the lack of production of secondary metabolites, because growth of the mutant in media with such metabolites excreted by the wild type were sufficient to revert this phenotype, as well as the fluffy aspect of the colony. A plausible explanation is that the peptaibols produced by *Trichoderma* spp. can affect its own plasma membrane functions, and that the lack of production of these metabolites by the mutant potentiates growth, leading to the production of more aerial mycelium. Although it has never been reported that *T. virens*'s own antibiotics could affect its growth or conidiation, Howell and Stipanovic (1983) reported that a vir-

idiol overproducing mutant of *T. virens* (formerly *Gliocladium virens*) grew more slowly than the parental strain.

When grown in media with low or high iron concentration, conidia of the $\Delta ppt1$ mutant showed low germination index. In this respect, trihydroxamates such as ferricrocin are essential to store iron intracellularly, which aids spore germination in iron-poor media, as demonstrated for *sidC* in *A. fumigatus* (Schrettl et al. 2007), *A. nidulans* (Wallner et al. 2009), and *Neurospora crassa* (Berthold et al. 1987; Charlang and Williamst

Fig. 5. Protection of *Solanum lycopersicum* seeds by *Trichoderma* spp. **A**, Effect of inoculation of the indicated *Trichoderma virens* strains on germination of seeds in soil infested with *Rhizoctonia solani*. **B**, Effect of inoculation of *T. virens* on germination of seeds in sterile soil. **A** and **B**, Control corresponds to uninoculated seed. Bars indicate standard deviation. Means with different letter in a column are statistically different ($P \leq 0.05$). The experiment was repeated twice with similar results.

Fig. 4. Colonization of *Solanum lycopersicum* roots. Top: Confocal microscopy representative photographs of **A**, roots without *Trichoderma* spp. or roots colonized by **B**, *Tv* 29.8; **C**, *Tv* 10.4; or **D**, $\Delta ppt1-1$ strains. Bottom: *Trichoderma* strains recovered from root fragments; **E**, $\Delta ppt1-1$ and **G**, *Tv* 10.4 recovered and grown in Rose Bengal selective medium; **F**, $\Delta ppt1-1$ and **H**, *Tv* 10.4 strains recovered and grown in potato dextrose agar.

1977). On the other hand, when mycelial growth of the null mutant of *T. virens* was analyzed under iron-limiting conditions, it grew at approximately the same rate as observed for the wild-type strain, although mycelium was scarce. Similar results were also observed in *cfwA* and *PPT1* mutants in *A. nidulans* and *Colletotrichum graminicola*, respectively (Horbach et al. 2009; Oberegger et al. 2003). The observed phenotype might be due to the lack of *cis/trans* fusarinine and dimerum acid, which trap extracellular iron, and might be substituted by the reductive iron assimilation (RIA) pathway, a two-step process that involves the extracellular reduction of Fe^{3+} to Fe^{2+} followed by high-affinity uptake of Fe_2 . In this sense, several orthologous genes to the three components of RIA (*Fre1*, *FetC*, and *FtrA*) were found in the *T. virens* genome.

One of the main attributes of the genus *Trichoderma* is the production of secondary metabolites that inhibit the growth of or kill phytopathogenic fungi and some gram-positive bacteria in vitro (Fravel 1988; Neuhoef et al. 2007; Reino et al. 2007; Wiest et al. 2002). Among the main antibiotics produced by *T. virens*, peptaibols have deserved more attention. Peptaibols of class 11, 14, and 18 mer were produced by the wild-type strain of *T. virens* as well as the strain used for transformation (Tv10.4), which correspond to those classified as short (11 to 16 amino acids) and long (18 to 20 amino acids) sequences. These peptaibols belong to the TvA class, which is a mix of 11-amino-acid peptides similar to Harzianines HB, and class TvBI Trichorzins type, with 18 residues (Wiest et al. 2002). These peptaibols were reported in the study of the function of the *T. virens* 29.8 NRPS encoding gene *tex1*, where the corresponding mutants failed to produce 18-, 14-, and 11-amino-acid residue peptides peptaibols (Viterbo et al. 2007; Wiest et al. 2002). Our results showed that the *Appt1-1* mutant did not

produce peptaibols, thus indicating that the NRPS that participate in their synthesis require PPT1 for their activation.

The role of nonribosomal peptides and polyketides in antagonism by the different *T. virens* strains analyzed here was evaluated through their effects on the growth of several phytopathogenic fungi. Overexpression of *ppt1* did not result in increased inhibitory capacity. In contrast, *Appt1* mutants showed strongly decreased antagonistic activity against phytopathogens, suggesting that normal PPT1 function is an important factor in biocontrol by *T. virens*, and that higher levels of PPT1 do not seem to result in increased antagonistic activity by *T. virens*.

An important application of microbial antagonists is seed protection, which allows plant germination in pathogen-infested soils. However, the role of antibiotics produced by *Trichoderma* spp. in plant protection in soil has remained speculative. The fact that the *Appt1-1* mutant of *T. virens* conferred greater protection to seed of *S. lycopersicum* in soil infested with *R. solani* than the wild-type and parental strains indicates that non-ribosomal peptide and polyketide antibiotics play a minor role in seed protection by *T. virens*. The higher protection observed with the *Appt1-1* mutant could be due to the fact that the mutant grows faster, increasing its competition capacity, although we cannot discard the possibility that other antimicrobial compounds are overproduced by *Trichoderma* spp. in the absence of active NRPS or PKS, which might aid in seed protection. Our findings suggest that hydrolytic enzymes and mycoparasitism are more relevant than antibiotics in the control of *R. solani* during seed protection. A similar observation was made in the case of a *T. virens* mutant that did not produce gliotoxin but remained efficient in the protection of plants against infection by *R. solani* (Howell and Stipanovic 1995).

Fig. 6. Effect of *Trichoderma virens* on expression of the pathogen-related response markers *pPr1a:uidA* and *pLox2:uidA*. Transgenic *Arabidopsis thaliana* seedlings carrying the marker constructs were germinated and grown for 4 days on 0.2× Murashige and Skoog plates, transferred to 0.2× Murashige and Skoog medium supplemented with 300 μM Lys, and then co-cultivated with the *T. virens* strains indicated on top. β -Glucuronidase (GUS) expression in seedlings was determined 6 days after transfer. **A to J**, Plants carrying the *pPr1a:uidA* construct. **K to S**, Plants carrying the *pLox2:uidA* construct. **A, F, K, and O**, Axenically grown plants. **A to E and K to N**, GUS expression in aerial parts of the plant. **F to J and O to S**, GUS expression in roots. Photographs show representative individuals of at least 20 stained seedlings. The experiment was repeated twice with similar results.

As in other fungi, *T. virens* $\Delta ppt1$ mutants are auxotrophic to lysine. This represented an obstacle in evaluating the role of PPT1 in biocontrol. To overcome this obstacle, we analyzed the possibility of allowing colonization of the root system of *S. lycopersicum* plantlets in vitro and then transferring them into soil. The root system was colonized after 48 h and the fungus remained in plants up to 3 weeks. This is explained by the fact that root exudates of *S. lycopersicum* contain essential amino acids such as lysine, arginine, aspartic acid, and glutamic acid, among others, that provide the requirements for growth (Simons et al. 1997).

Some natural isolates of *Trichoderma* spp. have been found to colonize not only plant roots but also other parts of the plant, as in the case of *Theobroma cacao*, colonizing roots, stem, leaves, and even seed (Bailey et al. 2006). By using an in vitro system, we showed that the $\Delta ppt1$ -1 mutant colonized the

root system as well as the wild-type strain. Interestingly, after a longer period of time, the mutant continues growing, invading the stem and leaves, which did not occur when the plant interacts with the wild-type strain. This may be interpreted as either a miscommunication effect or as a simple defect in the interaction due to the accelerated growth of the mutant.

Jasmonate and ET mediate the main defense responses of plants during infection by necrotrophic fungal pathogens, whereas SA-dependent responses and SAR were initially not predicted to play a role (Bent 2006). In the interaction of *Trichoderma* spp. with common bean (*Phaseolus vulgaris*), tomato (*S. lycopersicum*), and *A. thaliana*, the fungus promotes accumulation of PR proteins (Salas-Marina et al. 2011; Woo et al. 2006). In agreement with these findings, we found that wild-type *T. virens* activated *pPr1a:uidA* and induced a four-fold increase in the levels of SA. These responses were absent in plants co-cultivated with the $\Delta ppt1$ -1 mutant. Notably, the levels of JA and the expression of the JA-induced marker *pLox2:uidA* remained similarly induced by the wild type and the $\Delta ppt1$ -1 mutant, suggesting a specific role for PPT1 function during SAR.

Antimicrobial compounds can be produced as part of normal plant growth and in response to pathogens. Camalexin production has been found to be elicited by bacterial and fungal phytopathogens and possess antimicrobial activity (Glazebrook and

Fig. 7. Effect of *Trichoderma virens* on salicylic acid (SA) and jasmonic acid (JA) accumulation in *Arabidopsis thaliana*. Wild-type *A. thaliana* (Col-0) seedlings were germinated and grown for 4 days on 0.2× Murashige and Skoog plates, then co-cultivated with the indicated *T. virens* strains in 0.2× Murashige and Skoog medium supplemented with 300 μ M Lys for 8 additional days. Free A, SA or B, JA in *A. thaliana* shoots. Control corresponds to axenically grown seedlings. Error bars represent the standard error. Different letters are used to indicate means that differ significantly ($P < 0.05$). The experiment was repeated twice with similar results.

Fig. 8. Effect of *Trichoderma virens* inoculation on camalexin accumulation in *Arabidopsis thaliana*. Wild-type *A. thaliana* (Col-0) seedlings were germinated and grown for 4 days on 0.2× Murashige and Skoog plates, transferred to 0.2× Murashige and Skoog medium supplemented with 300 μ M Lys, and then co-cultivated with the indicated *T. virens* strains for 8 additional days. **A**, Representative chromatogram showing camalexin levels in leaves of wild-type *A. thaliana* seedlings. Control corresponds to axenically grown seedlings. **B**, Mass spectra from camalexin standard. **C**, Camalexin quantification from *Arabidopsis* leaves. The bars show the mean $\pm$ standard deviation of three independent biological replicates. Different letters are used to indicate means that differ significantly ($P < 0.05$). The experiment was repeated twice with similar results.

Ausubel 1994; Ferrari et al. 2003; Thomma et al. 1999). Here, we showed that *A. thaliana* seedlings colonized with wild-type *T. virens* accumulated higher levels of camalexin than axenically grown seedlings. This response was compromised in plants co-cultivated with the $\Delta ppt1$ -1 mutant.

In agreement with the role of PPT1 in activating defense-signaling pathways, a *Trichoderma* mutant lacking PPT1 failed to confer protection against the fungal necrotizing pathogen *B. cinerea*. In this regard, it has been previously shown that *Arabidopsis* resistance to *B. cinerea* involves SA and camalexin (Ferrari et al. 2003). *Arabidopsis pad2-1* and *pad3-1* mutants, which accumulate low levels of camalexin, are highly susceptible to *B. cinerea*, and purified camalexin inhibits growth of this pathogen in a dose-dependent manner (Ferrari et al. 2003; Glazebrook et al. 1997). The relation found between reduced SA and camalexin production and much lower protection in plants colonized by the *T. virens* $\Delta ppt1$ -1 mutant confirmed that the combined activation of SA-dependent pathways and camalexin production are important to confer plant immunity against a fungal necrotizing pathogen. Based on these analyses, we conclude that *T. virens* is capable of regulating multiple defense responses. In addition to the defense gene induction and hormone biosynthesis, we now provide chemical evidence supporting a role for *ppt1* from *T. virens* in phytoalexin induction, another important defense response.

In conclusion, we have shown the important role of the 4-phosphopantetheinyl transferase PPT1 from *T. virens* in secondary metabolism and plant defense responses. Among the several mechanisms activated in *A. thaliana* by *T. virens* to confer immunity against *B. cinerea*, there is a role of *ppt1* in a resistance mechanism involving SA and camalexin production.

MATERIALS AND METHODS

Fungal strains and culture conditions.

The *T. virens* wild-type strain Tv29.8 and an arginine auxotroph (Tv10.4) derived from it were used in this study (Baek and Kenerly 1998). *T. virens* strains were grown in potato dextrose agar (PDA) medium (Difco Laboratories, Detroit) or in Vogel's minimal medium supplemented with 2 mM arginine. Pathogenic strains of *R. solani*, *F. oxysporum*, *Fusarium* spp., *Alternaria solani*, *Sclerotium rolfsii*, *S. cepivorum*, and *Phytophthora capsici* were grown in PDA medium (Difco Laboratories). *T. virens* $\Delta ppt1$ mutants were grown in Vogel's minimal medium supplemented with 10 mM lysine or 50% siderophore-containing conditioned medium. To prepare siderophore-containing medium, 1×10^9 conidia from strain Tv29.8 were used to inoculate Vogel's liquid minimal medium without iron (Marquz-Fernndez et al. 2007).

Cloning and overexpression of *ppt1*.

Based on the *T. virens* genome sequence, forward primer *F_orf_ppt1* and reverse primer *R_orf_ppt1* (Table 1) were

designed and used to amplify by PCR the 4-phosphopantetheinyl transferase-encoding gene (*ppt1*) from the wild-type strain Tv29.8 using genomic DNA as template and the following conditions: an initial cycle of 95C for 3 min; 30 cycles of

Fig. 9. Protection against *Botrytis cinerea* in *Arabidopsis thaliana* seedlings is compromised in $\Delta ppt1$ mutants. **A**, *A. thaliana* plants (12 days old) grown axenically or co-cultivated for 6 days with the indicated *Trichoderma virens* strains in vitro were placed in petri dishes containing 0.2 Murashige and Skoog medium, and the shoot was treated with sterilized deionized water or inoculated with 1×10^6 *B. cinerea* spores, distributing the inoculum over the leaf surface. Each treatment was applied to 30 plants. **A**, Number of symptomatic leaves per plant was scored 3 days post-infection and **B**, the percentage of dead plants was scored 5 days post-infection. Bars show the mean $\pm$ standard deviation of 30 *A. thaliana* seedlings. Different letters are used to indicate means that differ significantly ($P < 0.05$). The experiment was repeated twice with similar results.

Table 1. List of primers of *Trichoderma virens*

Name	Sequence	Temperature (��C)	Size (bp) ^a
<i>F_orf_ppt1</i>	5'-TCGCAGCCATGGCTCAGCCCA-3'	60.8	987
<i>R_orf_ppt1</i>	5'-AATCGCCATCTCACTCGCCCAGC-3'	...	...
<i>ppt1_A</i>	5'-GAGACGCAGTAGGTTCTTTC-3'	60.8	1,006
<i>ppt1_B</i>	5'-CGCCGTGGGGCTCTGTATCGACAAGCTGGCTGCAGCGATCATTTATATGTCG-3'	...	...
<i>ppt1_C</i>	5'-CGCAACGAATAATCCTCCCTGTAGAAGGCAAGATCACCGACAAGTTGGTTCG-3'	60.8	857
<i>ppt1_D</i>	5'-TAAGCATGGGGCGCCACAGTTGC-3'	...	...
<i>ppt1_E</i>	5'-TTGATATCGAGCCCCAGGCG-3'	53.8	2,929
<i>ppt1_F</i>	5'-GGAATTGGGTCTGGCACG-3'	...	...
<i>ppt1_G</i>	5'-CGCTTTAAACGGCGTCTGTTC-3'	65	4,792
<i>ppt1_H</i>	5'-GGGGACTTTGAGCTGAAGTGGATG-3'	...	...

^a Size of the amplified fragment using the corresponding pair of oligonucleotides.

94°C for 45 s, 60.8°C for 45 s, and 72°C for 1 min; and a final cycle of 72°C for 7 min. The PCR product was cloned into PCR 2.1 TOPO (Invitrogen, Carlsbad, CA, U.S.A.). To generate the overexpression plasmid, one of the clones was selected and plasmid DNA was digested with *EcoRI* and subsequently ligated into plasmid pUE08 (Esquivel-Naranjo and Herrera-Estrella 2007). DNA of the resulting plasmid (pOEptt) was used in polyethylene glycol-mediated transformation of protoplasts of the Tv29.8 strain, as described previously (Baek and Kenerly 1998). Hygromycin-resistant transformants were subjected to three consecutive monospore passes. Southern blot analysis was used to confirm plasmid integration, using as a probe a fragment of the *ppt1* gene containing the complete open reading frame.

Replacement of the phosphopantetheinyl transferase gene.

Gene *ppt1* was replaced in strain Tv10.4 by the *T. virens* *arg2* gene using the double-joint PCR procedure described by Yu and associates (2004). In a first round of PCR, 5' forward primer *ppt1_A*, 5' reverse primer *ppt1_B*, 3' forward primer *ppt1_C*, and 3' reverse primer *ppt1_D* (Table 1) were used to generate the upstream and downstream regions flanking the *ppt1* gene. In addition, to generate the marker cassette, the following primers were used: *arg2* forward primer *ppt1_E*, and *arg2* reverse primer *ppt1_F* (Table 1). In the third and final round of amplification, we used the nested forward primer *ppt1_G* and reverse primer *ppt1_H* (Table 1). The final product was used to transform protoplasts. Selection of transformants was carried out in Vogel's minimal medium. For the selection of transformants, it was considered that, initially, transformants could require lysine and siderophores, which were added to the medium for the recovery of transformed protoplasts. All arginine prototrophs were subjected to three rounds of monospore culture. Gene replacement was confirmed by PCR using primers *ppt1_G* and *ppt1_H* (Table 1). Putative gene replacement mutants were then confirmed by Southern blot analysis, using as probe a 1-kb fragment of the 5' upstream region covering up to the translation start codon of the *ppt1* gene.

Complementation of the null mutant.

The complete *ppt1* gene, including the 5' and 3' flanking regions, was amplified by PCR with oligonucleotides 5' forward primer *ppt1_A* and 3' reverse primer *ppt1_D* (Table 1) using genomic DNA of the wild-type strain. The PCR conditions were an initial cycle at 95°C for 3 min; 30 cycles of 45 s at 94°C, 3 min at 55.7°C, and 1 min at 72°C; followed by a final cycle at 72°C for 7 min. The PCR product was cloned into TOPO PCR 2.1 (Invitrogen). The resulting plasmid, named *ppt1*-complement (7 kb), was used in co-transformation experiments using, as selectable plasmid, pCB1004 (FGSC), which carries a hygromycin resistance cassette, and protoplasts of the gene replacement mutant (*Δppt1*-1). Hygromycin-resistant transformants were then transferred to medium without lysine and siderophores and were subjected to three rounds of monospore culture. These transformants were designated *T. virens* complemented (TvC). Confirmation of transformation was carried out by PCR amplification of the *ppt1* gene followed by Southern blot analysis.

Phenotypic analysis of mutants.

Overexpressing strains, gene-replacement mutants, complemented strains, and Tv29.8 and Tv10.4; were grown in the dark on PDA, and radial growth of the colonies measured at 24, 48, and 72 h. To determine the effect of the manipulation of the *ppt1* gene in the production and development of conidiophores, all strains were grown in Vogel's medium and incubated at 28°C for

5 days with white light illumination. For analysis of conidial germination, the medium used was Grimm-Allen (Vittone 2008) with and without ferric chloride and ferrous sulfate.

Antibiosis.

For antibiosis tests, all strains were inoculated in plates containing PDA covered by a sterile cellophane membrane and incubated for 48 h in total darkness. The cellophane was removed together with the mycelium, an agar disk carrying mycelium of the indicated fungus placed on the antagonist-free medium, and the plates further incubated for 48 h in total darkness.

Determination of peptaibols.

T. virens strains were inoculated in VMS medium as described by Viterbo and associates (2007), with some modifications. In this case, fermentation was allowed to proceed for 9 days with constant agitation and sucrose as carbon source to 1.5%. Mycelium was harvested and lyophilized. In total, 250 µl of mixture A (methanol, water, an acetonitrile in a 1:1:1 ratio) or mixture B (5% acetonitrile and 0.1% formic acid) was added to 5 mg of dry mycelia. The suspension was homogenized for 15 min in vortex, with subsequent sonication for 15 min. The mixture was then centrifuged at 12,000 rpm for 15 min and the supernatant collected and concentrated in a vacuum evaporation system to a final volume of 50 µl. The extract (10 µl) was taken and passed through a C18 micro-column (Zip Tip, Millipore, Bedford, MA, U.S.A.) as recommended by the supplier, and eluted with 5 µl of a mixture of solvents (60% acetonitrile and 1% formic acid), for ESI-QTOF analysis.

Root colonization.

Solanum lycopersicum var. Rio Grande (Emerald) seeds were surface disinfected and germinated on water agar (1.5%) in a climate chamber at 25°C for 3 days with a photoperiod of 16 h of light and 8 h of darkness. Germinating seeds were then transferred to 150-mm-diameter petri dishes (three per plate), containing 0.5% Murashige and Skoog medium (Murashige and Skoog basal salts mixture; Sigma-Aldrich, St. Louis) and 0.5% agar, placed at a 65-degree angle to prevent root burial into the medium. Seedlings were incubated at 25°C until the development of two true leaves. A disk of mycelium of strains Tv29.8, Tv10.4, *Δppt1*, and TvC was placed near the root area. Media were supplemented with 10 mM lysine or 2 mM arginine in the case of the *Δppt1* mutant and the Tv10.4 strain, respectively. The root system overgrown by *Trichoderma* spp. was collected with the help of a scalpel. Samples were placed in two dyes, one that stains the nuclei of root cells (propidium iodide to 20 µg/ml) and one that stains chitin in fungal cell wall (WGA Alexa Flour 488; 10 µg/ml) (Invitrogen). Samples were visualized at the National Institute of Neurobiology Campus Juriquilla, Mexico, in a Nikon Eclipse E-600 PCM 2000 confocal microscope. Images were obtained with the ×40 objective.

Isolation of *T. virens* from roots of *S. lycopersicum*.

According to the methodology described above, seedlings once colonized in vitro by *Trichoderma* spp. were transplanted into sterile soil, and allowed to grow for an additional 15-day period. The roots were then collected, fragmented, and placed on Rose Bengal selective medium for the isolation of *Trichoderma* spp. (Ahmed et al. 1999). In the case of roots colonized by auxotrophic strains, lysine or arginine was added to the medium. Root fragments were incubated at 28°C until the appearance of colonies. Genomic DNA was extracted from each of the recovered colonies, as reported by Raeder and Broda (1985), to detect the strains, and analyzed by PCR using primers *ppt1_G* and *ppt1_H* (Table 1).

Seed protection assays.

One-liter polypropylene pots containing sterile soil were inoculated with *R. solani* according to Brunner and associates (2005). Seeds were surface sterilized with 95% (vol/vol) ethanol for 5 min and 20% (vol/vol) sodium hypochlorite for 7 min, followed by five washes in distilled water. Ten *S. lycopersicum* seeds were placed per pot, with three replicates per treatment. Four discs of mycelium of the indicated *T. virens* strain were added near the area where the seeds were sown. Seeds were incubated in a growth chamber at 28°C until the emergence of two true leaves. The results were validated with analysis of variance statistical analysis with a Tukey-Kramer multiple comparison test ($\alpha = 0.05$), using the Statistical Analysis and Graphics software package (version NCSS 2007).

Arabidopsis co-cultivation experiments.

A. thaliana Columbia-0 (Col-0) ecotype wild type, a transgenic line carrying a JA-inducible *pLox2:uidA* construct (Schommer et al. 2008), and a transgenic line carrying an SA-inducible *pPr1a:uidA* construct (Shah et al. 1997) were used throughout this work. Seeds were surface sterilized as described above, germinated, and grown on agar plates containing 0.2× Murashige and Skoog medium. Plates were settled vertically at an angle of 65° to allow root growth along the agar surface and to allow unimpeded aerial growth of the hypocotyls. Plants were placed in a Percival AR95L growth chamber with a photoperiod of 16 h of light and 8 h of darkness, light intensity of 200 $\mu\text{mol m}^{-2} \text{s}^{-1}$, and temperature of 24°C.

Arabidopsis inoculation experiments.

The *T. virens* 29.8 and $\Delta\text{p}t1$ -1 mutant strains were evaluated in vitro for their ability to elicit defense responses in *A. thaliana*. Fungal spore densities of 1×10^6 conidia were inoculated by placing a drop of a spore suspension at 4 cm in the opposite ends of agar plates containing 4-day-old germinated *Arabidopsis* seedlings (10 seedlings per plate). Plates were arranged in a completely randomized design. The seedlings were cultured for six additional days in a Percival AR95L growth chamber. The percentages of primary roots colonized by *T. virens* were determined with a ruler by measuring the primary root length and the surface covered by fungal hyphae. In the fungal co-cultivation experiments, the Murashige and Skoog 0.2× medium was supplemented with 300 μM lysine to allow fungal growth.

Histochemical analysis.

For histochemical analysis of GUS activity, *Arabidopsis* seedlings were incubated 12 to 14 h at 37°C in a GUS reaction buffer (5-bromo-4-chloro-3-indolyl-b-D-glucuronide at 0.5 mg/ml in 100 mM sodium phosphate, pH 7). The stained seedlings were cleared using the method of Malamy and Benfey (1997). For each marker line and for each treatment, at least 20 transgenic plants were analyzed. A representative plant was chosen and photographed using a Leica MZ6 stereomicroscope.

SA and JA extraction and measurement.

The SA and JA extraction and determination were performed in *A. thaliana* (ecotype Col-0) shoots at 8 days after co-cultivation with *Trichoderma* spp. in vitro. For sample preparation, plants were sectioned at the root/shoot interface. Plant tissues were frozen and ground in liquid nitrogen. Each sample (300 mg) was placed in a polypropylene microtube, homogenized with 500 μl of isopropanol/ H_2O /concentrated HCl (2:1:0.002, vol/vol), supplemented with 200 ng of orto-anisic acid (OA) (Sigma-Aldrich) as internal standard for SA, and shaken for 30 s. The tubes were centrifuged at 11,500 rpm for 3 min. The supernatants were collected and subjected to SA extraction

with 200 μl of dichloromethane. SA and JA were derivatized with acetyl chloride in methanol (1 ml per 250 μl), sonicated for 15 min, and heated for 1 h at 75°C. After cooling, the derivatized sample was evaporated and resuspended in 25 μl of ethyl acetate for GC-MS analysis. A retention time and selected ion monitoring (SIM) were established for SA-methyl ester (ME) (2.3 min, m/z 152, respectively), OA-ME (3.2 min, m/z 166), and JA-ME (7.5 min, m/z 224). JA was quantified by comparison with a standard curve obtained by using purified methyl jasmonate (Sigma-Aldrich).

Camalexin determination.

Camalexin was extracted from leaves of wild-type *A. thaliana* seedlings 8 days after *T. virens* inoculation. Camalexin levels were determined as described by Glazebrook and Ausubel (1994) and GC-MS analysis performed. For GC-MS analysis, 100 mg per sample of shoot material was submerged in 800 μl of methanol and kept at 80°C for 20 min. The supernatant was transferred to a vial, evaporated under a stream of nitrogen, and redissolved in 10 μl of methanol for GC-SIM-MS analysis. The volume of injected sample was 2 μl . The molecular ions with m/z 58, 142, and 200 $[\text{M}]^+$ were monitored to verify the presence of camalexin in the sample. Camalexin (retention time 18.44 min) was quantified with a standard curve, using purified camalexin provided by J. Glazebrook (University of Minnesota) and dissolved in 100 μl of methanol for chemical analysis.

MS analysis.

Identification and determination of all investigated compounds were performed using a GC-MS system. The samples were injected in an Agilent 6850 Series II gas chromatograph equipped with an Agilent MS detector model 5973 and a 5% phenyl methyl silicone capillary column, 30 m by 0.2 mm by 0.25 mm (HP-5 MS). Operating conditions used helium 1 ml min^{-1} as carrier gas, detector temperature of 300°C, and injector temperature of 250°C. The column was held for 3 min at 80°C and programmed at 6°C min^{-1} to a final temperature of 230°C for 5 min.

Bioassays for *Trichoderma* spp.-induced resistance against *B. cinerea*.

To test plant protection conferred by the wild-type and $\Delta\text{p}t1$ *T. virens* strains against *B. cinerea*, *A. thaliana* seedlings were inoculated with a fungal density of approximately 1×10^6 spores by placing the spores at 1 cm at the opposite ends of agar plates containing 12-day-old germinated seedlings (10 seedlings per plate). *Trichoderma* spp. were co-cultivated for 6 days with the plant to elicit defense responses by the physical contact of the mycelium with the root system. *A. thaliana* shoots were inoculated with a density of *B. cinerea* spores of 1×10^6 , distributing drops of the inoculum over leaf surfaces. Induced disease resistance in *A. thaliana* seedlings was evaluated 3 or 5 days after pathogen inoculation by scoring symptomatic leaves or percentage of dead plants, respectively, for a total of 30 plants. Plants were placed in a plant-growth chamber with a photoperiod of 16 h of light and 8 h of darkness, light intensity of 200 $\mu\text{mol m}^{-2} \text{s}^{-1}$, and temperature of 24°C.

Data analysis.

Experiments were statistically analyzed in the SPSS 10 program (SPSS, Chicago). Univariate and multivariate analyses with a Tukey's post hoc test were used for testing differences in the biochemical analysis for SA, ICAld, camalexin measurements, number of lesions, and percentage of dead plants in wild-type *A. thaliana*. Different letters are used to indicate means that differ significantly ($P \leq 0.05$).

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AUTHOR-RECOMMENDED INTERNET RESOURCE

Joint Genome Institute *T. virens* genome webpage:
genome.jgi-psf.org/TriviGv29_8_2/TriviGv29_8_2.home.html