Journal of Nematology 33(2–3):116–120. 2001.

Mi-1.2 Transcripts Accumulate Ubiquitously in Resistant *Lycopersicon esculentum*

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Abstract: The tomato *Mi-1.2* gene confers resistance against both root-knot nematodes and the potato aphid. Plants are resistant to nematodes early in root development. However, plants as old as 4 weeks are susceptible to aphid infestation. We monitored *Mi-1.2* expression at the transcriptional level in resistant (*Mi/Mi*) and susceptible (*mi/mi*) tomato cultivars by means of RT-PCR. *Mi-1.2* transcripts accumulated in seeds, roots, stems, leaves, flowers, and green fruits of uninfected 10-week-old resistant plants but were not expressed in the same organs from similar-age susceptible plants. *Mi-1.2* RNAs in roots and leaves can be detected very early in development, and levels of transcripts do not change after either root-knot nematode or aphid attack.

Key words: gene expression, *Macrosiphum euphorbiae, Meloidogyne spp., Mi,* root-knot nematode, RT-PCR, signal transduction.

Resistance against three species of root-knot nematodes, *Meloidogyne arenaria, M. incognita,* and *M. javanica,* is conferred by the *Mi* locus in tomato (Roberts and Thomason, 1986). In addition, resistance to the potato aphid, *Macrosiphum euphorbiae,* is linked to this locus (Kaloshian et al., 1995). *Mi* was introgressed into cultivated tomato (*Lycopersicon esculentum*) from its wild relative *Lycopersicon peruvianum* (Smith, 1944). Recently, this locus was cloned and found to contain two highly homologous genes, *Mi-1.1* and *Mi-1.2.* Only *Mi-1.2* was shown to confer simultaneous resistance against root-knot nematodes (Milligan et al., 1998) and potato aphid (Rossi et al., 1998). The protein predicted to be encoded by this gene contains a leucine zipper, a nucleotide binding site, and a leucine-rich repeat. The highest protein similarity corresponds to proteins encoded by resistance genes of the Solanaceae including *Prf* (Salmeron et al., 1996), *Gpa2* (van der Vossen et al., 2000), *Rx* (Bendahmane et al., 1999), and *Sw-5* (Brommonschenkel et al., 2000), conferring resistance against *Pseudomonas syringae, Globodera pallida,* potato virus X, and several tospoviruses, respectively. Dual specificity has been described for the *Arabidopsis thaliana* gene *Rpm1,* which confers resistance to two strains of *P. syringae* with different avirulence genes, *avrRPM1* and *avrB* (Grant et al., 1995). However, *Mi-1.2* constitutes the first known case where a single gene is responsible for resistance against two diverse organisms.

Mi-1.2 is a member of a multigene family present in both resistant and susceptible tomato plants (Milligan et al., 1998). RNA gel blot analysis showed that *Mi*-related transcripts are present in both roots and leaves of 7-week-old resistant tomato but only in roots of similar-age, susceptible plants. In addition, transcripts from several individual members of this gene family have been detected in both leaves and roots of resistant plants using Rapid Amplification of cDNA Ends (RACE) and cDNA library screening (Milligan et al., 1998; Rossi et al., 1998).

Although resistance to root-knot nematodes and potato aphid is conferred by the same gene, the resistance to these organisms is regulated differently. *Mi/Mi* plants are resistant to nematodes early in root development. In contrast, the *Mi-1.2*-mediated resistance against aphids is developmentally regulated, with fully expanded leaves becoming resistant only when plants are approximately 5 weeks old (Kaloshian et al., 1995). In these plants, all fully expanded leaves are resistant to aphids irrespective of their position on the plant (Kaloshian et al., 1997). However, expanding leaves remain aphid-susceptible throughout the life of the plant (Kaloshian and Williamson, unpubl.).

Here we report the study of *Mi-1.2* expression in various organs of resistant and susceptible tomato, in response to nematode inoculation in roots and aphid infestation in leaves, and its developmental regulation.

Materials and Methods

Plant material and growth conditions: Near-isogenic tomato cv. Moneymaker (*mi-1/mi-1*) and Motelle (*Mi-1/Mi-1*), susceptible and resistant to root-knot nematodes, respectively, were used. Plants were grown in plastic cups (10-cm-diam., 17-cm-deep) in soil, except for nematode inoculations, which are described below. Plants were supplemented with Osmocote (17-6-10) (Sierra Chemical Company, Milpitas, CA), fertilized biweekly with Tomato Miracle-Gro (18-18-21) (Stern's Miracle-Gro Products, Port Washington, NY), and maintained in the greenhouse at temperatures ranging between 22 to 27 °C.

Plant organs: Samples were taken from roots, stems, fully expanded leaves, whole flowers, and green fruits of 10-week-old Motelle and Moneymaker tomato plants. In addition, samples of roots and leaves from Motelle plants were collected at 2, 3, 6, and 8 weeks after planting. Tissues were immediately frozen in liquid nitrogen and stored at −80 °C until processed.

Response to nematode inoculation: Resistant (Motelle) tomato plants were grown in 50-round-cell seedling trays (4.8-cm-diam., 5.9-cm-deep) in steam-sterilized loamy sand. Four-week-old seedlings were individually

Received for publication 08 November 2000.

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The authors thank Linda Walling, Donald Cooksey, and Cynthia LeVesque for critical reviews of the manuscript. This work was supported by USDA-NRICGP grant 9802562.

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This paper was edited by E. L. Davis.

inoculated, and roots were collected at 3, 5, 7, and 10 days after inoculation and stored as above. Time points were chosen considering that, in our system, the hypersensitive response (HR) is not observed until 5–7 days after inoculation (data not shown).

Response to aphid infestation: Eight-week-old Motelle plants were transferred to a growth chamber under continuous light at 24 °C. Leaflets were collected at 3, 6, 9, 12, and 24 hours after aphid infestation and stored as above.

Nematode culture and inoculation: Meloidogyne javanica isolate VW4 was maintained on tomato cv. UC82-B in the greenhouse. Eggs were extracted in 10% bleach as described previously (Hussey and Barker, 1973) and hatched using a modified Baermann system. Wire mesh baskets were lined with two layers of paper towels, set in a glass petri dish, and filled with the egg mixture. The setup was incubated at 25 °C for 48 hours, and each seedling was inoculated with 1,000 hatched second-stage juveniles.

Aphid infestation: A colony of the parthenogenetic aphid, *M. euphorbiae,* was clonally propagated from a single individual and maintained on tomato cv. UC82-B in a cage in the greenhouse. For the aphid induction experiment, five adult aphids were caged onto fully expanded tomato leaves and aphids were allowed to feed on the abaxial leaf surface.

RNA extraction: Total RNA was extracted as in Jones et al. (1985). Plant tissues were ground in liquid nitrogen, nucleic acids were extracted with phenol, and RNA was selectively precipitated in 2M LiCl. Aliquots of RNA were run on an agarose/formaldehyde gel to check quality and quantified using a spectrophotometer.

Reverse transcriptase-polymerase chain reaction (RT-PCR): Five μg of total RNA was treated with 1 U RNase-free DNase I (Promega, Madison, WI) to eliminate genomic DNA contamination. DNase I was removed by phenol/chloroform extraction, and cDNAs were synthesized using a First Strand cDNA Synthesis Kit (MBI Fermentas, Amherst, NY) according to the manufacturer's instructions, with oligo-dT as the primer. For *Mi-1.2*-specific amplification, the primers C1/2Do (5′-GAGAGGAATCCTTCCCCAATCT-3′) and C2S4 (Milligan et al., 1998) were used. As an internal control, the primers ubi3-dir (5′-GAAAACCCTAACGGGGAAG-3′) and ubi3-rev (5′-GCCTCCAGCCTTGTTGTAAA-3′) were designed to amplify the tomato ubiquitin *ubi3* gene transcripts (Hoffman et al., 1991). Both pairs of primers were simultaneously used in the amplification reactions. PCR conditions were 3 minutes at 94 °C, 20 cycles (94 °C, 45 seconds; 64 °C, 1 minute; 72 °C, 1 minute), followed by 3 minutes at 72 °C. Twenty cycles of amplification were performed to ensure amplification was within the exponential range. As a control to confirm lack of genomic DNA contamination, 200 ng of DNase-treated RNA was used as template.

DNA cloning and sequencing: RT-PCR amplifications were performed using C1/2Do and C2S4 primers as described earlier, except 30 cycles were used. Amplification products were purified using the Concert Purification System (Gibco BRL, Baltimore, MD) and directly ligated into a pGEM-T-Easy vector (Promega, Madison, WI). Bacterial transformation and DNA preparation followed established protocols (Sambrook et al., 1989). Purified recombinant plasmids were sequenced by Davis Sequencing (Davis, CA).

DNA blot analysis: One to 5 μl of amplification products was separated by electrophoresis on a 2% agarose gel and blotted onto a Nytran membrane (Schleicher & Schuell, Keene, NH). *Mi-1.2* and *ubi3* probes were labeled with ^{32}P-α-dCTP using the Redi-prime labeling kit (Amersham, Arlington Heights, IL). A mixture of *Mi-1.2* and *ubi3* probes (relative activities 100:1) was used. Hybridization was performed for 16 hours at 42 °C as described in Kaloshian et al. (1998). Final wash was in 0.5X SSC (75 mM NaCl and 7.5 mM Na citrate, pH 7.0) and 0.1% sodium dodecyl sulfate for 30 minutes at 65 °C.

DNA blots were exposed to an Imaging Screen-K and quantified on a Molecular Imager FX System using the Quantity One Analysis Software (Bio-Rad, Hercules, CA). Signal intensities were measured as mean values. To obviate individual differences in RT-PCR efficiency, *Mi-1.2* expression was correlated to the constitutive expression of the tomato ubiquitin gene *ubi3*. *Mi-1.2* expression was normalized by calculating the ratio between *Mi-1.2* and *ubi3* signal intensity from each sample. This ratio was plotted and its variation among different samples analyzed to monitor changes in *Mi-1.2* expression. Arbitrary values of ''one'' were given to the ratios for samples taken at the beginning of each experiment, and the ratios of the related samples were proportional to this ratio. Results are the average of two independent experiments.

Results

Expression of Mi-1.2 in different organs of resistant and susceptible tomato plants: We compared levels of *Mi-1.2* RNAs in seeds, roots, stems, leaves, flowers, and green fruits from uninfected 10-week-old Motelle and Moneymaker tomato plants. Total RNA was treated with DNase I to avoid amplification of contaminating genomic DNA. No amplification products were detected when DNase-treated total RNA was used as template, demonstrating elimination of genomic DNA from these samples (data not shown). Using *Mi-1.2* and *ubi3* specific primers, two DNA fragments of the expected sizes of 300 and 500 bp, respectively, were amplified from every resistant tomato cDNA (Fig. 1). Only the *ubi3* amplification product was obtained using cDNAs synthesized from total RNAs isolated from susceptible tomato organs. However, a *Mi-1.2* hybridization signal from a DNA fragment of the expected size was obtained

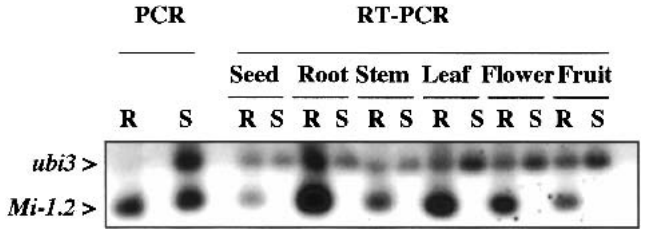

FIG. 1. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of *Mi-1.2* expression in different organs of noninoculated 10-week-old resistant Motelle (*Mi/Mi*) and susceptible Moneymaker (*mi/mi*) tomato plants. DNA transfer-hybridization analysis of DNAs and cDNAs amplified with *Mi-1.2* and ubiquitin *ubi3*-specific primers. The filter was hybridized to a mixture of *Mi-1.2* and *ubi3* probes and visualized using a Molecular Imager (Bio-Rad, Hercules, CA). Templates used in ''PCR'' and ''RT-PCR'' are genomic DNA and cDNAs, respectively. R = Motelle, S = Moneymaker. Twenty times greater ''PCR'' product was loaded for Moneymaker genomic DNA than that for Motelle.

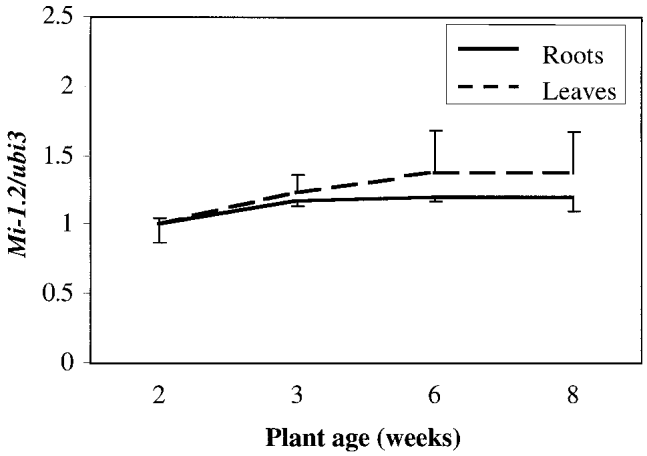

FIG. 2. Graphic comparison of *Mi-1.2* hybridization signal intensity data from noninoculated roots and fully expanded leaves of different-age tomato plants cv. Motelle (*Mi/Mi*) after normalization to ubiquitin controls. The ratio from 2-week-old samples was given a value of 1.0 and the remaining samples calculated as a proportion of the 2-week-old ratio. Error bars show standard deviation in two independent experiments.

when using genomic DNA from susceptible tomato as template, indicating the presence of a gene(s) highly related to *Mi-1.2* in the susceptible tomato genome (Fig. 1). *Mi-1.2* signal was significantly fainter when using susceptible tomato genomic DNA as a template. To obtain a comparable signal intensity of *Mi-1.2* product using resistant and susceptible tomato genomic DNA, 20 times more PCR product from the susceptible DNA was loaded. In addition, since the probe used was a mixture of *Mi-1.2/ubi3* at 100:1 relative activities, *ubi3* signal intensity in the resistant genomic lane was greatly reduced.

The *Mi-1.2* cDNA amplification products from resistant leaves and roots were cloned, and the nucleotide sequences from three randomly chosen clones from each organ were determined. Sequences were found to be identical to the corresponding region of *Mi-1.2*. We have repeated these experiments using different resistant (Sun6082, Roma VFN) and susceptible (Castlerock II, Pixie, UC82-B) tomato cultivars and obtained identical results (data not shown).

Expression of Mi-1.2 in roots and leaves of different age-resistant tomato plants: To determine if the resistance to aphids is the result of developmentally regulated changes in *Mi-1.2* gene expression, *Mi-1.2* mRNA levels in roots and fully expanded leaves from Motelle plants of different ages were monitored. *Mi-1.2* transcripts were present in all samples from roots and leaves, and no significant changes in transcript levels were detected in both organs from plants ranging between 2 to 8 weeks of age (Fig. 2).

Expression of Mi-1.2 in roots and leaves of resistant tomato plants after nematode and aphid attack, respectively: No significant change in *Mi-1.2* transcript levels was detected in roots inoculated with root-knot nematodes over a 10-day period (Fig. 3). Similarly, no significant change in transcript levels was detected in aphid-infested leaves during the 24 hours following infestation (Fig. 4).

DISCUSSION

Limited work has addressed plant-resistance gene regulation and expression patterns. Most resistance genes are expressed at very low levels and are members of gene families where only the expression of specific members confers resistance (Michelmore and Meyers, 1998). Hybridization-based methods do not discriminate between the accumulation of transcripts of a specific gene family member and those of other family members. We chose RT-PCR to study *Mi-1.2* expression in tomato because the technique is extremely sensitive and primers could be designed to discriminate between the transcripts of the highly homologous genes *Mi-1.1*

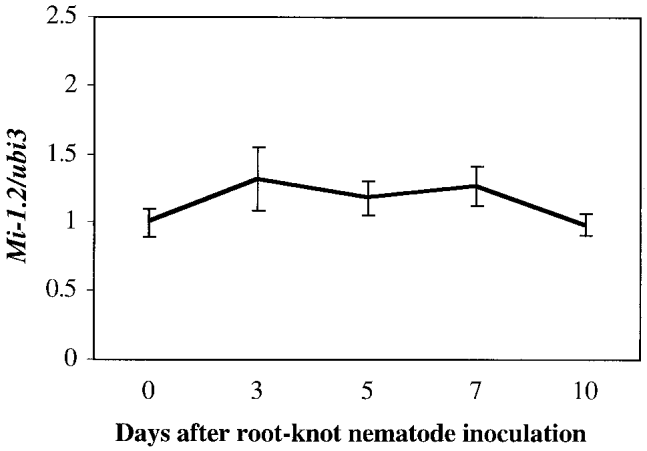

FIG. 3. Graphic comparison of *Mi-1.2* hybridization signal intensity data from roots of resistant tomato plants cv. Motelle (*Mi/Mi*) inoculated with 1,000 second-stage juveniles of *M. javanica* after normalization to ubiquitin controls. Samples were taken over 10 days following inoculation. The ratio at inoculation time was given a value of 1.0 and the remaining samples calculated as a proportion of the inoculation time ratio. Error bars show standard deviation in two independent experiments.

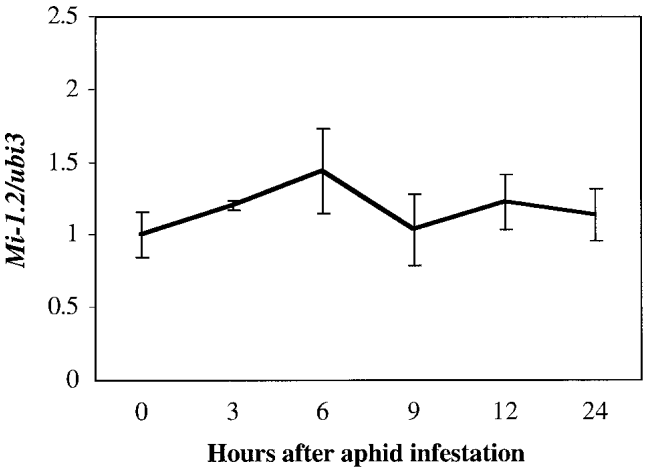

FIG. 4. Graphic comparison of *Mi-1.2* hybridization signal intensity data from leaves of resistant tomato cv. Motelle (*Mi/Mi*) infested with potato aphids after normalization to ubiquitin controls. Five adult aphids were caged on 8-week-old tomato leaves. Samples were collected at various time points during the 24 hours after infestation. The ratio at infestation time was given a value of 1.0 and the remaining samples calculated as a proportion of the infestation time ratio. Error bars show standard deviation in two independent experiments.

and *Mi-1.2* (Milligan et al., 1998). To consider RT-PCR as a reliable semi-quantitative technique, amplification reactions were kept in the exponential phase using only 20 cycles. In addition, *ubi3* transcripts were simultaneously amplified as an internal control to which the amount of *Mi-1.2*-amplified product was correlated.

Using RT-PCR, a constitutive level of resistance gene transcripts in uninfected plants has been observed for the tomato *I-2* fungal resistance gene (Mes et al., 2000), the flax *L6* rust resistance gene (Ayliffe et al., 1999), and the rice *Xa21* bacterial blight resistance gene (Century et al., 1999). In contrast, transcripts of the rice bacterial resistance gene *Xa1* accumulate only after bacterial inoculation or wounding and cannot be detected in uninfected leaves (Yoshimura et al., 1998). Similar to *1-2, L6,* and *Xa21* genes, *Mi-1.2* transcripts are present in uninfected resistant plants. Moreover, we have detected the presence of *Mi-1.2* RNA in every tissue of resistant tomato analyzed, including seeds and green fruits, upon which neither nematodes nor aphids feed. Although our results indicate that *Mi-1.2* transcript levels are higher in roots and leaves than in the other organs tested, these results must be carefully interpreted because transcripts of the control gene used in this study, *ubi3,* accumulated to different levels in different plant organs (Hoffman et al., 1991).

Mi-1.2-mediated resistance is functional against nematodes very early in development, while resistance against aphids is acquired around the fifth week after germination. Data from *Mi-1.2* transcript levels in roots and leaves at different ages showed that *Mi-1.2* transcripts are present in very young roots and leaves, suggesting that *Mi-1.2* is post-transcriptionally regulated differently in leaves and roots. Alternatively, another component, which is developmentally regulated, is required for aphid resistance but not for root-knot nematode resistance. Once Mi-1.2-specific antiserum is developed, the first hypothesis can be addressed. The relative amount of Mi-1.2 protein can be determined in leaves and roots from plants of different ages. This might shed light on the role of translational control or RNA stability in aphid resistance. However, if the alternative hypothesis is the case, protein blot analysis would result in no differences in protein levels between samples of different ages. These results would suggest that a second component of the *Mi-1.2* signal transduction pathway is expressed early in roots and later in fully expanded leaves.

Developmental regulation of resistance has been described for the wheat gene *Lr35,* which confers resistance to *Puccinia recondita* (Kolmer, 1997), and for a number of rice genes conferring resistance to various isolates of *Xanthomonous oryzae* (Qi and Mew, 1985; Mew, 1987; Mazzola et al., 1994). Among these, the only cloned gene is *Xa21.* Results from *Xa21* expression studies using RT-PCR indicate that *Xa21* transcripts are also present in young rice leaves before they become resistant to the bacteria (Century et al., 1999), suggesting a similar scenario for regulation as with aphid resistance from *Mi-1.2.*

The possible effects of root-knot nematode and aphid attack on the expression levels of *Mi-1.2* were studied. Previous data had demonstrated that root-knot nematodes are able to penetrate roots of both resistant and susceptible tomato plants and that they fail to establish feeding sites in resistant roots as a result of the onset of an HR (Dropkin et al., 1969). In the conditions described in this work, HR is seen within 5 to 7 days after inoculation (I. Kaloshian, unpubl.). No differences in *Mi-1.2* expression were detected during 10 days following inoculation, indicating that *Mi-1.2* RNA levels do not change upon nematode infection. Similarly, *Mi-1.2* expression in leaves was monitored after aphid infestation. Potato aphids preferentially pierce the abaxial surface of leaves shortly after access. During the 24-hour infestation period, no changes in *Mi-1.2* RNA levels were detected.

Our experiments indicate that *Mi-1.2* transcripts accumulate before pathogen attack and that RNA levels are not altered during the resistant response. Interestingly, sequence similarities exist between a number of plant defense genes and genes related to the *Drosophila* innate immune system (Wilson et al., 1997). It is tempting to speculate that *Mi-1.2* and other resistant gene products may act as an innate immune system and that through recognition of pathogen avirulence determinants-either by themselves or with the mediation of additional factors—are able to elicit a resistant response and prevent infection.

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