INSTITUTO POTOSINO DE INVESTIGACIÓN CIENTÍFICA Y TECNOLÓGICA, A.C.

POSGRADO EN CIENCIAS EN BIOLOGIA MOLECULAR

La deacetilasa de histonas HDA-2 del hongo simbionte *Trichoderma atroviride* modula múltiples respuestas en *Arabidopsis thaliana*

Tesis que presenta

Magnolia Estrada Rivera

Para obtener el grado de

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Director de la Tesis:

Dr. J. Sergio Casas Flores

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Constancia de aprobación de la tesis

La tesis ***"La deacetilasa de histonas HDA-2 del hongo simbionte Trichoderma atroviride modula múltiples respuestas en Arabidopsis thaliana"*** presentada para obtener el Grado de Doctora en Ciencias en Biología Molecular fue elaborada por **Magnolia Estrada Rivera** y aprobada el veinticuatro de enero del dos mil diecinueve por los suscritos, designados por el Colegio de Profesores de la División de Biología Molecular del Instituto Potosino de Investigación Científica y Tecnológica, A.C.

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DOCTORA EN CIENCIAS EN BIOLOGÍA MOLECULAR

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Magnolia Estrada Rivera

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La deacetilasa de histonas HDA-2 del hongo simbionte Trichoderma atroviride modula múltiples respuestas en Arabidopsis thaliana

que se desarrolló bajo la dirección de

Dr. J. Sergio Casas Flores

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Dedicatorias

***Dedico este trabajo a Dios y a mi familia*:**

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Resumen

La deacetilasa de histonas HDA-2 del hongo simbionte *Trichoderma atroviride* modula múltiples respuestas en *Arabidopsis thaliana*

Trichoderma spp. es un gran productor de metabolitos secundarios y compuestos orgánicos volátiles (COVs), los cuales pueden activar la resistencia sistémica y promover el crecimiento en plantas. En los hongos filamentosos, las modificaciones de la cromatina juegan un papel relevante en la regulación del metabolismo secundario. En este trabajo investigamos el efecto de la deleción del gen *hda-2*, que codifica para una deacetilasa de histonas del hongo *Trichoderma atroviride* y su contribución en la promoción del crecimiento y la inducción de la resistencia sistémica contra patógenos. La producción de COVs por la cepa Δ*hda-2* y la función de estos en el desarrollo de *A. thaliana* también fue analizada. La ausencia de HDA-2 afectó la capacidad de la cepa para colonizar las raíces y como consecuencia su efecto en la promoción de crecimiento, así como de modular las respuestas de defensa contra los fitopatógenos *Botrytis cinerea* y *Pseudomonas syringae*. Los COVs de Δ*hda-2* fueron incapaces de inducir el sistema de defensa en la planta. La cepa Δ*hda-2* resultó ser una sobreproductora del volátil 6-pentil-2H-2-piranona (6-PP), el cual fomentó el desarrollo de raíces laterales y llevó a una regulación diferencial de genes relacionados a las fitohormonas en *A. thaliana.* Un análisis de diez COVs entre ellos el 6-PP, llevó al descubrimiento de tres compuestos que regulan el crecimiento positivamente, mientras que seis de ellos mostraron un efecto opuesto. Los genes relacionados con el metabolismo secundario, la desintoxicación y la comunicación con la planta fueron analizados y los resultados revelaron que HDA-2 posee un papel doble en la regulación génica de *T. atroviride* durante la interacción con la planta. Mediante experimentos de inmunoprecipitación de la cromatina en las regiones promotoras de los genes que responden a la planta *epl-1*y *abc-2*, presentaron bajos niveles de H3 acetilada, mientras que *ctf-1* presento niveles constitutivos en la cepa Δ*hda-2*. Esto sugiere una regulación indirecta de la HDA-2 en el metabolismo secundario, defensa y comunicación con la planta. Este trabajo destaca la importancia de las modificaciones de la cromatina en *Trichoderma* y su modulación en múltiples respuestas en Arabidopsis.

PALABRAS CLAVE: Deacetilasa de histonas HDA-2, Promoción de crecimiento, Resistencia Sistémica, *Trichoderma atroviride,* Compuestos Orgánicos Volátiles (COVs), 6-pentil-2H-2-piranona (6-PP)

Abstract

Histone deacetylase HDA-2 is essential in *Trichoderma* to modulate multiple responses in Arabidopsis

Trichoderma spp. are a rich source of secondary metabolites (SM) 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 investigate the impact of the absence of the histone deacetylase HDA-2 in *Trichoderma atroviride* to modulate plant growth, and plant defenses against foliar pathogens. VOCs production and their impact on *Arabidopsis thaliana* growth and development were assessed as well. The absence of HDA-2 impaired *Trichoderma* in its capability to colonize Arabidopsis roots, therefore, to promote growth and modulate plant defenses against foliar pathogens *Botrytis cinerea* and *Pseudomonas syringae*. Furthermore, Δ*hda-2* VOCs were incapable of triggering plant defenses to counterattack such pathogens. Δ*hda-2* overproduced the VOC 6-pentyl-2H-pyran-2-one (6-PP), which showed an enhanced capability to promote root branching, and differentially regulated phytohormones related-genes. Analysis of ten VOCs (including 6-PP) led to discover of three positive plant-growth regulators, whereas six of them provoked the opposite effect. Assessment of SM, detoxification, and communication with the plant-related genes showed a dual role for HDA-2 in gene regulation in *T. atroviride* during its interaction with plants. Chromatin immunoprecipitations of acetylated histone H3 on the promoters of plant responsive genes in Δ*hda-2*, in the presence of Arabidopsis, showed low levels of acetylated H3 of *epl-1* and *abc-2* compared with the WT, whereas *ctf-1* presented high constitutive levels, supporting the 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.

KEY WORDS: Histone Deacetylase HDA-2, Plant Growth Promotion, Systemic Resistance, *Trichoderma atroviride*, Volatile Organic Compounds (VOCs), 6-pentyl-α-pyrone, 6-PP

Introduction

The nomenclature of *Trichoderma* spp. exists in two morphologically and physiologically different stages: sexual (teleomorphic) known by the generic name *Hypocrea*, whereas the asexual (anamorphic or mitosporic) is called *Trichoderma* (Webster and Rifai 1968; Papavizas 1985; Srivastava et al. 2014). Here we will refer to the genus collectively as *Trichoderma*. *Trichoderma* isolates can be found inhabiting a variety of substrates such as: Mediterranean sponges *Psammocinia* (Irciniidae) (Gal-hemed et al. 2011; Paz et al. 2010), agricultural fields (Hagn et al. 2003; Kredics et al. 2012; Hernández-Mendoza et al. 2011), living plants (endophytes of cacao plants (Samuels et al. 2006; Hanada et al. 2008) and *Taxus mairei* (Zhang et al. 2007)), mushrooms (Pukahuta et al. 2000; Hatvani et al. 2007; Wang et al. 2011) and even from liver transplants (Ranque et al. 2008), human lung tissues (Druzhinina et al. 2007), blood serum, skin lesions, sputum, and throat of pediatric patients (Kantarcioğlu et al. 2009),

Trichoderma diversity of lifestyles is versatile and refers to two major nutritional modes: saprotrophy and biotrophy which are commonly not in disagreement for many of *Trichoderma* species (Atanasova 2014), however *Trichoderma* spp. lifestyle has also been defined as mycotrophic to include bio- and sapro- trophic nutritional strategies (Druzhinina et al. 2011). *Trichoderma* spp. mycoparasitism-life style can be dated to at least 400 million years ago by fossil evidence (Taylor et al. 2017), this view is supported by a recent survey of >1,100 *Trichoderma* strains from 75 molecularly defined species, which showed that all the species tested possess mycoparasitic potential against three causative agents of plant diseases: *Alternaria alternata*, *Botrytis cinerea* and *Sclerotinia sclerotiorum* (Druzhinina et al. 2011). Necrotrophic mycoparasites are highly aggressive and destructive, killing their host cells slightly before or after invasion and absorbing nutrients from dying or dead host cells. Details of the mycoparasitism mode are complex and include chemotrophic growth towards its host, host recognition, coiling around the host, penetration and lysis of the host (Binder 1973; Manocha, M. S. and Sahai 1993; Herrera-estrella and Chet 2004). *Trichoderma* antagonistic activities against phytopathogens also include: competition, parasitism, production of volatile or nonvolatile antibiotics, and induction of plant defense responses (Harman 2000). *Trichoderma* spp. can also effectively antagonize the nematodes *Heterodera*

cajani (Siddiqui and Mahmood 1996), and *Meloidogyne javanica* (Spiegel, Yitzhak and Chet 1998; Sharon et al. 2008).

Root colonization by *Trichoderma* influence root development, enhancing secondary roots proliferation and growth promotion. Plant-growth stimulation has been observed in petunia, chrysanthemums (Chang et al. 1986), bean, radish, tomato, pepper, cucumber (Kleifeld 1992), lettuce (Lynch et al. 1991), and the plant model *A. thaliana* (Salas-Marina et al. 2011), however not all *Trichoderma* species are capable to promote plant growth. In this respect, plant-growth promotion assays tested the ability of several *Trichoderma* isolates, *T. longipile* 65r4 and 3Sr4-2 and *T. tomentosum* 5Sr2-2 increased leaf area (58-71%), shoot dry weight (91-102%), and root dry weight (100- 158%), however *T. virens* B19 and G24 were incapable to promote plant-growth in cabbage plants (Rabeendran et al. 2000). It must also be remembered that some *Trichoderma* species have been reported as pathogens of maize seeds, in this analysis *Trichoderma* was isolated from pre-treated maize seeds with captan, etridiazole, thiram or combinations of these fungicides (Falloon 1982). *Trichoderma* plant-growth promotion is highly variable, studies have compared more than 500 documented field trials from seed treated and untreated with *T. harzianum* T22, average yield increases was about 5 bu/acre. However, there were yield decreases through to +50% increases, these results showed a tremendous variability in plant-growth promotion by *Trichoderma*. The greatest variability in yield increases where observed when the test variety had some genetic weakness or where there were biotic (disease) or abiotic (soil compaction, drought, nutrient) constraints (Harman 2006). Thus, inconsistency could be due to several limiting factors including sites/seasons, crop type, growing conditions, inoculum rate/concentration and/ or formulation type (Stewart and Hill 2014). For years, research groups have attributed the plant-growth promotion activity of *Trichoderma* to the following mechanisms: a) Modulation of synthesis of phytohormones by the plant: application of *T. harzianum* T-22 in *Prunus cerasus* × *P. canescens* significantly increased the levels of indole-3-acetic acid (IAA), and gibberellic acid (GA3) in both leaves and roots, this work proved that the change in plant-phytohormones levels is one of the direct mechanisms to promote rooting and shoot growth by T22 (Sofo et al. 2011); b) Synthesis of phytohormones by the microbe, numerous studies have reported that *T. atroviride* produces IAA and even investigators have been able to stimulate its production after the addition of L-tryptophan, tryptamine and tryptophol (Gravel et al. 2007; Salas-Marina et al. 2011). IAA production is not unique of *T. atroviride,* as a

further analysis of 101 *Trichoderma* isolates from Colombia were evaluated, and sixty percent of these isolates produced IAA or auxin analogues, however the ability to produce these metabolites was not directly correlated with the plant-growth promotion of *Trichoderma* (Hoyos-carvajal et al. 2009). It seems that IAA production is strain dependent and diverse stimuli are associated with its production (Nieto-Jacobo et al. 2017); c) increased uptake and translocation of nutrients; in 2001, experiments carried out under axenic hydroponic systems demonstrated a significant increase in the concentration of Cu, P. Fe, Zn, Mn in root-inoculated cucumber with *T. harzianum* (Yedidia et al. 2001); d) enhanced solubilization of soil nutrients: *T. harzianum* T-22 was the first reported strain capable to solubilize insoluble or sparingly soluble minerals by means of chelation an reduction (Altomare et al. 1999); e) photosynthesis: *Trichoderma* can also improve photosynthetic efficiency, especially in plants subjected to various stresses; endophytic *T. hamatum* DIS 219 delayed drought-induced changes in stomatal conductance, net photosynthesis, and green fluorescence emissions in Cacao plants (Bae et al. 2009). Furthermore, proteomic approaches in maize plants root-inoculated with *T. harzianum* T22 have identified a large portion of up-regulated proteins involved in carbohydrate metabolism, photosynthesis or stress related mechanisms (Shoresh and Harman 2008). Maize roots inoculated with *T. virens*, showed an increase in the photosynthetic rate in leaves. In agreement with this, the steady-state levels of mRNA for Rubisco small subunit and the oxygen-evolving enhancer 3-1 was accumulated after roots inoculation with *T. virens* (Vargas et al. 2009).

The genome size of *T. atroviride, T. virens* and *T. reesei* is 36.1, 38.8, and 34.1 Mbp, and contain around 11,865, 12,518 and 9,143 predicted genes, respectively (Kubicek et al. 2011). The eukaryotic DNA, is several meters long, and must be packaged into a highly organized structured termed chromatin. (Kornberg and Thomas 1973; Kornberg and Lorch 1999). Chromatin consists of eight core histones (two each of H2A, H2B, H3 and H4), which are responsible for folding 146 base pairs of DNA into nucleosomes, and are highly conserved throughout evolution (Baxevanis and Landsman 1996). In contrast, to their counterparts H1 and H5 linker histones which are conserved to a much lower extent (Baxevanis and Landsman 1996). A flexible and dynamic chromatin is essential for cells to respond and adapt to physiological and environmental changes. *T. reesei*, *T. atroviride*, and *T. virens* have 22 to 28 predicted proteins related to the SWI/SNF ATPase/helicase domain (Schmoll et al. 2016). Interestingly, most of them belong to the "Rad 5/16-like" group (Schmoll et al. 2016).

Helicases are nucleic acid dependent ATPases that are capable of unwinding DNA or RNA duplex substrates (Singleton et al. 2007). The helicases are classified into six superfamilies: SF1, SF2, SF3, SF4, SF5, and SF6 (Corbalenya and Koonin 1993; Singleton et al. 2007). The DEAD-box and Snf2-like enzymes families belong to the helicase superfamily II (SF2) one of the largest superfamilies, the SF2 proteins contain seven conserved motifs distributed along sequence regions ranging in length from ~200 to ~700 amino acid residues (Corbalenya and Koonin 1993).

In *S. cerevisiae* the *rad16* deletion mutants are UV sensitive, however results indicate that *RAD16* encodes a non-essential function and is not absolutely required for excision repair (Schild et al. 1992). Rad5 protein of *S. cerevisiae* plays a key role in the protection of protruding single-strand ends and for the avoidance of non-homologous end joining during repair of double-strand gaps in budding yeast (Ahne et al. 1997). In humans the SWI/SNF family of genes, has been implicated in cancer, the helicase-like transcription factor gene (*HLTF),* a member of the SWI/SNF protein family, was specifically silenced by hypermethylation in 43% of colon cancers, thus *HLTF* functions as a common target for methylation and epigenetic gene silencing in colon cancer (Moinova et al. 2002). In addition, homozygous mutations at highly conserved positions of *RAD54B,* a member of the SNF2 family, were observed in human primary lymphoma and colon cancer, these findings suggest that some cancers arise through alterations of the *RAD54B* function (Hiramoto et al. 1999). In plants, scarce information is available concerning the function of these Rad5/16-like group (Knizewski et al. 2008). In 2018, *AtRAD5a* and *AtRAD5b* two homologs in the *A. thaliana* genome, sharing 30% identity and 45% similarity to yeast Rad5 were discovered (Chen et al. 2008). Interestingly *Atrad5a* mutants were hypersensitive to the cross-linking agents mitomycin C and cis-platin and to a lesser extent to the methylating agent, methyl methane sulfonate, whereas the *Atrad5b* mutants did not exhibit any sensitivity to all DNA-damaging agents (Chen et al. 2008). These results suggest that the RAD5-dependent error-free branch of postreplication repair is conserved between yeast an plants (Chen et al. 2008).

Histone tails can be modified and are the site of interaction for diverse classes of enzymatic machineries capable of covalently modify the tails through acetylation, phosphorylation, sumoylation, ubiquitination, and methylation (Shilatifard 2006). Acetylation is a reversible process that depends on two antagonizing enzymes: histone acetyltransferases (HATs) and histone deacetylases (HDACs) (Brosch et al. 2008). HATs catalyze the transfer of acetyl

groups from acetyl coenzyme A (acetyl-CoA) to lysine residues ,which leads to a more open and permissive chromatin and therefore to a greater transcription (Workman and Kingston 1998; Roth et al. 2001). HDACs remove acetyl groups of lysine residues from histone amino tails, and in general are associated with hypoacetylation and therefore transcriptional repression (Vaquero et al. 2003). HDACs have been divided into three different classes: I, II and III. The first two classes are sensitive to the HDAC inhibitor TSA (trichostatin A), and are named as classic HDACs (Yoshida et al. 1990), whereas the third class are insensitive, and their activity requires the coenzyme NAD^+ (nicotinamide adenine dinucleotide) as cofactor, and are named as Sirtuins (Moazed 2001). The genomes of *T. reesei, T. atroviride*, and *T. virens* contain four classical HDACs (Schmoll et al. 2016). In recent years, the importance of the physical modification of the chromatin, has become evident in the research field of plant-pathogen interactions (Ramirez-Prado et al. 2018). In 1995 and 1997 was reported that the HC-toxin (host selective toxin) produced by *Cochliobolus carbonum* a maize pathogen inhibited the histone deacetylases HD1-A, HD1-B, and HD2 of maize (Brosch et al. 1995; Ransom and Walton 1997). Similarly, depudecin an eleven-carbon linear polyketide produced by the pathogenic fungus *Alternaria brassicicola* was found to inhibit HDACs as well (Kwon et al. 1998; Wight et al. 2009). Besides of inhibiting HDAC compounds, research has also analyzed the role of chromatin modifications in the regulation of infection-related genes. In *Fusarium graminearum* and *Magnaporthe oryzae*, wheat and rice phytopathogens respectively, the deletion of a transducin β-like gene (*FTL1* and Tig1, respectively), homologous to yeast *SIF2* component of the Set3 HDAC complex, caused a diminished virulence and conidiation in both phytopathogens (Ding et al. 2009, 2010). Later studies showed that *hdf1* deletion in *F. graminearum*, a major class II HDAC gene, resulted in a reduced virulence and conidiation in the fungus, *HDF1* is also homologous of the yeast Set3 complex, which physically interacts with the *FTL1* protein (Li et al. 2011). Furthermore, accumulating evidence has convincingly demonstrated the role of HDACs in secondary metabolism regulation. In *Aspergillus nidulans* the deletion of histone deacetylase *hdaA*, caused transcriptional activation of two telomere-proximal gene clusters-and subsequent increased levels of the corresponding molecules (toxins and antibiotics) (Shwab et al. 2007). In *Fusarium fujikuroi*, deletion of -*ffhda1*, *ffhda2*, Zn^{2+}-dependent HDACs-encoding genes impaired the production of secondary metabolites, and both genes are required for gibberellic acid (GA)-induced bakanae disease on plant rice (Studt et al. 2013).

Trichoderma spp. are well-known producers of Volatile Organic Compounds (VOCs), VOCs comprise a very diverse chemically group of compounds with a high vapor pressure under ambient conditions and a molecular weight in the range of 50-200 Daltons (Rowan 2011). VOCs are formed during primary and secondary metabolism and can serve as signaling molecules (semiochemicals) passing information within and between organisms (Korpi et al. 2009; Rowan 2011). Approximately, 479 *Trichoderma* VOCs have been identified and classified as: hydrocarbons, heterocycles, aldehydes, ketones, alcohols, phenols, thioalcohols, thioesters and their derivatives, including, among others, benzene derivatives, and cyclohexanes (Siddiquee 2014). *Trichoderma* VOCs functions remain to be explored, however, some of them have proven to be plant growth promoting or inhibition molecules for plant pathogens. Since 1956, Bilai has reported the presence of volatile metabolites possessing antifungal and antibacterial properties (Bilai, 1956). In 1997, the VOCs inhibitory effect of two *Trichoderma* isolates against four common wood decay fungi was reported (Wheatley et al. 1997). Later work, also tested the antibiotic activity of *Trichoderma* VOCs against *Rhizoctonia solani, Fomes annosus, Fusarium oxysporum, Pyronema domesticum, Mucor hiemalis* and *Pythium ultimum*, the inhibitory effect was dependent of the isolates and phytopathogens tested (Dennis and Webster 1971). Interestingly all the active metabolites had the same effect in morphology of all susceptible tested fungi, resulting in stunted mycelial growth (Dennis and Webster 1971). More recent works in *T. gamsii* YIM PH30019, displayed antagonistic activities against the pathogenic fungi of the plant *Panax notoginseng* via production of VOCs, dimethyl disulfide, dibenzofuran, methanethiol. Furthermore ketones were produced by *T. gamsii* during cocultivation with the pathogenic fungus, and are probably the responsible VOCs for pathogen growth inhibition (Chen et al. 2016). In 2008, researchers isolated and evaluated the 6-n-pentyl-6H-pyran-2-one (6-PP), one of the major secondary metabolites produced by *T. harzianum* (T22, T39 and A6) and its effect in wheat-growth promotion. 6-PP wheat seedlings treated showed a diminished dry weight, however tomato plants treated with 6-PP showed an increase in plant height and leaf area, suggesting a 6-PP role in plant growth promotion and eventually an auxin-like activity (Vinale et al. 2008). In 2013, researchers reported *A. thaliana* as a model plant to test *Trichoderma* VOCs effect, they also compared Arabidopsis development with or without *T. viride* physical contact, demonstrating that plants cocultured with *T. viride* VOCs were taller, bigger, flowered earlier, and had more lateral roots (Hung et al. 2013). To date, VOCs effect in plant

growth promotion has also been reported for *T. virens* (Contreras-Cornejo et al. 2014), *T. atroviride, T.* sp. "*atroviride B", T. virens* and *T. asperellum* (Nieto-Jacobo et al. 2017). Detailed analysis of 6-PP effect in Arabidopsis root morphogenesis showed that 6-PP promotes and regulates root architecture, inhibiting primary root growth and inducing lateral root formation (Garnica-Vergara et al. 2015). 6-PP modulates expression of *PIN* auxin-transport proteins in a dose-dependent manner (Garnica-Vergara et al. 2015). Besides VOCs and their effects on the plant-growth promotion, studies have revealed that *Trichoderma* VOCs can successfully turn-on plant immunity responses. VOCs from *T. virens* caused an induction of *pLox2:uidA* a JA responsive-marker in Arabidopsis, which was accompanied with an increase in JA accumulation (Contreras-Cornejo et al. 2014). Other groups have observed a salicylic acid (SA) and abscisic acid (ABA) phytohormone accumulation in *A. thaliana* exposed to *T. asperellum* volatiles, whereas jasmonic acid (JA), phytohormone levels remained unchanged (Kottb et al. 2015). Plants not only respond to VOCs exposure but also to *Trichoderma* physical contact, which triggers a defense response in plants. During root colonization, *T. harzianum* grows in the epidermis and outer cortex, mainly through intercellular space of Cucumber roots (Yedidia et al. 1999). Root-colonization by *Trichoderma* is coupled to epidermal and cortical cell strengthening (Yedidia et al. 1999). The molecular mechanisms that govern recognition and association between *Trichoderma* and its host are still unknown, however sucrose has been defined as a key element in the symbiotic association between *Trichoderma* and plants (Koch 2004; Vargas et al. 2009). In 2009, reverse genetics experiments demonstrated that an intracellular invertase from *T. virens* (TvInv) was crucial for symbiotic association and fungal growth in the presence of sucrose (Vargas et al. 2009). In 2012, a plant-like sucrose transporter was identified in *T. virens*, suggesting an active sucrose transference from the plant to fungal cells during their beneficial interaction (Vargas et al. 2011). *Trichoderma* root-colonization is a complex process, which follows a series of relevant steps including: root-adherence and plant penetration. In *T. asperellum* root surface adherence can be mediated by *TasHyd1* a Class I hydrophobin. Lack of this gene impaired root attachment and colonization of cucumber plants (Viterbo and Chet 2006). In *T. asperellum* a Swollenin *Taswo*, an expansin-like protein with a cellulose-binding domain able to recognize cellulose and modify plant cell wall architecture, is necessary for root colonization (Brotman et al. 2008). Plant cell-wall degrading enzymes are also required for active root colonization, *Thpg1*-silenced transformants were affected in plant-root

colonization (Morán-Diez et al. 2009). Once inside the roots, *Trichoderma* spp. must be able to suppress or tolerate plant defense mechanisms. The *T. harzianum* SQR-T037 strain for example is able to degrade phenolic compounds (PAs) isolated from cucumber rhizosphere, including: 4-hydroxybenzoic acid, vanillic acid, ferulic acid, benzoic acid, 3-phenylpropionic acid, and cinnamic acid (Chen et al. 2011). On the other hand, *Taabc2*, an ATP binding cassette transporter cell membrane pump from *T. atroviride*, functions as an extensive and powerful cell detoxification system in the fungus (Ruocco et al. 2009).

Plants, unlike mammals cannot move to evade biotic stresses caused by potential phytopathogens such as: fungi, bacteria, nematodes, viruses, and insects (Dangl and Jones 2001). In turn, plants deploy sophisticated strategies to perceive common features of the microbes and to translate this recognition into an adaptive response specifically directed against the invader (Dangl and Jones 2001). The plant defense response against pathogen attack, begins with the production of antimicrobial metabolites and physical reinforcement of plant cell walls through production of callose and lignin to prevent or attenuate invasion (Glazebrook 2005; Hückelhoven 2007). Once the interior has been breached, and the microbes have penetrated the leaf or root surface, microbes will be exposed to the host plasma membrane, where they will encounter extracellular surface receptors that recognize pathogen-/microbe- associated molecular pattern (PAMP/MAMP). PAMs or MAMPs are highly conserved motifs or domains characteristic of a whole class of microbes but absent from the host. This primary immune response is referred to as PAMP- MAMP-triggered immunity (PTI, MPI) (Boller and Felix 2009; Chisholm et al. 2006). However, pathogenic microbes have evolved the means to suppress PTI by interfering with recognition at the plasma membrane or by secreting effector proteins into the plant cell cytosol. Interestingly, once the pathogens acquired the capacity to suppress PTI, the plants developed a more specialized mechanism to detect microbes, referred to as effector triggered immunity (ETI) (Chisholm et al. 2006). Once plant defenses are activated at the site of infection, the systemic defense needs to be triggered in distal parts to protect the undamaged tissues, this long-lasting and broad spectrum induced disease resistance is divided into two, the systemic acquired resistance (SAR) and induced systemic resistance (ISR) (Pieterse et al. 2009). SAR requires the signal molecule salicylic acid (SA), and is characterized by the activation of specific *PR* genes, which encode proteins with antimicrobial activity (Loon et al. 2006). SAR is effective against pathogens with a biotrophic lifestyle, which feed on living host tissue, although there

are exceptions (Durrant and Dong 2004; Glazebrook 2005). ISR is effective against pathogens with a necrotrophic and hemibiotrophic lifestyle, and is regulated by jasmonate (JA) and ethylene (ET) dependent signaling pathways (Pieterse et al. 2009). Necrotrophs kill host tissue and feed on the remains, whereas hemibiotrophs display both lifestyles, depending on the sage of their life cycle (Glazebrook 2005).

Results Part I

Histone deacetylase HDA-2 is essential in *Trichoderma* to modulate multiple responses in Arabidopsis

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One sentence summary:

HDA-2 histone deacetylase plays a pivotal role in the symbiont fungus *Trichoderma atroviride* by modulating multiple responses in *Arabidopsis thaliana* to establish a beneficial relationship.

List of author contributions:

M.E.R., O.G.R.P, and S.C.F. planned and designed the research. M.E.R., O.G.R.P., M.C.G.L., and D.A.P.R. performed the experiments. M.E.R., O.G.R.P. and M.C.R.M. analyzed data. M.E.R. and S.C.F. wrote the manuscript with contributions from all authors.

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Introduction

Phytohormones are small signaling molecules that occur at low concentrations and play pivotal roles in plants influencing plant growth and development. Classical phytohormones comprise auxins, abscisic acid (ABA), cytokinins (CKs), ethylene (ET), and gibberellic acid (GA). However, brassinosteroids (BRs), jasmonates (JAs), and salicylic acid (SA) are considered phytohormones as well. These signaling molecules not only integrate, but also transmit environmental signals and modulate responses to abiotic and biotic stresses (Pieterse et al. 2009; De Bruyne et al. 2014). In their natural environments plants interact with a plethora of microorganisms, establishing pathogenic or beneficial relationships. The plant response against these microbes lies primarily in an array of structural barriers or inducible defenses. The plant immune system has the ability to perceive non-self by recognizing pathogen- or microbe-associated molecular patterns (PAMPs or MAMPs, respectively), and translate this perception into an appropriate adaptive response (Pieterse et al. 2009). Plants have developed the ability to enhance their basal resistance after PAMPs or MAMPs are detected, by triggering the systemic acquired resistance (SAR) or the induced systemic resistance (ISR), which are phenotypically similar, but different at the biochemical level. The SAR is associated with the accumulation of SA, and is accompanied by the direct activation of pathogenesis related (PR) proteins, encoding genes (Dong 2004), whereas in the ISR, JA and ET are accumulated, modulating the expression of the pathogen-inducible genes *HEL* (Potter et al. 1993), *CHIB* (Samac et al. 1990), and *PDF1.2* (Penninckx et al. 1996). The SA-related defense response is triggered against biotrophic and hemi-biotrophic phytopathogens 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 like 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). Volatile organic compounds (VOCs), released by *Trichoderma* spp., play a pivotal role in plant growth promotion as well (Lee et al. 2015, 2016; Hung et al. 2013). These "semiochemicals" are low molecular mass and usually hydrophobic compounds with high vapor pressure (Effmert et al. 2012). Approximately 479 *Trichoderma* VOCs have been reported (Siddiquee 2014). 6-PP is one of the most common secondary metabolites, and the responsible VOC for the coconut odor in some *Trichoderma* species. 6-PP promotes plant growth and regulates root architecture, inhibiting primary root growth and inducing lateral root formation (Garnica-Vergara et al. 2015; Kottb et al. 2015; Vinale et al. 2008). 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 secondary metabolites (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 (HATs) and histone deacetylases (HDACs) activities (Tribus et al. 2005; Shwab et al. 2007; Lee et al. 2009). It is well known that addition of acetyl groups to histones by HATs 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; Tribus et al. 2010; Lee et al. 2009; Ding et al. 2010). Recently, the histone deacetylase *hda-2* encoding gene from *T. atroviride* was reported to be induced by light and reactive oxygen species (ROS), and its product regulates growth, conidiation, blue-light perception, and oxidative stress responses (Osorio-concepción et al. 2017). In this work, we study the *hda-2* deletion mutant, whose gene codes in the wild-type (WT) for the histone deacetylase HDA-2, during its interaction with *Arabidopsis thaliana* plants. Here, we show that Δ*hda-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 in presence of Δ*hda-2* or its VOCs promoted a strong lateral root branching. We characterized the VOCs profile emitted by Δ*hda-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 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 Δ*hda-2* increased 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 co-culture, 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 (Fig. S1).

To analyze *in vitro* the effect of Δ*hda-2* on the root system architecture of Arabidopsis, plants were inoculated with the wild-type (WT) or Δ*hda-2*. Plants treated with either the WT or Δ*hda-2* showed a statistically significant increase in lateral root number, however the Δ*hda-2* inoculated seedlings showed more lateral roots than the WT treated plants (Fig. 1A, B). Primary root length of plants treated with the WT was barely but significantly larger than those treated with Δ*hda-2* (Fig. 1C); however, no significant differences were observed in fresh weight of plants treated with these fungi (Fig. 1D). Moreover, dry weight of WT-treated plants was higher than Δ*hda-2*-treated seedlings, and the latter showed higher dry weight than 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 Δ*hda-2*.

Histone deacetylase HDA-2 from *T. atroviride* is necessary to effectively colonize and promote plant growth in *A. thaliana*

To study the role of the histone deacetylase HDA-2 from *T. atroviride* on the promotion of plant growth in pots, and to determine if it is compatible with the *in vitro* approach, Arabidopsis seedlings grown in pots were root inoculated with mycelia of *T. atroviride* WT or Δ*hda-2*. Plants inoculated with Δ*hda-2* showed diminished fresh and dry weights compared to those treated with the WT; however, the plants treated with Δ*hda-2* showed significant growth compared to the control plants (Fig. 2A, B, C). To investigate the capability of Δ*hda-2* to colonize Arabidopsis roots, seedlings were root inoculated with the WT or Δ*hda-2*. Thereafter, the roots were detached; total DNA was extracted, and *T. atroviride tef-1* gene *versus* the Arabidopsis *ACT2* gene were quantified by real-time PCR

(qPCR). The Δ*hda-2* capability to colonize the Arabidopsis roots was impaired compared to the WT (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 RT-qPCR. The expression of *PR-1a* and *PDF1.2* was strongly induced by the WT, whereas Δ*hda-2* barely triggered the expression of *PDF1.2* at 72 and 96 h (Fig. 3A, B). Indeed, the mutant *PR-1a* was not detected at any tested time (Fig. 3A, B).

Based on the expression analysis of *PR-1a* and *PDF1.2* in response to the Δ*hda-2*, we asked whether the mutant provides protection against the fungal pathogen *B. cinerea* and the bacterial pathogen *Pst* DC3000. Plants treated with Δ*hda-2* exhibited an enhanced disease susceptibility to both pathogens compared to plants treated with the WT (Fig. 3C, D, 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. 3C, D). On the other hand, WT-treated seedlings and post- inoculated with *Pst* DC3000 showed $3.895\times10^{6}\pm1.054\times10^{6}$CFU/ml, whereas plants treated with Δ*hda-2* and inoculated with the bacterial pathogen showed similar levels of CFU/ml ($7.985\times10^{6}\pm1.75\times10^{6}$) as the control seedlings ($9.232\times10^{6}\pm2.454\times10^{6}$) (Fig. 3E).

The Δ*hda-2* VOCs enhance the growth of Arabidopsis seedlings

To assess whether the VOCs of Δ*hda-2* promote growth in Arabidopsis, seedlings were grown on MS and co-cultured with mycelia of the WT or Δ*hda-2* grown in small plates within a large MS plate (Fig. 4A). Plants exposed to VOCs of Δ*hda-2* exhibited a significant increase in lateral root number (Fig. 4B) and in fresh and dry weights (Fig. D, E) compared to those exposed to the WT VOCs, whereas no differences in primary root length inhibition were observed with both strains (Fig. 4C).

The Δ*hda-2* VOCs did not trigger the SAR and ISR responses in *A. thaliana*

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

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

The Δ*hda-2* strain overproduced 6-PP

To analyze the VOCs produced by Δ*hda-2* and to compare them with those of the WT, fresh inoculums of both strains were grown on MS plates for 5 and 6 days. 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 WT, whereas

the 2-undecanone, unknown ketone, two unknown terpenes and β-curcumene were only detected in the Δ*hda-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 Δ*hda-2* and WT grown on MS at 5 and 6 days (Table 1). This was confirmed by quantification of 6-PP, where Δ*hda-2* synthesized 970.4 and 847.7 ppm (parts per million) at day 5 and 6, respectively; whereas the WT produced 129.1 and 89.9 ppm at the same times (Table 2).

The effect of 6-PP on root system architecture depended 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. 7A, 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 ppm respectively). After 13 days of treatments with 6-PP, lateral root number was increased in a dose- dependent manner (Fig. 7A, B, C). A strong primary root growth inhibition was detected when 50, 75, and 100 µM of 6-PP were supplied into 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. 7A, B, D). Regarding the fresh weight, both treatments followed a similar behavior, except when 50 µM was applied as a volatile or into 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 plants treated with 6-PP, supplied as volatile, did not differ significantly between 25, 50, 75 µM in that order, whereas 100 µM presented a negative effect on plant growth. However, plants treated with 75 µM showed significant differences in biomass gain compared to 25 µM-treated seedlings (Fig. 7B, C, E, F). To determine whether the age of Arabidopsis plays a key role in the plant growth promotion effect by 6-PP, 7-day-old Arabidopsis plants were exposed to 25, 50, 75, and 100 µM of 6-PP or ethanol as control. After 13 days, the treated plants showed an increased lateral root number (Fig. 7G, H), 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 µM 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 Δ*hda-2* and WT strains grown on PDA. To this end, both strains were grown for 5 to 7 days. 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 WT were ketones: 2-heptanone and 3-octanone, whereas for the Δ*hda-2* it was 6-PP. Four exclusive VOCs were identified for the WT, comprising: 3-octanone, 2-heptanol, 3-octanol, and 1-octen-3-ol (Table 1), whereas 17 volatiles were found in the Δ*hda-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 the WT and Δ*hda-2* (Table 1). Because Arabidopsis exposed to Δ*hda-2* VOCs displayed a remarkable phenotype in plant growth, we decided to investigate in more detail the VOCs profile of the Δ*hda-2* growing on PDA. 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 WT and Δ*hda-2* (Table 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 ppm. Addition of 2-heptanol (100 and 1000 ppm), 3-octanol (100 ppm), and 2-heptanone (10, 100, and 1000) as VOCs led to a significant effect on plant growth (Fig. 8A-D). In contrast, 3-octanol, 1-octen-3-ol, 2-octanone, 3-octanone, 2-nonanone, 2-undecanone, and 2-pentylfuran were highly phytotoxic at 10000 ppm; the plants showed a yellow color and a bleaching phenotype (Fig. 8A-D). Most of the VOCs did not show a growth effect at 10 ppm, whereas at 1000 ppm the plants started to show a detrimental effect (Fig. 8A-D). Interestingly, the 2-heptanol and 2-heptanone were the only VOCs that did not bleach the plants at 10,000 ppm (Fig. 8A, B).

Δ*hda-2* is misregulated in the expression of secondary metabolism-, defense-, and communication with the plant-related genes

To know whether Δ*hda-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, Δ*hda-2* and WT were co-cultured 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. 9A-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. 9E-H). Indeed, *epl-1*, *epl-2*, *pbs-1* (Fig. 9A-C), and *abc-2* (Fig. 9D) showed dropped expression levels in Δ*hda-2* in presence of Arabidopsis seedlings, compared to the WT, whereas *ctf-1*, *tps-2*, *pbs-1*, *ggp-1,* and *fpp-1* showed enhanced levels of transcription as compared to the WT (Fig. 9E-H).

HDA-2 is required in *T. atroviride* for proper acetylation of histone H3 on the promoter region of plant responsive genes

To determine the acetylation pattern of histone H3 at Lys9/Lys14/Lys18/Lys23/Lys27 on the promoter regions of *epl-1*, *ctf-1,* and *abc-2* in WT and Δ*hda-2* backgrounds in the presence or in the absence of Arabidopsis, chromatin immunoprecipitation (ChIP) assays were performed. The co-culture of WT 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), compared to the WT grown in the absence of the plant (Fig. 10 A-C). However, histone H3 acetylation on *epl-1* (Fig. 10A), *ctf-1* (Fig. 10B), and *abc-2* (Fig. 10C) promoters in Δ*hda-2* was barely higher than when grown alone compared to the WT (Fig. A-C); but, during its interaction with plants, it dropped to 1.83, 2.55, and 2.81%, respectively, as compared to the WT grown under the same conditions (Fig. A-C).

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 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, Δ*hda-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 Δ*hda-2* was affected in its capability to colonize Arabidopsis roots. Root colonization assays revealed that Δ*hda-2* does not colonize properly the plant roots. Furthermore, additional data of colony forming units (CFU) showed that the mutant colonized at a really low frequency compared with the WT (data not shown). This evidence suggests that HDA-2 plays an important role in plant root colonization. In this regard, it has been reported for several phytopathogens that mutants in the orthologous gene to *hda-2* were strongly affected in virulence, as a result of reduced penetration efficiency (Proctor et al., 1995; Baidyaroy et al., 2001; Ding et al., 2010; Li et al., 2011). Furthermore, Δ*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; Brotman et al. 2008; Morán-Diez et al. 2009; Salas-Marina et al. 2015). Importantly, some of these MAMPs are also proteins (swollenins, endopolygalacturonases, and hydrophobins) that work as colonization factors (Brotman et al. 2008; Morán-Diez et al. 2009; Guzmán-Guzmán et al. 2017), or help the fungus to tolerate antimicrobial compounds accumulated by the plant (Ruocco et al. 2009). Here, the Δ*hda-2* was affected in its capability to induce the SAR and ISR in Arabidopsis, probably as a consequence of its impairment to colonize Arabidopsis roots, therefore, to induce protection against the biotrophic and necrotrophic pathogens *Pst* DC3000 and *B. cinerea*. Based on these results, it is tempting to hypothesize that HDA-2 is a positive regulator of MAMPs and/or colonization factors recognized as elicitors of plant defenses and transporters to tolerate the plant antimicrobial compounds as well. Indeed, Δ*hda-2* was severely affected in the expression of *epl-1*, *-2*, and *pbs-1*, which code for the cerato-platanin elicitor proteins -1, -2, and for the peptaibol synthetase PBS-1, respectively. These proteins are involved on the induction of ISR and SAR in plants (Djonovick et al., 2006; Viterbo et al., 2007; Salas-

Marina et al., 2015). These results explain in part the loss of induction of ISR and SAR in Arabidopsis against the foliar pathogens *B. cinerea* and *Pst* DC3000. Furthermore, the *abc-2* gene that codes for an ABC transporter, involved in cell detoxification, showed dropped transcription levels, which could explain the diminished capability of Δ*hda-2* to colonize Arabidopsis roots.

Recently, it was reported that *hda-2* codes for a class I HDACs, like HDA-2 and Hos2p of *N. crassa* and *S. cerevisiae*, which putatively remove acetyl groups from histone tails, leading to a compacted chromatin, 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 Δ*hda-2* was dropped, 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 its orthologous Rpd3 and Hos2p in *S. cerevisiae* (Eulàlia de Nadal et al. 2004; Sharma et al. 2007). In agreement with such results, the lack of *hda-2* led to the downregulation 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 Δ*hda-2* (compared to *epl-1* and *abc-2* promoters) in both 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; Bee 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 WT VOCs. Interestingly, decreased levels of *PR-1a* and *PDF1.2* were observed at 72 h of Arabidopsis exposure to the WT VOCs, compared to 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, 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, the Δ*hda-2* VOCs failed to properly induce ISR and SAR. Probably, some VOCs in the Δ*hda-2* were missing or diminished their amount by 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, in our analysis of 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, were upregulated both in the presence or 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*, where 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, the Δ*hda-2* produced 7.5 to 9.5-fold more 6-PP than the WT growing on MS, which cannot explain the impairment of Δ*hda-2* to induce SAR and ISR, 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 on the promoters 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 Δ*hda-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.

Δ*hda-2* presented a diminished activity in promoting plant growth and biomass gain in Arabidopsis; this might be the result of Δ*hda-2* inability to colonize the plant root; however, Arabidopsis seedlings treated with Δ*hda-2* VOCs presented a greater biomass gain compared to Arabidopsis treated with WT VOCs. Stimulation of lateral root number in plants treated with the Δ*hda-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 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 days 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 days of co-cultivation (Nieto-Jacobo et al. 2017; Pelagio-Flores et al. 2017). Nieto-Jacobo et al. (2017) attributed such phenotype to an 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, here we inoculated the Arabidopsis seedlings at the root tips with the WT and Δ*hda-2*, observing the inhibition of primary root, 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), since addition of water to an acidic solution turns 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 the Δ*hda-2* VOCs, we attribute the overstimulation of the emergence of lateral roots mainly to the over-production of 6-PP, however, we do not discard that other VOCs could be involved. In this respect, WT and Δ*hda-2* VOCs were more effective in stimulating the emergence of lateral roots, compared with the different concentrations of 6-PP applied as VOC or in the growing medium. The WT produced from 5.3 to 7.7 fold compared with the highest concentration of 6-PP used (100 μM or 16.62 ppm), whereas Δ*hda-2* synthesized from 50.9 to 58.3 fold. Recent results showed 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., 2015). Our results support such works and add novel knowledge, since 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), 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 root 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, IAA, 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 WT or Δ*hda-2* strains or exposed to their VOCs, 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 Δ*hda-2* and/or its VOCs are promoting this phenotype in the plant by modulating a different pathway.

Here, the ET response and biosynthesis-related genes (*ACO2, ETR1, ERS1,* and *EIN2*) were upregulated by WT and Δ*hda-2* VOCs and during direct contact, however, Δ*hda-2* did it to a minor extent. The ET receptor-encoding genes *ERS1* and *ETR1* were upregulated by the WT and Δ*hda-2* through their VOCs and by direct-contact, together with the upregulation of *ACO2,* whose products are involved in the synthesis of ET, probably promoting 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 points to a key role of ET in the primary root inhibition in Arabidopsis seedlings and to a different modulation of this pathway

by Δ*hda-2*. In agreement with our results, where ISR and ET-related genes were co-expressed with *EBP* (ethylene-responsive element binding protein) (data not shown) in plants inoculated with *T. atroviride*, or in the presence of the WT 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-ethylene pathways in Arabidopsis to promote root branching. In this regard, it has been demonstrated that ethylene–auxin interactions regulate lateral root initiation and emergence and elongation in Arabidopsis (Ivanchenko et al. 2008). Moreover, it was described that, at low doses, ethylene promotes auxin biosynthesis leading to lateral root initiation (Ivanchenko et al. 2008). Furthermore, IAA and ethylene 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 secondary metabolites, many of which are part of large biosynthetic gene clusters tightly regulated by chromatin modifications (Schmoll et al. 2016). Here, we report for the first time 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 *Aspergilli* resulted in overproduction of several secondary metabolites, 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 WT and Δ*hda-2* are dependent on the fungal age and the composition of the culture medium. Sixteen compounds were produced only by Δ*hda-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.

To our knowledge, this is the first investigation where 2-heptanol, 3-octanol, and 2-heptanone are reported 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). 3-octanol, 3-octanone, and 2-pentylfuran were identified in promoting and neutral strains, 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 resulted detrimental to Arabidopsis growth at concentrations from 10 to 100 ppm, 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 resulted phytotoxic at 10,000 ppm. The phytotoxic effects of 1-octen-3-ol, 3-octanol, and 3-octanone were already demonstrated by Splivallo et al. (2007). They also reported that the phytotoxic effect of the 1-octen-3-ol was due to an increased ROS-scavenging enzyme activity and/or increased H_2O_2 concentrations.

Conclusions

Our results revealed that histone deacetylase HDA-2 from *T. atroviride* is necessary to effectively colonize and promote plant growth in *A. thaliana,* as well as to induce the systemic disease resistance against foliar pathogens. Moreover, Δ*hda-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 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 revealed that 2-heptanol, 2-heptanone, and 3-octanol promoted plant growth in Arabidopsis seedlings. Our expression analysis led us to conclude that the expression of auxin- and ET-related genes is differentially modulated in Arabidopsis by Δ*hda-2* direct contact or by exposure to its VOCs, which may explain the different phenotype of WT and Δ*hda-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 *A. thaliana* seedlings.

Materials and methods

Trichoderma–plant interaction on MS medium 1× (Murashige and Skoog, 1962) (PhytoTechnology Laboratories®). *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 days at 4 °C, germinated on MS agar plates, and grown under 16/8 h light/dark photoperiod at 22 ± 1 °C, 65% relative humidity, and 150 µmol m^{-2} s^{-1} light.

Trichoderma–plant interaction in pots. Arabidopsis seeds were sown in pots containing peat moss (Lambert peat moss) as substrate and stratified for 2 days at 4 °C. One-day-old seedlings were transplanted into pots containing sterile peat moss and grown as described above.

Trichoderma atroviride IMI 206040 WT, its isogenic Δ*hda-2* mutant (Osorio-Concepción et al., 2016), and *Botrytis cinerea* B05.10 (Amselem 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 12/12 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 (Elizabeth O. King et al. 1954).

Effect of *T. atroviride* WT and Δ*hda-2* on plant growth promotion and root colonization in soil

Fifteen 10-day-old Arabidopsis plants were root inoculated with mycelium of the WT or Δ*hda-2*, as described above. 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 tef-1* gene (Table S1), which codes for the translation elongation factor, and the Arabidopsis *ACT2* gene that codes for the actin protein 2 (Table S1).

Effect of the WT and Δ*hda-2* on plant biomass and root system architecture *in vitro*

Arabidopsis seeds were germinated and placed on Petri dishes containing MS 1×. Eleven days thereafter, the seedlings were inoculated with mycelial plugs of the WT or Δ*hda-2*, sealed with plastic film, and co-cultured for 10 days under a 16/8 h light/dark photoperiod at 22 ± 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 Δ*hda-2* does not undergo conidiation, flasks containing 100 ml of potato dextrose broth (PDB, DIFCO) were inoculated with three mycelial plugs of the WT or Δ*hda-2* and grown at 25 °C, 200 rpm, in the dark for 72 h. Mycelia of the WT or Δ*hda-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-day-old Arabidopsis seedlings, and allowed to interact for 2 weeks. Three Arabidopsis leaves per plant were inoculated with 10 μl of 5×10^5 conidia/ml of *B. cinerea* diluted in inoculation buffer (per 40 ml of stock solution: 1.37 g sucrose, 400 μl of 1 M KH_2PO_4, 80 μl of 12.5% Tween-20). Lesioned areas of infected leaves were quantitatively measured at 3 and 6 days 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 WT or Δ*hda-2* as described above. Thereafter, three leaves per plant were infiltrated with *Pst* DC3000 in 10 mM $MgCl_2$ (OD_{600} = 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 $MgCl_2$. Samples were serial-diluted and plated onto King's B medium containing the appropriate antibiotics to determine the colony-forming-units (CFU).

Influence of the WT and Δ*hda-2* VOCs on plant biomass and root system architecture *in vitro*

Exposure of Arabidopsis plants to the WT and Δ*hda-2* VOCs was achieved using a double plate-within-a-plate system (Olmedo-Monfil and Casas-Flores, 2014). Small Petri dishes containing MS 1× (35 × 10 mm) were embedded into large Petri dishes containing MS 1× 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 WT or Δ*hda-2* onto the small Petri dishes, sealed with plastic film, and grown as described above. Control

plants were inoculated with an MS plug. Root length, number of lateral roots, fresh and dry weights were determined as described before.

Analysis of gene expression by quantitative reverse transcription PCR (RT-qPCR)

Total RNA extraction was performed by the Trizol® method, as described by the vendor (Invitrogen). Total RNA was DNase I (RNase-free) (Ambion) treated, followed by cDNA synthesis using SuperScript II Reverse Transcriptase (Invitrogen), following manufacturer's recommendations. cDNAs were used as template for RT-qPCR reactions with gene-specific primers (Table S1) and the Fast SYBR Green Master Mix (Applied Biosystems) according to manufacturer's recommendations.

The reaction mixtures were: 10 µl SYBR Green Master Mix (Applied Biosystems), 200 ng cDNA template, and 0.3 µl of gene-specific primers (150 nM). The qPCR program consisted of: one cycle at 95 °C for 5 minutes, 40 cycles at (95 °C for 30 s, 65 °C for 30 s, and 72 °C for 40 s). Relative expression was normalized against the level of *tef-1* for *Trichoderma* samples or *ACT2* for Arabidopsis samples using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001).

Expression analysis of auxin, ET, and defense related genes in Arabidopsis inoculated with the WT or Δ*hda-2* or exposed to their VOCs

Plants were germinated and grown for 9 days on Petri dishes containing MS 1×. For direct contact, the root tips were inoculated with disks of fresh mycelium of the WT or Δ*hda-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 hpi (hours post-inoculation), frozen in liquid nitrogen, and stored at -80 ºC 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 above.

Expression analysis of *Trichoderma* genes in co-culture with Arabidopsis seedlings

Arabidopsis Col-0 seedlings were germinated and grown for 10-days on Petri plates containing MS medium. At day 10, the seedlings were root-inoculated with the WT or Δ*hda-2* and the fungi-samples were collected at 72 and 96 hpi. WT and Δ*hda-2* growing in MS

medium alone were included as control. Total RNA extraction, cDNA synthesis, and RT-qPCR were performed as described above.

Effect of 6-PP on plant growth of 2- and 7-day-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) (Garnica-Vergara et al., 2016), whereas 2- or 7-day 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 µl 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 5 plants were closed with sealing film and incubated for 13 days under a 16/8 h light/dark photoperiod at 22 ± 1 °C.

Identification of VOCs through GC-MS

For VOCs analysis, *T. atroviride* WT and Δ*hda-2* were grown on PDA plates at 25 °C for 5 and 7 days, respectively. Non-inoculated PDA plates were included as controls. Compounds were collected as described (Fig. S2) for 1 h with a blue SPME fiber (PDMS/DVB; Supelco Inc., Bellefonte, PA, USA), and desorbed at 180 °C for 30 s in the injector port of a gas chromatograph (Agilent 7890B; Agilent, Foster City, CA, USA), equipped with a mass spectrometry detector (5977A; Agilent) and Mass Hunter Workstation Software (Agilent Technologies, Santa Clara, CA, USA) for data acquisition 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 ppm (parts per million) 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 above. The different VOCs were supplied in a sterile cotton as described in the 6-PP section; 200 µl of 10, 100, 1000, and 10,000 ppm of each selected VOC was applied to the cottons. Water was included as control since it was used as solvent for most VOCs (except 6-PP). Plates were closed with plastic film and incubated at 22 ºC under 12-h-light/12-h-dark cycles for 7 days. 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 above. 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 EGTA [pH 8], 50 µl of 0.5 M EDTA [pH 8, 1.25 ml of 1M HEPES [pH 8], 7.45 ml of 37% formaldehyde, 15.75 m H_2O. Mycelia were incubated for 10 min at room temperature on a rocking platform and neutralized by adding 1.25 M glycine for 5 min at 4 °C. Cross-linked chromatin was immunoprecipitated using 2 µl of anti-H3 antibody (Abcam; Ab1791), and 5 µl of anti-histone-H3 acetyl K9K14K18K23K27 (Abcam; ab47915); 10% of the chromatin was used as input. Immunoprecipitated chromatin was analyzed by qPCR using specific primers (Table S1) on the promoter region of *epl-1, ctf-1, and abc-2*.

Data analysis and statistics

For all Arabidopsis experiments treated with the WT and Δ*hda-2*, the overall data were statistically analyzed using SPSS 10 software (IBM Corp., Endicott, NY, USA). 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 expression of genes in pathogenesis assays, as well as in the plant growth promotion by 10 different VOCs.

Supplemental materials

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

Supplemental Figure S2. *Trichoderma* VOCs exposure system.

Supplemental Figure S3. Chromatin immunoprecipitation (ChIP) assay on the promoter regions of *epl-1* (A), *abc-2* (B) and *ctf-1* (C) genes

Supplemental Table S1. List of primers used in this study

Acknowledgments

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

Table 1. VOCs of the WT and Δ*hda-2* detected by GC-MS in MS 1× and PDA

		Relative area %					
		MS 1×				PDA	
		WT		Δ*hda-2*		WT	Δ*hda-2*
Compound name	**Rt (min)**	**5 dpi**	**6 dpi**	**5 dpi**	**6 dpi**	**5 dpi**	**7 dpi**
2-heptanone*	9.453					46.08 ± 0.02	0.20 ± 0.00
Unknown terpene	9.966						0.14 ± 0.00
2-pentylfuran*	12.976					0.05 ± 0.00	2.40 ± 0.00
3-octanone*	14.288					20.12 ± 0.03	
2-octanone*	15.952						0.29 ± 0.00
Unknown ketone	19.303						0.15 ± 0.00
2-heptanol*	18.972					3.66 ± 0.00	
2-nonanone*	21.926					9.22 ± 0.00	0.12 ± 0.00
3-octanol*	22.875					0.80 ± 0.00	
1-octen-3-ol*	25.704	1.01 ± 0.14				17.70 ± 0.04	
Unknown terpene	30.644	5.06 ± 1.62	7.24 ± 1.94	0.46 ± 0.00	1.79 ± 0.19	0.12 ± 0.00	2.63 ± .011
2-undecanone*	31.644			0.15 ± 0.02	0.03 ± 0.02	0.04 ± 0.00	0.72 ± 0.00
Unknown ketone	32.247				0.25 ± 0.05		0.36 ± 0.00
Unknown terpene	34.153	2.95 ± 0.74	3.27 ± 0.97		0.22 ± 0.06		
Unknown terpene	34.443		3.01 ± 1.16				2.05 ± 0.00
γ-terpinene	35.091						0.06 ± 0.00
α-zingiberene	36.371						0.30 ± 0.00
Unknown terpene	36.573						0.27 ± 0.00
β-Sesquiphellandrene	38.182						1.29 ± 0.00
Unknown ketone	42.392						0.10 ± 0.00
Unknown alcohol	42.569						0.30 ± 0.00
Unknown alkane	42.896						0.09 ± 0.00
Unknown phenol	44.06						0.39 ± 0.00
Unknown terpene	49.15						0.11 ± 0.00
Unknown terpene	51.374				2.22 ± 0.46		0.33±0.00
Unknown terpene	52.304				1.08 ± 0.24		
6-pentyl-α-pyrone *	53.22	90.90 ± 2.51	39.14 ± 2.07	90.58 ± 0.17	76.78 ± 2.42	0.44 ± 0.00	72.32 ± 0.02
β-curcumene	54.089					1.06 ± 0.00	1.43 ± 0.00
Unknown terpene	55.507		47.31 ± 2.05	8.79 ± 0.19	17.30 ± 2.12		13.22 ± 0.02
Unknown alcohol	59.214						0.39 ± 0.00

Mean values ± SE of the sum of three independent determinations are given. *Compounds confirmed and quantified with their respective standards.

Table 2. Quantification of the WT and Δ*hda-2* VOCs in MS and PDA by GC-MS

		Concentration (ppm)					
		MS 1×				PDA	
Compound name	**Calibration curve (r^2)**	**WT (5 dpi)**	**Δ*hda-2* (5 dpi)**	**WT (6 dpi)**	**Δ*hda-2* (6dpi)**	**WT (5 dpi)**	**Δ*hda-2* (7 dpi)**
2-heptanone	0.99					3189.6 ± 189.9	184.8 ± 17.6
2-pentylfuran	1.00					5.0 ± 1.9	43.6 ± 18.7
3-octanone	0.99					1388.0 ± 3.2	
2-octanone	0.99						221.4 ± 24.3
2-heptanol	0.99					771.7 ± 74.8	51.5 ± 2.7
2-nonanone	0.99					768.4 ± 22.2	38.3 ± 4.3
2-octanol	0.99					339.3 ± 80.2	
1-octen-3-ol	0.98	269.4 ± 0.5				3354.0 ± 238.6	
2-undecanone	0.98			262.9 ± 0.8	262.4 ± 0.9	387.0 ± 0.6	474.1 ± 12.2
6-pentyl-alpha-pyrone	0.99	129.1 ± 30.2	970.4 ± 42.6	89.9 ± 6.9	847.7 ± 148.3	53.2 ± 3.9	9579.1 ± 1945.8

Mean values ± SE of the sum of three independent determinations are given.

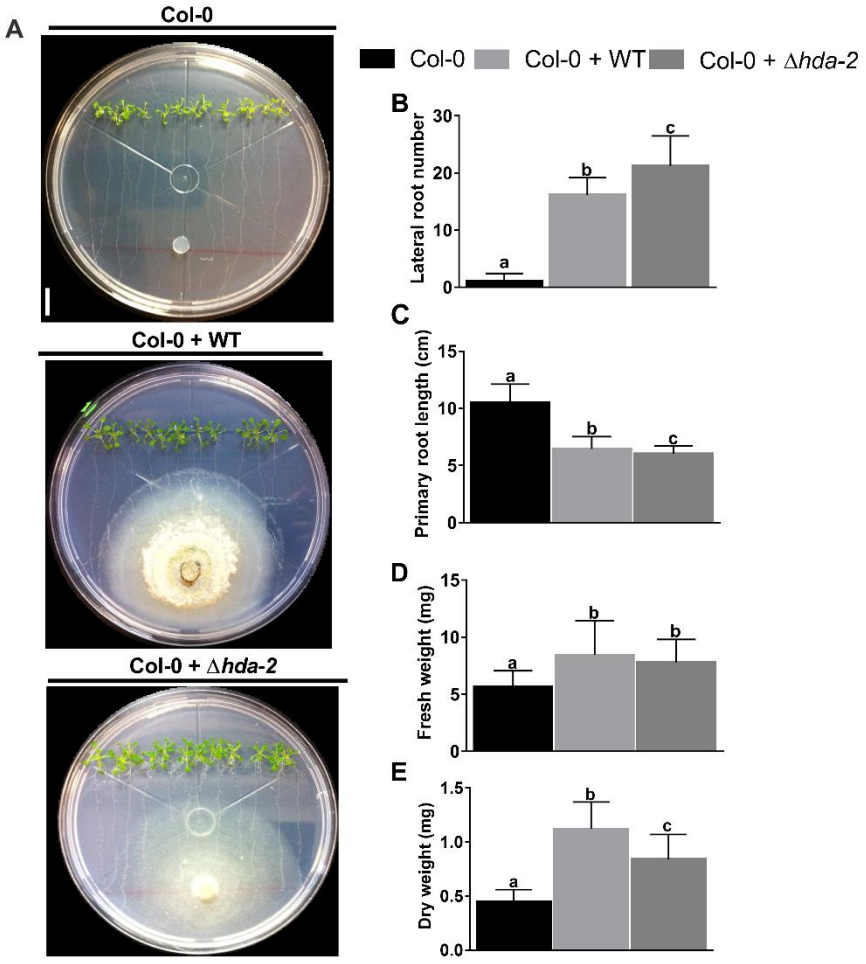


Figure 1. Δ*hda-2* increased the lateral root number in Arabidopsis. Eleven-day-old Arabidopsis seedlings grown on MS medium were co-incubated for 10 days with the WT or Δ*hda-2*. (A) Representative pictures of Arabidopsis grown under the indicated treatments. (B) Lateral root number. (C) Root length. (D) Fresh weight. (E) Dry weight. Data from (A-E) show the mean ± SD of two technical replicates (10 plates with 12 plants each). The experiment was repeated twice with similar results. Results were validated with an analysis of variance statistical analysis using a Tukey multiple comparison test ($\alpha = 0.05$), different letters represent means statistically different at the 0.05 level. Bar, 1.5 cm.

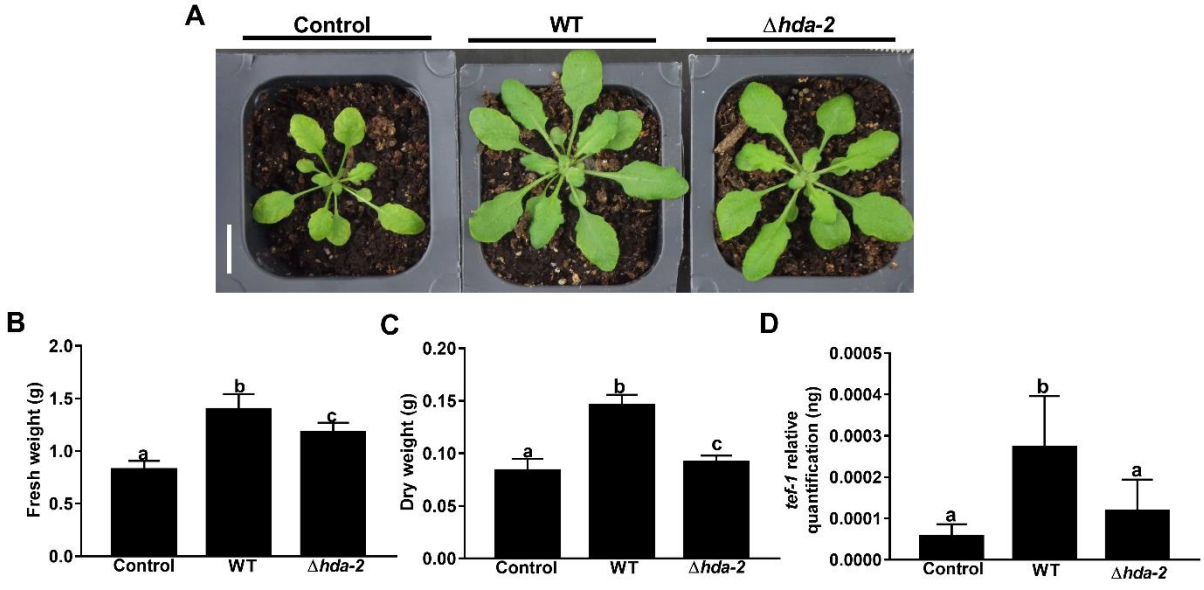


Figure 2. Histone deacetylase HDA-2 is necessary in *T. atroviride* to effectively colonize and promote growth in Arabidopsis. Ten-day old Arabidopsis seedlings grown in soil were inoculated with mycelium of the WT or *hda-2*, and co-cultured for 3 weeks for plant growth promotion assays or for 2 weeks for root colonization. (A) Representative pictures of 4-week-old Arabidopsis plants inoculated with mycelium of the WT or Δ*hda-2*. (B) Fresh weight. (C) Dry weight. (D) Arabidopsis root colonization by *Trichoderma* quantified by qPCR. The fungal DNA was quantified by real-time PCR (qPCR) using the *T. atroviride tef-1* gene *versus* the Arabidopsis *ACT2* gene. Photographs show representative individuals of 24 plants. Data from (A-C) show the mean ± SD of one technical replicate (12 groups with 2 plants each one). The experiment was repeated thrice with similar results. Data from D show the mean ± SD of two technical replicates (16 pooled plants for treatment). Results were validated with an analysis of variance using a Tukey multiple comparison test ($\alpha = 0.05$). Different letters represent means statistically different at the 0.05 level. Bar, 1.5 cm.

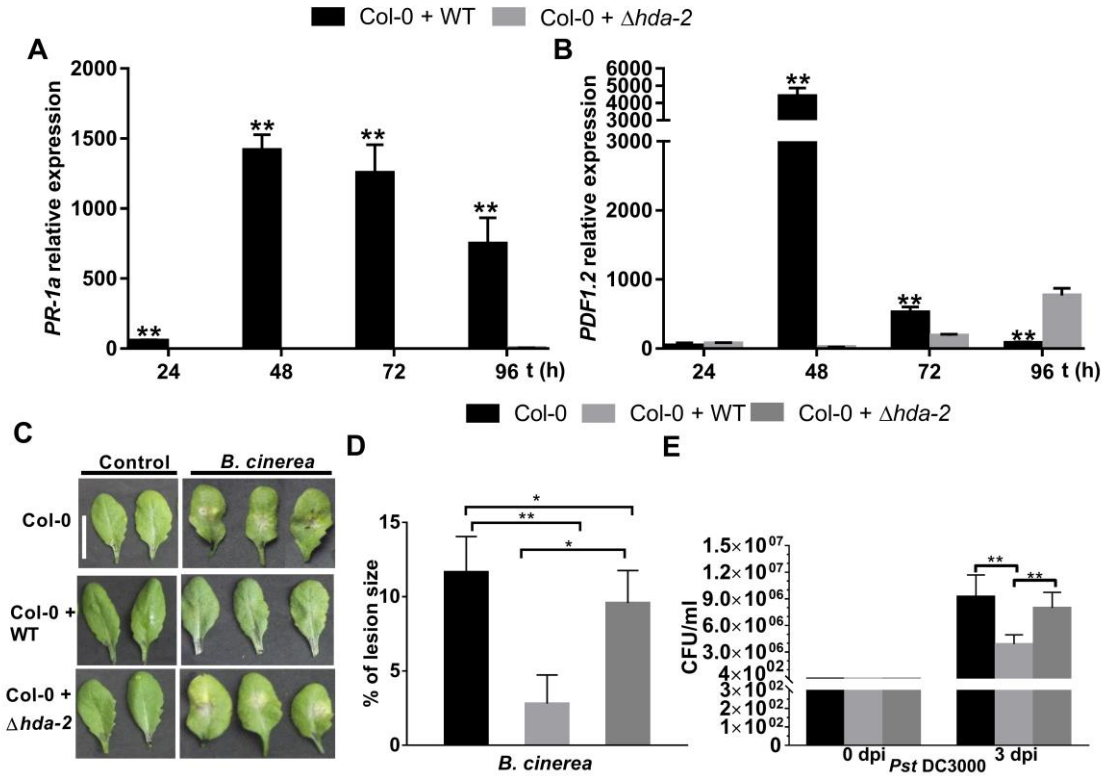


Figure 3. Deletion of *hda-2* in *T. atroviride* compromises the induction of Arabidopsis systemic disease resistance against foliar pathogens. Ten-day-old Arabidopsis seedlings grown on MS medium were root-inoculated with the WT or Δ*hda-2* and the expression levels of *PR-1a* (A) or *PDF1.2* (B) were analyzed by RT-qPCR at 24, 48, 72, and 96 hpi. RT-qPCR results are reported as fold-change compared to Arabidopsis grown without the fungi. Arabidopsis *ACT2* gene was used as control to normalize the expression of *PR-1a* and *PDF1.2* using the $2^{-\Delta\Delta Ct}$ method. Ten-day-old Arabidopsis seedlings grown in soil were inoculated with the WT or Δ*hda-2*, and 2 weeks later leaves were infected with *B. cinerea* (C) or inoculating buffer as control. (D) Lesion sizes of infected plants with *B. cinerea* were determined using ImageJ at 6 dpi**.** (E) Colony forming units of *Pst* DC3000 at 0 and 3 dpi in leaves of treated and untreated plants with WT or Δ*hda-2*. Data from (A-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 (C-D) show the mean ± SD of one technical replicate (12 leaves each). The experiment was repeated thrice with similar results. Asterisks indicate significant difference (independent t-test, *P < 0.05 and **P < 0.01). Bar, 1.5 cm.

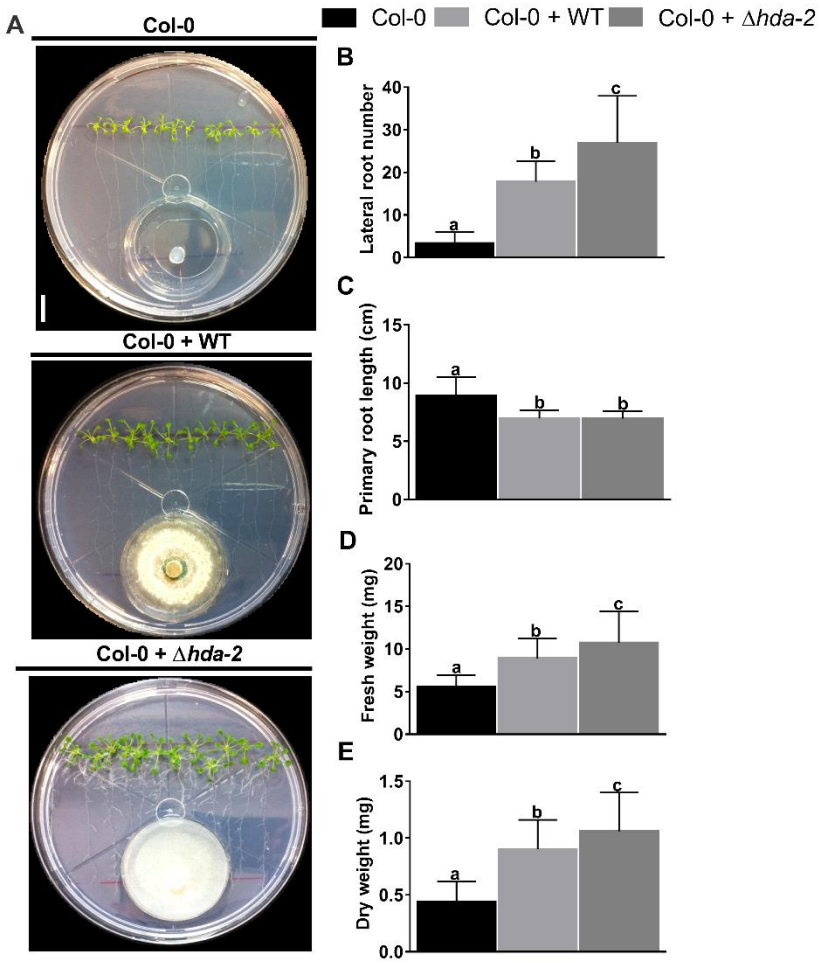


Figure 4. The Volatile Organic Compounds (VOCs) of Δ*hda-2* enhanced the plant growth in Arabidopsis. Eleven-day-old Arabidopsis seedlings grown on MS medium were exposed to WT or Δ*hda-2* VOCs for seven days. (A) Representative pictures of Arabidopsis grown under the indicated treatments. (B) Lateral root number. (C) Root length. (D) Fresh weight. (E) Dry weight. Data from (A-E) show the mean ± SD of two technical replicates (6 plates with 12 plants each). Results were validated with an analysis of variance statistical analysis using a Tukey multiple comparison test ($\alpha = 0.05$), different letters represent means statistically different at the 0.05 level. Bar, 1.5 cm.

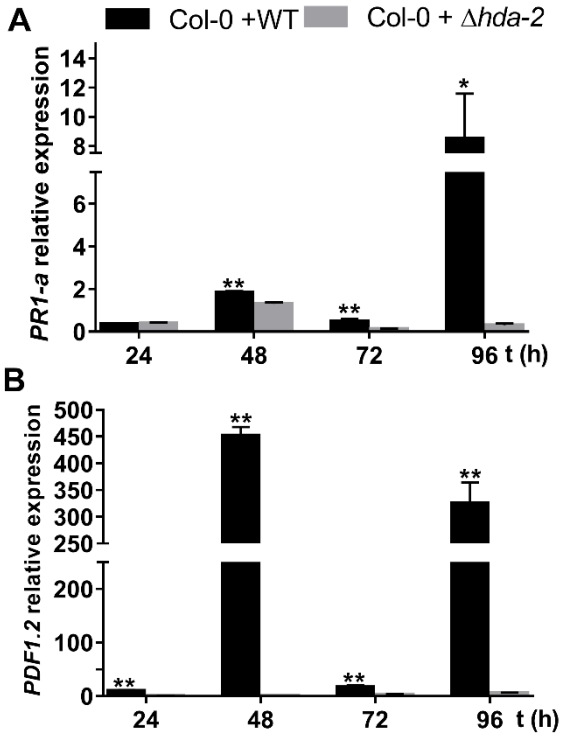


Figure 5. Δ*hda-2* VOCs failed to properly induce ISR and SAR in Arabidopsis. Ten-day-old Arabidopsis seedlings grown on Petri plates containing MS were exposed to the WT or Δ*hda-2* VOCs. The expression levels of defense-related genes *PR-1a* (A) and *PDF1.2* (B) were analyzed by RT-qPCR. Results are reported as fold-change compared to Arabidopsis grown without the fungi. Arabidopsis *ACT2* gene was used as control to normalize the expression using the $2^{-\Delta\Delta Ct}$ method. Data from (A-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 t-test, *P < 0.05 and **P < 0.01).

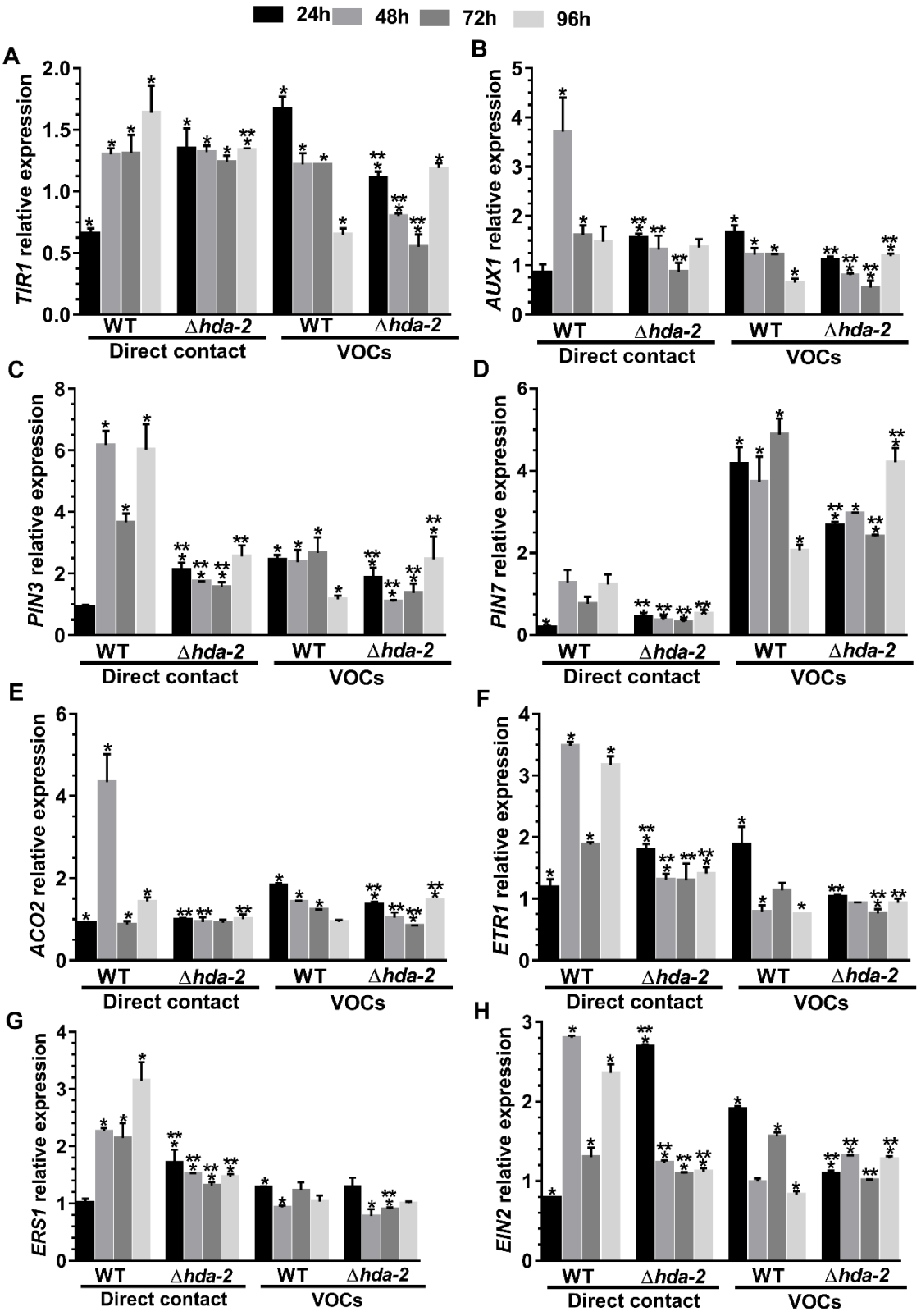

24h
48h
72h
96h
A
TIR1 relative expression
B
AUX1 relative expression
C
PIN3 relative expression
D
PIN7 relative expression
E
ACO2 relative expression
F
ETR1 relative expression
G
ERS1 relative expression
H
EIN2 relative expression
WT
Δhda-2
Direct contact
VOCs

Figure 6. The expression of auxin and ET synthesis and perception genes were altered in Arabidopsis by direct contact with Δ*hda-2* or exposed to its VOCs

Ten-day-old Arabidopsis seedlings grown on Petri plates containing MS were inoculated with the WT or Δ*hda-2* or exposed to their VOCs. The expression levels of: (A) *TIR1*, (B) *AUX1*, (C) *PIN3*, (D) *PIN7*, (E) *ACO2*, (F) *ETR1*, (G) *ERS1*, and (H) *EIN2* were analyzed by RT-qPCR. Results are reported as fold-change compared to Arabidopsis grown without the fungi. Arabidopsis *ACT2* gene was used as control to normalize the expression using the $2^{-\Delta\Delta Ct}$ method. Data from (A-H) 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 t-test, $P < 0.05$) * significant difference between Control vs. WT and vs. Δ*hda-2*. **significant difference between WT vs. Δ*hda-2*.

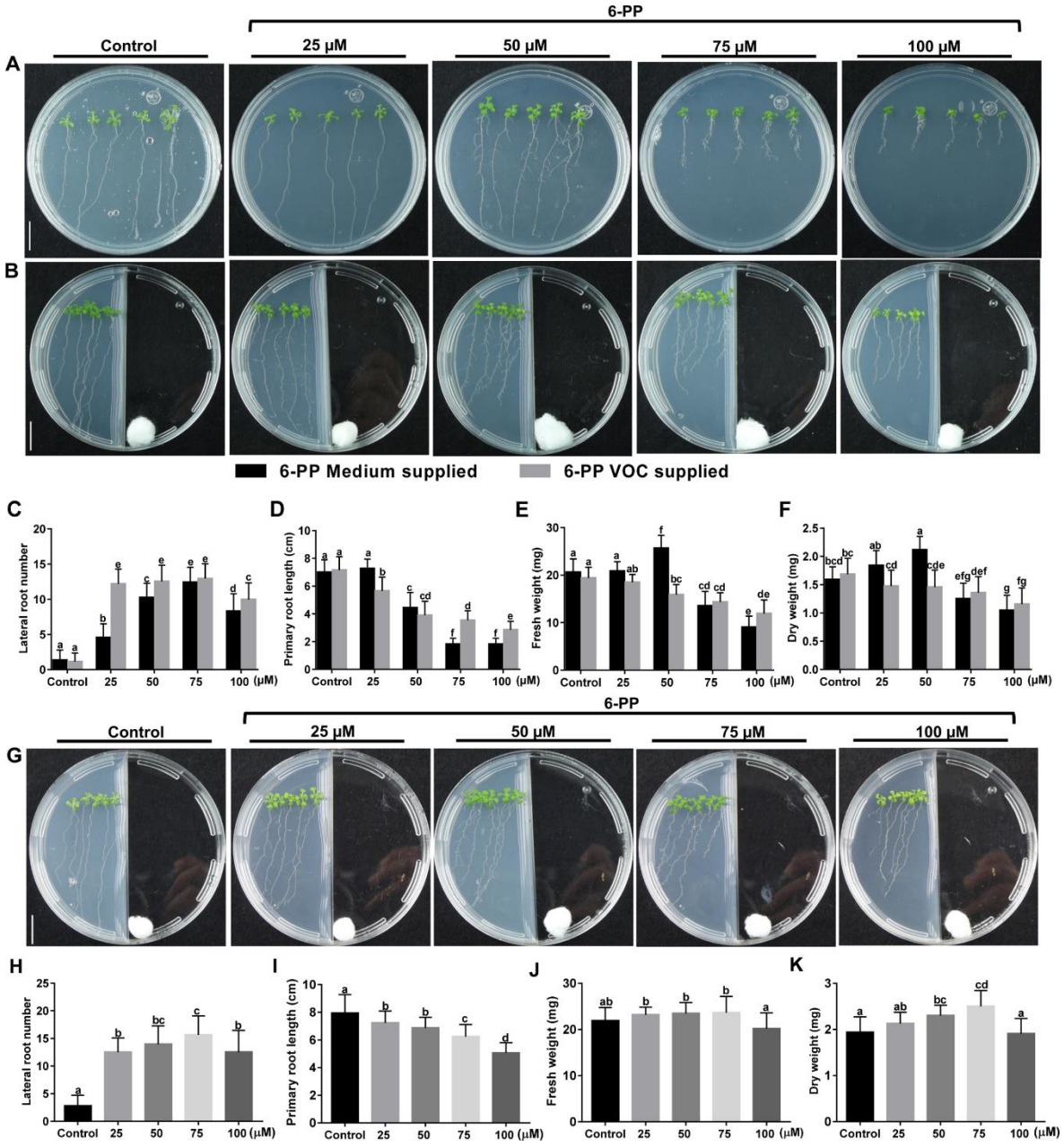

6-PP
Control
25 μM
50 μM
75 μM
100 μM
A
B
6-PP Medium supplied
6-PP VOC supplied
C
Lateral root number
D
Primary root length (cm)
E
Fresh weight (mg)
F
Dry weight (mg)
Control 25 50 75 100 (μM)
6-PP
Control
25 μM
50 μM
75 μM
100 μM
G
H
Lateral root number
I
Primary root length (cm)
J
Fresh weight (mg)
K
Dry weight (mg)
Control 25 50 75 100 (μM)

Figure 7. 6-PP effect on root system architecture was dependent on the plant age and the application method. Two-day- or seven-day-old Arabidopsis seedlings grown on MS 1× supplemented with increasing concentrations of 6-PP or onto sterile cottons of compartmented Petri dishes for 13 days. (A) Representative pictures of 2-day old Arabidopsis grown on supplemented medium with 6-PP at the indicated concentrations. (B) Representative pictures of 2-day old Arabidopsis exposed to the 6-PP VOC at the indicated concentrations. (C) Lateral root number. (D) Root length. (E) Fresh weight. (F) Dry weight. (G) Representative pictures of 7-day old Arabidopsis exposed to 6-PP at the indicated concentrations. (H) Lateral root number. (I) Root length. (J) Fresh weight. (K) Dry weight. Data from (A-K) show the mean ± SD of three technical replicates (15 plates with 5 plants each). Results were validated with an analysis of variance statistical analysis using a Tukey multiple comparison test ($\alpha = 0.\ 05$), different letters represent means statistically different at the 0.05 level. Bar, 1.5 cm.

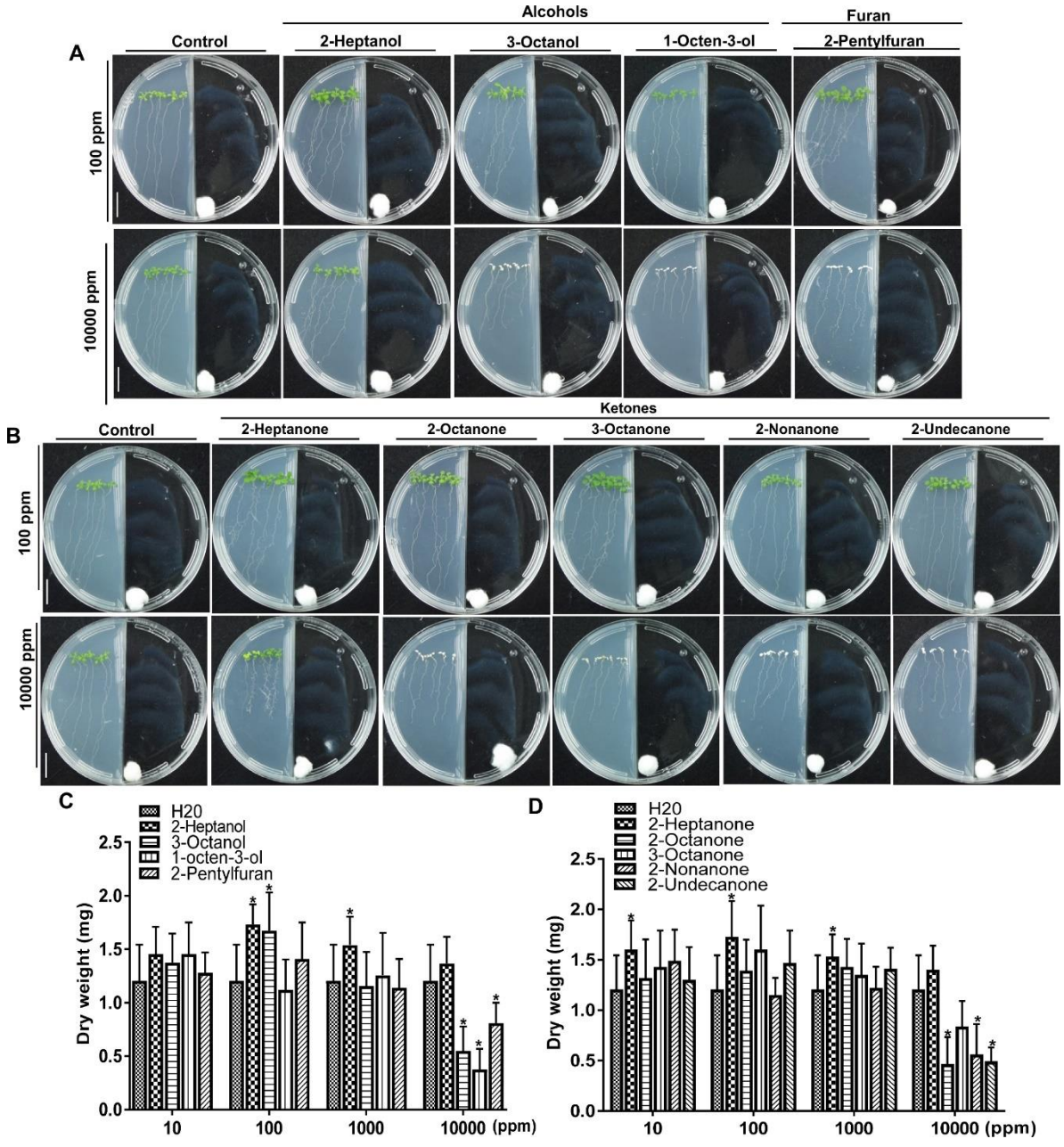

A
Alcohols
Furan
Control
2-Heptanol
3-Octanol
1-Octen-3-ol
2-Pentylfuran
100 ppm
10000 ppm
B
Ketones
Control
2-Heptanone
2-Octanone
3-Octanone
2-Nonanone
2-Undecanone
100 ppm
10000 ppm
C
H20
2-Heptanol
3-Octanol
1-octen-3-ol
2-Pentylfuran
Dry weight (mg)
10
100
1000
10000 (ppm)
D
H20
2-Heptanone
2-Octanone
3-Octanone
2-Nonanone
2-Undecanone
Dry weight (mg)
10
100
1000
10000 (ppm)

Figure 8. 2-Heptanol, 3-octanol, and 2-octanone VOCs promoted plant growth in Arabidopsis *seedlings.* Seven-day-old Arabidopsis seedlings, grown on split-plates containing MS on one side were exposed for 7 days to the indicated VOC by placing an impregnated cotton at the opposite side of the split-dish. (A) Representative pictures of Arabidopsis exposed to increasing concentrations of the indicated ketones, alcohols, and furan. (B) Dry weight. (C) Representative pictures of Arabidopsis exposed to increasing concentrations of the indicated ketones. (D) Dry weight. Data from (A-D) show the mean ± SD of three technical replicates (15 plates with 5 plants each). Results were validated with an analysis of variance statistical analysis using a Tukey multiple comparison test ($\alpha = 0.05$), different letters represent means statistically different at the 0.05 level.

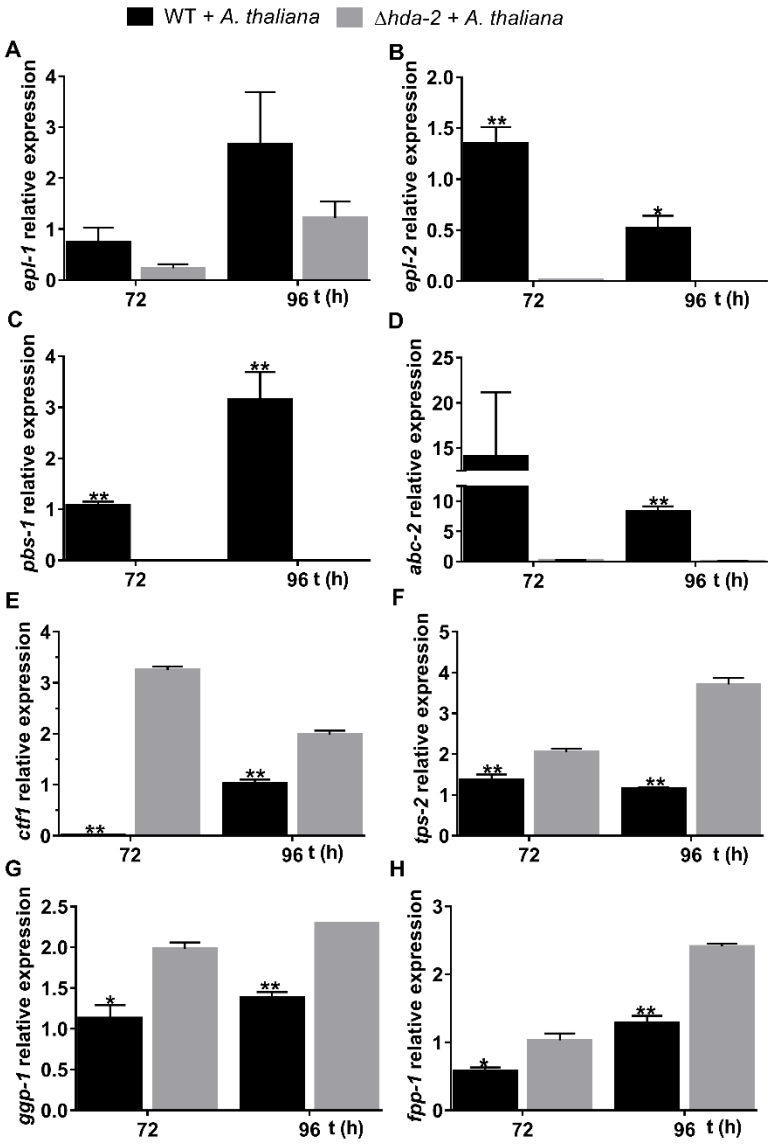


Figure 9. Δ*hda-2* is misregulated in the expression of genes related to secondary metabolism, defense, and communication with the plant. Ten-day-old Arabidopsis Col-0 seedlings were root-inoculated with the WT and Δ*hda-2* and the mycelia were collected at 72 and 96 hpi. WT and Δ*hda-2* grown in MS medium alone were included as control. The expression levels of: (A) *epl-1*, (B) *epl-2*, (C) *pbs-1*, (D) *abc-2*, (E) *ctf-1*, (F) *tps-2*, (G) *ggp-1*, and (H) *fpp-1* were analyzed by RT-qPCR. RT-qPCR results are reported as fold-change compared to the fungi grown without Arabidopsis. Data from (A-H) show the mean ± SD of one technical replicate (fungi mycelia pooled and collected from 5 plates with 7 plants each). The experiment was repeated twice with similar results. Asterisks indicate significant difference (independent t-test, *P < 0.05 and **P < 0.01).

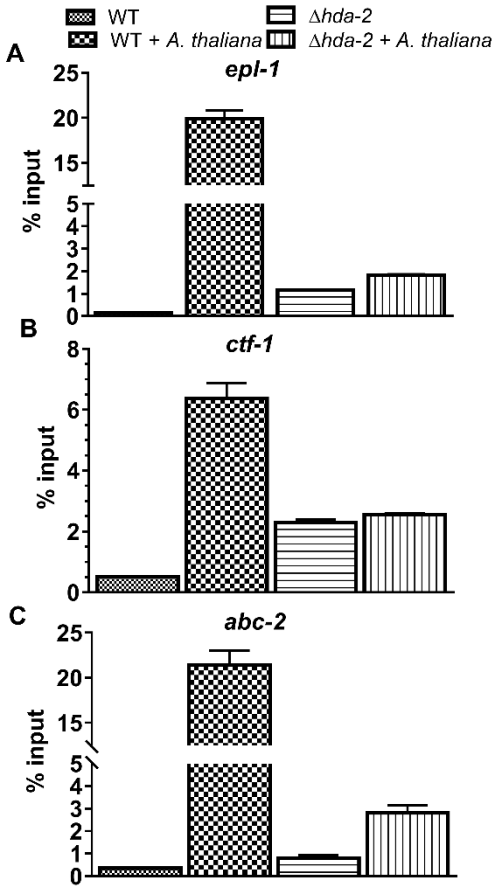
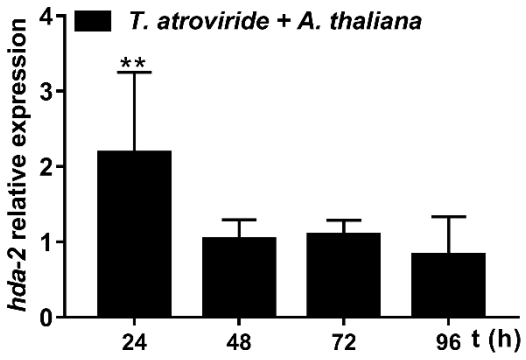
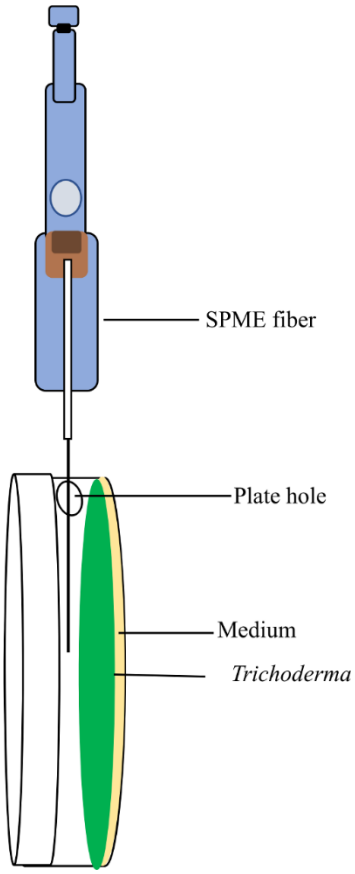


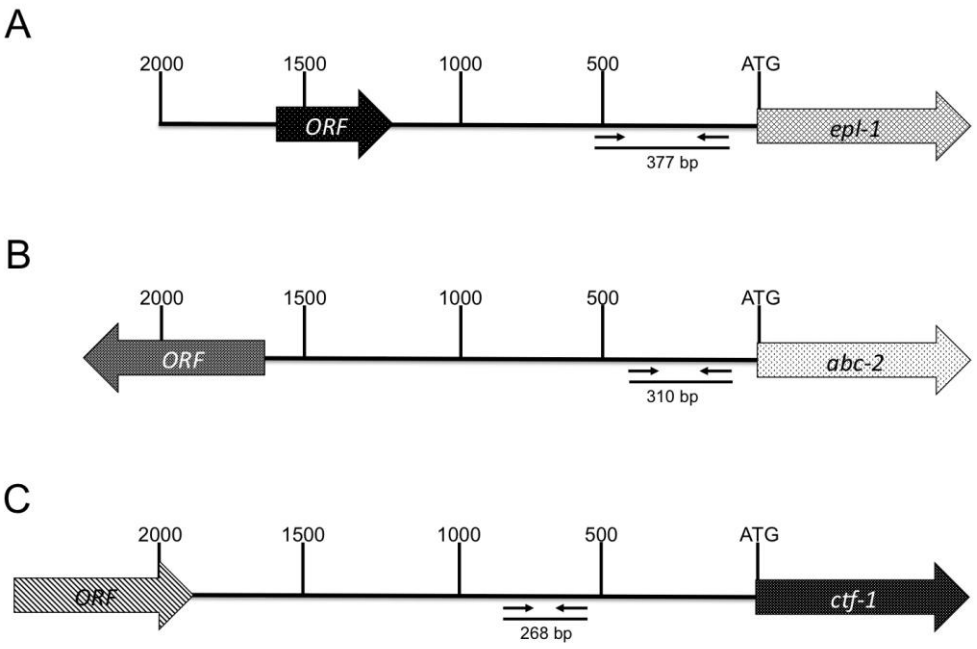
Figure 10. Lack of HDA-2 led to a misregulation of histone H3 acetylation of plant-responsive genes in *T. atroviride.* Cross-linked chromatin of WT or Δ*hda-2* after 96 h of co-culture with 10-day-old Arabidopsis seedlings was immunoprecipitated with antibodies against anti-H3, and anti-H3ac (acetylated-histone H3Lys9Lys14Lys18Lys23Lys27). Fungi grown in absence of the plant were used as controls. Specific primers were designed on conserved regions of (A) *epl-1,* (B) *ctf-1,* and (C) *abc-2* promoters to quantify input DNA and immunoprecipitated chromatin by qPCR. Enrichment was calculated as percentage of DNA control input. The experiment was repeated twice with similar results.



Supplemental Figure S1. The mRNA levels of *hda-2* were slightly increased in *Trichoderma* in the presence of Arabidopsis Col-0 seedling. Arabidopsis Col-0 seedlings were germinated and grown for 10-days on Petri plates containing MS medium. At day 10 the seedlings were root-inoculated with *T. atroviride*-wt mycelia were collected at 24, 48, 72 and 96 h post inoculation (hpi). *Trichoderma* growing in MS medium alone was included as control. The expression levels of *hda-2* were analyzed by RT-qPCR. The graphs show the mean expression levels ± SD. RT-qPCR results are reported as fold-change compared to the fungi growing without Arabidopsis. *tef-1* was used as control gene to normalize the expression of *hda-2* using the $2^{-\Delta\Delta Ct}$ method.



Supplemental Figure S2. *Trichoderma* VOCs exposure system. Schematic representation of WT and *hda-2* growing on MS or PDA plates with a hole, the plates were sealed with a plastic film and at the indicated times the plates were perforated with the SPME fiber and exposed for 1 hour.



Supplemental Figure S3. Chromatin immunoprecipitation (ChIP) assay on the promoter regions of *epl-1* (A), *abc-2* (B) and *ctf-1* (C) genes. One pair of primers was used for each gen on the indicated regions (black lines under arrows). Promoter regions were selected based on the alignment of each promoter gene with its orthologous of *Trichoderma citrinoviride* TUCIM 6016 v4.0, *T. asperellum* CBS 433.97 v1.0, *T. virens* Gv29-8 v2.0 and *T. harzianum* CBS 226.95 v1.0. Primer pair F and R (black arrows) covered highly conserved promoter regions between the five *Trichoderma* species genes. F and R pair of primers are located upstream of the translation initiation codon ATG. For *epl1-1* promoter, the F primer aligned from -140 to -163 and the R primer from -495 to 517. For *abc-2* promoter, the F primer aligned from -140 to -161 and the R primer from -431 to 450. For *ctf-1* promoter, the F primer aligned from -535 to -558 and the R primer from -783 to 800. The different *T. atroviride* orthologous genes were named as follow, based on JGIDB ID: ***epl-1***, Tc_1134502, Tas_141461, Tv_110852, Th_ 508110; ***ctf-1***, Tc_1157619, Tas_381009, T. v_73221 and Th_469719; ***abc-2***, Tc_1121966, Tas_146459, Tv_52608, Th_97417.

Supplemental Table S1 List of primers used in this study

TAIR Locus or JGI id	Gene	Primer sequences (5′-3′)
AT3G18780	*ACTIN 2*	F: TGTGACAATGGTACCGGTATG R: CAGCCCTGGGAGCATCAT
AT2G14610	*PR-1a*	F: ATCTAAGGGTTCACAACCAGGCAC R: TGCCTCTTAGTTGTTCTGCGTAGC
AT5G44420	*PDF1.2*	F: CACCCTTATCTTCGCTGCTC R: GGAAGACATAGTTGCATGATCC
AT3G62980	*TIR*	F: CTGGTGTGCAAGTCATGGTA R: CACTTTCGGGAACCTCCTAATC
AT2G38120	*AUX1*	F: TCAGCTGCCGCCGTTTA R: GCGTTGGAGTGGTCGAGAAG
AT2G01420	*PIN-4*	F: CCGTGGCGCTAAGCTTCTTA R: CGAAACAATAGACGCACCAGTCT
AT2G20610	*SUR1*	F: CGTCCTGGCTTCCCTCACTA R: GCGAACCTCGAGACCACTGT
AT4G14560	*AUX/IAA*	F: TGAAAGGATCCGAAGCTCCTACT R: TGCCTCGACCAAAAGGTGTT
AT1G62380	*ACO2*	F: CACGGTTCGCAGCAATGA R: CGGCTGCTGTAGGATTCAGTT
AT2G40940	*ERS1*	F: TCTGTGGAGCTACGCATTTCA R: CAACGGCTTTGGAATGCAT
AT3G16770	*EBP*	F: GCGAGTTAGCTCAGCCGAGTT R: TCTTCCCCCTTTCCAAATCC
AT1G70940	*PIN3*	F: TCAGCAGACGACTCTTCCAAC R: CTTGGTCAGATCTTCCGCCTT
AT1G23080	*PIN-7*	F: GCTCTTGTTGCTTTCAGGTGG R: CACGCAATAGGTCTCCACGTA
AT1G66340	*ETR1*	F: TGGAAAAGGATGCACGGCTAT R: TGAACTCACCGTGGTCACTTC
AT5G03280	*EIN2*	F: GTTTGGTGTAGCGGAGAGGAA R: CAGCTACCCGGTCAATCAGTT
300828	*tef-1*	F: CAGGTCGGTGCCGGATAC R: CCATCTACGTCTTGGCTGTTGA
212638	*hda-2*	F: AAGCCCTGGCGTCTGACACTCTCC R: GGCCACCTCCCCACGCAATAG
302952	*epl-1*	F: AGCGGCAAGACCATCTACGT R: TCGAGGCCAATGTTGAATCC
88590	*epl-2*	F: CACTCGTCACAGCCATCACT R: CTGAACGCCGCCAATGTATG
317938	*pbs-1*	F: CCGAGACAAGCGTCAAGGA R: CGAAGTGATGAAGGGCATGTAG
53468	*abc-2*	F: TGGATAACGTTCAGGGTTGGG R: GCAGAGAACTCCAAAGCCTC

145197	*ctf1*	F: ATCCCTGGCTCACACCATTG R: CTAAAAGCATCGGCACGTCG
146906	*tps-2*	F: ATTCAGTGTACGGAGGCACAG R: GAAGCGCCTTCAACAGCATAG
284926	*ggp-1*	F: CGTCAACCCAATGTCGAACAC R: TCTTGTTGCCAAACTCCAGGT
134836	*fpp-1*	F: TCAGGACGACTACCTCGACAA R: TAGAGCTTCTTGACGACCTGC
302952	Pepl1-ChIP	F: CAGGGATGTTGTGTTGCTATTT R: GAGGACGACTCTGGTCTTTATAC
145197	Pctf1-ChIP	F: TCCGCACAAAGAGTCAAGAG R: AGGAACAGAGACGAGATGGA
53468	Pabc2-ChIP	F: TGCGCTTCAGACGTTATCC R: GGCGAAAGGAGCTTAGAGATT

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Results Part II

Transcriptome landscape of the beneficial fungus *Trichoderma virens* in response to *Arabidopsis thaliana*

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Introduction

The *Trichoderma* genus comprises ascomycete-filamentous fungi, cosmopolitan inhabitants of soil, decaying wood and vegetable matter. *Trichoderma* spp. can been isolated from crop fields, pastures, forests, salt marshes, prairies, and deserts (Kubicek et al. 2008). This may be attributable to the diverse metabolic capability of *Trichoderma* spp. and their aggressively competitive nature. The potential of *Trichoderma* species as biocontrol agents of phytopathogenic fungi, oomycetes and nematodes has been well documented (Aluko and Hering 1970; Elad and Kapat 1999; Lifshtz et al. 1986; Howell 1982; Wells et al. 1972; Kerry 1988; Sharon et al. 2008). Various mechanisms for the biocontrol activity of *Trichoderma* spp. includes: antibiosis, competition, mycoparasitism and enzymatic hydrolysis (Howell 2003; Harman 2000). Indirectly *Trichoderma* spp. are also capable of protecting plants through the induction of their defense responses (Yedidia et al. 1999). Recent advances have unraveled that plant systemic acquired resistance (SAR), and induced systemic resistance (ISR) are simultaneously triggered by plant root colonization by *Trichoderma* spp. (Salas-Marina et al. 2011; Perazzolli et al. 2012). Triggering of SAR requires the phytohormone salicylic acid (SA) as signal molecule, and is characterized by the accumulation of pathogenesis-related proteins encoding genes (PR) (Durrant and Dong 2004; Van Loon 1975; Van Loon and Van Strien 1999). SA-signaling cascade leads to a long-lasting disease resistance effective against biotrophic (*Hyaloperonospora arabidopsidis*) or hemibiotrophic (*Pseudomonas syringae*) phytopathogens (Heil and Bostock 2002; Pieterse et al. 2009). Moreover, ISR elicitation relies on the phytohormones jasmonate (JA) and ethylene (ET), as signal molecules, and is associated with an enhanced expression of *HEL* (Potter et al. 1993), *CHIB* (Samac et al. 1990), and *PDF1.2* (Penninckx et al. 1996) genes which code for hevein-like protein, chitinase B and plant defensing respectively. ISR is effective against necrotrophic (*Botrytis cinerea*) phytopathogens and herbivore insects (Pieterse et al. 2009; Van Oosten et al. 2008). *Trichoderma* strains also induce a physiological state called "priming", which results in a faster and/or stronger activation of defense responses when plants are re-exposed to biotic or abiotic stresses (Goellner and Conrath 2008; Conrath et al. 2006). Furthermore, activation of the mutually antagonistic SA and JA pathways by *Trichoderma*, implies a cost in the plant ecological fitness causing a phenomenon called

cross-talk (Van Oosten et al. 2008). *Trichoderma*-plant interactions occur mainly in the rhizosphere, where fungi produce and release a diverse repertoire of microbe-associated molecular patterns (MAMP's) and/or elicitors of plant responses such as: polygalacturonases (*Thpg1*) (Morán-Diez et al. 2009), xylanases (*Eix*) (Rotblat et al. 2002), cerato-platanins (Sm1/Epl1) (Djonović et al. 2006; Seidl et al. 2006), cellulases (Martinez et al. 2001), and swollenins (*Swo1*) (Brotman et al. 2008). Plant growth promotion by *Trichoderma* has been studied for many years in numerous crops including: chrysanthemum, tomato, cacao, pinus and model plants (Gravel et al. 2007; Hohmann et al. 2011; Bae et al. 2009; Mackenzie and Starman 1995; Salas-Marina et al. 2011). One of the most recognized biological control agents is *Trichoderma*, its ability to control pests and promote plant growth has spread worldwide. *T. virens* genome size is 38.8 Mbp (mega base pairs), with a total of 12,428 predicted genes (Kubicek et al. 2011). Genomic DNA in the eukaryotic cell nucleus, is compacted in a highly organized structure termed chromatin (Brosch et al. 2008). Chromatin is organized in nucleosomes, which consist of 147 base pairs of DNA wrapped around a histone octamer consisting of 2 copies each of the core histones (H2A, H2B, H3 and H4) (Luger 2003). Chromatin is not inert and actually undergoes several chemical modifications by enzymes at histone amino tails, leading to a dynamic chromatin (Vaquero et al. 2003; Fry and Peterson 2001; Tsukiyama 2002). Recently, chromatin dynamics in plant-pathogen interactions has gained the researchers attention (Gómez-Díaz et al. 2012; Ramirez-Prado et al. 2018). The deletion of *PFP1* a subunit of a HAT complex in *Rhynchosporium commune* the causal agent of leaf scald disease led to nonpathogenic strains (Siersleben et al. 2014). In *Fusarium fujikuroi* deletion of two HDAC-encoding genes, *ffhda1* and *ffhda2* revealed that both HDACs are required for gibberellic acid GA-induced bakanae disease of rice (Studt et al. 2013). In *Fusarium graminearum* the lack of *FTL1* gene, which codes for a component of the Set3 complex, failed to colonize the vascular tissues of rachis or cause necrosis on the rachis of inoculated wheat heads (Ding et al. 2009). Chromatin-remodeling complexes are ATP-dependent and necessary to rearrange or to mobilize nucleosomes (Lusser and Kadonaga 2003). Chromatin remodelers can participate in transcriptional regulation, DNA repair, homologous recombination and chromatin assembly (Lusser and Kadonaga 2003). There are currently four different families of chromatin remodeling complexes. All four utilize ATP hydrolysis to alter histone-DNA contacts and share a similar ATPase domain (Clapier and Cairns 2009), these families comprise: SWI/SNF (switching defective/sucrose

nonfermenting), ISWI (imitation switch), CHD (chromodomain, helicase, DNA binding), and the INO80 (inositol requiring 80) family remodelers (Lusser and Kadonaga 2003; Clapier and Cairns 2009). The SWI/SNF family remodelers complexes were initially purified from *Saccharomyces cerevisiae* and are composed of 8 to 10 subunits. The SWI/SNF family are defined by the presence of an N-terminally located HSA (helicase-SANT), which is known to recruit actin and actin-related proteins, and a C-terminal located bromodomain, known to bind to the acetylated-lysine of histones (Clapier and Cairns 2009; Längst and Manelyte 2015). Remodeler complexes belonging to this family have been shown to slide/or evict nucleosomes from DNA, but lack chromatin assembly activities (Längst and Manelyte 2015). The Snf2 family of helicase-related proteins fall into 24 subfamilies based on the primary sequence of the helicase-like region (Eisen et al. 1995; Flaus and Owen-Hughes 2011). The subfamily Rad5/16 proteins is one of them and includes, Rad5p, Rad16p, Ris1, Lodestar, and SHPRH frequently involved in DNA repair pathways (Flaus et al. 2006). A genomic analysis of *T. atroviride*, *T. reesei* and *T. virens* species showed that they contain six, five and five SNF2 related proteins respectively. The coming of the Genomic Era has facilitated the investigation of the transcriptomic responses in *Trichoderma*. One of the first studies reported the transcriptomic response of *T. harzianum* T34 in the presence of tomato plants, this work highlighted the importance of the expression of fungal genes related to redox reactions, lipid metabolism, detoxification, and sugar or amino-acid transport (Chacón et al. 2007). In 2012, a comparative analysis with high-density oligonucleotide (HDO) microarray compared the transcriptomic response of *T. harzianum* T34, *T. virens* T87 and *T. hamatum* T7 in presence or absence of tomato plants, this analysis showed that 3.15 % of the total sets deposited showed a significant change of at least 2-fold in expression in the presence the plant (Morán-Diez et al. 2012). In this paper, we focus on analyzing the transcriptomic response of *T. virens* to the presence of *A. thaliana* seedlings as well as in generating mutants that help to understand the plant-*Trichoderma* interaction.

Materials and Methods

Organisms and growth conditions

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 days at 4 °C, germinated on MS 0.7× (PhytoTechnology Laboratories®) (Murashige and Skoog, 1962) agar plates, and grown under 16/8 h light/dark photoperiod at 22 ± 1 °C, 65% relative humidity, and 150 μmol m^{-2} s^{-1} light. *Trichoderma virens* Gv29-8 WT (wild-type) (Baek and Kenerley 1998), its isogenic mutants Δ*snf2-23*, Δ*snf2-24* (this work), *Botrytis cinerea* B05.10 (Amselem et al. 2011), and *Rhizoctonia solani* were used throughout this study. The Δ*snf2-23* strains was selected and used for further studies unless otherwise specified. All fungal strains were routinely grown at 28 °C on potato dextrose agar (PDA; DIFCO), under a 12/12 h light/dark regime. 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 (Elizabeth O. King et al. 1954).

RNA-Seq libraries preparation and sequencing

To construct the RNA-seq libraries, twenty ten-day-old Arabidopsis seedlings were root inoculated with *T. virens* WT (wild-type), and mycelia were collected for 48, 72 and 96 hpi (hours post inoculation). *T. virens* growing in MS 1× plates were used as control. Mycelial samples were frozen in liquid nitrogen and stored at -80ºC until their processing. Total RNA was extracted by the TRIzol method (Invitrogen), following the protocol recommended by manufacturer. DNA contamination was eliminated with TURBO DNase (Life Technologies). RNA samples were quantified by measuring the absorbance ratio at A260/280 and A260/230 using an Epoch Spectrophotometer (Biotek). RNA samples were assessed for quality and quantity using an Agilent 2100 Bioanalyzer (Agilent Technologies). RNA with an integrity number (RIN) ≥ 8.0 was used for libraries construction. RNA-Seq libraries preparation and sequencing were performed at the Genomics Core Facility at the Laboratorio Nacional de Genómica para la Biodiversidad, CINVESTAV (Irapuato, Gto., Mexico). The RNA-seq libraries were prepared from 3 biological RNA replicates for each treatment, using SOLiD Kit, and sequenced with SOLiD platform (Applied Biosystem), following the manufacturer's instructions. The raw reads were deposited in the NCBI Sequence Read Archive (SRA).

RNA-Seq data analysis and functional annotation

The sequenced reads (csfasta and qual files) were converted into the fastq format using the SOLiD system XSQ Tools and solid2fastq.pl script, and their quality was checked with FastQC (Andrews, 2010). Reads were mapped to the predicted genes of the *T. virens* genome version 2 (Kubicek et al. 2011) using the Burrows-Wheeler Aligner (BWA) software (Li and Durbin 2009) allowing 4% of mismatches. Visualization and quantification of reads per gene was carried out using the Tablet tool (Milne et al. 2009), only reads with unique hits were maintained and used for the quantification of reads number per gene. Raw data count of all the samples and biological replicates were normalized together by trimmed mean of M values (TMM) using the edgeR (Robinson et al. 2010). Correlation and biological coefficient of variation between samples and biological replicates were determined with a Pearson´s correlation and multidimensional scaling analyzes (MDS) using the normalized expression data by TMM. Only the biological replicates with a Pearson´s correlation factor ≥ 0.9 between them were considered for subsequent analyzes. Almost all biological replicates had a Pearson´s correlation factor greater than 0.9, except for the biological replicate 2 of *T. virens* growing in MS media (Tv48h.2). To assess the transcriptional response of *T. virens* to the presence of *A. thaliana*, we determined the differentially expressed genes by comparison of *T. virens* growing in MS medium versus *T. virens* in co-culture with *A. thaliana* at 48, 72 and 96 hpi, using the edgeR package (Robinson et al. 2010), using a statistical methods based on pairwise comparisons and a common dispersion calculated as suggested by edgeR manual. Genes that exhibited a false discovery rate (FDR) 0.05 and a log2 Fold Change > 1 (log2FC) were considered as differentially expressed genes (DEG). Functional annotation of DEG based on gene ontology terms and the enrichment analysis (Fisher´s exact test, FDR < 0.05) were performed using BLAST2GO suite (Conesa et al. 2005). Hierarchical clustering of DEG by log2FC and the heat map of the GO terms enriched were created using the hclust and heatmap.2 functions with the agglomeration method of ward.D, and the gplots R library (http://cran.r-project.org/web/packages/gplots/index.html).

RNA-Seq validation by RT-qPCR

Transcriptome validation of *T. virens* was performed with one of the biological replicates. cDNA was synthesized using 5 μg of total RNA with a SuperScript II Reverse Transcriptase

(Invitrogen), following the manufacturer's recommendations. The oligonucleotides were designed using the software Primer 3 (Applied Biosystems) (Supplementary Table S1), and the RT-qPCR (Reverse transcription polymerase chain reaction), reactions were performed using the Fast Syber Green Master Mix kit (Applied Biosystem), with 100 ng of cDNA from each sample. The Abiprism 7500 Fast Real-Time PCR system (Applied Biosystem) was used throughout the study. *tef-1* gene was used as a reference gene for the normalization of the measured genes, by using the formula $2^{-\Delta\Delta CT}$ (Livak and Schmittgen 2001).

Generation of *T. virens* protoplasts

Protoplast generation was performed according to the protocol described in (Baek and Kenerley 1998) with modifications: 1×10^8 conidia/ml were inoculated in 100 ml of PDYCB medium (per litre: 24 g potato dextrose broth, 2 g yeast extract and 1.2 g casein hydrolysate medium) and incubated for three days at 28 °C under shaking at 250 rpm. Mycelium was collected by filtration, washed with 0.01 M phosphate buffer (pH 7.2), and 0.5 g was resuspended in osmoticum (50 mM CaCl2, 0.5 M mannitol, 50 mM MES, pH 5.5) with 15 mg/ml lytic enzymes of *Trichoderma harzianum* (Sigma, L1412). The mixture was incubated at 28 °C under gentle shaking for 5 h. For protoplast regeneration, a selective medium (potato dextrose broth DIFCO™, 0.8% Agarose (Nara Biotec), and 0.5 M sucrose) containing 200 µg/mL hygromycin was used.

Genetic transformation of *T. virens* and stable transformants screening

Deletion of *snf2* gene and primer design was performed according to double joint PCR technology (Yu et al. 2004). In a first round of PCR, the 5 '(5'SNF2-F and 5' SNF2-R-Fusion) and 3' (3 SNF2-F-Fusion and 3 SNF2-R) modules of *snf2* were amplified with specific primers (Supplementary Table S2), using *T. virens* genomic DNA was as template. The *hph* cassette was amplified by PCR with DJhph-F and DJhph-R specific primers (Supplementary Table S2)and using 1519 lacZ vector bearing the hph as template (Cervantes-Badillo et al. 2013). In a second round of PCR the three fragments were fused, and then purified with Wizard® Genomic DNA Purification Kit. Herculase II Fusion DNA Polymerase (Agilent) was used for all PCR reactions following manufacturer's recommendations. The final product was used to transform *T. virens* WT strain protoplasts. Stable transformants were

selected by three consecutive transfers of a single colony to PDA medium supplemented with 200 µg/ml hygromycin. For the screening of gene-replacement events, total DNA from hygromycin-resistant colonies was subjected to PCR using 5'SNF2-F'-Upstream and 3'SNF2-R'-Downstream oligonucleotides (Supplementary Table S2), corresponding to sequences on the genome ≈100 bp up and downstream of the 5' and 3' modules used in the fusion PCR, respectively. For the screening of gene-replacement events, as well as to test for ectopic insertions of *hph* cassette in the *T. virens* genome, DNA from the hygromycin-resistant colonies was subjected to qPCR using specific primers for both genes (Supplementary Table S2). The copy number of *hph* and *snf2* in the genome of the transformants was calculated using the $2^{-\Delta\Delta Ct}$ method (Bubner and Baldwin 2004).

Phenotype analysis and conidia quantification

WT, Δ*snf2-23* and Δ*snf2-24* strains were grown on Petri dishes containing PDA at 28 ºC with 12/12 h light-dark photoperiod. Phenotype of each strain were photographed and evaluated qualitatively by visual inspection every 24 h. The radial growth of the colonies was also measured every 24 h, and the conidia were harvested with water and quantified at 24, 48, 72 and 96 h with a Neubauer chamber (Marienfeld, Germany) under microscope (Zeizz).

Dual cultures of *T. virens* WT and Δ*snf2* strains versus *R. solani* and *B. cinerea*

WT and Δ*snf2* capability to growth over the phytopathogens *R. solani and B. cinerea* was assessed in dual cultures confrontation assays. Agar plugs of actively growing mycelia of *Trichoderma* strains and the phytopathogens were placed on the opposite sides of PDA plates and incubated at 28 °C by 7 days and photographed.

Antimicrobial activity of *T. virens* WT and Δ*snf2* mycelium-free culture filtrates (MFCF) versus *R. solani* and *B. cinerea*

To determine the antifungal properties of Δ*snf2* MFCF against *R. solani* and *B. cinerea* B05.10, 1×10^7 conidia of the WT or Δ*snf2* were inoculated in 150 mL of PDB and grown by 4 days at 28 °C and 200 r.p.m. Subsequently the WT and Δ*snf2* cultures were filtered under vacuum through 0.20 µm filter papers, and PDA plates 1× containing 40% (v/v) of the MFCF were prepared. Mycelial discs of *R. solani* or *B. cinerea* were placed at one edge of the PDA plates 1× containing 40% and incubated at 28 °C for 4 and 7 days respectively.

Phytopathogens growing on PDA 1× without MFCF served as controls. Colony diameters were measured every 24 h.

Effect of Δ*snf2* on plant growth promotion

Arabidopsis seeds were sown in pots containing peat moss (Lambert peat moss) as substrate and stratified for 2 days at 4 °C. One-day-old seedlings were transplanted into pots containing sterile peat moss and grown at the same conditions as described above. One week later, 15 Arabidopsis plants were root inoculated with 500 µl of 1×10^6 conidia/ml of the WT or Δ*snf2* in 0.3×MS. Plants growing without the fungi were used as control. Three weeks postinoculation, fresh and dry weights were determined on an analytical scale.

Expression analysis of defense related genes in Arabidopsis inoculated with the WT or Δ*snf2*

Arabidopsis seedlings were grown 9 days on Petri dishes containing MS 1×. Thereafter, seedlings were root-inoculated with disks of actively growing mycelium of the WT or Δ*snf2*-23 -24. Plants were harvested at 0, 48, 72, and 96 hpi, frozen in liquid nitrogen, and stored at -80 ºC 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 above, and specific oligonucleotides were used for the PCR (Supplementary Table S3). Relative expression was normalized against the level of *ACT2* using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

***Botrytis cinerea* pathogenesis assay**

Ten-day-old Arabidopsis seedlings were root inoculated with 500 µl of 1×10^6 conidia/ml of *T. virens* or Δ*snf2* in 0.3× MS liquid medium. Two weeks later Arabidopsis leaves were infected with 10 µl of a spore suspension of *B. cinerea*. The spore suspension was diluted in 40 ml of inoculation buffer (Sucrose 1.37 g, 1M KH2PO4 400µL, 12.5% Tween20: 80µL) and adjusted to 5×10^5 conidia/ml. Lesion sizes of infected plants were quantitatively measured at 6 days post inoculation (dpi) using ImageJ software [http://rsb.info.nih.gov/ij/index.html].

***Pseudomonas syringae* infection assay**

Ten-day-old Arabidopsis seedlings were root inoculated with 500 µl of a 1×10^6 conidia/ml of *T. virens* or Δ*snf2* in 0.3× MS liquid medium. Two weeks later three leaves per plant were infiltrated with *Pst* DC3000 in 10 mM $MgCl_2$ (OD_{600} = 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 $MgCl_2$. Samples were serial-diluted and plated onto King's B medium containing the appropriate antibiotics to determine the colony-forming unit (CFU).

Results

Overview of the transcriptome sequencing

To assess the transcriptional response of *T. virens* to the presence of *A. thaliana* seedlings, total RNA was extracted at 48, 72 and 96 hpi and validated by the expression of *sm-1* (small protein 1) gene, which is induced by the presence of the plant (Djonović et al. 2006; Salas-Marina et al. 2015) by RT-qPCR. The *sm-1* expression was induced by the presence of Arabidopsis, showing its highest peak at 48 hpi (Supplementary Fig. S1). Once the induction of *sm-1* was verified, eighteen RNA libraries from three biological replicates for each sample were sequenced using the SOLiD platform. In total, 219 million reads with an average length of 75 nt were obtained (~16.4 Gb sequenced), with approximately 12 million reads per library. Reads of each library were mapped to the predicted genes of the *T. virens* genome V2 (see materials and methods for details). Reads with more than one hit were eliminated. Our results showed that roughly 40.9% of reads per library were aligned to the predicted genes with only one hit (Table 1).

TMM Pearson's correlation coefficient analysis revealed a high correlation (an average of 0.95) between biological replicates of the same sample (Fig. 1A). Furthermore, MDS analysis showed the formation of 4 groups with similar expression profiles. The first group consisted of *T. virens* control sample collected at 48 hpi (Tv_48h). The second group included the control samples of *T. virens* at 72 and 96 hpi (Tv_72h and Tv_96h), whereas the third group included the *T. virens* sample growing in co-culture with Arabidopsis at 48 hpi (TvAt_48h). Interestingly, the fourth group was comprised by *T. virens*- Arabidopsis samples at 72 and 96 hpi (TvAt_72h and TvAt_96h) (Fig. 1B).

Transcriptional response of *Trichoderma virens* to the presence of *Arabidopsis thaliana*

To identify the differentially expressed genes (DEG) in *T. virens* during its interaction with Arabidopsis at 48, 72 and 96 hpi, pairwise comparisons were carried-out against the control samples (the fungus growing alone at 48, 72 and 96 h) using edgeR package (see Materials and Methods). This analysis showed 691 responsive fungal genes to *A. thaliana*, corresponding to 5.5% of the predicted genes of the fungus (Supplementary Table S4). As shown in Fig. 2A, the largest transcriptional changes occurred at 48 hpi, with 518 genes, of which, 434 were repressed and only 84 induced. At 72 hpi, 236 genes were differentially expressed including 138 induced and 98 repressed. Finally, at 96 hpi, only 153 genes were differentially expressed with 128 induced and 25 repressed (Supplementary Table S4).

A comparison of up-regulated genes showed that the transcriptional response of *T. virens* is specific to each time point because only 31 genes were common to all points, whereas 44, 59 and 52 genes were specifically up-regulated at 48, 72 and 96 hpi, respectively. However, a higher number of up-regulated genes were shared at 72 and 96 h compared to 48 h, confirming that the transcriptional response observed in MDS analysis at 72 and 96 hpi is closely related. The repression of *T. virens* genes during the interaction was more specific at early times. Interestingly, 357 genes (77.9%) were specifically repressed at 48 hpi, compared to 25 (17.6%) and 8 (4.5%) genes at 72 and 96 hpi, respectively (Fig. 2B; Supplementary Table S4), suggesting that the transcriptional response of *T. virens* mainly involves gene repression at early stages of the interaction with Arabidopsis.

To identify which biological processes are involved in the response of *T. virens* during the interaction with *A. thaliana*, functional annotations of differentially expressed genes based on Gene Ontology (GO) terms were performed. Subsequently, an enrichment analysis of more specific GO terms using a Fisher's exact test with an FDR <0.05 was carried out. A total of 87 GO terms were enriched during the *T. virens*-Arabidopsis interaction, of which 49 GO terms were enriched at 48 hpi, including 9 for up-regulated and 40 for down-regulated genes, whereas 21 were enriched at 72 hpi (13 for up- and 8 for down-regulated genes) and only 17 GO terms were enriched in the up-regulated genes at 96 hpi (Supplementary Table S5) Analysis of the more specific GO terms showed that the transcriptional response of *T. virens* at early times mainly involved repression of genes related to extracellular activity, lysosome activity, transport and metabolism of carbohydrates, sphingolipid metabolism, endochitinase activity, cellulose binding, and hydrolysis of polysaccharides activity, among others. Moreover, four GO terms enriched for up-regulated genes at 48 and 72 hpi, included

processes related to integral components of the membrane, copper ion transport, and ferric-chelate reductase activity. In addition, the transcriptional response of *T. virens* at 96 hpi comprised the induction of genes related to copper ion transport, iron ion binding, ferric-chelate reductase activity, and flavin adenine dinucleotide binging (Fig. 3 and Supplementary Table S5).

Hierarchical clustering by expression levels of 907 differentially expressed genes, grouped them in 7 clusters with similar expression patterns. Clearly, the largest transcriptional activity of *T. virens* during the interaction with Arabidopsis was observed at 48 hpi, involving mainly gene repression (Fig. 4). Cluster 1 comprised 130 genes specifically repressed at 48 hpi, related to carbohydrate metabolic process, hydrolase activity of O-glycosyl compounds and glucosidase activity. Cluster 2 included 30 genes strongly repressed at 48 and 72 hpi related to carbohydrate metabolic process, lysosome and vacuole organization, cellulose binding and hydrolase activity of glycosyl compounds. Like cluster 1, cluster 5 comprised 230 repressed genes exclusively at 48 hpi, which are involved in carbohydrate metabolic process, catalytic activity, oxidoreductase activity and monooxygenase activity among others. Cluster 7 included 119 induced genes at 48, 72 and 96 hpi, involved in copper ion transport, ferric chelate reductase activity, oxidoreductase activity, FAD and iron ion binding activity, among others. Moreover, cluster 3 formed by 81 slightly repressed genes at 48, 72 and 96 hpi. Cluster 4 comprised 75 slightly induced genes at 72 hpi, and cluster 6 formed by 53 genes specifically induced at 48 hpi, however, their functions were not clear, since no enriched GO terms were found (Fig. 4; Table 2).

Validation of RNA-seq by real-time RT-PCR analyses

To validate the bioinformatic analysis, nineteen genes were selected and assessed by RT-qPCR based on their expression pattern at 48, 72 and 96 hpi as follow: three genes, two of them induced (*gst-1*, hypothetical-Glutathione-S-transferase and *pfp*-1, hypothetical-Glutamine amidotransferase) and one repressed (*gh7*, Glycoside hydrolase family 7), were selected because they were regulated at the three times of interaction with the plant. Most of RT-qPCR results agreed with the RNA-Seq analysis, except for *pfp-1* that was only induced at 96 h (Fig 5A-C). Six genes regulated at two interaction times, were selected as follow: one repressed gene at 48 hpi and induced at 72 hpi (*oxrdtase*, hypothetical-Oxidoreductase NAD-binding domain); other one induced gene at 48 and 72 hpi (3*hcd*, hypothetical-3-

hydroxyacyl-CoA dehydrogenase); other repressed gene at 48 and induced at 96 hpi (*hsp23*, small heat-shock protein); and 3 induced genes at 72 and 96 hpi (hydrophobin, hypothetical-hydrophobin; *pfp-2*, hypothetical-Glutamine amidotransferase, and *slac-1*, hypothetical-unknown uptake transporter). Five of the six genes tested showed an expression profile similar to the RNA-Seq analysis, however 3*hcd* was not induced in the fungi at any of the tested times, therefore 3*hcd* was considered as a false positive (Fig 5D-I). Finally, 10 genes were selected, because they were regulated just in one interaction time. Three of these genes were induced at 48 hpi (*set*, hypothetical-lysine methyltransferase [HMKT], *snf2*, hypothetical-chromatin remodeler, and *ras*, hypothetical-small Ras GTPase); 3 repressed at 48 hpi (*gt4*, Glycosyltransferase family 4 protein; *gh18*, Glycoside Hydrolase Family 18 protein, and *kin*, hypothetical- Serine/threonine protein kinase); 1 induced at 72 hpi (*gst-2*, hypothetical-Glutathione-S-transferase); 2 induced at 96 hpi (*nox*, hypothetical-Ferric reductase-like transmembrane component and *rta-1*, hypothetical-binding protein to unknown toxic compounds), and 1 repressed at 96 hpi (*aqp*, hypothetical-Aquaporin). Seven genes showed the expression profile detected by SOLiD (*set, ras, gt4, gh18, kin, gst-2*, and *rta-1*), whereas, *snf2* and *nox* were induced at 72h, and *aqp* was repressed at 48 and 72 hpi (Fig. 5J-S). Although the extent of modulation revealed by RT-qPCR and RNA-Seq may differ, the real-time RT-qPCR expression profiles in our analysis were in most of the cases in agreement with the RNA-Seq data (18 of 19 genes).

The Δ*snf-2* is not necessary for growth or conidiation in *T. virens*

SNF2 was previously annotated and predicted by (Schmoll et al. 2016). In a multiple alignment analysis of SNF2 from *T. virens, T. atroviride, T. reesei, N. crassa and G. zeae* revealed that SNF2 belongs to the Rad5/16 like proteins subfamily with a 100% degree of identity (Schmoll et al. 2016). By analyzing the *snf2* domains through the Pfam database (http://pfam.sanger.ac.uk/) (Bateman et al. 2004), we found a domain involved in a variety of processes including transcription regulation, DNA repair, DNA recombination, and chromatin unwinding. The nucleotide sequence of the open reading frame (JGI ID: 113458) is 2,043 bp in length and codes for a 663-amino-acid protein. The *snf2* gene was induced in *T. virens* by the presence of Arabidopsis (Fig. 5K), which suggested a role of *snf2* during *Trichoderma*-Arabidopsis interaction. Therefore, to test our hypothesis *snf2* was deleted and replaced by a hygromycin-resistance cassette in the Gv29-8 wild-type background. We

obtained several independent-hygromycin resistant colonies, which were tested by qPCR to corroborate the *snf2* gene replacement, as well as the copy number of *hph* (Supplementary Table S2). *snf2* was detected in the WT but not in Δ*snf2-23* and Δ*snf2*-24 transformants, whereas the *hph* cassette was detected in just one copy (Supplementary Table S2), therefore, we used these strains for further experiments (Supplementary Table S2). Conidiation and radial growth was evaluated in the Δ*snf2*-23 and Δ*snf2*-24 mutant strains at 24, 48, 72 and 96 h as well as in the WT, however the Δ*snf-2*-23 and 24 were not affected in conidiation or radial growth (Supplementary Fig. S2).

The absence of *snf2* did not impair the ability of *T. virens* to grow over *R. solani* and *B. cinerea*

To determine the capability of Δ*snf2* to grow over *R. solani* or *B. cinerea*, dual cultures of the WT and Δ*snf2* strains against such phytopathogens were performed. Disks of actively growing mycelium of the phytopathogens and *Trichoderma* strains were placed on the opposite sides of PDA plates and allowed to interact by 7 days. The WT and Δ*snf2* grew over the two phytopathogens tested and no differences were observed between the WT and the Δ*snf2* (Supplementary Fig. S3).

Mycelial free culture Filtrates (MFCF) of Δ*snf2* showed diminished inhibition of *R. solani* radial growth

To better understand the role of Δ*snf2* in secondary metabolism and antimicrobial activity in *T. virens*, WT and Δ*snf2* were grown in PDB by 4 days. Then PDA plates 1× containing 40% of MFCF were prepared. Subsequently, a plug of actively growing mycelium of *R. solani* was inoculated on PDA amended with the-MFCF plates or in PDA as control. The Δ*snf2* MFCF showed an enhanced inhibition effect on the growth on *R. solani* compared to the WT MFCF (Fig. 6). In contrast, there was no difference in between the WT and Δ*snf2* MFCF in the radial growth inhibition of *B. cinerea* (Supplementary Fig. S4).

Deletion of *snf2* in *T. virens* did not affect plant growth-promotion on Arabidopsis

To study the role of *snf2* in plant-growth promotion induced by *T. virens*, Arabidopsis seedlings were grown in pots and their roots were inoculated with the WT or Δ*snf2*. Plants inoculated with the Δ*snf2* showed statistically similar fresh and dry weights compared to

those treated with the WT, suggesting that the absence of *snf2* did not affect the promoting activity of *T. virens* (Supplementary Fig. S5).

Deletion of *snf2* resulted in an enhanced plant protection against *P. syringae*, but not against *B. cinerea*

To determine if SNF2 is necessary to elicit the plant defense responses by *T. virens,* the expression profiles of the well-known Arabidopsis marker genes *PR-1a* (SAR) and *PDF1.2* (ISR) were assessed by RT-qPCR in co-cultures with the WT and Δ*snf2*. The expression profile of *PR-1a* and *PDF1.2* was induced by the WT and Δ*snf2*, however the extent of the responses was higher in plants treated with Δ*snf2* (Fig. 7A-B).

Based on the expression profiles of *PR-1a* and *PDF1.2* in response to the Δ*snf2*, we asked whether the mutant provides more protection against the fungal pathogen *B. cinerea* and the bacterial pathogen *Pst* DC3000. Plants treated with Δ*snf2* exhibited an enhanced resistance to *Pst* DC3000 compared to plants treated with the WT (Fig. 7E), however, plants infected with *B. cinerea* showed no significant differences in the % of lesion size in between the WT and Δ*snf2* (7.8 and 7.02, respectively) treated plants (Fig. 7C-D).

Discussion

Here, we analyzed the transcriptomic response of *T. virens* to Arabidopsis plants at 48, 72 and 96 h by SOLiD (Sequencing by Oligonucleotide Ligation and Detection). Six hundred ninety one out of 12, 427 predicted genes in the *T. virens* genome (Kubicek et al. 2011) were differentially regulated by the presence of Arabidopsis, corresponding to a 5.5% of the *T. virens* genome. RNA-Seq validation by qRT-PCR revealed a 94% of accuracy in the data as compared with RT-qPCR, with only one gene out of 19 showing a different profile from that found in RNA-Seq. The differences in sensitivity in between RNA-Seq and RT-qPCR may be a plausible explanation of the discrepancies between these two analyses.

Enriched metabolic categories in *T. virens* transcriptome indicated cellular processes prioritization in the presence of Arabidopsis seedlings. The biological processes of carbohydrate transport (48h), and carbohydrate metabolic process (48 and 72h) were repressed in the fungus, as well as the molecular functions of Monooxygenase activity, Endochitinase activity, β-glucosidase activity, Glucan endo- 1,3-α-glucosidase activity, Hydrolase activity, O-glycosyl compounds, and Cellulase binding, these enzymes have been

previously categorized into different families by www.cazy.org as Carbohydrate-Active enzymes, or 'CAZymes' which function degrading, modifying, or creating glycosidic bonds (Levasseur et al. 2013). During plant root colonization *Trichoderma* deploy a broad array of specialized 'CAZymes' for plant cell-wall polymers degradation, which can be classified as: Glycoside Hydrolases (GH), Glycosyltransferases (GT), Polysaccharide Lyases (PL), Carbohydrate Esterases (CE), Carbohydrate-Binding Modules (CBMs), and Auxiliary Activities (AAs) (Martinez et al. 2008; Schmoll et al. 2016). In this analysis, a plethora of CAZymes encoding-genes were remarkably down-regulated by *T. virens* at 48 h in the presence of *A. thaliana*, the next list includes the following CAZymes: putative glycoside hydrolases, GH1 (JGI ID: 74123 and 41540), GH2 (JGI ID: 48076 and 181866), GH3 (JGI ID: 74688, 151663, 29366, 83777, 58670, and 192759), GH5 (JGI ID: 35701, 61403, 76400), GH6 (JGI ID: 78675), GH7 (JGI ID: 182161, 90504), GH11 (JGI ID: 8282 and 59409), GH13 (JGI ID: 53956, and 53515), GH16 (JGI ID: 55235), GH18 (85551, 83539, 80909, 194859, 89999, 213202, 42107,and 52337), GH31 (JGI ID: 34797), GH45 (JGI ID: 59335) , GH71 (JGI ID: 77550), and GH74 (JGI ID: 59360); putative Glycosyltransferase, GT4 (JGI ID: 83336); putative Polysaccharide lyases, PL7 (JGI ID: 70901 and 52707), and PL8 (JGI ID: 62770); putative Carbohydrate esterases, CE1 (JGI ID: 64949), CE5 (JGI ID: 51211), CE10 (JGI ID: 190792, 84609, 50483, 40764, 195940, 118304, and 151341), and CE16 (JGI ID: 58493); and finally putative Carbohydrate-binding modules: CBM1 (JGI ID: 46583, 49838), CBM13 (JGI ID: 61360), and CBM50 (JGI ID: 128337). In addition to this, 18 putative monooxygenases encoding-genes were down-regulated as well by *T. virens* at 48 h. The Polysaccharide Monooxygenases (PMOs), have been recently assigned to the novel category of AAs, which is divided into 10 families or subfamilies according to the catalytic reaction mechanism or specific substrate (Levasseur et al. 2013). AAs are responsible for generating highly reactive-non-specific free radicals, which then catalyze the cleavage of carbon-carbon and ether inter-unit bonds using a metal-dependent oxidative mechanism (Beeson et al. 2011; Medie et al. 2012; Levasseur et al. 2008). With that in mind, it would be reasonable to attribute the down-regulation of the CAZymes to the lack of metals as cofactors for PMOs in the organism. Interestingly, the down-regulation of enzymes involved in carbohydrate metabolism was only found at 48 h, remaining unchanged in later times of the interaction (72 and 96 h). Whereas, the biological process of Copper Ion Transport (48, 72, and 96h), as well as the molecular functions of Iron ion binding (96h), Ferric-chelate

reductase activity (48, 72 and 96 h), Copper ion transmembrane transport activity (48, 72, and 96h), were induced by *T. virens* in the presence of *A. thaliana*, suggesting a role of these pathways in providing the necessary metals to *T. virens* PMOs. In support of this idea, three putative CTR transporters (JGI ID: 49075, 50748, and 53545) were found induced at 48, 72, and 96 h in the fungus. On the other hand, the repression of CAZymes during *T. virens* coculture with Arabidopsis, may have been due to a phenomenon called carbon catabolite repression (CCR). CCR functions switching off certain enzymes required to utilize less-favored carbon sources when a more readily available carbon source is present in the medium (Glass et al. 2013; Adnan et al. 2018), this mechanism has been previously reported in *Aspergilli* (Ruijter and Visser 1997), *Neurospora crassa* (Sun and Glass 2011), and *T. reesei* (Nakari-Setälä et al. 2009). The reduction of CAZymes involved in the degradation of plant cell-wall in the *T. virens* transcriptome, may also be the result of a self-defense mechanism of *Trichoderma* in an attempt to escape or minimize the plant defense responses. Plenty of investigations have found a decreased repertoire of carbohydrate-active enzymes in the genome of diverse biotrophic life style microorganisms such as: *Laccaria biccolor* (Martin et al. 2008), Périgord black truffle (Martin et al. 2010), *Hyaloperonospora arabidopsidis* (Baxter et al. 2010), and *Blumeria graminis* (Spanu et al. 2010), the authors claimed that the diminished number of CAZyme illustrate the genetic basis of symbiosis-related traits of the most ancient lineage of plant biotrophs. Unlike our data, in *T. hamatum* T7, *T. harzianum* T34 and *T. virens* T87 the presence of tomato plants triggers a significant activation of fungal carbohydrate metabolism and transport processes in the mutualistic *Trichoderma*–tomato interaction. However, it must be taken into account that the experimental conditions used in each analysis, as coculture times, host plant and *Trichoderma* species varied greatly between each study (Rubio et al. 2012).

To our knowledge this is one of the first works analyzing a chromatin remodeler mutant of *T. virens* and their impact and involvement in plant responses, including: plant growth promotion, resistance systemic triggering, and plant protection assays against *B. cinerea* and *P. syringae*. Our data showed that the lack of *snf2* caused an overexpression of the marker genes of the ISR and SAR pathways in Arabidopsis, and consequently plant roots-inoculated with Δ*snf2* and challenged with *P. syringae* show a greater resistance to the pathogen. The general question in chromatin remodelers analysis is whether these proteins act regulating general or specific transcriptional regulators (Tsukiyama 2002; Kwon and Wagner 2007).

Our work demonstrates that the *snf2* deletion does not affect cell viability, conidiation, development, antagonism, antibiosis against *B. cinerea,* and plant growth promotion activity in *T. virens*, pointing to a specific regulation in the organism. Our data suggests that SNF2 may function as a negative regulator of genes involved in turning off the SAR and ISR pathways in *T. virens.* The specificity of the *Trichoderma* SNF2, resembles the one observed in *A. thaliana*, where a SWI/SNF class chromatin remodeling ATPase SPLAYED (SYD), regulates the expression of genes downstream of the jasmonate (JA) and ethylene (ET) signaling pathways where it was demonstrated that SYD is required for resistance against the necrotrophic pathogen *Botrytis cinerea* but not for the biotrophic pathogen *Pseudomonas syringae* (Walley et al. 2008). Another possibility is that SNF2 is required to allow DNA methyltransferases, or other necessary factors to gain access to the chromatin, a mechanism already reported in Arabidopsis where a *DDM1* (decrease in DNA methylation) encoding a SWI2/SNF2-like protein (Jeddeloh et al. 1999) and $Lsh^{-/-}$ showed substantial loss of methylation throughout the genome (Dennis et al. 2001). Interestingly, we also found that the MFCF from *snf2*, showed a diminished antimicrobial effect on *R. solani*, but was unaffected against *B. cinerea.* The antagonistic activity of *T. virens* has been widely recognized, a recent survey of >1,100 *Trichoderma* strains from 75 molecularly defined species, showed that all the species tested possess mycoparasitic potential against three causative agents of plant diseases: *Alternaria alternata*, *Botrytis cinerea* and *Sclerotinia sclerotiorum* (Druzhinina et al. 2011). However, the poor *R. solani* radial growth inhibition by *snf2* MFCF, suggest that *snf2* is affected in the production of a metabolite(s) important to inhibit or kill this phytopathogen.

Acknowledgments

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Table 1. Alignment statistics to the predicted genes in *T. virens* genome version 2

Sample	Total reads	Total mapped reads	%	Filtered reads with unique hit	%
Tv_48h.1	10,638,040	4,742,561	44.6	4,670,185	43.9
Tv_48h.2	8,948,382	3,998,400	44.7	3,922,972	43.8
Tv_48h.3	16,175,272	6,620,122	40.9	6,513,256	40.3
Tv_72h.1	10,587,525	4,420,421	41.8	4,339,249	41
Tv_72h.2	9,912,382	4,174,130	42.1	4,105,547	41.4
Tv_72h.3	29,927,732	11,940,497	39.9	11,735,297	39.2
Tv_96h.1	10,585,610	4,368,118	41.3	4,280,790	40.4
Tv_96h.2	9,116,693	3,728,951	40.9	3,657,705	40.1
Tv_96h.3	16,969,904	6,738,066	39.7	6,627,744	39.1
TvAt_48h.1	9,373,116	3,947,810	42.1	3,871,633	41.3
TvAt_48h.2	9,660,475	4,087,627	42.3	4,010,811	41.5
TvAt_48h.3	12,018,028	5,140,130	42.8	5,048,819	42
TvAt_72h.1	11,255,646	4,760,713	42.3	4,667,094	41.5
TvAt_72h.2	9,887,273	4,011,968	40.6	3,933,627	39.8
TvAt_72h.3	14,219,218	5,656,869	39.8	5,562,212	39.1
TvAt_96h.1	11,747,581	4,949,453	42.1	4,851,428	41.3
TvAt_96h.2	5,848,710	2,512,642	43.0	2,464,191	42.1
TvAt_96h.3	12,328,096	4,941,085	40.1	4,855,695	39.4

Table 2. List of more specific GO terms enriched in the genes belonging to each cluster created by hierarchical clustering

Cluster	#Genes	GO Cat[a]	GO ID	GO name	%Seqs[b]	FDR[c]
1	130	BP	GO:0005975	carbohydrate metabolic process	18.4	2.0E-03
		MF	GO:0004553	hydrolase activity, hydrolyzing O-glycosyl compounds	22.4	1.1E-05
		MF	GO:0016798	hydrolase activity, acting on glycosyl bonds	22.4	1.1E-05
		MF	GO:0015926	glucosidase activity	10.5	2.0E-03
2	30	BP	GO:0005975	carbohydrate metabolic process	56.5	3.0E-10
		BP	GO:0080171	lytic vacuole organization	8.7	3.4E-02
		BP	GO:0007040	lysosome organization	8.7	3.4E-02
		BP	GO:0007033	vacuole organization	8.7	4.3E-02
		MF	GO:0001871	pattern binding	39.1	1.5E-14
		MF	GO:0030248	cellulose binding	39.1	1.5E-14
		MF	GO:0030247	polysaccharide binding	39.1	1.5E-14
		MF	GO:0030246	carbohydrate binding	39.1	3.0E-11
		MF	GO:0004553	hydrolase activity, hydrolyzing O-glycosyl compounds	56.5	2.0E-10
		MF	GO:0016798	hydrolase activity, acting on glycosyl bonds	56.5	2.6E-10
		MF	GO:0004348	glucosylceramidase activity	8.7	3.4E-02
		CC	GO:0005576	extracellular region	39.1	1.5E-10
		CC	GO:0005764	lysosome	8.7	3.4E-02
		CC	GO:0000323	lytic vacuole	8.7	4.3E-02
3	81	No enriched GO terms were found				
4	75	No enriched GO terms were found				
5	203	BP	GO:0005975	carbohydrate metabolic process	13.0	2.7E-02
		MF	GO:0003824	catalytic activity	86.1	3.5E-04
		MF	GO:0016491	oxidoreductase activity	35.7	2.2E-03
		MF	GO:0018734	butyrolactone hydrolase activity	3.5	1.8E-02
		MF	GO:0004497	monooxygenase activity	11.3	1.8E-02
		MF	GO:0018733	3,4-dihydrocoumarin hydrolase activity	3.5	1.8E-02
		MF	GO:0018732	sulfolactone hydrolase activity	3.5	1.8E-02
		MF	GO:0018731	1-oxa-2-oxocycloheptane lactonase activity	3.5	1.8E-02
		MF	GO:0046906	tetrapyrrole binding	10.4	2.2E-02
		MF	GO:0020037	heme binding	10.4	2.2E-02
6	53	No enriched GO terms were found				

7	119	BP	GO:0006825	copper ion transport	4.3	1.2E-02
		MF	GO:0000293	ferric-chelate reductase activity	8.6	1.0E-06
		MF	GO:0050660	flavin adenine dinucleotide binding	12.9	2.4E-03
		MF	GO:0005506	iron ion binding	14.3	4.2E-03
		MF	GO:0005375	copper ion transmembrane transporter activity	4.3	1.8E-02
		CC	GO:0016021	integral component of membrane	32.9	5.7E-06

[a] Gene Ontology categories: BP, Biological process; MF, Molecular function; CC, Cellular component.
[b] Percentage of genes belonging to each GO term of the test set.
[c] FDR < 0.05.

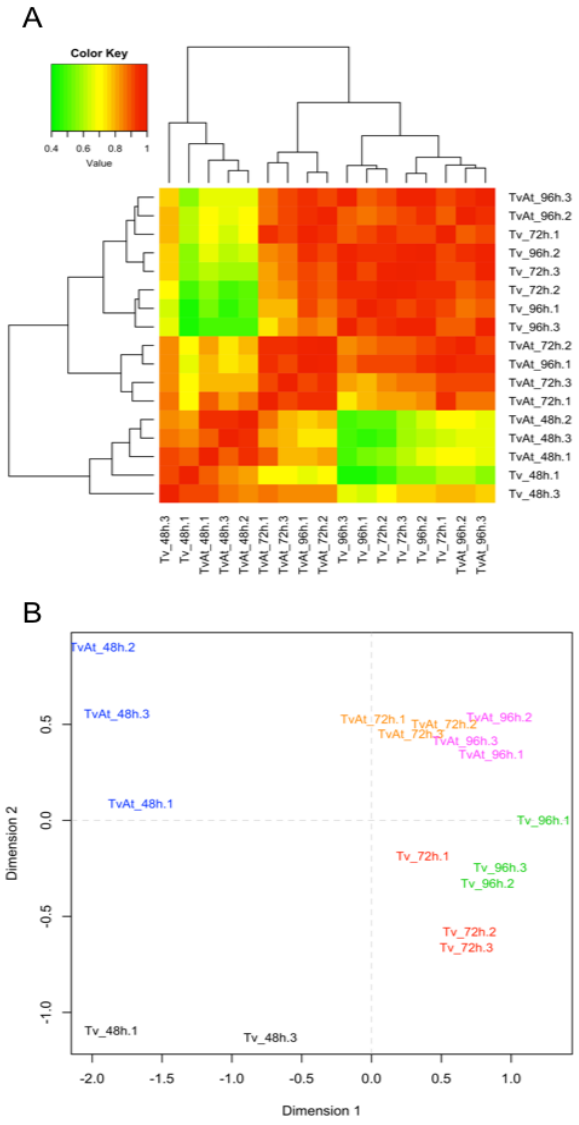


Fig. 1. Overview of gene expression profiles of biological replicates. A, Heat-map of Pearson's correlation of the transcript expression levels of control and treated samples at 48, 72 and 96 hpi. Color key indicates the Pearson's correlation values from 0.4 to 1, Red – highly correlated samples, green – low correlation. B, Multidimensional scaling plot showing the distances of the biological coefficient of variation between each pair of samples. Tv (*T. virens* control); TvAt, (Interaction *T. virens-A. thaliana*). The biological replication number is indicated after the dot. Raw count data were normalized using the edgeR bioconductor package (Robinson et al. 2010).

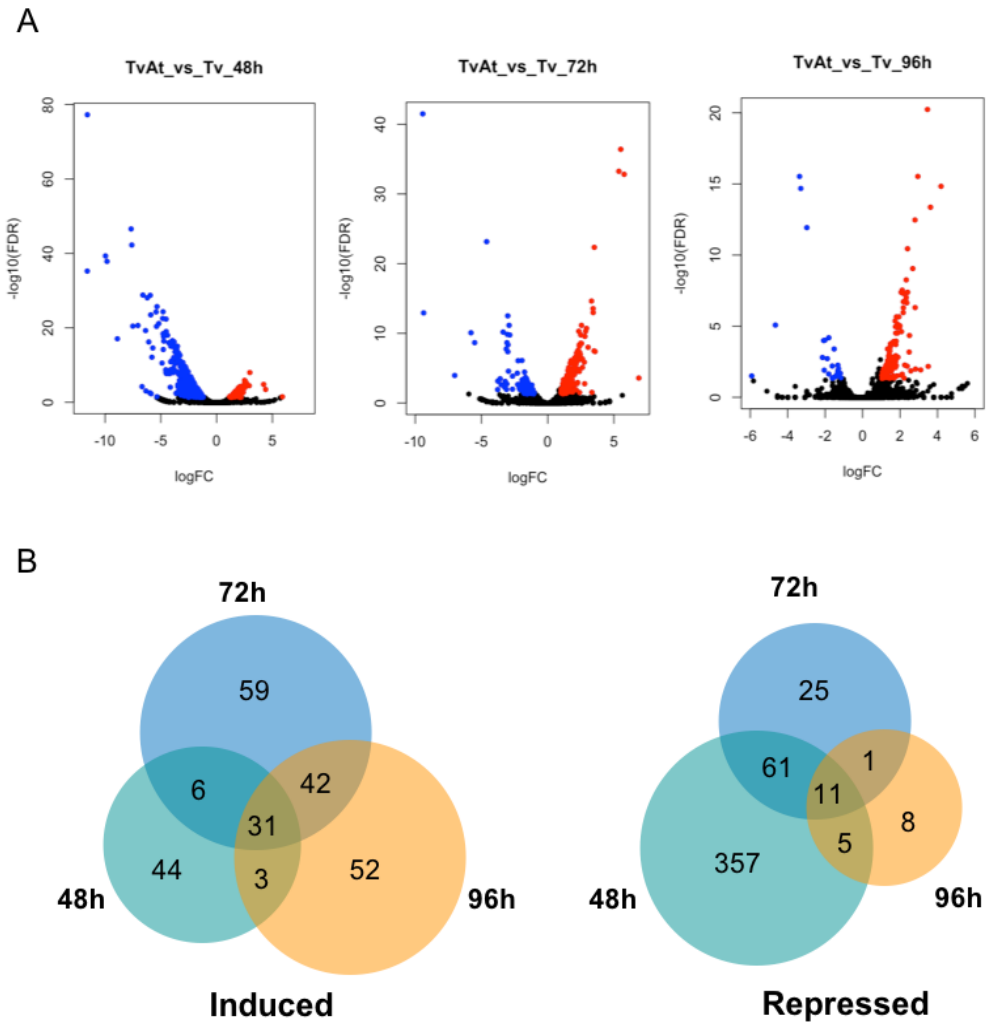


Fig. 2. Transcriptional response of *T. virens* to *Arabidopsis thaliana* seedlings. A, Differential expression of *T. virens* genes during its interaction with *A. thaliana* (TvAT) vs *T. virens* control at 48, 72 and 96 hpi. Differentially expressed genes with an FDR <=0.05 are shown in red and blue, up-regulated genes (log2FC> 1) are shown in red and down-regulated (log2FC<-1) are shown in blue. B, Venn diagram showing the distribution of induced and repressed genes (TvAt *vs* Tv) at 48, 72 and 96 hpi.

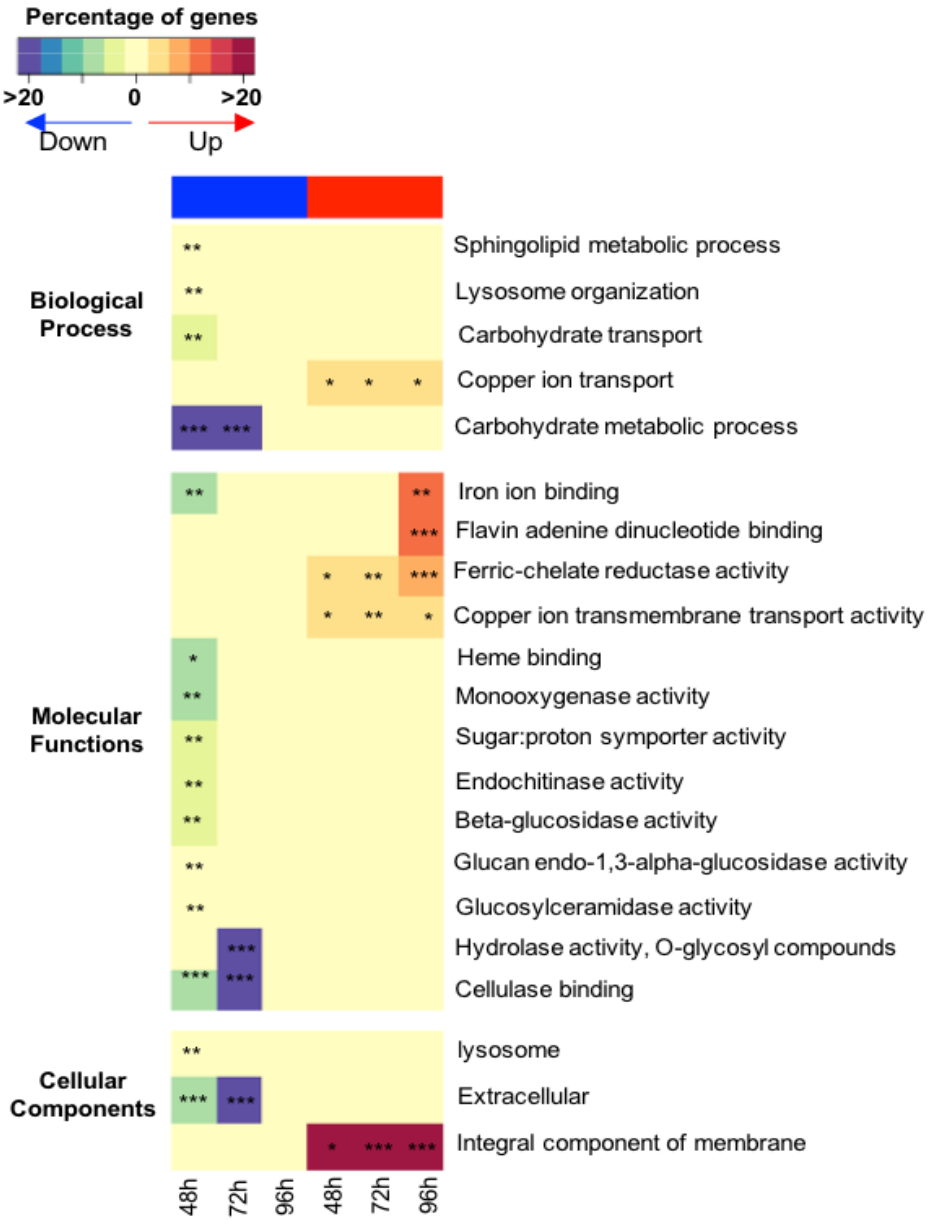


Fig. 3. Heatmap of gene ontology enrichment of *T. virens* up- and down-regulated genes during its interaction with Arabidopsis seedlings. Each column contains the up- and down-regulated GO terms. Only the most specific GO terms are shown (FDR< 0.05). Color scale represents the percentage of genes belonging to each GO term. Asterisks represent the statistical significance of enrichment (*FDR<0.05, **FDR<0.01, ***FDR<0.001).

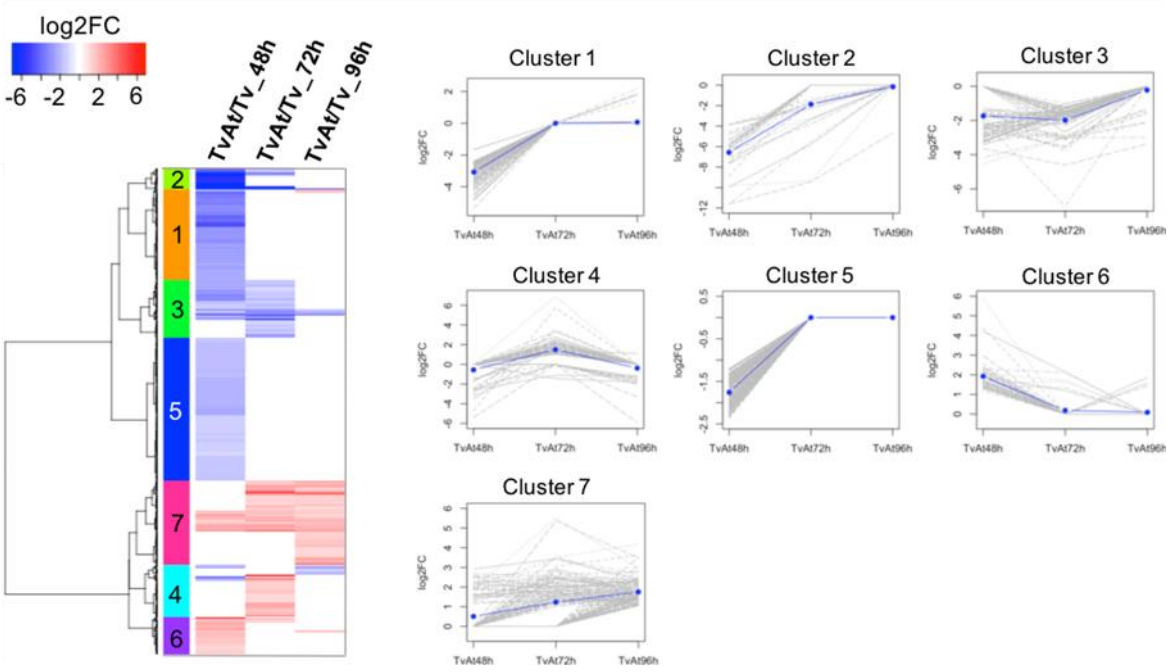


Fig. 4. Hierarchical clustering of the differential gene expression profiles. Heatmap showing clustering of the DEG during *T. virens*-Arabidopsis interaction. The dendrogram indicates the relationship between gene profiles using the agglomeration method ward.D. Left column colors indicate the clusters created of genes with similar expression profiles, the number of each cluster is indicated. The color key shows the log2 of fold change values (TvAT vs Tv at 48, 72 and 96 hpi). Colors red and blue indicate the up- and down-regulated genes using an FDR $\leq$ 0.05 and log2FC > 1. The expression patterns for each cluster generated are shown on the right. Each graph shows the expression profiles for each gene with gray lines and the average for all genes belonging in the cluster is showing in the blue line.

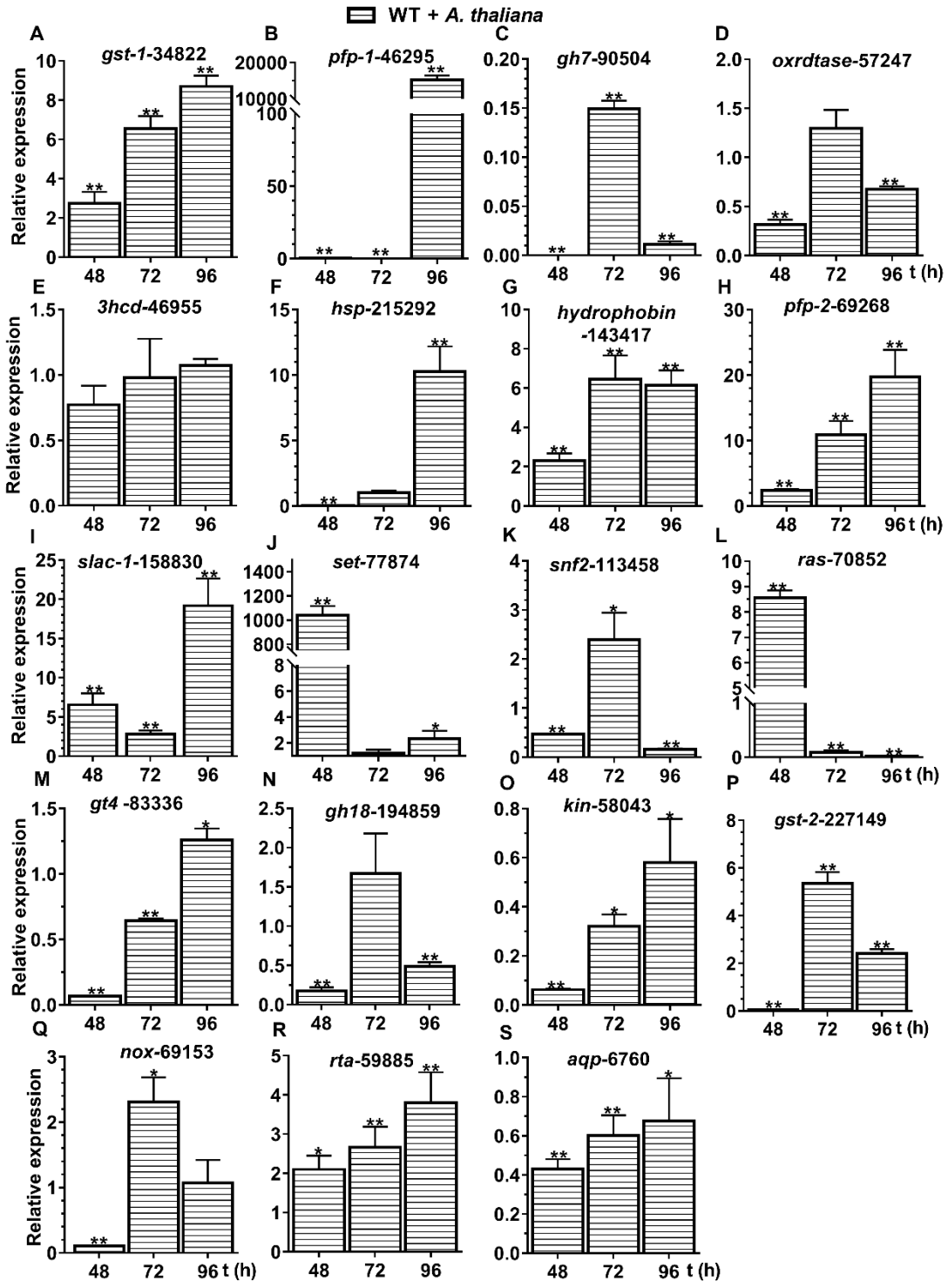


Fig. 5. Validation of RNA-seq by RT-qPCR. *T. virens* was co-cultured with Fifteen-day-old Arabidopsis seedlings and mycelium was collected at 48, 72 and 96 hpi. *Trichoderma* growing alone in MS medium was included as control. The expression levels of: A, putative *gst*-1 (JGI ID: Tv_34822). B, putative *pfp-1* (JGI ID: Tv_46295). C, *gh7* (JGI ID:90504). D,

putative *oxrdtase* (JGI ID:57247). E, putative *hcd* (JGI ID:46955). F, putative *hsp* (JGI ID:215292), G, putative *hydrophobin* (JGI ID:143417). H, putative *pfp-2* (JGI ID:69268). I, putative *slac-1* (JGI ID:158830). J, putative *set* (JGI ID:77874). K, putative *snf2* (JGI ID:113458). L, putative *ras* (JGI ID:70852). M, *gt4* (JGI ID:83336). N, *gh18* (JGI ID:194859). O, putative *kin* (JGI ID:58043). P, putative *gst-2* (JGI ID:227149). Q, putative *nox* 69153). R, *rta-2* (JGI ID:59885). S, putative *aqp* (JGI ID:6760)., were analyzed by RT-qPCR. Results are reported as fold-change compared to the fungi growing without Arabidopsis. *tef-1* was used as control gene to normalize the expression level of the genes tested using the $2^{-\Delta\Delta Ct}$ method. The RNA-Seq results were validated with one the biological replicates of the transcriptome.

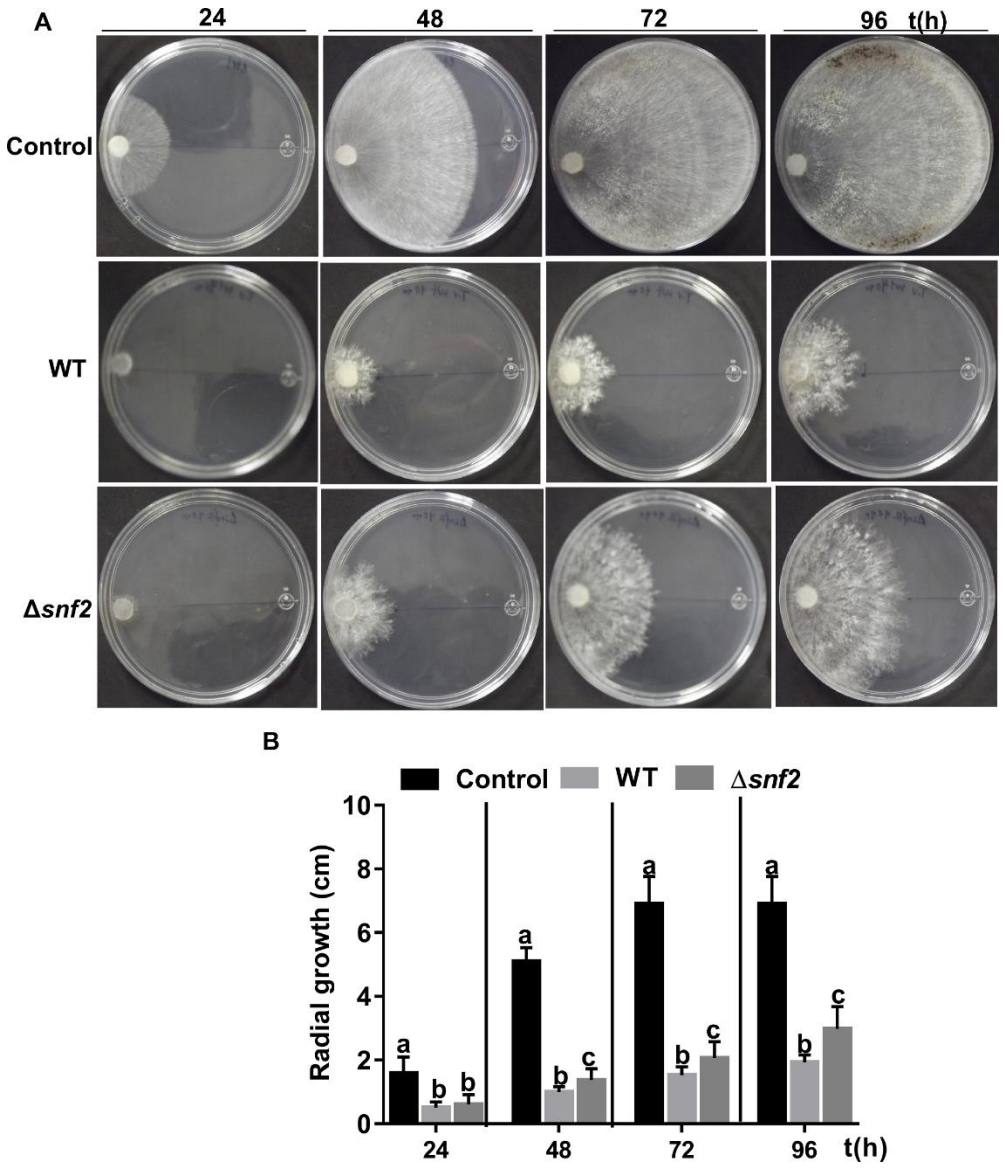


Fig. 6. Mycelial free culture Filtrates (MFCF) of Δ*snf2* showed diminished inhibition of *R. solani* radial growth. The WT and Δ*snf2* strains were grown in PDB media for 4 days and MFCF were used to prepare PDA plates at 40%. A, Representative pictures of *R. solani* radial growth at 24, 48, 72 and 96h on MFCF WT and Δ*snf2* strains. B, Radial growth of *R. solani* at 24, 48, 72 and 96 h PDA plates amended with MFCF WT and Δ*snf2* strains. Data from B show the mean ± SD of three independent experiments (3 plates for treatment). Results were validated with an analysis of variance statistical analysis with a Tukey multiple comparison test (α= 0. 05), different letters represent means statistically different at the 0.05 level.

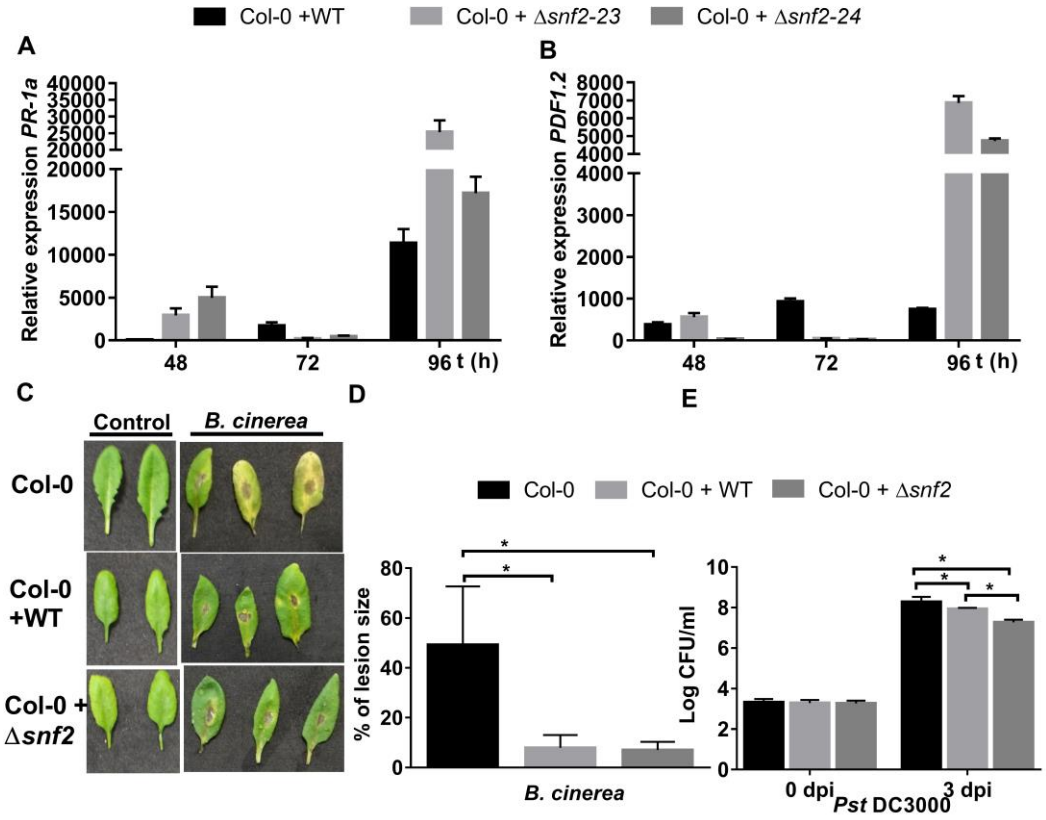
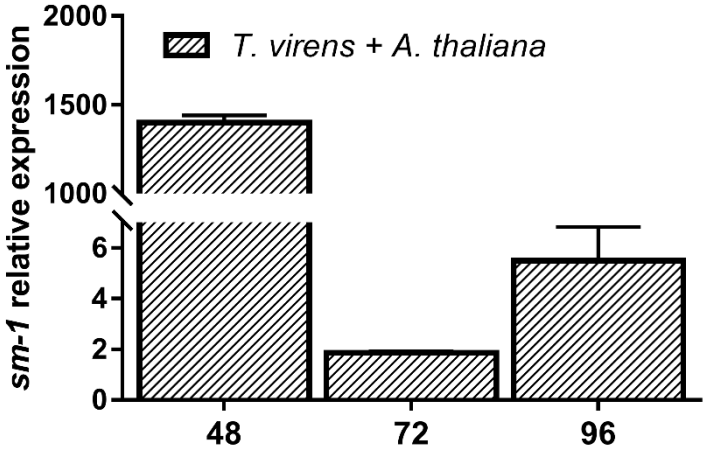
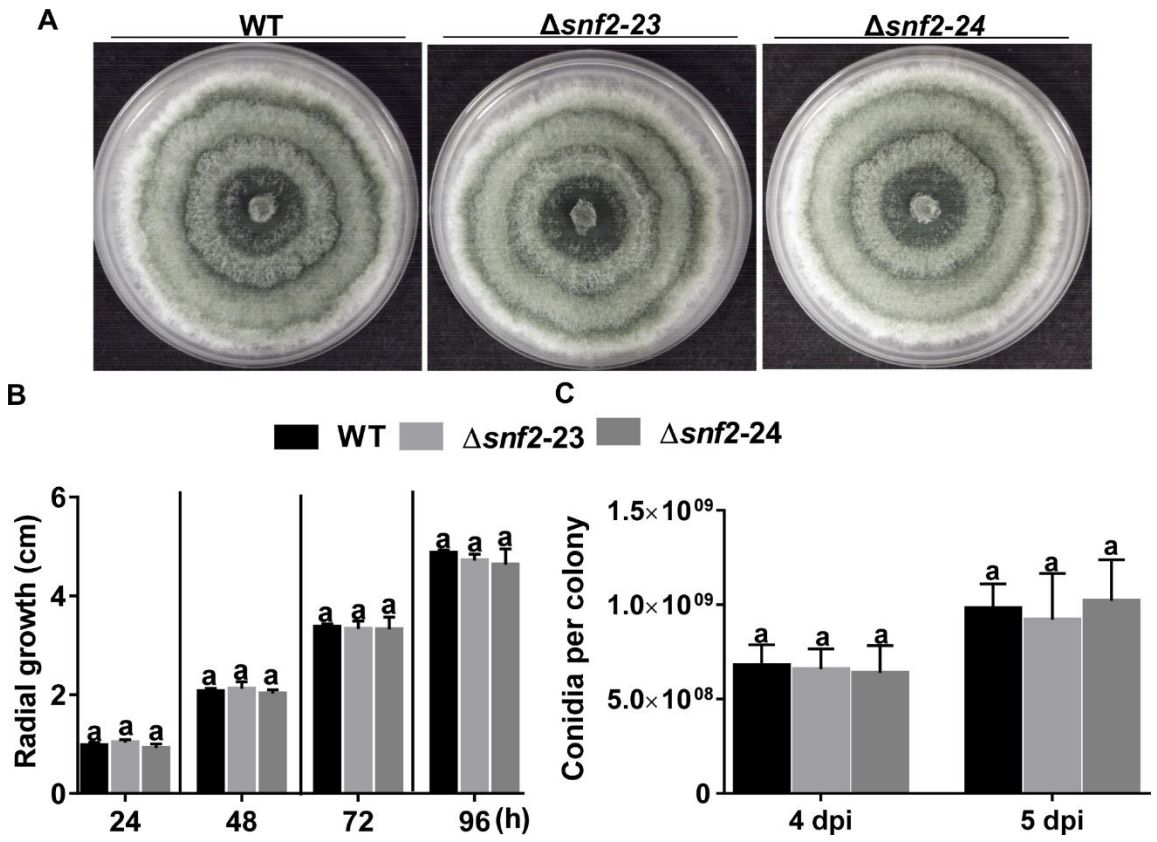


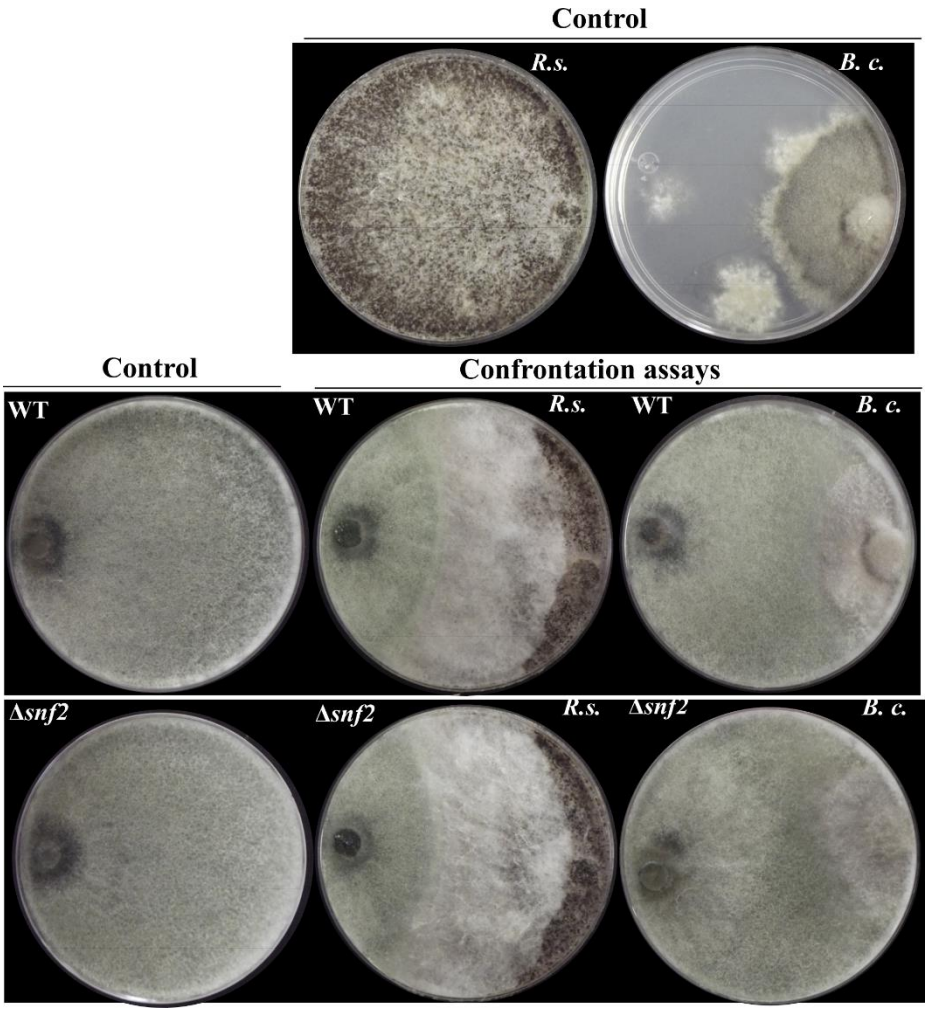
Fig. 7. Deletion of *snf2* resulted in an enhanced plant protection against *P. syringae*. Ten-day-old Arabidopsis seedlings grown on MS medium were root-inoculated with the WT or Δ*snf2* and the expression levels of A, *PR-1a* or B, *PDF1.2* were analyzed by RT-qPCR at 24, 48, 72 and 96 hpi. RT-qPCR results are reported as fold-change compared to Arabidopsis growing without the fungi. Arabidopsis *ACT2* gene was used as control to normalize the expression of *PR-1a* and *PDF1.2* using the $2^{-\Delta\Delta Ct}$ method. Ten-day-old Arabidopsis seedlings grown in soil and inoculated with the WT or Δ*snf2*, and 2 weeks later leaves were infected with C *B. cinerea* or inoculating buffer as control. D Lesion sizes of infected plants with *B. cinerea* were determined using ImageJ at 6 dpi**.** E Colony forming units of *Pst* DC3000 at 0 and 3 dpi in leaves of treated and untreated plants with WT or Δ*snf2*. Data from (A-B) show the mean ± SD of at least 5 plates with 7 plants. The experiment was repeated twice with similar results. Data from (C-D) show the mean ± SD of at least 12 leaves. The experiment was repeated twice with similar results. Asterisks indicate significant difference (independent t-test, *P < 0.05 and **P < 0.01).



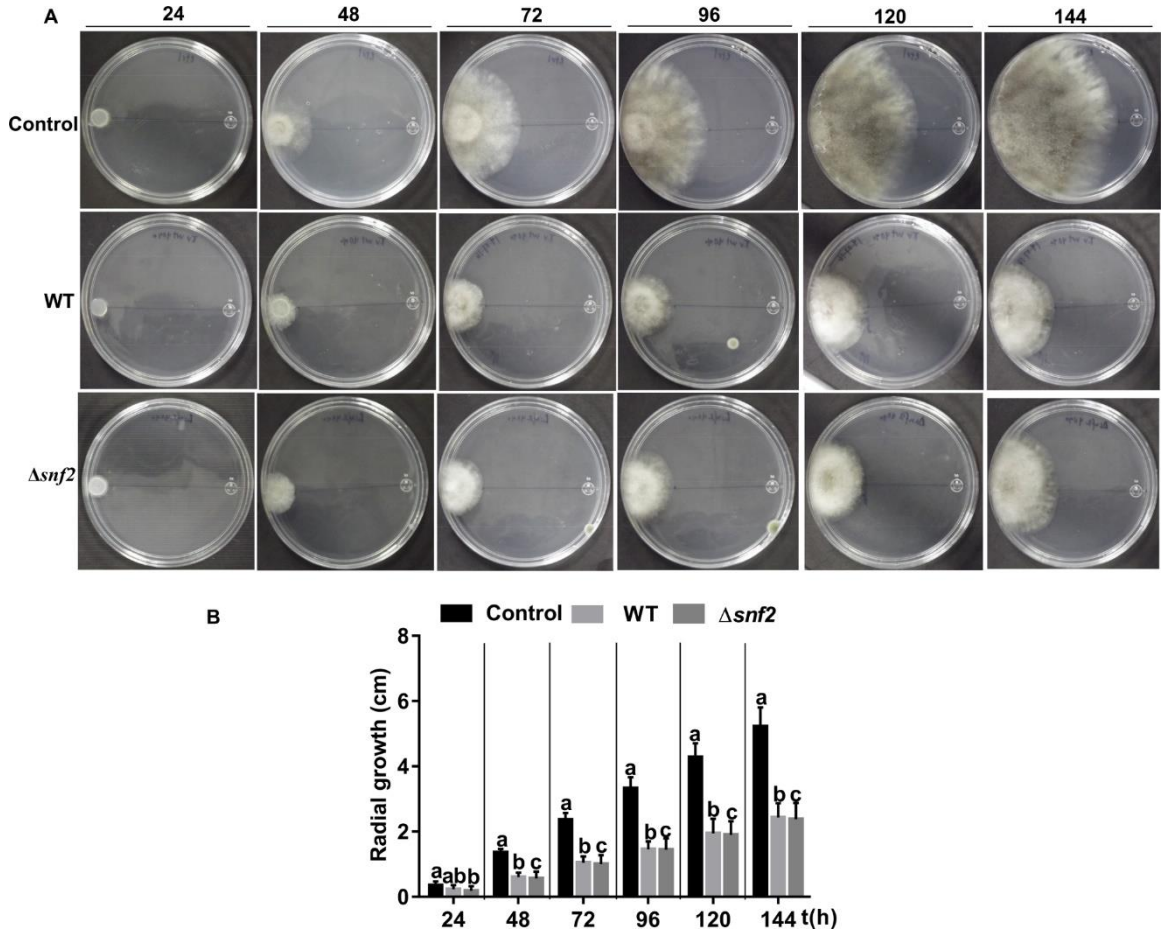
Supplementary Fig. S1. The mRNA levels of *sm-1* were induced in *Trichoderma* in the presence of Arabidopsis Col-0 seedling. 15-day-old Arabidopsis Col-0 seedlings were root-inoculated with *T. virens*-WT actively growing mycelium. *Trichoderma* growing in MS medium alone was included as control. The mycelium were collected at 48, 72 and 96 hpi. The expression levels of *sm-1* were analyzed by RT-qPCR. The graphs show the mean expression levels ± SD. RT-qPCR results are reported as fold-change compared to the fungi growing without Arabidopsis. *tef-1* was used as control gene to normalize the expression of *hda-2* using the $2^{-\Delta\Delta Ct}$ method.



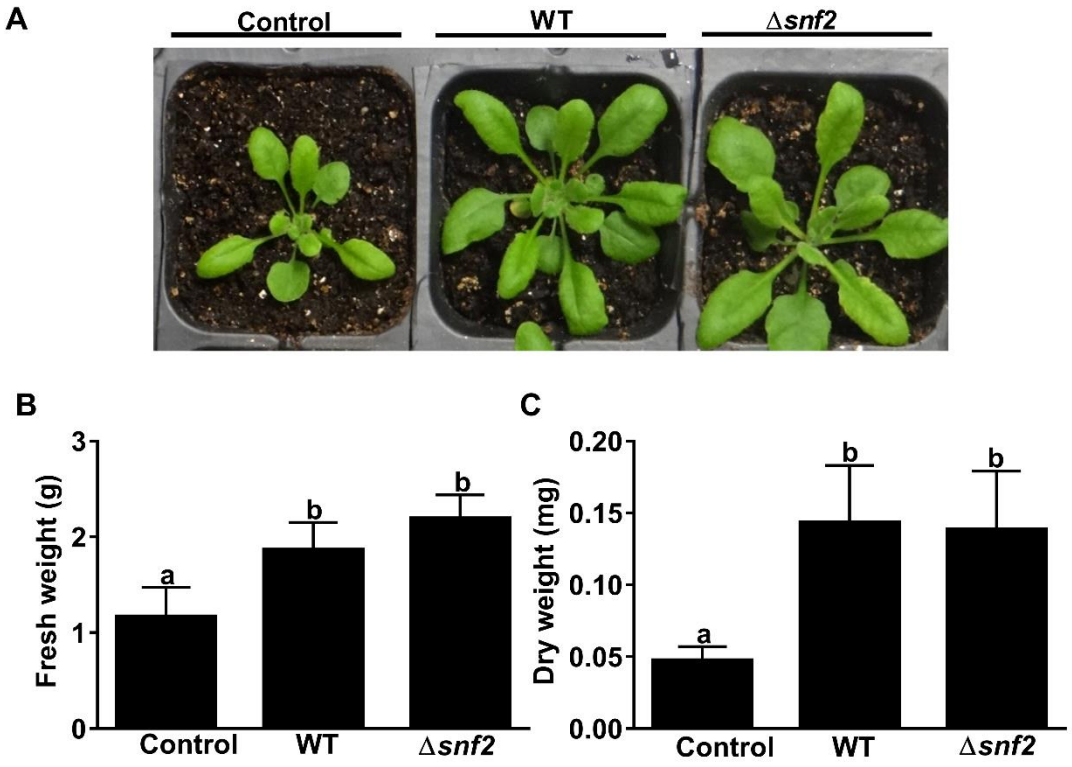
Supplementary Fig. S2. *T. virens* deletion mutants in *snf2* are not affected in growth or conidiation. A Representatives photographs showing the growth of the WT, Δ*snf2-23* and -24 after 72h of growing on PDA. B Radial growth of the WT, Δ*snf2-23* and -24 at 24, 48, 72 and 96 h of growing on PDA. C Production of conidia of WT, Δ*snf2-23* and -24 strains after 96 and 120 h of growing on PDA. Data from (A-C) show the mean ± SD of three independent experiments, with at least 3 plates per condition. Results were validated with an analysis of variance statistical analysis with a Tukey multiple comparison test (α= 0. 05), different letters represent means statistically different at the 0.05 level.



Supplementary Fig. S3. The absence of *snf2* did not impair the ability of *T. virens* to grow over the phytopathogens *R. solani* and *B. cinerea.* Confrontations assays were carried out by 7 days at 28 °C in darkness. Representative photographs of dual confrontation plates with the WT and Δ*snf2* (always left) against *R. solani* and *B. cinerea* plant-pathogens (always right). The dual confrontation was repeated thrice with similar results.



Supplementary Fig. S4 Mycelial free culture Filtrates (MFCF) of Δ*snf2* did not affect *B. cinerea* radial growth. The WT and Δ*snf2* strains were grown in PDB media for 4 days and MFCF were used to prepare PDA plates at 40%. A Representative pictures of *B. cinerea* radial growth at 24, 48, 72, 96, 120 and 144h of culture on MFCF. (b) Radial growth of *B. cinerea* at 24, 48, 72, 96, 120 and 144h of culture on PDA plates containing MFCF. Data from (B) show the mean ± SD of three independent experiments (3 plates for treatment). Results were validated with an analysis of variance statistical analysis with a Tukey multiple comparison test (α= 0. 05), different letters represent means statistically different at the 0.05 level.



Supplementary Fig. S5 Deletion of *snf2* did not affect the plant growth-promoting activity of *T. virens*. Ten-day-old Arabidopsis seedlings grown on MS medium, and co-incubated for 14 days with the WT or Δ*snf2*. A Representative picture of Arabidopsis grown under the indicated treatments. B Fresh weight. C Dry weight. Data from (A-C) shows the mean ± SD of two independent experiments (30 plants for each treatment). Results were validated with an analysis of variance statistical analysis with a Tukey multiple comparison test (α= 0. 05), different letters represent means statistically different at the 0.05 level.

Supplementary Table S1. RNA-Seq Validation by RT-qPCR

Primer Name/Id	Primer Forward (5'-3')	Primer Reverse (5'-3')
EF-1-300828	CAGGTCGGTGCCGGATAC	TCAGAGAACTTGCAGGCAATGT
SM1	CCATCTACGTCTTGGCTGTTGA	GCATCGAGCGCAATGTTG
GTPase-70852	TGCCAATGTGGCGAGTATCA	CGTGGCTGGCTAGTTGACACT
SET-77874	GAATGGCGGCCTTGGATT	TCCACTCAGTTCTCGTGACGTT
GT4-83336	AGTTGCACGAGCGAATGTCA	TCCAACTTCGCACGAAACAC
GH18-194859	TGAACTTTCTGGGCATTGCA	CGCTCCCCCTTCTGATAGC
GST S.V.-227149	GCTCTGCTTGAGGCTGGACTT	GTCACGCTCCCAATTTGGA
NOX-69153	CCCTCGTCTCGCCTCTAGTCT	CGCTGCACACTCGTCAAGA
RTM1-59885	CTGGATGCACTGCCAATGTT	TCGCCAGGGTATTTCCAATG
AQPs-6760	GATGGCGGGAAGCTGGAT	CCCAAAAGACGACGCAATGT
GATase1-46295	GGCCGAACCCACTCACTTT	CATGGCGACGGATGAACTC
GST L.V.- 34822	AAGCAATCACCCCGTTGGT	CCAAAACATCGGGAGAATGAA
FNR-57247	CCAGGAGAGCCGCTGCTA	TGGCCGTTTCCGTCGAT
3HCDH_N-46955	CGC CTG GAG CTC ACG AA	TTGTCACCCACTCGTGTTTCA
HSP-215292	GCACGAGTCGGGCTTTGA	CCTTGTTCTCGGGCTTCTTCT
TDT-158830	TTGCCCGTTGCACCTCTAG	AGACTTTTCCCAGCTGCATGA
Hydrophobin-143417	CAAGGCCCCGAGGTTGA	TCCCCCAGAGAAGGAATGC
GATase-69268	TCATGGAAGCCGTAAAAACGT	GCCTTTGCCACGGTTGAGT
HSP-215292	GCACGAGTCGGGCTTTGA	CCTTGTTCTCGGGCTTCTTCT
KN-58043	GCCCAAGCCGATAAGAAAGG	ATTATTCCGTATTTGCGCCATT
SNF2-113458	GCCGTGGGATTAGCAGCAT	GGCGAAAGGGCAGAAAGTCT

Supplementary Table S2. Primers used for deletion, replacement confirmation and copy number determination of *snf2*

***snf2* deletion**	
Primer Name	**Primers (*5'-3'*)**
5'SNF2-F	TCTCTCATATCTTTTGACTCGGATAGG
5' SNF2-R-Fusion	CTCCTTCAATATCAGTTAACGTCGATC ATCCCATGAAATGACAAACTCCATTTA
3 SNF2-F-Fusion	GCGCACTCGTCCGAGGGCAAAGGAATAG CTAGAGGTTCGTATAACAGTATCATGGC
3 SNF2-R	CTTGAAGAACGCTGGATATAGGGTC
DJhph-F	GATCGACGTTAACTGATATTGAAGGA
DJhph-R	CTATTCCTTTGCCCTCGGACGAGTGC
***snf2* replacement confirmation**	
5'SNF2-F'-Upstream	TTTTGCCGCTCCCAACGCAGC
3'SNF2-R'-Downstream	ACTTGGATCGAGGCAAGGCAG
Id-Mut R	ACAGCGGGCAGTTCGGTTTCA
Id-Mut F	ATAGTGGAAACCGACGCCCC
Copy number determination of *snf2* and *hph*	
SNF2-TR-F	GCCGTGGGATTAGCAGCAT
SNF2-TR-R	GGCGAAAGGGCAGAAAGTCT

Supplementary Table S3. Primers used to analyze the marker genes of plant defense response of *A. thaliana*

Primer Name/ TAIR Locus	**Primer Forward (5'-3')**	**Primer Reverse (5'-3')**
ACTIN 2/AT3G18780	TGTGACAATGGTACCGGTATG	CAGCCCTGGGAGCATCAT
PR-1a/AT2G14610	ATCTAAGGGTTCACAACCAGGCAC	TGCCTCTTAGTTGTTCTGCGTAGC
PDF1.2/ AT5G44420	CACCCTTATCTTCGCTGCTC	GGAAGACATAGTTGCATGATCC

Conclusions

Overall Conclusions

The histone deacetylase HDA-2 is a global regulator in *T. atroviride*, which mutation caused pleiotropic effects within the organism and during the establishment of the symbiotic relationship with *A. thaliana* seedlings.

The SNF2 chromatin remodeler of *T. virens* is a highly specific regulator, which is not involved in growth, conidiation and development in the fungus, however it plays a role in the plant-defense responses in *A. thaliana* seedlings.

Trichoderma-Arabidopsis interaction and chromatin modifications

1. The HDA-2 of *T. atrovide* acts as a positive regulator of root-colonization and plant-growth promotion in *A. thaliana* seedlings, and as a consequence, the plants root-inoculated with Δ*hda-2* weighed less than the WT root-treated.

2. The SNF2 of *T. virens* it is not relevant to promote plant-growth, therefore *A. thaliana* seedling root-inoculated by the WT or Δ*snf2* presented the same plant-growth.

3. The HDA-2 of *T. atroviride* acts as a positive regulator of the plant-defense response in *A. thaliana* against *B. cinerea* and *P. syringae,* therefore Arabidopsis root-inoculated by the Δ*hda-2* showed a compromised induction of the *PR-1a* and *PDF1.2* plant-defense marker genes, and a susceptible phenotype against those phytopathogens.

4. The SNF2 of *T. virens* is a negative regulator of the plant-defense response in *A. thaliana*. The results showed that Arabidopsis root-inoculated by the Δ*snf2* displayed an enhanced gene transcription of *PR-1a* and *PDF1.2* gene markers compared to their parental.

Secondary Metabolism and chromatin modifications

1. The HDA-2 of *T. atroviride* plays a positive role in the regulation of plant-responsive genes, including those related to secondary-metabolism. The results showed that the level expression of *epl-1*, *epl-2* and *pbs-1* (plant communication), *abc-2* (defense against toxic compounds), and *ctf-1*, *tps-2*, *pbs-1*, *ggp-1* and *fpp-1* (secondary metabolism) related-genes was dropped in the Δ*hda-2* strain co-culture with *A. thaliana* seedlings.

2. The HDA-2 plays a dual role in the maintenance of the acetylation levels in *T. atroviride.* ChIP assays of the H3acK9/K14/K18/K23/K27 on the promoter regions of *epl-1* and *abc-2* presented low levels, whereas *ctf-1* showed high constitutive levels.

3. The HDA-2 is a global regulator of secondary metabolites in *T. atroviride.* The results showed an altered production of VOCs by the Δ*hda-2*, some of these included: 6-PP, 2-heptanone, 2-pentylfuran, 3-octanone, 2-octanone, 2-heptanol, 2-nonanone, 3-octanol, 1-octen-3-ol, 2-undecanone.

4. The SNF2 is necessary for the antagonistic activity of *T. virens* against the phytopathogen *R. solani*. The results showed that Δ*snf2* antagonistic activity against *R. solani* was diminished as a consequence of an impaired metabolite-production.

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Annexes

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