Shifting from priming of salicylic acid- to jasmonic acid-regulated defences by *Trichoderma* protects tomato against the root knot nematode *Meloidogyne incognita*

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Summary

- Beneficial root endophytes such as *Trichoderma* spp. can reduce infections by parasitic nematodes through triggering host defences. Little is currently known about the complex hormone signalling underlying the induction of resistance. In this study, we investigated whether *Trichoderma* modulates the hormone signalling network in the host to induce resistance to nematodes.
- We investigated the role and the timing of the jasmonic acid (JA)- and salicylic acid (SA)-regulated defensive pathways in *Trichoderma*-induced resistance to the root knot nematode *Meloidogyne incognita*. A split-root system of tomato (*Solanum lycopersicum*) was used to study local and systemic induced defences by analysing nematode performance, defence gene expression, responsiveness to exogenous hormone application, and dependence on SA and JA signalling of *Trichoderma*-induced resistance.
- Root colonization by *Trichoderma* impeded nematode performance both locally and systemically at multiple stages of the parasitism, that is, invasion, galling and reproduction. First, *Trichoderma* primed SA-regulated defences, which limited nematode root invasion. Then, *Trichoderma* enhanced JA-regulated defences, thereby antagonizing the deregulation of JA-dependent immunity by the nematodes, which compromised galling and fecundity.
- Our results show that *Trichoderma* primes SA- and JA-dependent defences in roots, and that the priming of responsiveness to these hormones upon nematode attack is plastic and adaptive to the parasitism stage.

Introduction

In nature, plants are constantly subjected to a dynamic range of detrimental microbes and insects that challenge the plant's capability for growth and survival. To prevent consumption by attackers, plants can activate their defence arsenal upon recognition of the attacker encountered (Pieterse *et al.*, 2009). Phytohormones coordinate the induction of plant defences. In general, salicylic acid (SA)-dependent signalling is activated to protect plants from infection by biotrophic pathogens that feed on living plant tissues. By contrast, jasmonic acid (JA)-dependent signalling is generally effective against plant cell-killing necrotrophic pathogens and leaf-chewing insects (Pieterse *et al.*, 2009). The SA and JA signalling pathways often interact in an antagonistic manner in diverse plants, a phenomenon referred to as 'crosstalk' (Pieterse *et al.*, 2012). Moreover, other hormones, such as abscisic acid and ethylene, can antagonize or synergize the SA and JA signalling pathways (Robert-Seilaniantz *et al.*, 2011; Pieterse *et al.*, 2012; Thaler *et al.*, 2012). The hormone interactions within the plant immune signalling network determine the specific nature of the defence response that is triggered. Much of the knowledge about hormone signal interactions in defence stems from research conducted with leaf tissue, whereas little information is available for the roots (Tytgat *et al.*, 2013; Lu *et al.*, 2015). More specifically, very little information is available about hormone-coordinated defensive responses that take place in complex long-term parasitic relationships such as root nematode interactions.

Root knot nematodes are small parasitic animals that can manipulate plant defences and reprogram feeding cells in the roots to supply them with nutrients (Gheysen & Mitchum, 2011). They enter the root cortex near the elongation zone as juveniles, and migrate towards the root tip into the vascular cylinder, where they select four to eight cells to induce their feeding site. Repeated rounds of nuclear division and cell growth lead to the formation of multinucleate, hypertrophied giant cells. In addition, hyperplasy of surrounding cells causes the formation of

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macroscopically visible root knots or galls, in which the nematodes are embedded (Kyndt *et al.*, 2014b). As obligate endoparasites that complete most of their life-cycle within plant roots, the ability of root knot nematodes to overcome plant defences and maintain their feeding cells relies on continuous modulation of plant defence signalling (Schaff *et al.*, 2007; Caillaud *et al.*, 2008; Barcala *et al.*, 2010). The plant defence mounted in response to the nematode attack results therefore from the dynamic interplay between the active manipulation of host defences by nematode effectors secreted in the plant tissue to promote susceptibility and defence responses activated by the plant to reduce the infection (Goverse & Bird, 2011; Goverse & Smant, 2014). Several studies indicated that both SA and JA coordinate plant resistance and susceptibility to root knot nematodes (Bhattarai *et al.*, 2008; Fujimoto *et al.*, 2011; Hamamouch *et al.*, 2011; Nahar *et al.*, 2011; Molinari *et al.*, 2014; Fan *et al.*, 2015). However, the dynamic regulation of these hormones and their contribution to the success of the root knot nematodes during the infection progress are largely unexplored.

In addition to these detrimental interactions, plant roots are commonly engaged in multiple beneficial associations that comprise, among others, colonization of plant roots by beneficial endophytic and mycorrhizal fungi. Of these, species belonging to the genus *Trichoderma* are among the most commonly isolated saprotrophic fungi in the rhizosphere (Harman *et al.*, 2004). Plants have gained various advantages from these types of intimate relationships with root-associated microorganisms. For example, they can provide the plant hosts with enhanced levels of nutrition, an increased growth rate and protection from abiotic stressors (Goh *et al.*, 2013). In addition, beneficial root-associated microbes induce plant resistance in systemic tissues against a wide range of pathogens and herbivores that attack the roots or the shoots (Pieterse *et al.*, 2014). Induced systemic resistance (ISR) by beneficial microbes is characterized by priming of the plant for a more efficient activation of defence responses upon subsequent encounter with pathogens or insects (Conrath *et al.*, 2015; Martinez-Medina *et al.*, 2016). In most cases ISR is regulated by JA/ethylene (ET) signalling, but dependence on SA signalling has been reported as well (Van Wees *et al.*, 2008; Martinez-Medina *et al.*, 2013).

The vast majority of evidence supporting a central role of JA/ET signalling in beneficial microbe-mediated ISR is based on studies on above-ground resistance to attackers. It is as yet unknown to what extent this model applies to protection of the roots against complex long-term parasitic relationships like that of the roots with root knot nematodes, in which the attacker is able to change its behaviour and strategy with the progress of the infection (Goverse & Smant, 2014; Kyndt *et al.*, 2014a). Several studies demonstrated that root colonization by mycorrhizal fungi, endophytic fungi and nonpathogenic rhizobacteria accelerates the expression of defence responses in their host upon subsequent attack by different pathogenic nematodes (Siddiqui & Shaukat, 2004; Hao *et al.*, 2012; Vos *et al.*, 2012; Adam *et al.*, 2014). Among them, *Trichoderma* has been suggested to induce systemic resistance to the root knot nematode *Meloidogyne javanica* by mediating an increased accumulation of different antagonistic compounds such as peroxidase and phenoloxidase (Selim *et al.*, 2014). However, studies on the hormonal network responsible for the induced protection driven by beneficial root-associated microbes are scarce and often fragmented. For example, mycorrhizal symbiosis did not boost the expression of SA- or JA-dependent defences in response to infection by the nematode *Xiphinema index* (Hao *et al.*, 2012). In contrast, Vos *et al.* (2013) suggested that the protective effect of mycorrhizas against the root knot nematode *Meloidogyne incognita* could be associated with the observed enhanced expression of genes related to the phenylpropanoid pathway, suggesting involvement of SA signalling. Furthermore, Paparu *et al.* (2007) found potentiated pathogenesis-related protein 1 (*PR-1*) expression in plants inoculated with beneficial *Fusarium* after infection with the burrowing nematode *Radopholus similis*, which again might suggest a role for the SA-related pathway.

The root interaction with root knot nematodes is highly dynamic and, hence, the plant responses differ significantly between the initial and advanced stages of nematode infection. Initially, plant defence responses (i.e. callose deposition and typical hypersensitive response) are related to recognition, invasion and migration of the nematodes (Melillo *et al.*, 2006; Goverse & Smant, 2014). In later stages of the infection, plant defence is targeted towards gall development and reproduction (Portillo *et al.*, 2013). Accordingly, here we hypothesize that the cost-effective induction of defences by beneficial microbes such as *Trichoderma* is a plastic phenomenon, adaptive to the temporal regulation of the infection. Using split-root systems, we found that the ISR-inducing fungus *Trichoderma harzianum* T-78 (Martinez-Medina *et al.*, 2013) protected tomato (*Solanum lycopersicum*) roots against the root knot nematode *M. incognita* at the different stages of the infection (i.e. root invasion, gall development and reproduction) in local and systemic root tissues. Expression of either SA- or JA-responsive marker genes was primed in distinct stages of root knot nematode infection, highlighting the capability of *T. harzianum* to display plasticity in priming of either SA- or JA-dependent defence responses. Finally, to validate the biological relevance of the hormone-mediated plant defence signalling by the endophyte, we analysed the induced protection of *T. harzianum* against the root knot nematode in different hormone signalling mutants. Our results highlight that the impact of the beneficial endophytic fungus *T. harzianum* on SA- and JA-dependent induced defences against *M. incognita* is a plastic phenomenon, which effectively wards off the long-term parasitic relationship of tomato with the root knot nematode.

Materials and Methods

Endophyte, plant and nematode material

The endophytic fungus *Trichoderma harzianum* T-78 (T-78; CECT 20714, Spanish Type Culture Collection; provided by J. A. Pascual, CEBAS-CSIC) was cultured on a solid mixture containing commercial oat, bentonite and vermiculite according to Martinez-Medina *et al.* (2009). Tomato (*Solanum lycopersicum*) cultivar Moneymaker was used in the bioassays unless indicated

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otherwise. Additionally, the wild-type cultivar Castlemart, the JA-impaired mutant *def1* (Howe *et al.*, 1996) in the background Castlemart (provided by G. Howe, Michigan State University, USA), and the SA-deficient transgenic line *NahG* (Brading *et al.*, 2000) in the background Moneymaker (provided by J. Jones, John Innes Centre, UK) were used. *Meloidogyne incognita* (provided by A. Verhage, Rijk Zwaan) was maintained as a glasshouse stock culture on tomato cultivar Moneymaker. Inoculum was initiated from a single egg mass and after 8 wk egg masses were extracted from heavily infected tomato roots according to Van Bezooijen (1999). Eggs were counted and adjusted to a suspension of 3000 eggs ml^{-1} water.

Plant growth conditions and inoculation with *T. harzianum*

Tomato seeds were surface-sterilized in 4% sodium hypochlorite, rinsed thoroughly with sterile water and germinated for 1 wk in vermiculite at 25°C. For the split-root experiments, tomato seedlings were first grown in vermiculite for 3 wk in a growth chamber with a 16-h light (24°C) and 8-h dark (20°C) cycle at 70% relative humidity. During the course of the experiment, plants were watered three times a week, alternately with tap water and half-strength Hoagland solution (Hoagland & Arnon, 1938) containing 10 µM sequestreen. After 3 wk the plants were transferred to the split-root set-up (Fig. 1), consisting of two 400-ml adjacent pot compartments that were filled with a sterile soil–sand mixture (12 : 5). Inoculation with T-78 was achieved by mixing the inoculum through the soil to a final density of 1×10^6 conidia g^{-1} before transplanting (Martinez-Medina *et al.*, 2009). The plants were then placed in a completely randomized design in a glasshouse compartment under conditions of 25 ± 3°C, 16-h light : 8-h dark, and 70% relative humidity. After 3 wk, plants were used for experiments.

Nematode infection

Three weeks after inoculation with T-78, roots in one of the two pots of the split-root set-up were inoculated with ~3000 fresh *M. incognita* eggs per root system by injecting 1 ml of an egg suspension (3000 eggs ml^{-1}) into the soil. The split-root experiments consisted of three main treatments (Fig. 1): (1) half of the root system was challenged with *M. incognita* (Fig. 1a), (2) half of the root system was preinoculated with T-78 and challenged with *M. incognita* (Fig. 1b) to study local effects of T-78 on nematode infection, and (3) half of the root system was preinoculated with T-78 and the other half challenged with *M. incognita* (Fig. 1c) to assess systemic effects of T-78 on the nematode infection. Two control treatments were included: (4) both halves of the root system were uninoculated (Fig. 1d), and (5) one half of the root system was inoculated with T-78 (Fig. 1e). All plants were placed in a completely randomized design and 10 biological replicates of each treatment per time-point were used. At 0 (before the inoculation with the nematode), 3, 7, 21, 35 and 42 d after the nematode inoculation (dai), roots were harvested, root and shoot weight were measured and the severity of the infection was determined. Root material was collected and stored at −80°C. Roots

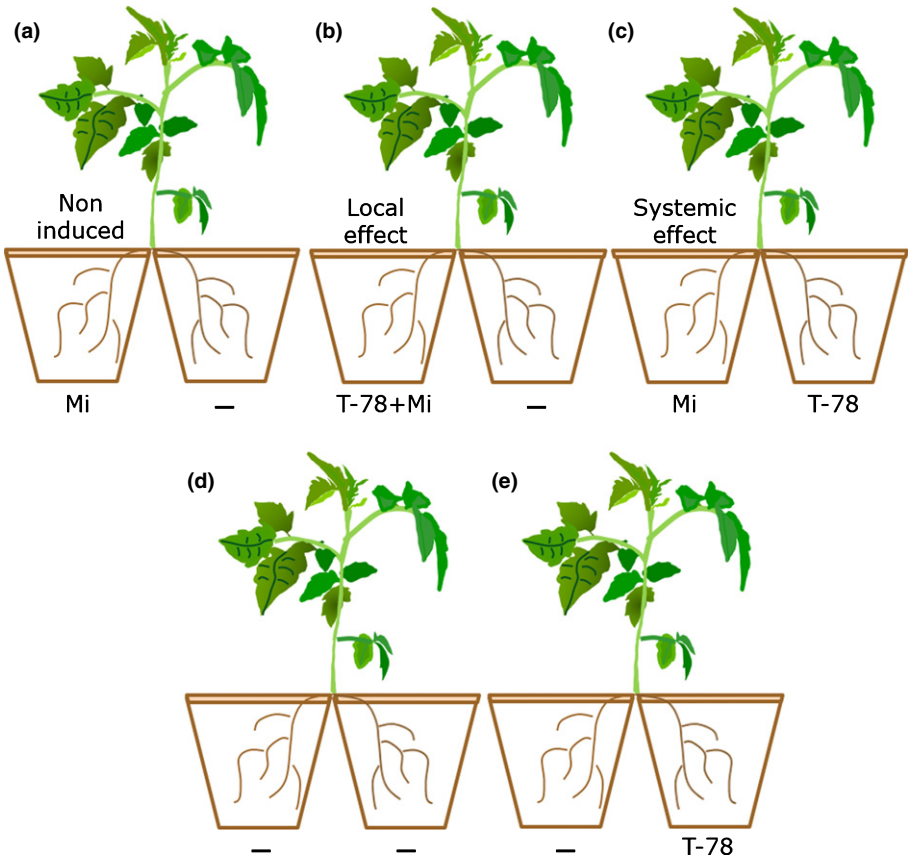

Fig. 1 Experimental design of the tomato split-root system to study *Trichoderma harzianum*-induced local and systemic resistance against *Meloidogyne incognita*. Three-week-old tomato plants were transferred to two adjacent pots, with half of the root system grown in each pot. Soil in the pots was mixed with *T. harzianum* (T-78) or not (–). Three weeks after transplanting, roots were inoculated with *M. incognita* (Mi). Local (b) and systemic (c) *Trichoderma*-induced resistance against *M. incognita* was investigated, and compared with that in T-78 noninduced roots (a). Two control treatments without *M. incognita* inoculation were included: (d) both halves of the root system were uninoculated and (e) one half of the root system was preinoculated with T-78.

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galls (20–30 per replicate) were collected with a razor blade from each root system and stored at −80°C. The substrate attached to the root system was considered to represent the rhizosphere substrate and reserved for microbiological analyses. Before effects of systemic treatments were analysed, all samples were checked for contamination by T-78: non-T-78 preinoculated root halves were tested for the presence of *Trichoderma* RNA by qRT-PCR (as described under 'Quantitative RT-PCR analysis') using Tef1 primers: Tef1-F: 5′-GGTACTGGTGAGTTCGAGGCTG-3′ and Tef1-R: 5′- GGGCTCGATGGAGTCGATAG3′, which are specific for the constitutively expressed *Trichoderma translation elongation factor-1*α gene, and only negative samples were used for further analysis.

Assessment of nematode behaviour

Root systems were carefully washed with tap water. To study the effect of T-78 on nematode root invasion, the expression level of the constitutively expressed *Actin* gene from *M. incognita* was analysed in roots at 3 and 7 dai. Nematode performance was analysed by counting gall numbers on roots at each time-point by visual inspection. Fecundity was determined by counting the number of egg clusters and the number of eggs per egg cluster at 35 and 42 dai. The number of egg clusters was analysed by visual inspection of galls, and the percentage of galls showing egg clusters was calculated. The number of eggs per egg cluster was determined by selecting 20 egg clusters randomly from each root system and soaking them in 1% bleach solution for 5 min. The suspension of eggs was then sieved using a 20-μm sieve. Released eggs were collected in 5 ml of water and the number of eggs was counted in 1 μl with the aid of a light microscope (×10). Moreover, the developmental stage of the eggs was recorded, including single-cell, multi-cell and juvenile J1 (Dutta *et al.*, 2011).

Trichoderma quantification

Serial dilutions of the rhizosphere substrate were used for quantifying T-78 colony-forming units (CFU) by a plate count technique using potato dextrose agar amended with 50 mg l^{-1} rose bengal and 100 mg l^{-1} streptomycin sulphate (Martinez-Medina *et al.*, 2009). Plates were incubated at 28°C and CFU were counted after 5 d.

Chemical treatments

In the split-root set-up, as described under 'Plant growth conditions', half of the root system (roots in one pot) was treated with 20 ml of a 0.1 mM methyl jasmonate (MeJA; Serva, Brunschwig Chemie, Amsterdam, the Netherlands) solution, which was prepared from a 100 mM MeJA stock solution in 96% (v/v) ethanol. The experiment consisted of three main treatments: (1) half of the root system was treated with MeJA, (2) half of the root system was preinoculated with T-78 and treated with MeJA, to study local effects of T-78 on JA-mediated defences, and (3) half of the root system was preinoculated with T-78 and the other half treated with MeJA to study systemic effects of T-78 on JA-mediated defences. All plants were placed in a completely randomized design and five biological replicates of each treatment at each time-point were used. Root material was collected at 0 (before treatment), 1, 4, 8 and 24 h after the application of the hormone and stored at −80°C.

Quantitative RT-PCR analysis

Total DNA was extracted using the DNeasy plant kit (Qiagen) according to the manufacturer's instructions. Total RNA was extracted using the GenElute™ Plant total RNA kit (Sigma) according to the manufacturer's instructions and treated with DNase (Thermo Scientific, Waltham, MA, USA). First-strand cDNA was synthesized from 1 μg of purified total RNA using the Revert Aid H Minus RT (Thermo Scientific) according to the manufacturer's instructions. Real-time quantitative RT-PCR (qPCR) reactions and relative quantification of specific mRNA levels were performed according to Vos *et al.* (2015), using the gene-specific primers described in Supporting Information Table S1. The data were normalized using the housekeeping gene *SlEF* (X14449) encoding the tomato translation elongation factor-1α, which has been reported to be one of the most stable reference genes for data normalization during root infection with *M. incognita* (Miranda *et al.*, 2013).

Statistical analysis

The statistical analysis was performed with the ANOVA General Linear Model (SPSS software, v.20; SPSS Inc., Chicago, IL, USA). Following two-way and three-way ANOVAs, one-way ANOVAs were performed for each time-point and/or genotype to analyse the effect of T-78 preinoculation. Tukey's test was used for overall comparisons. When ANOVA assumptions were not met, Dunnett's test was performed to detect differences among treatments. Student's *t*-test was used to detect differences in shoot biomass between nematode-infected and noninfected plants. A chi-square test was used to compare the distribution of nematode embryonic stage categories between treatments. All the experiments were repeated at least twice with similar results.

Results

Trichoderma protects local and systemic root tissue of tomato against nematode infection by inhibiting invasion and reducing galling

To study whether T-78 protects roots locally and systemically against *M. incognita*, a split-root system of tomato was used (Fig. 1). Five weeks after challenge with the nematode, root and shoot symptoms were evaluated. All the *M. incognita*-infected plants showed visible signs of root galling (Fig. 2a) and a reduction in shoot weight ($P < 0.001$; Student's *t*-test; Fig. 2b). Preinoculation with T-78 led both locally and systemically to a decreased density of root galls and a smaller reduction in shoot biomass compared with non T-78 plants. Treatment with T-78 alone (without *M. incognita* infection) did not affect shoot growth (Fig. 2b).

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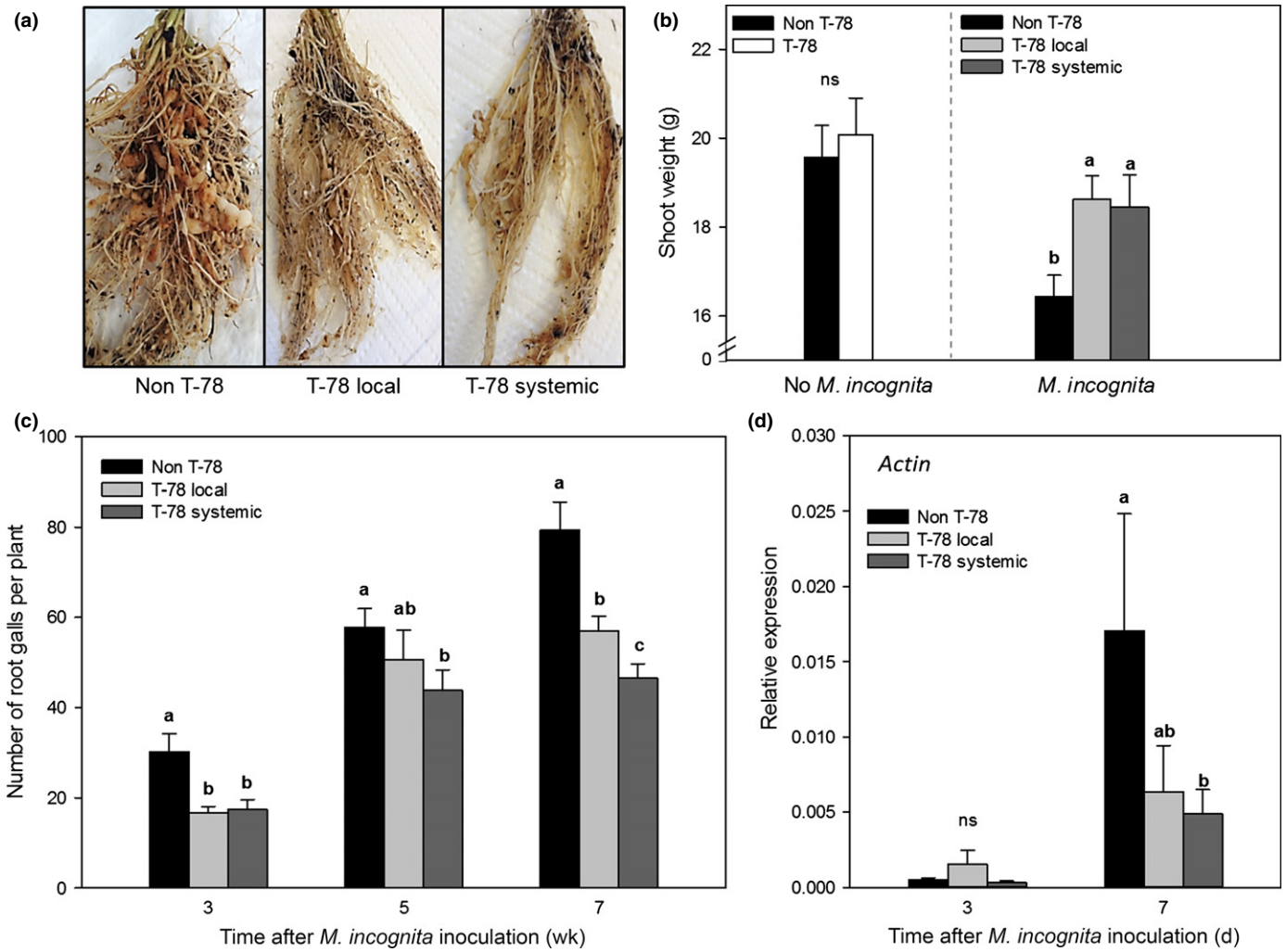

Fig. 2 *Trichoderma harzianum* reduces locally and systemically *Meloidogyne incognita* infection. Five weeks after inoculation with *M. incognita*, root knots (a) and shoot weight (b) of *T. harzianum* (T-78) uninoculated (Non T-78), and locally or systemically T-78-preinoculated plants were evaluated. (b) Different letters for *M. incognita*-inoculated plants indicate significant differences ($P < 0.05$; $n = 10$) between treatments according to Tukey's test following one-way ANOVA with the factor T-78 preinoculation. (c) The number of galls was monitored 3, 5 and 7 wk after inoculation with *M. incognita* in roots of T-78-uninoculated and locally or systemically T-78-preinoculated plants. At each time-point, different letters indicate significant differences between treatments ($P < 0.05$; $n = 10$) according to Tukey's test following two-way ANOVA, with factors T-78 preinoculation and time after *M. incognita* inoculation (Supporting Information Table S2) and one-way ANOVA at each time-point with the factor T-78 preinoculation. (d) The relative expression of the *Actin* gene from *M. incognita* was determined inside the roots at 3 and 7 d after infection with *M. incognita*. The results were normalized with the tomato *SlEF* translation elongation factor1-α gene. At each time-point, different letters indicate significant differences between treatments ($P < 0.05$; $n = 5$) according to Tukey's test following two-way ANOVA, with factors T-78 preinoculation and time after *M. incognita* inoculation (Table S2) and one-way ANOVA at each time-point with the factor T-78 preinoculation. Data are mean ± SE. ns, not significant.

To study the dynamics of the protective effect of T-78 on *M. incognita* infection, the formation of root galls was measured at different time-points after nematode inoculation. In the monitored time frame between 3 and 7 wk after nematode inoculation, preinoculation with T-78 reduced the amount of root galls in both local and systemic root tissue, whereby the systemic effects were significantly stronger than the local effects at 7 wk after inoculation (Fig. 2c; Table S2). To determine whether T-78 is effective already at the early nematode infection stages, nematode biomass was estimated by following the expression of the *Actin* gene of *M. incognita* inside the roots at 3 and 7 dai. After 3 d, *M. incognita Actin* transcript levels were low in both T-78-treated and nontreated roots and no significant differences were found (Fig. 2d; Table S2). However, 7 d after *M. incognita* inoculation, the amount of *M. incognita Actin* transcripts increased by 15-fold in non-T-78 *M. incognita*-inoculated control roots. A 3-fold lower level of *M. incognita Actin* mRNA was observed in roots that were locally or systemically preinoculated with T-78. These results demonstrate the ability of T-78 to protect roots against nematode infection from the early infection stages.

Trichoderma disrupts the fecundity of the nematode in systemic root tissue

The effect of T-78 on the fecundity of *M. incognita* in tomato roots was assessed at 5 and 7 wk after inoculation with

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M. incognita. No egg clusters were observed at 3 wk after inoculation. First, the percentage of galls that contained egg clusters was determined, but no significant differences between non-T-78 and T-78 preinoculated plants were observed at 5 and 7 wk after inoculation with *M. incognita* ($P < 0.05$; data not shown). However, a significant reduction (up to 50%) in the number of eggs per egg cluster was observed in systemic roots of T-78-treated plants, but not locally in T-78-preinoculated roots (Fig. 3a). Moreover, the developmental stage of the eggs in the egg clusters was delayed in the T-78 systemic root tissue, as relatively more eggs were found at the single-cell stage and fewer at the juvenile stage (Fig. 3b). These results indicate that the fecundity of *M. incognita* is impeded in the systemic parts of T-78-preinoculated roots.

Trichoderma primes for SA-related defences at the invasion stage of nematode infection

The hormones SA and JA have been suggested to play a key role in coordinated defence reactions to root knot nematodes, but how and at which infection stage SA and JA signalling are engaged is not clear. We investigated whether SA-dependent defence signalling during infection of tomato roots by *M. incognita* was modulated by pretreatment with T-78. Therefore, the expression profile of the SA-responsive marker genes *Pathogenesis-related protein 1a* (*PR1a*) and *Pathogenesis-related protein P6* (*PR-P6*; Fig. S1) was assessed in *M. incognita*-infected root tissue at different infection stages. In non-nematode-infected roots, T-78 inoculation locally resulted in a moderate repression of *PR1a*, which could be related to the ability of T-78 to modulate SA-mediated defences for successful root colonization (A. Martinez-Medina *et al.*, unpublished data). Infection with *M. incognita* resulted in a transient activation of *PR1a* and *PR-P6* at 7 dai ($P < 0.005$; Fig. 4a,b). At 21 dai, *PR1a* and *PR-P6* transcripts had declined to basal levels, suggesting that *PR1a* and *PR-P6* expression is associated with the early induction stage of infection. In T-78 local and systemic roots, a faster activation of *M. incognita*-induced *PR1a* and *PR-P6* expression was observed, which was apparent at 3 d after challenge with *M. incognita*, which is during the invasion stage. Accordingly, after nematode challenge SA concentrations in T-78-pretreated plants were higher compared with nonpretreated plants (Fig. S2).These results suggest that T-78 primes local and systemic root tissues for accelerated activation of SA-dependent defences during the early stage of *M. incognita* infection.

Trichoderma antagonizes the suppression of JA-dependent defences by nematode infection

Expression analysis of the JA-responsive genes *Proteinase inhibitor II* (*PI II*) and *Multicystatin* (*MC*) of tomato after *M. incognita* infection revealed that JA signalling was already significantly down-regulated 3 dai in non-T-78-induced plants ($P < 0.005$). This down-regulation increased during the later stages of the infection, namely at 7 and 21 dai (Fig. 5a,b). These later time-points correspond to the sedentary feeding stage of nematode infection. When T-78 was preinoculated in both local and systemic root compartments, a significant reduction in the suppression of *PI II* and *MC* by *M. incognita* was observed. This started at 3 dai and was still detected at 21 dai for *MC*. In accordance, the concentration of different jasmonates remained higher in T-78-treated roots, compared with nontreated roots, after nematode challenge (Fig. S2). Interestingly, the systemic root parts of T-78-preinoculated plants displayed a more consistent and longer lasting reduction of the suppressive ability of *M. incognita* than local root parts (Fig. 5a,b).

A challenge in studies of the effects of resistance inducers on hormone-regulated defence signalling during attack by harmful pathogens or insects is that the decrease in the infection or infestation may itself lead to a reduced effect on defence signalling.

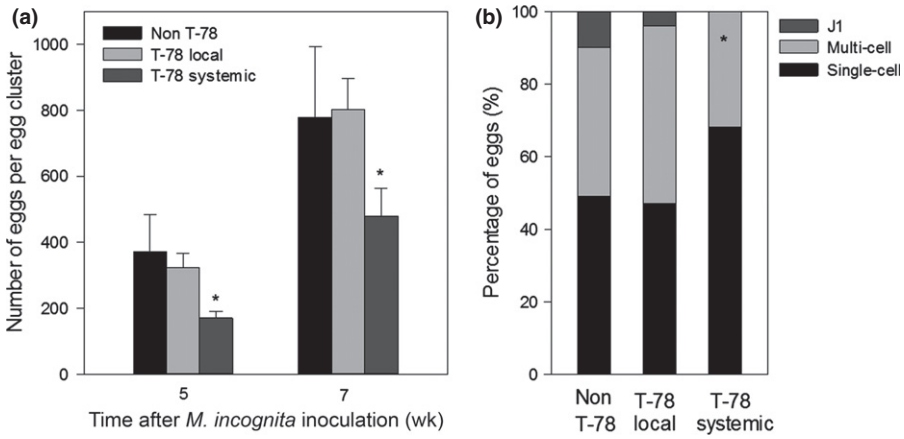

Fig. 3 *Trichoderma harzianum* reduces the reproductive ability of *Meloidogyne incognita* in systemic root tissue. (a) Number of eggs per egg cluster. Egg clusters of *M. incognita* were collected from tomato root tissue at 5 and 7 wk after inoculation with *M. incognita*. Roots had been preinoculated locally or systemically with *T. harzianum* (T-78) or not inoculated (Non T-78). Data are mean ± SE ($n = 20$). Statistically significant differences compared with T-78-uninoculated plants at a given time-point were determined according to Dunnett's test: *, $P < 0.05$. (b) Percentage of eggs belonging to the following embryonic stages: single-cell, multi-cell and juvenile (J1) stages. Eggs were collected from roots at 5 wk after inoculation with *M. incognita*. Significantly different distributions of embryonic stages compared with T-78-uninoculated plants were determined: *, $P < 0.05$; $n = 50$.

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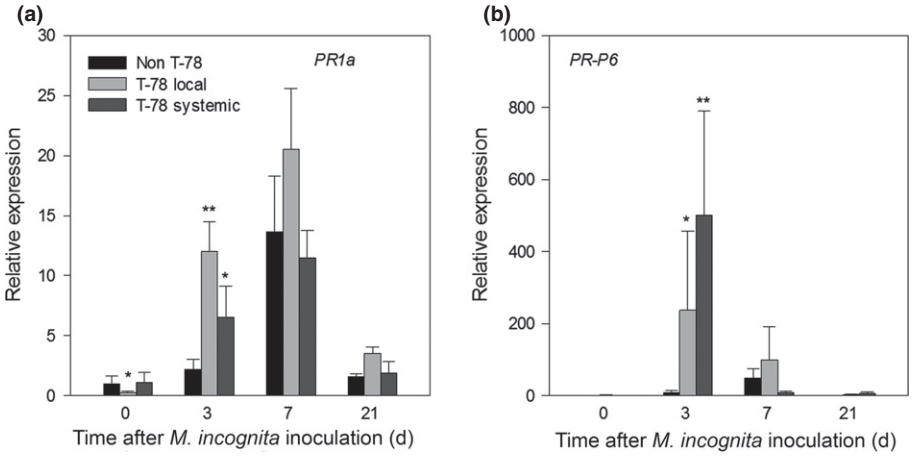

Fig. 4 *Trichoderma harzianum* primes roots for enhanced expression of the salicylic acid (SA) marker genes *Pathogenesis-related protein 1a* (*PR1a*) and *Pathogenesis-related protein P6* (*PR-P6*) upon *Meloidogyne incognita* infection. Expression levels of the SA-responsive marker genes *PR1a* (a) and *PR-P6* (b) were analysed in roots of *T. harzianum* (T-78)-uninoculated (Non T-78) plants and in locally or systemically T-78-preinoculated plants at 0, 3, 7 and 21 d after nematode inoculation. The results were normalized to *SlEF* encoding for the tomato elongation factor-1α gene expression levels and expressed relative to those found in T-78-uninoculated plants before nematode infection, which were arbitrarily given a value of 1. Data are mean ± SE ($n = 5$). At each time-point, the asterisk indicates significant differences from non-T-78-inoculated plants according to Dunnett's test ($P < 0.05$). *, $P < 0.05$; **, $P < 0.01$.

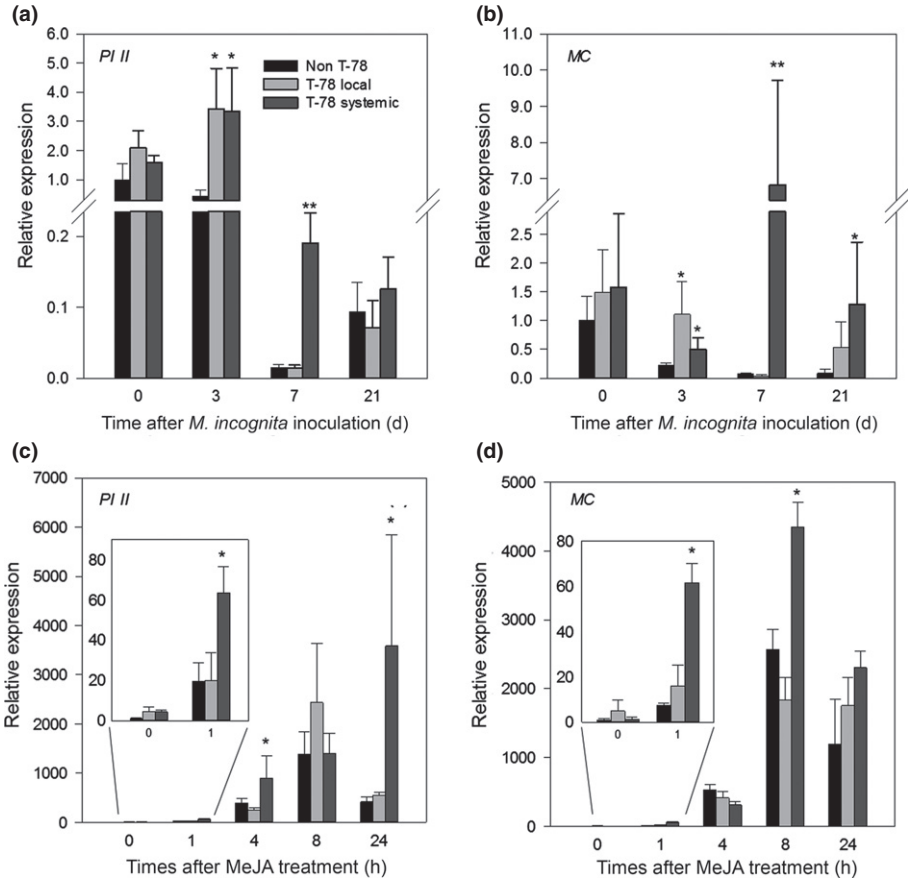

Fig. 5 *Trichoderma harzianum* antagonizes the suppression of the jasmonic acid (JA) marker genes *Proteinase inhibitor II* (*PI II*) and *Multicystatin* (*MC*) by *Meloidogyne incognita* infection. Expression levels of the JA-responsive marker genes *PI II* (a) and *MC* (b) were analysed in roots of *T. harzianum* (T-78)-uninoculated (Non T-78) plants and in locally or systemically T-78-preinoculated plants at 0, 3, 7 and 21 d after nematode inoculation. *PI II* (c) and *MC* (d) were also analysed in T-78-uninoculated roots and in roots preinoculated with T-78 locally or systemically at 0, 1, 4, 8 and 24 h after exogenous application of methyl jasmonate (MeJA). The results were normalized to *SlEF* encoding for the tomato elongation factor-1α gene expression levels and expressed relative to those found in T-78-uninoculated roots before nematode infection (a, b) or hormonal treatment (c, d), which were arbitrarily given a value of 1. Data are mean ± SE. At each time-point, the asterisk indicates a significant difference from T-78-uninoculated plants according to Dunnett's test ($P < 0.05$; $n = 5$). *, $P < 0.05$; **, $P < 0.01$.

To investigate whether the reduced alteration of JA signalling by nematode infection in T-78-preinoculated plants is a cause or a consequence of the reduced nematode infection in the T-78-preinoculated roots (Fig. 2c), we investigated the ability of T-78 to prime JA signalling after exogenous hormone application. We were able to evoke induced expression of *PI II* and *MC* by exogenously applying MeJA to the roots without applying a stress (Fig. 5c,d). We analysed mRNA levels of *PI II* and *MC* after 1, 4, 8 and 24 h of treatment of the roots with MeJA in T-78 local and systemic compartments. In general, in systemic root tissues,

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preinoculation with T-78 led to an enhanced and more durable activation of *PI II* and *MC* after MeJA application compared with non-T-78 and T-78 local roots (Fig. 5c,d). These findings support the notion that T-78 primes JA-dependent defence signalling in systemic root tissue, which probably counteracts the suppression of JA-mediated signalling by *M. incognita*.

Trichoderma primes SA-related defences triggered by nematode oviposition in differentiated root galls

After successful establishment of infection by *M. incognita*, the eggs that are produced during the reproduction stage may present new elicitors that trigger defence responses in the plant. We investigated whether roots are capable of detecting the nematode eggs on the gall surface and whether T-78 influences the activation of defences after this recognition. To this end, we studied the expression of *PR1a* and *PI II* in dissected galls of roots colonized by T-78, at different stages of gall maturity (3, 5 and 7 wk after challenging with *M. incognita*). No significant differences in *PR1*a expression were found in the youngest galls, collected 3 wk after challenging with *M. incognita*, compared with noninfected roots. In more developed galls, collected 5 wk after challenge with the nematode, a slight, 2-fold up-regulation of *PR1a* was observed, which coincided with the first appearance of egg clusters (Fig. 6a; Table S2). *PR1a* expression was further up-regulated in the oldest galls, collected 7 wk after nematode inoculation, when there were more egg clusters as well as more eggs per egg cluster. Interestingly, when plants were preinoculated with T-78, a stronger increase of *PR1a* expression was detected in galls collected from both local and systemic T-78 tissues at 5 and 7 wk after nematode inoculation, showing that T-78 enhanced the SA-mediated plant response to the nematode eggs in root galls. Remarkably, the enhancement of SA-dependent *PR1a* by T-78 in mature galls at 5 and 7 wk was accompanied by a down-regulation of the JA-marker gene *PI II* (Fig. 6b; Table S2), which suggests antagonistic crosstalk between the SA- and JA-defence pathways following egg recognition specifically in root galls. Galls collected from T-78-treated plants showed an enhanced down-regulation of *PI II*, suggesting that the enhanced SA signalling by T-78 resulted in an enhanced antagonism of JA signalling in root galls after oviposition.

Differential requirement of SA and JA signalling for *Trichoderma*-induced resistance against different nematode infection stages

To gain further insight into the role of SA and JA signalling in the protective effect induced by T-78 against *M. incognita*, nematode infection was assessed in roots of the SA-deficient transgenic *NahG* tomato line, the JA-deficient mutant *def1* and their corresponding background wild-type tomato lines. As shown in Fig. 7(a,b), the protective effect triggered by T-78 against nematode invasion at 7 d after nematode inoculation was stronger in the cultivar Castlemart (CM) than in Moneymaker (MM), where significant protection levels were observed only in systemic root parts of T-78-preinoculated plants. However, this systemic protection was lost in SA-deficient *NahG* plants (MM background), indicating a crucial role for SA signalling in systemic induction of resistance by T-78 against *M. incognita* invasion (Fig. 7a; Table S3). In plants carrying the *NahG* transgene, catechol accumulates as a result of the activity of salicylate hydroxylase (Brading *et al.*, 2000), which could have a pleiotropic effect on the system studied (Van Wees & Glazebrook, 2003). Exogenous application of catechol to wild-type plants allowed us to discriminate effects of SA deficiency and of catechol accumulation in *NahG* plants. Catechol treatment did not alter plant susceptibility to nematode parasitism (Fig. S3). In contrast to *NahG* plants, *T. harzianum* was still able, both locally and systemically, to

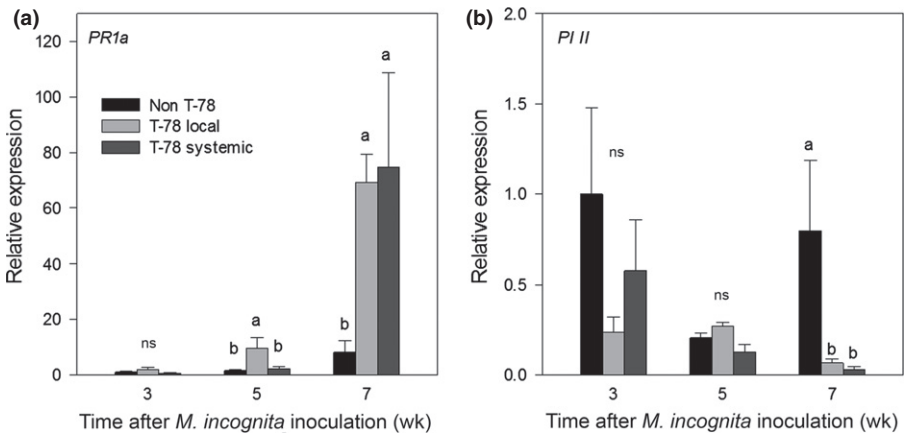

Fig. 6 *Trichoderma harzianum* primes *Pathogenesis-related protein 1a* (*PR1a*) expression triggered in mature galls after oviposition by *Meloidogyne incognita*. Expression levels of the salicylic acid (SA)- and jasmonic acid (JA)-responsive genes *PR1a* (a) and *Proteinase inhibitor II* (*PI II*) (b) were analysed in hand-dissected galls isolated from *T. harzianum* (T-78)-uninoculated (Non T-78) roots and locally or systemically T-78-preinoculated roots at 3, 5 and 7 wk after inoculation with *M. incognita*. Expression levels were normalized to *SlEF* encoding for the tomato elongation factor-1α gene expression levels and expressed relative to those found in T-78-uninoculated plants before nematode infection, which were arbitrarily given a value of 1. Data are mean ± SE. At each time-point different letters indicate significant differences between treatments ($P < 0.05$; $n = 5$) according to Tukey's test following two-way ANOVA, with factors T-78 preinoculation and time after nematode inoculation (Table S2), and one-way ANOVA at each time-point with the factor T-78 preinoculation. ns, not significant.

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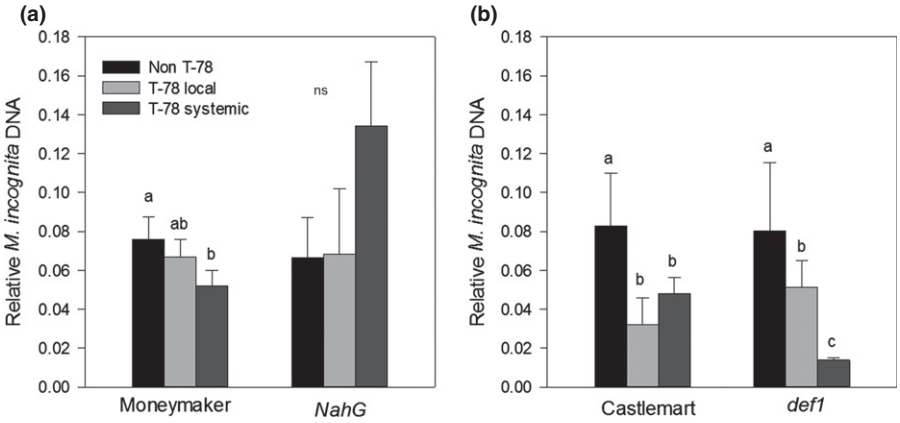

Fig. 7 *Trichoderma harzianum*-mediated protection against nematode invasion requires salicylic acid (SA) but is independent of jasmonic acid (JA) signalling. The infection level was calculated as the relative amount of nematode DNA in relation to the amount of plant DNA at 7 d after infection with *Meloidogyne incognita* in roots of *T. harzianum* (T-78)-uninoculated (Non T-78) plants and locally or systemically T-78-preinoculated plants in two independent experiments including (a) the wild-type cv Moneymaker (MM) and the SA-deficient transgenic line *NahG*, and (b) the wild-type Castlemart (CM) and the JA-impaired mutant *def1*. Data are mean ± SE. For each tomato genotype, different letters indicate significant differences between treatments ($P < 0.05$; $n = 5$) according to Tukey's test following two-way ANOVA, with factors T-78 preinoculation and plant genotype (Table S3), and one-way ANOVA in each genotype with the factor T-78 preinoculation. ns, not significant.

protect the roots of the JA-deficient *def1* mutant (background CM) against nematode invasion (Fig. 7b; Table S3), indicating that JA signalling is not required for T-78-mediated protection against *M. incognita* invasion. Remarkably, a stronger systemic protection by T-78 was observed in *def-1* compared with that observed in its wild-type background (Fig. 7b). Accordingly, T-78 systemic induction of SA accumulation in *def1* plants was up to 3-fold higher compared with its background (data not shown).

The role of SA and JA signalling in T-78-induced resistance was also investigated at later stages of the infection. Root galling was significantly reduced by preinoculation with T-78 in both local and systemic root tissue of the two tomato cultivars MM and CM at all the time-points investigated (Fig. 8a,c). This induced protection against root galling was still fully expressed in the SA-deficient *NahG* (Fig. 8b; Table S4), but it was blocked in the JA-deficient *def1* (Fig. 8d; Table S4). This suggests that in our experimental set-up T-78-primed JA signalling significantly contributes to protection against gall induction and/or development, while the role of SA signalling is less obvious at this advanced level of nematode infection.

We further studied the involvement of SA and JA signalling in the impairment of nematode fecundity by T-78 during the latest stage of a well-established infection. The number of eggs per egg cluster in the root galls was measured in *NahG* and *def1* tomato lines at 5 wk after inoculation with *M. incognita*. Fig. 9 shows that systemic preinoculation with T-78 reduced the amount of eggs per egg cluster in both the MM and CM cultivars. Interestingly, this protective effect was impeded not only in JA-deficient *def1* plants (Fig. 9b), but also in SA-deficient *NahG* plants (Fig. 9a). This suggests that, while SA-dependent defences do not seem to play a role in T-78-mediated protection against galling, they are involved in impeding nematode fecundity. Taken together, these findings suggest that the systemic ability of T-78 to interfere with the fecundity of *M. incognita* relies on both SA and JA signalling.

Discussion

In this study, we demonstrated that the endophytic fungus *T. harzianum* T-78 impacted the plant interaction with the root knot nematode *M. incognita* throughout the entire infection cycle (i.e. invasion, galling and reproduction) by boosting specific host defences, depending on the parasitism stage. By using a split-root experimental design, we showed that, in addition to possible direct effects of T-78 on the nematode population, that is, direct nematicidal activity of the fungus (Sahebani & Hadavi, 2008), plant-mediated effects induced by T-78 also impacted the nematode parasitism (Figs 2, 3). Similarly, previous studies have reported the ability of other beneficial fungal endophytes to protect plants against different parasitic nematodes through plant-mediated mechanisms (Elsen *et al.*, 2008; Hao *et al.*, 2012; Vos *et al.*, 2012, 2013; Martinuz *et al.*, 2015).

Many beneficial microbes, including *Trichoderma* species, protect plants from attack by pathogens or insects by priming plant tissue for enhanced and/or accelerated expression of defence responses (Pieterse *et al.*, 2014). Induction of JA/ET- and/or SA-inducible defences following pathogen attack is boosted in leaves by colonization of the roots by *Trichoderma* spp. (Segarra *et al.*, 2009; Contreras-Cornejo *et al.*, 2011; Salas-Marina *et al.*, 2011; Mathys *et al.*, 2012; Yoshioka *et al.*, 2012; Martinez-Medina *et al.*, 2013). The ability of *Trichoderma* spp. to modulate JA/ET- and/or SA-inducible defences in the same plant species indicates the presence of diverse transduction pathways regulating *Trichoderma*-induced resistance. This ability for alternative activation of different resistance-regulating mechanisms might increase the performance of plants inoculated with *Trichoderma* spp. in response to a broad spectrum of pathogens (Salas-Marina *et al.*, 2011; Nawrocka & Malolepsza, 2013).

In order to unravel the signalling network governing the protective effect of T-78 throughout the nematode infection process, we first analysed the expression of known marker genes for

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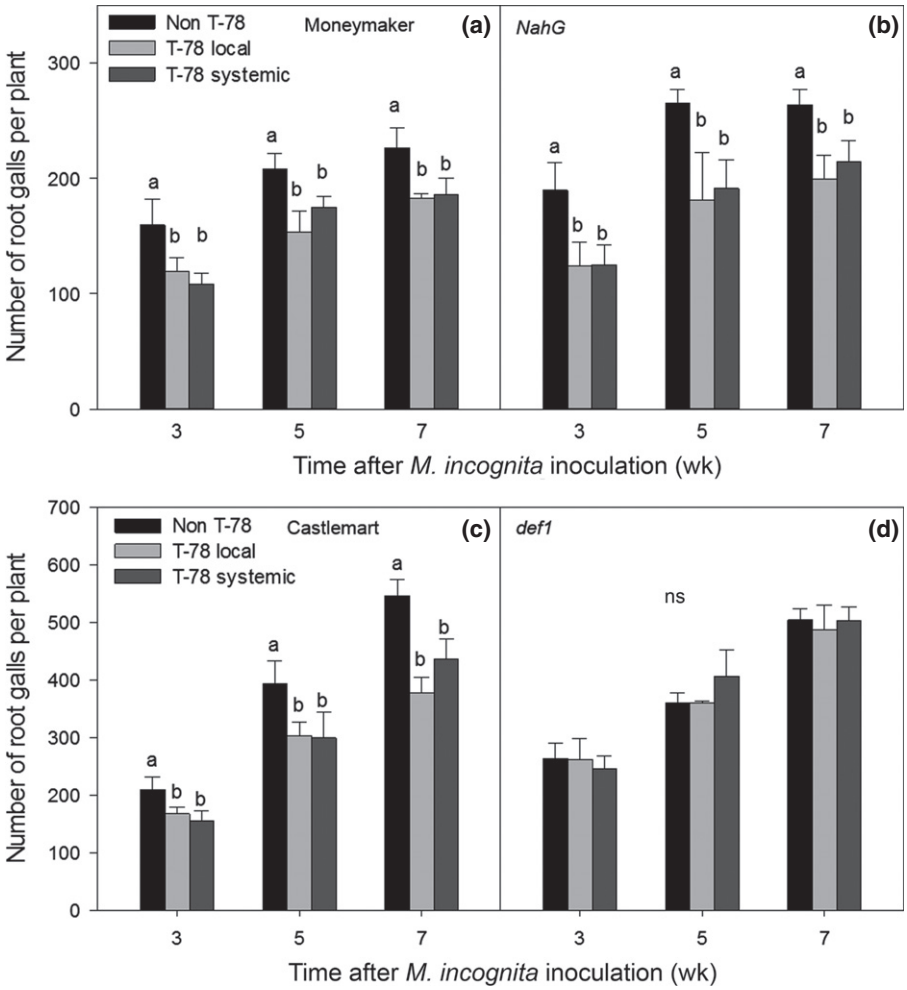

Fig. 8 *Trichoderma harzianum*-mediated protection against root galling relies on jasmonic acid (JA) signalling, but is independent of salicylic acid (SA) signalling. The number of *Meloidogyne incognita* galls was monitored at 3, 5 and 7 wk after nematode inoculation in roots of *T. harzianum* (T-78)-uninoculated (Non T-78) plants and locally or systemically T-78-preinoculated plants, in two independent experiments including the wild-type cv Moneymaker (MM) (a), the SA-deficient transgenic line *NahG* (b), the wild-type Castlemart (CM) (c), and the JA-impaired mutant *def1* (d). Data are mean ± SE. For each tomato genotype, different letters at each time-point indicate significant differences ($P < 0.05$; $n = 5$) between treatments according to Tukey's test following three-way ANOVA, with factors T-78 preinoculation, plant genotype and time after *M. incognita* inoculation (Supporting Information Table S4) and one-way ANOVA in each genotype and time-point with the factor T-78 preinoculation. ns, not significant.

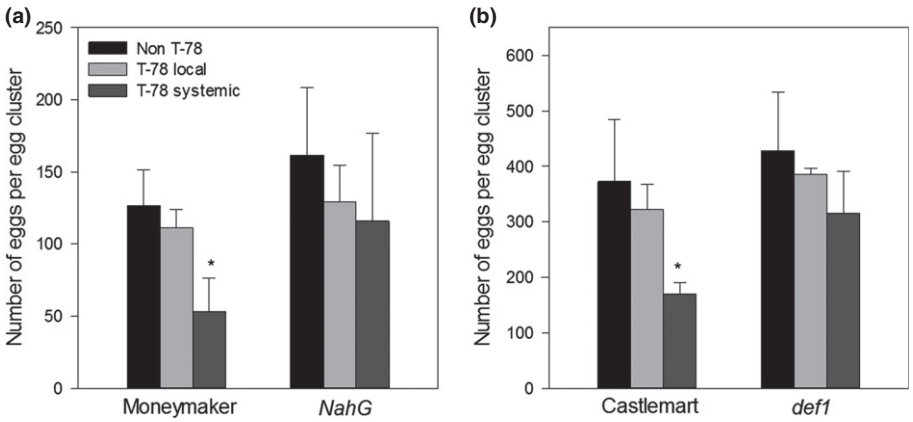

Fig. 9 *Trichoderma harzianum* interference with nematode fecundity requires salicylic acid (SA) and jasmonic acid (JA) signalling. The number of *Meloidogyne incognita* eggs was monitored at 5 wk after inoculation with the nematode in dissected galls from roots of *T. harzianum* (T-78)-uninoculated (Non T-78) plants and locally or systemically T-78-preinoculated plants in two independent experiments including (a) the wild-type cv Moneymaker (MM) and the SA-deficient transgenic line *NahG*; and (b) the wild-type Castlemart (CM) and the JA-impaired mutant *def1*. Data are mean ± SE. For each tomato genotype, the asterisk indicates a significant difference from non-T-78-preinoculated roots according to Dunnett's test ($P < 0.05$; $n = 20$). *, $P < 0.05$.

SA- and JA-mediated defence signalling at different stages of the root–nematode interaction. *Meloidogyne incognita* triggered a complex series of hormonal-coordinated responses in the host roots, which varied depending on the nematode infection stage. A transient up-regulation of *PR1a* and *PR-P6* (SA markers) was observed at the induction/early nutrient acquisition stages (Fig. 4), while *PI II* and *MC* (JA markers) were down-regulated by the nematode throughout the entire parasitism process (Fig. 5a,b), and more strongly during later stages of the parasitism (the feeding and reproduction stages). These findings point

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to specific kinetics and signatures of the SA- and JA-modulated pathways upon *M. incognita* attack (Fig. 10). Similarly, Kammerhofer *et al.* (2015) found a 'strict dependence' of the hormonal-coordinated responses triggered by the cyst nematode *Heterodera schantii* on the parasitism stage.

Colonization with T-78 was not associated with direct transcriptional activation of SA- or JA-marker genes in local or systemic root tissues (Figs 4, 5). However, a faster activation of *PR1a* and *PR-P6* was observed in T-78 plants upon nematode invasion, starting already at 3 d after the inoculation with the nematode (Fig. 4). This was associated with priming for SA production and SA sensitivity (Figs S1, S2). Elicitation of SA-mediated defences by application of exogenous SA or synthetic analogues such as benzothiadiazole has previously been shown to decrease the infection of different species of root knot nematodes (Nandi *et al.*, 2003; Molinari & Baser, 2010; Vieira dos Santos *et al.*, 2013; Melillo *et al.*, 2014; Nikoo *et al.*, 2014). Further, *PR1*-overexpressing Arabidopsis plants exhibit reduced susceptibility to *M. incognita* infection (Hamamouch *et al.*, 2011). Thus, the observed boost of SA-dependent defences by T-78 at the invasion stage of *M. incognita* infection putatively contributed to the observed enhancement of host immunity against the nematode. The critical role for SA signalling in T-78-mediated protection against invasion by *M. incognita* was confirmed using the SA-impaired *NahG* plants, as T-78-mediated protection against nematode invasion was completely blocked in *NahG* plants (Fig. 7a). Accordingly, T-78 failed to increase SA concentrations and to prime *NahG* plants for enhanced *PR1a* gene expression (Fig. S4). However, later on in the parasitism cycle, SA-dependent signalling became less important in T-78-induced protection, as *NahG* plants developed a similar level of enhanced resistance to galling as wild-type plants (Fig. 8a,b). This indicates that priming of SA-dependent defences by T-78 is required to limit *M. incognita* invasion by increasing nematode mortality or delaying the penetration, but does not contribute to the induced protection during the sedentary feeding stage. Accordingly enhancement of the SA-related phenylpropanoid pathway has been proposed as one of the possible mechanisms underlying mycorrhizal protection against root knot nematode infection (Vos *et al.*, 2012, 2013). However, how boosting of the SA-dependent pathway by T-78 reduced invasion of nematodes remains unknown.

JA-dependent defence signalling has been reported to play an important role in host susceptibility to root parasitic nematodes (Bhattarai *et al.*, 2008). In line with our observations, previous studies found that root knot nematodes can suppress JA-mediated defences from very early stages after penetration, probably to promote parasitism success (Nahar *et al.*, 2011; Kyndt *et al.*, 2012; Ji *et al.*, 2013). Interestingly, reduced suppression of the JA-dependent defence genes *PI II* and *MC* was observed in T-78-treated roots, from nematode invasion to the sedentary feeding stage (Fig. 5a,b). Remarkably, this reduction of suppression lasted longer in systemic tissue of T-78-inoculated plants compared with local tissue. It has been demonstrated for some *Trichoderma* isolates that they are capable of modulating JA-related defences in leaf tissue following pathogen attack (Segarra *et al.*, 2009; Martinez-Medina *et al.*, 2013). However, the ability of root knot nematodes to actively manipulate host defences renders it difficult to discern whether the reduced suppression of JA-dependent defences in T-78-treated roots results from the ability of T-78 to boost JA-related defences, or rather from the reduced nematode infection in T-78 roots, which might interfere with the ability of the nematode to deregulate host immunity. The *PI II* and *MC* expression analysis after exogenous application of MeJA showed that, similar to systemic leaves, T-78 primes systemic root tissues for faster, stronger and more durable activation of JA-related defences (Fig. 5c,d). In addition, production of jasmonates is primed by T-78 (Fig. S2). Remarkably, T-78 did not prime JA-related defence gene expression in local root tissues, suggesting that the protective effect in local roots against *M. incognita* parasitism (Fig. 2) relies on mechanisms other than enhancement of JA-dependent defences. Such mechanisms might

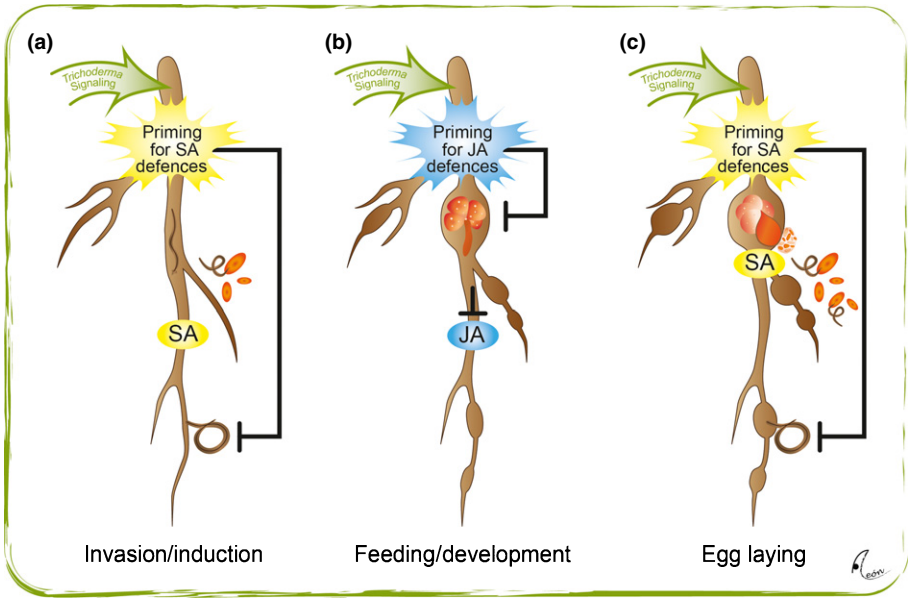

Fig. 10 Model for the plasticity of priming of salicylic acid (SA)- and jasmonic acid (JA)-related defence responses by *Trichoderma harzianum* against *Meloidogyne incognita*. (a) During invasion by the nematode *M. incognita*, *Trichoderma* boosts SA-dependent defences in roots, leading to higher resistance to nematode invasion. (b) Subsequently, during the feeding stage, *Trichoderma* antagonizes the suppression of JA signalling by the nematode by increasing jasmonate concentrations, leading to reduced nematode development and reproduction. (c) In the mature root galls that develop upon successful parasitism, nematode egg-induced SA-dependent defences are primed by *Trichoderma*, which contributes to host resistance in the early stage of successive nematode invasion.

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include direct effects of T-78 on the nematode and/or elicitation of multiple signalling pathways in the roots other than the JA pathway. Although we do not have an explanation for the differences between T-78-induced local and systemic defences, previous studies have also shown differences in local and distal root defense responses triggered by other beneficial fungi (Pozo *et al.*, 2002; Hao *et al.*, 2012). In contrast tissues locally inoculated with T-78, the long-lasting T-78-induced interference with the suppression of JA-dependent defences by the nematode found in systemic tissues is probably linked to the ability of T-78 to prime JA-dependent defences.

Previous experiments showed that elicitation of the JA pathway by external MeJA application enhances resistance to root knot nematode infection in different plants species, including tomato (Cooper *et al.*, 2005; Fujimoto *et al.*, 2011; Nahar *et al.*, 2011; Vieira dos Santos *et al.*, 2013; Fan *et al.*, 2015). This protective effect has been linked to increased concentrations of compounds that are toxic to nematodes, such as phytoectosteroids, flavonoids, and proteinase inhibitors (Soriano *et al.*, 2004a,b; Cooper *et al.*, 2005; Fujimoto *et al.*, 2011) in MeJA-elicited plants. Therefore, activation of JA-dependent defences by T-78 might underlie the systemic protection against *M. incognita* observed in our study. Interestingly, the phenotypic analysis of the *M. incognita* infection in roots of JA-impaired *def1* plants indicated that JA signalling is not required for T-78-mediated protection against invasion by the nematode in either systemic or local root tissue, as determined at 7 d after infection with *M. incognita* (Fig. 7b). However, JA signalling plays a critical role in T-78-induced protection at later stages of *M. incognita* infection, as local and systemic protection against root galling as determined at 3, 5 and 7 wk after nematode inoculation was absent in *def1* mutants (Fig. 8a,b). Accordingly, T-78 failed to increase JA concentrations and to prime *PI II* gene expression in *def1* plants (Fig. S4). These findings indicate that, in contrast to priming of SA-dependent defences, regulation of the JA-dependent defences seems to play a role in T-78 systemic protection at later stages of nematode parasitism (i.e. during nematode feeding and reproduction). In line with our observations, JA-inducible protease inhibitors and cystatins have been shown to affect the growth and development of root knot nematodes, interfering with different processes such as nutrition, reproduction and embryogenesis, without affecting in the ability of nematodes to invade roots (Chan *et al.*, 2010; de Souza *et al.*, 2013). Accordingly, a reduced number of eggs and a retardation in embryogenesis were found only when T-78 was inoculated systemically, supporting the idea that regulation of JA-dependent defences plays an important role in systemic protection during later stages of the parasitism. Remarkably, the ability of T-78 to interfere with *M. incognita* fecundity (as measured by the amount of eggs per egg cluster) in systemic root tissue was absent in both the JA-impaired *def1* and the SA-impaired *NahG* lines (Fig. 9). These findings indicate that, in addition to JA-dependent defences, SA signalling is also required for T-78 inhibition of *M. incognita* reproduction.

The nematode eggs that are present in egg clusters on root galls represent a threat to the plant, as newly hatched juveniles will rapidly re-invade new root tissues. We have shown that *PR1a* transcription was up-regulated in mature galls at the time of egg deposition (5 and 7 wk after inoculation with *M. incognita*; Fig. 6). Plants can detect the presence of insect eggs on their leaves and respond to them by activating SA-related defences (Hilker & Fatouros, 2015). Our results suggest that roots might also detect *M. incognita* eggs, the gelatinous matrix or other associated elicitors, and consequently activate SA-dependent defences. In plants preinoculated with T-78, a boosted up-regulation of *PR-1a* was found in root galls (Fig. 6a). These findings show again the ability of T-78 to fine-tune the different hormone-regulated defence responses depending on the stage of *M. incognita* infection. According to our observations, boosting of the SA pathway by T-78 might play a defensive role against newly hatched J2, during the early stages of nematode invasion.

Based on our results, we suggest that the T-78-induced protection against *M. incognita* in systemic root tissue proceeds in three phases (Fig. 10). In the first phase, T-78 primes tomato root tissues for faster SA-regulated defence responses to protect roots against *M. incognita* invasion (Fig. 10a). In the second phase, when *M. incognita* suppresses JA-related defences in the roots, T-78 switches its activity to priming for expression of JA-dependent defences, thereby antagonizing the *M. incognita*-mediated suppression, leading to a reduction in nematode development and reproduction (Fig. 10b). Once the *M. incognita* parasitism is established, T-78 boosts the activation of SA-dependent defences which are activated in the gall, probably through recognition of the eggs. This may contribute to enhanced defences against invasion by new juveniles (Fig. 10c). Overall, we have shown that the cost-efficient *Trichoderma*-induced systemic resistance against a complex root attacker is a plastic phenomenon, which adapts its priming of SA- and JA-related defence responses according to the stage of the dynamic *M. incognita* infection cycle.

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Author contributions

A.M.-M., S.C.M.V.W. and C.M.J.P. planned and designed the research. A.M-M., I.F. and G.B.L. performed experiments. A.M-M., I.F., G.B.L., M.J.P. and S.C.M.V.W. analysed data. A.M-M., M.J.P., C.M.J.P. and S.C.M.V.W. wrote the manuscript.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information tab for this article:

Fig. S1 *Trichoderma harzianum* primes roots for enhanced expression of the SA-marker gene *PR-P6*.

Fig. S2 Impact of *Trichoderma harzianum* preinoculation on hormone levels in *Meloidogyne incognita*-infected roots.

Fig. S3 Exogenous application of catechol does not affect tomato resistance to *Meloidogyne incognita* infection.

Fig. S4 Impact of *Trichoderma harzianum* preinoculation on JA and SA accumulation and *PR1a* and *PI II* expression in tomato *NahG* and *def1* plants infected with *Meloidogyne incognita*.

Table S1 Primer sequences used for the real-time qPCR analysis

Table S2 Results of two-way ANOVAs for the response of *Meloidogyne incognita* galling and the relative expression of *M. incognita Actin*, and the response of *PR1a* and *PI II* relative expression in hand-dissected galls to *Trichoderma harzianum* preinoculation and time after *M. incognita* inoculation

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Table S3 Results of two-way ANOVAs for the response of the relative amount of *Meloidogyne incognita* DNA and number of eggs per egg cluster to *Trichoderma harzianum* preinoculation and plant genotype

Table S4 Results of three-way ANOVAs for the response of *Meloidogyne incognita* galling to *Trichoderma harzianum* inoculation, plant genotype, and time after infection

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