Salicylic acid prevents *Trichoderma harzianum* from entering the vascular system of roots

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SUMMARY

Trichoderma is a soil-borne fungal genus that includes species with a significant impact on agriculture and industrial processes. Some *Trichoderma* strains exert beneficial effects in plants through root colonization, although little is known about how this interaction takes place. To better understand this process, the root colonization of wild-type *Arabidopsis* and the salicylic acid (SA)-impaired mutant *sid2* by a green fluorescent protein (GFP)-marked *Trichoderma harzianum* strain was followed under confocal microscopy. *Trichoderma harzianum* GFP22 was able to penetrate the vascular tissue of the *sid2* mutant because of the absence of callose deposition in the cell wall of root cells. In addition, a higher colonization of *sid2* roots by GFP22 compared with that in *Arabidopsis* wild-type roots was detected by real-time polymerase chain reaction. These results, together with differences in the expression levels of plant defence genes in the roots of both interactions, support a key role for SA in *Trichoderma* early root colonization stages. We observed that, without the support of SA, plants were unable to prevent the arrival of the fungus in the vascular system and its spread into aerial parts, leading to later collapse.

Keywords: *Arabidopsis thaliana*, callose deposition, plant defence, root colonization, salicylic acid, *Trichoderma*.

INTRODUCTION

Trichoderma is an ascomycete fungal genus that includes species able to express multiple activities, with a significant impact on agriculture and industrial processes (Lorito *et al.*, 2010). Some *Trichoderma* spp. are commonly used as biocontrol agents owing to their capability to antagonize soil-borne pathogens (Druzhinina *et al.*, 2011; Howell, 2003). They also have direct beneficial effects

on plants by promoting growth and development, stimulating defences against pathogens and increasing tolerance to abiotic stress (Hermosa *et al.*, 2012; Shores *et al.*, 2010). These effects are a consequence of the different metabolic changes elicited in plants by the intimate interaction between *Trichoderma* and roots. In such an interaction, the fungus acts as an endophytic plant symbiont (Harman *et al.*, 2004), plant tissues are not destroyed (Chacón *et al.*, 2007) and an advantageous molecular dialogue for both organisms is established (Hermosa *et al.*, 2013).

An initial study addressing the interaction of *Trichoderma asperelloides* (formerly *Trichoderma harzianum* T203) with cucumber plants revealed that fungal root colonization was followed by a systemic induction of defence responses, and transmission electron microscopy was employed to confirm that the fungus had indeed penetrated into the epidermis and the upper cortex of the root (Yedidia *et al.*, 1999). It was surmised that plant defences did not reach their full potential to allow *Trichoderma* colonization, although they were sufficient to prevent an indiscriminate spread of the fungus into the root. The thickening of plant cell walls and callose deposition on the inner surface of root cell walls were observed, acting as a physical barrier that limited the dissemination of *T. asperelloides* in the root tissues (Yedidia *et al.*, 1999). A later confocal microscopy study also revealed that *T. harzianum* was able to colonize the epidermis and cortex of tomato roots, this penetration being restricted to the apoplast as a consequence of the cell wall reinforcement (Chacón *et al.*, 2007). More recently, a similar behaviour has been reported for *Trichoderma virens* in tomato roots (Velázquez-Robledo *et al.*, 2011).

Recognition between plants and their attackers, including beneficial microorganisms, involves several molecular responses. A pioneer study showed that *Trichoderma* root colonization elicits systemic responses in plants (De Meyer *et al.*, 1998). Later, such responses were linked to an induction of defence genes through the jasmonic acid (JA)/ethylene (ET) signalling pathways (Korolev *et al.*, 2008; Shores *et al.*, 2005; Yedidia *et al.*, 2003). Several studies have also shown that *Trichoderma* spp. are able to activate plant defences both locally (Brotman *et al.*, 2008) and systemically (Morán-Díez *et al.*, 2009; Shores *et al.*, 2010; Van Wees *et al.*,

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2008), even for relatively long periods of time (Tucci *et al.*, 2011), suggesting that a delayed and overlapping expression of defence-related genes of the salicylic acid (SA) and JA/ET pathways can occur (Mathys *et al.*, 2012; Rubio *et al.*, 2014; Salas-Marina *et al.*, 2011). Thus, *Trichoderma* would be perceived as hostile by the plant and, by activating its defences, the plant achieves a successful limitation of fungal penetration to the first few layers of root cortical cells. Recent microarray studies of *Arabidopsis thaliana* colonized by *T. harzianum* (Morán-Díez *et al.*, 2012) or *T. asperelloides* (Brotman *et al.*, 2013) have revealed that, a few hours after *Trichoderma* inoculation, widespread gene transcript reprogramming occurs, preceded by a transient repression of the plant immune responses, presumably to allow root colonization.

In a similar manner to phytopathogens, beneficial microbes need to evade the hormone-regulated immune signalling network of plants to establish a mutualistic association (Zamioudis and Pieterse, 2012). SA plays key roles in plant defence against biotrophic pathogens, which usually live in the intercellular spaces, but its transient accumulation is also necessary during the early stage of root colonization by beneficial microbes (Herrera-Medina *et al.*, 2003). It has been indicated that a partial suppression of SA-dependent responses in plants is necessary for the occurrence of the symbiotic association between mycorrhiza and plants (López-Ráez *et al.*, 2010; Pozo and Azcón-Aguilar, 2007), for the interaction between the plant beneficial basidiomycete *Piriformospora indica* and *Arabidopsis* (Jacobs *et al.*, 2011) and for the establishment of the symbiotic *Rhizobium*–legume association (Peleg-Grossman *et al.*, 2009; Stacey *et al.*, 2006).

In this study, we analysed the role of SA in the colonization of *Arabidopsis* roots by *T. harzianum*. For this purpose, a green fluorescent protein (GFP)-expressing transformant of *T. harzianum* and the *Arabidopsis* SA induction deficient 2 (*sid2*) mutant, with a defect in the isochorismate synthase 1 (*ICS1*) gene, which is unable to accumulate SA after microbial inoculation, were used. Confocal microscopy visualization of this interaction, callose deposition in root cell walls, quantification of fungal root colonization by real-time polymerase chain reaction (PCR) and changes in expression in several plant defence-related genes at root level

revealed that SA is necessary in the plant to limit *Trichoderma* root colonization to the apoplastic space of the epidermis and cortex tissues.

RESULTS

Root colonization by *T. harzianum*

A set of experiments was performed in *Arabidopsis* plants to analyse the role of SA in root colonization by *T. harzianum*. The results obtained from the *Arabidopsis* Col-0 genotype– and *sid2* mutant–*T. harzianum* GFP22 interactions were compared. As shown in Fig. 1a, after 72 h of interaction, *T. harzianum* was able to penetrate the outer layers of *Arabidopsis* Col-0 roots. After longer periods of time, *T. harzianum* underwent further morphological changes, switching to the yeast-like cell type described previously (Chacón *et al.*, 2007) (data not shown). In contrast (Fig. 1b), in the *Arabidopsis* SA-deficient mutant *sid2*, *T. harzianum* was able to penetrate the vascular tissue, as revealed by the fluorescence emitted by GFP22.

Furthermore, abundant callose deposition was detected after aniline blue staining in the epidermal cells, cortex and vascular tissue of Col-0 roots inoculated with *T. harzianum* (Fig. 2a,c), but was not visualized in either the *sid2* mutant (Fig. 2b,d) or controls (Fig. 2e,f).

The effect of SA production in the colonization of the *Arabidopsis* rhizosphere by *Trichoderma* was evaluated by real-time PCR in Col-0 and *sid2* mutant seedlings challenged with *T. harzianum* GFP22. The amount of GFP22 DNA obtained from Col-0 roots was more than ten-fold lower than that of *sid2* mutant roots (Fig. 3).

To check whether *T. harzianum* was able to invade plant organs other than the roots, two leaf segments per plant from 20 plants per treatment were placed on potato dextrose agar (PDA). As shown in Fig. S1 (see Supporting Information), fungal mycelium was able to emerge from 100% of the 40 leaf segments taken from the aerial parts of *sid2*, but not from those of Col-0. The identity of the emerged fungus was confirmed as *T. harzianum* by fluorescence microscope observations and growth in *Trichoderma*-

Fig. 1 Confocal microscopy analysis of *Arabidopsis* roots from hydroponic cultures at 72 h post-inoculation of 17-day-old seedlings with *Trichoderma harzianum* GFP22. (a) Wild-type Col-0. (b) Salicylic acid (SA)-impaired *sid2* mutant. co, cortex; ep, epidermis, vt, vascular tissue.

Fig. 2 Callose localization in Col-0 (a, cross-section; c, longitudinal section; e, control without *Trichoderma*) and *salicylic acid* induction deficient 2 (*sid2*) mutant (b, cross-section; d, longitudinal section; f, control without *Trichoderma*) *Arabidopsis* roots from hydroponic cultures at 72 h post-inoculation of 17-day-old seedlings with *Trichoderma harzianum* GFP22. co, cortex; ep, epidermis, p, parenchyma, vt, vascular tissue. Scale bar. 200 μ m.

specific medium, again indicating that GFP22 is able to reach the vascular tissue when it colonizes *sid2* roots.

Effect of GFP22 on *Arabidopsis* plant growth and development

The results of hydroponic culture assays revealed that there were significant differences in plant phenotypes between *Arabidopsis* Col-0 and the *sid2* mutant when treated with *T. harzianum* GFP22 (Fig. 4), whereas these differences were not observed in the absence of *Trichoderma*. GFP22 treatment exerted an effect on the developmental precocity of Col-0 plants, in which an advance in the reproductive phase was observed in comparison with its corresponding control. By contrast, a detrimental effect on plant size was elicited by GFP22 in the *sid2* mutant (Fig. 4). No inflorescences were observed in the mutant treated with *Trichoderma*

and, in addition, a significant reduction in rosette diameter was detected in comparison with the wild-type (1.16 ± 0.22 and 1.97 ± 0.56 cm, respectively). Moreover, the roots of the *sid2* mutant were affected by treatment with *T. harzianum*, where a marked degree of root rot was observed (Fig. 5d).

Expression of defence-related genes

After the observation that the *Arabidopsis sid2* mutant allowed the penetration of *T. harzianum* to the vascular tissues and that a significantly higher fungal colonization was quantified in *sid2* mutant roots than in Col-0 roots, real-time PCR was used to analyse the expression levels of different genes related to defence responses in the roots of both *Trichoderma*–*Arabidopsis* interactions. As shown in Fig. 6, at 72 h post-inoculation of Col-0 hydroponic cultures with *T. harzianum* GFP22, a significant

Fig. 3 Quantification of *Trichoderma harzianum* GFP22 in *Arabidopsis* roots from Col-0 and salicylic acid induction deficient 2 (*sid2*) mutant plants by real-time polymerase chain reaction (PCR). *Trichoderma harzianum* and *Arabidopsis actin* genes were used. Bars represent standard deviations of the means of three biological replicates. Asterisks denote significant differences at $P \leq 0.05$ between *Arabidopsis* Col-0 and the *sid2* mutant.

up-regulation of the lipoxygenase 1 (*Lox1*) gene, involved in JA biosynthesis, the *ICS1* gene, involved in SA biosynthesis, and callose synthase 5 (*Cals5*), involved in callose biosynthesis, was observed in roots. The SA-response pathogenesis-related protein (*PR-1*) gene expression level was not affected significantly. In the case of the SA-deficient mutant, an even greater increase in the level of expression of *Lox1* was triggered by *T. harzianum*. Similarly, an increase in *ICS1* transcript levels was also observed 72 h after inoculation of *T. harzianum*, this being indicative that the fungus induces the expression of an SA biosynthetic gene in roots, regardless of whether or not the plant would be able to produce this hormone. By contrast, a down-regulation of the *Cals5* gene was observed in the *sid2* mutant roots challenged with *T. harzianum*.

DISCUSSION

The establishment of beneficial associations between plants and microbes requires the recognition of both organisms and a high degree of coordination between them. *Trichoderma* has been reported to be cosmopolitan, as members of this genus have been recovered from all continents (Druzhinina *et al.*, 2010; Hermosa *et al.*, 2004), and some species, such as *T. harzianum*, are abundant in many root ecosystems. A recent comparative genome analysis has shown mycoparasitism to be the ancestral lifestyle of *Trichoderma* (Kubicek *et al.*, 2011), and this fungus would have later colonized the roots of land plants (Druzhinina *et al.*, 2011), as the pathogens and compounds secreted by plants that enable the growth of this fungus are abundant in the rhizosphere (Rubio *et al.*, 2012, 2014; Vargas *et al.*, 2009). The *Trichoderma* colonization process requires recognition and adherence to roots for the

plant to be penetrated, and must be able to withstand the toxic metabolites produced by the host in response to invasion (Hermosa *et al.*, 2012).

Recent work has shown that partial suppression of the SA defence response is needed to allow symbiotic associations between *Trichoderma* and plants to occur (Brotman *et al.*, 2013; Morán-Díez *et al.*, 2012). This plant response has also been observed in other beneficial plant–microbe interactions (Herrera-Medina *et al.*, 2003; Jacobs *et al.*, 2011; López-Ráez *et al.*, 2010).

Taking into account this background and our previous findings in a *T. harzianum*–*Arabidopsis* transcriptome analysis (Morán-Díez *et al.*, 2012), which revealed a down-regulation of SA-mediated defence genes, such as flavin-dependent monooxygenase 1 (*FMO1*) (Mishina and Zeier, 2007) and *PR-1* in leaf tissue after 24 h of hydroponic incubation with the fungus (also observed in roots after 6 h of treatment with *Trichoderma*), the aim of this work was to gain further insight into the role of SA in *Trichoderma*–root colonization and to demonstrate that this hormone is involved in preventing this fungus from entering the vascular system of the roots. A GFP-expressing transformant of *T. harzianum* inoculated in hydroponic *Arabidopsis* cultures of the Col-0 ecotype and the SA-impaired mutant *sid2* allowed us to follow the penetration of *T. harzianum* GFP22 into root tissues. As expected, GFP22 achieved only a limited colonization, restricted to the epidermis and cortex, in Col-0, this being in agreement with the notion that *Trichoderma* is able to penetrate into the outer layers of roots, as observed previously in cucumber or tomato (Chacón *et al.*, 2007; Velázquez-Robledo *et al.*, 2011; Yedidia *et al.*, 1999), and that, after root colonization, the fungus is subjected to additional morphological changes (Chacón *et al.*, 2007). We also observed the formation of yeast-like cells in GFP22 when it colonized Col-0 (data not shown) and fungal penetration along the intercellular spaces, recently described in *T. harzianum*-colonized tomato roots (Samolski *et al.*, 2012). We failed to detect fungal structures within the vascular tissues of *Arabidopsis* Col-0, in agreement with other *Trichoderma*–plant interaction studies (Chacón *et al.*, 2007; Rubio *et al.*, 2014; Samolski *et al.*, 2012; Velázquez-Robledo *et al.*, 2011; Yedidia *et al.*, 1999), as the plant limits fungal growth by activating defence responses that involve plant cell wall reinforcement and the accumulation of antimicrobial compounds in the root (Hermosa *et al.*, 2012). By contrast, the *sid2* mutant, which does not accumulate SA even after microbial inoculation, allowed GFP22 colonization not only at the epidermal and cortical level, but also in the vascular vessels. *Trichoderma* invasion had a detrimental effect on plant growth and a drastic reduction in the rosette diameter was detected (Fig. 4). Moreover, root rot was evident (Fig. 5d) and a subsequent collapse of *sid2* mutant plants was observed at 10 days post-inoculation of GFP22. In other reports, no aberrant phenotypes were observed when *sid2* mutants were challenged with *T. hamatum* or *T. asperellum* (Mathys *et al.*, 2012; Segarra *et al.*,

Fig. 4 Effect of *Trichoderma harzianum* GFP22 treatment on *Arabidopsis* Col-0 (c, d) and the *salicylic acid induction deficient 2* (*sid2*) mutant (a, b) grown in hydroponic culture. Photographs were taken 10 days after inoculation of 2×10^7 *Trichoderma* spores (b, d).

Fig. 5 Effect of *Trichoderma harzianum* GFP22 on *Arabidopsis* Col-0 (a, b) and *salicylic acid induction deficient 2* (*sid2*) mutant (c, d) roots grown in hydroponic culture, treated (b, d) or not (a, c) with the fungus. Representative sections of roots of both *Arabidopsis* lines treated or not with GFP22 were photographed with a Leica stereomicroscope. Scale bars, 0.15 mm.

2009). We have realized that these phenotypes are observed when plants are grown in hydroponic cultures, whereas they are not always detected when *Arabidopsis* is grown in soil. The observed developmental precocity effect of Col-0 plants exerted by *Trichoderma* is in agreement with the plant growth and development promotion described previously (Hermosa *et al.*, 2012; Shores *et al.*, 2010). The advance in the Col-0 reproductive phase triggered by *Trichoderma* (Fig. 4) could be responsible for the senescence symptoms observed in these plants.

The different behaviours of Col-0 and *sid2* to GFP22 challenge could be explained in terms of the observed differences in callose deposition, this being indicative of the key role of SA in plant cell wall reinforcement and the limitation of *Trichoderma* colonization to the outer layers of roots. Similarly, *Arabidopsis* plants blocked in SA accumulation or synthesis failed to elicit high levels of callose-associated defence and allowed the successful multiplication of

plant pathogenic bacteria (DebRoy *et al.*, 2004). Recently, it has been shown in *Arabidopsis* that callose can strongly support resistance to penetration when deposited in elevated amounts at early time points during powdery mildew infection (Ellinger *et al.*, 2013). Thus, it seems that *Trichoderma* must cope with plant defence responses during the early stages of the interaction by overcoming the innate immunity of the plant, although an SA-dependent defence response will limit the endophytic growth of *T. harzianum*, preventing its access to the vascular system. In support of this hypothesis, we detected a higher level of root colonization in the *sid2* mutant than in Col-0 plants, underlining the importance of SA in the first steps of the *Trichoderma*–plant interaction (Fig. 3). This latter result is in agreement with the findings recently reported for roots of the *Arabidopsis* knockout mutant *FMO1* colonized by *T. asperelloides* (Brotman *et al.*, 2013) and several SA-deficient mutants colonized by *P. indica* (Jacobs *et al.*, 2011).

Fig. 6 Quantitative real-time polymerase chain reaction (PCR) of the lipoxygenase 1 (*Lox1*), pathogenesis-related protein-1 (*PR-1*), isochlorogenic acid synthase 1 (*ICS1*) and callose synthase 5 (*CalS5*) genes in *Arabidopsis* Col-0 and *salicylic acid induction deficient 2* (*sid2*) mutant plant roots colonized or not with *Trichoderma harzianum* GFP22. Values correspond to relative measurements against *Arabidopsis* incubated without GFP22 ($2^{-\Delta\Delta Ct} = 1$). The *Arabidopsis actin* gene was used as an internal reference gene. Bars represent standard deviations of the means of three biological replicates. Asterisks denote significant differences at $P \leq 0.05$ between *Arabidopsis* Col-0 and the *sid2* mutant.

As well as the activation of physical barriers, plants activate a complex immune phytohormone-modulated system against their attackers. It is recognized that *Trichoderma* induces the activation of the JA- and ET-mediated defence pathways in a similar way to those triggered by plant growth-promoting rhizobacteria (Shores *et al.*, 2010). Although JA and SA have been described to be reciprocally antagonistic (Thaler *et al.*, 2012), it has been shown that *Trichoderma* spp. enhance SA accumulation (Velázquez-Robledo *et al.*, 2011) and induce JA- and SA-dependent defence responses in plants (Salas-Marina *et al.*, 2011; Woo *et al.*, 2006). We also observed that GFP22 induced an up-regulation of the JA-biosynthesis gene *Lox1* in Col-0 roots at 72 h after fungal inoculation.

Although there is evidence that leaf and root immunities are similar (Jacobs *et al.*, 2011), pathogens activate tissue-specific root responses (Millet *et al.*, 2010). Little is known about the innate immune responses to beneficial microorganisms in roots (Zamioudis and Pieterse, 2012) and, in particular, to *Trichoderma*. Recently, a transcriptome approach has also revealed an increase in JA-related gene expression in *Arabidopsis* roots colonized by *T. asperelloides* (Brotman *et al.*, 2013). The significant increase in *Lox1* gene up-regulation elicited by GFP22 in the *sid2* mutant at 72 h would be consistent with its impairment in SA biosynthesis.

We observed that GFP22 induces callose deposition in Col-0 roots. Previously, it has been described that callose and callose synthase negatively regulate the SA pathway (Nishimura *et al.*, 2003). However, we observed that, at 72 h of interaction of Col-0 with GFP22, the up-regulation of the callose synthase *CalS5* gene occurred simultaneously to an increased expression of the

SA-biosynthesis gene *ICS1*, whereas no significant changes in expression were detected in the SA-response gene *PR-1*. We have reported previously that *PR-1* levels are down-regulated in *Arabidopsis* roots during the early hours of interaction with *T. harzianum*, but that its levels increase after 48 h of interaction (Morán-Díez *et al.*, 2012). Transgenic *Arabidopsis* seedlings co-cultivated with *T. virens* showed an increased expression of the gene markers of SA and JA defences, which was correlated with an enhanced accumulation of SA, JA and camalexin in the plant (Velázquez-Robledo *et al.*, 2011). In the *sid2* mutant, unable to produce SA, the observed down-regulation of *CalS5* in 72-h GFP22-treated plants, together with the absence of callose accumulation in response to fungal colonization, is indicative that SA might at least control callose deposition. Other studies have shown that pathogen attack triggers callose deposition in roots in an indole glucosinolate biosynthesis- and ET-signalling-dependent manner (Clay *et al.*, 2009; Millet *et al.*, 2010), indicating that the responses to MAMPs in the roots are SA independent (Millet *et al.*, 2010). These results further complicate the existing controversy with regard to the complex and poorly understood mechanisms of callose deposition regulation in response to attackers (Luna *et al.*, 2011).

The up-regulation of *ICS1* in *sid2* mutant roots colonized by GFP22 indicates that the plant produces SA in response to *T. harzianum* inoculation, as occurs in the Col-0 plants. However, owing to a T-DNA insertion in the *ICS1* gene (Alonso *et al.*, 2003), the corresponding protein is not functional in *sid2* and SA is not produced.

Taken together, our data indicate, as previously suggested for other mutualists (Jacobs *et al.*, 2011), that *Trichoderma* has to evade, at least partially, the innate plant immune system in order to colonize roots, minimizing the stimulation of the immune system of the host. Subsequently, a reactivation of defences mediated by SA is necessary to avoid root invasion by *Trichoderma*, as seen from the different root colonization assays performed with the SA-impaired mutant *sid2*. Although *T. harzianum* is a plant beneficial microbe, it is recognized as an invader and SA clearly plays a crucial role in its interaction with the plant, as, without the support of this hormone, the plant is unable to prevent the fungus from entering the vascular system and its later collapse.

EXPERIMENTAL PROCEDURES

Fungal strain

The GFP-marked strain *T. harzianum* GFP22 was used in this work. GFP22 was kindly provided by Dra. Ana Rincón (Seville, Spain) and was obtained from *T. harzianum* T34 (CECT2413, Spanish Type Culture Collection, Burjassot, Spain) as described previously (Chacón *et al.*, 2007). GFP22 was grown on PDA medium (Sigma-Aldrich, St. Louis, MO, USA) and was maintained at -80°C in a 20% glycerol solution. Spores were harvested from PDA plates after culture at 28°C for 7 days, and then diluted in sterile distilled water.

Plant material

Arabidopsis thaliana Col-0 ecotype and the SA-impaired mutant *sid2* were used in this research. The *sid2* mutant SALK_088254 was kindly provided by Dra. P. García-Agustín (University Jaume I, Castellón, Spain).

Trichoderma–*Arabidopsis* hydroponic cultures

Trichoderma–*Arabidopsis* hydroponic cultures were grown as described previously (Rubio *et al.*, 2012), with some modifications. Col-0 and *sid2* *Arabidopsis* seeds were placed inside Phytatray II boxes (Sigma) on a sterile gauze sheet over a sterile stainless steel screen and kept for 20 days at 22 °C in a plant growth chamber with 40% humidity under long daylight conditions (16 h light/8 h dark) (light intensity, 80–100 µE/µm²/s). Spores of *T. harzianum* GFP22 (10⁷ spores) were used to inoculate 250-mL flasks containing 100 mL of minimal medium (MM; Penttilä *et al.*, 1987), and cultures were maintained at 25 °C and 200 rpm for 48 h. Mycelia were harvested by filtration, washed with sterile water and used to inoculate the Phytatray boxes containing Col-0 or *sid2* mutant *Arabidopsis* plants. Hydroponic cultures were maintained at 22 °C and 80 rpm for 3 or 10 days. For confocal microscopy experiments, plants were recovered after 3 days and washed with sterile water. For nucleic acid extractions, roots were recovered, washed with sterile water, frozen in liquid nitrogen and lyophilized. In addition, after 10 days of hydroponic culture, roots were collected and photographed with a Leica MZ95 stereomicroscope (Leica Microsystems AG, Solms, Germany).

Detection of *Trichoderma* in plant tissue

To check whether *T. harzianum* T34 was able to colonize *Arabidopsis* leaves, we performed an experiment following the procedure described by Segarra *et al.* (2009). Briefly, two 2–3-mm leaf segments from one leaf per plant, cut from 20 17-day-old *Arabidopsis* plants, incubated for 24 h in the presence or not of *T. harzianum* as indicated above, were surface sterilized by immersing them in 95% ethanol (2 min), followed by sodium hypochlorite (4% available chlorine; 5 min), and then washed three times with sterile water. Samples were then allowed to dry on sterile filter paper for 10 min in a sterile laminar flow chamber. The two segments were placed horizontally on PDA medium and the plates were incubated for 7 days in order to check for the presence or not of fungal emergence from *Arabidopsis* leaf tissues. Twenty leaves of each treatment from three biological replicates were analysed. Subsequently, to confirm that the fungus growing out of the tissue was the target organism, mycelia were observed under a fluorescence microscope. Moreover, PDA plugs were taken from the edge of the fungal colonies and deposited on a *Trichoderma*-specific medium, as described previously by Williams *et al.* (2003).

Gene expression studies

RNA was isolated from *Arabidopsis* roots using a Nucleospin RNA Plant kit (Macherey-Nagel, Duren, Germany) followed by quantification using a NanoDrop spectrophotometer (ND 1000; ThermoFisher Scientific, Wilmington DE, USA). cDNAs were synthesized from 1 µg of total RNA

using the Prime Script™ RT reagent kit (Takara Inc., Tokyo, Japan) with an oligo(dT) primer. Then, 0.5 µL of the cDNA was used in the subsequent PCR. Quantitative real-time PCR was performed using an AB PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) in a total volume of 10 µL employing the Brilliant SYBR Green QPCR Master Mix (Roche Diagnostics, Mannheim, Germany) and a final primer concentration of 300 nM each, as described previously (Montero-Barrientos *et al.*, 2010). Real-time PCRs were performed with the cDNA from four pooled biological replicates for each condition. PCRs were performed in triplicate for 40 cycles under the following conditions: denaturation, 95 °C for 15 s; annealing, 60 °C for 1 min; extension, 72 °C for 1 min.

The following specific primers were used and checked for dimer formation: for *Cal55* (At2g13680), 5'-CTTTGCTGGTTTCAACTCAACTC-3' and 5'-AATGTTTGCTCTCCGTTCC-3'; for *Lox1* (At1g55020), 5'-GTAA GCTCTGATGTTACTGATTC-3' and 5'-CTGCGGTTAACGACGTGATTG-3'; for *PR-1* (At2g14610), 5'-GGCTAACTACAACACGCTG-3' and 5'-GGCTTCTC GTTCACATAATTC-3'; for *ICS1* (At1g74710), 5'-GATCTAGCTAACGAGAA CGG-3' and 5'-CATTAACTCAACCTGAGGGAC-3'; and for *actin* (At3g18178), 5'-CTCCCGCTATGTATGTCGCC-3' and 5'-TTGGCAGAGTGT GAGACACAC-3'.

Quantification of *Trichoderma* root colonization

To quantify root colonization by *T. harzianum* GFP22, we used Col-0 and *sid2* *Arabidopsis* roots from hydroponic cultures incubated as described above. DNA isolation was performed essentially with a cetyltrimethylammonium bromide (CTAB) extraction method, as reported previously (Dellaporta *et al.*, 1983). Fungal quantification was carried out by real-time PCR as described previously (Morán-Díez *et al.*, 2009), with modifications, using Act-F (5'-ATGGTATGGGTCAGAGGA-3') and Act-R (5'-ATGTC AACACGAGCAATGG-3') primers, which are specific for the amplification of a fragment of the *Trichoderma actin* gene, and the *Arabidopsis actin* gene primers indicated above. The mix was prepared in a 10-µL volume, using 10 ng of DNA and the forward and reverse primers at a final concentration of 100 nM. PCR conditions were as indicated above. For relative quantitative PCR, we used the Ct (cycle threshold) method, described in the manufacturer's instruction manual (Applied Biosystems), where Ct is the cycle number when the fluorescence of the sample exceeds the background fluorescence. Ct values were calculated and the amount of fungal DNA was estimated using standard curves. Values were normalized to the amount of *Trichoderma* DNA in the samples. Each sample was tested in triplicate.

Confocal scanning microscopy

Arabidopsis roots colonized by *T. harzianum* GFP22 were taken from hydroponic cultures and visualized with a Leica SP2 microscope. The excitation wavelength was 488 nm (argon/krypton laser) and the emission wavelengths were 500–550 nm. Images were visualized with a 40× objective and were acquired with Leica LSM Image Browser software.

Callose staining method

This procedure was performed as described previously (Clay *et al.*, 2009). *Arabidopsis* seedlings from hydroponic cultures were fixed in ethanol–

glacial acetic acid (3 : 1) for a short period of time under a vacuum. Subsequently, seedlings were shaken for several hours with several changes of fixative. Seedlings were rehydrated in 70% ethanol for 2 h and in 50% ethanol for another 2 h, washed twice with water and maintained in water overnight on a shaking platform. After several washes with water, seedlings were maintained in 150 mM K₂HPO₄ (pH 9.5) solution containing 0.01% aniline blue (Sigma) for ≥4 h, mounted on slides with 50% glycerol and viewed with a Leica DMLS2 optical microscope under UV illumination with a broad-band 4',6-diamidino-2-phenylindole (DAPI) filter set (excitation filter, 390 nm; dichroic mirror, 420 nm; emission filter, 460 nm).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1 Detection of *Trichoderma* in *Arabidopsis* leaves. Surface-sterilized leaf segments collected from 17-day-old *Arabidopsis* Col-0 and *salicylic acid induction deficient 2 (sid2)* mutant plants and cultured in liquid medium in the presence of *Trichoderma harzianum* GFP22 (a) or not (b). Plant segments were placed on a fungal growth medium and plates were incubated for 7 days at 24 °C.