Trichoderma Histone Deacetylase HDA-2 Modulates Multiple Responses in Arabidopsis

Magnolia Estrada-Rivera,a,2 Oscar Guillermo Rebollo-Ludovico,a,2 Doris Arisbeth Pérez-Robles,a Ma. del Carmen Rocha-Medina,b María del Carmen González-López,a and Sergio Casas-Floresa,3,4

aDivisión de Biología Molecular, Instituto Potosino de Investigación Científica y Tecnológica (IPICYT), Camino a la presa San José No. 2055, Colonia Lomas 4a sección. C.P. 78216, San Luis Potosí, Mexico
bLaboratorio Nacional de Biotecnología Agrícola, Médica y Ambiental, Instituto Potosino de Investigación Científica y Tecnológica (IPICYT), Camino a la presa San José No. 2055, Colonia Lomas 4a sección. C.P. 78216, San Luis Potosí, Mexico

ORCID IDs: 0000-0002-3579-2111 (M.E.-R.); 0000-0002-1042-5630 (O.G.R.-P.); 0000-0002-8314-9796 (M.d.C.R.-M.); 0000-0002-5407-4158 (M.d.C.G.-L.); 0000-0002-9612-9268 (S.C.-F.).

Trichoderma spp. are a rich source of secondary metabolites and volatile organic compounds (VOCs), which may induce plant defenses and modulate plant growth. In filamentous fungi, chromatin modifications regulate secondary metabolism. In this study we investigated how the absence of histone deacetylase HDA-2 in the Trichoderma atroviride strain Δhda-2 impacts its effect on a host, Arabidopsis (Arabidopsis thaliana). The production of VOCs and their impact on plant growth and development were assessed as well. The Δhda-2 strain was impaired in its ability to colonize Arabidopsis roots, thus affecting the promotion of plant growth and modulation of plant defenses against foliar pathogens Botrytis cinerea and Pseudomonas syringae, which normally result from interaction with T. atroviride. Furthermore, Δhda-2 VOCs were incapable of triggering plant defenses to counterattack foliar pathogens. The Δhda-2 overproduced the VOC 6-pentyl-2H-pyran-2-one (6-PP), which resulted in enhanced root branching and differentially regulated phytohormone-related genes. Analysis of ten VOCs (including 6-PP) revealed that three of them positively regulated plant growth, whereas six had the opposite effect. Assessment of secondary metabolites, detoxification, and communication with plant-related genes showed a dual role for HDA-2 in T. atroviride gene expression regulation during its interaction with plants. Chromatin immunoprecipitation of acetylated histone H3 on the promoters of plant-responsive genes in Δhda-2 showed, in the presence of Arabidopsis, low levels of epl-1 and abc-2 compared with that in the wild type; whereas ctf-1 presented high constitutive levels, supporting a dual role of HDA-2 in gene regulation. This work highlights the importance of HDA-2 as a global regulator in Trichoderma to modulate multiple responses in Arabidopsis.
that feed on living host tissue, such as Pseudomonas syringae (Dong, 2004); whereas the JA/ET-related defense response is boosted by necrotrophic microorganisms, such as Botrytis cinerea (Pieterse et al., 1996), which kill host tissue at early stages of the invasion. Fungi belonging to the plant-beneficial Trichoderma genus are cosmopolitan inhabitants of soil. Some Trichoderma species confer beneficial effects to plants by means of mycoparasitism, and the synthesis of antibiotics against phytopathogens, inducing systemic disease resistance, and promoting growth and fitness (Shoresh et al., 2010; Hermosa et al., 2012; Olmedo-Monfil and Casas-Flores, 2014). During root colonization, Trichoderma spp. produce and secrete a diversity of MAMPs, such as xylanases (EIX), cellulases, and the proteinaceous Sm1/Epl1 (Martinez et al., 2001; Rotblat et al., 2002; Djonović et al., 2006; Salas-Marina et al., 2015) or secondary metabolites (SMs) such as alamethicin and trichokonin (Engelberth et al., 2001; Luo et al., 2010). Trichoderma MAMPs are specifically recognized by triggering simultaneously SAR and ISR responses (Salas-Marina et al., 2011; Perazzolli et al., 2012). The plant growth promotion capability of Trichoderma has been linked to its ability to colonize the plant roots (Hermosa et al., 2013). Some of the Trichoderma mechanisms to promote plant growth include synthesis of phytohormones, solubilization of soil nutrients, increased uptake and translocation of nutrients, and enhanced root development (Baker, 1989; Harman, 2000, 2006). VOCs released by Trichoderma spp. play a pivotal role in plant growth promotion as well (Hung et al., 2012). Approximately 479 hydrophobic compounds with high vapor pressure have been reported (Siddiquee, 2014). One of the VOCs that feed on living host tissue, such as Pseudomonas syringae (Dong, 2004); whereas the JA/ET-related defense response is boosted by necrotrophic microorganisms, such as Botrytis cinerea (Pieterse et al., 1996), which kill host tissue at early stages of the invasion. Fungi belonging to the plant-beneficial Trichoderma genus are cosmopolitan inhabitants of soil. Some Trichoderma species confer beneficial effects to plants by means of mycoparasitism, and the synthesis of antibiotics against phytopathogens, inducing systemic disease resistance, and promoting growth and fitness (Shoresh et al., 2010; Hermosa et al., 2012; Olmedo-Monfil and Casas-Flores, 2014). During root colonization, Trichoderma spp. produce and secrete a diversity of MAMPs, such as xylanases (EIX), cellulases, and the proteinaceous Sm1/Epl1 (Martinez et al., 2001; Rotblat et al., 2002; Djonović et al., 2006; Salas-Marina et al., 2015) or secondary metabolites (SMs) such as alamethicin and trichokonin (Engelberth et al., 2001; Luo et al., 2010). Trichoderma MAMPs are specifically recognized by triggering simultaneously SAR and ISR responses (Salas-Marina et al., 2011; Perazzolli et al., 2012). The plant growth promotion capability of Trichoderma has been linked to its ability to colonize the plant roots (Hermosa et al., 2013). Some of the Trichoderma mechanisms to promote plant growth include synthesis of phytohormones, solubilization of soil nutrients, increased uptake and translocation of nutrients, and enhanced root development (Baker, 1989; Harman, 2000, 2006). VOCs released by Trichoderma spp. play a pivotal role in plant growth promotion as well (Hung et al., 2012). Approximately 479 hydrophobic compounds with high vapor pressure have been reported (Siddiquee, 2014). One of the most common SMs is 6-PP, and the responsible VOC for the coconut odor in some Trichoderma species. Plant growth is promoted by 6-PP, and it regulates root architecture, inhibiting primary root growth and inducing lateral root formation (Vinale et al., 2008; Garnica-Vergara et al., 2016 Kottb et al., 2015). VOCs appear during both primary and secondary metabolism (from intermediates of the primary metabolism; Korpi et al., 2009). The genes encoding for enzymes involved in the synthesis of SMs are typically arrayed in gene clusters in filamentous fungi (Keller and Hohn, 1997; Walton, 2000; Keller et al., 2005), frequently regulated by chromatin modifications, such as histone acetylation and deacetylation, performed by histone acetyltransferases and histone deacetylases (HDACs) activity (Tribus et al., 2005; Shwab et al., 2007; Lee et al., 2009). It is well known that addition of acetyl groups to histones by histone acetyltransferases promote a relaxed chromatin state, leading to gene expression, whereas removal of such groups by HDACs drives chromatin compaction and represses transcription. In this regard, it has been reported that HDACs are involved in growth and development, synthesis of SM, virulence, and invasive growth of plant pathogenic fungi (Baidyaroy et al., 2001; Lee et al., 2009; Ding et al., 2010; Tribus et al., 2010). The histone deacetylase hda-2 encoding gene from Trichoderma atroviride can be induced by light and reactive oxygen species (ROS). Its product regulates growth, conidiation, blue-light perception, and oxidative stress responses (Osorio-Concepción et al., 2017). In this work, we study the T. atroviride hda-2 deletion mutant (Δhda-2), whose gene codes in the wild type for the histone deacetylase HDA-2, during its interaction with Arabidopsis (Arabidopsis thaliana) plants. We show that Δhda-2 is impaired in its ability to colonize Arabidopsis roots, as well as in triggering SAR and ISR responses by direct contact or through VOCs. An analysis of the root system architecture of Arabidopsis showed that the presence of Δhda-2 or its VOCs promoted a strong lateral root branching. We characterized the VOCs profile emitted by Δhda-2 using gas chromatography-mass spectrometry (GC-MS). Interestingly, the absence of hda-2 impaired the VOCs metabolism, resulting in an overproducing-6-PP strain. In vitro assays in a medium amended with 6-PP (or supplied as VOC) demonstrated that the plant growth-promoting effect is dependent on the Arabidopsis age and the application protocol. Split-plate assays with nine different VOCs resulted in the discovery of three different VOCs with a plant growth-promoting effect in Arabidopsis seedlings, whereas six of them provoked the opposite effect. Transcription analyses and chromatin immunoprecipitations of acetylated histone H3 of plant-responsive genes in T. atroviride showed that HDA-2 could be regulating these genes directly or indirectly by its HDAC activity. Together these results indicate that HDA-2 is a global regulator in T. atroviride, which modulates multiple responses in Arabidopsis.

RESULTS

Root Treatment of Arabidopsis with Δhda-2 Increases the Lateral Root Number

To investigate whether the product of the hda-2 gene is involved in the interaction of the fungus with Arabidopsis, plants were root inoculated with T. atroviride. The mycelium was collected at the indicated times of cocolulture, total RNA was extracted, and the transcript level of hda-2 was determined. The mRNA level of hda-2 was induced early (24 h) by the presence of Arabidopsis (Supplemental Fig. S1).

To analyze in vitro the effect of Δhda-2 on the root system architecture of Arabidopsis, plants were inoculated with the wild type or Δhda-2. Plants treated with either the wild type or Δhda-2 showed a statistically significant increase in lateral root number; however, the Δhda-2-inoculated seedlings showed more lateral roots than that of the wild-type–treated plants (Fig. 1, A and B). Primary root length of plants treated with the wild type was barely but significantly larger than those treated with Δhda-2 (Fig. 1C); however, no significant

Downloaded on May 31, 2021. - Published by https://plantphysiol.org
Copyright (c) 2020 American Society of Plant Biologists. All rights reserved.
differences were observed in fresh weight of plants treated with these fungi (Fig. 1D). Moreover, dry weight of the wild-type–treated plants was higher than that of Δhda-2–treated seedlings, and the latter showed higher dry weight than that of control plants (Fig. 1E). These data show that the balance between the extension of the primary root and production of lateral roots is responsible for biomass gaining in plants inoculated with Δhda-2.

The Δhda-2 VOCs Enhance the Growth of Arabidopsis Seedlings

To assess whether the VOCs of Δhda-2 promote growth in Arabidopsis, seedlings were grown on MS and cocultured with mycelia of the wild type or Δhda-2–grown in small plates within a large MS plate (Fig. 4A). Plants exposed to VOCs of Δhda-2 exhibited a significant increase in lateral root number (Fig. 4B) and in fresh and dry weights compared with those treated with the wild type (Fig. 4D). Moreover, dry weight of the wild-type–treated plants was higher than that of Δhda-2–treated plants, and the latter showed signiﬁcantly diminished fresh and dry weights compared with those treated with the wild type; however, the plants treated with Δhda-2 showed signiﬁcantly enhanced growth compared with that of the control plants (Fig. 2, A to C). To investigate the capability of Δhda-2 to colonize Arabidopsis roots, seedlings were root inoculated with the wild type or Δhda-2. Thereafter, the roots were detached; total DNA was extracted, and abundance of the T. atroviride tef-1 gene versus the Arabidopsis ACT2 gene were quantiﬁed by real-time polymerase chain reaction (PCR; quantitative PCR, qPCR). Δhda-2 capability to colonize the Arabidopsis roots was impaired compared with that of the wild type (Fig. 2D).

Deletion of hda-2 in T. atroviride Compromises its Capability to Induce the Arabidopsis Systemic Disease Resistance Against Foliar Pathogens

To determine if HDA-2 is necessary to elicit the plant defense responses by Trichoderma, the expression profiles of the well-known Arabidopsis marker genes PR-1a (SAR) and PDF1.2 (ISR) were assessed by reverse transcription-qPCR (RT-qPCR). The expression of PR-1a and PDF1.2 was strongly induced by the wild type, whereas Δhda-2 barely triggered the expression of PDF1.2 at 72 h and 96 h (Fig. 3, A and B). Indeed, mutant-induced expression of PR-1a was not detected at any tested time (Fig. 3, A and B).

Based on the expression analysis of PR-1a and PDF1.2 in response to Δhda-2, we asked whether the mutant provides protection against the fungal pathogen B. cinerea and the bacterial pathogen Pst DC3000. Plants treated with Δhda-2 exhibited an enhanced disease susceptibility to both pathogens compared with that in plants treated with the wild type (Fig. 3, C to E); however, in the case of B. cinerea, the plants treated with the mutant did not reach the disease susceptibility of the Trichoderma–untreated plants (Fig. 3, C and D). On the other hand, the wild-type–treated seedlings postinoculated with Pst DC3000 showed 3.895 × 10⁶ ±1.054 × 10⁶ colony forming units (CFU)/mL; whereas plants treated with Δhda-2 and inoculated with the bacterial pathogen showed similar levels of CFU/mL (7.985 × 10⁶ ± 1.75 × 10⁶) as that for the control seedlings (9.232 × 10⁶ ± 2.454 × 10⁶; Fig. 3E).
The **D**hda-2 VOCs Do Not Trigger the SAR and ISR Responses in Arabidopsis

We next tested the effect of **D**hda-2 VOCs on the triggering of ISR and SAR responses in Arabidopsis seedlings. The VOCs of the wild type increased the expression levels of both **PR-1a** and **PDF1.2** (Fig. 5, A and B), although to a lesser extent compared with that of the root-inoculated plants (Fig. 3, A and B). Similar to the results of the **D**hda-2 root-inoculated Arabidopsis seedlings (Fig. 3, A and B), the plants exposed to **D**hda-2 VOCs barely induced **PR-1a** or **PDF1.2** at any of the tested times (Fig. 5).

The Expression of Auxin- and ET-Related Genes Is Differentially Modulated in Arabidopsis by **D**hda-2 Direct Contact or by Exposure to its VOCs

We analyzed the expression of ET- and auxin-related genes in Arabidopsis. Arabidopsis seedlings were root inoculated or exposed to the wild-type or **D**hda-2 VOCs for 24, 48, 72, and 96 h. Overall, the auxin-related genes **TIR1** (auxin receptor), **AUX1** (auxin importer), and **PIN3** and **PIN7** (auxin efflux carriers) were upregulated to a different extent in the wild-type or **D**hda-2 root-treated plants (with the exception of **PIN7**, which was downregulated by the presence of **D**hda-2; Fig. 6, A to D). Exposure of Arabidopsis to the wild-type VOCs upregulated **TIR1** at 24, 48, and 72 h, but **TIR1** expression downregulated at 96 h, whereas **D**hda-2 VOCs barely induced that gene. Furthermore, **AUX1** was barely induced by both the wild-type and **D**hda-2 VOCs, whereas **PIN3** and **PIN7** were upregulated by the wild-type and **D**hda-2 VOCs to a different extent (Fig. 6, A to D). On the other hand, the ET pathway-related genes **ACO2** [1-aminocyclopropane-1-carboxylic acid (ACC) oxidase], **ETR1** (ET receptor 1), **ERS1** (ET response sensor 1), and **EIN2** (ET insensitive 2) were upregulated by the presence of the wild type, whereas roots exposed to **D**hda-2 induced **EIN2** at 24 h, although **ACO2**, **ETR1**, and **ERS1** were barely induced or unaltered (Fig. 6, E to H). Exposure of Arabidopsis to the wild-type VOCs led to the up-regulation of **ETR1** and **EIN2**, whereas **ERS1** and **ACO2** showed basal levels. **D**hda-2 VOCs-treated seedlings showed basal or barely up-regulation of all four ET pathway-related genes (Fig. 6, E to H).

The **D**hda-2 Strain Overproduces 6-PP

To analyze the VOCs produced by **D**hda-2 and to compare them with those of the wild type, fresh inoculums of both strains were grown on MS plates for 5 and 6 d. The analysis of VOCs was performed through GC-MS. The VOCs identified were assigned to alcohols, ketones, unknown terpenes, and pyrones. Two unknown terpenes and 6-PP (Table 1) were present in both strains.
The 1-octen-3-ol and an unknown terpene was only found in the wild type, whereas the 2-undecanone, unknown ketone, two unknown terpenes, and β-curcumene were only detected in Δhda-2 (Table 1). Furthermore, based on the percentage of relative area, 6-PP together with an unknown terpene were the most abundant VOCs produced by Δhda-2 and the wild type grown on MS for 5 and 6 d (Table 1). This was confirmed by quantification of 6-PP, whereby Δhda-2 synthesized 970.4 and 847.7 μg mL⁻¹ at day 5 and 6, respectively, whereas the wild type produced 129.1 and 89.9 μg mL⁻¹ by the same time (Table 2).

The Effect of 6-PP on Root System Architecture Depends on the Plant Age and the Application Method

To evaluate the effect of 6-PP on the plant’s growth promotion and the root system architecture of Arabidopsis, 6-PP was supplied into the medium or provided as volatile in split-petri dishes (Fig. 7, A and B). Two-day-old Arabidopsis plants were treated with ethanol (control treatment) or with 25, 50, 75, and 100 μM 6-PP (4.15, 8.30, 12.46, and 16.62 μg mL⁻¹ respectively). After 13 d of treatments with 6-PP, lateral root number was increased in a dose-dependent manner (Fig. 7, A to C). A strong primary root growth inhibition was detected when 50, 75, and 100 μM of 6-PP were supplied in the growing medium. A similar behavior was observed when 6-PP was applied as VOC, but, to a minor extent, at concentrations from 25 to 100 μM of 6-PP (Fig. 7, A, B, and D). Regarding the fresh weight, both treatments followed a similar behavior, except when 50 μM was applied as a volatile or in the growing medium, showing a better biomass gain when the medium was amended with 6-PP (Fig. 7E). In contrast, the lateral root number and dry weight of

Figure 3. Deletion of hda-2 in T. atroviride compromises the induction of Arabidopsis systemic disease resistance against foliar pathogens. A and B, Ten-day-old Arabidopsis seedlings grown on MS medium were root-inoculated with the wild type (WT) or Δhda-2, and the expression levels of PR-1a (A) or PDF1.2 (B) were analyzed by RT-qPCR at 24, 48, 72, and 96 hpi. RT-qPCR results are reported as fold-change expression compared with that in Arabidopsis grown without the fungi. Arabidopsis ACT2 gene was used as control to normalize the expression of PR-1a and PDF1.2 using the 2⁻ΔΔCt method. C, Representative images of leaves from 10-d-old Arabidopsis seedlings grown in soil that were inoculated with the wild type or Δhda-2; and two weeks later, leaves were infected with B. cinerea or the inoculating buffer as a control. Bar = 1.5 cm (applies to all images). D, Lesion sizes of infected plants with B. cinerea were determined using ImageJ at 6 dpi. E, CFU of Pst DC3000 at 0 and 3 dpi in leaves of treated and untreated plants with the wild type or Δhda-2. Data from A and B show the mean ± SD of one technical replicate (5 plates with 7 plants each). The experiment was repeated twice with similar results. Data from D show the mean ± SD of one technical replicate (12 leaves each). The experiment was repeated three times with similar results. Data from E show the mean ± SD of one technical replicate (12 leaves each). The experiment was repeated twice with similar results. Asterisks indicate significant difference (independent Student’s t test, *P < 0.05 and **P < 0.01).
plants treated with 6-PP, supplied as volatile, did not differ significantly between 25, 50, 75 mM in thia treatment order, whereas 100 mM caused a negative effect on plant growth. However, plants treated with 75 mM showed significant differences in biomass gain compared with that of 25 mM-treated seedlings (Fig. 7B). To determine whether the age of Arabidopsis plays a key role in the plant growth promotion by 6-PP, 7-d-old Arabidopsis plants were exposed to 25, 50, 75, and 100 mM of 6-PP or ethanol as a control. After 13 d, the treated plants showed an increased lateral root number (Fig. 7G), whereas the primary root growth was inhibited in a dose-dependent manner (Fig. 7I). However, we observed a dry weight gain only at 50 and 75 mM of 6-PP (Fig. 7K). In summary, the reduction in primary root length and the aerial part of the plant by 6-PP seems to be compensated by an increase in lateral root number, leading to a biomass gain at the indicated concentrations.

2-heptanol, 2-heptanone, and 3-octanol VOCs Promoted Plant Growth in Arabidopsis Seedlings

Next, we analyzed the VOCs profile produced by the Δhda-2 and wild-type strains grown on PDA. To this end, both strains were grown for 5 to 7 d. A total of 28 VOCs were detected. The VOCs were assigned to the compounds classes of alcohols, ketones, mono- di- and sesquiterpenes, alkanes, and pyrones (Table 1). The most abundant compounds for the wild type were the ketones 2-heptanone and 3-octanone, whereas for Δhda-2 it was 6-PP. Four exclusive VOCs were identified for the wild type, comprising 3-octanone, 2-heptanol, 3-octanol, and 1-octen-3-ol (Table 1), whereas 17 volatiles were found

![Figure 4. The VOCs of Δhda-2 enhance plant growth in Arabidopsis.](image)

Eleven-day-old Arabidopsis seedlings grown on a MS medium were exposed to the wild type (WT) or Δhda-2 VOCs for seven days. A, Representative pictures of Arabidopsis grown under the indicated treatments. Bar = 1.5 cm (applies to all images). B, Lateral root number. C, Root length. D, Fresh weight. E, Dry weight. Data from B to E show the mean ± SD of two technical replicates (6 plates with 12 plants each). Results were validated with an ANOVA statistical analysis using a Tukey multiple comparison test (α = 0.05). Lower case letters a, b, and c (B to E) represent means statistically different at the 0.05 level.

![Figure 5. Δhda-2 VOCs fail to properly induce ISR and SAR in Arabidopsis.](image)

A and B, Ten-day-old Arabidopsis seedlings grown on petri plates containing MS were exposed to the wild-type (WT) or Δhda-2 VOCs. A and B, The expression levels of defense-related genes PR-1a (A) and PDF1.2 (B) were analyzed by RT-qPCR. Results are reported as fold-change expression compared with that in Arabidopsis grown without the fungi. The Arabidopsis ACT2 gene was used as a control to normalize the expression using the 2^(-ΔΔCt) method. Data from A and B show the mean ± SD of one technical replicate (5 plates with 7 plants each). The experiment was repeated twice with similar results. Asterisks indicate significant difference (independent Student’s t-test, *P < 0.05 and **P < 0.01).
Figure 6. The expression of auxin and ET synthesis and perception genes are altered in Arabidopsis by direct contact with Δhda-2 or exposure to its VOCs. A to H, Ten-day-old Arabidopsis seedlings grown on petri plates containing MS were inoculated with the wild type (WT) or Δhda-2 (direct contact), or exposed to their VOCs. The expression levels of TIR1 (A), AUX1 (B), PIN3 (C), PIN7 (D), ACO2 (E), ETR1 (F), ERS1 (G), and EIN2 (H) were analyzed by RT-qPCR. Results are reported as fold-change expression compared with that in Arabidopsis grown without the fungi. The Arabidopsis ACT2 gene was used as control to normalize the expression using the 2-ΔΔCt method. Data from A to H show the mean ± so of one technical replicate (5 plates with 7 plants each).
in Δhda-2, including six unknown terpenes, 2-octanone, three unknown ketones, γ-terpinene, α-zingiberene, β-sesquiphellandrene, two unknown alcohol, one unknown alkane, and one unknown phenol (Table 1). Compounds such as 2-heptanone, 2-pentylfuran, 2-nonanone, unknown terpene, 2-undecanone, 6-PP, and β-curcumene were common to both the wild type and Δhda-2 (Table 1). Because Arabidopsis exposed to Δhda-2 VOCs displayed a remarkable phenotype in plant growth, we decided to investigate the VOCs profile of Δhda-2 growing on PDA in more detail. We found striking differences in the production of 2-heptanol, 3-octanol, 1-octen-3-ol, 2-heptanone, 2-octanone, 3-octanone, 2-nonanone, 2-undecanone, and 2-pentylfuran between the wild type and Δhda-2 (Tables 1 and 2). Therefore, these compounds were tested in plant-growth assays in vitro. Arabidopsis plants were exposed to individual VOCs at four different concentrations: 10, 100, 1000, and 10,000 μg mL⁻¹. Addition of 2-heptanol (100 and 1000 μg mL⁻¹), 3-octanol (100 μg mL⁻¹), and 2-heptanone (10, 100, and 1000) as VOCs led to a significant effect on plant growth (Fig. 8). In contrast, 3-octanol, 1-octen-3-ol, 2-octanone, 3-octanone, 2-nonanone, 2-undecanone, and 2-pentylfuran were highly phytotoxic at 10,000 μg mL⁻¹; the plants showed a yellow color and a bleaching phenotype (Fig. 8). Most of the VOCs did not result in a growth effect at 10 μg mL⁻¹, whereas at 1000 μg mL⁻¹ the plants started to show a detrimental effect (Fig. 8). Interestingly, 2-heptanol and 2-heptanone were the only VOCs that did not bleach the plants at 10,000 μg mL⁻¹ (Fig. 8, A and B).

**Δhda-2 Has Misregulated Expression of Secondary Metabolism-, Defense-, and Plant Communication-Related Genes**

To know whether Δhda-2 is affected in the expression of genes described as important in *Trichoderma* to establish a beneficial relationship with plants, as well as in the synthesis of SM, Δhda-2, and the wild type were cocultured with Arabidopsis seedlings for 72 and 96 h. Then, we analyzed the expression of genes involved in different traits of *T. atroviride*, such as communication with the plant (epl-1, epl-2, and pbs-1; Fig. 9, A to C), defense against toxic compounds (abc-2; Fig. 9D), and synthesis of SM (ctf-1, tps-2, pbs-1, ggp-1, and fpp-1; Fig. 9, E to H). Indeed, epl-1, epl-2, pbs-1 (Fig. 9, A to C), and abc-2 (Fig. 9D) showed reduced expression levels in Δhda-2 in the presence of Arabidopsis seedlings compared with that in the wild type, whereas ctf-1, tps-2, pbs-1, ggp-1, and fpp-1 showed enhanced levels of transcription in Δhda-2 compared with that in the wild type (Fig. 9, E to H).

**Δhda-2 Is Required in *T. atroviride* for Proper Acetylation of Histone H3 on the Promoter Region of Plant-Responsive Genes**

Chromatin immunoprecipitation (ChIP) assays were performed to determine the acetylation pattern of histone H3 at Lys-9/Lys-14/Lys-18/Lys-23/Lys-27 on the promoter regions of epl-1, ctf-1, and abc-2 (Supplemental Fig. S3) in the wild-type and Δhda-2 backgrounds in the presence or absence of Arabidopsis. The coculture of the wild type with Arabidopsis increased the acetylation of histone H3 to 19.88%, 6.37%, and 21.38% on the promoters of epl-1 (Fig. 10A), ctf-1 (Fig. 10B), and abc-2 (Fig. 10C), respectively, compared with that in the wild type grown in the absence of the plant (Fig. 10). However, histone H3 acetylation on epl-1 (Fig. 10A), ctf-1 (Fig. 10B), and abc-2 (Fig. 10C) promoters in Δhda-2 was barely higher when grown alone compared with that in the wild type (Fig. 10); however, during Δhda-2 interaction with plants, epl-1, ctf-1, and abc-2 histone H3 acetylation dropped to 1.83%, 2.55%, and 2.81%, respectively, as compared with that in the wild type grown under the same conditions (Fig. 10).

**DISCUSSION**

Here, the *hda-2* gene, which codes for an HDAC class I in *T. atroviride*, was induced by the presence of Arabidopsis. This result may indicate that the product of this gene could be involved in the Arabidopsis-*T. atroviride* interaction. Indeed, the absence of *hda-2* in *Trichoderma* led to the loss of some of its beneficial effects on Arabidopsis. For instance, Δhda-2 showed a reduced effect in promoting plant growth and biomass gain, both when grown in vitro or in pots. This prompted us to hypothesize whether Δhda-2 was affected in its capability to colonize Arabidopsis roots. Root colonization assays revealed that *hda-2* is highly sensitive to oxidative stress (Osorio-Concepción et al., 2017), which may affect the colonization process. At the beginning of plant root colonization by *Trichoderma*, the fungus is recognized as foreign through its MAMPs by the plant, triggering systemic disease resistance (Djonović et al., 2006; Viterbo et al., 2007;...
These proteins are involved in the induction of ISR and SAR, respectively, and for the peptaibol synthetase PBS-1, respectively, which code for the cerato-platanin elicitor proteins -1 and -2, and for HDA-2 putative orthologs Rpd3 and Hos2p in S. cerevisiae (De Nadal et al., 2004; Sharma et al., 2007). In agreement with these results, a class I HDACs, such as HDA-2 and Hos2p of N. crassa and S. cerevisiae, respectively, which putatively remove acetyl groups from histone tails, leading to a compacted chromatin and therefore to gene repression (Osorio-Concepción et al., 2017). Interestingly, the presence of Arabidopsis increased the acetylation of H3 on the promoters of epl-1, ctf-1, and abc-2; however, the acetylation of H3 N-terminal tail on the promoters of Hda-2 was reduced, which suggests a positive role of HDA-2 on the transcription of epl-1 and abc-2, as recently reported for blue light-responsive genes (Osorio-Concepción et al., 2017), and for HDA-2 putative orthologs Rpd3 and Hos2p in S. cerevisiae (De Nadal et al., 2004; Sharma et al., 2007). In agreement with

### Table 1. VOCs of the wild type and Δhda-2 detected by GC-MS in 1 × MS and PDA

Mean values ± SE of the sum of three independent determinations are given. (–), No data; Rt, retention time.

<table>
<thead>
<tr>
<th>Compound Name</th>
<th>MS 1 ×</th>
<th>Relative Area %</th>
<th>PDA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild Type</td>
<td>Δhda-2</td>
<td>Wild Type</td>
</tr>
<tr>
<td></td>
<td>5 dpi</td>
<td>6 dpi</td>
<td>5 dpi</td>
</tr>
<tr>
<td>2-heptanone</td>
<td>9.453</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Unknown terpene</td>
<td>9.966</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>2-pentylfuran</td>
<td>12.976</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>3-octanone</td>
<td>14.288</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>2-octanone</td>
<td>15.952</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Unknown ketone</td>
<td>19.303</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>2-heptanol</td>
<td>18.972</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>2-nonanone</td>
<td>21.926</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>3-octanol</td>
<td>22.875</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>1-octen-3-ol</td>
<td>25.704</td>
<td>1.01 ± 0.14</td>
<td>–</td>
</tr>
<tr>
<td>Unknown terpene</td>
<td>30.644</td>
<td>5.06 ± 1.62</td>
<td>7.24 ± 1.94</td>
</tr>
<tr>
<td>2-undecanone</td>
<td>31.644</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Unknown ketone</td>
<td>32.247</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Unknown terpene</td>
<td>34.153</td>
<td>2.95 ± 0.74</td>
<td>3.27 ± 0.97</td>
</tr>
<tr>
<td>Unknown terpene</td>
<td>34.443</td>
<td>3.01 ± 1.16</td>
<td>–</td>
</tr>
<tr>
<td>γ-terpinene</td>
<td>35.091</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>α-zingiberene</td>
<td>36.371</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Unknown terpene</td>
<td>36.573</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>β-Sesquiphellandrene</td>
<td>38.182</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Unknown ketone</td>
<td>42.392</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Unknown alcohol</td>
<td>42.569</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Unknown alkane</td>
<td>42.896</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Unknown phenol</td>
<td>44.06</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Unknown terpene</td>
<td>49.15</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Unknown terpene</td>
<td>51.374</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Unknown terpene</td>
<td>52.304</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>6-pentyl-2H-pyran-2-one</td>
<td>53.22</td>
<td>90.90 ± 2.51</td>
<td>39.14 ± 2.07</td>
</tr>
<tr>
<td>β-curcumene</td>
<td>54.089</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Unknown terpene</td>
<td>55.507</td>
<td>47.31 ± 2.05</td>
<td>8.79 ± 0.19</td>
</tr>
<tr>
<td>Unknown alcohol</td>
<td>59.214</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>
such results, the lack of hda-2 led to the down-regulation of epl-1 and abc-2 transcripts. On the other hand, acetylation of H3 N-terminal tail on the promoter of ctf-1 reached high basal levels in Δhda-2 (compared with epl-1 and abc-2 promoters) in either the presence or absence of the plant, which correlates with high levels of transcription, suggesting a direct regulation of ctf-1 by HDA-2.

Several reports on plant growth-promoting rhizobacteria and plant growth-promoting fungi, including Trichoderma, have shown the role of their VOCs in triggering SA- and JA-signaling pathways (Ryu et al., 2004; Park et al., 2013; Naznin et al., 2014; Kottb et al., 2015). In agreement with these reports, we demonstrated the induction of SAR and ISR by the wild-type VOCs. Interestingly, decreased levels of PR-1a and PDF1.2 were observed at 72 h of Arabidopsis exposure to the wild-type VOCs, compared with that at 48 and 96 h, a phenomenon that did not happen by direct contact with the fungus. In this regard, it is known that the circadian clock controls secondary metabolism in fungi, and therefore accumulation of VOCs might not be continuous through all developmental stages (Bayram and Braus, 2012). Circadian rhythms have not been described in Trichoderma spp.; however, their genomes contain the central components of the circadian clock (Casas-Flores and Herrera-Estrella, 2013, 2016).

Conversely, Δhda-2 VOCs failed to properly induce ISR and SAR. Probably some VOCs in Δhda-2 were absent or in diminished amounts owing to the absence of HDA-2, which points to a positive role of HDA-2 on the synthesis of VOCs that could be functioning as elicitor molecules to induce plant responses. Interestingly, we analyzed several SM metabolism-related genes, such as ggp-1 (hexaprenyl pyrophosphate synthase); fpp-1 (farnesyl pyrophosphate synthase), involved in the biosynthesis of isoprenoids; ctf-1 (cutinase transcription factor 1 beta protein), a putative positive regulator of the biosynthesis of 6-PP (Rubio et al., 2009); and tps-2 (terpene synthase-2), whose product is putatively involved in the synthesis of terpenes. All of these were upregulated both in the presence and absence of the plant, which supports our hypothesis stated above. A striking observation was the magnitude of responses between plants exposed to Trichoderma VOCs and Arabidopsis roots treated with Trichoderma, whereby the latter provoked stronger responses. These data indicate that Trichoderma triggers the plant defense responses mainly by root colonization (probably delivering elicitors of ISR and SAR), and secondly by the secretion of VOCs.

Previous studies have shown that 6-PP triggers the systemic resistance (Vinale et al., 2008; Kottb et al., 2015). Interestingly, Δhda-2 produced 7.5 to 9.5-fold more 6-PP than that in the wild type growing on MS, which cannot explain the impairment of Δhda-2 to induce SAR and ISR, thus suggesting that 6-PP does not play or could be playing a minor role on triggering such pathways. In addition, we propose that HDA-2 could be exerting its role as a negative regulator of the promoter activity of 6-PP biosynthetic genes or through a positive regulator, whose transcription depends on HDA-2. Here, we demonstrated that ctf-1 transcript, which codes for a positive regulator of the synthesis of 6-PP (Rubio et al., 2009), was upregulated in a Δhda-2 background at all tested conditions. These results strongly support our hypothesis about the regulation of a positive regulator of 6-PP biosynthesis by HDA-2.

Δhda-2 presented a diminished ability to promote plant growth and biomass gain in Arabidopsis. This reduction might be the result of Δhda-2 inability to colonize the plant root; however, Arabidopsis seedlings treated with Δhda-2 VOCs presented a greater biomass gain compared with that of Arabidopsis treated with the wild-type VOCs. Stimulation of lateral root number in plants treated with Δhda-2 or exposed to its VOCs was consistent in both treatments, together with a shortening of the primary root length. Together, these results indicate that in our working conditions, root colonization plays a minor role in plant growth.

Table 2. Quantification of the wild type and Δhda-2 VOCs in MS and PDA by GC-MS

<table>
<thead>
<tr>
<th>Compound Name</th>
<th>Calibration Curve</th>
<th>MS 1 ×</th>
<th>Wild Type</th>
<th>Δhda-2</th>
<th>PDA</th>
<th>Wild Type</th>
<th>Δhda-2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(5 dpi)</td>
<td>(6 dpi)</td>
<td>(7 dpi)</td>
<td></td>
<td>(5 dpi)</td>
<td>(6 dpi)</td>
</tr>
<tr>
<td>2-heptanone</td>
<td>0.99</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>3189.6</td>
<td>184.8</td>
</tr>
<tr>
<td>2-pentylfluorane</td>
<td>1.00</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>5.0</td>
<td>1.9</td>
</tr>
<tr>
<td>3-octanone</td>
<td>0.99</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>1388.0</td>
<td>3.2</td>
</tr>
<tr>
<td>2-octanone</td>
<td>0.99</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>771.7</td>
<td>4.8</td>
</tr>
<tr>
<td>2-heptanol</td>
<td>0.99</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>768.4</td>
<td>2.2</td>
</tr>
<tr>
<td>2-nonanone</td>
<td>0.99</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>339.3</td>
<td>80.2</td>
</tr>
<tr>
<td>2-octanol</td>
<td>0.99</td>
<td>–</td>
<td>–</td>
<td>262.9</td>
<td>0.9</td>
<td>3354.0</td>
<td>238.6</td>
</tr>
<tr>
<td>1-octen-3-ol</td>
<td>0.98</td>
<td>269.4</td>
<td>0.5</td>
<td>–</td>
<td>–</td>
<td>387.0</td>
<td>0.6</td>
</tr>
<tr>
<td>2-undecanone</td>
<td>0.98</td>
<td>–</td>
<td>–</td>
<td>262.9</td>
<td>0.8</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>6-pentyl-2-H-pyran-2-one</td>
<td>0.99</td>
<td>129.1</td>
<td>± 30.2</td>
<td>970.4</td>
<td>42.6</td>
<td>51.2</td>
<td>3.9</td>
</tr>
</tbody>
</table>

Mean values ± se of the sum of three independent determinations are given. (–), No data.
Figure 7. The 6-PP effect on root system architecture is dependent on plant age and the application method. Two- or seven-d-old Arabidopsis seedlings were grown on 1× MS supplemented with increasing concentrations of 6-PP or onto sterile cottons of compartmented petri dishes for 13 d. A, Representative pictures of 2-d-old Arabidopsis grown on supplemented medium with 6-PP at the indicated concentrations. Bar = 1.5 cm (applies to all images). B, Representative pictures of 2-d-old Arabidopsis exposed to the 6-PP VOC at the indicated concentrations. Bar = 1.5 cm (applies to all images). C, Lateral root number. D, Root length. E, Fresh weight. F, Dry weight. G, Representative pictures of 7-d-old Arabidopsis exposed to 6-PP at the indicated concentrations. Bar, 1.5 cm (applies to all images). H, Lateral root number. I, Root length. J, Fresh weight. K, Dry weight. Data for C to F and H to K show the mean ± SD of three technical replicates (15 plates with 5 plants each). Results were validated with an ANOVA statistical analysis using a Tukey multiple comparison test (α = 0.05). Lower case letters a, b, and c (C to F and H to K) represent means statistically different at the 0.05 level.
Figure 8. In Arabidopsis seedlings, 2-heptanol, 3-octanol, and 2-octanone VOCs promote plant growth. Seven-day-old Arabidopsis seedlings, grown on split-plates containing MS on one side, were exposed for 7 d to the indicated VOC by our placing an impregnated cotton swab at the opposite side of the split-dish. A, Representative pictures of Arabidopsis exposed to increasing concentrations of the indicated alcohols and furan. Bar = 1.5 cm (applies to all images). B, Representative pictures of Arabidopsis exposed to increasing concentrations of the indicated ketones. Bar = 1.5 cm (applies to all images). C, Dry weight of seedlings exposed to the indicated alcohols and furan. D, Dry weight of seedlings exposed to the indicated ketones. Data from C and D show the mean ± SD of three technical replicates (15 plates with 5 plants each). Results were validated with an ANOVA statistical analysis using a Tukey multiple comparison test (α = 0.05). Different letters represent means statistically different at the 0.05 level. Asterisks indicate significant difference (independent Student’s t-test, *P < 0.05), between control H2O versus VOC treatment.
promotion; but this process could play an important role in ISR and SAR induction, pointing to a more relevant role of *T. atroviride* VOCs in the promotion of plant growth and biomass gain.

In this respect, it has been reported that Arabidopsis root colonization by *T. atroviride* promotes plant growth associated with short root length and lateral root growth (Salas-Marina et al., 2011; Hermosa et al., 2013). Contreras-Cornejo et al. (2009) proposed that auxin-like molecules from *Trichoderma* promote plant growth and lateral root branching. They detected transcriptional activity of the DR5::GUS auxin reporter in Arabidopsis after 5 d of fungus-plant interaction in both primary and lateral roots. However, contradictory reports show that *Trichoderma* represses the auxinic primary root tip responses at 5 d of cocultivation (Nieto-Jacobo et al., 2017; Pelagio-Flores et al., 2017). Nieto-Jacobo et al. (2017) attributed such phenotype to impaired auxin signaling, whereas Pelagio-Flores et al. (2017) proposed that acidification of the medium by *Trichoderma* leads to the loss of root meristem functionality (after 72–96 h of interaction). Interestingly, they also showed that the beneficial effects provided by *T. atroviride* took place during the first 60 h. Contrastingly, we inoculated Arabidopsis seedlings at the root tips with the wild type and Δhda-2, and observed the inhibition of primary roots and the emergence of lateral roots and branching, but not the negative effects reported by Pelagio-Flores et al. (2017). Additionally, the medium amended with 50% and 25% of free-mycelium culture filtrates of *Trichoderma* showed an enhanced inhibition of plant growth, whereas 12.5% and 6.25% showed enhanced...
biomass gain in pepper plants (Olmedo-Monfil and Casas-Flores, 2014). These results support, at least in part, the proposal of Pelagio-Flores et al. (2017), because addition of water to an acidic solution renders it less acidic and raises the pH; however, plant growth and biomass gain have been observed using low concentrations of mycelium-free culture filtrates (Olmedo-Monfil and Casas-Flores, 2014), indicating that soluble compounds could be acting also as plant growth regulators or as molecules that modify hormone homoeostasis provoking such phenotypes.

Based on our results using Δhda-2 VOCs, we attribute the overstimulation of lateral root emergence mainly to the overproduction of 6-PP; however, we do not discard that other VOCs could be involved. In this respect, the wild-type and Δhda-2 VOCs were more effective in stimulating the emergence of lateral roots, compared with that of the different concentrations of 6-PP applied as VOC or in the growing medium. The wild type produced from 5.3- to 7.7-fold compared with that of the highest concentration of 6-PP used (100 μM or 16.62 μg mL⁻¹), whereas Δhda-2 synthesized from 50.9- to 58.3-fold. Recent studies show that 6-PP regulates the root architecture, formation of lateral roots, plant growth, and inhibition of the primary root (Kottb et al., 2015; Garnica-Vergara et al., 2016. Our results support such works and add further knowledge, because we showed that the effect of 6-PP is dependent on both the age of the plant and how it is applied. We propose that 6-PP applied as VOC could be acting as an effector molecule, which modulates plant phytohormones, such as auxins and ET, to promote a beneficial relationship with Trichoderma; whereas 6-PP applied in the culture medium exerts a role as a stressor. In this regard, stresses such as phosphorus limitation, exposure to heavy metals, irradiation with UV light, mechanical stress (Potters et al., 2007), and medium salt stress (Zolla et al., 2010) reduce primary root length and induce lateral roots formation.

Auxins and ET coordinately regulate several developmental programs in plants. These phytohormones regulate apical hook formation (Lehman et al., 1996; Raz and Ecker, 1999), root hair differentiation (Masucci and Schiefelbein, 1994), root hair elongation (Pitts et al., 1998), root growth (Rahman et al., 2001), and hypocotyl phototropism (Harper et al., 2000). On the other hand, changes in the root architecture have been attributed to diffusible Trichoderma compounds such as ET, indole-3-acetic acid, and indole-3-acetaldehyde (Gravel et al., 2007; Contreras-Cornejo et al., 2009; Hoyos-Carvajal et al., 2009; Salas-Marina et al., 2011; Nieto-Jacobo et al., 2017). In this work, most of the auxin pathway-related genes (TIR1, AUX1, PIN3, and PIN7), whose products participate in the perception, efflux, influx, and homeostasis of auxins in Arabidopsis, were up-regulated in the presence of the wild-type or Δhda-2 strains, or following exposure to their VOCs (albeit to a different extent), pointing to a positive regulation of this pathway in Arabidopsis at the beginning of the interaction with the fungus. Although loss of HDA-2 induced the auxin-related genes only to a minor extent, this result indicates that such increased transcription is enough to promote root branching. Another possible explanation is that 6-PP produced by Δhda-2 and/or its VOCs are promoting this phenotype in the plant by modulating a different pathway.

In this study, the ET response and biosynthesis-related genes (ACO2, ETR1, ERS1, and EIN2) were

Figure 10. A lack of HDA-2 leads to a misregulation of histone H3 acetylation of plant-responsive genes in T. atroviride. A to C, The cross-linked chromatin of the wild type (WT) or Δhda-2 after 96 h of coculture with 10-d-old Arabidopsis seedlings was immunoprecipitated with antibodies against anti-H3, and against anti-H3ac (acetylated-histone H3Lys9Lys14Lys18Lys23Lys27). Fungi grown in absence of the plant were used as controls. Specific primers were designed on conserved regions of epl-1 (A), ctf-1 (B), and abc-2 (C) promoters to quantify input DNA and immunoprecipitated chromatin by qPCR. Enrichment was calculated as a percentage of DNA control input. The experiment was repeated twice with similar results.
upregulated by the wild-type and Δhda-2 VOCs and during direct contact; however, Δhda-2 caused this response to a minor extent. The ET receptor-encoding genes ERS1 and ETR1 were upregulated by the wild type and Δhda-2 through their VOCs and by direct-contact, together with the up-regulation of ACO2, whose products are involved in the synthesis of ET. This likely promotes the dephosphorylation and cleavage of the EIN2 C terminus and translocation to the nucleus to exert positive regulation on EIN3, leading to the ET responses, including a reduction in primary root growth and emergence of lateral roots. This situation points to a key role of ET in primary root inhibition in Arabidopsis seedlings and to a different modulation of this pathway by Δhda-2. In agreement with our results, whereby ISR and ET-related genes were coexpressed with EBP (ET-responsive element binding protein; Supplemental Fig. S4) in plants inoculated with T. atroviride, or in the presence of the wild-type VOCs, it has been reported that ACC, MeJA, or infection with B. cinerea induced the expression of EBP, which correlates with the induction of PDF1.2 (Li et al., 2008). Taken together, these results suggest that T. atroviride modulates the auxin–ET pathways in Arabidopsis to promote root branching. In this regard, it has been demonstrated that ET–auxin interactions regulate lateral root initiation, emergence, and elongation in Arabidopsis (Ivanchenko et al., 2008). Moreover, it was described that, at low doses, ET promotes auxin biosynthesis leading to lateral root initiation (Ivanchenko et al., 2008). Furthermore, indole-3-acetic acid and ET synthesized by Tuber spp. act additively on plant roots, provoking root shortening, increased branching, and root hair elongation (Splivallo et al., 2009). 

Trichoderma spp. are major producers of numerous bioactive SMs, many of which are part of large, biosynthetic gene clusters tightly regulated by chromatin modifications (Schmoll et al., 2016). In this study, we report that the absence of the histone deacetylase HDA-2 in a beneficial microorganism resulted in an altered production of VOCs. Our results suggest both a negative and a positive role for HDA-2 on VOCs metabolism in T. atroviride. In this regard, deletion of genes encoding for HDACs class II in Aspergillus resulted in overproduction of several SMs, which correlated with increased gene expression of secondary metabolism-related genes (Shwab et al., 2007). Here, we demonstrated that the production and amount of the VOCs emitted by the wild type and Δhda-2 are dependent on the fungal age and the composition of the culture medium. Sixteen compounds were produced only by Δhda-2 in PDA, whereas 6-PP was overproduced. Furthermore, the expression of genes involved in the synthesis of SM was upregulated. These results suggest that HDA-2 is a key global positive and negative regulator of the synthesis of VOCs. In our results, we report 2-heptanol, 3-octanol, and 2-heptanone as plant growth promoters; whereas 3-octanol, 1-octen-3-ol, 2-pentylfuran, 3-octanone, 2-nonane, and 2-undecanone showed a phytotoxic effect at higher concentrations. In 2016, the VOCs emitted by 20 Trichoderma strains were identified and the strains classified based on their impact on plant growth as promoters, neutrals, or detrimental (Lee et al., 2016). Identified in promoting and neutral strains were 3-octanol, 3-octanone, and 2-pentylfuran, whereas 2-heptanone, 2-octanone, 2-nonanone, and 2-undecanone were identified in promoting, neutral, and negative strains; however, none of the VOCs tested in this work were detrimental to Arabidopsis growth at concentrations from 10 to 100 μg mL⁻¹, suggesting that the dose applied in the plant environment will determine the outcome of the plant. Furthermore, all the C8 compounds and furan tested in this work were phytotoxic at 10,000 μg mL⁻¹. The phytotoxic effects of 1-octen-3-ol, 3-octanol, and 3-octanone were demonstrated by Splivallo et al. (2007), who also reported that the phytotoxic effect of 1-octen-3-ol was due to an increased ROS-scavenging enzyme activity and/or increased H₂O₂ concentrations.

CONCLUSION

Our results revealed that histone deacetylase HDA-2 from T. atroviride is necessary to effectively colonize and promote plant growth in Arabidopsis, as well as to induce the systemic disease resistance against foliar pathogens. Moreover, Δhda-2 VOCs enhanced the growth of Arabidopsis seedlings and did not trigger the SAR and ISR responses in the plant. The absence of hda-2 impaired VOCs metabolism, resulting in an overproduction of 6-PP. In vitro assays using medium amended with 6-PP or 6-PP supplied as VOC demonstrated that the plant growth-promoting effect is dependent on the Arabidopsis age and the application protocol. Split-plate assays with nine different VOCs revealed that 2-heptanol, 2-heptanone, and 3-octanol promoted plant growth in Arabidopsis seedlings. Our expression analysis led to the conclusion that the expression of auxin- and ET-related genes is differentially modulated in Arabidopsis by Δhda-2 direct contact or by exposure to its VOCs, which may explain the different phenotype of the wild-type–treated and Δhda-2-treated plants. Our work highlights the importance of HDA-2 as global regulator of multiple processes in T. atroviride and highlights the relevance of maintaining a histone acetylation balance to properly respond to the presence of Arabidopsis seedlings.

MATERIALS AND METHODS

Organisms and Growth Conditions

A Trichoderma–plant interaction was fostered on a 1× MS medium (Murashige and Skoog, 1962; PhytoTechnology Laboratories). Arabidopsis (Arabidopsis thaliana) ecotype Col-0 was used in this study. Arabidopsis seeds were sterilized by soaking in 75% ethanol for 3 min, treated with 10% bleach (HOCl) in water for 7 min, and rinsed three times with sterile, distilled water. Seeds were stratified for 2 d at 4°C, germinated on MS agar plates, and grown under a 16-h/8-h light/dark photoperiod at 22°C ±1°C, 65% relative humidity, and 150 μM m⁻² s⁻¹ light.

Downloaded on May 31, 2021. - Published by https://plantphysiol.org
Copyright (c) 2020 American Society of Plant Biologists. All rights reserved.
Trichoderma–plant interaction in pots: Arabidopsis seeds were sown in pots containing peat moss (Lambert peat moss) as a substrate and stratified for 2 d at 4°C. One-day-old seedlings were transplanted into pots containing sterile peat moss and grown as just described.

The *Trichoderma atroviride* IMI 206040 wild type, its isogenic Δhda-2 mutant (Osorio-Concepción et al., 2017), and *Botrytis cinerea* B05.10 (Amsellem et al., 2011) were used throughout this study. All fungal strains were routinely grown at 25°C on potato dextrose agar (PDA; DIFCO), under a 12-h/12-h light/dark regime, unless otherwise specified. The bacterium *Pseudomonas syringae pv. Tomato*, strain DC3000 (Cuppels, 1986) was grown at 28°C in Kings B medium, supplemented with rifampicin 50 μg/mL (King et al., 1954).

Effect of the *T. atroviride* Wild Type and Δhda-2 on Plant Growth Promotion and Root Colonization in Soil

Fifteen 10-d-old Arabidopsis plants were root inoculated with mycelium of the wild type or Δhda-2, as described in the ‘Organisms and Growth Conditions’ section. At three weeks postinoculation, fresh and dry weights were determined. For root colonization, the roots were detached from at least 10 plants and rinsed with sterile distilled water. Total DNA was extracted according to (Dellaporta et al., 1983) and subjected to relative quantification (qPCR) of the *Trichoderma inf-1* gene (Supplemental Table S1), which codes for the translation elongation factor, and the *Arabidopsis ACT2* gene, which codes for the actin protein 2 (Supplemental Table S1).

Effect of the Wild Type and Δhda-2 on Plant Biomass and Root System Architecture In Vitro

Arabidopsis seeds were germinated and placed on petri dishes containing 1× MS. Eleven days thereafter, the seedlings were inoculated with mycelial plugs of the wild type or Δhda-2, sealed with plastic film, and cocultured for 10 d under a 16-h/8-h light/dark photoperiod at 22°C ±1°C. Control plates were inoculated with a PDA plug without the fungi. The length of primary roots and the number of lateral roots were determined. Fresh and dry weights were also determined on an analytical scale.

*Botrytis cinerea* Infection Assay

Taking into account that Δhda-2 does not undergo conidiation, flask containing 100 mL of potato dextrose broth (PDB; DIFCO) were inoculated with three mycelial plugs of the wild type or Δhda-2, and grown at 25°C, 200 rpm, in the dark for 72 h. Mycelia of the wild type or Δhda-2 were vacuum-harvested onto 0.2 μm membrane filters (Whatman). The collected mycelium was cut with a 0.6 mm diameter cork borer and used to inoculate the roots of 10-d-old Arabidopsis seedlings, and allowed to interact for two weeks. Three Arabidopsis leaves per plant were inoculated with 10 μL of 5×10⁶ conidia/mL of *B. cinerea* diluted in inoculation buffer (per 40 mL of stock solution: 1.37 g Suc, 400 μL of 1 M KH₂PO₄, 80 μL of 12.5% Tween 20). Lesioned areas of infected leaves were quantitatively measured at 3 and 6 d postinoculation (dpi) using ImageJ software (http://rsb.info.nih.gov/ij/index.html).

*Pseudomonas syringae* Infection Assay

Two-week-old Arabidopsis seedlings were grown and inoculated with mycelium of the wild type or Δhda-2 as described in the ‘Organisms and Growth Conditions’ section. Thereafter, three leaves per plant were infiltrated with *Pst DC3000* in 10 mM MgCl₂ (OD₅₀₀ = 0.0004) using a needleless syringe. Twelve leaves of control and treated plants were collected at 0 and 3 dpi, and ground in 10 mM MgCl₂. Samples were serial-diluted and plated onto a King’s B medium containing the appropriate antibiotics to determine the CFU.

Influence of the Wild Type and Δhda-2 VOCs on Plant Biomass and Root System Architecture In Vitro

Exposure of Arabidopsis plants to the wild-type and Δhda-2 VOCs was achieved using a double plate-within-a-plate system (Olmedo-Monfil and Casas-Flores, 2014). Small petri dishes containing 1× MS (35 × 10 mm) were embedded into large petri dishes containing 1× MS as well (100 × 15 mm). Ten-day-old Arabidopsis seedlings were grown onto large petri dishes. Ten days thereafter, the seedlings were inoculated with a mycelial plug of the wild type or Δhda-2 onto the small petri dishes, sealed with plastic film, and grown as described in the ‘Organisms and Growth Conditions’ section. Control plants were inoculated with an MS plug. Root length, number of lateral roots, fresh and dry weights were determined as described in the ‘Effect of the Wild Type and Δhda-2 on Plant Biomass and Root System Architecture In Vitro’ section.

Expression Analysis of Auxin, ET, and Defense-Related Genes in Arabidopsis Inoculated with the Wild Type or Δhda-2 or Exposed to their VOCs

Plants were germinated and grown for 9 d on petri dishes containing 1× MS. For direct contact, the root tips were inoculated with disks of fresh mycelium of the wild type or Δhda-2, whereas for exposure to VOCs, the mycelial disk was inoculated into the opposite compartment of Arabidopsis seedlings grown in split-petri dishes. Plants were harvested at 0, 24, 48, 72, and 96 h (hours postinoculation), frozen in liquid nitrogen, and stored at −80°C until total RNA extraction. Plants growing without the fungi were used as controls. Total RNA extraction, cDNA synthesis, and RT-qPCR were performed as described in the ‘Analysis of Gene Expression by Quantitative Reverse Transcription PCR (RT-qPCR)’ section.

Expression Analysis of *Trichoderma* Genes in Coclure with Arabidopsis Seedlings

Arabidopsis Col-0 seedlings were germinated and grown for 10 d on petri plates containing a MS medium. At day 10, the seedlings were root-inoculated with the wild type or Δhda-2, and the fungi-samples were collected at 72 and 96 hpi. The wild type and Δhda-2 growing in MS medium alone were included as controls. Total RNA extraction, cDNA synthesis, and RT-qPCR were performed as described in the ‘Analysis of Gene Expression by Quantitative Reverse Transcription PCR (RT-qPCR)’ section.

Effect of 6-PP on Plant Growth of 2- and 7-D-Old Plants

Two-day-old seedlings were placed on petri dishes containing MS amended with 25, 50, 75, and 100 μM 6-PP (Sigma Aldrich; Garmnia-Vergara et al., 2016); whereas 2- or 7-d-old seedlings’ exposure to 6-PP, as VOC, was performed according to Splivallo et al. (2007), with some modifications. Briefly, seedlings were placed in split-petri dishes containing MS in one side to sow the seedlings, and 300 μM 6-PP was added to a piece of sterile cotton at the opposite side at the same concentrations as above. Ethanol (Sigma Aldrich) was used as solvent at a final concentration of 1.3% in water (higher concentrations promote plant growth) and used as control as well. Petri plates containing five plants were closed with sealing film and incubated for 13 d under a 16-h/8-h light/dark photoperiod at 22°C ±1°C.

Identification of VOCs through GC-MS

For VOCs analysis, the *T. atroviride* wild type and Δhda-2 were grown on PDA plates at 25°C for 5 and 7 d, respectively. Noninoculated PDA plates were included as controls. Compounds were collected as described (Supplemental Fig. S2) for 1 h with a blue SPME fiber (PDMS/DVB; Supelco Inc.), and desorbed at 180°C for 30 s in the injector port of a gas chromatograph (Agilent 7890B; Agilent), equipped with a mass spectrometry detector (5977A; Agilent) and Mass Hunter Workstation Software (Agilent Technologies) for data collection.
accession and processing. In the operating conditions, helium was used as the carrier gas (1 mL min⁻¹) and the detector temperature was 250°C. The column was held for 1 min at 60°C, and then programmed to rise at a rate of 3°C min⁻¹ to a final temperature of 180°C.

Quantification of Fungal VOCs

The concentration of fungal VOCs (Table 2) contained in the cultures was calculated with their corresponding standards (all from Sigma-Aldrich), based on the calibration curves determined independently for each compound. The SPME vials were filled with 1 mL of the corresponding standard diluted in methanol. The compounds were adjusted to 10, 100, 1000, 5000, and 10,000 µg mL⁻¹ and analyzed under the same conditions as used for the quantification of the fungal VOCs mixtures.

Exposure of Arabidopsis to Individual VOCs

The effect of Trichoderma individual VOCs on plant growth promotion was assessed using a split-plate system. Arabidopsis plants were grown, sterilized, and stratified as described in the "Organisms and Growth Conditions" section. The different VOCs were supplied in sterile cottons as described in the "Effect of f-PP on Plant Growth of 2- and 7-D-Old Plants" section; 200 µL of 10, 100, 1000, and 10,000 µg mL⁻¹ of each selected VOC was applied to the cottons. Water was included as control because it was used as solvent for most VOCs (except 6-PP). Plates were closed with plastic film and incubated at 22°C under 12-h-light/12-h-dark cycles for 7 d. Fresh and dry weights were determined for each group on an analytical scale.

Chromatin Immunoprecipitation Assays

The Trichoderma–Arabidopsis interaction experiment was carried out as described in the "Expression Analysis of Trichoderma Genes in Coculture with Arabidopsis Seedlings" section. Mycelia from interaction with Arabidopsis and control in absence of the plant were fixed in 10 mL of 1× crosslinking buffer (10 × crosslinking buffer: 0.5 mL of 5 M NaCl, 25 µL of 0.5 M EDTA [pH 8], 50 µL of 0.5 M EDTA [pH 8], 1.25 mL of 1 M HEPES [pH 8], 7.45 mL of 37% formaldehyde, 15.75 mL water). Mycelia were incubated for 10 min at room temperature on a rocking platform and neutralized by adding 1.25 M Gly for five min at 4°C. Cross-linked chromatin was immunoprecipitated using 2 µL of anti-H3 histone antibody (Abcam; Ab1791), and 5 µL of antihistone-H3 acetyl K9K14K18K23K27 (Abcam; ab7915); 10% of the chromatin was used as input. Immunoprecipitated chromatin was analyzed by qPCR using specific primers (Supplemental Table S1) on the promoter region of Atldh-2, cif-1 and abc-2.

Data Analysis and Statistics

For all Arabidopsis experiments treated with the wild type and Δldh-2, the overall data were statistically analyzed using SPSS 10 software (IBM Corp.). Tukey’s post hoc test was used to assess the significance of differences in plant growth promotion and root system architecture between treatments. Different letters are used to indicate means that differ significantly (P < 0.05). Student’s t test was used to evaluate differences in the relative level of expression of genes in pathogenesis assays, as well as in the plant growth promotion by 10 different VOCs.

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers (Supplemental Table S1).

Supplemental Material

The following supplemental materials are available.

Supplemental Figure S1. The mRNA levels of ldh-2 were slightly increased in Trichoderma in the presence of Arabidopsis Col-0 seedlings.

Supplemental Figure S2. Trichoderma VOCs exposure system.

Supplemental Figure S3. Chromatin immunoprecipitation (ChIP) assay on the promoter regions of T. atroviride plant-responsive genes.

Supplemental Table S1. List of primers used in this study

Supplemental Figure S4. The expression of ERF was induced in Arabidopsis by direct contact with Δldh-2 or exposure to its VOCs.

ACKNOWLEDGMENTS

The authors wish to thank Nicolás Gómez-Hernández, Norma Angelica Ramírez Pérez, Mituzako Dautt Castro, Edith Elena Uresti-Rivera, and María Guadalupe Ortiga Salazar for their technical support. We also thank the National Biotechnology, Agricultural and Medical Laboratory for providing access to LANBAMA’s GC-MS.

Received September 10, 2018; revised December 10, 2018; accepted January 9, 2019; published January 22, 2019.

LITERATURE CITED


Copyright (c) 2020 American Society of Plant Biologists. All rights reserved.