Salicylic Acid-Dependent Restriction of *Tomato ringspot virus* Spread in Tobacco Is Accompanied by a Hypersensitive Response, Local RNA Silencing, and Moderate Systemic Resistance

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Tomato ringspot virus (ToRSV, a *Nepovirus* sp.) systemically infects many herbaceous plants. Viral RNA accumulates in symptomatic leaves and in young, asymptomatic leaves that emerge late in infection. Here, we show that systemic infection by ToRSV is restricted in tobacco. After an initial hypersensitive response in inoculated leaves, only a few plants showed limited systemic symptoms. Viral RNA did not usually accumulate to detectable levels in asymptomatic leaves. ToRSV-derived small-interfering RNAs and *PR1a* transcripts were only detected in tissues that contained viral RNA, indicating local induction of RNA silencing and salicylic acid (SA)-dependent defense responses. Lesion size and viral systemic spread were reduced with SA pretreatment but enhanced in NahG transgenic lines deficient in SA accumulation, suggesting that SA-dependent mechanisms play a key role in limiting ToRSV spread in tobacco. Restriction of virus infection was enhanced in transgenic lines expressing the P1-HC-Pro suppressor of silencing. Knocking down the SA-inducible RNA-dependent RNA polymerase 1 exacerbated the necrotic reaction but did not affect viral systemic spread. ToRSV-infected tobacco plants were susceptible to reinoculation by ToRSV or *Tobacco mosaic virus*, although a small reduction in lesion size was observed. This moderate systemic resistance suggests inefficient induction or spread of RNA silencing and systemic acquired resistance signal molecules.

Plants mount several layers of defense in response to viral infection, including RNA silencing and specific defense responses directed by dominant resistance genes (Carr et al. 2010; Kachroo et al. 2006; Moffett 2009). The pathways regulating these defense mechanisms are complex and often inter-

connected. RNA silencing targets viral RNAs in a sequence-specific manner (Alvarado and Scholthof 2009; Ding 2010; Mlotshwa et al. 2008; Ruiz-Ferrer and Voinnet 2009). Double-stranded (ds)RNAs present in viral replication intermediates or in secondary structures within the genomic RNAs are cleaved by dicer-like (DCL) enzymes into small-interfering (si)RNAs. One strand of the siRNAs, the so-called guide strand, is incorporated into RNA-induced silencing complexes, and Argonaute (AGO) proteins within these complexes direct specific degradation or translational repression of RNA molecules harboring complementary sequences. Host RNA-dependent RNA polymerases (RDR) also play a role in antiviral defense by amplifying viral RNAs for a second round of siRNA formation. RNA silencing is initially induced at the site of infection and is normally followed by the establishment of a systemic silencing state in which distant tissues are protected from subsequent viral infection. Small RNAs, probably in the form of duplexes, constitute the mobile systemic silencing signal (Dunoyer et al. 2010a and b; Molnar et al. 2010).

Dominant resistance genes directly or indirectly recognize specific viral proteins and trigger a cascade of defense responses (production of reactive oxygen species, cell wall reinforcement, and induction of defense genes, such as those encoding pathogenesis-related [PR] proteins) (Carr et al. 2010; Kachroo et al. 2006; Maule et al. 2007; Moffett 2009). Defense responses at the site of infection are often accompanied by cell death resulting in necrotic lesions. This is generally referred to as the hypersensitive response (HR). Induction of local defense responses is usually followed by a broad-spectrum resistance state in upper, noninoculated leaves termed systemic acquired resistance (SAR). Increasing evidence suggests that the development of necrotic lesions at the site of infection does not directly inhibit pathogen replication or spread and is not required for the development of SAR (Cole et al. 2001; Komatsu et al. 2010; Liu et al. 2010a; Mishina and Zeier 2007; Mur et al. 2008). Salicylic acid (SA) is an important signal molecule of defense responses directed by resistance genes, and a methylated form of SA has been implicated in the establishment of SAR (Park et al. 2007; Singh et al. 2004; Tsuda et al. 2008; Vlot et al. 2009). The role of SA in virus resistance was first established for the interaction between *Tobacco mosaic virus* (TMV) and the tobacco N resistance gene (Gaffney et al. 1993). Tobacco (NN) transgenic lines, in which the level of endogenous SA is reduced due to the expression of the bacterial *salicylate hydroxylase* (*NahG*) gene, inefficiently restrict TMV movement, and establishment of SAR is inhibited (Friedrich et

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al. 1995; Gaffney et al. 1993; Murphy et al. 1999). On the other hand, exogenous treatment of plants with SA results in the induction of an antiviral state similar to SAR (Naylor et al. 1998; Ward et al. 1991). Biosynthesis of SA is also induced (although at lower levels) during some compatible interactions, suggesting that SA-dependent pathways may be involved in basal defense responses (Carr et al. 2010). Finally, virus resistance is influenced by the age of the plant in both compatible and incompatible interactions (Holmes 1938; Ji and Ding 2001; Mas and Pallas 1996; Yalpani et al. 1993). In the case of the tobacco–TMV interaction, this age-dependent resistance was correlated with elevated levels of SA in older leaves (Yalpani et al. 1993).

Several lines of evidence indicate a cross-talk between SA-mediated defense responses and RNA silencing. First, viral silencing suppressors are often virulence factors and symptom determinants (Diaz-Pendon and Ding 2008) and have been shown to either interfere with SA-mediated defense responses (e.g., *Cucumovirus* 2b and *Tobamovirus* replicase proteins) or enhance these responses (e.g., *Potyvirus* HC-Pro protein) (Endres et al. 2010; Ji and Ding, 2001; Lewsey et al. 2010; Pruss et al. 2004; Shams-Bakhsh et al. 2007; Wang et al. 2009a). Second, expression of RDR1, an enzyme implicated in antiviral RNA silencing, is induced by SA (Garcia-Ruiz et al. 2010; Rakhshandehroo et al. 2009; Yang et al. 2004). Third, translational repression mediated by AGO4 was shown to be required for effective dominant resistance against several viruses (Bhattacharjee et al. 2009). Environmental conditions (e.g., temperature) influence the induction of RNA silencing and SA-dependent defense responses (Szittya et al. 2003; Wang et al. 2009b).

Nepovirus spp. induce diseases in grapevine, small fruit, and stone fruit crops that cause considerable losses (Rochon and Sanfacon 2001). However, little is known about the host–pathogen interactions underlying *Nepovirus* spp.-induced diseases. In herbaceous hosts, *Nepovirus* infection is often characterized by the development of symptoms on inoculated leaves and on upper noninoculated leaves, followed by the emergence of symptom-free leaves. This symptom recovery phenomenon, first described over 80 years ago (Wingard 1928), has been linked to the induction of virus-specific RNA silencing, which results in the drastic reduction of virus titer in the recovered leaves (Ratcliff et al. 1997). In a previous study, we have shown that infection of *Nicotiana benthamiana* plants with *Tomato ringspot virus* (ToRSV) results in the induction of several defense responses, including a systemic HR-like necrotic response and RNA silencing (Jovel et al. 2007). Surprisingly, although ToRSV-specific RNA silencing is active in young, asymptomatic leaves, viral RNA does accumulate to high concentration. Thus, ToRSV is able to overcome both the necrotic defense response and RNA silencing to systemically invade the plant.

In this study, we investigated the interaction of ToRSV with defense responses in tobacco. As in *N. benthamiana*, we found that initial symptom induction is associated with hallmarks of the HR. However, in contrast to *N. benthamiana*, spread of ToRSV in tobacco plants was generally restricted to a few lesions in one or two upper, noninoculated leaves in a fraction of the plants, and viral RNA was generally not detected in asymptomatic upper leaves. Collectively, our results suggest that ToRSV infection of tobacco plants triggers multiple layers of defense, including RNA silencing, a necrotic response, and other SA-dependent defense responses. SA-dependent mechanisms were implicated in the restriction of viral spread. Induction of defense responses was only detected in tissues that also contained viral RNA and was accompanied with moderate systemic resistance to reinoculation.

RESULTS

Temperature influences local and systemic symptoms in ToRSV-infected tobacco plants.

Development of symptoms following inoculation of *N. tabacum* ('Petite Gerard') with ToRSV was influenced by temperature. At 34°C, visual symptoms did not develop on the inoculated leaves or on upper, noninoculated leaves and neither viral RNA nor viral-derived siRNAs were detected in samples collected randomly (data not shown). At lower temperatures, lesions developed on the inoculated leaves 2 to 3 days postinoculation (dpi). At 21°C, lesions were clearly defined and necrotic (Fig. 1A, panel 1). Typical symptoms included a central necrotic spot surrounded by a dark-gray concentric ring. At 27°C, lesions initially appeared as chlorotic spots or as chlorotic rings surrounding green areas. At later time points (6 dpi), brown halos developed around the lesions (Fig. 1A, panel 5). Occasionally, necrotic spots were also observed in the center of larger lesions at 27°C (Fig. 1C, panel 1). Intermediate symptoms were observed at 24°C, where a combination of necrotic and chlorotic lesions was observed (Fig. 1A, panel 3). The average lesion diameter was 36% smaller at 21 than at 27°C ($2.44 \text{ mm} \pm 0.74$ at 21°C versus $3.84 \text{ mm} \pm 1.43$ at 27°C, $n = 300$ over six independent experiments, $P < 0.001$, t test). At all temperatures, development of symptoms was accompanied by the induction of polyphenolic compounds, visible under UV illumination of the leaves (Fig. 1A, panels 2, 4, and 6). Reactive oxygen species were also induced around ToRSV-induced lesions at 27 and 21°C (Fig. 1B; data not shown), indicating an HR-like response. Trypan blue staining revealed that the brown halos surrounding lesions at 27°C were generally not accompanied by cell death. However, cell death was confirmed in association with the necrotic lesions occasionally observed at 27°C (Fig. 1C).

We have previously reported that extensive systemic symptoms develop in *N. benthamiana* leaves immediately above the inoculated leaves. These symptoms are characterized by vein clearing and are observed in 100% of ToRSV-infected plants but this is later followed by the emergence of asymptomatic young leaves (Jovel et al. 2007). Development of symptoms in upper noninoculated leaves was substantially less extensive in tobacco. The percentage of plants showing any sign of infection in upper noninoculated leaves varied among experiments but was much lower than that observed in *N. benthamiana* (between 10 and 60%). When observed, systemic infection was characterized by the development of lesions on leaves immediately above the inoculated leaves. The size of lesions and the extent of necrosis in upper noninoculated leaves were clearly influenced by temperature. At 21°C, lesions were punctual and necrotic (Fig. 1D, panels 1 and 4, 13 dpi), although more extensive symptoms (larger necrotic lesions sometimes accompanied by limited vein necrosis on a section of the leaf) did develop later (Fig. 1D, panel 5, 40 dpi). At 27°C, lesions on upper noninoculated leaves were characterized by chlorotic line patterns surrounding green areas of the leaves (Fig. 1D, panels 3, 7, and 8). In most cases, the systemic symptoms induced at 27°C were difficult to visualize under bright light but visualization of fluorescent polyphenolic compounds under UV light rendered the lesions easily detectable (Fig. 1D, compare panels 7 and 8). In rare cases, the systemic symptoms were more extensive. The chlorotic line patterns encompassed larger section of the leaves and could be found in a larger number of leaves in the plant (not shown). At the intermediate temperature (24°C), lesions were also characterized by chlorotic rings surrounding small sections of the leaves. However, lesions were tighter than those observed at 27°C (Fig. 1D, panels 2 and 6). At all temperatures, systemic symptoms were usually restricted to one or two leaves directly

above the inoculated leaves, and newly emerged leaves appeared symptom free later in infection. It is noteworthy that, although lesions were tighter and more necrotic on the inoculated leaves at 21°C, this was not accompanied by a reduction in the percentage of plants that became systemically infected ($53\% \pm 30$ at 21°C versus $34\% \pm 22$ at 27°C; six independent experiments; $P > 0.05$, t test). Similar results were observed in other tobacco cultivars (data not shown).

ToRSV RNAs and siRNAs are detected in symptomatic but not asymptomatic tissues.

We have previously shown that, in ToRSV-infected *N. benthamiana*, young asymptomatic leaves contain a high concentration of viral RNA (Jovel et al. 2007). To determine whether this was also the case in tobacco, we used Northern blotting and nucleic acid hybridization experiments. Viral RNA was readily detected in symptomatic tissues from inoculated leaves at 6 or 13 dpi but not in adjacent green tissues (Fig. 1E; data not shown). Upper symptomatic leaves immediately above the inoculated leaves also contained viral RNA. However, upper asymptomatic leaves contained very low or undetectable levels of viral RNA. Virions were also not detectable by enzyme-linked immunosorbent assay in upper asymptomatic leaves (data not shown). Similar results were observed in all tobacco cultivars tested (data not shown).

We have previously determined that ToRSV-specific siRNAs are predominantly derived from the regions of RNA2 that code for the movement protein (MP) and coat protein (CP) and from the 3' untranslated region (Jovel et al. 2007). Using probes corresponding to these regions of the viral genome, virus-specific

siRNAs were detected in symptomatic tissues, indicating that ToRSV RNAs are targeted by DCL enzymes. Accumulation of ToRSV siRNAs in symptomatic tissues was greater at 27 than at 21°C (Fig. 1E). This result is consistent with previous reports on other RNA viruses (Szittyá et al. 2003) and suggests that either RNA silencing is more active at higher temperature or that replication of the virus is more efficient at 27°C, thus providing more abundant DCL substrates. ToRSV siRNAs were not detected in asymptomatic leaves that did not contain viral RNAs (Fig. 1E). Although siRNAs have been shown to be the mobile signal of systemic silencing, previous studies have indicated that, in the absence of the target RNA (in this case, viral RNA), siRNAs are 1,000-fold less abundant in distal tissues than in the source tissues (Molnar et al. 2010). Thus, our inability to detect siRNAs in asymptomatic tissues does not rule out the possibility that systemic silencing occurred.

Systemic defense responses are inefficiently mounted in ToRSV-infected tobacco plants.

To determine whether SAR was induced during the course of virus infection in tobacco, we first examined the expression of the *PR1a* gene, which is a SAR molecular marker known to be induced by SA. Induction of *PR1a* expression was observed in inoculated and upper symptomatic leaves at all temperatures tested but was not detectable in upper asymptomatic leaves in which viral RNA was not detected (Fig. 1E). In classical dominant resistance cases, such as in the TMV-N gene interaction, establishment of SAR is linked with the induction of the expression of defense genes (e.g., *PR1a*) in upper asymptomatic leaves (Ward et al. 1991). The lack of significant *PR1a* expres-

Fig. 1. Effect of temperature on the development of symptoms in *Tomato ringspot virus* (ToRSV)-infected tobacco plants. **A**, Close-up of lesions on the inoculated leaves of ToRSV-infected tobacco plants ('Petite Gerard') grown at 21°C (panels 1 and 2), 24°C (3 and 4), or 27°C (5 and 6) photographed at 6 days postinoculation (dpi) under bright light (1, 3, and 5) or UV light (2, 4, and 6). Reference bar under panel 6 represents 5 mm. **B**, Colocalization of hydrogen peroxide production with virus-induced lesions. Inoculated leaves (6 dpi, 27°C) were stained with 3,3'-diaminobenzidine and photographed before (panel 1) and after (panel 2) staining. Reference bar represents 5 mm. **C**, Colocalization of cell wall collapse and necrotic symptoms. Inoculated leaves (6 dpi, 27°C) were stained with trypan blue and photographed before (panel 1) and after (panel 2) staining. Reference bar represents 5 mm. **D**, Development of symptoms at 13 dpi. Whole plants grown at 21°C (panel 1), 24°C (panel 2), or 27°C (panel 3) were photographed under UV light. The open arrowheads point to the inoculated leaves. Symptoms on upper noninoculated leaves (when present) are shown with the arrows. Detached upper, noninoculated leaves from plants grown at 21°C (panels 4 and 5), 24°C (panel 6), or 27°C (panels 7 and 8) were photographed under bright light (panels 4 to 7) or under UV light (panel 8) at 13 dpi (panels 4 and 6 to 8) or at 40 dpi (panel 5). The same leaf is shown in panels 7 and 8. Arrows point to necrotic or chlorotic symptoms on the leaves. At 27°C, symptoms on upper noninoculated leaves were often difficult to see under bright light but were accompanied by the induction of polyphenolic compounds which were readily detected under UV light (compare panels 7 and 8). **E**, Detection of viral RNAs, viral-derived small-interfering RNA (siRNAs), and *PR1a* transcripts during the course of infection. Total RNA was extracted at 6 or 13 dpi from mock-inoculated plants (M), inoculated leaves (i), upper noninoculated symptomatic leaves (us), or upper asymptomatic leaves (ua) of ToRSV-infected plants. Ethidium bromide staining of rRNAs and tRNAs are shown as a loading control for the detection of large and small RNAs, respectively.

sion in upper asymptomatic leaves of ToRSV-infected tobacco plants prompted us to test whether upper asymptomatic leaves were susceptible to reinoculation by ToRSV. For this experiment, we used two tobacco cultivars (Petite Gerard and ‘Samsun’) that differ slightly in their response to ToRSV. During the initial infection, the necrotic response was somewhat more acute in Samsun and the onset of symptoms occurred faster (data not shown). However, the outcome of viral infection was very similar for both cultivars. As observed in Petite Gerard, systemic infection occurred in only a portion of the ToRSV-infected Samsun plants and was restricted to discrete lesions on one or two upper leaves. Reinoculation experiments were conducted at 27°C, a temperature at which both RNA silencing and SA-dependent defense responses are triggered in symptomatic tissues. Plants were first inoculated either with ToRSV or with inoculation buffer (mock). At 13 dpi, upper asymp-

omatic leaves were reinoculated with ToRSV. In three separate experiments, the number of lesions induced after ToRSV reinoculation was similar in plants previously infected with ToRSV or in plants previously mock inoculated (Fig. 2A). Lesions on reinoculated leaves of plants previously inoculated with ToRSV were, on average, 11 to 18% smaller than lesions observed on plants previously mock inoculated (Fig. 2B). This moderate reduction in size was reproducible and statistically significant (*t* test, *P* < 0.05). Often, lesions were similar in appearance regardless of whether or not plants had been previously inoculated with ToRSV or mock inoculated. In a few cases, we observed a more acute induction of the typical brown halo (Petite Gerard) or necrotic rings (Samsun) surrounding the lesions (Fig. 2A, panels 3, 6, 9, and 12) in plants that had been previously inoculated with ToRSV. In reinoculated leaves, ToRSV RNA was detected in association with lesions but not

Fig. 2. Susceptibility of upper asymptomatic leaves to reinoculation by *Tomato ringspot virus* (ToRSV). **A**, Tobacco plants (‘Petite Gerard’ [PG, panels 1 to 6] or ‘Samsun’ [panels 7 to 12]) were either mock inoculated (panels 1, 4, 7, and 10) or inoculated with ToRSV (panels 2, 3, 5, 6, 8, 9, 11, and 12). At 13 days postinoculation (dpi), upper asymptomatic leaves were reinoculated with ToRSV. Development of symptoms on the reinoculated leaves was observed under bright light at three (panels 1 to 3 and 7 to 9) and six (panels 4 to 6 and 10 to 12) days post-reinoculation (dpi). Reference bar under panel 12 represents 5 mm. **B**, Quantification of lesion size after ToRSV reinoculation. Diameter of individual lesions on the reinoculated leaves of ToRSV-infected or mock-infected tobacco (PG or Samsun grown at 27°C) were measured at 3 and 6 dpi (*n* = 30 and 75 for PG 3 dpi mock+ToRSV and ToRSV+ToRSV, 100 for PG 6 dpi, 200 for Samsun 3 dpi, and 140 for Samsun 6 dpi). Values represent the average diameter and the standard deviation. Differences between the two treatments were significantly different at *P* < 0.05 (PG, 3 dpi), *P* < 0.01 (PG, 6 dpi) and *P* < 0.001 (Samsun, 3 and 6 dpi) (*t* test). Results were gathered from a single experiment and similar results were obtained in two other independent experiments. **C**, Analysis of the concentration of viral RNA and *PR1a* mRNA after reinoculation of healthy plants previously mock-inoculated (M) or ToRSV-infected plants (T). Sampling was done at 3, 6, or 13 dpi on entire inoculated leaves (first eight lanes) or on sections of the reinoculated leaves (last six lanes) containing visible lesions (il) or adjacent green tissues (ig). Total RNA preparations were loaded on gel and ethidium bromide staining of rRNAs is shown as a loading control. **D**, Quantification of lesion size after *Tobacco mosaic virus* (TMV) reinoculation. Tobacco plants (PG) were inoculated with ToRSV or TMV. The average number of lesions observed on leaves inoculated with various dilutions of the ToRSV or TMV inocula is indicated (N les./leaf). Young asymptomatic leaves were reinoculated with TMV at 8 dpi and the diameter of individual lesions were measured at 4 dpi for plants grown at 27°C (upper panel) and at 6 dpi for plants grown at 21°C (lower panel). Twenty lesions were measured for each reinoculated leaf (two reinoculated leaves per plant, 8 to 14 plants for each treatment). Differences between treatments and the corresponding mock control treatments were significantly different (*P* < 0.001, *t* test, *n* = 750 to 950). Results were gathered from a single experiment and similar results were obtained in three independent experiments.

in adjacent asymptomatic green tissues (Fig. 2C). The pattern of detection for *PR1a* transcripts mirrored that of ToRSV RNA. Expression of *PR1a* was induced in association with the necrotic lesions after reinoculation. In some cases, the induction of *PR1a* was slightly stronger in plants that had been previously inoculated with ToRSV (Fig. 2C). ToRSV-derived siRNAs were also detected in association with lesions in reinoculated leaves (data not shown). These results suggest that, upon virus challenge, defense responses were induced in reinoculated leaves. The susceptibility of upper asymptomatic leaves to ToRSV reinoculation implies that systemic RNA silencing is not efficient in these leaves. This suggests that the movement of the systemic silencing signal is blocked in ToRSV-infected tobacco plants or that siRNAs are rapidly degraded in upper asymptomatic leaves.

Although modest in magnitude, the observed reduction in lesion size and increase in the necrotic response in plants reinoculated with ToRSV suggest that SAR was induced upon initial infection with ToRSV. To confirm this, we used TMV as a challenging pathogen. The presence of the N resistance gene in the Petite Gerard cultivar also allowed us to compare the induction of SAR after inoculation with ToRSV or with TMV. Petite Gerard plants were inoculated with ToRSV, TMV (U1 strain), or inoculation buffer (mock) and grown at 27 or 21°C. Necrotic lesions rapidly developed (1 to 2 dpi) on TMV-inoculated leaves at both temperatures. Consistent with the known temperature dependence of the N resistance gene (Malamy et al. 1992), some TMV-infected plants became systemically infected at 27 but not at 21°C. These plants were excluded from our experiments. Because the strength of SAR has been shown to depend on the concentration of the initial inoculum (Ross 1961), several dilutions of the ToRSV and TMV inocula were tested to obtain comparable levels of initial infection. Young, asymptomatic leaves were reinoculated with TMV at 8 dpi and the size of TMV-induced lesions was measured 4 to 6 days post-reinoculation. ToRSV-infected plants showed a reduction in lesion size of 8 to 15% compared with the mock-infected control at both temperatures (Fig. 2D). These results were similar to those observed after reinoculation with ToRSV (Fig. 2B). TMV-infected plants showed a stronger reduction of lesion size after reinoculation with TMV (Fig. 2D). These results confirm that SAR is induced in ToRSV-infected tobacco plants but to a lesser degree than plants initially infected with TMV.

One possible explanation for the moderate level of SAR in ToRSV-infected tobacco is that ToRSV might interfere with SA-mediated defense responses. To investigate this, we used a detached-leaf assay (Ji and Ding 2001) in which mock-inoculated or ToRSV-inoculated tobacco leaves were floated on a

solution of SA or on water at 6 dpi. As expected, induction of defense genes (*PR1a*, *RDR1*, and *S-adenosyl methyl-transferase* [*SAMT1*]) was observed after treatment of mock-inoculated detached leaves with SA (Fig. 3). The SAMT1 enzyme is responsible for the methylation of SA, thereby producing methyl-salicylate (MeSA), the proposed systemic signal for SAR (Liu et al. 2010b; Park et al. 2007). These genes were also induced in ToRSV-inoculated leaves prior to the SA treatment, although at a low level. Induction of the expression of defense genes by SA was not inhibited in ToRSV-infected leaves. Instead, we observed enhanced induction of *PR1a*, *RDR1*, and *SAMT1* by SA in ToRSV-infected leaves compared with healthy ones. Thus, ToRSV does not seem to exert a strong antagonistic effect on local SA-mediated defense responses in tobacco.

SA-dependent responses influence local lesion size and systemic spread of ToRSV.

To test whether SA-dependent pathways are involved in the necrotic response or in the restriction of systemic movement of ToRSV in tobacco, we used two different approaches. First, wild-type tobacco plants (Petite Gerard) were treated with a solution of SA prior to inoculation with ToRSV and compared with control plants. Second, we used a previously characterized transgenic tobacco line (Petite Gerard) expressing the bacterial *NahG* gene that degrades endogenous SA (Friedrich et al. 1995). At 27°C, pretreatment of wild-type plants with SA resulted in an average 50% reduction in lesion size on the inoculated leaves (Fig. 4A, panels 1 and 3, and B). The percentage of plants that became systemically infected was also significantly reduced in SA-treated plants ($9\% \pm 6$ in SA-treated plants versus $49\% \pm 15$ in control plants; three independent experiments; $P < 0.01$, *t* test). In contrast, lesions on inoculated leaves were larger and more diffuse in the *NahG* plants at this temperature (Fig. 4A, panels 5 and 6). Appearance of brown halos or necrosis in association with local lesions was infrequent in the *NahG* plants at 6 dpi and lesions were often only visible by observing the induction of polyphenolic compounds under UV illumination (Fig. 4A, panels 5 and 6). The large local lesions became more visible later in infection (13 dpi) and were accompanied by a limited necrotic response (not shown). The average percentage of plants that became systemically infected was slightly higher for *NahG* plants than for control plants, although the difference was not considered statistically significant ($62\% \pm 16$ for *NahG* plants versus $40\% \pm 23$ for control plants, *t* test, $P > 0.05$). Appearance of systemic symptoms was delayed in *NahG* plants (20 dpi). However, after this initial delay, the extent of systemic infection on individual plants was substantially increased in *NahG* plants compared with wild-type ones and lesions were observed on a much larger number of leaves (Fig. 4D, panels 3 and 4). Collectively, these results suggest that the local lesion size, development of symptoms, and extent of systemic infection are influenced by SA-dependent pathways at 27°C.

At 21°C, the size of local lesions was also affected by SA although to a lesser extent (26% reduction in SA-treated plants) (Fig. 4A, panels 7 to 12, and B). Interestingly, the necrotic reaction was not delayed or prevented in *NahG* plants at this temperature. Neither SA pretreatment nor the presence of an *NahG* transgene significantly affected the percentage of plants that became systemically infected (data not shown), although the extent of systemic infection (number of systemic leaves with symptoms and number of lesions per symptomatic systemic leaves) was somewhat increased in *NahG* plants compared with the control Petite Gerard plants (Figs. 4D, panels 1 and 2, and 1D, panels 1 and 4). Thus, SA-dependent mechanisms may influence local lesion size and extent of systemic infection at 21°C, but only moderately. There are at least two

Fig. 3. Effect of *Tomato ringspot virus* (ToRSV) on salicylic acid (SA)-dependent induction of defense responses in a detached leaf assay. Tobacco plants ('Samsun') were inoculated with ToRSV. Six days postinoculation, inoculated leaves (or mock-inoculated leaves) were detached and floated in water (–) or in a solution of SA. Total RNAs extracted from these leaves were probed for *PR1a*, *RDR1*, and *SAMT1* mRNAs and ToRSV RNAs. Probing for *eIF1α* mRNAs and ethidium bromide staining of rRNAs are shown as loading controls. A single blot was successively striped and re-probed with each probe.

possible interpretations of these results. First, the induction of SA-dependent defense responses is reduced or slowed at lower temperature. Second, the restriction of ToRSV spread by SA-dependent mechanisms is less efficient at 21 than at 27°C.

The levels of ToRSV RNA and *PR1a* transcripts were compared in wild-type plants, plants pretreated with SA, and NahG plants (Fig. 4C and E). In all treatments, ToRSV RNAs were detected only in association with visible symptoms (Fig. 4C and E; data not shown). The levels of ToRSV RNAs were higher in NahG plants, probably as a result of the larger local lesions and enhanced systemic spread of the virus (Fig. 4E). As expected, in SA-treated plants, induction of *PR1a* (and *RDR1*) was enhanced at early time points in both mock- and

ToRSV-inoculated plants (Fig. 4C). *PR1a* induction was observed in NahG plants in response to ToRSV infection but the accumulation of *PR1a* transcripts was generally lower and delayed compared with wild-type plants at both temperatures (Figs. 4E and 5E). Similar moderate induction of *PR1a* transcripts in inoculated leaves was also described in TMV-infected NahG tobacco (NN) lines and may be due to low levels of residual SA present in these leaves (Friedrich et al. 1995). In all cases, *PR1a* transcripts were only detected in tissues in which ToRSV RNAs were also present (Fig. 4E; data not shown). Taken together, these results suggest that SA-dependent defense responses play an important role in restricting the spread of ToRSV in tobacco, especially at 27°C.

Fig. 4. Effect of the *NahG* transgene and salicylic acid (SA) pretreatment on symptom development at various temperatures. **A**, Close-up of lesions on the inoculated leaves of *Tomato ringspot virus* (ToRSV)-infected tobacco plants grown at 27°C (panels 1 to 6) or at 21°C (panels 7 to 12) photographed at 5 days postinoculation (dpi) under bright light (1, 3, 5, 7, 9, and 11) or UV light (2, 4, 6, 8, 10, and 12). Plants were either wild-type tobacco ('Petite Gerard' [PG]) noninduced (panels 1, 2, 7, and 8) or induced with SA treatments (PG + SA) (panels 3, 4, 9 and 10), or tobacco lines (PG) expressing the *NahG* transgene (NahG) (panels 5, 6, 11 and 12). Reference bar under panel 12 represents 5 mm. **B**, Quantification of lesion size on inoculated leaves. Diameter of individual lesions was measured at 7 dpi. Values represent the mean with standard deviation. Mean values were statistically different at $P < 0.001$ (t test, $n = 77$ to 134 over three separate experiments). **C**, Detection of viral RNAs and *PR1a* and *Rdr1* transcripts during the course of infection following SA pretreatment of tobacco plants (PG). Total RNA was extracted at 3, 6, and 13 dpi from mock-inoculated plants or from inoculated leaves (i) or upper noninoculated symptomatic leaves (us) of ToRSV-infected plants. **D**, Development of systemic symptoms in NahG transgenic lines. At 20 dpi, whole plants grown at 21°C (panel 1), or 27°C (panel 3) were photographed under UV light and detached upper noninoculated leaves were photographed under bright light (panels 2 and 4). **E**, Detection of viral RNAs and *PR1a* transcripts in NahG transgenic lines or control plants. Total RNA was extracted at 6, 13, and 21 dpi from inoculated leaves (i), upper noninoculated symptomatic leaves (us), or upper asymptomatic leaves (ua) of ToRSV-infected NahG or wild-type PG plants. Ethidium bromide staining of rRNAs is shown as a loading control in C and E.

Expression of P1-HC-Pro increases resistance to ToRSV while silencing of RDR1 does not affect viral systemic spread.

In an attempt to investigate the role of RNA silencing in the restriction of ToRSV systemic spread in tobacco, we used the U-6B transgenic line, which expresses the *Tobacco etch virus* (TEV) P1-HC-Pro protein, a known suppressor of silencing (Pruss et al. 2004). At both 21 and 27°C, inoculated leaves of U-6B plants exhibited fewer lesions than those of the corresponding control lines (tobacco ‘Havannah’ transformed with an empty vector) (Fig. 5A). Lesions on U-6B plants were, on average, 50 to 60% smaller than those observed on the control plants at both temperatures (Fig. 5C). The percentage of plants that showed any sign of systemic infection was also significantly reduced in the U-6B line ($3 \pm 6\%$ at 27°C and $4 \pm 7\%$ at

21°C for the U-6B lines compared with $32 \pm 28\%$ at 27°C and $45 \pm 9\%$ at 21°C for the control lines). The HC-Pro suppressor of silencing has been shown to enhance both SA-dependent and SA-independent antiviral responses (Endres et al. 2010; Pruss et al. 2004). To investigate whether the enhanced resistance observed in the U-6B lines would be affected by depletion of SA-mediated antiviral responses, we took advantage of a double transgenic line that expresses simultaneously the *U-6B* and *NahG* transgenes (U × N lines). At 21 and 27°C, the local lesion size on inoculated leaves and the systemic movement of the virus (as judged by the rate and extent of systemic infection) was higher in the U × N lines than in the U-6B line (Fig. 5A to C; data not shown). There were increased levels of ToRSV genomic RNAs and siRNAs in the U × N plants, especially at 27°C (Fig. 5D and E). Thus, the concentration of

Fig. 5. Effect of the HC-Pro transgene on symptom development at various temperatures. **A**, Close-up of lesions on the inoculated leaves of *Tomato ringspot virus* (ToRSV)-infected tobacco plants grown at 27°C (panels 1 to 6) or at 21°C (panels 7 to 12) photographed at 6 days postinoculation (dpi) under bright light (1, 3, 5, 7, 9, and 11) or UV light (2, 4, 6, 8, 10, and 12). Leaves were from wild-type tobacco (‘Havannah’) transformed with the empty vector (panels 1, 2, 7, and 8), U-6B transgenic lines expressing the *U-6B* transgene (panels 3, 4, 9, and 10), or double transgenic lines expressing both the *U-6B* and *nahG* transgenes (U × N) (panels 5, 6, 11, and 12). Reference bar under panel 12 represents 5 mm. **B**, Development of systemic symptoms in double U × N transgenic lines. Whole plants grown at 21 or 27°C (panel 1 or 3, respectively) were photographed under UV light at 20 dpi. Detached upper, noninoculated leaves from plants grown at 21 or 27°C (panel 2 or 4, respectively) were photographed under bright light. **C**, Quantification of lesion size on inoculated leaves. Values represent the mean diameter with the standard deviation. Percentage of reduction in lesion diameter for the U-6B lines and U × N lines was calculated compared with the control lines (Havannah transformed with empty vector). Comparison of the results from the different lines at either 21 or 27°C revealed that they were significantly different ($P < 0.001$, t test; $n = 298$ for Havannah [Hav]-21°C, $n = 50$ for U-6B-21°C, $n = 157$ for U × N-21°C, $n = 154$ for Hav-27°C, and $n = 59$ for U-6B-27°C and U × N-27°C; measurements are collated from three separate experiments). **D** and **E**, Detection of viral RNAs, virus-derived small-interfering (si)RNA, and *PR1a* transcripts during the course of infection. Total RNA was extracted at 6 and 13 dpi from inoculated leaves (i) or upper noninoculated symptomatic leaves (us) of ToRSV-infected plants. All samples shown in E were from symptomatic inoculated leaves collected at 6 or 13 dpi. Please note that only symptomatic inoculated leaves were collected in E whereas all inoculated leaves were collected in D. Because very few inoculated leaves were symptomatic in the U-6B plants, the relative concentration of ToRSV RNAs and siRNAs in these lines is higher in E than in D. Ethidium bromide staining of rRNAs and tRNAs are shown as a loading control for the detection of large and small RNAs, respectively.

ToRSV RNAs correlated with the size of the local lesions and the extent of systemic infection observed. These results indicate that attenuation of the antiviral response mediated by SA apparently reduced the effectiveness of the enhanced-resistance phenotype induced by P1-HC-Pro in U-6B plants.

Among genes induced during the SA-dependent defense response, *RDR1* has been implicated in resistance to many viruses, possibly by enhancing antiviral RNA silencing. In tobacco, *RDR1* expression is induced by infection with many viruses (Xie et al. 2001). Tobacco lines silenced for *RDR1* have increased susceptibility to a number of viruses in compatible or incompatible interactions (Hirai et al. 2008; Rakhshandehroo et al. 2009; Xie et al. 2001). In this study, we have shown that *RDR1* is induced in tobacco upon infection with ToRSV (Figs. 3 and 4). To test the effect of *RDR1* on ToRSV-infected tobacco plants, we used a previously described transgenic line (RDR1i) in which an intron-hairpin transgene reduces the transcript level to 10 to 15% of that observed in the corresponding wild-type Samsun (Hirai et al. 2008). Indeed, low levels of *RDR1* transcripts were induced in ToRSV-infected control Samsun plants but were apparently degraded in the RDR1i plants (Fig. 6C). In RDR1i plants grown at 27°C, the initial necrotic response was enhanced in the inoculated leaves compared with control tobacco lines (Samsun transformed with the empty vector) (Fig. 6A). Local lesions were also slightly smaller in the RDR1i lines (reduction of 16%) (data not shown). As observed in wild-type plants at 21°C, the increased necrotic reaction and smaller local lesion size in RDR1i plants at 27°C were not accompanied by reduced systemic spread of the virus (Fig. 6B; data not shown). Upper asymptomatic leaves of ToRSV-infected RDR1i plants did not contain significant levels of ToRSV RNAs or *PR1a* transcripts (Fig. 6C). Thus, in spite of a more acute initial necrotic response, the outcome of the infection was not significantly altered in RDR1i plants.

DISCUSSION

Plants are exposed to a multitude of exogenous stimuli that include attack by pathogens and herbivores and changes in environmental conditions. As a consequence, they have acquired a vast array of defense responses which are energetically costly and, hence, are selectively activated and regulated according to the nature and intensity of the stimulus (Jones and Dangl 2006). As shown in this and previous studies (Szittyá et al. 2003; Wang et al. 2009b; Zhu et al. 2010), temperature is a major modulator of such responses. We have previously shown that ToRSV can overcome an HR-like necrotic response and RNA silencing to systemically invade *N. benthamiana* plants (Jovel et al. 2007). We now show that systemic spread of ToRSV is much more limited in tobacco and that SA-mediated mechanisms play a key role in restricting the spread of ToRSV in tobacco. ToRSV infection of tobacco plants was accompanied with the induction of local RNA silencing and with moderate systemic resistance to reinoculation.

Possible mechanisms mediating restriction of ToRSV systemic spread in tobacco.

The extent and rate of systemic infection caused by ToRSV in tobacco was low under all conditions evaluated in this study, suggesting that effective defense responses are activated upon virus attack. A necrotic response and the concomitant transcriptional induction of defense genes, including *PR1a*, *RDR1*, and *SAMT1*, are footprints of a triggered immune response against ToRSV. We did not observe a strong correlation between the intensity of the necrotic response and the rate of viral systemic spread in ToRSV-infected tobacco. A more acute

necrotic response and smaller lesion size were observed in wild-type plants at 21°C or in RDR1i lines at 27°C but this was not accompanied by enhanced restriction of viral systemic spread (Figs. 1 and 6). The more acute necrotic response at 21°C was also not accompanied by a stronger induction of SAR (Fig. 2). Although the rate and extent of systemic infection and the size of local lesions are influenced by SA-dependent mechanisms, the necrotic response itself was largely SA independent, at least at lower temperatures. Our results are in agreement with an increasing body of evidence suggesting that, for some host–pathogen interactions, i) cell death observed in association with HR responses is not directly responsible for restricting pathogen movement or multiplication, ii) cell death and inhibition of viral movement or multiplication are regulated by independent pathways, and iii) cell death is not a prerequisite for the induction of SAR (Cawly et al. 2005; Chandra-Shekara et al. 2004; Kiraly et al. 2008; Komatsu et al. 2010; Liu et al. 2010a; Wang et al. 2010a).

Fig. 6. Effect of silencing RNA-dependent RNA polymerase (*RDR1*) on symptom development. **A**, Close-up of lesions on the inoculated leaves of *Tomato ringspot virus* (ToRSV)-infected wild-type tobacco plants ('Samsun', panel 1) or transgenic tobacco plants silenced for *RDR1* expression (RDR1i, panel 2). Plants were grown at 27°C and photographed at 5 days postinoculation (dpi) under bright light. Reference bar under panel 2 represents 5 mm. **B**, Development of systemic symptoms. Symptomatic upper noninoculated leaves of wild-type plants (panels 1 and 2) or RDR1i transgenic plants (panels 3 and 4) grown at 27°C were photographed at 20 dpi under bright light (panels 1 to 3) or UV light (panels 2 to 4). **C**, Analysis of the concentration of viral RNAs and of *PR1a* and *RDR1* mRNAs during the course of infection. Samples were analyzed at the indicated time. Total RNA purified from mock-inoculated leaves (M), inoculated leaves (i), upper asymptomatic leaves (ua), or regions of upper symptomatic leaves with visible lesions (us) were analyzed. Ethidium bromide staining of rRNAs is shown as a loading control.

Our results suggest that SA-mediated defense responses play a key role in limiting ToRSV systemic infection. SA-mediated pathways encompass multiple defense responses, which can arrest viruses at the steps of genome replication, cell-to-cell movement, or systemic movement (Singh et al. 2004). Systemic movement of viruses can be hindered during uploading into the vasculature bundles in the inoculated leaves or during viral egress from minor veins into the mesophyll cells of systemic leaves (Oparka and Cruz 2000). In ToRSV-infected tobacco, the rate of systemic infection was low and symptoms in systemic leaves were restricted to a few lesions, suggesting a reduced ability of the virus to reach the vasculature in the initially inoculated leaves. Local defense reactions induced during HR responses (such as callose deposition) can harden the cell wall and prevent movement toward the vascular bundle (Beffa et al. 1996).

RNA silencing has also been identified as an important defense response blocking the upload of *Turnip crinkle virus* into the vascular system but not the egress from veins in the systemic leaves (Cao et al. 2010). ToRSV-derived siRNAs were detected in inoculated leaves, indicating that viral RNAs are at least partially decimated by the RNA-silencing machinery. This raised the possibility that RNA silencing acts in conjunction with other defense mechanisms to restrict virus spread as previously suggested for the *Plum pox virus*–tobacco interaction (Alamillo et al. 2006). Expression of *RDR1* is induced by SA and in response to infection by many viruses, including ToRSV, and has been implicated in antiviral immunity by allowing the production of secondary siRNAs (Garcia-Ruiz et al. 2010; Xie et al. 2001; Yang et al. 2004; Yu et al. 2003). In a previous study, we found that systemic infection of ToRSV is efficiently accomplished in *N. benthamiana* plants (Jovel et al. 2007), wherein *RDR1* is a loss-of-function mutant due to an insertion that introduces early stop codons (Yang et al. 2004). Therefore, we tested the contribution of tobacco *RDR1* to the restriction of ToRSV spread. Tobacco plants silenced for *RDR1* did not show any obvious increase in the rate or extent of systemic infection (Fig. 6). Similarly, expression of the *Medicago truncatula RDR1* gene in *N. benthamiana* did not alter the systemic infection of ToRSV in this host (Jovel et al. 2007). Although the effect of expressing the *N. tabacum RDR1* gene in *N. benthamiana* has not been tested, our results suggest that the difference between the restricted ToRSV systemic movement in *N. tabacum* and the extensive ToRSV systemic movement in *N. benthamiana* cannot be solely explained by the presence or absence of a functional *RDR1* gene. The tobacco *RDR1* was recently reported to have silencing suppression activity, which could enhance susceptibility of this host to some viruses (Ying et al. 2010). It is also possible that silencing of a single *RDR* gene is not enough to affect virus accumulation, because antiviral activity has also been attributed to *RDR6* (Schwach et al. 2005; Wang et al. 2010b), which could compensate for silencing of *RDR1*. For a *Cucumber mosaic virus* (CMV) mutant that lacks the suppressor of silencing 2b, it has been shown that *Arabidopsis thaliana RDR1* is involved in the biogenesis of secondary viral siRNAs generated from the 5' end of the viral RNAs while *RDR6* mainly targets the 3' moiety of the viral RNA3, in the absence of *RDR1*. Accordingly, rescue of the CMV mutant is observed in *rdr1 rdr6* lines but not in *rdr1* or *rdr6* lines (Wang et al. 2010b). Parallel high-throughput sequencing of ToRSV siRNAs from infected wild-type and *rdr* plants may help elucidate a putative role of *RDR* against ToRSV accumulation. It is not clear why the initial symptomatic response to ToRSV was exacerbated in the *RDR1i* lines (increased necrosis and tighter lesion). We see at least two possible explanations. First, silencing of *RDR1* may allow for an increased accumulation of viral RNA (and pro-

teins) in initially infected cells, triggering a more acute necrotic response. Second, *RDR1* may somehow contribute to the downregulation of the necrotic response at 27°C. A role for *RDR1* in regulating other defense pathways, possibly by enhancing silencing of defense gene regulators, has been proposed (Rakhshandehroo et al. 2009).

We also tested a transgenic tobacco line (U-6B) that expresses the P1-HC-Pro protein from TEV, a known suppressor of silencing. Interestingly, ToRSV spread was even more restricted in these lines compared with wild-type tobacco. Although ToRSV spread was increased in the U-6B × NahG transgenic line compared with the U-6B line, these results do not exclude the possibility that SA-independent responses also play a role in the enhanced resistance observed in the U-6B lines. Indeed, U-6B plants show a distorted vascular system (Anandalakshmi et al. 2000; data not shown), that could impede viral systemic movement. The HC-Pro proteins from TEV and from *Turnip mosaic virus* have been shown to enhance the expression of many genes implicated in biotic and abiotic defense responses (Endres et al. 2010). These include the gene coding for SNC1, an SA-dependent positive regulator of several resistance genes and two endogenous suppressors of silencing (Endres et al. 2010; Yi and Richards 2007). Our results contrast with those obtained with two other *Nepovirus* spp. and similar HC-Pro transgenic lines. Tobacco transgenic lines expressing the *Potato virus Y* HC-Pro protein were shown to enhance systemic movement of *Tobacco ring-spot virus* (Siddiqui et al. 2008). Systemic spread of *Tomato black ring virus* was also enhanced in U-6B lines despite an initial decrease of virus accumulation in inoculated leaves (Pruss et al. 2004). However, in both cases, the enhancement of viral systemic spread by potyviral HC-Pro transgenes was observed under conditions in which viral infection was asymptomatic. The possible role of HR-like responses and other SA-dependent or SA-independent defense responses in restricting viral spread was not rigorously analyzed. In the case of the ToRSV–tobacco interaction, it is possible that enhancement of viral systemic spread by HC-Pro mediated inhibition of viral RNA silencing was shadowed by the apparent enhancement of other defense responses that restrict virus spread.

Although our results could not directly establish a role for RNA silencing in the restriction of ToRSV movement in tobacco, the interaction between ToRSV and the silencing machinery is likely to be complex. RNA silencing may play multiple roles, including controlling the concentration of viral RNA in the initially infected cells and possibly preventing the virus from moving systemically. RNA silencing could also modulate other defense responses (Katiyar-Agarwal and Jin 2010). For instance, some defense responses are downregulated by virus-induced endogenous miRNA or trans-acting siRNAs (He et al. 2008; Varallyay et al. 2010; Yi and Richards 2007). Virus-derived siRNAs have also been proposed to target host genes (Moissiard and Voinnet 2006). Furthermore, specific silencing enzymes (e.g., AGO4) may be required to enable dominant resistance against plant viruses (Bhattacharjee et al. 2009). Additional experiments aimed at silencing or inactivating silencing enzymes in tobacco may help clarify the role of RNA silencing in the outcome of the ToRSV–tobacco interaction.

Moderate systemic defense responses induced in ToRSV-infected tobacco.

It has been recently suggested that viral siRNAs constitute the systemic silencing signal and move through the vascular system following essentially the same route as the virus (Dunoyer et al. 2010a and b; Molnar et al. 2010). The susceptibility of young asymptomatic leaves to ToRSV reinoculation suggests that the movement of ToRSV siRNAs is inhibited

in infected plants or that these siRNAs are rapidly degraded in upper asymptomatic leaves. Transport of siRNAs (and viral RNAs) from the primary inoculated leaves may be inhibited as a consequence of a plant defense mechanism. We have previously shown that systemic silencing of a green fluorescent protein transgene is hindered in ToRSV-infected *N. benthamiana* plants, yet movement of the viral RNA is not inhibited (Jovel et al. 2007). Thus, it is also possible that ToRSV encodes a suppressor of silencing that hinders the systemic movement of the siRNAs. Testing the effect of individual viral proteins on systemic silencing will be necessary to resolve this issue.

Local HR reactions are usually associated with dominant resistance, prevent systemic spread of the pathogen, and are normally followed by the establishment of SAR (Carr et al. 2010; Moffett 2009). In the ToRSV–tobacco interaction, the local HR-like response only partially restricted virus spread, and a fraction of the plants showed limited systemic movement of the virus. SAR was observed but was moderate compared with that induced in the tobacco–TMV interaction. Partial evasion of local HR responses directed by dominant resistance genes has been reported for some plant–virus interactions. For example, systemic TMV infection of tobacco plants harboring the N gene was reported when young plants were inoculated (Holmes 1938). Other cases of systemic or trailing necrosis have been associated with various dominant resistance genes and may be due to inefficient recognition of the viral proteins or impaired signaling of the defense responses; for example, in plants altered in the SA signaling pathway (Chandra-Shekara et al. 2004; Collmer et al. 2000; Kachroo et al. 2000; Moffett 2009). Thus, the incomplete resistance to ToRSV in tobacco may be due to the presence of a dominant resistance gene. However, induction of various defense responses, including SA-dependent defense responses, has also been noted in several compatible plant–virus interactions (Atsumi et al. 2009; Huang et al. 2005; Love et al. 2005; Xu et al. 2003). Thus, it is also possible that basal SA-dependent defense responses account for the partial resistance to ToRSV observed in tobacco. Similarly, the SAR induced in ToRSV-infected tobacco plants could be mediated by a dominant resistance gene or by basal defense responses. The relatively low level of SAR could be due to interference with SA induction of the defense response, blockage of the systemic movement of the SAR signal by a viral protein, or, indirectly, as a consequence of the plant defense reaction. The CMV 2b and TMV replicase proteins interfere with SA induction of various defense genes, including *PR1a* (Ji and Ding 2001; Wang et al. 2009a). Using a detached-leaf assay, we did not see an inhibition of SA-dependent induction of *PR1a*, *RDR1*, or *SAMT1* in ToRSV-infected leaves (Fig. 3). MeSA is the suggested systemic SAR signal synthesized from free SA by SAMT1 (Liu et al. 2010b; Park et al. 2007). SA-binding protein 2 (SABP2) is a MeSA esterase that enables conversion of MeSA into SA in the systemic leaves (Vlot et al. 2008). SA binds to the active site of SABP2 and inhibits its activity (Forouhar et al. 2005). This product inhibition of SABP2 was suggested to allow accumulation of MeSA in inoculated leaves and dissemination of the SAR signal to systemic leaves. Although the expression of *SAMT1* was not inhibited in ToRSV-infected leaves, it is possible that ToRSV interferes with other steps of this signaling pathway. Finally, we cannot exclude the possibility that the HR-like response observed in the inoculated leaves of ToRSV-infected tobacco is intrinsically different from that induced by dominant resistance genes in other plant–virus interactions. Further investigation of the signaling pathways regulating the ToRSV–tobacco interaction will help distinguish between these possibilities.

MATERIALS AND METHODS

Plant material.

Homozygous *N. tabacum* transgenic lines NahG, U-6B, and U-6B × NahG and the corresponding control plants were a generous gift from V. Vance (Department of Biological Sciences, University of South Carolina, Columbia) and have been previously described (Pruss et al. 2004). U-6B and U-6B × NahG transgenic lines had an altered phenotype (stunting and altered leaf shape) which was used to screen transgenic plants. Because these plants grew more slowly than the corresponding control plants, they were seeded 1 week earlier to allow them to reach the same developmental stage as control plants at the time of inoculation. F1 transgenic *N. tabacum* RDR1i lines and the corresponding control plants were kindly provided by T. Meshi (National Institute of Plant Sciences, Tsukuba, Japan) and have also been described (Hirai et al. 2008). From these, F2 plants were generated and screened for the presence of the transgene by polymerase chain reaction. These plants did not show any phenotypical abnormality. All plant lines were grown under glasshouse conditions and transferred postinoculation to individual Conviron units set at 21, 24, and 27°C with a 16-h photoperiod.

Virus inoculations and monitoring of symptoms.

ToRSV Raspl isolate initially propagated in cucumber cotyledons was used as inoculum (Jafarpour and Sanfacon 2009; Wang and Sanfacon 2000). Inoculum was prepared by macerating virus-infected tissue with inoculation buffer (50 mM NaPO₄, pH 7.2) at a ratio of 1:3 (grams of tissue/milliliters of buffer). TMV (strain U1) inoculum was kept as lyophilized infected plant tissue. Extracts were rub inoculated onto two leaves of each Carborundum-dusted 4- to 5-week-old plants. Plants or detached leaves were photographed under bright or UV light. Lesion diameter was quantified using the freely available ImageJ program and a metric ruler for calibration. At 27°C, ToRSV-induced lesions on wild-type plants were often chlorotic and surrounded by a brown halo. In these cases, the outer edge of this brown halo was used to define the lesion size. Comparison of lesion size in separate treatments was determined only between experiments in which all the treatments considered were conducted in parallel. Because the number and size of lesions could vary with the age of the leaf, as reported for other tobacco–virus interactions (Yalpani et al. 1993), we only compared leaves that were at a similar developmental stage. Unless otherwise indicated, measurements presented are the average of at least three independent experiments. The number of lesions measured for each treatment is indicated in the figure legends. With the exception of U-6B plants, which developed very few lesions on the inoculated leaves, at least 100 individual lesions per treatment were included in our measurements. The percentage of reduction in lesion diameter of various treatments was calculated using the following equation: $100 \times (1 - [\text{lesion diameter of treatment} / \text{lesion diameter of control}])$. Quantification of systemic infection was conducted by visually inspecting individual plants at 13 and 20 dpi. In some cases, systemic symptoms were difficult to visualize under bright light (mild chlorosis) but were easily detectable by examining the plants under UV light. Therefore, the percentage of plants that showed any symptoms on upper noninoculated leaves was routinely evaluated by observation of the plants under UV light. This percentage does not take into account the number of leaves that displayed symptoms or the extent of these symptoms. Thus, it purely refers to incidence and not to severity of the disease in systemic leaves. Comparisons between treatments were carried out only for experiments in which all treatments were conducted in parallel.

Each experiment included at least 10 to 15 plants per treatment but most experiments included 30 to 50 plants per treatment.

Characterization of HR response hallmarks and SA-induction experiments.

Detection and photography of reactive oxygen species by 3,3'-diaminobenzidine staining and cellular death by trypan blue staining were conducted as previously described (Jovel et al. 2007). SA induction of host defense genes was performed by pretreating plants with SA using a previously described protocol with a few modifications (Lewsey and Carr 2009). Briefly, 4-week-old plants were pretreated by spraying and soil drenching with a solution of 1.1 mM SA in 0.11% ethanol for 4 days prior to inoculation. On day 5, plants were inoculated in the morning and leaves were sprayed with a final SA treatment 4 h later. Control plants were sprayed with 0.11% ethanol. To assess the effect of ToRSV infection and SA on induction of defense genes, symptomatic ToRSV-inoculated leaves or mock-inoculated leaves were detached from plants and floated on a solution of 1 mM SA or distilled water overnight and then frozen at -70°C until analysis.

RNA blots.

For Northern blots and siRNA detection, total RNA was extracted with Trizol reagent as recommended by the manufacturer (Invitrogen, Carlsbad, CA, U.S.A.). Northern blots and siRNA analysis were conducted as previously described (Jovel et al. 2007), with the following exceptions. For siRNAs detection, 100 μg of total RNA was fractionated by denaturing polyacrylamide gel electrophoresis and then transferred to Hybond+ membranes (GE Healthcare, Piscataway, NJ, U.S.A.). Membranes for Northern blots and siRNA blots were cross-linked under UV light (254 nm, 8 W, 120,000 $\mu\text{J}/\text{cm}^2$; HL-2000 Hybrilinker, UVP LLC, Upland, CA, U.S.A.) and baked for 2 h at 80°C . Hybridizations were conducted in the presence of DNA probes labeled with [α - ^{32}P]dCTP (Perkin-Elmer, Norwalk, CT, U.S.A.) using the Rediprime II DNA labeling system (GE Healthcare). Two cDNA fragments corresponding to the region of the ToRSV genome coding for the MP and CP were amplified for labeling using specific primer pairs (for the MP: 5'-AAATGCTGCTCCGTACCG-3' and 5'-TCTTCAAAA GCGGCGTTC-3'; for the CP and 3' untranslated region 5'-GCCTTTTAGTCCCAAAG-3' and 5'-TCGTCTCCTAGACTA GTC-3') and were mixed together. Similarly, oligonucleotides (5'-GGAGGAATTGGAGACATTAGC-3' and 5'-CTGTCT GTGCTTTAGACATGCATTC-3') were used to amplify a cDNA fragment complementary to *SAMT1* transcripts from purified tobacco total RNA. For detection of *RDR1* transcripts, two separate cDNA fragments were amplified from tobacco total RNA using two primer pairs (5'-CACCATGCAAAGTTT ATTTTGTGGTCCAGA-3' and 5'-GCGGGAAGTTTGC AGCATATTGAAAGAAA-3'; 5'-CACTTGGAAATTTGGCT ACATG-3' and 5'-CACCTCACTGGATCATAACAAC-3') and were mixed together. For detection of *PR1a* transcripts, a cDNA fragment was amplified from purified *N. benthamiana* total RNA using specific oligonucleotides (5'-CTCCTATAGTC ATGGGATTTG-3' and 5'-AAGCTTGTTCTTAAGGCATG-3').

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In the article ‘Salicylic Acid-Dependent Restriction of *Tomato ringspot virus* Spread in Tobacco Is Accompanied by a Hypersensitive Response, Local RNA Silencing, and Moderate Systemic Resistance’ by J. Jovel, M. Walker, and H. Sanfaçon, the original Figure 3 was assembled incorrectly. The images for ToRSV, *PR1α*, and *RdR1* were derived from a single blot, which was stripped and reprobed consecutively. The image for *SamT1* was derived from a different blot, and, as such, a distinct loading control should have been shown. A revised figure has now been assembled showing a replicate experiment in which a single blot was successively reprobed for all the genes tested (including the housekeeping gene *eIF1α* as an additional loading control). This experiment was repeated four times with similar results. Please note that this correction does not affect any of the conclusions of the paper. The corresponding author takes full responsibility for this mistake. All authors wish to apologize for the inconvenience caused.

Revised Fig. 3

Fig. 3. Effect of *Tomato ringspot virus* (ToRSV) on salicylic acid (SA)-dependent induction of defense responses in a detached leaf assay. Tobacco plants (‘Samsun’) were inoculated with ToRSV. Six days postinoculation, inoculated leaves (or mock-inoculated leaves) were detached and floated in water (–) or in a solution of SA. Total RNAs extracted from these leaves were probed for *PR1α*, *RdR1*, and *SAMT1* mRNAs and ToRSV RNAs. Probing for *eIF1α* mRNAs and ethidium bromide staining of rRNAs are shown as loading controls. A single blot was successively stripped and reprobed with each probe.

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Fig. 3. Effect of *Tomato ringspot virus* (ToRSV) on salicylic acid (SA)-dependent induction of defense responses in a detached leaf assay. Tobacco plants (‘Samsun’) were inoculated with ToRSV. Six days postinoculation, inoculated leaves (or mock-inoculated leaves) were detached and floated in water (–) or in a solution of SA. Total RNAs extracted from these leaves were probed for *PR1α*, *RdR1*, and *SAMT1* mRNAs and ToRSV RNAs. Ethidium bromide staining of rRNAs is shown as a loading control.