Functional Characterization of TvCyt2, a Member of the p450 Monooxygenases From *Trichoderma virens* Relevant During the Association With Plants and Mycoparasitism

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Secondary metabolites are crucial for the establishment of interactions between plants and microbes, as in the case of *Trichoderma*-plant interactions. In the biosynthetic pathway of secondary metabolites, specific enzymes participate in the formation of hydroxyl and epoxy groups, belonging to the p450 monooxygenases family. Here, we show that the product of the gene *TvCyt2* from *Trichoderma virens* encodes a new protein homologous to the cytochrome p450, which is down-regulated at the beginning of *Trichoderma-Arabidopsis* interaction. To investigate its role in the interactions established by *Trichoderma* spp., we analyzed the metabolic profile obtained from the overexpressing (OETvCyt2) and null mutant ($\Delta tvcyt2$) strains, observing that the OETvCyt2 strains produce a higher concentration of some metabolites than the wild-type (WT) strain. $\Delta tvcyt2$ strains showed a decreased antagonistic activity against *Rhizoctonia solani* in antibiosis assays. *Arabidopsis* plants cocultivated with the OETvCyt2 strains showed stronger induction of systemic acquired resistance than plants cocultivated with the WT strain, as well as increases in biomass and fitness. Our data suggest that the product of the *TvCyt2* gene is involved in secondary metabolite biosynthesis, which can increase antagonistic activity with phytopathogenic fungi and the capacity to promote plant growth.

Plants and microorganisms establish molecular dialogs that involve recognition of conserved signaling molecules that play key roles during this communication (Zeilinger et al. 2016). Some of these molecules are produced by secondary metabolism, determining the type of association to be established, either beneficial or harmful interactions (Bais et al. 2006; Ortiz-Castro et al. 2009). The pathways leading to the synthesis of secondary metabolites include the participation of different enzymatic activities.

Among the main enzymatic activities involved in the biosynthesis of secondary metabolites are the polyketide synthases, non-ribosomal peptide synthases, dimethylallyltryptophan synthases, and cytochrome p450 monooxygenases (CYP) (Werck-Reichhart

and Feyereisen 2000; Mukherjee et al. 2006; Deepika et al. 2016). In the fungal kingdom, CYPs have attracted attention due to reports linking them with new industrial and biotechnological applications. In fungi, CYPs participate in primary and secondary metabolism and are involved in detoxification and degradation of toxic compounds (Durairaj et al. 2016). This class of enzymes catalyze regio- and stereospecific conversions of a wide range of lipophilic compounds to more hydrophilic derivatives by introducing an oxygen atom and, in some cases, p450 enzymes can catalyze hydroxylation or epoxidation reactions (Črešnar and Petrič 2011).

The production of secondary metabolites, regarding quantity and time, is crucial for triggering a perception and interaction in biological associations (Sharma et al. 2017; Vinale et al. 2016; Zhai et al. 2017). According to the role of these molecules during plant-fungus interactions, some are identified as effector-like molecules because they are capable of altering the host cell structure and function either facilitating infection or triggering defense responses (Hogenhout et al. 2009). In *Magnaporthe oryzae*, a polyketide synthase encoded by the *ACE1* gene is related to the synthesis of secondary metabolites that might function as effectors recognized by resistance proteins (Collemare et al. 2008; Yi and Valent 2013). In *Colletotrichum higginsianum*, a gene cluster implicated in secondary metabolite biosynthesis is reported; compounds produced by this pathway are predicted to function in host-response manipulation, as protein effectors do (O'Connell et al. 2012). In nonpathogenic fungus, little is known about the role of effector molecules and, therefore, secondary metabolite involvement in plant-fungi interactions.

Trichoderma species are known, because of their use as biological control agents, to antagonize fungal plant pathogens. This characteristic is mainly attributed to the production of metabolites with antimicrobial activity and the secretion of several hydrolytic enzymes (Lorito et al. 2010). Furthermore, *Trichoderma* spp. establish interactions with plants, colonizing the epidermis and root cortex, this penetration being restricted to the apoplast as a consequence of the cell-wall reinforcement (Chacón et al. 2007). The interaction enhances root and plant growth as well as activates the defense responses mediated by salicylate (SA), jasmonate (JA), and ethylene (ET) (Contreras-Cornejo et al. 2009; Salas-Marina et al. 2011). *Trichoderma* spp. are able to establish such interactions, inducing massive changes at transcriptomic and metabolomic level (Morán-Díez et al. 2015; Rubio et al. 2012), and some of these metabolites have been found, not only to directly inhibit the growth of pathogenic microorganisms, but also increase

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disease resistance triggering the defense system in plants (induced systemic resistance) (Vos et al. 2015). Furthermore, metabolite-pretreated plants responded to a pathogen attack much faster or more intensively (Hermosa et al. 2012; Shores et al. 2010; Verhagen et al. 2011), a mechanism known as priming (Conrath 2011).

During the establishment of the association of *Trichoderma* spp. with plants, a specific molecular communication, as in other biological interactions, must be involved, and these molecules either are recognized or trigger, or both, physiological changes in both organisms, allowing the establishment of the association. Among the compounds released by *Trichoderma* spp., which are related to the interaction with plants, effector-like molecules have been reported, such as cellulases, cerato-platanins, and secondary metabolites such as antibiotics, hormones, mycotoxins, and terpenes (Atanasova et al. 2013; Brito et al. 2014; Hermosa et al. 2012; Horwitz et al. 2013). In the interaction of *Trichoderma* spp. with common bean (*Phaseolus vulgaris*), tomato (*Solanum lycopersicum*), and *Arabidopsis thaliana*, the presence of the fungus promotes accumulation of pathogenesis-related proteins (Salas-Marina et al. 2011; Woo et al. 2006). Contreras-Cornejo and collaborators (2011) observed that the presence of *T. virens* increases the levels of SA and JA in *A. thaliana*.

In other *Trichoderma* species, the participation of secondary metabolites during plant-*Trichoderma* interactions has been demonstrated. In *T. brevicompactum*, the overexpression of the gene *tri5*, which encodes for a p450 protein, increased the production levels of trichodermin and other metabolites, leading to an exacerbated antimicrobial activity (Tijerino et al. 2011).

Studies on *T. arundinaceum* showed that biocontrol activity against *Botrytis cinerea* is, in part, due to harzianum A (HA), an analog of compounds belonging to a family of sesquiterpenes known as trichothecenes. HA is involved in the biocontrol activity by *Trichoderma* spp. as well as in its ability to upregulate plant defense-related genes (Malmierca et al. 2012). *tri* genes are also present in *Fusarium* species and are involved in the biosynthesis of mycotoxins (McCormick et al. 2004; Meek et al. 2003). The importance of the concentration of these molecules in the establishment of the interaction between *Trichoderma* spp. and plants is evidenced by the effect of harzianic acid and harzianolide, which either promote plant growth, induce plant resistance at low concentrations or both, while at high concentrations, they exhibit the opposite effect, inhibiting plant growth (Cai et al. 2013; Vinale et al. 2009).

In this work, we selected the *T. virens* gene *TvCyt2* from a catalog of effectors proposed by Guzmán-Guzmán and collaborators (2017), because *TvCyt2* is predicted to be a p450 family member that could be relevant in the establishment of biological interactions (Carpenter et al. 2008; Malmierca et al. 2012). We determined its differential transcriptional response in the presence of the plant host and fungal competitors. Additionally, we confirmed the participation of *TvCyt2* in the synthesis of secondary metabolites, by comparing metabolic profiles between wild-type (WT), mutants, and overexpressing strains of the *TvCyt2* gene. Moreover, we evaluated the impact of the differential production of secondary metabolites in antibiosis assays against phytopathogens. Finally, we determined their relevance during the establishment of symbiotic interactions with plants, focusing on plant defense responses and plant total mass.

RESULTS

In silico analysis and expression patterns of *TvCyt2* from *T. virens*.

To demonstrate if *TvCyt2* could participate during the biological associations established by *T. virens* with plants, we first analyzed its expression pattern in interactions with *A. thaliana*. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) assays using mycelia collected from the vicinity with *A. thaliana* seedlings, after 3, 5, and 7 days of interaction (Fig. 1A), showed that the gene was down-regulated at the first time of interaction, when there was not a physical contact between roots and hyphae; the *TvCyt2* expression level increased gradually during the two subsequent timepoints analyzed, until reaching the level shown by the control condition, in which *T. virens* was grown without plant interaction (Fig. 1B).

Trichoderma spp. are widely used as biocontrol agents because of their mycoparasitic activity against phytopathogenic fungi. To analyze if *TvCyt2* could be involved in this process, we determined expression levels of the *TvCyt2* gene in a confrontation with the anastomosis groups AG2 and AG5 of *Rhizoctonia solani*. *Trichoderma* mycelium was collected before contact between both fungi, during initial contact, and by the time *Trichoderma* overgrew the phytopathogens. *TvCyt2* expression was not modified in a confrontation with the AG2 strain. Interestingly, in confrontation with *R. solani* AG5, *TvCyt2* was up-regulated during the initial contact and overgrowth conditions (Fig. 2). These results suggest that the expression of *TvCyt2* gene depends on the phytopathogen with which *T. virens* is confronted.

The Joint Genome Institute database reports that the *TvCyt2* gene encodes for a protein member of the p450 family (ID 190045); the open reading frame is composed of 1,680 bp and contains two introns that, after being processed, should generate a 1,581-bp sequence. Nevertheless, when the *TvCyt2* cDNA was amplified, cloned, and sequenced, we observed that the putative first intron is not processed, obtaining a sequence of

Fig. 1. The expression of *TvCyt2* from *Trichoderma virens* is down-regulated in the earlier interaction time with *Arabidopsis thaliana*. **A**, Representative photograph of *Arabidopsis* seedlings cocultivated with *T. virens* at 3, 5 and 7 days. Mycelia between the dashed lines were collected to the expression assays and *Trichoderma* cultured alone was used as control, collecting a similar quantity of mycelia. **B**, Determination of *TvCyt2* expression by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) in mycelia interacting with *Arabidopsis* at 3, 5 and 7 days. Glyceraldehyde-3-phosphate dehydrogenase gene (*gpd*) was used as endogenous expression control; for each time tested, the expression level determined for *T. virens* growing alone was considered as 1. The qRT-PCR assay was done in triplicate with three technical replicates each. Data were analyzed using an analysis of variance nonparametric test with $P < 0.05$. Only the expression of *TvCyt2* at 3 days had significant differences with respect to the control.

1,614 bp that encodes for a protein of 537 amino acids, maintaining the open reading frame. The retained intron added 33 bp and interrupted the region that encodes the putative signal peptide, indicating that it is not functional. Upon expression of the reporter *mCherry* fused to *TvCyt2* encoding sequence, red fluorescence was only observed inside the hypha (Supplementary Fig. S1). This result, together with the absence of the signal peptide and the predicted function, indicate that *TvCyt2* protein is not secreted. The *TvCyt2* protein sequence contains five potential *N*-glycosylation sites (analyzed with HIV database) and five predicted transmembrane domains (TMpred server and “DAS”, the transmembrane prediction server), suggesting that the protein resides in some membrane, as is generally reported for p450 proteins, which are found primarily in the endoplasmic reticulum or the mitochondrial membrane (Park et al. 2014, 2015). The final deduced protein sequence has two characteristics found in p450 enzymes, an amino terminal domain (PERFL) related to membrane anchoring (Nebert and González 1987) and a motif (FXXGXXXCXC), which contains a conserved cysteine residue, as part of the heme-binding domain (Bairoch 1991; Larkin 1994) (Supplementary Fig. S2). We aligned the *TvCyt2* protein and observed that it has a similarity close to 75% with other hypothetical p450 proteins from *Trichoderma* species and less than 50% with some p450 from *Trichoderma* and *Fusarium* species (analyzed with Clustal Omega online) (Supplementary Fig. S3).

To analyze the presence of putative regulatory motifs in the promoter of *TvCyt2*, a sequence 1,500 bp upstream of the ATG was analyzed. We found two CreA motifs for glucose repression, two ACEI recognition sequences for cellulose repression, and six mycoparasitic-related motifs MYC (Aro et al. 2001, 2003; Cortés et al. 1998; Cubero and Scazzocchio 1994; Lorito et al. 1996). We analyzed *TvCyt2* expression in culture media supplemented with glucose, cellulose, root exudates, or chitin. We did not observe a relevant change in the expression levels of *TvCyt2* (data not shown), suggesting that the expression of the gene could be affected for other molecules released by the fungus or the plant.

***TvCyt2* is involved in the biosynthesis of soluble secondary metabolites.**

To evaluate the possible role of *TvCyt2* in *T. virens* strains, overexpressing (OETvCyt2) and null mutants ($\Delta tvcyt2$) were generated. For null mutants *TvCyt2* was replaced with the selection marker *hph*, which confers hygromycin resistance. For overexpression, we generated a construct bearing *TvCyt2* gene under the control of the pyruvate kinase (*pki*) constitutive promoter from *T. reesei* (Yu et al. 2004; Zeilinger et al. 1999); gene replacement for the null mutants and construction integration for overexpression strains were confirmed by PCR (Supplementary Figs. S4 and S5). We selected two independent transformants for each type of strain and their *TvCyt2* expression levels were corroborated by qRT-PCR (Supplementary Fig. S6). The null mutants and the overexpressing strains did not present phenotypic significant differences on growth rate, conidiation, or colony appearance compared with the WT (data not shown).

Trichoderma strains were grown on Murashige-Skoog minimal medium and the supernatants were analyzed using a gas chromatography mass spectrometry (GC-MS) system. The obtained chromatograms from each strain were compared to identify differences among the secreted secondary metabolites. These assays allowed us to determine if both OETvCyt2 strains have differential metabolites at higher concentrations in comparison with the parental and null mutant strains (Fig. 3A). According to the mass spectra obtained from the chromatograms of the OETvCyt2 strains (Supplementary Fig. S7), peaks a, b, and c correspond to

the compounds viridiflorol, tau-muurolol, and α -cadino, respectively (Fig. 3B); whereas peaks d and e were unknown compounds of the sesquiterpene type (Fig. 3C). Compound f (pyrazine [1,2-a] indole-1,4-diene, 2,3-dihydro-2-methyl-3-methylene) was the most abundant; however, this compound is present in all strains (Fig. 3A). These results suggest that *TvCyt2* is involved, either directly or indirectly, in the biosynthesis of different secondary metabolites.

***TvCyt2* plays a role in the *T. virens* antagonistic interactions.**

Previously, we observed a different expression pattern of *TvCyt2* when *T. virens* was confronted with two anastomosis groups from *R. solani*, suggesting that the compounds produced through the pathway in which *TvCyt2* is participating could play a role in the antagonistic activity against *R. solani*, with different degrees of impact.

Considering that the overexpressing strains produce more secondary metabolites, we decided to analyze the impact of secondary metabolite production in these strains on the antagonistic activity against *R. solani*. As shown in Figure 4, in antibiosis assays, we observed that both anastomosis groups formed similar-sized colonies in the media in which *T. virens* WT had previously been grown. The same result was observed when we used plates on which OETvCyt2 strains were grown. While the colonies formed by the AG2 strain were bigger in the media in which *TvCyt2* null mutant strains had been cultivated, this effect was not observed in the AG5 strain. These results indicate that *TvCyt2* is involved in the production of molecules necessary to control *R. solani* AG2 strain.

***TvCyt2* is involved in the production of metabolites that differentially modulate plant defense responses.**

The plant defense pathways that *Trichoderma* spp. are capable of inducing in plants involve both JA and SA (Martínez et al. 2001; Salas-Marina et al. 2011). To examine the possible role for the metabolites produced by the pathway on which *TvCyt2* participates to modulate plant defense responses, we examined the null mutants, the overexpressing strains, and WT

Fig. 2. Expression of *TvCyt2* is differentially controlled in response of two antagonistic groups of *Rhizoctonia solani*. Direct confrontations between *Trichoderma* and two anastomosis groups from *R. solani* were carried out. The AG2 group is represented with triangles and the AG5 group with circles. Mycelia were collected before contact between the two fungi (BC), during initial contact (C), and at the time when *Trichoderma virens* overgrew the phytopathogens (OG). The glyceraldehyde-3-phosphate dehydrogenase gene (*gpd*) was used as endogenous expression control. The quantitative reverse transcription-polymerase chain reaction assay was done in triplicate with three technical replicates each. Data were analyzed using an analysis of variance nonparametric test with $P < 0.05$. Only the expression of *TvCyt2* in confrontation with AG5 during the initial contact (C) and the overgrown (OG) had significant differences with respect to the control.

in interaction with two *A. thaliana* transgenic lines containing the *uidA* gene reporter, which encodes for the β -glucuronidase (GUS) enzyme. One marker line contained the *uidA* gene under the control of the promoter of *Lox2*, a gene activated by JA (Schommer et al. 2008). In the second transgenic line, the reporter gene was under the control of the promoter of *Pr1a*, which is activated by SA (Shah et al. 1997). *A. thaliana* transgenic seedlings carrying those markers were cocultivated with each of the strains generated in this work. Although the GUS staining shown by seedlings of the *pPr1a:uidA* and *pLox2:uidA* lines without interacting with *T. virens* was low (Fig. 5A), the signal was slightly activated in shoots of plants cocultivated with the WT strain; the OETvCyt2 strain activated *pPr1a:uidA* expression slightly more than the WT strain, while the null mutant strain did not activate *pPr1a:uidA* expression (Fig. 5F to 5H). The JA-activated marker *pLox2:uidA* was expressed at a slightly lower level in plants cocultivated with the null mutant strain, as compared with those cocultivated with the WT strain. Interestingly,

the OETvCyt2 strain activated *pLox2:uidA* expression at a higher level than the WT strain (Fig. 5B to D). These data indicate that the products generated by TvCyt2 are involved in triggering *A. thaliana* responses mainly mediated by JA.

TvCyt2 has a role in plant growth enhancement mediated by *T. virens*.

Trichoderma spp. have the ability to promote plant growth through different compounds, including secondary metabolites (Contreras-Cornejo et al. 2009; Salas-Marina et al. 2011; Vinale et al. 2016). To evaluate if TvCyt2 played a relevant role during plant growth promotion, we analyzed *Arabidopsis* seedlings that had been cocultivated with the overexpressing strains, compared with those cultivated in the presence of the WT strain of *T. virens*. We observed that the OETvCyt2 strain stimulated lateral root and root-hair formation and increased shoot biomass, indicating a better effect on plant growth than the parental strain at 7 days postinoculation (Supplementary Fig. S8). To corroborate

Fig. 3. The alteration in the levels of the product of the *TvCyt2* gene modifies the production of secondary metabolites in *Trichoderma virens*. **A**, Representative gas chromatography mass spectrometry chromatogram showing different metabolites profile. **B**, Peaks a, b, and c correspond to the compounds viridiflorol, tau-murolol, and α -cadinol, respectively; **C**, peaks d and e were unknown compounds of the sesquiterpene type. Compound f was pyrazine [1,2-a] indole-1, 4-diene, 2,3-dihydro-2-methyl-3-methylene. The experiment was carried out twice, obtaining similar results. Secondary metabolite profiles obtained from the wild-type strain, two independent null mutants ($\Delta tvcyt2$) and two independent overexpressing strains (OETvCyt2) are shown.

that the metabolites produced by the OETvCyt2 strains influence plant growth differences, *A. thaliana* seedlings were transferred to plates on which *T. virens* strains had been previously grown for 36 h and the fungi were then removed. We found that the seedlings exposed to metabolites released by OETvCyt2 strains showed higher density and length of root hairs compared with seedlings exposed to metabolites produced by the mutant and WT strains (Supplementary Fig. S9). To validate these results and analyze if the effect of TvCyt2 on plant growth promotion can occur in other plant systems, tomato seedlings were cocultivated

with the strains described above. As we observed with *Arabidopsis*, tomato plants in interaction with the OETvCyt2 strains showed an increase in plant growth, as determined by the size of roots and leaves, and total fresh weight after 3 and 6 weeks postinoculation (Fig. 6). The OETvCyt2 strains enhanced the number, length, and area of leaves from 3 weeks of interaction (Fig. 6A and D) and the roots were 75% longer than the roots treated with the WT and null mutant strains as well as the control plants (Fig. 6B). Moreover, the fresh weight was almost double in plants treated with the OETvCyt2 strains, as compared with the

Fig. 4. The overexpression of the TvCyt2 gene improves the antagonistic capacity of *Trichoderma virens* against *Rhizoctonia solani* AG2. Antibiosis assays with *Trichoderma* strains (wild-type, null mutants, or overexpressing strains) and AG2 and AG5 anastomosis groups of the phytopathogen *R. solani* were carried out. Photographs were taken after 72 h and are representative of three independent experiments.

Fig. 5. The metabolites differentially produced by the TvCyt2 overexpressing strain activate the jasmonate (JA)-mediated pathway with higher intensity in *Arabidopsis thaliana*. Transgenic *Arabidopsis* seedlings containing the β -glucuronidase (GUS) reporter gene responsive to JA (pLox2:uidA) and salicylate (pPR1a:uidA) were **A** and **E**, axenically grown as negative controls or cocultivated with **B** and **F**, *Trichoderma virens* wild type, **C** and **G**, overexpressing strains, or **D** and **H**, null mutants. GUS activity in seedlings was determined at 7 days of plant-fungi interaction. Photographs show representative individuals of at least 20 stained seedlings.

other treatments (Fig. 6C). Our results indicate that *TvCyt2* is involved in the synthesis of secondary metabolites and these have an important role in both growth and promoting plant defense capabilities, tested in plants commonly used in the laboratory as well as in species of agricultural importance.

DISCUSSION

The rhizosphere is a complex and dynamic environment shared by a great diversity of organisms that have developed mechanisms to interact with others to ensure their survival. Among other factors, the nature of these interactions will depend on the combination, quality, and quantity of molecular signals that each participant perceives and produces (Bais et al. 2006; Zeilinger et al. 2016). Secondary metabolites play a major role in chemical communication between organisms. In the case of the beneficial associations that *Trichoderma* spp. establish with plants, a diverse number of molecules are produced (Brotman et al. 2008; Gomes et al. 2015; Mukherjee et al. 2013). It is important to note that, although *Trichoderma* does not cause plant disease, it shares ortholog genes with pathogenic fungi that could have evolved under selection to allow an optimal *Trichoderma*-plant interaction and elicit plant defense response without triggering it completely (Horwitz et al. 2013). How the *Trichoderma*-plant association is controlled on the edge between a beneficial interaction and a pathogenic one is a primary interest in the area.

In this work, we analyzed the participation of p450 monooxygenase enzyme *TvCyt2* during the interaction of *T. virens* with plants or phytopathogens. We confirmed its role in the synthesis of secondary metabolites, finding that the most abundant

compound was pyrazine [1,2-a] indole-1,4-diene, 2,3-dihydro-2-methyl-3-methylene; however, this was found in all tested strains, suggesting that *TvCyt2* activity is not related to its synthesis. We found five terpene-like molecules in the overexpressing strains that were absent or present in very low amounts in the WT and null mutant strains. Several studies have demonstrated the properties of sesquiterpenes; their oxygenated forms have a wide spectrum of biological activities, participating as inducers of plant defense mechanisms as well as important antimicrobial properties. This activity has been related with the presence of one or both an unsaturated double bond or oxygen-containing functional group (Kramer and Abraham 2012; Petrelli et al. 2016; Wu et al. 2005). In plant-microbe associations, sesquiterpenes are crucial for the establishment of the interaction, an example is when *Laccaria bicolor* interacts with *Populus* or *Arabidopsis* plants (Ditengou et al. 2015), highlighting the relevance of secondary metabolites in biological interactions.

To determine if those secondary metabolites participate in the associations between *Trichoderma* spp. and phytopathogens, we analyzed the expression of *TvCyt2* in a confrontation with two anastomosis groups of *R. solani*. There were no differences when analyzing anastomosis group AG2, while the gene was up-regulated in the interaction with AG5 until both fungi established contact. Carpenter and collaborators (2008) reported a monooxygenase gene in *T. hamatum* and its homologs in *T. atroviride* and *T. virens* that were expressed specifically in response to the plant pathogens *Sclerotinia sclerotiorum*, *S. minor*, and *S. cepivorum* but not in response to *Botrytis cinerea* and the expression of the gene did not occur until contact had been established between the two fungal species. These results

Fig. 6. The *TvCyt2* overexpressing strain promotes greater growth in tomato plants. Tomato plants were cocultivated in pots with *Trichoderma virens* (wild-type strain, null mutants, and overexpressing strains); plants without fungi were used as control. **A**, Plant size was observed at 3 and 6 weeks posttreatment with the different *Trichoderma* strains. **B**, Length of primary root, **C**, fresh weight, and **D**, length of leaves were also registered. Data were analyzed using an analysis of variance nonparametric test with $P < 0.05$. In all cases, only the treatments with the overexpressing (OETvCyt2) strain had significant differences with respect to the control.

show that this class of genes seems to be regulated in response to specific molecules produced by the host during confrontations with phytopathogens. Nevertheless, the nature or identity of those molecules remains unknown. Additionally, we evaluated whether the loss of function and the overexpression of the *TvCyt2* gene had a physiological effect against *R. solani* AG2 and AG5 strains. Antibiosis assays showed that the null mutants were less effective to restrict the growth of the AG2 strain. The expression of *TvCyt2* was not increased by the presence of AG2, suggesting that the basal production of metabolites could be enough to antagonize this strain. On the other hand, although *T. virens* increases the expression of *TvCyt2* in confrontation with AG5, this one is not susceptible to the secondary metabolites produced through the pathway involving *TvCyt2*. More antibiosis analyses are needed to determine the range of fungi sensitivity to these metabolites.

During *T. virens* and *A. thaliana* interactions, we observed that, even before there was physical contact between both participants, the expression of *TvCyt2* was down-regulated, subsequently increasing until reaching the basal expression levels observed in the control condition. This behavior in gene expression has been observed in *epl1* and *Tatrx2* genes reported by Guzmán-Guzmán et al. (2017). Moreover, a transcriptomic analysis of *T. atroviride* in interactions with *A. thaliana* showed a larger number of down-regulated genes than upregulated genes, suggesting that the presence of the plant provides signals participating in the down-regulation of some genes normally expressed in the vegetative state, thus starting a chemical communication that contributes to the establishment of the symbiosis (*A. Herrera-Estrella personal communication*). Once the association has been established, it is probable that the expression of some of these genes returns to their basal levels to contribute to promotion of the interaction.

In other *Trichoderma* species, it is reported that the disruption of different p450 genes may either induce or reduce the plant defense response, depending on the pathway in which the gene product participates (Cardoza et al. 2014; Malmierca et al. 2012). In our case, the higher activation of the JA-mediated defense response shown by plants that interacted with the OETvCyt2 strains could represent an advantage for the plant to respond to challenges. Interestingly, Kottb and collaborators (2015), concluded that *A. thaliana* perceives the *Trichoderma* metabolites as stress compounds and subsequently initiates multilayered adaptations including activation of signaling cascades to withstand this environmental influence. A similar mechanism might be used by *Arabidopsis* when it is in the presence of *Trichoderma* OETvCyt2 strains.

Secondary metabolites synthesized by *TvCyt2* are important for the biocontrol activity of *T. virens* and they participate in inducing plant defense responses. The presence of both fungi and plants has an impact on the expression of the *TvCyt2* gene, showing how these processes can establish a network between the organisms and how secondary metabolites play an important role in *Trichoderma*-host interactions. However, it is necessary to determine the biological behavior in interactions with three or more participants, aimed at representing what could happen in the field. Given the proposed role for gene product *TvCyt2*, it is important to highlight that the modification of the expression of only this enzyme leads to a drastic change in the interaction of *Trichoderma* spp. with plant hosts or fungal antagonists, revealing a fine-tuning orchestrated by different molecular signals that work together, leading to successful interactions.

MATERIALS AND METHODS

Fungal strains and plant species.

The *T. virens* WT strain Tv29.8 and the phytopathogenic strains of *R. solani* AG2 and *R. solani* AG5 were used in this study and

were propagated on potato dextrose agar (PDA) (Difco) at 28°C. Seeds of *A. thaliana* Col-0, a transgenic line, carrying a JA-inducible *pLox2:uidA* construct (Schommer et al. 2008) and the SA-inducible *pPr1a:uidA* construct (Shah et al. 1997) were used.

Obtaining *TvCyt2* null mutants and overexpressing strains.

Knockout mutants of *TvCyt2* were generated following double-joint protocol (Yu et al. 2004). A linear DNA sequence containing a hygromycin B expression cassette flanked by two DNA fragments, homologous to the *TvCyt2* 5' and 3' regions was obtained. Primers to amplify the open reading frame from *TvCyt2* were 5'-GGCATACACGGCACTACGCAC-3' and 5'-GGCATACACGGCACTACGCAC-3', respectively. Nest primers were 5'-CGTGGCAACAGTCTCCACTG-3' and 5'-GCACAA GTCGACAGCGAGGTC-3'. Chimeric primers were 5'-GCCTG TTCCTCTTGTCGGCTTTGCTTAAATGTGCTGCAACCTCT CTCAGGCA3'- and 5'-ACCGTCTCCACGCCGCCGTCAATC TCCTTGGTCGATGGCGATGAAGTTGAAACACG-3'. To over-express the *TvCyt2* gene, the complete *TvCyt2* encoding sequence (1.6 kb) was amplified from cDNA and was cloned into the *Bam*HI and *Hind*III sites of the pUE08 plasmid (Esquivel-Naranjo and Herrera-Estrella 2007). The primers *TvCyt2*-F (5'-G GATCCATCATGTTCAATATCCTTGTCGC-3') and *TvCyt2*-R (5'-AAGCTTCATGTGCTCATTCGTGAGTC-3') were used. Protoplasts of *T. virens* 29.8 were transformed with the purified plasmid. All transformants were subjected to at least three rounds of monospore culture. The identification and selection of the transformants were performed by PCR using the specific internal primers to *TvCyt2* and *hph* genes mentioned in the next section. Additionally, two more fragments were amplified, a 5' region amplified using a direct primer localized outside of the replacement construction and a reverse primer localized inside the *hph* gene (*TvCyt2* ctrl F: 5'-CCGTGCTACCCAATGTGATAGC-3' and *hphC* R: 5'-CCACTAGCTCCAGCCAAGCC-3'), amplifying a product of 3,799 bp. The 3' region, amplified using a reverse primer localized outside of the replacement construction and a direct primer inside the *hph* gene (*hphC* F: 5'-CGTCCGAGG GCAAAGGAATAG-3' and *TvCyt2* ctrl R: 5'-GGGTCTCGA GAACCTCTAGATGG-3') and amplifies a product of 3,857 bp.

Gene analysis by PCR.

PCR reactions were carried out with 50 ng of DNA as template and 25 amplification cycles. We used the *TvCyt2* primers Forward 5'-AAGCATCTACCCGATGGGTTTCCA-3' and Reverse 5'-CGTTTGCCATTTGCCTCTCAACGA-3'. The glyceraldehyde-3-phosphatedehydrogenase gene (*gpd*), a constitutive gene (Carreras-Villaseñor et al. 2013), was used as a control of expression (Forward 5'-GCTGCCGATGGTGAGCTCAAGGG-3' and Reverse 5'-GAGGTCGAGGACACGGCGGGA-3'). To amplify *hph* gene, the primers HygF (5'-GATCGACGTTAAC TGATATTGAAGGAG-3') and HygR (5'-CTATTCCTTTGCCC TCGGACGAGTGCTG-3') were used.

A. thaliana-*Trichoderma* interaction assays.

Seeds of *A. thaliana* Col-0 were surface-sterilized with ethanol four times and were then placed in 0.2x Murashige-Skoog agar plates, 10 seeds per plate, and were incubated at 24°C in a plant growth chamber (MRClab) with 16-h light and 8-h dark cycles. *T. virens* strains were inoculated 4 days after seed germination (Contreras-Cornejo et al. 2009). Plates were incubated for 3, 5, and 7 days, corresponding to the stage of interaction analyzed, i.e., before contact, contact, and overgrowth. At each time, mycelia from the culture edge (1 cm in width) were collected, were frozen, and were subjected to total RNA extraction and further cDNA synthesis. *T. virens* was grown alone as control. Three independent experiments were performed.

Tomato-*Trichoderma* interactions.

Tomato seeds were germinated on vermiculite, and after three weeks, the roots were incubated, for 90 min, on a 1×10^6 spore suspension with each one of the *Trichoderma* strains or with water as control. After that, seedlings were transplanted to plots containing sterile soil and were incubated at room temperature. The plants were irrigated with water for 3 weeks. Tomato plants were carefully removed from containers. Plant length was measured with a ruler and fresh weight was measured on an analytical scale. Plants were returned to their pot and, after 1 week, were irrigated with a spore suspension (1×10^6 conidia per milliliter) and, 3 weeks later, plant length was measured again. The area of the leaves was measured using the software ImageJ 3.0 (NIH Image for the Macintosh).

Direct fungal confrontation assays.

For mycoparasitic confrontation assays to analyze the expression of *TvCyt2*, *T. virens* Gv29-8 was inoculated on PDA plates and was grown at 28°C in total darkness, confronted with *R. solani* AG5 and AG2 or with itself as a control. The confrontations were carried out on plates covered with a double cellophane membrane, and mycelia from *Trichoderma* strains were collected before contact occurred between the two fungi, during contact of both strains, and after *Trichoderma* had overgrown *R. solani*. Three independent experiments for each confrontation were performed.

Antibiosis assays.

T. virens Gv29-8, null mutants, and overexpressing strains were inoculated on PDA plates covered with a double cellophane membrane and were grown at 28°C for 48 h. After that, the cellophane membrane with the mycelia was removed. On the same medium, *R. solani* AG5 and AG2 were grown at 28°C for 72 h. *R. solani* AG5 and AG2 grown on PDA were used as controls. Three independent experiments for each assay were performed.

RNA extraction and cDNA synthesis.

The collected mycelia from plant interactions and from fungal confrontations were frozen immediately in liquid nitrogen. Mycelia were ground to a fine powder and total RNA was isolated using the TRIzol method. cDNA was synthesized with RevertAid H Minus first-strand cDNA synthesis kit (Thermo Scientific), following the recommendations of the manufacturer.

Gene expression analysis by qRT-PCR.

qRT-PCR reactions were performed with Fast SYBR green master mix (Applied Biosystems), using 150 ng of cDNA as template. The *gpd* gene was used as a housekeeping gene and three technical replicates were analyzed for each type of interaction. The cycle threshold ($\Delta\Delta C_t$) method was used to analyze the data obtained from the qRT-PCR assays, using StepOne software (Applied Biosystems) to determine the expression of the *TvCyt2* gene. The primers for *TvCyt2* and *gpd* genes were the same as those used in PCR assays.

Primer design.

Primer sequences were designed using the QuantPrime and Primer Quest online tools and the SnapGene program (GSL Biotech, LLC). Primers for qRT-PCR were designed to produce amplicons around 200 bp.

Determination of soluble metabolites from *T. virens*.

Conidia from *T. virens* strains (1×10^6) were inoculated in 100 ml of 0.2× Murashige-Skoog and were incubated at 28°C for 48 h. The medium was filtered and lyophilized. The samples were recovered, were resuspended in 10 ml of water, and were

vortexed. Samples of 5 ml were taken and 2 ml of ethyl acetate was added to each. The organic phase was transferred to new tubes, the liquid was evaporated, and a second extraction was done, using 200 μ l of ethyl acetate. The extracts were analyzed using a GC-MS system (Bruker 456 GC) equipped with a DB-5MS column (30 m, 0.2 mm ID $\times$ 0.25 μ m) capillary column and connected to a SCION TQ mass spectrometer (Bruker). The column temperature program started at 40°C for 2 min and was increased to 320°C at a rate of 10°C per minute, and the final temperature was maintained for 5 min. The transfer line temperature and the ion source were set to 250°C. The carrier gas (helium, 5.0) was set to 1 ml per minute. Internal electron ionization (70 eV) was performed and mass spectra were recorded in the range 40 to 500 *m/z*. Data were processed using Bruker MSWS software and were re-evaluated using AMDIS software. Soluble compounds were identified by comparison of the mass spectra with the NIST 11MS library data.

Statistical analysis.

A Kruskal-Wallis and a Dunn's post hoc test was carried out to compare the data obtained from the assays, using GraphPad Prism 4 software (GraphPad Software, Inc.). Experiments were done at least in triplicate with three technical replicates.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

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