

The *Mi-1*-Mediated Pest Resistance Requires *Hsp90* and *Sgt1*^{1[OA]}

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The tomato (*Solanum lycopersicum*) *Mi-1* gene encodes a protein with putative coiled-coil nucleotide-binding site and leucine-rich repeat motifs. *Mi-1* confers resistance to root-knot nematodes (*Meloidogyne* spp.), potato aphids (*Macrosiphum euphorbiae*), and sweet potato whitefly (*Bemisia tabaci*). To identify genes required in the *Mi-1*-mediated resistance to nematodes and aphids, we used tobacco rattle virus (TRV)-based virus-induced gene silencing (VIGS) to repress candidate genes and assay for nematode and aphid resistance. We targeted *Sgt1* (suppressor of G-two allele of *Skp1*), *Rar1* (required for *Mla12* resistance), and *Hsp90* (heat shock protein 90), which are known to participate early in resistance gene signaling pathways. Two Arabidopsis (*Arabidopsis thaliana*) *Sgt1* genes exist and one has been implicated in disease resistance. Thus far the sequence of only one *Sgt1* ortholog is known in tomato. To design gene-specific VIGS constructs, we cloned a second tomato *Sgt1* gene, *Sgt1-2*. The gene-specific VIGS construct TRV-*SlSgt1-1* resulted in lethality, while silencing *Sgt1-2* using TRV-*SlSgt1-2* did not result in lethal phenotype. Aphid and root-knot nematode assays of *Sgt1-2*-silenced plants indicated no role for *Sgt1-2* in *Mi-1*-mediated resistance. A *Nicotiana benthamiana* *Sgt1* VIGS construct silencing both *Sgt1-1* and *Sgt1-2* yielded live plants and identified a role for *Sgt1* in *Mi-1*-mediated aphid resistance. Silencing of *Rar1* did not affect *Mi-1*-mediated nematode and aphid resistance and demonstrated that *Rar1* is not required for *Mi-1* resistance. Silencing *Hsp90-1* resulted in attenuation of *Mi-1*-mediated aphid and nematode resistance and indicated a role for *Hsp90-1*. The requirement for *Sgt1* and *Hsp90-1* in *Mi-1*-mediated resistance provides further evidence for common components in early resistance gene defense signaling against diverse pathogens and pests.

Plant resistance (R) proteins recognize pathogen avirulence (Avr) determinants and activate plant defenses. The carefully orchestrated active defense involves the regulation of a large number of genes that often results in a hypersensitive response (HR), a form of programmed cell death (Schenk et al., 2000; Glazebrook et al., 2003; Nimchuk et al., 2003). The cell death is presumed to stop the invasion of the pathogen at the point of penetration or stop the feeding of the pest and limit the damage caused by the attack. Preceding the HR, a series of metabolic changes are observed including the accumulation of reactive oxygen and nitrogen species (Nimchuk et al., 2003).

During the past decade, a number of R and Avr genes have been cloned from a variety of host pathogen

systems. Although similarities among Avr gene products are limited, in general, plant disease R genes share a number of known structural motifs (Martin et al., 2003). The largest class of R genes encodes proteins with nucleotide-binding site (NBS) and Leu-rich repeat (LRR) motifs. Members of this group confer resistance to a number of pathogens including bacteria, viruses, fungi, nematodes, and insects, suggesting the existence of a common signal transduction pathway that results in resistance to these diverse organisms (Martin et al., 2003). The NBS-LRR class of R genes could be subdivided into two major groups based on the presence of domains similar to the Toll and interleukin-1 receptor or coiled-coil (CC) domain at the amino terminus.

Several common components that interact with R proteins or are required for R function have been recently identified (Schulze-Lefert, 2004). Using mutational analysis, *Rar1* was originally isolated from barley (*Hordeum vulgare*) and identified as a requirement for resistance to powdery mildew (*Blumeria graminis* f. sp. *Hordei*) mediated by *Mla12* (Torp and Jorgensen, 1986). *Rar1* encodes a predicted cytosolic protein with two highly similar but distinct Cys- and His-rich (CHORD) zinc-binding domains (Shirasu et al., 1999). *Rar1* homologs are present in eukaryotes, except for yeast (*Saccharomyces cerevisiae*), and recently have been predicted to function as a cochaperone in plants. In addition, *Rar1* is required for a subset of R-gene-mediated resistance responses in monocot and dicot plant species

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(Shirasu et al., 1999; Liu et al., 2002b; Muskett et al., 2002; Scofield et al., 2005). Another protein, SGT1, which interacts with RAR1, also contributes to *R*-gene-mediated resistance (Austin et al., 2002; Azevedo et al., 2002; Liu et al., 2002c, 2004). *Sgt1* has multiple functions and associates with complexes including the Skp1-Cullin/F-box type E3 ubiquitin ligase complexes that target regulatory proteins for degradation by the 26S proteasome (Muskett and Parker, 2003). Both RAR1 and SGT1 interact with chaperon HSP90, a cytosolic abundant protein that functions in various signal transduction networks (Picard, 2002; Hubert et al., 2003; Takahashi et al., 2003; Liu et al., 2004).

Distinct isoforms of *Arabidopsis* (*Arabidopsis thaliana*) *HSP90* are required for specific *R*-gene-mediated resistance responses. For example, *AtHSP90.1* is required for the full function of *RPS2* that confers resistance to *Pseudomonas syringae* expressing *AvrRpt2* (Takahashi et al., 2003), while *AtHSP90.2* is required for the function of *RPM1* resistance to *P. syringae* expressing *AvrRPM1* (Hubert et al., 2003). Similarly, the requirement for *Hsp90* in *R*-gene-mediated resistance in solanaceous plants has been shown using virus-induced gene silencing (VIGS). These include *Rx*-mediated resistance to *Potato virus X*, *N*-mediated resistance to *Tobacco mosaic virus*, and *Pto*-mediated resistance to *P. syringae* expressing *AvrPto*. Thus, *Hsp90* plays an important role in disease resistance signaling (Lu et al., 2003; Liu et al., 2004).

The tomato (*Solanum lycopersicum*) resistance gene *Mi-1* encodes a protein with CC-NBS-LRR motifs (Milligan et al., 1998). *Mi-1* is a unique *R* gene conferring resistance to root-knot nematodes (*Meloidogyne* spp.), potato aphids (*Macrosiphum euphorbiae*), and sweet potato whitefly (*Bemisia tabaci*; Milligan et al., 1998; Rossi et al., 1998; Nombela et al., 2003). Although four other nematode resistance genes have been cloned, *Mi-1* remains the only cloned root-knot nematode *R* gene (Williamson and Kumar, 2006). Similarly, to date, *Mi-1* is the only cloned insect *R* gene (Kaloshian, 2004). The resistance mediated by *Mi-1* acts in a gene-for-gene manner. *Mi-1* confers resistance to the root-knot nematodes *Meloidogyne arenaria*, *Meloidogyne incognita*, and *Meloidogyne javanica*, but it does not confer resistance to *Meloidogyne hapla*, a nematode present in overlapping geographic locations (Roberts and Thomason, 1989). Likewise, the resistance to potato aphids is limited to specific biotypes of the aphid (Rossi et al., 1998). It is not clear whether potato aphid, whitefly, and the three root-knot nematode species share similar *Avr* determinants as no nematode or insect *Avr* determinant has been conclusively isolated.

The resistance mediated by *Mi-1* is manifested differently against nematodes and aphids. Infective-stage juveniles (J2) of the nematode are able to penetrate and migrate through resistant tomato roots to initiate feeding near the vascular element. However, HR develops in the area near the head of the feeding juvenile, which is presumed to inhibit nematode feeding (Paulson and Webster, 1972). In resistant leaves, however, aphid feed-

ing is not associated with HR (Martinez de Ilarduya et al., 2003). Potato aphids are able to access the phloem tissue in resistant leaves and initiate feeding, however phloem feeding is extremely limited and aphid seem to die from starvation (Kaloshian et al., 2000). The resistance mediated by *Mi-1* to nematodes is early in plant development while the resistance to both insects is developmentally regulated. Seedlings up to 4 to 5 weeks of age, with four expanded leaves, are susceptible to both aphids and whiteflies (Kaloshian et al., 1995; Pascual et al., 2000). In spite of the developmental

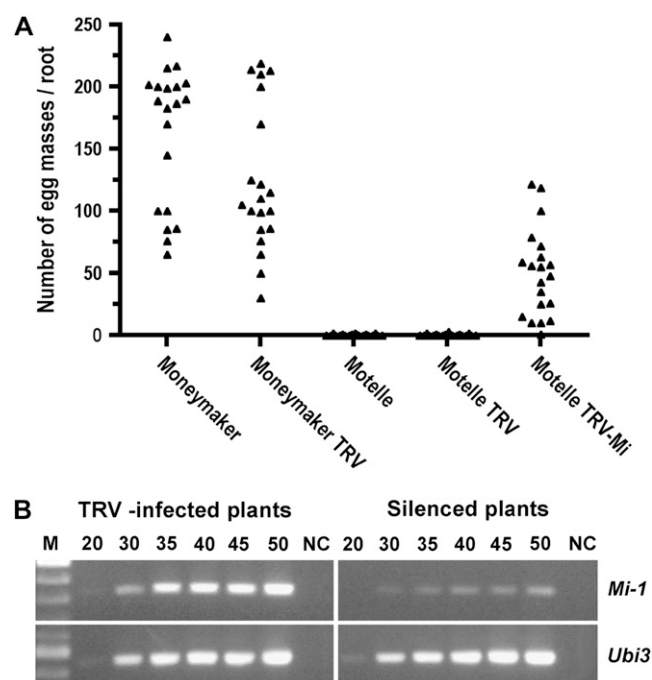


Figure 1. Root-knot nematode reproduction and *Mi-1* expression in tomato roots silenced for *Mi-1* and in control roots. Near isogenic tomato 'Motelle' (*Mi-1/Mi-1*) and 'MoneyMaker' (*mi-1/mi-1*) were used. Cultures of *Agrobacterium tumefaciens* strain GV3101 containing pTRV1, pTRV2-*Mi-1*, or pTRV2 empty vector were used. The pTRV1 plus pTRV2-*Mi-1* is referred to as TRV-*Mi* and pTRV1 plus pTRV2 empty vector is referred to as TRV. Controls were infiltrated with buffer. Four weeks after infiltration, plants were infected with root-knot nematodes. A, Nematode reproduction on control plants and plants agroinfiltrated with TRV-*Mi*. Triangles represent the number of egg masses on a single root system. Twenty plants per genotype treatment were used in each experiment. Ten weeks after inoculation, plants were evaluated by counting the number of egg masses on each root. Four independent experiments were performed all of which gave similar results. Data from one experiment are presented. B, Effect of TRV-*Mi* on *Mi-1* transcript levels in tomato roots 'Motelle.' Ethidium bromide stained 1.5% agarose gels with RT-PCR products. cDNA was synthesized from total RNA isolated from roots of plants agroinfiltrated with TRV or TRV-*Mi* supporting egg masses. For *Mi-1*-specific amplification, the primers VIGS-F and C2S4 (see Table I) were used. PCR amplification from cDNA from a single representative sample is presented. Amplification of the tomato ubiquitin *Ubi3* gene was used as an internal control for equal cDNA use from control and silenced plants. PCR cycles are indicated on the top of the sections. Lane M indicates DNA ladder and NC indicates negative control where RNA was used as template in the absence of reverse transcriptase.

regulation of *Mi-1*-mediated resistance to these insects, *Mi-1* transcripts accumulate to similar level in leaflets of young and old plants (Martinez de Ilarduya and Kaloshian, 2001). However, it is not clear whether *Mi-1*-mediated resistance is posttranscriptionally regulated differently in roots and leaves or that another member of the signal transduction pathway is developmentally regulated.

Limited information exists about the signal transduction pathway mediated by *Mi-1*. Recent work using mutational approaches identified the requirement of another gene, *Rme1*, for *Mi-1*-mediated resistance to nematodes, aphids, and whiteflies (Martinez de Ilarduya et al., 2001, 2004). Although *Rme1* is nec-

essary for *Mi-1* function it is not required for *Pto* or *I-2*-mediated resistance against *Fusarium oxysporum* f. sp. *lycopersici* race 2 (Martinez de Ilarduya et al., 2001, 2004). In addition to *Rme1*, *Mi-1* resistance requires the salicylic acid (SA) signaling pathway and mitogen-activated protein kinase (MAPK) cascades (Branch et al., 2004; Li et al., 2006). The tomato MAPK kinase MKK2 and MAPKs *LeMPK1*, *LeMPK2*, and *LeMPK3* are required for *Mi-1*-mediated aphid resistance (Li et al., 2006). Their role in root-knot nematode resistance has not yet been identified.

VIGS has emerged as an important tool to assess gene function in systems where mutational, tagging, and cloning approaches require significant expenditure of time

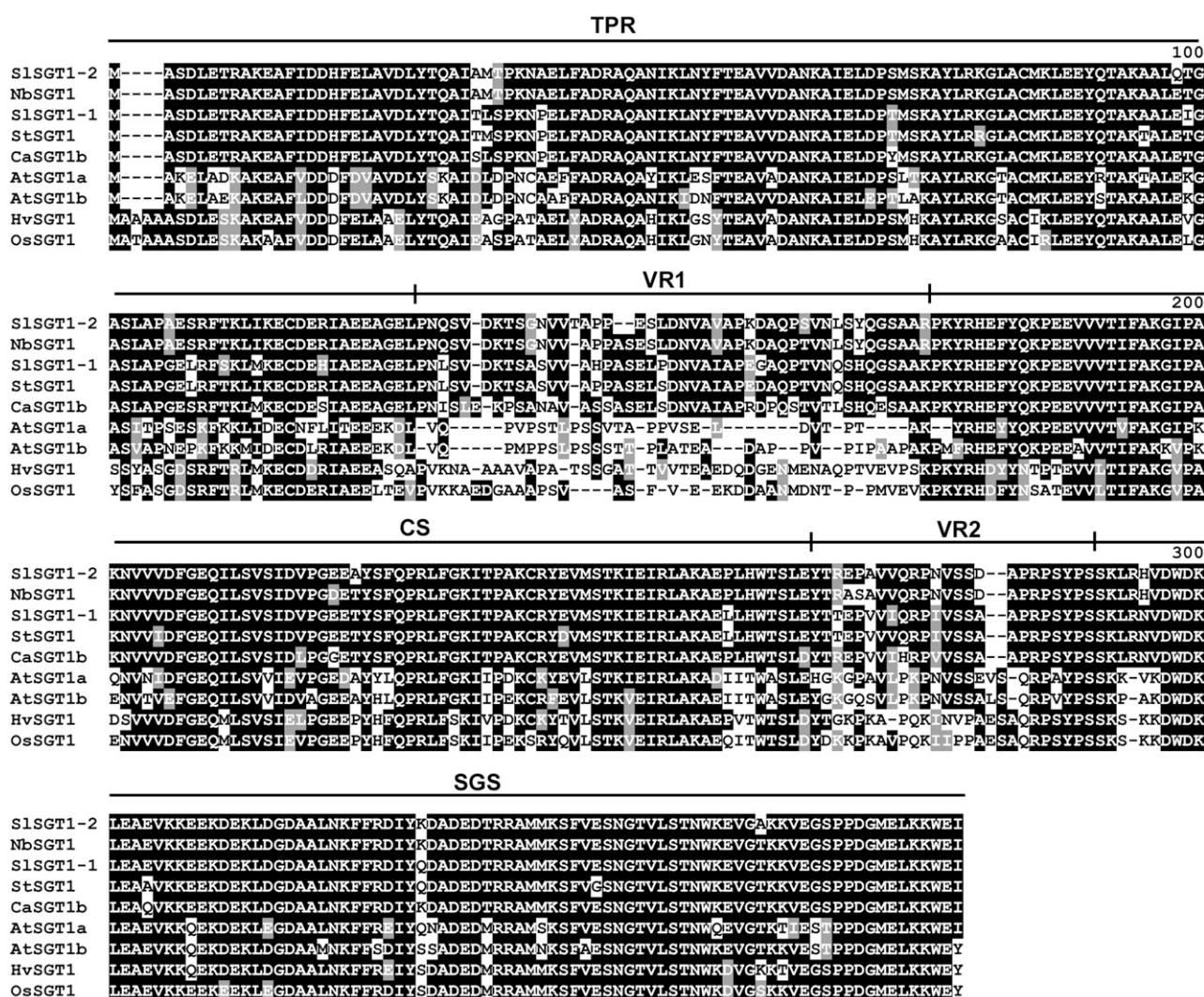


Figure 2. Alignment of deduced amino acid sequences of tomato (*Sl*) SGT1-1 and SGT1-2 with SGT1 sequences from potato (*St*), pepper (*Capsicum annuum*, *Ca*), *N. benthamiana* (*Nb*), Arabidopsis (*At*), barley (*Hv*), and rice (*Oryza sativa*, *Os*). Peptide sequences were from GenBank or The Institute of Genomic Research tomato gene index. *NbSGT1*, AAO85509; *S1SGT1-1*, TC162726; *S1SGT1*, AAU04979; *CaSGT1*, AAX83943; *AtSGT1a*, AAL33611; *AtSGT1b*, AAL33612; *HvSGT1*, AAL33610; and *OsSGT1*, AAF18438. Black and gray shades indicate identical and highly conserved amino acids, respectively. Lines above sequences indicate consensus domains. TPR, Tetratricopeptide repeat domain; VR1 and VR2, variable domains; CS, CHORD and SGT1-specific domain; SGS, SGT1-specific domain.

and resources. Tobacco rattle virus (TRV)-based VIGS has been used to assess the function of a number of genes in tomato and *Nicotiana* spp., including those that play a role in disease resistance (Liu et al., 2002b, 2002c, 2004, 2005; Ekengren et al., 2003; Lu et al., 2003; Li et al., 2006). In this article, we have used TRV-VIGS to determine whether *Hsp90*, *Sgt1*, and *Rar1* are required for *Mi-1*-mediated aphid and nematode resistance. In the process we cloned *Sgt1-2*. In addition, we demonstrated that silencing *Sgt1-1* in tomato results in lethality and that *Sgt1* is required for *Mi-1* resistance. Our results also indicated that *Hsp90-1* is also required for *Mi-1*-mediated resistance but no role for *Rar1* in this resistance was identified.

RESULTS

Optimization of *Mi-1* Silencing in Tomato Roots

The use of the bipartite TRV vector, pTRV1 plus pTRV2, for VIGS in above-ground parts of tomato has been previously demonstrated (Liu et al., 2002a). To test the use of this vector for VIGS in tomato roots, we targeted the *Mi-1* gene for silencing using TRV and assayed for root-knot nematode resistance. We used a similar procedure we had previously optimized for TRV-VIGS to silence *Mi-1* in tomato leaves of 'Motelle' and 'Money-maker,' resistant and susceptible to root-knot nematodes, respectively (Li et al., 2006). *Agrobacterium* cultures (strain GV3101) containing either pTRV1 plus pTRV2 carrying a fragment of *Mi-1* (TRV-*Mi*) or empty vector (TRV) were used to agroinfiltrate 2-week-old tomato seedlings. Four weeks after agroinfiltration, plants were inoculated with J2 of *M. javanica*. Initially, we used 3,000 J2 to infect an individual plant, an inoculum level typically used in our laboratory for nematode assays. However, this inoculum level resulted in a very low level of nematode infection and reproduction on 'Motelle' plants silenced for *Mi-1* (data not shown). It is likely that in tomato roots as in leaves, virus spread and VIGS is not

uniform, resulting in patchy silenced and nonsilenced regions. To overcome the lack of uniformity of silencing, we increased the nematode inoculum level to 10,000 J2 to more thoroughly expose the root system to nematode infection.

Evaluation of roots from the susceptible 'Money-maker' tomato infiltrated with buffer or agroinfiltrated with TRV resulted in similar numbers of egg masses, indicating that neither TRV nor *Agrobacterium* hindered nematode infection (Fig. 1A). In general, no egg masses were present on roots of 'Motelle' plants infiltrated with buffer or agroinfiltrated with empty vector TRV, indicating that neither TRV or *Agrobacterium* interfered with *Mi-1*-mediated nematode resistance (Fig. 1A). In contrast, the number of egg masses on 'Motelle' roots agroinfiltrated with TRV-*Mi* ranged from 5 to 122, indicating that TRV-*Mi* attenuated *Mi-1*-mediated root-knot nematode resistance (Fig. 1A).

To confirm *Mi-1* transcript degradation in TRV-*Mi* roots, 'Motelle' root portions with egg masses were used as a source of RNA for semiquantitative analysis of the relative abundance of *Mi-1* transcript levels. More than 10 root samples were used in reverse transcription (RT)-PCR analysis. All samples indicated reduction in *Mi-1* transcript levels in TRV-*Mi* agroinfiltrated roots compared to empty vector TRV agroinfiltrated roots (Fig. 1B).

Cloning Tomato *Sgt1-2* Gene

Two copies of the *Sgt1* gene, *SGT1a* and *SGT1b*, exist in Arabidopsis (Azevedo et al., 2002). Mutant analysis indicated a role for *SGT1b* in plant defense (Muskett and Parker, 2003). No role for *SGT1a* has yet been identified. However, *sgt1a;sgt1b* double mutation is lethal, suggesting a redundant role for *SGT1a* and *SGT1b* (Muskett and Parker, 2003). Mutations in tomato *Sgt1* gene(s) have not been identified, also suggesting the possibility of a lethal phenotype. To avoid the lethality observed in the Arabidopsis double mutation and to

Table 1. Primers used in RT-PCR analyses

Genes targeted in VIGS and gene-specific primers used in RT-PCR for transcript evaluations.					
Tomato Genes	Accession or Tentative Consensus No.	Gene-Specific Primers	Primer	Fragment Length	Source
<i>Mi-1</i>	AF039682	5'-CTTGCGTCTACTGACTCTTTCC-3' 5'-CTAAGAGGAATCTCATCACAGG-3'	VIGS-F C2S4	330	Li et al. (2006)
<i>Rar1</i>	TC159170	5'-GATGTCAGAGGATCGGTTGTAACG-3' 5'-ATCTAGAACAGGCTCTTTCCGGTG-3'	RAR-1RF RAR-1RR	300	This article
<i>Sgt1-1</i>	TC162726	5'-ACATCTGTCATCTGAGTTACC-3' 5'-AAGCGATGTCCAGTGTAAAC-3'	SGT1-1RF SGT1-1RR	358	This article
<i>Sgt1-2</i>	EF011105	5'-GCTCCCCCTGAGTCTTTG-3' 5'-TCGAGAGATGTCCAGTGTAAAGG-3'	SGT1-2RF SGT1-2RR	356	This article
<i>Hsp90-1</i>	AY368906	5'-TTGAGGAGACTGAAGATGAGAAG-3' 5'-CATGTCCAGATGGTGGAGCTGAG-3'	HSP90-F HSP90-1R	608	This article
<i>Hsp90-2</i>	AY368907	5'-TTGAGGAGACTGAAGATGAGAAG-3' 5'-CGTTGAACTGCCATTCTAAAAG-3'	HSP90-F HSP90-2CR	639	This article

construct gene-specific tomato *Sgt1* VIGS constructs, we identified the two tomato *Sgt1* genes, *Sgt1-1* and *Sgt1-2*. *Sgt1-1* sequence information was available in The Institute of Genomic Research database but no information existed about *Sgt1-2* in public databases. We cloned the full-length cDNA of *Sgt1-2* using RT-PCR with OYL538 and OYL1091 primers. The tomato *Sgt1-2* has 89% nucleotide sequence identity with tomato *Sgt1-1*. The predicted tomato SGT1-2 (*SISGT1-2*) protein encodes 369 amino acids and shares 97% identity with *NbSGT1*, 89% with *SISGT1-1*, 90% with *SISGT1*, 88% with *CaSGT1*, 64% with *HvSGT1*, 65% with *OsSGT1*, 63% with *AtSGT1a*, and 64% with *AtSGT1b* (Fig. 2).

Silencing *Sgt1-1* and *Sgt1-2*

We developed *Sgt1-1* and *Sgt1-2* gene-specific TRV-VIGS constructs, TRV-*SISgt1-1* and TRV-*SISgt1-2*, and used them in VIGS in *Mi-1* tomato. The TRV-*SISgt1-1* and TRV-*SISgt1-2* constructs have a maximum of 16 nucleotides identity stretches with *SISgt1-2* and *SISgt1-1*, respectively. Eight days after agroinfiltration, we noticed that plants infiltrated with TRV-*SISgt1-1* construct were unhealthy and developed brown lesions on stems and the crown area. Soon after, these plants started to die (data not shown). In contrast, plants agroinfiltrated with TRV-*SISgt1-2* construct were healthy and no plant died from this treatment (data not shown). A possible explanation for the plant death with TRV-*SISgt1-1* construct could be silencing both *Sgt1-1* and *Sgt1-2* genes, similar to the lethality observed in the Arabidopsis *sgt1a;sgt1b* double mutant. To confirm that the gene-specific TRV-*SISgt1-1* construct silenced only *Sgt1-1*, and not *Sgt1-2*, we assessed *Sgt1-1* and *Sgt1-2* transcript levels in TRV-only and TRV-*SISgt1-1*-silenced plants. The relative abundance of *Sgt1-1* and *Sgt1-2* transcripts was determined using semiquantitative RT-PCR and gene-specific primers (Table I). Reduction in the *Sgt1-1* transcript level was observed in TRV-*SISgt1-1*-silenced plants compared to TRV-only treated plants (Fig. 3A). No reduction in the relative abundance of *Sgt1-2* transcript was observed in these plants (Fig. 3A). These analyses also indicated that the *Sgt1-2* transcripts were less abundant compared to *Sgt1-1* transcripts in TRV-only control plants. Similarly, TRV-*SISgt1-2*-silenced plants showed reduction in the relative abundance of *Sgt1-2* transcript and not in *Sgt1-1* transcript levels (Fig. 3B).

The limited differences between the two *Sgt1* genes compelled us to design gene-specific primers for *Sgt1-1* and *Sgt1-2* that resulted in similar size RT-PCR amplification products (Table I). To confirm the identity of the amplified products, the RT-PCR products from both gene-specific primers were cloned and sequenced. Sequence information indicated that the gene-specific primers indeed amplified the expected transcripts (data not shown).

To test the effect of TRV-*NbSgt1* in silencing the tomato *Sgt1* genes, we used the existing TRV-*NbSgt1* construct

(Liu et al., 2002c). We agroinfiltrated tomato seedlings with the three constructs TRV-*NbSgt1*, TRV-*SISgt1-1*, and TRV-*SISgt1-2*, and monitored plant growth and survival. At day 10, seedlings agroinfiltrated with TRV-*SISgt1-1* showed clear symptoms of reduced plant health compared to those treated with TRV-*SISgt1-2* and TRV-*NbSgt1* (Fig. 4). At day 15, plants agroinfiltrated with TRV-*SISgt1-1* started to die (Fig. 4). At this time, seedlings agroinfiltrated with TRV-*NbSgt1* began to develop necrotic lesions on stems and the crown area while plants agroinfiltrated with TRV-*SISgt1-2* remained healthy. At day 21, most of the plants agroinfiltrated with TRV-*SISgt1-1* were dead and by the end of the experiment at day 25, all plants were dead (Fig. 4; data not shown). Also at day 21, a number of

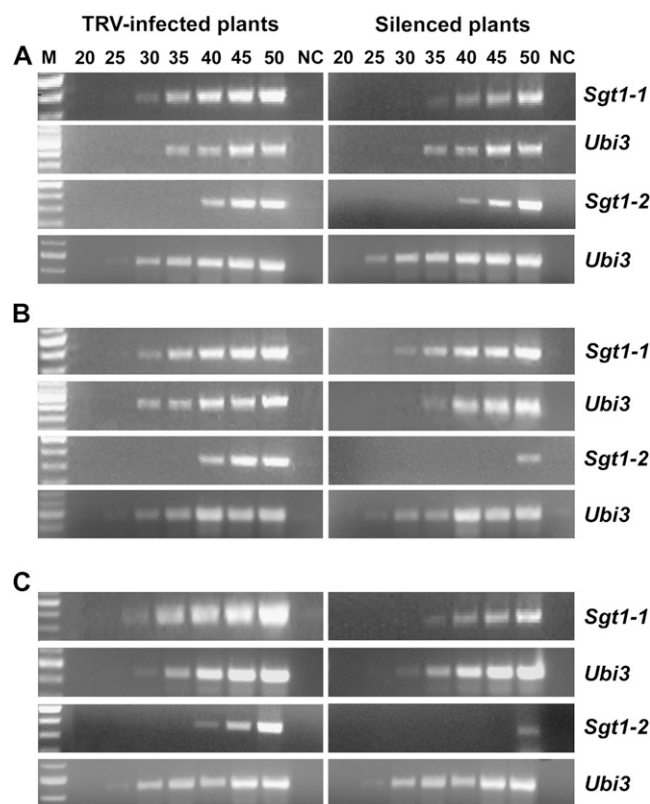


Figure 3. Effect of TRV-VIGS on transcript levels of *Sgt1-1* and *Sgt1-2* in control and silenced 'Motelle' (*Mi-1/Mi-1*) tomato. Plants were agroinfiltrated with pTRV1 plus pTRV2 empty vector (TRV) or with TRV-*SISgt1-1*, TRV-*SISgt1-2*, or TRV-*NbSgt1* constructs. Two weeks after agroinfiltration, leaflets were used for RNA extraction and RT-PCR. Ethidium bromide stained 1.5% agarose gels with RT-PCR products. For each VIGS construct, PCR amplification from cDNA from a single representative sample is presented. For gene-specific amplification, the primers listed in Table I were used. Amplification of the tomato ubiquitin *Ubi3* gene was used as an internal control for equal cDNA use from control and silenced plants. PCR cycles are indicated on the top of the sections. Lane M indicates DNA ladder and NC indicates negative control where RNA was used as template in the absence of reverse transcriptase. A, Leaflets of plants agroinfiltrated with TRV-*SISgt1-1*. B, Leaflets of plants agroinfiltrated with TRV-*SISgt1-2*. C, Leaflets of plants agroinfiltrated with TRV-*NbSgt1*.

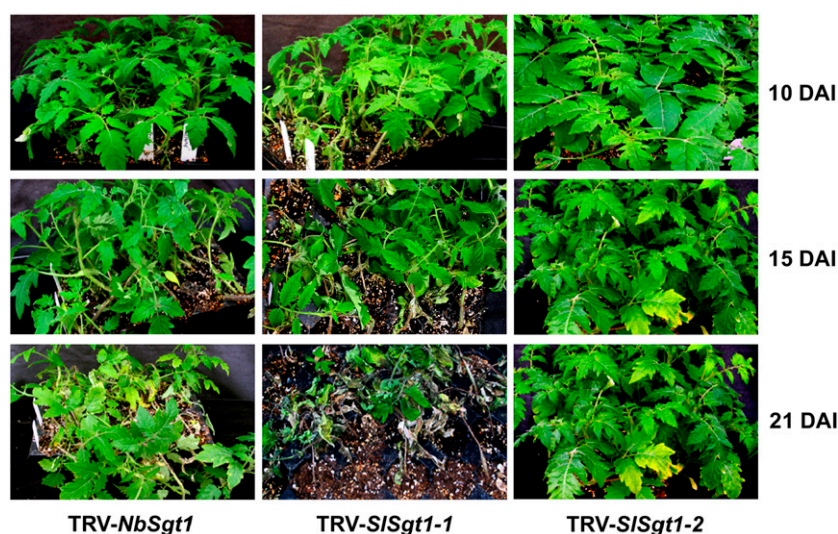


Figure 4. Phenotype of 'Motelle' (*Mi-1/Mi-1*) tomato plants agroinfiltrated with TRV-*NbSgt1*, TRV-*SISgt1-1*, or TRV-*SISgt1-2* constructs. Photographs were taken at the indicated days after treatment (DAI).

plants agroinfiltrated with TRV-*NbSgt1* started to die but 15% to 25% of the plants remained alive at the end of the experiment in spite of the presence of necrotic lesions on their stems. No adverse effects were observed in plants agroinfiltrated with TRV-*SISgt1-2* throughout the experiment (Fig. 4).

Assessing the transcript levels of *Sgt1-1* and *Sgt1-2* in surviving TRV-*NbSgt1* infiltrated plants indicated a reduction in the relative abundance of both *Sgt1-1* and *Sgt1-2* transcripts (Fig. 3C).

Evaluation of *Mi-1*-Mediated Resistance in *Sgt1*-Silenced Plants

To assess the role of *Sgt1* in *Mi-1*-mediated resistance, we used plants agroinfiltrated with TRV-*SISgt1-2* and TRV-*NbSgt1* in aphid and nematode assays. In the aphid assays, insect survival was also monitored on 'Motelle' and 'Moneymaker' control plants agroinfiltrated with TRV. Two weeks after aphid exposure, all aphids were dead on leaflets of 'Motelle' plants agroinfiltrated with TRV and the TRV-*SISgt1-2* VIGS construct (Fig. 5, A and B). In contrast, aphids were alive on leaflets of 'Motelle' agroinfiltrated with TRV-*NbSgt1* and on 'Moneymaker' agroinfiltrated with TRV (Fig. 5, A and B).

Nematode assays with 'Motelle' plants agroinfiltrated with TRV, TRV-*SISgt1-2*, and TRV-*NbSgt1* VIGS constructs resulted in no nematode infection and development (Fig. 6A).

Silencing of *Rar1* and *Hsp90*

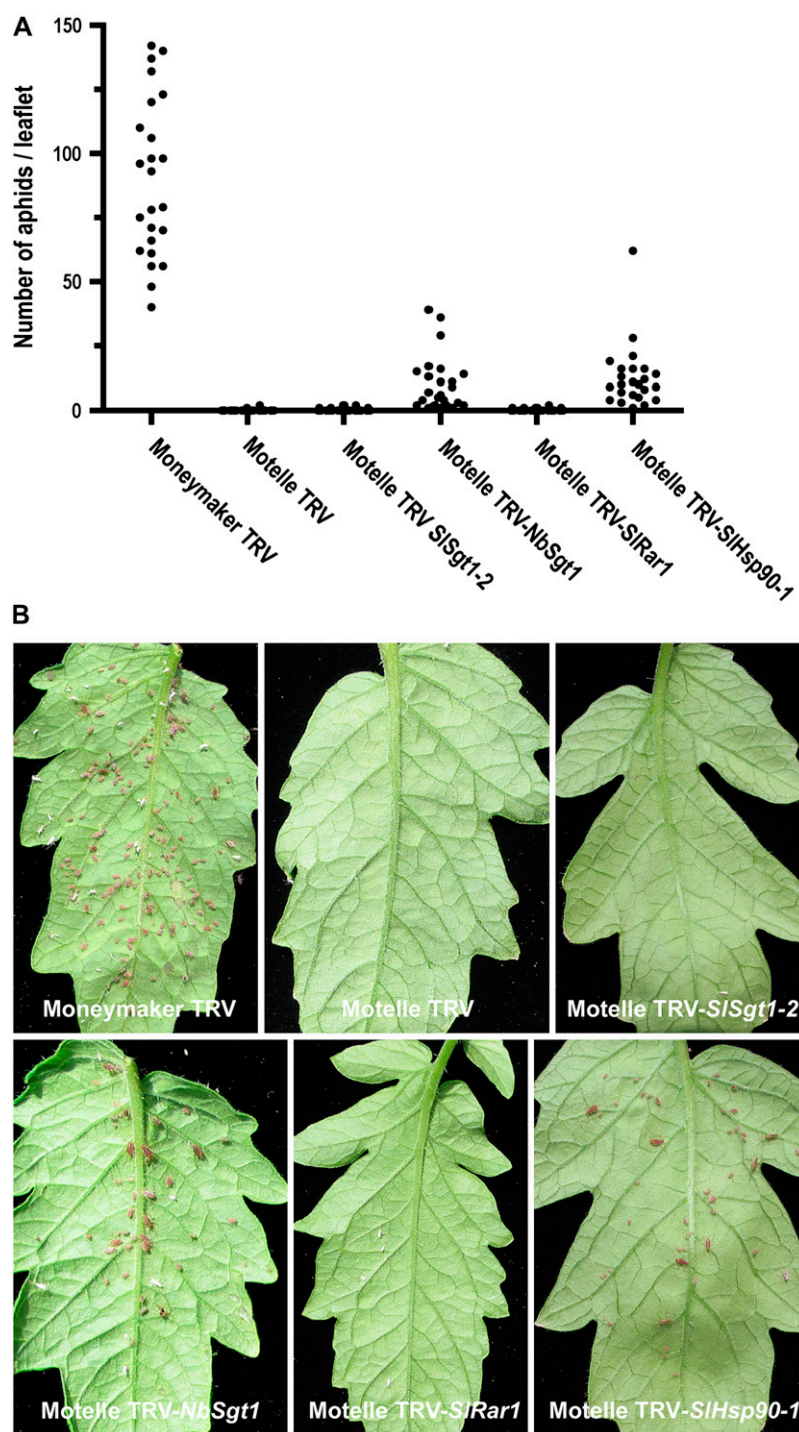
Since RAR1 interacts with SGT1 and is sometimes required for *R*-mediated resistance, we evaluated the role of *Rar1* in *Mi-1*-mediated aphid and nematode resistance. We developed a tomato *Rar1* TRV construct, TRV-*SlRar1*, and used it in VIGS. No aphids survived on 'Motelle' plants agroinfiltrated with the TRV-*SlRar1* construct (Fig. 5, A and B), although RT-PCR results demonstrated that *Rar1* transcripts were less abundant

in TRV-*SlRar1* leaflets compared to TRV 'Motelle' control leaflets (Fig. 7A). Similarly, root-knot nematodes were not able to infect and reproduce on TRV-*SlRar1* plants, indicating no attenuation in *Mi-1*-mediated resistance (Fig. 6A). We also used a TRV-*NtRar1* construct in VIGS in 'Motelle' tomato with similar results.

The molecular chaperon HSP90 is required for *R*-mediated resistance and interacts with RAR1 and SGT1 as well as R proteins. We therefore evaluated the role of *Hsp90* in *Mi-1*-mediated resistance. Both aphid and nematode assays with 'Motelle' plants infiltrated with TRV-*SlHsp90-1* VIGS construct resulted in attenuation of *Mi-1*-mediated resistance. Aphids survived on leaflets from the genetically resistant 'Motelle' plants agroinfiltrated with the TRV-*SlHsp90-1* VIGS construct (Fig. 5, A and B). Similarly, root-knot nematodes were able to penetrate, develop, and deposit egg masses on 'Motelle' roots agroinfiltrated with TRV-*SlHsp90-1* (Fig. 6, A and B).

The TRV-*SlHsp90-1* construct used has regions of 21 to 28 nucleotide stretches with perfect sequence identity with *Hsp90-2*, also known as *Hsp80* (Koning et al., 1992; Liu et al., 2004). This sequence identity might allow *Hsp90-2* silencing by the TRV-*SlHsp90-1* construct. To evaluate the effect of TRV-*SlHsp90-1* VIGS construct on transcript abundance of both *SlHsp90-1* and *SlHsp90-2*, we designed gene-specific primers for each and evaluated their transcript abundance in TRV-*SlHsp90-1* agroinfiltrated 'Motelle' leaflets harboring aphids and roots with nematode egg masses. Our results indicated that *Hsp90-1* transcripts were less abundant in 'Motelle' leaflets supporting aphid growth compared to transcript levels in control leaflets (Fig. 7B). No change in abundance of *Hsp90-2* transcripts was observed in these TRV-*SlHsp90-1* agroinfiltrated 'Motelle' leaflets harboring aphids (Fig. 7B). Similarly, in 'Motelle' root portions with egg masses *Hsp90-1* transcripts were less abundant compared to control roots with no egg masses (Fig. 7C). No change in *Hsp90-2* transcript abundance was detected in these roots (Fig. 7C).

Figure 5. Aphid survival on ‘Motelle’ (*Mi-1/Mi-1*) tomato leaflets silenced for *Sgt1*, *Rar1*, and *Hsp90*. Plants were agroinfiltrated with pTRV1 plus pTRV2 empty vector (TRV) or with TRV-*SlSgt1-2*, TRV-*NbSgt1*, TRV-*SlRar1*, or TRV-*SlHsp90-1* constructs used for silencing. Aphid infestations were performed using leaf cages. A, Aphid survival on leaflets agroinfiltrated with the indicated constructs. Dots represent number of aphids (adult and nymphs) on a single infested leaflet. For each construct, six plants were used in each experiment and four leaflets per plant were assayed with aphids. For each construct, three independent experiments were performed all of which gave similar results. B, Phenotype of leaflets used in VIGS experiments. Photographs were taken 7 to 10 d after aphid infestations and representative leaflets are shown.



Sgt1-1 and *Hsp90-1* Expression after Aphid Infestation in *Mi-1* Tomato

Our results indicate that *Sgt1* and *Hsp90-1* are required for *Mi-1*-mediated aphid resistance (Figs. 5A and 6A). To determine whether *Sgt1-1* and *Hsp90-1* transcripts are induced after aphid infestation, we examined *Sgt1-1* and *Hsp90-1* transcript levels using RT-PCR. No change in abundance of *Sgt1-1* and *Hsp90-1* transcripts

was observed in ‘Motelle’ tomato leaflets after 6, 12, 24, and 48 h infestation with potato aphids (Fig. 8).

DISCUSSION

Our results indicated that the pTRV vector could be used to efficiently silence genes in tomato roots. Using the TRV-*Mi* VIGS construct we were able to completely

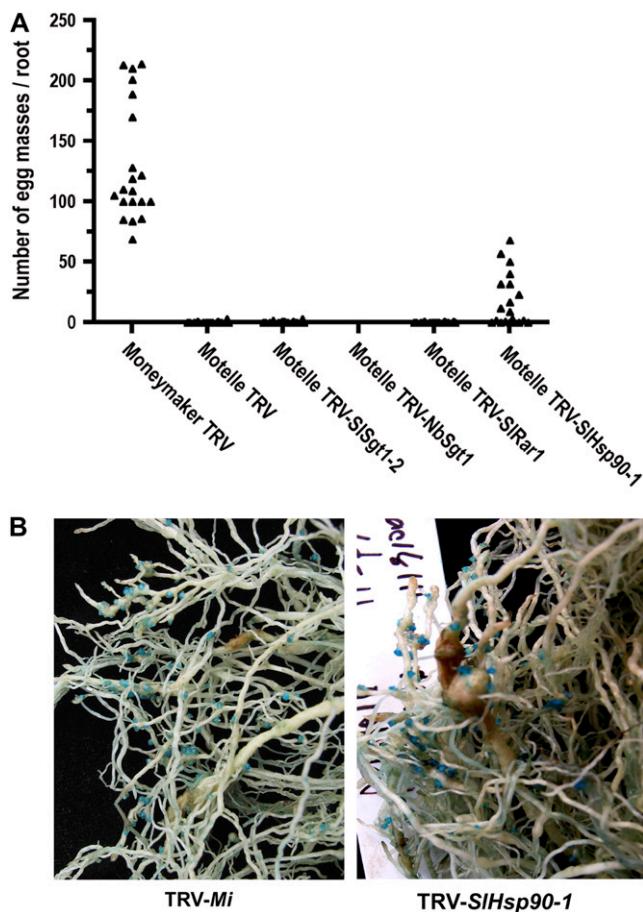


Figure 6. Root-knot nematode reproduction in tomato roots silenced for *Sgt1*, *Rar1*, or *Hsp90* and controls. Plants were agroinfiltrated with pTRV1 plus pTRV2 empty vector (TRV) or with TRV-*SlSgt1-2*, TRV-*NbSgt1*, TRV-*SlRar1*, or TRV-*SlHsp90-1* constructs used for silencing. Four weeks after infiltration, plants were infected with root-knot nematodes. A, Nematode reproduction on control plants and plants used in VIGS with the indicated constructs. Triangles represent the number of egg masses on a single root system. Twenty plants per genotype treatment were used in each experiment. Ten weeks after inoculation, plants were evaluated by counting the number of egg masses on each root. Three or four independent experiments were performed all of which gave similar results. Data from one experiment are presented. B, Phenotype of roots silenced for *Mi-1* (TRV-*Mi*) or *Hsp90-1* (TRV-*SlHsp90-1*). Blue dots are stained root-knot nematode egg masses.

abolish *Mi-1*-mediated root-knot nematode resistance and obtain comparable numbers of egg masses on resistant 'Motelle' as on the near isogenic susceptible 'MoneyMaker.' To achieve the complete susceptible phenotype in TRV-*Mi* agroinfiltrated 'Motelle' plants, high levels of nematode inoculum were necessary. Although elimination of *Mi-1* resistance was achieved, the genetically resistant 'Motelle' roots silenced for *Mi-1* exhibited a range of disease severity, measured as the number of nematode egg masses. This variation in nematode infection suggests that VIGS in tomato roots is not uniform. VIGS is known to result in variable silencing within a plant and between plants. To overcome

these variations, larger numbers of plants are needed in VIGS experiments addressing phenotypes in roots. Nevertheless our experiments indicate that pTRV can be used to identify *R*-gene signaling components in roots.

Similar to Arabidopsis, tomato also has two *Sgt1* genes, *Sgt1-1* (*SlSgt1-1*) and *Sgt1-2* (*SlSgt1-2*). The tomato *Sgt1-2* is more closely related to *NbSgt1* than tomato *Sgt1-1*. In addition, *Sgt1-2* transcripts are less abundant than *Sgt1-1* transcripts, which may explain the reason for the absence of the sequences of this gene in the public databases. Our data also indicates distinct roles for *Sgt1-1* and *Sgt1-2*. The lethal phenotype obtained by silencing *Sgt1-1* indicates an essential role for *Sgt1-1* in tomato. The Arabidopsis *Sgt1* genes appear to have evolved differently than the tomato orthologs. Although mutations in either *SGT1a* or *SGT1b* are not lethal, the *sgt1a;sgt1b* double mutant is lethal, suggesting redundant but essential roles for both these genes. A lethal phenotype has not been observed by silencing *Sgt1* using VIGS in *Nicotiana benthamiana* (Liu et al., 2002c) and barley (Scofield et al., 2005), suggesting that *Sgt1* does not play an essential role in these plants. Alternatively, more than one *Sgt1* gene exists in

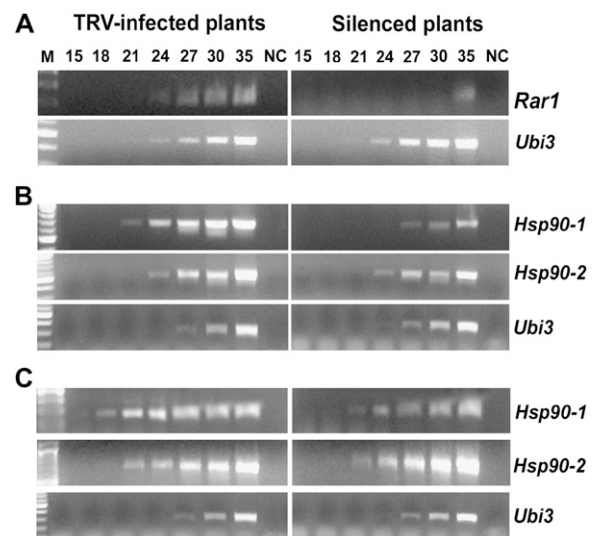


Figure 7. Effect of TRV-VIGS on transcript levels of *Rar1* and *Hsp90* in control and silenced 'Motelle' (*Mi-1/Mi-1*) tomato. Plants were agroinfiltrated with pTRV1 plus pTRV2 empty vector (TRV) or with TRV-*SlRar1* or TRV-*SlHsp90-1* constructs used for silencing. Ethidium bromide stained 1.5% agarose gels with RT-PCR products. cDNA was synthesized from total RNA isolated from leaflets or roots from plants agroinfiltrated with TRV or TRV containing the indicated constructs. PCR amplification from cDNA from a single representative sample is presented. A, *Rar1* expression in leaflets of plants agroinfiltrated with TRV or TRV-*SlRar1*. Gene-specific expression of *Hsp90* genes in plants agroinfiltrated with TRV or TRV-*SlHsp90-1* in leaflets supporting aphid growth (B) or in roots with egg masses (C). For gene-specific amplification, primers listed in Table I were used. Amplification of the tomato ubiquitin *Ubi3* gene was used as an internal control for equal cDNA use from control and silenced plants. PCR cycles are indicated on the top of the sections. Lane M indicates DNA ladder and NC indicates negative control where RNA was used as template in the absence of reverse transcriptase.

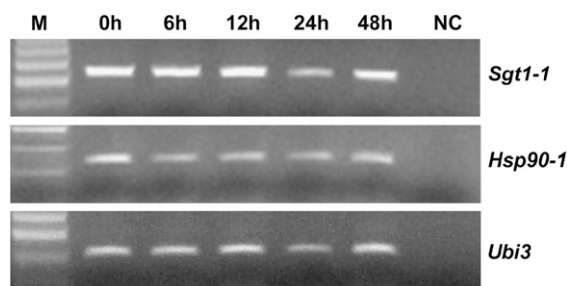


Figure 8. Expression of *Sgt1-1* and *Hsp90-1* in 'Motelle' (*Mi-1/Mi-1*) tomato leaflets after aphid infestation. Ethidium bromide stained 1.5% agarose gels with RT-PCR products. Leaflets were infested using leaf cages and tissue was harvested at 0, 6, 12, 24, and 48 h after aphid infestation. Total RNA was reverse transcribed and first-strand cDNAs were used in 20 cycles of PCR with gene-specific primers listed in Table I. Amplification of the tomato ubiquitin *Ubi3* gene was used as an internal control for equal cDNA use.

these species and the VIGS constructs target transcripts from only one member for degradation.

The generation of stable mutants in tomato is time consuming and requires significant resources. VIGS not only provided a fast and effective means to generate loss-of-function phenotypes, but also allowed us to identify the role of an essential gene like *Sgt1* in *Mi-1*-mediated aphid resistance. Although no plants agroinfiltrated with the TRV-*SlSgt1-1* construct survived in our experiments, a number of plants agroinfiltrated with the TRV-*NbSgt1* construct did. The longest stretch of nucleotide identity that the TRV-*NbSgt1* construct has with tomato *Sgt1-1* is 55 bases and 150 bases with *Sgt1-2*, indicating that this construct is able to silence both genes. Indeed, RT-PCR results demonstrated that the abundance of both transcripts was lower in TRV-*NbSgt1* agroinfiltrated plants compared to the TRV control, indicating that both genes were targeted. However, the TRV-*SlSgt1-1* construct must have been more efficient in silencing *Sgt1-1* than the TRV-*NbSgt1* construct. This is demonstrated by the fact that the initial symptoms of lethality using the TRV-*SlSgt1-1* construct were very fast, within 10 d, compared to TRV-*NbSgt1*, within 14 d.

Silencing *Sgt1-2* did not result in attenuation in *Mi-1*-mediated resistance, suggesting no role for *Sgt1-2* in this pathway. Alternatively, *Sgt1-1* and *Sgt1-2* play redundant roles in resistance and silencing both genes is required for the attenuation of *Mi-1* resistance as suggested by the *NbSgt1* VIGS experiments. Since silencing *Sgt1-1* results in lethality, we cannot conclusively determine whether *Sgt1-1* alone is required for *Mi-1* resistance. In Arabidopsis, *Sgt1b* is required for the function of only a subset of *R* genes and no role for *Sgt1a* has been identified. If *Sgt1* is universally required for the function of the major classes of plant *R* genes then it is possible that *Sgt1a* and *Sgt1b* or *Sgt1-1* and *Sgt1-2* have redundant roles in some *R*-mediated resistances (Muskett and Parker, 2003).

Although TRV-*NbSgt1* agroinfiltrated plants resulted in attenuation of *Mi-1*-mediated potato aphid re-

sistance, these plants were not altered in the *Mi-1*-mediated root-knot nematode resistance. The lack of nematode development on the TRV-*NbSgt1* agroinfiltrated roots maybe the result of inefficient silencing of *Sgt1-1* combined with inefficient VIGS in roots compared to leaves. TRV VIGS is not as efficient in roots as it is in above-ground parts of plants (Valentine et al., 2004; I. Kaloshian, unpublished data). Even in samples showing attenuation of *Mi-1* resistance, a reduction in transcript levels was not consistently detected when the entire root system was used for RNA extraction and transcript evaluation. Reduction of transcript levels was detected consistently in portions of the root system with nematode egg masses. In the absence of nematode infection and development, it is impossible to know which root portion to target for RNA isolation and transcript evaluation. Therefore, portions of roots containing nematode egg masses were used in all root RT-PCR evaluations of transcript levels in silenced plants. Consequently, inefficient silencing could be one of the reasons for the lack of resistance attenuation phenotype in our experiments. Therefore, the lack of root-knot nematode infection on TRV-*NbSgt1* agroinfiltrated plants does not necessarily indicate the lack of a role for *Sgt1* in *Mi-1*-mediated nematode resistance. For these reasons, it is likely that *Sgt1* is required for *Mi-1*-mediated resistance to both aphids and nematodes.

The lack of aphid and nematode growth and multiplication on *Rar1*-silenced plants indicates no role for

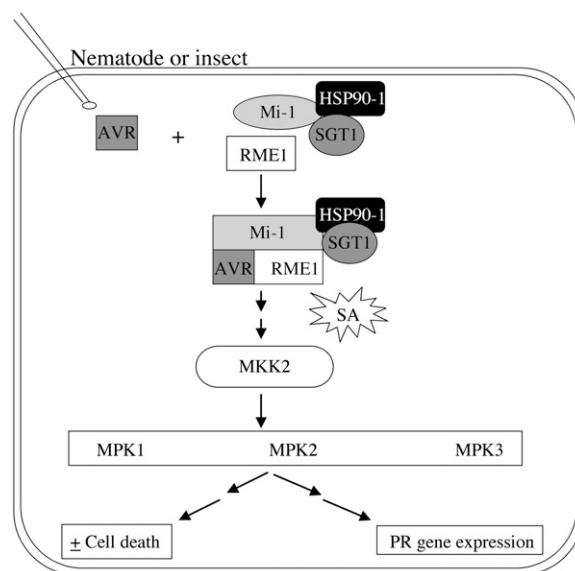


Figure 9. A model for *Mi-1* signal transduction pathway. The interactions in this model are based on other plant NBS-LRR *R* proteins. *Mi-1*, *HSP90-1*, and *SGT1* form a *R*-signaling complex. *Mi-1* guards *RME1*, which possibly represents the host target for the nematode and insect *Avr* determinant(s). The animal *Avr* determinant modifies *RME1* and this modification is detected by *Mi-1*, which causes a conformational change in *Mi-1* and activates *R*-signaling pathway. SA and MAPK cascade(s) follows, resulting in activation of *PR* genes and presence or absence of cell death in root-knot nematodes and potato aphid interactions, respectively.

this gene in *Mi-1*-mediated resistance. The lack of a role for *Rar1* in *Mi-1* resistance is probably not because of inefficient silencing, since two distinct TRV constructs, TRV-*SlRar1* and TRV-*NtRar1*, gave similar results. In addition, *Rar1* transcript levels were lower in TRV-*SlRar1* agroinfiltrated leaflets compared to TRV control leaflets. Since VIGS does not eliminate all the targeted gene transcripts, it is possible that the presence of any level of transcripts, and consequently the protein, facilitates the function of RAR1. Therefore, our experiments cannot conclusively exclude a role for *Rar1* in *Mi-1* resistance. However, the absence of a role for *Rar1* in other NBS-LRR *R*-gene-mediated resistances have been previously reported using stable mutants, which indicates a differential requirement for *Rar1* in *R* signaling (Warren et al., 1999; Azevedo et al., 2002; Tornero et al., 2002; Leister et al., 2005).

Our results indicate that like many *R* genes, *Mi-1* function also requires the chaperon *Hsp90-1*. HSP90 and other heat shock proteins play roles in proper folding of peptides, degradation of misfolded peptides, and regulating of signal pathways (Picard, 2002; Pratt and Toft, 2003). Unlike plants silenced for both *Sgt1-1* and *Sgt1-2*, resistant 'Motelle' plants silenced for *Hsp90-1* were compromised in both aphid and nematode resistance. The attenuation of root-knot nematode resistance suggests that either *Hsp90-1* is silenced more efficiently than *Sgt1-1* and *Sgt1-2* or the threshold level for HSP90-1 required for *Mi-1* resistance to root-knot nematodes is lower than that for SGT1-1 and SGT1-2. Silencing *Hsp90-1* did not result in complete attenuation of *Mi-1*-mediated resistance to both pests. A higher nematode infection rate was observed on 'Motelle' roots silenced for *Mi-1* compared to roots silenced for *Hsp90-1*. Earlier, we demonstrated that silencing *Mi-1* using TRV VIGS also resulted in complete attenuation of aphid resistance (Li et al., 2006). However, silencing either *Hsp90-1* or *Sgt1-1* and *Sgt1-2* only partially attenuated *Mi-1*-mediated aphid resistance. These observations are consistent with previous findings that HSP90 and SGT1 contribute quantitatively to the function of various NBS-LRR *R* proteins (Austin et al., 2002; Hubert et al., 2003).

The TRV-*SlHsp90-1* construct used in our experiments selectively silenced *Hsp90-1* and not *Hsp90-2*. Although this construct has a noteworthy nucleotide identity (up to 28 nucleotide stretches with 100% identity) with *Hsp90-2*, it does not appear to reduce *Hsp90-2* transcript levels. Although 23 nucleotides identity to a targeted gene is sufficient to initiate VIGS, other reports have also indicated the requirement for longer stretches of nucleotide identity for silencing to occur (Thomas et al., 2001; Ekengren et al., 2003).

In summary, results in this report have identified new components of *Mi-1*-mediated resistance to aphids and nematodes. Previous information has demonstrated that intramolecular interaction of Mi-1 protein is important for regulation of HR signaling (Hwang et al., 2000; Azevedo et al., 2002; Hwang and Williamson, 2003). In addition, Mi-1 binds and hydro-

lyzes ATP (Tameling et al., 2002). Based on this and other information, a model for *Mi-1* signal transduction is presented (Fig. 9). The model also takes into account information from other NBS-LRR proteins. In this model the Mi-1 signaling complex, which includes HSP90-1 and SGT1 (representing SGT1-1 and SGT1-2), guards RME1. Upon detection of modification(s) to RME1 by the animal Avr determinant(s), ATP binds to the Mi-1 NBS domain and ATP hydrolysis assists in generating a conformational change in Mi-1, which in turn activates defense responses. Alternatively, in an inactive form, the Mi-1 C-terminal LRR domain is bound to its NBS domain. Upon detection of RME1 modifications by the animal Avr determinants, ATP binding and hydrolysis activates Mi-1, which recruits HSP90-1 and SGT1 to form a signalosome. Downstream signals include SA and MAPK cascades and activation of PR proteins.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Tomato (*Solanum lycopersicum*) 'UC82B' (*mi-1/mi-1*) and near isogenic lines 'Motelle' (*Mi-1/Mi-1*) and 'Moneymaker' (*mi-1/mi-1*) were used. Seeds were sown in seedling trays in organic planting mix, supplemented with Osmocote (17-6-10; Sierra Chemical Company), and maintained in a mist room. After germination, seedlings were transferred to plant growth chambers and maintained at 24°C and with 16-h-light and 8-h-dark photoperiod and 200 $\mu\text{mol m}^{-2} \text{s}^{-1}$ light intensity unless otherwise stated. Two to 3 weeks after germination, seedlings with a pair of newly emerged leaves were used in VIGS and maintained at 19°C. Ten days later, seedlings used in pest assays were transplanted into plastic cups (10-cm diameter, 17-cm deep) filled with University of California mix II or sand and maintained at 19°C until bioassay. Plants were supplemented with Osmocote and fertilized biweekly with Tomato MiracleGro (18-18-21; Stern's MiracleGro Products).

Cloning of *Sgt1-2* and Sequence Analysis

Tomato *Sgt1-2* was PCR amplified using tomato VF36 cDNA as template and primers OYL538 (5'-GGGGACAAAGTTGTACAAAAAGCAGGCTCTACCATGGCGTCCGATCTGGAGACTAG-3') and OYL1091 (5'-GGGGACACATTTGTACAAAGAAAGCTGGGTCTAGATCTCCCATTTCTTCAGTCAT-3'), and was recombined into pDONR201 through GATEWAY BP reaction (Invitrogen). This primer set amplified both *Sgt1-1* and *Sgt1-2*. We identified *Sgt1-2* by sequence comparison with tomato *Sgt1-1*. The full-length cDNA sequence was deposited in GenBank as accession number EF011105. Alignment of predicted protein sequences was performed using ClustalX (Thompson et al., 1997) and edited manually using GENEDOC (<http://www.psc.edu/biomed/genedoc>).

RNA Isolation and RT-PCR

Total RNA was extracted either as in Li et al. (2006) or using Trizol (Invitrogen). Five micrograms of total RNA were treated with *RNAse-free DNase I* (Promega). *DNase I* was removed by phenol/chloroform extraction and cDNAs were synthesized using a 0.5 μg oligo(dT)₁₈ primer and Superscript II reverse transcriptase (Invitrogen Life Technologies Co.) to a final volume of 20 μL . For PCR experiments, the tomato ubiquitin *Ubi3* gene transcripts were amplified as an internal control for equal cDNA use from control and silenced plants as described in Li et al. (2006). Except for *Sgt1-2*, PCR was performed in 15 μL volume using 0.8 μL cDNA, denaturation at 94°C for 45 s, annealing at 60°C for 45 s, and elongation at 72°C for 1 min. For *Sgt1-2* evaluation, two independent RT reactions were performed from a single RNA template as described above. The cDNAs from the two reactions were pooled before further use. For PCR, 2.0 μL cDNA was used in 15 μL volume for *Sgt1-2* amplifications and 1.5 μL cDNA was used for the *Ubi3*

control amplifications. The number of cycles used for each transcript is indicated on the gel figures. To confirm lack of genomic DNA contamination, 200 ng of *DNase* I-treated RNA was also used as template. The amplified products were analyzed on 1.5% (w/v) agarose gels. When needed, amplification products were purified using the Concert Purification system (Gibco BRL) and ligated into a pGEM-T-Easy vector (Promega). Purified recombinant plasmids were sequenced.

For the time-course expression studies, 5 µg of total RNA isolated from leaflets from each time point was reverse transcribed as described above, and 5 µL of the first-strand cDNAs was used in PCR for 20 cycles.

VIGS Constructs

The TRV-*Mi* construct used in this work was described earlier (Li et al., 2006). The TRV-VIGS constructs for tomato *SlHsp90-1*, tobacco (*Nicotiana tabacum*) *NtRar1*, and *Nicotiana benthamiana* *NbSgt1* were also described earlier (Liu et al., 2002b, 2002c, 2004).

To obtain a tomato *Rar1* TRV-VIGS construct, a 407 bp fragment was amplified with primers *Rar1*-SF (5'-ACGAATTCCTGGGTGAAGACAG-GAAAGCAC-3') and *Rar1*-SR (5'-ACGGATCCTTTCATCCGGTCATGGAA-GATAG-3') using tomato EST clone cLET23B21. Primers *Rar1*-SF and *Rar1*-SR introduced *EcoRI* and *Bam*HI restriction sites at the 5' and 3' ends of the amplified fragment, respectively. The PCR product was restricted with *EcoRI* and *Bam*HI and inserted into the same site of TRV vector pYL156, resulting in TRV-*Rar1* construct.

For silencing *Sgt1-1*, a fragment of 195 bp was amplified with the primers SGT1-1F2 (5'-ACGAATTCAGAATACCAAACTGC-3') and SGT1-1R (5'-ACG-GATCCCAAC ATTGTCGGTA-3') using tomato leaf cDNA as template. Primers SGT1-1F2 and SGT1-1R introduced *EcoRI* and *Bam*HI restriction sites at the 5' and 3' ends of the amplified fragment, respectively. The PCR product was restricted with *EcoRI* and *Bam*HI and inserted into the same site of TRV vector pYL156, resulting in TRV-*Sgt1-1* construct.

TRV-*Sgt1-2* gene-specific clone was constructed in two steps using two sets of nested primers. First, a 385 bp fragment was amplified with primers SGT1-2CF (5'-GACCTTTACACTCAAGCCATAGCCAT-3') and SGT1-2CR (5'-CGC-AACAGCAACATTGTCCAAAG-3') using tomato leaf cDNA as template. The resulting fragment was cloned into the pGEM-T-Easy vector resulting in plasmid KB100 (Promega). Plasmid KB100 was sequenced to confirm the identity of the clone. A 164 bp subclone fragment was subsequently amplified using primers SGT1-2VF (5'-ACGAATTCGAGTACCAAACTGCAAAAGC-AGC-3'), SGT1-2VR (5'-ACGGATCCGCCGTAACGACATTTCCCGAGG-3'), and pKB100 as template. Primers SGT1-2VF and SGT1-2VR introduced *EcoRI* and *Bam*HI restriction sites at the 5' and 3' ends of the amplified fragment, respectively. The PCR product was restricted with *EcoRI* and *Bam*HI and inserted into the same site of TRV vector pYL156, resulting in TRV-*Sgt1-2* construct. The identity of all clones in pYL156 plasmid was confirmed by sequencing. All TRV-VIGS clones were transformed into *Agrobacterium tumefaciens* strain GV3101.

Agrobacterium-Mediated Virus Infection

Cultures of *A. tumefaciens* strain GV3101 containing each of the constructs derived from pTRV2, empty vector control, and pTRV1 were grown as described earlier (Li et al., 2006). *Agrobacterium* cultures were pelleted, resuspended in infiltration buffer, and adjusted to an O.D.₆₀₀ of 1.0. Cells were incubated at room temperature for 3 h before use. Equal volume of pTRV1 *Agrobacterium* culture was mixed with one of the pTRV2 cultures before infiltration.

Leaflets of two to 3-week-old seedlings were infiltrated with *Agrobacterium* cultures (agroinfiltration) using a 1-mL needleless syringe. Plants were maintained at 19°C in a growth chamber.

Nematode Culture and Nematode Inoculation

A *Mi-1*-avirulent culture of the parthenogenetic *Meloidogyne javanica* (VW4) was maintained on susceptible tomato 'UC82B' in a greenhouse. Root-knot nematode eggs and J2 were obtained as described earlier (Martinez de Ilarduya et al., 2001). Nematodes were collected every 48 h and used immediately or stored at room temperature for an additional 48 h with aeration.

Two to 3 weeks after transplanting agroinfiltrated seedlings, individual plants were inoculated with 10,000 J2 using a modified pipette and maintained at 22°C to 26°C. In each experiment, 18 to 25 plants per construct were infected with nematodes. Eight weeks after inoculation, nematode reproduc-

tion was evaluated by staining roots in 0.001% (w/v) erioglaucine (Sigma-Aldrich). Seedlings were evaluated by counting the egg masses on individual root systems. For each construct, nematode assays were performed three or four times.

Aphid Colony and Bioassay

A *Mi-1*-avirulent colony of the parthenogenetic potato aphid (*Meloidogyne euphorbiae*) was maintained on susceptible tomato 'UC82B' (*mi-1/mi-1*) in insect cages in a pesticide-free greenhouse. Individual leaflets of 8- to 9-week-old tomato plants were infested with about 25 apterous (wingless) adults and nymphs of potato aphids using leaf cages as described in Li et al. (2006). Four leaf cages were used per plant and eight to 10 plants were used for each construct. Assays were performed in a pesticide-free greenhouse maintained at temperature ranging between 23°C and 26°C. Ten days after infestation, the number of aphids in each cage was counted. Experiments were performed three times.

Time-Course Aphid Experiment

Thirty apterous adults and nymphs of potato aphids were caged onto a tomato leaflet on the fourth or fifth leaf of 7-week-old tomato plants as described above. Three cages were used per plant and two plants were infested for each time point and tissue pooled. Leaflets were collected at 0, 6, 12, 24, and 48 h after aphid infestation. Cages were removed and leaflets were sprayed with 1% (w/v) SDS to force aphids to withdraw their stylets prior to careful removal using a paintbrush. Tomato leaflets were excised using a razor blade, immediately frozen in liquid nitrogen, and stored at -80°C. Two independent experiments were performed and tissue was pooled before RNA extraction.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number EF011105.

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