Agrobacteria Enhance Plant Defense Against Root-Knot Nematodes on Tomato

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ABSTRACT

The increased incidence of the crown gall disease caused by *Agrobacterium tumefaciens* has long been associated with activities of root-knot nematodes (*Meloidogyne* spp.). Pot experiments on tomato were designed to assess plant vitality, nematode reproduction, and crown gall incidence in combined infection with *Agrobacterium* and *Meloidogyne* spp. on tomato roots. Results suggest that tomato plants infected with pathogenic *A. tumefaciens* 2 days before the nematodes show enhanced plant defense against *M. ethiopica* resulting in lower egg and gall counts on roots 45 and 90 days postinoculation (dpi); no significantly enhanced defense was observed when the plant was inoculated with bacteria and nematodes at the

same time. Split-root experiments also showed that the observed interaction was systemic. Reverse-transcription quantitative polymerase chain reaction analysis that targeted several genes under plant hormonal control suggests that the suppression was mediated via systemic acquired resistance by the pathogenesis-related protein 1 and that *M. ethiopica* did not enhance the defense reaction of tomato against *Agrobacterium* spp. Nematodes completely inhibited tumor growth in a 45-day experiment if inoculated onto the roots before the pathogenic bacteria. We conclude that the observed antagonism in the tested pathosystem was the result of initially strong plant defense that was later suppressed by the invading pathogen and pest.

Combined infection of crown gall-causing bacteria (e.g., *Agrobacterium* spp.) and root-knot nematodes (RKN) (*Meloidogyne* spp.) results in increased frequency and severity of crown gall disease (Rubio-Cabetas et al. 2001) and stimulated RKN reproduction (El-Sherif and Elwakil 1991) on host plants. However, no consensus exists as to whether synergism of the pest and pathogen or an additive effect is responsible for the increased disease phenotype. Although RKN evade and modify host defense (Smant and Jones 2011), pathogenic or nonpathogenic bacteria may activate pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) (Jones and Dangl 2006), and most phytopathogenic bacteria suppress plant-related immune responses (Abramovitch et al. 2006).

The hypothesis of the synergism in the *Agrobacterium*–*Meloidogyne* pathosystem is based on reports from several authors that observed a positive correlation between the incidence of crown gall and the incidence of galling induced by RKN. Griffin et al. (1968) noticed higher incidence of raspberry crown gall when RKN were present in the soil. Similar observations were made by Orion and Zutra (1971) and Zutra and Orion (1982) on almond and cotton, Dhanvantari et al. (1975) on peach, Süle et al. (1995) on grapevine, and Rubio-Cabetas et al. (2001) on *Prunus cerasifera*. In a split-root experiment, *Agrobacterium tumefaciens* stimulated reproduction and development of *Meloidogyne incognita* in parts of tomato roots accessible only to the nematodes (El-Sherif and Elwakil 1991). Synergism was also inferred when Karimi et al. (2000) demonstrated that RKN transported agrobacteria into the roots that resulted in an increased transformation frequency of T-DNA from agrobacteria. In two reports, however, authors observed nonsynergistic effects of nematodes and bacteria. Fakhouri et al. (1996) described reduced disease symptoms with combined infection on tomato after 90 days, and El-Beltagi et al. (2011) showed that *A. tumefaciens* reduced *M. incognita* gall and egg production on grapevine roots after 1 year.

Agrobacteria and RKN cause damage on numerous plant species worldwide. Crown gall is economically more important on woody

hosts such as nut and fruit trees (Epstein et al. 2008; Kennedy and Alcorn 1980) and polyphagous RKN can significantly reduce crop yields in vegetables within one growing season (Nicol et al. 2011). *M. ethiopica* caused significant damage on grapevine, actinidia, and potato crops in Chile (Carneiro et al. 2007) and was detected for the first time in 2003 in Slovenia under sub-Mediterranean climate conditions (Širca et al. 2004). Later, it was also discovered in Greece (Conceição et al. 2012) and Turkey (Aydinli et al. 2013). RKN and crown-gall bacteria are difficult to control because efficient and environmentally friendly management strategies are limited. Recently, it has been recognized that soils accommodating a high microbial diversity possess high suppressiveness against RKN (Adam et al. 2014), with different rhizobacteria known to antagonize RKN (Aballay et al. 2013; Hallmann et al. 2001, 2009; Martinuz et al. 2013; Radwan et al. 2012; Siddiqui et al. 2007). In addition, rhizobacteria, mycorrhizal fungi, bioproducts of microbial origin, and derivatives of salicylic acid (SA) and jasmonic acid (JA) can induce resistance against various pathogens (Bakker et al. 2007; Fujimoto et al. 2011; Van Wees et al. 2008; Yan et al. 2002).

Plant defense is activated as soon as the host recognizes an invading pathogen. This first and basal line of defense is activated upon recognition of the PAMP and leads to PTI. PTI is part of the innate immune system and associated, among others, with early oxidative burst (ROS), defense gene induction, and callose deposition on the cell walls (Jones and Dangl 2006; Zipfel 2009). Many rhizobacteria trigger PTI and resistance-inducing mechanisms such as systemic acquired (SAR) and induced systemic resistance (ISR) (Jones and Dangl 2006; Van Wees et al. 2008). Rhizobacteria-induced ISR is manifested in upregulation of JA- and ethylene (ET)-controlled genes (Van der Ent et al. 2009). To overcome plant defense, pathogenic agrobacteria have evolved systems to suppress plant defense mechanisms to effectively integrate a segment of a tumor-inducing plasmid (Ti) called T-DNA into the plant genome (Lee et al. 2009; Veena et al. 2003; Xu and Pan 2000). The underlying mechanism was identified as a rapid local plant defense gene suppression on *Arabidopsis thaliana* (Ditt et al. 2006; Lee et al. 2009) and *Nicotiana tabacum* (Veena et al. 2003) and on cultured cells of *Ageratum conyzoides* (Ditt et al. 2005). Expression of bacterial tumorigenic genes may disturb plant hormonal balances, enabling uncontrolled division of

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transformed cells and tumor growth (Veselov et al. 2003). According to the model of Veena et al. (2003), the strength of plant defense rises again after *Agrobacterium* spp. had successfully transformed the plant cells. This model can explain why SA, albeit known to enhance plant's resistance to *Agrobacterium* (Anand et al. 2008), was found in tumors (Deeken et al. 2006; Lee et al. 2009).

Local plant defense is also suppressed by RKN that are known to modulate host immune responses. Infective second-stage juveniles secrete proteins designed to evade, prevent, and manipulate host defense mechanisms during root penetration and feeding site formation (Davis et al. 2008; Dubreuil et al. 2011; Jaubert et al. 2002). However, host resistance genes in tomato may recognize nematode effectors and activate an effector-triggered immunity (ETI) response, resulting in strong hypersensitive-like reactions against the pest (Smant and Jones 2011). Evidence for the suppression of the PTI or ETI signaling by RKN is slowly emerging. Two recent studies suggest that *M. incognita* and *M. javanica* nematodes secrete effector proteins interfering with the host PTI response (Jaouannet et al. 2013; Lin et al. 2016).

The research presented here aims at (i) determining the nature of the *Agrobacterium*–*Meloidogyne* interaction (synergistic versus antagonistic) in biological tests on a susceptible tomato hybrid and (ii) measuring the involvement of the systemic plant response in split-root experiments. To determine the plant defense mechanisms involved in the *Agrobacterium*–*Meloidogyne* interaction, a reverse-transcription quantitative polymerase chain reaction (RT-qPCR) analysis of JA/ET-regulated (*ACO1*, *ACS6*, *COI1*, *ERF5*, *JA3*, and *SIWRKY11*) and SA-regulated (*PR1* and *NIM1*) tomato genes was conducted.

MATERIALS AND METHODS

Single-pot experiments to evaluate pest–pathogen combined activity on the host plant, nematode reproduction, and crown gall incidence. Tomato (*Solanum lycopersicum* L.) hybrid ‘Arawak’ (Syngenta) seed were used in pot experiments (Table 1). Before planting, seed were surface sterilized in 70% ethanol for 15 min, soaked in 2.5% NaOCl with a drop of Tween 20 for 30 min, and washed in sterile water. Efficiency of surface sterilization was tested on King’s B (KB) medium for 1 week where aliquots of seed were germinated. Dry seed were planted into a mixture of autoclaved sand (0.25 to 1.0 mm) and hydroculture granules (1 to 4 mm) at a 3:1 (vol/vol) ratio. After 2 weeks, seedlings were transplanted into pots with 600 cm³ of sterilized sand and watered daily with sterile nutrient solutions for hydroponic growth (General Hydroponics) at pH 5.5 ± 0.2. The plants were grown in a growth chamber at an average daily temperature of 23.3°C and 14-h photoperiod. Four-week-old seedlings were used in pot experiments.

A population of *M. ethiopica* (Širca et al. 2004) from Slovenia (Table 1) was maintained on susceptible ‘Saint Pierre’ tomato plants in a greenhouse. Egg inoculum was obtained from the infested tomato roots (Hussey and Barker 1973) and cleansed following the modified protocol of McClure et al. (1973). Nematode eggs retrieved from the 32-μm sieve and collected into 50-ml polycarbonate centrifuge tubes

were centrifuged at 952 × g for 5 min. The pellets were resuspended in 45.4% (wt/vol) sucrose solution and centrifuged at 635 × g for 1 min. The supernatant containing eggs was poured over a 32-μm sieve and rinsed with tap water. A clean suspension of eggs was collected from the sieve. Eggs were surface sterilized in 0.002% HgCl₂ for 4 min and rinsed three times in sterile water. Egg concentrations were estimated with a stereomicroscope.

Agrobacterium tumefaciens (Smith and Townsend 1907) Conn 1942 strains 06-424-7 and 50-1 from Slovenia were used (Table 1). The isolates were maintained at –80°C, revitalized on KB plates for 2 days at 27°C, and subcultured. A loopfull of inoculum was transferred from a 1-day-old culture into 100 ml of liquid KB medium and incubated at 27°C for 16 to 18 h on a laboratory shaker. The culture was pelleted at 1,000 × g for 15 min and washed two times in sterile water. A bacterial inoculation suspension of approximately 10⁸ CFU/ml was prepared by adjusting the transmittance to 80%. Transmittance was measured with a turbidimeter (BioLog). Pathogenicity of reisolated *A. tumefaciens* from experimental pots was assessed on tomato plants at the end of the experiment. The tomato stem was punctured three times using a sterile entomological needle dipped into 1-day-old culture colonies grown on KB medium at 27°C. After 3 to 6 weeks, the plants were visually inspected for tumor formations.

Roots of 4-week-old tomato seedlings were inoculated with 2,000 *M. ethiopica* eggs and *A. tumefaciens* strain 06-424-7 at 10⁶ CFU/g of sand, or water (mock control). Before the experiment, the pathogenic strain 06-424-7 was tested for its capability to form tumors at 10⁶ CFU/g of sand on the roots of tomato. Inoculations were performed with a syringe to inject the inoculation suspension directly into substrate through three holes positioned around the tomato plant. The experimental design and treatments are described in Table 2. The experiment was conducted twice; six and five replicates per treatment were used in the first and second pot experiments, respectively. We added an additional treatment (N2npB1) to the second experiment, where a pathogenic *A. tumefaciens* strain was replaced by a non-pathogenic one (50-1) (Tables 1 and 2). Both experiments ended 45 days postinoculation (dpi) according to an equation model for the nematode reproduction cycle at different temperatures (Strajnar et al. 2011). Yet another experiment was set up to evaluate the interaction after the second reproduction cycle of the *M. ethiopica* nematode (90 dpi) designed in four different treatments (Table 2). This experiment was conducted once with five replicates per treatment. In all three experiments, the pots were randomly arranged in the growth chamber and, once a week, randomly rearranged to provide fairly similar conditions to the plants.

Tomato plants were harvested 45 or 90 days after the onset of the experiments. Shoots were cut, their length measured, and fresh weight recorded. The shoots were dried at 65°C for 72 h and weighed. Roots were washed by soaking and rinsing with tap water. Tumors were counted and excised. Fresh root weight was assessed by cutting the root into 1-cm strips that were thoroughly mixed in water, passed through filter discs (40 g/m²; Munketell) to remove gravitational water, and then weighed. A root subsample of 0.5 g was used for gall and egg sac enumeration by staining the roots in

TABLE 1. Organisms used in this study

Organism	Cultivar, strain	Characteristics	Source
<i>Solanum lycopersicum</i> L.	Arawak F1	Tomato hybrid, susceptible to RKN ^y	Syngenta
<i>Meloidogyne ethiopica</i>	Slovenian population	Root-knot nematode	Laboratory collection
<i>Agrobacterium tumefaciens</i>	06-424-7	Pathogenic, isolated from rose tumor, group G1 ^z	Laboratory collection
	50-1	Nonpathogenic soil isolate, group G1 ^z	Laboratory collection (Lamovšek et al. 2014)
	C58	Pathogenic, group G8 ^z	INRA
	C58C1	Nonpathogenic, cured from pTiC58	INRA
<i>Escherichia coli</i>	JM109	Nonpathogenic	Promega Corp.

^y RKN = root-knot nematodes.

^z Genomic species of *A. tumefaciens* species complex (Costechareyre et al. 2010).

Phloxine B (15 mg/liter) for 20 min. The total number of eggs was estimated according to Hussey and Barker (1973). The roots were chopped in a kitchen blender in 1% NaOCl for 3 to 4 min. The macerated roots were poured through three sieves, each carefully washed, to gather the eggs on a 32- μ m sieve, then collected in a 50-ml centrifuge tube. Appropriate dilutions were used to determine the egg concentration by counting eggs under a binocular stereomicroscope. The *M. ethiopica* reproduction was evaluated by calculating the reproduction factor (Rf), represented as ratio between final nematode egg number on host roots and initial inoculated number of eggs.

In treatments with bacteria, a sample of sandy substrate was taken to assess the bacterial concentration in each pot after the experiment. Sand (10 g) was mixed with 90 ml of sterile water and shaken for 30 min at 300 rpm. Four 10-fold serial dilutions were prepared from the sand suspension and streaked onto selective medium 1A (Schaad et al. 2001) supplemented with tellurite (Mougel et al. 2001) in three replicates per sample. The plates were incubated at 27°C for 3 to 4 days. According to visual inspection, the number of CFU per gram of sand was determined (Mougel et al. 2001). Data from two experiments were pooled.

Split-root experiment to evaluate systemic response of tomato roots to agrobacteria and *M. ethiopica* reproduction. For evaluating the systemic plant response, a split-root technique was used. One week after seed germination in sterile sand, seedlings were uprooted and the lateral root growth stimulated by cutting off the root tip with a sterile blade. The seedlings were repotted in sterilized sand substrate and maintained on a 14-h light cycle at the average daily temperature of 23.3°C. After an additional 2 weeks of growth, the seedlings were uprooted, the root system was divided into two parts, each part planted into 250 g of sterile sandy substrate in two

separate pots that had been glued together, and a plastic stick was placed between the pots for the support of the tomato stem. After two additional weeks, the roots of one pot were inoculated with 1,000 *M. ethiopica* eggs and the roots in the other pot with *A. tumefaciens* at 10⁶ CFU/g of sand. The split-root experiment was designed in six different treatments with four replicates (Table 2). In addition to *A. tumefaciens* strain 06-424-7, we included treatments with heat-killed strain 06-424-7 and living nonpathogenic bacteria *Escherichia coli* JM109 (Table 1). The inoculations were performed with a syringe injecting the inoculation suspension directly into the sand close to the roots. Experimental design and treatments are explained in Table 2.

***M. ethiopica* juvenile penetration into tomato roots.** To assess the effect of *A. tumefaciens* on *M. ethiopica* nematode penetration, an experiment using the split-root tomato plants was designed involving the treatment with 1,000 *M. ethiopica* eggs and pathogenic *A. tumefaciens* strain 06-424-7 at 10⁶ CFU/g of sand. The experimental design included three treatments in six replicates: (i) nematodes but no bacteria to roots of both pots at 0 days, (ii) nematodes to roots in one pot at 0 days and bacteria and nematodes to roots (NB) in the other pot at 0 days, and (iii) inoculation of strain 06-424-7 into the plant stem for tumor induction at 0 days and nematodes into both pots at 14 days (Table 3). To count the juveniles that entered the roots, plants from each treatment were uprooted and roots were rinsed and stained with acid fuchsin (Byrd et al. 1983) after 8 and 12 dpi.

Statistical analysis of pot experiment data. Statistical analysis was performed with R Software (v. 3.0.0.) and the Rcmdr package (v. 1.9-6) (R Development core team, www.r-project.org/). The homogeneity of variances was confirmed (Levene's test) prior to analysis of variance ($P < 0.05$). The Tukey's honestly significant

TABLE 2. Treatment description of pot trials for evaluating interactions of *Agrobacterium tumefaciens*, *Meloidogyne ethiopica*, and tomato in single-pot and split-root (two-pot) experiments

Treatment	Root part	Inoculation		Replicates (n)		End ^r	Note
		Time	Inoculum	Date 1	Date 2		
Single pot							
Control	Whole	0	Water (mock)	6	5	45	Data pooled
	Whole		Water (mock)	5		90	
N ^s	Whole	0	<i>M. ethiopica</i>	6	5	45	Data pooled
	Whole		<i>M. ethiopica</i>	5		90	
B ^t	Whole	0	<i>A. tumefaciens</i> strain 06-424-7	6	5	45	Data pooled
NB ^u	Whole	0	<i>M. ethiopica</i>	6	5	45	Data pooled
	Whole	0	<i>A. tumefaciens</i> strain 06-424-7				
N1B2 ^v	Whole	0	<i>M. ethiopica</i>	6	5	45	Data pooled
	Whole	8	<i>A. tumefaciens</i> strain 06-424-7	5		90	
N2B1 ^w	Whole	0	<i>A. tumefaciens</i> strain 06-424-7	6	5	45	Data pooled
	Whole	2	<i>M. ethiopica</i>	5		90	
N2npB1 ^x	Whole	0	<i>A. tumefaciens</i> strain 50-1	5	5	45	Data pooled
	Whole	2	<i>M. ethiopica</i>		5	45	
Split root							
N ^s	A	0	<i>M. ethiopica</i>	4	...	45	...
	B		Water		...		...
NB ^u	A	0	<i>M. ethiopica</i>	4	...	45	...
	B	0	<i>A. tumefaciens</i> strain 06-424-7	4	...	45	...
N1B2 ^v	A	0	<i>M. ethiopica</i>	4	...	45	...
	B	8	<i>A. tumefaciens</i> strain 06-424-7	4	...	45	...
N2B1 ^w	A	0	<i>A. tumefaciens</i> strain 06-424-7	4	...	45	...
	B	2	<i>M. ethiopica</i>	4	...	45	...
N2hkB1 ^y	A	0	Heat-killed <i>A. tumefaciens</i> strain 06-424-7	4	...	45	...
	B	2	<i>M. ethiopica</i>	4	...	45	...
N2Ec1 ^z	A	0	<i>Escherichia coli</i> JM109	4	...	45	...
	B	2	<i>M. ethiopica</i>	4	...	45	...

^r Trial end (days).

^s Treatment with *M. ethiopica* (2,000 eggs/pot; 1,000 eggs/pot in split root).

^t Treatment with *A. tumefaciens* water suspension (10⁶ CFU/g of soil).

^u Simultaneous treatment with *M. ethiopica* (2,000 eggs/pot; 1,000 eggs in split root) and *A. tumefaciens* (10⁶ CFU/g of soil).

^v Treatment with *M. ethiopica* (2,000 eggs/pot; 1,000 eggs in split root) 8 days before *A. tumefaciens* (10⁶ CFU/g of soil).

^w Treatment with *A. tumefaciens* (10⁶ CFU/g of soil) 2 days before *M. ethiopica* (2,000 eggs/pot; 1,000 eggs in split root).

^x Treatment with nonpathogenic strain of *A. tumefaciens* (10⁶ CFU/g of soil) 2 days before *M. ethiopica* (2,000 eggs/pot).

^y Treatment with heat-killed strain of *A. tumefaciens* (10⁶ CFU/g of soil) 2 days before *M. ethiopica* (2,000 eggs/pot; 1,000 eggs in split root).

^z Treatment with a strain of *Escherichia coli* (10⁶ CFU/g of soil) 2 days before *M. ethiopica* (2,000 eggs/pot; 1,000 eggs in split root).

difference test was applied when significant differences between the treatments were observed. Data from single-pot experiments that ended after 45 days were pooled (Table 2). Where appropriate, the data were log-transformed to ensure normality of the variances.

Split-root experiment for evaluation of systemic plant response based on gene expression of selected target tomato genes. RT-qPCR based on SYBR Green I as a reporter molecule was used to quantify gene expression of 10 selected target genes. We used split-root tomato pots where only one pot was inoculated; the other pot was used for root sampling. Two *A. tumefaciens* strains were used in this experiment: a pathogenic strain C58 and commonly used nonpathogenic strain C58C1 that is cured of its pTiC58 plasmid (Table 1). The experimental design was similar to the 45-dpi experiment (Table 4) and included six treatments with four replicates per time point (Table 4): (i) Control (water in one pot, 0 days); (ii) 1,000 *M. ethiopica* eggs in one pot, 0 days; (iii) C58 (*A. tumefaciens* strain C58 at 10^6 CFU/g of sand in one pot, 0 days); (iv) C58C1 (*A. tumefaciens* strain C58C1 at 10^6 CFU/g of sand in one pot, 0 days), (v) N2B1 (in the same pot, *A. tumefaciens* strain C58 was inoculated at 0 days and *M. ethiopica* eggs 2 days later); and (vi) N1B2 (in the same pot, *M. ethiopica* eggs were inoculated at 0 days and *A. tumefaciens* strain C58 8 days later). Roots were sampled at 0, 4, and 12 dpi. A noninoculated part of the root was rinsed gently in tap water to remove the sand and subsequently crushed in liquid nitrogen. Meanwhile, the inoculated roots and the stem remained attached to the plant. The first sampling at 0 dpi was

performed within 15 min after the first inoculations. Pulverized root samples were stored at -80°C until they were processed. According to the manufacturer's instructions for the RNeasy Plant Mini Kit (Qiagen), we used 100 mg of root tissue to extract total RNA. The quality and quantity of the extracted RNA was assessed on the Agilent 2100 Bioanalyzer (Agilent Technologies) with Agilent RNA 6000 Nano Chips, according to the manufacturer's instructions. All root samples had RNA integrity numbers above 9.0. Genomic DNA was degraded using the TURBO DNase free kit (Life Technologies) on samples containing 2 μg of RNA in a reaction volume of 25 μl . The absence of genomic DNA was assessed with the Eukaryotic 18S ribosomal RNA endogenous control (VIC/MGB probe, primer limited; Life Technologies) and RNA was reverse transcribed to complement DNA (cDNA) with the high-capacity cDNA RT kit, according to the manufacturer's instructions (Life Technologies). Synthesis was completed in triplicate, with 25- μl reactions (500 ng of total RNA) pooled into one sample and stored at -20°C .

Genes involved in the JA/ET or SA defense signalization were selected as targets (Bhattarai et al. 2010; Journot-Catalino et al. 2006; Mantelin et al. 2013; Pozo et al. 2008; Son et al. 2012; Ward et al. 1991; Weyman et al. 2006; Wu et al. 2012; Xie et al. 1998) and amplified with designed primers (Table 5). Previous studies were used for selecting four reference genes (Table 6) and their amplification primers (Table 5) (Fernández et al. 2014; Fowler et al. 2009; Løvdaal and Lillo 2009) for data normalization according to gene stability

TABLE 3. Number of *Meloidogyne ethiopica* nematode juveniles (RKN) that penetrated into the roots of tomato 8 and 12 days after joined inoculations with nematodes and agrobacteria (*Agrobacterium tumefaciens* strain C58) in split-root experiment^x

Treatment ^y	Pot	Inoculation		<i>n</i>	Number of juveniles		
		Time	Inoculum		8 days	<i>n</i>	12 days
N	A	0	RKN	3	...	3	...
	B	0	RKN	3	20.0 ± 3.14^z	3	38.7 ± 4.13^z
N_NB	A	0	RKN	6	30.2 ± 7.73	6	36.5 ± 5.88
	B	0	RKN + C58	6	18.2 ± 6.01	6	33.3 ± 3.68
T	A	0	RKN	0	n.a.	3	...
	B	0	RKN	0	n.a.	3	34.0 ± 6.52^z
<i>P</i> (treatment)	...	...	...	...	0.333 (n.s.)	...	0.880 (n.s.)

^x Data are represented as average number of counted juveniles inside the roots $\pm$ standard error ($n = 6$); n.s. = not significant and n.a. = not applicable.

^y Treatment designations: N = 1,000 RKN eggs in A and B pots, N_NB = 1,000 RKN eggs in pot A and RKN eggs in suspension of pathogenic *A. tumefaciens* (strain C58 in 10^6 CFU/g of soil) in pot B, and T = 1,000 RKN eggs on tomato plants exhibiting a 2-week-old stem tumor due to *A. tumefaciens* (strain C58) infection.

^z Average juvenile count from six replicates (pots).

TABLE 4. Treatment description of split-root pot experiment for evaluating systemic plant response to *Agrobacterium tumefaciens* and *Meloidogyne ethiopica* at the level of target gene expression in roots of tomato

Treatment	Pot	Inoculation		Replicates (<i>n</i>)	Sampling (dpi) ^u
		Time	Inoculum		
Control	A	0	Sterile water	4	0, 4, 12
	B	0	Sterile water	4	
N ^v	A	0	<i>M. ethiopica</i>	4	0, 4, 12
	B	0	Sterile water	4	
C58 ^w	A	0	<i>A. tumefaciens</i> strain C58	4	0, 4, 12
	B	0	Sterile water	4	
C58C1 ^x	A	0	<i>A. tumefaciens</i> strain C58C1	4	0, 4, 12
	B	0	Sterile water	4	
N1B2 ^y	A	0	<i>M. ethiopica</i>	4	0, 4, 12
	A	8	<i>A. tumefaciens</i> strain C58	4	
N2B1 ^z	B	8	Sterile water	4	0, 4, 12
	A	0	<i>A. tumefaciens</i> strain C58	4	
	A	2	<i>M. ethiopica</i>	4	0, 4, 12
	B	2	Sterile water	4	

^u Sampling of roots in pot B at 0, 4, and 12 days postinoculation (dpi).

^v Treatment with *M. ethiopica* (1,000 eggs/pot A).

^w Treatment with *A. tumefaciens* strain C58 water suspension (10^6 CFU/g of soil in pot A).

^x Treatment with *A. tumefaciens* strain C58C1 (without plasmid Ti) (10^6 CFU/g of soil in pot A).

^y Treatment with *M. ethiopica* (1,000 eggs/pot A) 8 days before *A. tumefaciens* strain C58 (10^6 CFU/g of soil in pot A).

^z Treatment with *A. tumefaciens* strain C58 (10^6 CFU/g of soil in pot A) 2 days before *M. ethiopica* (1,000 eggs/pot A).

expression analysis (Hellems et al. 2007; Vandesompele et al. 2002). The target gene sequences were downloaded from the ENA database (EMBL-EBI) and primer sequences were constructed with the Primer3 tool (Untergasser et al. 2012). Primer pairs with optimal annealing temperature of 60°C and a length of 20 to 22 bp were selected. Primer specificity was assessed with the Primer-BLAST algorithm (National Center for Biotechnology Information) (Altschul et al. 1990) on all available tomato sequences.

The qPCR was performed on a 7500 Fast Real-Time PCR System (Life Technologies) under universal conditions (95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min) and a final dissociation step. Each cDNA sample was run in duplicate. The specificity of the reaction was assessed according to the shape of retrieved melting curves and the shape of the nontemplate control, and additionally verified on 2% agarose gel. The amplification efficiencies for each primer pair were calculated from standard curves on dilutions of a mixed cDNA sample in triplicate and analyzed with the 7500 Software v.2.0.6 (Life Technologies). The interrun variation was controlled by adding three interplate calibration samples to each plate in duplicate. The stability of reference gene expressions was evaluated according to the method of Vandesompele et al. (2002) as implemented in the SLqPCR R package (Kohl 2007).

Quantitative cycle values were tested for differential expression using the Bioconductor package unified WMWqPCR for analysis of RT-qPCR data in R that also incorporates normalization (De Neve et al. 2013). Gene expression was interpreted from values of log of odds ratio, giving the estimate of the probability that the target gene from the independent treatment is upregulated in treatment 1 but not in treatment 2.

RESULTS

Tomato growth in the presence of pathogenic agrobacteria and RKN on roots. Single-pot experiments were conducted to evaluate the effect of combined infection (*A. tumefaciens* and

M. ethiopica) on plant vitality (Table 2). *A. tumefaciens* strain 06-424-7 and *M. ethiopica* nematodes induced tumors and galls but did not affect plant vitality during the experiment (45 or 90 dpi) because of the low concentration of applied inocula. No statistically significant change in tomato root weight or change in fresh and dry shoot weight was recorded 45 dpi, although the treated plants were 3.5 cm shorter, on average, than control plants ($P < 0.001$) (Table 7). After 90 dpi, treated plants appeared healthy, although the water content of the shoots was reduced ($P < 0.05$). Infestations with *M. ethiopica* eggs alone caused the greatest reduction in fresh shoot weight (average = 9.2 g) and a reduced dry shoot weight of 1.8 g. Due to galls, the *M. ethiopica*-infested roots were heavier than noninfested roots at 90 dpi (Table 7).

***M. ethiopica* reproduction in the presence of agrobacteria.** Uprooted tomato plants from single-pot experiments (Table 2) were used for determination of *M. ethiopica* Rf. The Rf was lowest in treatment N2B1, where *A. tumefaciens* strain 06-424-7 was added 2 days prior to *M. ethiopica* nematode eggs (Table 7). In treatment N2B1, the final egg count was 59% lower than in control treatments. A statistically similar reduction (68%) was also received with nonpathogenic strain 50-1 (Table 7). Lower nematode reproduction also was determined in treatment N2B1 at 90 dpi. The highest *M. ethiopica* reproduction was observed in treatment N1B2 after 90 days ($P = 0.012$) (Table 7).

TABLE 6. Stability of reference genes expression^z

Gene	CV (%)	<i>M</i> value
BTUB	29.21	0.485
UBI	10.39	0.286
EF1L	15.44	0.362
RPL2	10.15	0.341
Average	11.63	0.368

^z CV = coefficient of variation (%) and *M* = gene expression stability ($M \leq 1.5$).

TABLE 5. List of target and reference genes with the primer pair sequences used in this study^z

Gene	Target gene name	Primer sequence (5'–3')	Amp (bp)	Accession number	Conc (nM)	Reference
PR1	Pathogenesis-related protein 1	TGTCCGAGAGGCCAAGCTATAAC	143	DQ159948	300	Song et al. 2011
...	...	AATGAACCAACATCCGTTGTTGC	...	...	300	...
NIM1	Noninducible immunity 1 (syn. NPR1)	GCTTTTTCGGATTCCAATGA	202	NM_001247629.1	600	This study
...	...	GATGCACCGGAATTTCTCTTA	...	...	600	...
ERF5	Ethylene responsive factor 5	TGGAGCAACCGATTCTAGC	247	AY600439.1	300	This study
...	...	AACCTACGCACCCCTCAATGC	...	...	300	...
ACO1	1-Amino-cyclopropane-1-carboxylic acid (ACC) oxidase	TGAGTTGGTGAACCATGGAA	190	NM_001247095.1	300	This study
...	...	AGTAGGAAGATGGCGCAAGA	...	...	300	...
ACS6	ACC synthase 6	CAACCGCGTTTAGTCAACCT	164	NM_001247235.1	300	This study
...	...	GACAACGGCATCATTTGTACG	...	...	300	...
JA3	Jasmonic acid 3 (MYC2 transcription factor)	CAGTTTTGCCTTCTTCGGGC	154	XM_004245847.1	300	This study
...	...	TTCGCTGGCTTTCTACCTCG	...	...	300	...
COI1	Coronatine-insensitive protein 1	GGCTGCTGTTTCAGTGAACG	212	NM_001247535.1	300	This study
...	...	GCTGGATGCTCCGAGACTAC	...	...	300	...
S/WRKY11	WRKY transcription factor 11	ATCCGGTTTCGTTTCATTTCA	200	XM_004244436.1	300	This study
...	...	GGATTGTGCAACGGCGATAG	...	...	300	...
S/WRKY72	WRKY transcription factor 72	AGCCCAGTAATGATCCAAGC	154	XM_004233876.1	300	This study
...	...	GTTGGGTAATCGCACTTGGT	...	...	300	...
DEA1	Arachidonic acid-induced DEA1	TGTTCTTGGAATTTGCTTGG	215	NM_001246895.1	300	This study
...	...	TAGGGCATTGAAAGCCAGAT	...	...	300	...
BTUB	β-Tubulin (reference gene)	AACCTCCATTCAAGAGATGTTT	180	DQ205342	300	Løvdaal and Lillo 2009
...	...	TCTGCTGTAGCATCCTGGTATT	...	...	300	...
UBI	Ubiquitin (reference gene)	ACCAAGCCAAAGAAGATCAAGC	200	X58253	300	Fernández et al. 2014
...	...	GTGAGCCCACTTACCACAGT	...	...	300	...
EF1L	Elongation factor 1 α (reference gene)	CTCCGCTTCTCCACTTCAGG	128	X14449	300	Fowler et al. 2009
...	...	TCAGTTGTCAAACCAAGTAGGG	...	...	300	...
RPL2	Ribosomal protein L2 (reference gene)	GTCATCCTTTTCAGGTACAAGCA	156	X64562	70	Løvdaal and Lillo 2009
...	...	CGTTACAAACAACAGCTCCTTC	...	...	70	...

^z Amp = amplicon length and Conc = primer concentration.

Reduced final *M. ethiopica* egg number in N2B1 directly corresponds to low number of galls and egg masses. The lowest number of galls and egg masses was observed in treatments N2B1 and N2npB1 at 45 dpi (Table 7). Compared with the control treatment, the roots in N2B1 (N2npB1) had 55% (65%) less galls. Only one egg mass, on average, extruded from the surface of each gall. After 90 days, the galls were larger and hosted more than one female nematode, with egg masses hidden inside the single gall.

Agrobacteria indirectly affecting RKN reproduction. A split-root pot experiment was set up to evaluate the influence of systemic plant response on the observed suppression in treatment N2B1 (Table 2). The reproduction of the nematode was reduced if agrobacteria were present on the other, physically separated root segments in treatment N2B1 (Table 8). The reduction in numbers of galls and egg masses was even higher when heat killed *A. tumefaciens* strain 06-424-7 (N2hkB1) or the nonpathogenic *E. coli* strain (N2Ec1) were added. On the other hand, in treatment N1B2, the nematodes produced 290% more galls than in treatment N2B1 and 62% more galls than in the control treatment (Table 8).

Penetration of *M. ethiopica* juveniles into the roots was not affected by agrobacteria. Split-root experiment described in Table 3 was set up to evaluate the penetration of *M. ethiopica* juveniles into roots of tomato when *A. tumefaciens* is present. The bacteria *A. tumefaciens* did not influence the penetration of *M. ethiopica* juveniles into the tomato roots. Neither direct physical presence nor indirect presence on other root segments affected the rate of *M. ethiopica* penetration into the roots at 8 and 12 dpi (Table 3). Furthermore, the presence of tumors on the plant stem did not affect the penetration of *M. ethiopica* nematodes into the roots.

Tumor development by agrobacteria was reduced in the presence of *M. ethiopica*. In single-pot experiments, in the treatment with pathogenic bacteria *A. tumefaciens* (treatment B), tumors did not develop on 3 of 11 plants. The other eight test plants from treatment B developed one or more tumors on the roots; however, no tumor was formed at the crown stem (Table 9). None of the plants from the control treatment that was treated with water developed tumors. The presence of *M. ethiopica* reduced tumor incidence by 18% when *A. tumefaciens* was inoculated before the *M. ethiopica* eggs (NB and N2B1). Tumor development was completely prevented in N1B2 when *M. ethiopica* juveniles invaded the roots and began forming feeding sites prior to root infection with bacteria. The density of *A. tumefaciens* in the growth

substrate of different treatments did not significantly decrease at 45 dpi (Table 9).

Differential expression of tomato JA/ET pathway target genes. A split-root experiment was set-up to sample only non-inoculated root segments (Table 4). All four reference genes had a steady expression in all treated and control root samples (Table 6). The majority of target genes did not show differences in gene expression (Table 10). According to the results, the most significant downregulation of selected target genes was induced by pathogenic *A. tumefaciens* strain C58 alone. Immediate systemic response of tomato to C58 consisted of a downregulation of ET synthesis gene 1-amino-cyclopropane-1-carboxylic acid (ACC) synthase (ACS) 6 (0 dpi). ACS enzymes are part of the ET biosynthesis pathway, together with ACC oxidases (ACO) that catalyze the final step in ET synthesis. Downregulation of gene *ACO1* was detected at 12 dpi, together with the positive detection of SA-inducible pathogenesis-related (PR) protein gene *PR1* (Fig. 1A). A similar pattern in gene expression was noted in C58- and C58C1-treated plants in the early stages of pathogenesis. However, at 12 dpi, the pathogenic C58 induced stronger downregulation of *PR1* and *ACO1* than C58C1, indicating

TABLE 8. Evaluation of *Meloidogyne ethiopica* reproduction in the split-root pot experiment (45 dpi)^y

Treatment ^z	Number of galls/g of root	Number of egg masses/g of root
N	72.0 ± 6.70 b	41.9 ± 8.21 bc
NB	66.5 ± 20.56 b	30.8 ± 7.51 ab
N1B2	116.4 ± 9.98 c	66.3 ± 14.76 c
N2B1	29.9 ± 6.04 ab	13.9 ± 6.51 ab
N2Ec1	16.2 ± 5.18 a	8.3 ± 3.57 a
N2hkB1	19.8 ± 2.97 a	5.5 ± 1.38 a
P (treatment)	<0.001	<0.001

^y Data are represented as mean ± standard error ($n = 4$). Within each column, values followed by different letters are significantly different ($P \leq 0.05$) according to one-way analysis of variance and Tukey's honestly significant difference test (a to c).

^z Treatment designations: N = infested with *M. ethiopica* eggs, NB = simultaneous inoculation with *M. ethiopica* and *A. tumefaciens*, N1B2 = *A. tumefaciens* inoculated 8 days after *M. ethiopica*, N2B1 = *M. ethiopica* inoculated 2 days after *A. tumefaciens*, N2Ec1 = *M. ethiopica* inoculated 2 days after *Escherichia coli*, and N2hkB1 = *M. ethiopica* inoculated 2 days after heat-killed pathogenic *A. tumefaciens*.

TABLE 7. Growth parameters of tomato plants and *Meloidogyne ethiopica* nematode reproduction parameters in pot experiments for evaluation of the *Agrobacterium-Meloidogyne* interaction on host plant vitality and nematode reproduction^w

Time (dpi), treatment ^x	Root weight (g)	Fresh shoot weight (g)	Dry shoot weight (g)	Shoot length (mm)	Rf = Pf/Pi ^y	Number of galls/g of root	Number of egg masses/g of root	Number of eggs/egg mass
45								
Control	4.9 ± 0.28	17.8 ± 1.36	1.8 ± 0.21	292.5 ± 7.24 b	n.a.	n.a.	n.a.	n.a.
N	5.1 ± 0.25	15.6 ± 1.05	1.6 ± 0.16	261.6 ± 4.07 a	29.7 ± 5.75 b	54.8 ± 11.69 b	51.2 ± 10.38 b	94.1 ± 22.35
B	5.0 ± 0.29	16.8 ± 1.00	1.6 ± 0.14	260.5 ± 4.19 a	n.a.	n.a.	n.a.	n.a.
NB	5.8 ± 0.32	16.2 ± 1.26	1.6 ± 0.17	254.8 ± 5.91 a	30.9 ± 4.86 b	48.0 ± 5.40 ab	44.4 ± 6.55 b	179.7 ± 19.73
N1B2	5.7 ± 0.27	15.8 ± 1.97	1.8 ± 0.18	260.3 ± 7.01 a	24.7 ± 2.63 b	37.2 ± 5.68 ab	30.4 ± 4.79 ab	252.1 ± 56.37
N2B1	5.7 ± 0.20	16.7 ± 1.26	1.8 ± 0.19	245.1 ± 5.04 a	12.2 ± 1.77 a	24.4 ± 5.23 a	24.4 ± 5.04 ab	109.0 ± 30.51
N2npB ^z	5.3 ± 0.24	17.7 ± 1.25	2.1 ± 0.09	260.4 ± 6.30 a	9.4 ± 1.03 a	19.2 ± 3.83 a	16.0 ± 3.85 a	232.4 ± 155.17
P (treatment)	n.s.	n.s.	n.s.	<0.001	<0.001	0.007	0.007	n.s.
90								
Control	5.4 ± 0.40 a	33.6 ± 1.38 b	4.5 ± 0.44 b	486.2 ± 42.70	n.a.	n.a.	n.a.	n.a.
N	13.9 ± 0.88 b	24.4 ± 1.27 a	2.7 ± 0.28 a	461.0 ± 35.42	148.6 ± 8.79 ab	178.8 ± 13.08	86.4 ± 4.53 c	254.1 ± 24.67
N1B2	12.9 ± 0.70 b	28.1 ± 1.01 ab	3.1 ± 0.17 a	454.6 ± 14.74	227.2 ± 71.70 b	150.0 ± 7.56	52.0 ± 2.28 b	464.6 ± 149.78
N2B1	13.2 ± 0.79 b	28.9 ± 2.48 ab	3.3 ± 0.27 ab	451.6 ± 12.70	82.5 ± 14.37 a	174.8 ± 10.40	31.6 ± 6.73 a	438.5 ± 92.64
P (treatment)	<0.001	0.010	0.004	n.s.	0.012	n.s.	<0.001	0.0576 (n.s.)

^w Data are represented as mean ± standard error ($n = 11$ for calculations after 45 dpi and $n = 5$ after 90 dpi). Values followed by lowercase letters are significantly different ($P \leq 0.05$) according to one-way analysis of variance and Tukey's honestly significant difference test; n.s. = not significant and n.a. = not available.

^x Treatment designations: Control = uninfecting, N = infested with *M. ethiopica* eggs, B = inoculated with pathogenic strain *A. tumefaciens*, NB = simultaneous inoculation with *M. ethiopica* and *A. tumefaciens*, N1B2 = *A. tumefaciens* inoculated 8 days after *M. ethiopica*, N2B1 = *M. ethiopica* inoculated 2 days after *A. tumefaciens*, and N2npB1 = *M. ethiopica* inoculated 2 days after nonpathogenic *A. tumefaciens*.

^y Reproduction factor (Rf) of *M. ethiopica*. Pf = total number of *M. ethiopica* eggs per root system and Pi = initial number of eggs (2,000 per treatment).

^z The N2npB1 treatment was conducted once ($n = 5$).

the involvement of pTi genes in the suppression of plant defenses (Fig. 1A).

The gene for *PR1* that is known to be SA-induced was upregulated in N2B1 at 12 dpi where strain C58 was used (Table 10), even though the same gene was downregulated by C58 in a similar experiment in the absence of *M. ethiopica* (Fig. 1A). No *PR1* differential expression was detected in N1B2. However, the downregulation of *ACS6* in N1B2 at 12 dpi could be attributed to the action of C58 (Fig. 1A).

To determine the contribution of *M. ethiopica* to C58-infected tomato roots on overall target gene expression in N2B1, we compared treatments N2B1 and C58 (Table 10). Two days after the *M. ethiopica* inoculation in N2B1, the general immunity gene *SIWRKY72* showed significantly higher systemic expression than in C58 at the same sampling time (4 dpi) (Fig. 1B). It should be noted that the *M. ethiopica* nematodes and strain C58 alone downregulated *SIWRKY72* at 4 dpi (Fig. 1B). Later, at 12 dpi, the expression of all target genes in N2B1 was comparable with expression in C58 ($P = 0.014$).

Different batches of *M. ethiopica* eggs were used in pot and split-root experiments for the N2B1 treatment. Similar percentages of

M. ethiopica egg populations were observed in the three pot experiments, and conclusions drawn from the pot experiments are also supported by gene expression analysis. Therefore, we assume that using different egg batches had no significant effect on the results.

DISCUSSION

Opinions regarding the *Agrobacterium*–*Meloidogyne* pathosystem have not changed considerably over the last 50 years. Based on field observations and a few field and lab experiments, it was concluded that synergism best describes the *Agrobacterium*–*Meloidogyne* relationship on host plants (Dhanvantari et al. 1975; Rubio-Cabetas et al. 2001; Süle et al. 1995; Zutra and Orion 1982). Recently, a considerable number of rhizobacteria have been identified as biocontrol agents against different plant-pathogenic bacteria and RKN (Hallmann et al. 2009). Given the fact that the genus *Agrobacterium* is part of the rhizosphere microbial community, it would not be presumptuous to assume that, despite being pathogenic, *A. tumefaciens* might suppress RKN. This hypothesis was tested in pot experiments on the RKN-susceptible tomato hybrid Arawak F1 (Syngenta). We opted for low inoculum sizes of both the pathogenic *A. tumefaciens* strain and pest *M. ethiopica* but kept the bacterial concentration above the density required for tumor formations ($>10^5$ CFU g⁻¹) (Li et al. 2015).

Agrobacteria and *M. ethiopica* nematodes did not severely affect tomato growth. All tomato plants infected with *A. tumefaciens* or infested with *M. ethiopica* were smaller and the level of water content in the shoots was significantly reduced after 90 days as a consequence of root galling that also was observed by Strajnar et al. (2012). However, the effect of agrobacteria on the plant growth indices was negligible. Our observations agree with results from Fakhouri et al. (1996), who adopted a similar experimental design using tomato, *A. tumefaciens*, and *M. javanica*.

Positive, negative, and systemic effects of agrobacteria on reproduction of *M. ethiopica* nematodes. The results from pot experiments suggest that antagonism and synergism could describe the *A. tumefaciens*–*M. ethiopica* pathosystem. Timing of

TABLE 9. Tumor development on tomato roots in treatments with joined inoculation of *Agrobacterium tumefaciens* and *Meloidogyne ethiopica* nematodes (45 dpi)^x

Treatment ^y	Plants with root tumors (%)	Tumor weight (mg)	Conc (logCFU) ^z
B	72.7 (8/11)	60 ± 19.7	5.8 ± 0.08
NB	54.5 (6/11)	130 ± 40.4	5.5 ± 0.09
N1B2	0 (0/11)	0	5.6 ± 0.10
N2B1	54.5 (6/11)	103 ± 49.2	5.5 ± 0.11

^x Average tumor weight is presented as mean ± standard deviation; logCFU values are presented as mean ± standard error ($n = 11$). The experiment was conducted twice, in six and five replicates; data were pooled.

^y Treatment designations: B = pathogenic *A. tumefaciens* (06-424-7), NB = simultaneous inoculation with *M. ethiopica* and *A. tumefaciens*, N1B2 = *A. tumefaciens* inoculated 8 days after *M. ethiopica*, and N2B1 = *M. ethiopica* inoculated 2 days after *A. tumefaciens*.

^z Concentration of *A. tumefaciens*.

TABLE 10. Summary of target gene differential expression probabilities (LogOr) compared with probabilities of upregulation in other independent treatments^y

Treatment ^z	Time (dpi)	Gene	LogOr	SE	Gene regulation	P value
N2B1 versus control	12	<i>PR1</i>	2.329	0.8753	Up	0.00781
N2B1 versus C58	4	<i>SIWRKY72</i>	2.968	1.1302	Up	0.00862
	4	<i>ERF5</i>	-2.458	1.0911	n.s.	0.02430
	12	<i>ACO1</i>	2.303	0.9371	n.s.	0.01401
	12	<i>JA3</i>	2.303	0.9371	n.s.	0.01401
	12	<i>SIWRKY11</i>	2.303	0.9371	n.s.	0.01401
	12	<i>NIM1</i>	1.792	0.8075	n.s.	0.02649
N2B1 versus C58C1	4	<i>SIWRKY72</i>	2.197	0.8749	n.s.	0.01202
	12	<i>JA3</i>	2.708	0.8879	Up	0.00229
N2B1 versus N1B2	12	<i>PR1</i>	2.808	0.9605	Up	0.00346
N1B2 versus control	12	<i>ACS6</i>	-2.321	0.8696	Down	0.00761
	12	<i>DEA1</i>	-2.061	0.8107	n.s.	0.01100
C58 versus control	0	<i>ACS6</i>	-3.424	0.8914	Down	0.00012
	4	<i>ERF5</i>	-1.991	0.9026	n.s.	0.02736
	4	<i>SIWRKY72</i>	-1.991	0.9026	n.s.	0.02736
	12	<i>ACO1</i>	-3.219	0.9721	Down	0.00093
	12	<i>PR1</i>	-2.303	0.8874	Down	0.00947
	12	<i>JA3</i>	-1.946	0.8683	n.s.	0.02503
	12	<i>SIWRKY11</i>	-1.946	0.8683	n.s.	0.02503
C58 versus C58C1	4	<i>PR1</i>	2.231	0.8881	n.s.	0.01201
	12	<i>PR1</i>	-2.708	0.9578	Down	0.00470
	12	<i>ACO1</i>	-2.197	0.8315	Down	0.00823
N versus control	4	<i>SIWRKY72</i>	-2.457	0.9266	Down	0.00802
	12	<i>ERF5</i>	-1.977	0.8704	n.s.	0.02311

^y Time = days postinoculation (dpi), LogOr = log of odds ratio (estimation of probability that selected gene is upregulated in the treatment), SE = standard error, and n.s. = not significant. Statistically significant gene regulation at $P \leq 0.01$.

^z Treatment designations: Control = noninfected, N2B1 = *M. ethiopica* inoculated 2 days after *A. tumefaciens* strain C58, C58 = inoculated with *A. tumefaciens* strain C58, C58C1 = inoculated with nonpathogenic strain C58C1, N1B2 = *A. tumefaciens* strain C58 inoculated 8 days after *M. ethiopica*, and N = infested with *M. ethiopica*.

the *M. ethiopica* introduction appears crucial. When *M. ethiopica* infested the roots prior to infection with bacteria, the egg counts were higher than in control treatments, but this positive effect was observed only after 90 days. This suggests that synergism between agrobacteria and *M. ethiopica* may apply only for the later stages of parasitism.

When *M. ethiopica* eggs were added on the *A. tumefaciens*-infected tomato roots, the egg counts were reduced after the first and second reproduction cycle, as were the numbers of galls and egg masses. However, the observed 59% reduction in *M. ethiopica* egg counts after 45 days still resulted in a high nematode population. The next generation of *M. ethiopica* nematodes was still high and caused significant root galling after 90 days, albeit less than in control treatments. In spite of similar experimental design, Fakhouri et al. (1996) noted reduced *M. javanica* reproduction in treatments where agrobacteria and nematodes were added simultaneously to the roots of tomato. The difference in results could be ascribed to different RKN and bacterial species, subtle variations in experimental designs, and test conditions applied. Suppression observed in treatment N2B1 could be the result of priming because plants usually switch to a primed state of enhanced defense after they were affected by a pathogen. For many plant hosts, it has been shown that rhizobacteria potentiate the plant defense upon subsequent pathogen recognition, and many authors recognize the value of priming in field applications (Conrath et al. 2006; Pozo et al. 2008; Van Wees et al. 2008).

Pathogenic and nonpathogenic agrobacteria can similarly induce early plant defense responses upon PAMP recognition (Ditt et al. 2006; Zipfel 2009). However, upon transformation and expression of the T-DNA tumorigenic genes, the host defense is silenced, enabling initiation of tumor growth (Veena et al. 2003). Our split-root experiment (treatment N2B1) showed that the pathogenic *A. tumefaciens* strain systemically suppressed the reproduction of *M. ethiopica* nematodes when physically separated, which could indicate the involvement of defense priming. The systemic plant defense response also was evident in treatments where part of the tomato root was primed with the heat-killed pathogenic agrobacteria or nonpathogenic *E. coli*. The extent of *M. ethiopica* egg count reduction was greater in treatments with a heat-killed than with a live pathogenic *A. tumefaciens* strain. PAMP molecules such as bacterial lipopolysaccharide (LPS) are known to activate the PTI response (Mishina and Zeier 2007). On its own, *E. coli* O157:H7 is capable of inducing a strong plant response in *Arabidopsis thaliana* (Seo and Matthews 2012) and, similarly, *Arabidopsis* activates defenses toward mutant agrobacteria impaired in attachment (Ditt et al. 2006) or upon recognition of peptide elf 26 derived from the N-terminus of bacterial elongation factor Tu (Zipfel et al. 2006).

Unhindered penetration of *M. ethiopica* nematode juveniles into the roots. The PTI defense involves, among other things, an

extensive oxidative burst that targets invading pathogens, and accelerated callose deposition onto cell walls. Based on our results from treatment N2B1, fewer galls and fewer egg masses could result from hindered *M. ethiopica* juvenile penetration due to active plant defenses or inability of juveniles to establish feeding sites and, eventually, galls. We confirmed that agrobacteria did not affect *M. ethiopica* penetration into the roots. Callose deposition, known as one of the first and basal defense mechanisms to prevent pathogens from spreading, is reportedly ineffective against lytic enzymes secreted by invading infective juveniles (Kaplan and Keen 1980). Not only RKN but also pathogenic agrobacteria secrete catalases to neutralize the ROS outburst (Xu and Pan 2000).

***M. ethiopica* reproduction was stimulated by pathogenic *Agrobacterium tumefaciens* but tumor formation was prevented.** A stimulating effect of *Agrobacterium tumefaciens* on *M. ethiopica* reproduction on infested tomato plants was recorded in treatment N1B2 from the pot (90 dpi) and split-root (45 dpi) experiments. However, tumor growth was absent only in N1B2. We hypothesize that *M. ethiopica* can modify the plant response for allowing feeding site formation, as described previously (Smant and Jones 2011), and that the altered physiology of plants does not allow the initiation of tumors by *A. tumefaciens*. Plant hormonal balance has been suggested to play a significant role in the ability of agrobacteria to transform plant cells and cause tumor growth (Deeken et al. 2006; Veselov et al. 2003). Contrary to this notion, our results on root tumor incidence suggest a stimulated tumor growth rate in the presence of *M. ethiopica* nematodes but only when tumorigenesis is initiated prior to *M. ethiopica* inoculation. Faster tumor growth also was noticed by Dhanvantari et al. (1975) on young peach trees, where crown gall appeared on all trees after 150 days but the rate of tumor appearance was higher on RKN-infested peach trees. The speed of tumor growth might be attributed to higher transformation frequency mediated in an undetermined way by the nematodes, as proposed by Karimi et al. (2000).

Gene expression of PR1 was upregulated when *A. tumefaciens* reduced *M. ethiopica* reproduction. The split-root technique allows sampling of noninoculated root tissue for the determination of gene expression caused by a systemic plant response. Results from such experiments are also not influenced by volatiles that affect gene expression (e.g., plant ET and microbe-derived organic volatiles) (Farag et al. 2013). To prove the hypothesis that pathogenic agrobacteria prime tomato, we focused on the expression of genes controlled by JA/ET signaling pathway that have been implicated in defense priming and ISR activation with other rhizobacteria.

The majority of target genes had been either downregulated or not affected, which is not unexpected in compatible plant–pathogen interactions (Schaff et al. 2007). However, we showed that plants exhibited an induced systemic downregulation of the ACS6 gene within minutes after the pathogenic C58 strain was inoculated. The nonpathogenic strain C58C1, lacking the pTi plasmid, did not

Fig. 1. Systemic gene regulation for **A**, *PR1*, *JA3*, *ACO1*, *ACS6*, and *SIWRKY72* at 12 days postinoculation (dpi) and **B**, *SIWRKY72* at 4 dpi in roots of tomato upon combined infection with *Meloidogyne ethiopica* and *Agrobacterium tumefaciens*. Significant differences in gene expression level are labeled with an asterisk (*) above the standard error bar.

provoke a similar plant response, suggesting the importance of the pTi in early pathogenesis.

M. ethiopica did not induce a strong plant response. Gene expression of transcription factor *SIWRKY72* was downregulated at the time of *M. ethiopica* penetration into the roots (4 dpi). *WRKY* transcription factors are associated with transcriptional reprogramming in plant immunity (Eulgem and Somssich 2007). A variant *WRKY72* in tomato (*SIWRKY72*) was found to contribute to basal (PTI) defense against RKN and potato aphids and also linked to *Mi-1*-mediated resistance in tomato (Bhattarai et al. 2010). Gene expression of *SIWRKY72* was not upregulated, as would be expected at the site of infection (Bhattarai et al. 2010). We hypothesize that this is the result of RKN early action to inhibit or modify the PTI response. The detection of *ACS6* downregulation 4 days after strain C58 was inoculated onto *M. ethiopica*-infected roots (N1B2) and not sooner may support this hypothesis. This suggests a slower—rather than otherwise fast—plant response to pathogenic *Agrobacterium* that was modified by RKN.

Our results suggest that SA is responsible for the observed antagonism between *Agrobacterium* and *Meloidogyne* spp. and not JA, as suggested for many ISR-activating rhizobacteria (Cooper et al. 2005; Martinuz et al. 2013). Gene *PR1*, a marker gene of SA pathway activation and SAR induction (Ward et al. 1991), showed significant upregulation in roots physically separated from *A. tumefaciens*-infected roots subsequently challenged with *M. ethiopica* nematodes (N2B1 treatment). It was only in this treatment that *PR1* was upregulated. It has been reported that flagellin and purified bacterial LPS can induce expression of *PR1* and activation of the SA defense pathway (Mishina and Zeier 2007; Seo and Matthews 2012). Interestingly, the JA-induced gene *JA3*, a synonym for the *MYC2* transcription factor gene in tomato, was downregulated in the roots inoculated with C58 but upregulated in N2B1 (Fig. 1A). This indicates that priming with C58 resulted not only in *PR1* upregulation after *M. ethiopica* recognition but also in a derepression of *MYC2* that was otherwise downregulated by C58. Two important findings are highlighted in this study: plants can be primed with pathogenic agrobacteria for enhanced defense against the RKN, and RKN do not prime plants for increased *Agrobacterium* infections. Our results suggest that the pathogenic agrobacteria activated PTI, which resulted in priming of the host defenses. Priming is manifested in a fast, strong defense response upon subsequent pathogen infection (Conrath et al. 2006), which we observed as the upregulation of *PR1* in systemic root tissue upon *M. ethiopica* recognition. The second significant finding is that RKN do not prime plants for *Agrobacterium* infection. For a long time, the PTI response in plant–RKN interactions was questionable. Recently, Lin et al. (2016) proposed that nematodes may secrete currently unknown effectors for the suppression of the host antioxidant system involved in immune response. This would explain why a subsequent infection with *A. tumefaciens* did not result in an increased defense activation.

The conclusion that the SA pathway is responsible for the observed suppression in the studied *Agrobacterium*–*Meloidogyne* pathosystem may allow for the development of appropriate control agents for crop protection. Plant defense mediated by SA and JA is effective in RKN management (Cooper et al. 2005; Fujimoto et al. 2011); however, only SA arrests tumor growth in crown gall disease (Anand et al. 2008). Our results suggest that foliar treatments targeting SAR activation might offer a better disease and pest management strategy in the simultaneous control of pathogenic agrobacteria and RKN.

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