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Salicylic Acid Is Part of the *Mi-1*-Mediated Defense Response to Root-Knot Nematode in Tomato

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The *Mi-1* gene of tomato confers resistance against three species of root-knot nematode in tomato (*Lycopersicon esculentum*). Transformation of tomato carrying *Mi-1* with a construct expressing *NahG*, which encodes salicylate hydroxylase, a bacterial enzyme that degrades salicylic acid (SA) to catechol, results in partial loss of resistance to root-knot nematodes. Exogenous SA was toxic to roots expressing *NahG* but not to control roots. This toxicity is most likely due to the production of catechol from SA, and we report here that 100 µM catechol is toxic to tomato roots. Benzothiadiazole, a SA analog, completely restores nematode resistance in *Mi-1* roots transformed with *NahG* but does not confer resistance to susceptible tomato roots. The localized cell death produced by transient expression in *Nicotiana benthamiana* of *Mi-DS4*, a constitutively lethal chimera of *Mi-1* with one of its homologs, was prevented by coexpression of *NahG*. These results indicate that SA is an important component of the signaling that leads to nematode resistance and the associated hypersensitive response.

Tomato (*Lycopersicon esculentum*) plants carrying the gene *Mi-1* are resistant to at least three species of root-knot nematode (RKN), *Meloidogyne javanica*, *M. incognita*, and *M. arenaria*, as well as to isolates of potato aphid and white flies (Milligan et al. 1998; Nombela et al. 2003; Rossi et al. 1998). *Mi-1* encodes a protein with a nucleotide-binding (NB) domain and a leucine-rich repeat (LRR) region, characteristics of many plant resistance (*R*) gene products (Dangl and Jones 2001; Milligan et al. 1998). Resistance is associated with a localized hypersensitive response (HR) at and surrounding nematode-challenged cells (Ho et al. 1992; Riggs and Winstead 1959). How the presence of *Mi-1* mediates the recognition of the nematode and the effective resistance response characteristic of this gene is not yet known.

Features of plant-nematode interactions differ from those of plant-pathogen interactions against which closely related NB-LRR resistance genes are effective. RKNs infect plants as motile second stage juveniles (J2) and feed only on the cytoplasm of specific living plant cells (Williamson and Gleason 2003). Infective juveniles penetrate roots near the zone of elongation, migrate intercellularly to the root apical meristem, and then up the vascular cylinder. Upon reaching the zone of differentiation, nematodes appear to inject substances from specialized secretory glands into selected host cells (Hussey 1989; Wyss et al. 1992). These host cells undergo several rounds of mitosis uncoupled from cytokinesis, resulting in the formation of five to seven hypertrophic multinucleate cells known as giant cells. In resistant plants, migration of nematodes to feeding sites occurs as in susceptible plants, but the giant cells do not develop. Instead, localized cell death similar to the hypersensitive response (HR) seen with other incompatible interactions occurs in the host near the anterior of the nematode, and the nematode does not develop into the enlarged, sedentary form (Ho et al. 1992). It has not yet been determined whether the localized cell death is the cause of resistance, and no HR has been observed associated with *Mi-1*–mediated aphid resistance (Martinez de Ilarduya et al. 2003). *Mi-1*–mediated resistance is temperature sensitive, and temperature shift experiments indicate that resistance is determined within the first day of the interaction (Dropkin 1969). If roots are maintained at high temperature for the first few days after infection, then shifted to a permissive temperature, nematode development and reproduction will proceed as in susceptible tomato. This suggests that the time window during which the resistance response must occur is narrow.

It is not yet clear whether the signal transduction pathways and defense strategies triggered by these microscopic animals are the same as those that are effective in resistance against more conventional microbes. In addition, most of the previous work on *R* gene signaling has been carried out on leaf rather than root pathogens, and the similarities and differences between defense responses in above and below ground plant parts has been little studied. Factors contributing to the difficulty of investigating the resistance response to nematodes include the limited number of cells at the interaction site between root and nematode, the inaccessibility of the interaction site, and the asynchronous nature of the infection. The necrotic response, when apparent, is limited to a few cells near the anterior of the nematode within the vascular system of the living root. Studies on gene induction in root tips after nematode infection have found that a similar spectrum of genes is induced in resistant and susceptible tomato (Lambert et al. 1999).

The 60-kb region of the genome to which *Mi-1* was localized carries two highly homologous genes, *Mi-1.1* and *Mi-1.2,* encoding NB-LRR proteins that are 91% identical in amino acid sequence (Milligan et al. 1998). Complementation assays showed that *Mi-1.2* but not *Mi-1.1* could confer resistance to nematodes and aphids (Milligan et al. 1998; Rossi et al. 1999). To investigate the mechanism of action of the Mi-1 protein, we created chimeric constructs between *Mi-1.1* and *Mi-1.2*. One of these chimeric constructs, *Mi-DS4*, was lethal when introduced into roots and, when transiently expressed by *Agrobacterium rhizogenes*–mediated infiltration in *Nicotiana benthamiana* leaves, produced confluent necrosis resembling a HR in

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the infiltrated area (Hwang et al. 2000). Both *Mi-1*–mediated RKN resistance and *Mi-DS4*–mediated leaf HR are lost at high temperatures, suggesting that the pathways leading to resistance and leaf necrosis are related. Additional support for a common response includes the strong correlation of phenotypes produced by single amino acid changes introduced into the LRR region of *Mi-1.2* and a constitutively lethal chimera (Hwang and Williamson 2003). That is, the same single amino acid changes resulted in loss-of-function for both root-knot nematode resistance and leaf HR assays.

In this paper, we investigate the role of salicylic acid (SA), a central player in many *R* gene–mediated resistance responses (Klessig et al 2000; Shah 2003), in nematode resistance, and in the leaf necrosis produced upon transient expression of *Mi-DS4*. Expression of the *NahG* gene, which encodes salicylate hydroxylase, a bacterial enzyme that degrades SA to catechol, has been a useful tool in examining the role of SA (Gaffney et al. 1993). *NahG* expression experiments have indicated that SA is required for full function of several NBS-LRR-type *R* genes (Brading et al. 2000; Delaney et al. 1994). However, *NahG* expression does not affect resistance to *Cladosporium fulvum* mediated by *Cf-2* or *Cf-9*, *R* genes that encode proteins with extracellular LRRs, a transmembrane region, and a short cytoplasmic tail (Brading et al. 2000). *NahG* expression has been associated with additional phenotypes, including loss of nonhost resistance to *Pseudomonas syringae* pv. *phaseolicola* in *Arabidopsis* and the appearance of spontaneous necrotic lesions in tomato plants (Brading et al. 2000; Van Wees and Glazebrook 2003). Here, we present evidence that SA is required for both the localized necrosis produced upon expression of *Mi-DS4* in *N. benthamiana* leaves and for *Mi*-mediated nematode resistance in tomato roots.

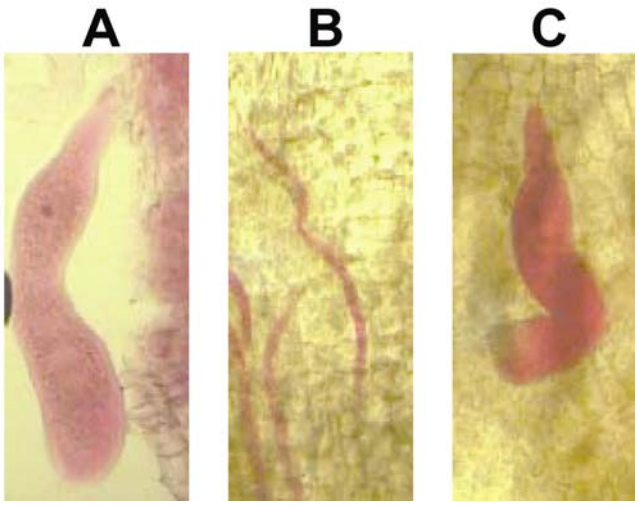

Fig. 1. *Meloidogyne javanica* stained with acid fuchsin two weeks after infection of transgenic tomato roots. **A,** Developed nematode from Motelle roots transformed with pTFS-NahG. **B,** Undeveloped nematodes from Motelle roots transformed with vector pTFS40. **C,** Developed nematode from Moneymaker transformed with vector pTFS40.

RESULTS

NahG expression reduces nematode resistance in transformed roots containing *Mi-1*.

The nematode resistant tomato line Motelle (homozygous for *Mi-1*) was transformed using *Agrobacterium rhizogenes* carrying pTFS-NahG, a binary vector with *NahG* under control of the cauliflower mosaic virus (CaMV) 35S promoter, to produce transformed "hairy" roots. As controls, both Motelle and the nearly isogenic susceptible tomato line Moneymaker were transformed with the vector pTFS40, which carries an intron-containing gene encoding β-glucuronidase (GUS) as a marker for DNA transfer to and expression in plants. Seven independent transformed root lines of Motelle that were transformed with pTFS-NahG and that stained strongly for GUS activity were transferred to new plates to expand the amount of root material. Roots from one plate of each line were frozen in liquid N_2 and were assayed for SA. Seven independent, putative *NahG* transgenic Motelle root culture lines and four different Moneymaker root culture lines were assayed. The average total SA for *NahG* lines was 76 ng per g of fresh weight (FW), significantly less than the average SA level of 2,977 ng per g of FW found for Moneymaker and Motelle plants transformed with the vector pTFS40 (analysis of variance, $p < 0.001$).

Freshly transferred root tips of a subset of the transformed root lines were inoculated with *M. javanica* juveniles and, after two weeks, were stained with acid fuschin to visualize nematodes. Individual nematodes inside roots were counted and scored for development. Previous studies had shown that, by two weeks after inoculation in susceptible roots, nematodes have initiated feeding sites and become sedentary and enlarged, whereas in resistant tomato roots, no development is observed (Ho et al. 1992; Hwang et al. 2000). Control

Table 1. Effect of *NahG* expression on salicylic acid (SA) levels and nematode resistance

			Nematodes inside roots[a]			
			No BTH added		+100 µM BTH	
Tomato line	Binary vector	SA (ng/g)[b]	No. counted	% Enlarged	No. counted	% Enlarged
Moneymaker (susceptible line)	pTFS40	2,959	63	100	63	100
Motelle[c]	PTFS40	2,952	39	0	nd[d]	nd
Motelle	NahG[e]	64	47	36	nd	nd
Motelle	NahG	76	44	36	56	0
Motelle	NahG	33	48	38	48	0
Motelle	NahG	36	68	20	nd	nd

[a] Differences between lines were established using a nonparametric 1-way analysis of variance (Kruskal-Wallis test, $p < 0.0001$) (SAS, Cary, NC, U.S.A.). For subsequent pairwise comparisons, a Bonferroni adjustment set $\alpha = 0.0125$. Motelle lines transformed with *NahG* in plates lacking benzothiadiazole (BTH) have a significantly higher percentage of enlarged nematodes than does Motelle transformed with the vector pTFS40 in plates lacking BTH ($p = 0.0006$) and Motelle transformed with *NahG* in plates with 100 µM BTH ($p < 0.0001$). Conversely, Motelle lines transformed with *NahG* in plates lacking BTH have a significantly lower percentage of enlarged nematodes than does Moneymaker transformed with pTFS40 regardless of BTH presence ($p = 0.0006$ without BTH, $p = 0.0006$ with BTH).

[b] Free plus bound salicylic acid (SA) (ng per gram wet weight of root tissue).

[c] The line Motelle is homozygous for *Mi-1*.

[d] nd = not determined

[e] Each row represents an independent transformed root line.

inoculations using resistant line Motelle and susceptible line Moneymaker transformed with the vector pTFS40 confirmed our previous findings (Fig. 1B and C). However, for Motelle roots transformed with pTFS-NahG, 20 to 38% of the nematodes had developed, a frequency intermediate between resistant and susceptible controls (Table 1). Developed nematodes were enlarged to the same degree and, to our eyes, were identical in appearance to those in the susceptible response (Fig. 1A and C).

Benzothiadiazole (BTH) is a functional analog of salicylic acid that is not metabolized by salicylate hydroxylase (Friedrich et al. 1996). We tested the ability of BTH to reverse the loss of *Mi*-mediated resistance in two lines of roots carrying the *NahG* construct. When 100 μM BTH was present in the medium, resistance was fully restored and no nematode development was observed (Table 1). However, the presence of BTH did not cause the susceptible roots to gain resistance, indicating that BTH alone is not sufficient to confer resistance.

NahG expression in tomato roots results in SA toxicity.

The correlation of reduced salicylic acid levels with loss of resistance suggested that SA is important in the signaling leading to *Mi-1*–mediated resistance. We tested the effect of exogenous SA on nematode susceptibility and resistance on untransformed cultured roots. When 100, 200, or 300 μM SA was added to the medium, Motelle roots remained resistant and Moneymaker roots remained susceptible to nematodes. However, when Moneymaker and Motelle roots transformed with pTFS-NahG were transferred to medium containing 300 μM SA, the roots became necrotic and died within 24 h of transfer (Fig. 2). The same concentration of SA did not kill roots transformed with the vector pTFS40. Since the primary product of SA metabolism is catechol, it seemed likely that the level of this compound generated by the enzymatic action of salicylate hydroxylase was toxic to the roots. In support of this, we found that medium containing 100 μM catechol was toxic to both Moneymaker and Motelle transformed with pTFS40, with root necrosis apparent within 24 h of transfer (data not shown).

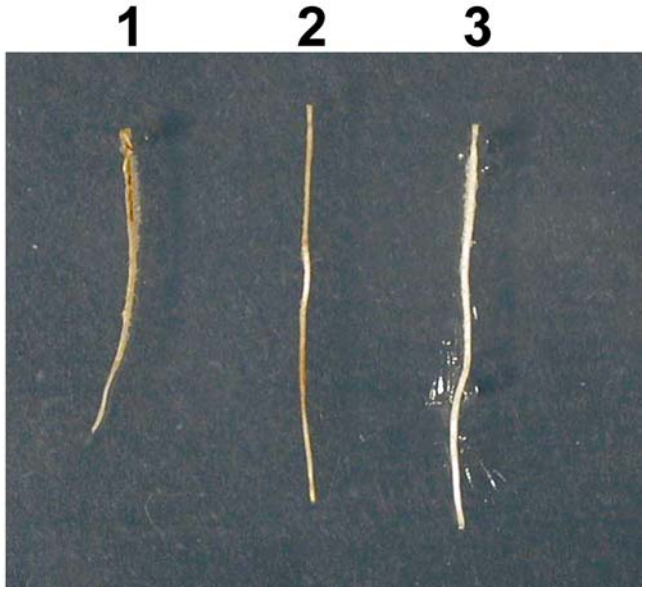

Fig. 2. Roots expressing *NahG* are sensitive to salicylic acid (SA). Roots of two independent transgenic root lines of Motelle expressing *NahG* (1 and 2) that had been placed on Murashige and Skoog medium with 300 μM SA for 24 h were killed. Control root Motelle transformed with pTFS40 (3) remained healthy.

NahG inhibits *Mi-DS4*-mediated cell death in *Agrobacterium* transient assay.

Mi-DS4, a chimeric construct comprising the LRR region from *Mi 1.2* and the remainder of *Mi-1.1*, is lethal when transformed into tomato and causes localized cell death resembling a HR if it is transiently expressed in *N. benthamiana* leaves (Hwang et al. 2000). To address whether SA is required for the cell death caused by transient expression of this construct, we coinfiltrated *A. rhizogenes* expressing *Mi-DS4* and *NahG* into *N. benthamiana* leaves. Leaves inoculated with *Mi-DS4* alone showed confluent necrosis in the infiltrated area after 72 h (Fig. 3). However, when *Mi-DS4* was coinfiltrated with *NahG*, no necrosis was observed in the infiltrated area, even at 10 days postinfiltration. Areas of the leaf inoculated with *A. rhizogenes* harboring either pTFS-NahG or pTFS40 alone did not display necrosis (Fig. 3). As previously noted, no necrosis was seen for any of the treatments at 24 or 48 h postinfection (Hwang et al. 2000).

Phenolic compounds produced during the HR can be detected by characteristic fluorescence under UV light (Bennett et al. 1996). When we examined infiltrated areas of the leaves with a fluorescent microscope with an excitation wavelength of 488 nm, we found that areas that had become necrotic due to transient expression of *Mi-DS4* fluoresced at 598 nm while areas inoculated with either pTFS40 or pTFS-NahG did not (Fig. 4). At 72 h postinfiltration, the leaf areas coinoculated with *A. rhizogenes*-carrying constructs expressing *Mi-DS4* and *NahG* displayed only spotty fluorescence (Fig. 4). The extent and general distribution of the phenolic deposits in the coinoculated leaf regions did not increase even up to 10 days postinfiltration. Few or no fluorescent spots were seen 24 or 48 h after any of the treatments.

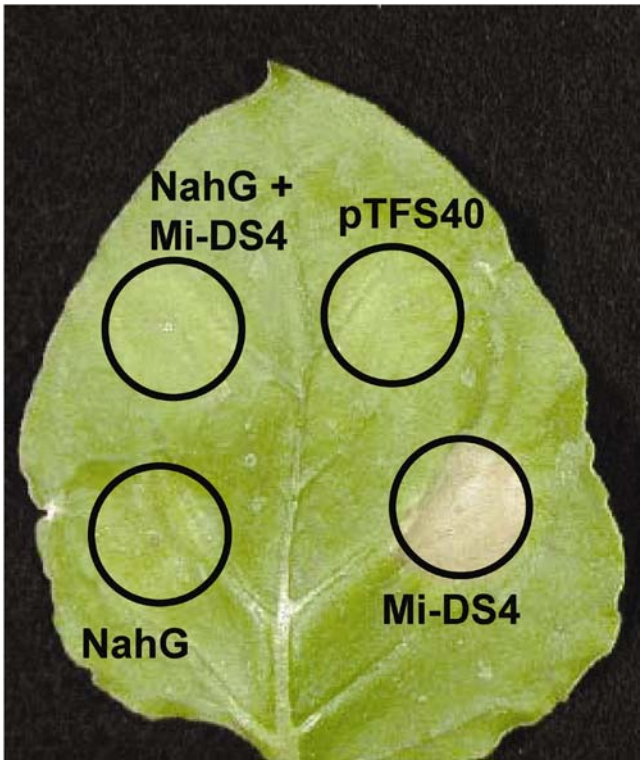

Fig. 3. Effect of *NahG* expression on localized necrosis upon infiltration of *Mi-DS4*. The circles indicate the boundaries of the infiltrated areas. Circles were infiltrated with *Agrobacterium rhizogenes* carrying the vector pTFS40, Mi-DS4, pTFS-NahG, or a mixture of equal amounts of *A. rhizogenes* expressing *NahG* and *Mi-DS4* as indicated. Leaf is shown 72 h after infiltration.

DISCUSSION

Our data demonstrate that expression of *NahG* reduces the *Mi-1*–mediated resistance against *M. javanica* in tomato. Several lines of evidence support that this is due to reduced SA levels. Total SA levels were dramatically reduced in the *NahG*-transformed roots in this study. The susceptibility of *Mi* roots due to *NahG* expression is reversed when BTH, a functional analog of SA, is supplied. This result suggests that one or more of the roles of SA that can be carried out by BTH are involved in the resistance response. Like SA, BTH inhibits the activity of catalase and ascorbate peroxidase and upregulates the expression of plant defense proteins PR-1, PR-2, PR-3, SAR 8.2, and phenylalanine ammonia lyase (Wendehenne et al. 1998). However, BTH does not induce SA production and is not degraded by the *NahG* product (Friedrich et al. 1996). The failure of exogenous BTH or SA to produce nematode resistance in susceptible roots indicates that the presence of either compound is not sufficient for resistance. In *Arabidopsis* the catechol produced by expression of *NahG* was found to be responsible for loss of nonhost resistance to *Pseudomonas syringae* pv. *phaseolicola* (Van Wees and Glazebrook 2003). The ability of BTH to restore resistance to *Mi-1* plants compromised by *NahG* expression and the repression of necrosis due to *Mi-DS4* expression in *N. benthamiana* leaves argue against a catechol effect.

We found that both nematode-resistant and -susceptible tomato roots expressing *NahG* are sensitive to exogenous SA at levels that do not affect control roots. Our finding that catechol is toxic to *A. rhizogenes*–transformed tomato roots is the likely explanation for this sensitivity and supports the presence of functional salicylate hydroxylase in the *NahG* roots. In *Arabidopsis*, no toxicity was found when catechol was infiltrated into leaves at concentrations up to 1 mM, substantially higher than the 100 μM that was tested in our experiments (van Wees and Glazebrook 2003). We also found that infiltration of 1 mM catechol into leaves of tomato produced localized death within 24 h, whereas infiltration of 1 mM catechol into leaves of *N.*

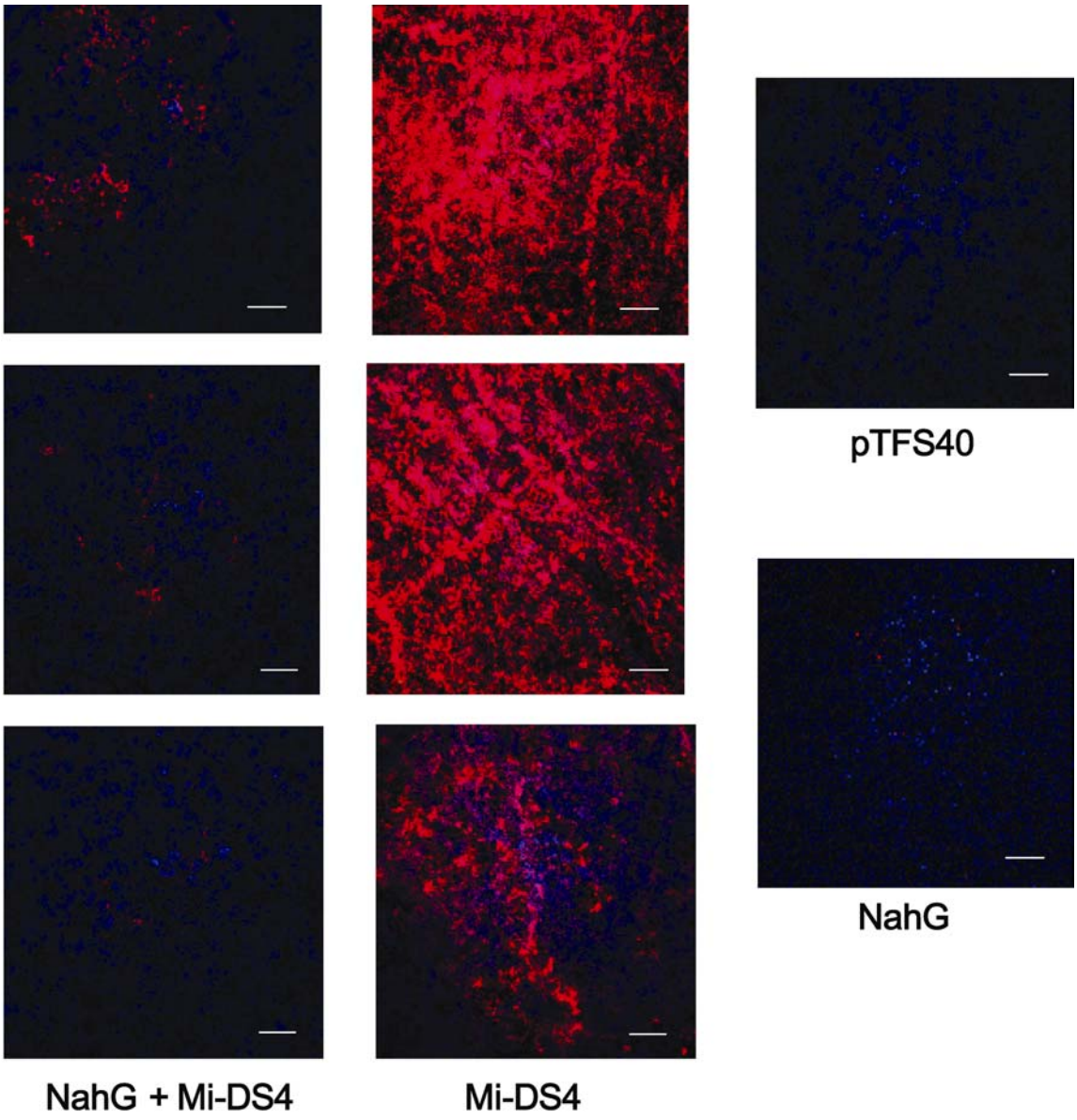

Fig. 4. Micrographs of *Nicotiana benthamiana* leaves 72 h after infiltration. Infiltrated areas are shown from three different leaves infiltrated to express *NahG* plus *Mi-DS4* or *Mi-DS4* alone. Comparable leaf areas are shown from controls infiltrated with pTFS40 or pTFS40-NahG. Leaves were viewed with excitation wavelength of 488 nm. The red areas correspond to fluorescence at 598 nm, expected wavelength for hypersensitive response-associated phenolic deposits (Bennett et al. 1996). White bars represent 100 μm.

benthamiana did not produce necrosis even after three days (results not shown). These results indicate that catechol is more toxic to tomato than it is to *Arabidopsis* or tobacco. The higher catechol sensitivity in tomato compared with some other plant species may explain the spontaneous necrotic lesions reported in transgenic tomato plants expressing *NahG* (Brading et al. 2000).

The loss of nematode resistance in roots expressing *NahG* is partial. Only 30% of the *M. javanica* that penetrated the root developed, compared with 100% in susceptible roots transformed with vector alone. Partial loss of resistance was also seen for the virus resistance gene *Tm-2*^{2}; that is, lesions were larger in tomato plants expressing *NahG*, but there was still no systemic spread of virus (Brading et al. 2000). Likewise, although *N* gene–mediated resistance to *Tobacco mosaic virus* is partially compromised when *NahG* is expressed, the systemic acquired resistance response is still initiated (Delaney et al. 1994). While fewer nematodes developed in the *Mi-1*-carrying roots expressing *NahG* than in susceptible roots transformed with vector alone, those that developed were indistinguishable from nematodes in the susceptible control. A proposed role for SA is to potentiate, or lower the threshold for, specific resistance responses in *R* gene–mediated resistance (Shirasu et al. 1997). Since the time period in the infection process during which nematode resistance can be effected is narrow, the higher threshold due to reduced SA level may allow initiation of resistance for only a fraction of the invading nematodes. It is not yet clear whether the HR response is required for nematode resistance. For *Cf-2* and *Cf-9* and for other fungal as well as bacterial or viral *R* genes resistance has been uncoupled from cell death (Richael and Gilchrist 1999). For RKNs, the death of the potential feeding cell would seem to be an effective mechanism to produce resistance, as these organisms can only feed on the cytoplasm of living plant cells and, once they initiate development at the feeding site, they become immobile. However, it is possible that the necrosis is a secondary effect of the resistance mechanism.

Using the *Agrobacterium* transient assay, we found that the HR-like necrosis induced in leaf tissue by *Mi-DS4* was inhibited by coexpression of *NahG*. This suggests that SA is also required for the death initiated by *Mi-DS4* at some time during the three-day period between infiltration and the visible death and phenolic deposits. Microscopic examination revealed that, in leaves coinoculated with constructs expressing *Mi-DS4* and *NahG*, some phenolic deposits were present, indicating that the repression of the *Mi-DS4*–mediated HR was not complete (Fig. 4). A response was initiated in a few cells but either was not initiated in all cells or was unable to spread, again consistent with a role for SA in potentiation of the response. The similarity of the responses to *NahG* expression in nematode-infected tomato roots and the cell death initiated by *Mi-DS4* in *N. benthamiana* leaves suggests that the latter may be a good model system for examining the signaling response leading to nematode resistance.

MATERIALS AND METHODS

Plant transformation and root propagation.

A 4.8-kb *Xba*I DNA fragment containing the *NahG* coding sequence with an enhanced CaMV 35S promoter and a *tml* 3′ terminator sequence (Gaffney et al. 1993) was inserted into the binary vector pTFS40 (British Sugar, Norwich, U.K.) to produce pTFS-NahG. The fragment containing *NahG* and regulatory sequences was provided in the vector p1761 by Ciba-Geigy (Research Triangle Park, NC, U.S.A). The construct was transferred into *A. rhizogenes* strain A4RS (Jouanin et al. 1986) by triparental mating (Bevan 1984).

The near-isogenic tomato lines Motelle (*Mi/Mi*) and Moneymaker (*mi/mi*) were used for the transformation experiments. Transformation was carried out as previously described (Hwang et al. 2000). Transgenic roots were initially screened by testing for GUS activity. The plasmid pTFS40 carries an intron-containing gene encoding GUS.

SA assays.

Actively growing roots (0.5 to 1 g) were removed from agar plates, were rinsed to remove agar, were blotted dry, and were frozen in liquid N_2. Tomato roots were pulverized in liquid nitrogen and a 300- to 500-mg sample was assayed for SA concentration, as described by Gaffney and associates (1993) with minor modifications. SA was analyzed on a Novapak C18 column using an Agilent 1100 high-performance liquid chromatography system and was detected fluorometrically. The isocratic mobile phase was 23% methanol and 77% 20 mM sodium acetate, pH 5.5, at a flow rate of 1 ml/min at 25°C. Recovery rates were determined using o-anisic acid as an internal standard.

Nematode assays.

Eggs of *M. javanica* strain VW4 were collected from heavily galled 12-week-old susceptible tomato plants. Roots were cut into pieces, then were shook vigorously for 3 min with 10% commercial bleach, and then were poured through a 250-micron mesh and collected on a 25-micron mesh screen. Eggs were further purified by centrifugation in 35% sucrose at 500 × *g* for 10 min. The supernatant containing the eggs was subjected to two 10-min treatments in 10% bleach, followed by centrifugation for 5 min at 500 × *g* and then several rinses in sterile water. Eggs were hatched at room temperature and juveniles were allowed to crawl through eight layers of Kimwipe tissues into sterile water.

For resistance assays, 1- to 2-cm root pieces including an actively growing tip were plated on 1× Murashige and Skoog medium containing 2% sucrose and 0.8% phytagel. Approximately 200 worms per petri dish were pipetted around root tips. The plates were kept at 25°C in the dark for two weeks. Nematodes within the roots were stained red with acid fuchsin and were scored as resistant or susceptible, as previously described (Ho et al. 1992). SA, catechol, and BTH were each separately mixed with water in a 10 mM stock solution, were filter-sterilized, and then were added to partially cooled, autoclaved medium. BTH was a gift from Syngenta (Greensboro, NC, U.S.A.).

Agrobacterium transient assay.

A. rhizogenes (approximately 1×10^9 cells) carrying the indicated binary constructs was infiltrated into leaves of *N. benthamiana* as described elsewhere (Hwang et al. 2000). Leaves were scored daily for necrosis. Infiltrated leaf areas were excised and placed on a slide in water and were covered with a cover slip. Leaf sections were photographed with a fluorescent microscope using a 488-nm excitation wavelength with a 598 ± 40 band pass.

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