Developmental Regulation of *Mi*-Mediated Aphid Resistance Is Independent of *Mi-1.2* Transcript Levels

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***Mi-1.2*, a member of the intracellular, nucleotide-binding site-leucine-rich repeat family of resistance genes, confers resistance in tomato (*Lycopersicon esculentum*) against both root-feeding nematodes and leaf-feeding aphids. Nematode resistance is effective in all life stages of the plant; in contrast, *Mi*-mediated aphid resistance is developmentally regulated, and protects mature plants but not seedlings against aphid infestation. To determine if the onset of aphid resistance is regulated by *Mi-1.2* transcript abundance, we compared aphid resistance and *Mi-1.2* transcript levels in seedlings and flowering plants. Paired bioassays and RNase protection assays revealed that *Mi-1.2* is transcribed in the leaves prior to the onset of aphid resistance, and that transcript levels are comparable in seedlings and flowering life stages. Furthermore, constitutive overexpression of *Mi-1.2* in transgenic plants did not hasten the onset of aphid resistance in seedlings, or boost the level of resistance observed in flowering plants. These data demonstrate that *Mi-1.2* transcript levels do not modulate the degree of aphid resistance in tomato leaves, or control the differences in regulation between aphid and nematode resistance.**

Additional keywords: insect resistance.

Mi-1.2 is a single, dominant gene in tomato (*Lycopersicon esculentum*) that confers resistance against three highly divergent agricultural pests: root-knot nematodes (*Meloidogyne* spp.), potato aphids (*Macrosiphum euphorbiae*), and tobacco whiteflies (*Bemisia tabaci*) (Milligan et al. 1998; Nombela et al. 2003; Rossi et al. 1998). This gene (also known as *Mi*) is the first described example of an insect resistance gene in the nucleotide-binding site-leucine-rich repeat family of plant resistance genes. Both the biological novelty of *Mi-1.2* and its practical value for crop protection have stimulated great interest in understanding how this gene functions. Little is known about the mode of action of either *Mi*-mediated nematode resistance or *Mi*-mediated insect resistance, and we cannot say whether these forms of resistance act through the same mechanism. To better understand the relationship between nematode and aphid resistance, we have compared the developmental regulation of these two forms of resistance.

Tomato plants that carry *Mi-1.2* are resistant to root-knot nematodes at all life stages. Seedlings inoculated with nema-

todes as early as 24 h after germination have proven resistant to infection (Dropkin 1969). In contrast, Kaloshian and associates (1995) have stated that *Mi*-mediated aphid resistance is developmentally regulated, and is not effective in plants until at least 5 weeks after germination. It has been reported that whitefly resistance also is influenced by plant development (Martinez de Ilarduya et al. 2004).

Many other forms of host plant resistance are developmentally regulated, or active in only certain life stages of the plant. There are, for example, forms of resistance against fungi (Abedon and Tracy 1996; Hugot et al. 1999; Kolmer 1997; Parniske et al. 1997), bacteria (Kus et al. 2002; Mazzola et al. 1994), and insects (Abedon and Tracy 1996; Karban and Baldwin 1997) that are present in mature plants but absent in seedlings. Developmental regulation in some cases limits the usefulness of resistance for crop protection, because it renders certain life stages of the plant susceptible to attack (Century et al. 1999). Many types of resistance potentially could be enhanced if we could manipulate their onset. Unfortunately, the mechanisms regulating the developmental control of resistance are not yet understood.

One hypothesis to explain why *Mi*-mediated nematode resistance and aphid resistance differ in onset is that *Mi-1.2* transcription may differ between above- and below-ground plant parts. In other words, the leaves of young plants could be susceptible to aphids due to insufficient *Mi-1.2* transcript levels. Transcription of at least one resistance gene complex, the *rp1* complex in maize, has been shown to vary with plant age, although the effects of transcript levels on resistance levels were not examined (Collins et al. 1999). In the case of *Mi-1.2*, results from reverse-transcriptase-polymerase chain reaction (PCR) have shown that the gene is transcribed in young leaves (Martinez de Ilarduya and Kaloshian 2001), but transcript levels were not quantified. The goals of the present study were to confirm that *Mi*-mediated aphid resistance is developmentally regulated, and to determine if *Mi-1.2* transcript abundance regulates the onset of resistance. Aphid resistance and *Mi-1.2* mRNA were quantified in individual seedlings and mature plants to determine if levels of resistance were correlated with levels of resistance-gene transcription. Aphid resistance and *Mi-1.2* transcripts also were quantified in transgenic plants that overexpress *Mi-1.2*, to determine if high, constitutive levels of *Mi-1.2* expression would confer constitutive aphid resistance.

RESULTS

Regulation of aphid resistance.

When the *Mi*-containing tomato cvs. Sun6082 and Motelle were challenged with aphids at the flowering stage, the num-

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bers of aphids that established on these cultivars were significantly lower than numbers on the near-isogenic susceptible controls, Castlerock II and Moneymaker (Table 1). In contrast, when the same cultivars were assayed at the seedling stage, aphids readily colonized all four lines, and there were no statistically significant differences in aphid numbers on plants with or without the resistance gene (Table 1). In tomato cultivars with both a determinant (Sun6082) and an indeterminate (Motelle) growth habit, *Mi*-mediated aphid resistance was evident in flowering plants but absent in seedlings. Similar results were obtained with 143-31, a transgenic line homozygous for a single introduced copy of *Mi-1.2* expressed from its endogenous promoter in a Moneymaker background. At flowering, this transgenic line was resistant to aphid infestation (Table 1), which confirmed our previous finding (Rossi et al. 1998) that *Mi-1.2* confers aphid resistance. At the seedling stage, 143-31 and Moneymaker were equally susceptible to aphid infestation.

Mi-1.2 transcript levels in seedlings versus flowering plants.

To determine if *Mi-1.2* transcript levels correlate with levels of aphid resistance, *Mi-1.2* mRNA was quantified using an RNase protection assay on a subset of the plants tested for aphid resistance (Figs. 1 and 2). Motelle seedlings that had not been exposed to aphids also were included in the study to confirm that prior aphid exposure did not influence *Mi-1.2* transcript levels (Fig. 2). *Mi-1.2* is expressed at low levels and is highly similar in sequence to several other genes in tomato; therefore, we chose to use the RNase protection technique because of its high sensitivity and specificity. Digestion with RNases resulted in two protected fragments of approximately the expected size (160 bp) that differed in size by fewer than 5 bp. These fragments were observed in all genotypes that carried *Mi-1.2*, but were absent in tomato cultivars lacking the gene (Figs. 1 and 2). The bands were present in the transgenic line 143-31 but absent in Moneymaker; therefore, we conclude that both bands correspond to *Mi-1.2* transcript. The intensity of the two bands taken together was used as a measure of *Mi-1.2* transcript levels. To test the ability of RNase protection to quantify *Mi-1.2* transcript levels, we assayed a dilution series of RNA from a mature Motelle plant. The intensity of the two protected fragments was proportionate to the amount of RNA used (Fig. 3).

For Sun6082, 143-31, and Motelle, *Mi-1.2* transcripts were detected in both seedlings and flowering plants, and the band intensities were comparable in seedlings and mature plants (Figs. 1 and 2). When aphid numbers were compared with *Mi-1.2* mRNA levels in the same plants for each genotype, there was no correlation between levels of aphid resistance and levels of *Mi-1.2* mRNA. Data from a representative experiment in which three seedlings of the transgenic line

143-31 were compared with three flowering plants of the same line is summarized in Table 2. Although aphid numbers were substantially higher on the seedlings than on the mature plants, the *Mi-1.2* mRNA levels for the two groups were comparable. Furthermore, the transcript level observed in each plant at flowering was not predictive of its degree of aphid resistance; for example, 143-31 #5 had a higher transcript level than the other plants, but bore slightly more aphids than the other two mature plants (Table 2). Levels of *Mi-1.2* mRNA in Motelle seedlings that had not been exposed to aphids were comparable to levels found in exposed seedlings, indicating that aphid feeding did not alter *Mi-1.2* transcript levels (Fig. 2).

Fig. 1. Relative *Mi-1.2* transcript levels in seedlings and flowering plants by RNase protection assays. **A**, *Mi-1.2* transcript levels are compared for three seedlings and three flowering plants of the *Mi-1.2* line Sun6082. Lane C contains DNA from CastlerockII, a near-isogenic line lacking *Mi-1.2*. **B**, Transcript levels in seedlings and flowering plants of 143-31, a transgenic line carrying *Mi-1.2* in a Moneymaker background. The negative control samples, CastlerockII ("C") and Moneymaker ("Mm"), each consist of RNA pooled from eight seedlings and eight mature plants. All other samples represent RNA from individual plants. The two protected fragments observed at approximately 160 bp represent *Mi-1.2* mRNA. Numbers below the lanes indicate the combined intensity of the two protected fragments relative to the negative controls, as measured by phosphorimager.

Table 1. *Mi*-mediated aphid resistance is present at flowering plants but absent at the seedling stage

Isolines used, life stage ^b	Average no. of aphids per plant (mean $\pm$ standard deviation) ^a		<i>P</i> > <i>F</i>
	Susceptible isolate (<i>mi/mi</i>)	Resistant isolate (<i>Mi/Mi</i>)	
Castlerock (<i>mi/mi</i>) and Sun6082 (<i>Mi/Mi</i>)			
Seedling	173 $\pm$ 39	174 $\pm$ 51	0.97
Flowering	149 $\pm$ 76	48 $\pm$ 41	0.0055
Moneymaker (<i>mi/mi</i>) and Motelle (<i>Mi/Mi</i>)			
Seedling	102 $\pm$ 16	97 $\pm$ 12	0.53
Flowering	58 $\pm$ 34	16 $\pm$ 18	0.0093
Moneymaker (<i>mi/mi</i>) and 143-31 (<i>Mi/Mi</i>)			
Seedling	117 $\pm$ 24	117 $\pm$ 21	0.97
Flowering	131 $\pm$ 30	72 $\pm$ 54	0.018

^a Plants were inoculated with 15 aphids each (8 plants/treatment), and total aphid numbers were measured 5 days after inoculation.

^b Seedling = inoculated at the seedling stage, two fully expanded leaves; Flowering = inoculated with aphids at the onset of flowering.

Overexpression of *Mi-1.2*.

To determine if the onset of aphid resistance could be hastened by increasing *Mi-1.2* transcript levels in seedlings, we examined aphid resistance in a tomato line derived from susceptible tomato line T30 transformed with a construct (*35S-Mi*) expressing *Mi-1.2* under control of the *Cauliflower mosaic virus* (CaMV) 35S promoter. RNase protection showed that *Mi-1.2* was transcribed in both seedlings and mature plants of the *35S-Mi* line 010, and that transcript levels in this line were nearly fivefold higher than the levels observed in Motelle, a cultivar that carries *Mi-1.2* with its endogenous promoter (Fig. 2). Aphid numbers 5 days after inoculation were significantly lower on the flowering transgenics than on the T30 control plants (one-way analysis of variance [ANOVA]: $df = 1, 14$; F

Table 2. Aphid resistance is not correlated with *Mi-1.2* mRNA levels

143-31	Aphid number	Band intensity ^a	Average band intensity
Seedling #1	98	16.0	...
Seedling #2	116	14.3	...
Seedling #3	118	12.7	14.3 ± 1.6
Flowering #4	38	12.6	...
Flowering #5	46	26.2	...
Flowering #6	40	8.5	15.7 ± 9.3

^a RNase protection was used to quantify relative *Mi-1.2* mRNA levels in the same individual plants that had been assayed for aphid resistance.

Fig. 2. Relative transcript levels of *Mi-1.2* in *35S-Mi* transgenic tomato compared with Motelle. “SA” designates aphid-infested seedlings, “FA” indicates aphid-infested flowering plants, and “SNA” indicates uninfested seedlings. Line 010 carries *35S-Mi-1.2* in a T30 genetic background, and Motelle carries *Mi-1.2* with its native promoter. T30 and Moneymaker (“Mm”) lack *Mi-1.2*. Yeast tRNA is a negative control. Samples T30 and Moneymaker each contain RNA pooled from eight seedlings and eight flowering plants. All other samples represent RNA from individual plants. Numbers below each lane represent the combined intensity of the two protected fragments (approximately 160 bp) relative to the negative controls, as measured by phosphorimager.

Fig. 3. RNase protection assay on a dilution series. RNase protection was performed on 0, 20, 40, 100, and 200 µg of RNA from a flowering Motelle plant. For each dilution, the combined intensity of the protected fragments (approximately 160 bp) is graphed on the Y axis. The R^2 value for a regression analysis is 0.98.

$= 7.16$; $P > F = 0.018$) (Fig. 4A). There was no significant difference in aphid numbers on 010 versus Motelle (one-way ANOVA: $df = 1, 14$; $F = 2.93$; $P > F = 0.11$). At the seedling stage, aphids successfully colonized 010, Motelle and the susceptible control, and there were no significant differences in aphid numbers among the three genotypes (one-way ANOVA: $df = 2, 21$; $F = 1.93$; $P > F = 0.17$) (Fig. 4B). Two additional lines, 038 and 002, derived from T30 plants independently transformed with *35S-Mi* also were assayed for aphid resistance at the seedling stage (average aphid numbers ± standard deviation: for 038, 75 ± 21 ; for 002, 72 ± 32 ; for T30, 92 ± 21). There were no statistically significant differences in aphid numbers on these lines versus T30 (one-way ANOVA: $df = 2, 21$; $F = 1.39$; $P > F = 0.27$). Lines 010, 038, and 002 were determined to be resistant to the root-knot nematode, *Meloidogyne javanica* (F. L. Goggin, G. Shah, V. M. Williamson, and D. E. Ullman, unpublished data).

DISCUSSION

Our results indicate that *Mi*-mediated aphid resistance develops with plant age but is not correlated with an increase in *Mi-1.2* transcript levels. Although seedlings lack aphid resistance, they have levels of *Mi-1.2* mRNA that equal or exceed those observed in resistant flowering plants (Figs. 2 and 3). Furthermore, aphid resistance is developmentally regulated even in transgenic plants that constitutively overexpress *Mi-1.2* (Fig. 4). This study adds to a growing body of evidence that resistance-gene function is regulated by mechanisms other than the transcriptional regulation of the resistance gene itself. Two pathogen resistance genes, *Xa21* in rice and *Cf-9B* in tomato, are tran-

Fig. 4. Comparison of aphid resistance in the *35S-Mi* line 010 and Motelle. Eight plants per genotype were inoculated with 15 aphids each at A, flowering and B, seedling stages, and the total number of aphids per plant was measured after 5 days. T30 was included as a susceptible control. Within each experiment, means labeled with different letters are significantly different according to Tukey's honestly significant difference test ($P < 0.05$).

scribed in seedlings, although they confer resistance only in mature plants (Century et al. 1999; Panter et al. 2002). Furthermore, *Cf-9B* failed to protect seedlings even when expressed under the control of a promoter with known activity in young plants (Panter et al. 2002). Not only the developmental regulation, but also the organ specificity of resistance can be independent of the resistance gene's promoter activity. *RPP1*, which mediates downy mildew resistance in *Arabidopsis* spp., is transcribed in both roots and leaves, although it protects only leaves against infection (Hermanns et al. 2003). In the case of *Mi-1.2*, it is not yet clear whether the onset of aphid and nematode resistance vary because of differences between roots and leaves or because of differences in the mode of action of these two forms of resistance.

One possible explanation for the developmental regulation of *Mi*-mediated aphid resistance is that the translation, processing, or stability of the *Mi-1.2* protein differ in the leaves of young versus mature plants. Unfortunately, *Mi-1.2* is expressed at very low levels, it bears strong homology to several other genes in tomato, and even minor modifications in its sequence have been found to eliminate gene function (Hwang and Williamson 2003). To date, these obstacles have stymied our attempts to generate an antibody to the *Mi-1.2* protein product or to detect the protein using epitope tags.

Another possibility is that young leaves lack some factor other than *Mi-1.2* that is necessary for resistance. It has been proposed that *Mi-1.2* is involved in a signaling cascade that induces a resistance response in the plant when avirulent nematodes or aphids are present (Hwang et al. 2000). Presumably, several plant genes in addition to *Mi-1.2* are involved in the resistance response. It also is possible that other genes are required for recognition of the aphid by the *Mi-1.2* resistance system. The *Rme1* locus in tomato is known to be required for *Mi*-mediated resistance to nematodes, aphids, and whiteflies, but not for function of two other resistance genes, *I-2* and *Pto* (Martinez de Ilarduya et al. 2001, 2004). *Rme1* has not yet been cloned, however; therefore, the effects of plant development on its expression are unknown. Comparisons between responses to aphid feeding in seedlings versus mature plants may help us to identify other critical genes and to understand the developmental regulation of resistance. Such studies also may help us to determine if *Mi*-mediated aphid and nematode resistance are dependent upon the same defensive pathways.

In addition to examining the developmental regulation of resistance, this study also indicates that artificial enhancement of *Mi-1.2* transcript levels does not enhance aphid resistance in mature plants. These findings contrast with those for two other tomato genes, *Prf* and *Pto*, which are required for resistance against *Pseudomonas syringae* pv. *tomato*. *Prf* renders plants susceptible to the insecticide fenthion and resistant to the bacterial pathogen. Transgenic plants with increased *Prf* mRNA have increased sensitivity to fenthion, and are resistant to a broader range of pathogens compared with plants with wild-type *Prf* transcript levels (Oldroyd and Staskawicz 1998). Using the CaMV 35S promoter to overexpress *Pto*, the *P. syringae* resistance gene, also resulted in broad-spectrum pathogen resistance, but was associated with spontaneous foliar lesions (Li et al. 2002; Xiao et al. 2001). In contrast, we have detected neither detrimental nor beneficial effects in plants with increased *Mi-1.2* transcript levels. It appears that low levels of *Mi-1.2* expression are sufficient to confer resistance, and that increased expression does not enhance *Mi-1.2* function. Further investigation is needed to identify the factors responsible for the developmental differences in aphid and nematode resistance in tomato. Identification of such differences may provide us with clues to the pathways leading to nematode and insect resistance mediated by this valuable gene.

MATERIALS AND METHODS

Plant materials.

Two pairs of near-isogenic tomato cultivars that differ in the presence of *Mi-1.2* were used in this study to examine whether plant determinacy influences the developmental regulation of resistance. Sun6082 (*Mi-1.2/Mi-1.2*) and CastlerockII (lacking *Mi-1.2*) have a determinate growth habit, and Motelle (*Mi-1.2/Mi-1.2*) and Moneymaker (lacking *Mi-1.2*) have an indeterminate growth habit. In addition, 143-31, a transgenic line carrying *Mi-1.2* in a Moneymaker background, also was included to confirm our previous finding that *Mi-1.2* confers aphid resistance. This line carries the *Mi-1.2* coding region and 4.62 kb of the 5' and 4.77 kb of the 3' flanking DNA sequences (Milligan et al. 1998). All 143-31 plants tested were from the T2 generation and were collected from a parent homozygous for a single insertion of the transgene.

Three additional transgenic lines also were used in this study to assess how overexpressing *Mi-1.2* would affect the developmental regulation of aphid resistance. These lines, 010, 002, and 038, were derived by independent transformation with *Mi-1.2* driven by the 35S CaMV promoter (G. Shah, unpublished data) of the nematode- and aphid-susceptible tomato line T30. Prior to their use in aphid bioassays, T2 plants from each line were screened for the presence of the insert using PCR primers specific for the NPTII kanamycin resistance gene (forward primer: 5'GCAATATCACGGGTAGCCAA3'; reverse primer: 5'GCCGTGTTCCGGCTGTCA3'). The size of the amplified product was 584 bp.

Aphid bioassays.

Potato aphid isolate WU12 was used for all bioassays (provided by S. Seah, University of California, Davis, U.S.A.). Aphids were reared on UC82 tomato seedlings and jimsonweed (*Datura stramonium* L.) in a Conviron growth chamber (20°C; 16 h of light, 8 h of dark per photoperiod; 70% relative humidity) (Controlled Environments, Inc., Winnipeg, Canada). All experimental plants were grown under greenhouse conditions, as previously described (Goggin et al. 2001).

For each plant genotype tested, one set of plants was assayed for resistance at the seedling stage, when the plants had only two true leaves (2 to 3 weeks after germination, depending upon the tomato cultivar tested). For all genotypes except 002 and 038, an additional set of plants was assayed at the early flowering stage, when the plants' first blooms began to open (5 to 7 weeks after germination, depending upon the cultivar tested). Bioassays were conducted in Conviron growth chambers (20°C; 16 h of light, 8 h of dark per photoperiod; 70% relative humidity). There were eight plants (replicates) per treatment. Each plant was inoculated with 15 potato aphids. On each flowering plant, the aphids were confined to a single leaflet using a sleeve cage, and the cage position was standardized from plant to plant. Sleeve cages proved to be damaging to seedlings; therefore, aphids were confined to seedlings using small cages that covered the entire plant. The total number of aphids per plant was counted 5 days after inoculation, and the numbers of aphids on plants with and without *Mi-1.2* were compared using one-way ANOVA (SAS Software Release 7.0; SAS Institute, Cary, NC, U.S.A.).

Tissue collection.

After the plants were scored for aphid resistance, three flowering plants and three seedlings each were sampled from the *Mi*-containing lines Sun6082, 143-31, Motelle, and 010. In flowering plants, leaf tissue was collected from the inoculated leaves and, in seedlings, tissue was taken from all fully expanded leaves. Genotypes lacking *Mi-1.2* (CastlerockII, Mon-

eymaker, and T30) were included as negative controls. For each of these control genotypes, tissue from all eight replicates per bioassay was pooled for RNA extraction. Tissue also was collected from three Motelle seedlings that had not been exposed to aphids, so that *Mi-1.2* transcript levels could be compared in uninoculated versus inoculated tissue. RNA was extracted using Trizol Reagent (Invitrogen, Carlsbad, CA, U.S.A.) according to the supplier's protocol, and RNA samples were quantified by measuring absorbance at 260 nm. RNA quality and quantity were verified by visualizing aliquots of the RNA samples on agarose gels stained with ethidium bromide.

RNase protection.

To produce an *Mi-1.2*-specific probe for RNase protection, a 160-bp *HindIII/EcoRI* fragment of the 5' cDNA clone pSM109-6 (Milligan et al. 1998) was inserted into Bluescript KS+. The plasmid was linearized with *HindIII*, and antisense RNA was synthesized by T7 RNA polymerase using [α^{32} P]UTP (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.). For RNase protection analysis, 20- μ g aliquots of total RNA per sample were hybridized to the radiolabeled probe as described by Melton and associates (1984). For negative control samples (genotypes lacking *Mi-1.2*), 10 μ g of RNA from seedlings was pooled with 10 μ g of RNA from mature plants. After an overnight hybridization, the hybrids were treated with RNase A/RNase T1 and the protected fragments resolved on a 6% polyacrylamide gel. Protected fragments were analyzed quantitatively with a PhosphorImager gel scanner and ImageQuant Software for Macintosh (Molecular Dynamics, Sunnyvale, CA, U.S.A.). The intensity of the two bands in each sample was measured relative to the same region of lanes containing negative controls.

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