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ARTICLE ADDENDUM

Impact of salicylic acid- and jasmonic acid-regulated defences on root colonization by *Trichoderma harzianum* T-78

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ABSTRACT

We recently found that the beneficial fungus *Trichoderma harzianum* T-78 primes tomato plants for salicylic acid (SA)- and jasmonic acid (JA)-regulated defenses, resulting in enhanced resistance against the root knot nematode *Meloidogyne incognita*. By using SA- and JA-impaired mutant lines and exogenous hormonal application, here we investigated whether the SA- and JA-pathways also have a role in T-78 root colonization of *Arabidopsis thaliana*. Endophytic colonization by T-78 was faster in the SA-impaired mutant *sid2* than in the wild type. Moreover, elicitation of SA-dependent defenses by SA application reduced T-78 colonization, indicating that the SA-pathway affects T-78 endophytism. In contrast, elicitation of the JA-pathway, which antagonized SA-dependent defenses, resulted in enhanced endophytic colonization by T-78. These findings are in line with our previous observation that SA-dependent defenses are repressed by T-78, which likely aids colonization by the endophytic fungus.

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Although the rhizosphere that surrounds the root system is among the most common ecological niches for fungal *Trichoderma* spp. (hereafter *Trichoderma*), several species have been found to be facultative endophytes that colonize the interior of the root.[1,2] Phylogenetic studies position endophytic *Trichoderma* isolates in a terminal position within their clades, suggesting that the development of the endophytism in this genus is evolutionary recent.[1] In analogy to plant pathogens, endophytic mutualists have to cope with the plant immune system to establish themselves within the plant tissues.[2-5] Indeed, a moderate suppression of host immune responses has been reported to be required for the establishment of mutualistic plant-microbe relationships.[3] For instance, repression of SA levels and SA-triggered responses occurs during root colonization by several endophytic mutualists like mycorrhizal fungi and *Rhizobium* bacteria.[6-9] Moreover, elicitation of plant immunity can negatively affect root interaction with their rhizobial or mycorrhizal partners.[10-12] However, the impact of plant defenses on root colonization by facultative endophytic *Trichoderma* has remained largely unexplored. Recently, we found that *Trichoderma harzianum* T-78 (T-78) is able to prime salicylic acid (SA)- and jasmonic acid (JA)-regulated defenses in tomato roots resulting in enhanced resistance against the root knot nematode *Meloidogyne incognita*.[13] Interestingly, in absence of the attacker, we found a moderate repression of the SA marker gene *PR1* in T-78-colonized roots. This could hint to a possible role of SA-dependent defenses in the establishment of T-78 in roots. In this addendum, by using the model plant *Arabidopsis thaliana* (hereafter *Arabidopsis*) we extend our investigation to better understand the role of SA- and JA-regulated defenses in root colonization by the mutualistic strain T-78.

T-78 colonizes endophytically the roots of Arabidopsis

To determine the endophytic colonization ability of T-78, we first imaged the fungus by confocal microscopy on roots of *Arabidopsis* plants grown for 5 weeks in soil inoculated with the fungus. Plants had been previously grown to the seedling stage for 2 weeks in river sand. Fig. 1A shows that T-78 was able to penetrate *Arabidopsis* roots and colonize endophytically the outer cell layers, as revealed by the presence of both external (Ex) and endophytic (En) T-78 mycelium. The amount of viable endophytic mycelium was further assessed 3 and 5 weeks after transplanting by analyzing the expression of the *Trichoderma* constitutively expressed gene *Trichoderma translation elongation factor-1*α (*Tef1*α), by using qPCR according to, ref. 13 and the specific primers in Table 1 (Fig. 1B). To selectively determine colonization of the endosphere compartment of the root, we used the sonication method as described in ref. 14. Sonication enabled removing T-78 mycelium from the epiphytic root region, without using chlorine bleach, which might enter roots and destroy RNA (Fig. 1D).[15] The absence of mycelium on the root surface in sonicated root fragments was verified by fluorescence microscopy (Fig. 1C). The viability of T-78 mycelium in sonicated roots was verified by plating fragments of sonicated

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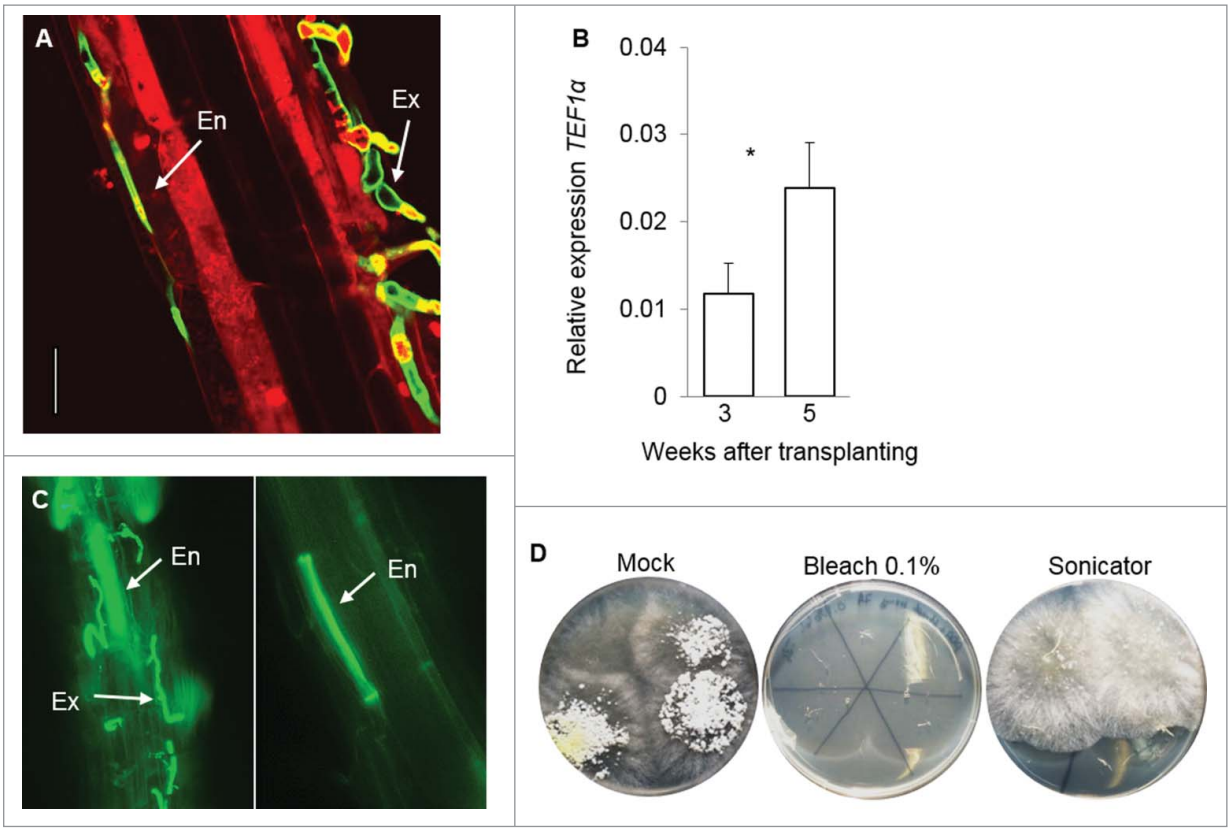

Figure 1. T-78 colonizes endophytically the roots of *Arabidopsis*. (A) T-78 endophytic colonization was assessed in roots of *Arabidopsis* grown for 5 weeks in soil inoculated with the fungus by using confocal microscopy and 0.05 mg mL-1 wheat germ agglutinin (WGA) Alexa Fluor 488 (green signal). Roots were counterstained with 10 μg mL-1 propidium iodide (PI, red signal). Chromophores were exited using the 488 nm Argon laser and fluorescence was detected at 495–519 (WGA) and 570–620 nm (PI). External (Ex) and endophytic (En) mycelium was observed in roots of inoculated plants. Scale bar = 25 μm. (B) Amount of T-78 endophytic mycelium was estimated by measuring the expression of *Tef1*α relative to the *Arabidopsis At1g13320* gene in sonicated roots at 3 and 5 weeks after transplanting. Values are means $\pm$ SE of 4 biological replicates. Each biological replicate consisted of pooled root tissue from 4 independent plants. Asterisk indicates significant difference according to Student's t-test ($P < 0.05$). These results are representative of 2 independent experiments. (C) Fluorescence microscopy (0.05 mg mL-1 WGA Alexa Fluor 488; green signal) showing (left) roots of *Arabidopsis* colonized by external (Ex) and endophytic (En) T-78 mycelium and (right) roots after sonication, where only endophytic colonization by T-78 is detected. (D) Multiple root fragments colonized by T-78 were placed on PDA after removing the rhizosphere soil (Mock) and treatments with either bleach (0.1%) or sonication, and 5 d later T-78 outgrow from inside roots was checked.

roots onto potato dextrose agar and checking T-78 outgrow from inside the roots (Fig. 1D). Expression of *Tef1*α was observed in the sonicated roots at 3 weeks and to a higher level at 5 weeks after transplanting (Fig. 1B), further corroborating the ability of T-78 to colonize endophytically *Arabidopsis* roots.

T-78 endophytic colonization is faster in the salicylic acid biosynthesis-impaired mutant sid2

To investigate the role of SA- and JA-regulated pathways in T-78 endophytic colonization, we studied the dynamics of T-78 colonization in the SA biosynthesis-impaired mutant *sid2* (*salicylic acid induction-deficient2*; defective in isochorismate synthase[16]) and in the JA biosynthesis-impaired mutant *dde2–2* (*delayed dehiscence2*; defective in allene oxide synthase[17]). *Tef1*α transcript levels were found to be 10 and 20 times higher inside the roots of the *sid2* mutant compared with the Col-0 wild type at 3 and 4 weeks after transplanting, respectively (Fig. 2). In line with this, strain T34 of *T. harzianum* has been recently observed to colonize *sid2* to higher levels in comparison to wild-type *Arabidopsis* plants.[18] However, 5 weeks after transplanting, *Tef1*α mRNA levels had also increased in wild type roots and reached a similar level to that observed in *sid2* plants. Hence, impairment of SA-defenses seems to allow an earlier and faster T-78 endophytic colonization. By contrast, no significant differences in *Tef1*α transcripts were found between the JA biosynthesis-impaired mutant *dde2–2* and the wild type in the analyzed time frame (Fig. 2), suggesting that the JA-regulated pathway does not play an important role in T-78 endophytic colonization.

Table 1. Primer sequences used for real time qPCR analysis.

ID	Target gene	Sequence (5′ → 3′)
AF456892	*Tef1*α	GGTACTGGTGAGTTCGAGGCTG GGGCTCGATGGAGTCGATAG
At5G24770	*VSP2*	CGGGTCGGTCTTCTCTGTTC CCAAAGGACTTGCCCTA
At2g14610	*PR1*	CTCGGAGCTACGCAGAACAACT TTCTCGCTAACCCACATGTTCA
At1g13320	*At1g13320*	TAACGTGGCCAAAATGATGC GTTCTCCACAACCGCTTGGT

Elicitation of salicylic acid-regulated defenses transiently impairs endophytic colonization by T-78

To study the impact of elicitation of plant defenses on T-78 endophytic colonization, the shoot of *Arabidopsis*

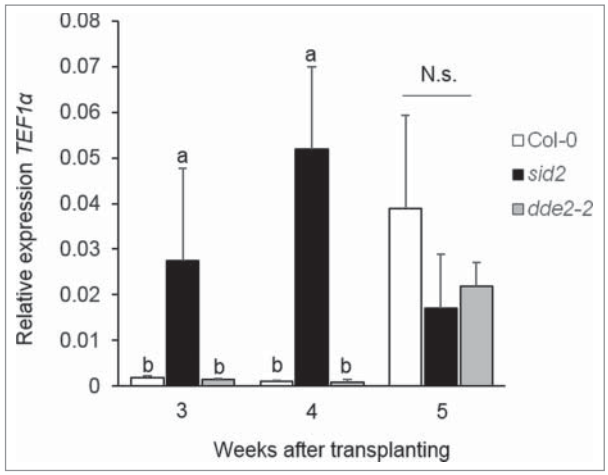

Figure 2. Quantification of T-78 endophytic colonization in roots of *Arabidopsis* Col-0, *sid2* and *dde2–2*. Amount of T-78 endophytic mycelium was estimated by measuring the relative expression of *Tef1*α relative to the *Arabidopsis At1g13320* gene in sonicated roots at 3, 4 and 5 weeks after transplanting. Values are means ± SE of 3 biological replicates. Each biological replicate consisted of pooled root tissue from 4 independent plants. For each time point, different letters indicate statistically significant differences between treatments (Tukey's HSD test; $P < 0.05$). N.s.: not significant. These results are representative of 2 independent experiments.

plants was treated with 1 mM of SA (Sigma-Aldrich, St. Louis, USA) or 100 μM of MeJA (Serva, Brunschwig Chemie, Amsterdam, the Netherlands), amended with 0.015% (v/v) Silwet L-77, according to ref. 19. Plants were treated twice: 3 and 4 weeks after transplanting to T-78-inoculated soil, according to ref. 11. Subsequently, T-78 endophytic mycelium was assessed in sonicated roots at 1 and 2 weeks after the last hormone application, and compared with mock-treated plants. Shoot application of SA led to the induction of *PR1* expression in *Arabidopsis* roots 1 week after hormone application, however after 2 weeks *PR1* levels had returned to basal levels (Fig. 3A). Interestingly, the systemic elicitation of SA-regulated defenses was associated with a strong decrease (about 150-fold) of *Tef1*α transcripts in sonicated roots 1 week after elicitation (Fig. 3B; endophytic colonization), without significantly affecting T-78 population in the rhizosphere (Fig. 3C; external colonization). However, 2 weeks after treatment, the amount of *Tef1*α transcripts in SA-elicited plants was similar to that in mock-treated controls (Fig. 3B). These observations indicate a transient negative effect of systemic elicitation of SA-regulated defenses on T-78 internal root colonization. Accordingly, previous studies have reported negative effects of elicitation of SA-regulated defenses on beneficial plant-microbe interactions.[10-12] Conversely to SA elicitation, shoot treatment with MeJA did not result in activation of JA-dependent defenses in roots (*VSP2* expression data not shown). Interestingly, a strong downregulation of *PR1* was observed in MeJA-treated plants (Fig. 3A), highlighting a systemic negative crosstalk between the JA- and the SA-regulated pathways.[20] According to the negative impact of activation of SA-regulated defenses on T-78 colonization, *PR1* downregulation might explain the increased endophytic colonization observed in MeJA-treated plants (Fig. 3B).

Altogether, our results show that elicitation of SA-regulated defenses has a transient negative effect on the endophytic root colonization by the beneficial fungus T-78. By contrast, our data point to an indirect positive effect of JA-triggered defenses on T-78 endophytic colonization, most likely via a JA/SA crosstalk-mediated downregulation of SA-dependent defenses.

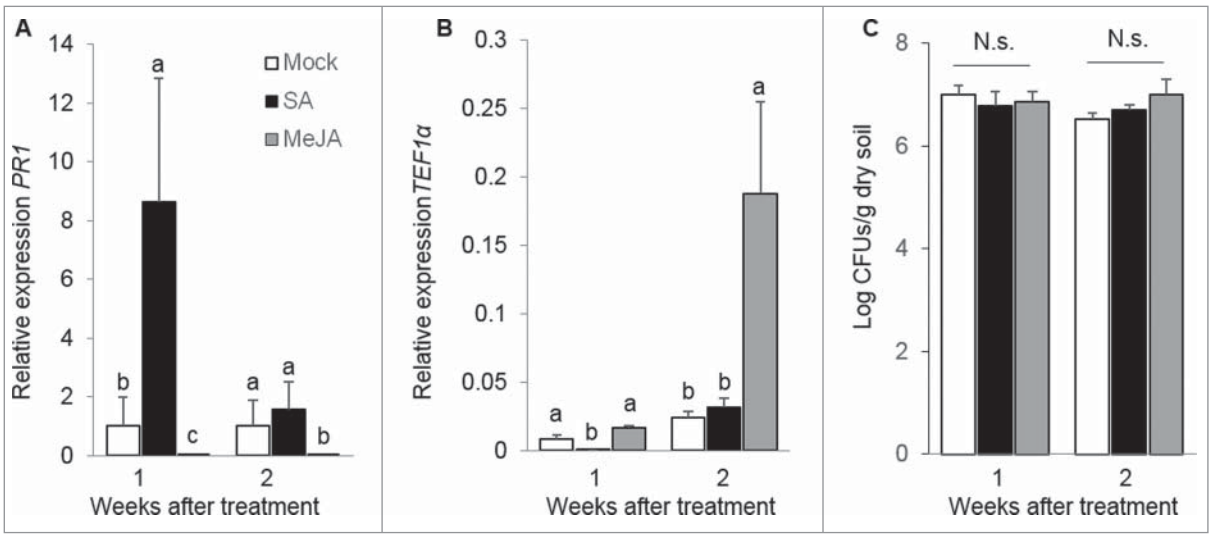

Figure 3. Impact of elicitation of SA- and JA-regulated defenses on T-78 root endophytic colonization. (A) Expression level of the SA-responsive marker gene *PR1* was analyzed in roots of *Arabidopsis* plants 1 and 2 weeks after application of SA or MeJA to the shoot, and in mock-treated plants. The results were normalized to the *Arabidopsis At1g13320* gene expression level and depicted relative to the mock-treated plants. (B) Amount of T-78 endophytic mycelium was estimated by measuring the expression of *Tef1*α relative to *At1g13320* in sonicated roots at 1 and 2 weeks after shoot application of SA or MeJA. (C) Population of T-78 in the rhizosphere of *Arabidopsis* 1 and 2 weeks after shoot application of SA or MeJA. Values are means ± SE of 4 biological replicates. Each biological replicate consisted of pooled root tissue from 4 independent plants. In (A) and (B) different letters indicate statistically significant differences between treatments per time point (Tukey's HSD test; $P < 0.05$). N.s.: not significant. These results are representative of 2 independent experiments.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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