The *Mi-1*-Mediated Pest Resistance Requires *Hsp90* and *Sgt1*[^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-S*gt1*-1 resulted in lethality, while silencing *Sgt1*-2 using TRV-S*gt1*-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.
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). Sgc1 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 isofoms 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 Plac-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 feeding is not associated with HR (Martinez de llarduy 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...
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 necessary for Mi-1 function it is not required for Pto or I-1-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.
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 ‘Moneymaker,’ 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 ‘Moneymaker’ 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 (Azavedo et al., 2002). Mutant analysis indicated a role for SGT1b in plant defense (Musket and Parker, 2003). No role for SGT1a has yet been identified. However, sgt1asgt1b double mutation is lethal, suggesting a redundant role for SGT1a and SGT1b (Musket 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

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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 (SI SGT1-2) 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 sgt1asgt1b 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 load. 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.
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-SlSgt1-2 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-SlSgt1-2 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-SlSgt1-2 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-SlSgt1-2, 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).
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...
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 Sgl1 genes, Sgl1-1 (SlSgl1-1) and Sgl1-2 (SlSgl1-2). The tomato Sgl1-2 is more closely related to NbSgt1 than tomato Sgl1-1. In addition, Sgl1-2 transcripts are less abundant than Sgl1-1 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 Sgl1-1 and Sgl1-2. The lethal phenotype obtained by silencing Sgl1-1 indicates an essential role for Sgl1-1 in tomato. The Arabidopsis Sgl1 genes appear to have evolved differently than the tomato orthologs. Although mutations in either SGT1a or SGT1b are not lethal, the sgt1asgt1b double mutant is lethal, suggesting redundant but essential roles for both these genes. A lethal phenotype has not been observed by silencing Sgl1 using VIGS in *Nicotiana benthamiana* (Liu et al., 2002c) and barley (Scofield et al., 2005), suggesting that Sgl1 does not play an essential role in these plants. Alternatively, more than one Sgl1 gene exists in

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**Figure 6.** Root-knot nematode reproduction in tomato roots silenced for Sgl1, Rar1, or Hsp90 and controls. Plants were agroinfiltrated with pTRV1 plus pTRV 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.

**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 pTRV 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. 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.
internal control for equal cDNA use.

I. Amplification of the tomato ubiquitin gene was 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 (Musket and Parker, 2003).

Although TRV-Nbsgt1 agroinfiltrated plants resulted in attenuation of Mi-1-mediated potato aphid resistance, 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 Rtr1-silenced plants indicates no role for

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**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.

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**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-SiRar1 and TRV-NiRar1, gave similar results. In addition, Rar1 transcript levels were lower in TRV-SiRar1 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-SiHsp90-1 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 hydrolyzes 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 signalsome. Downstream signals include SA and MAPK cascades and activation of PR proteins.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Tomato (Solanum lycopersicum) ‘UC202’ (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 μmol m⁻² 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 (Solanum lycopersicum) ‘UC202’ (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 μmol m⁻² 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).

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 Ubih3 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 Ubih3
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., 2002). The TRV-VIGS constructs for tomato STHsp90-1, tobacco (Nicotiana tabacum) NirR1, and Nicotiana benthamiana NRSgt1 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'-AGCGAATTCACGATCGACAG-GAACACAGC-3') and Rar1-SR (5'-AGCGAATTCCTTACCACATCGCATGAA-GATAAC-3') using tomato EST clone cLET25821. Primers Rar1-SF and Rar1-SR introduced EcoRI and BamHI restriction sites at the 5' and 3' ends of the amplified fragment, respectively. The PCR product was restricted with EcoRI and BamHI 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'-AGCGAATTCAGATACCACACTGC-3') and Sgt1-1R (5'-AGCGAATTCACGATCGACAG-GAACACAGC-3') using tomato leaf cDNA as template. Primers Sgt1-1F2 and Sgt1-1R introduced EcoRI and BamHI restriction sites at the 5' and 3' ends of the amplified fragment, respectively. The PCR product was restricted with EcoRI and BamHI 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 Sgt1-2RI and Sgt1-2CR (5'-AGCGAATTCACGATCGACAG-GAACACAGC-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'-AGCGAATTCAGATACCACACTGC-3') and Sgt1-2VR (5'-AGCGAATTCACGATCGACAG-GAACACAGC-3') using tomato leaf cDNA as template. The PCR product was restricted with EcoRI and BamHI 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 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-avrulent culture of the parthenogenetic Meloidogyne javanica (VW4) was maintained on susceptible tomato ‘UC82B’ in a greenhouse. Root-knot nematode egg and J2 were obtained as described earlier (Martínez de Iarduy et al., 2001). Nematodes were collected every 48 h and used immediately or stored at room temperature for an additional 4 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 reproduction was evaluated by staining roots in 0.001% (w/v) eriolugaine (Sigma-Aldrich). Seedlings were evaluated by counting the egg masses on individual root systems. For each construct, nematode assays were performed three to four times.

Aphid Colony and Bioassay

A Mi-1-avrulent colony of the parthenogenetic potato aphid (Meloidogyne euphorbeae) was maintained on susceptible tomato ‘UC82B’ (mi-1/mi-1) in insect cages in a pesticide-free greenhouse. Individual leaflets of 9- to 9-week-old tomato plants were infested with about 25 aphis (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 aphis 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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Hsp90-1 and SGT1 Are Required for Mi-1 Resistance