December 2005

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JOURNAL OF VIROLOGY, Dec. 2005, p. 15209–15217 Vol. 79, No. 24
0022-538X/05/$08.00+0 doi:10.1128/JVI.79.24.15209–15217.2005

RDR6 Has a Broad-Spectrum but Temperature-Dependent Antiviral Defense Role in *Nicotiana benthamiana*

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Received 29 June 2005/Accepted 27 September 2005

SDE1/SGS2/RDR6, a putative RNA-dependent RNA polymerase (RdRP) from *Arabidopsis thaliana*, has previously been found to be indispensable for maintaining the posttranscriptional silencing of transgenes, but it is seemingly redundant for antiviral defense. To elucidate the antiviral role of this RdRP in a different host plant and to evaluate whether plant growth conditions affect its role, we down-regulated expression of the *Nicotiana benthamiana* homolog, NbRDR6, and examined the plants for altered susceptibility to various viruses at different growth temperatures. The results we describe here clearly show that plants with reduced expression of NbRDR6 were more susceptible to all viruses tested and that this effect was more pronounced at higher growth temperatures. Diminished expression of NbRDR6 also permitted efficient multiplication of tobacco mosaic virus in the shoot apices, leading to serious disruption with microRNA-mediated developmental regulation. Based on these results, we propose that NbRDR6 participates in the antiviral RNA silencing pathway that is stimulated by rising temperatures but suppressed by virus-encoded silencing suppressors. The relative strengths of these two factors, along with other plant defense components, critically influence the outcome of virus infections.

RNA silencing is a surveillance system in eukaryotic organisms triggered by double-stranded RNA (dsRNA) that is subsequently digested by a dsRNA-specific RNase (Dicer or Dicer-like) into a small RNA species of 21 to 25 nucleotides (nt) long, called small interfering RNA (siRNA). The resultant siRNAs are then recruited into the RNA-induced silencing complex to direct the degradation of other RNAs with sequence complementarity to siRNAs (13). RNA silencing is thought to function primarily in defending eukaryotic cells against RNA molecular parasites, such as RNA viruses and transposon RNAs. Plant viruses, as well as some animal viruses, counteract this host defense mechanism by encoding suppressors of RNA silencing, which act at different steps of the pathway and with various strengths to ensure their successful systemic invasion of specific hosts (26, 31).

In addition to guarding the host against parasitic RNAs, recent studies have shown that processes highly related to RNA silencing are also involved in developmental regulation (22, 28), methylation of chromosomal DNA and histones, and chromatin maintenance (20, 43). miRNA-mediated regulation of gene expression in both animal and plant systems is a particularly interesting discovery. Unlike siRNAs, miRNAs are encoded by genomes of eukaryotes in the form of partially double-stranded precursor molecules, which are processed by Dicer-like RNase(s) to release mature miRNAs. The miRNAs then mediate degradation or translational repression of the target RNAs (15). One well-studied example in plants is miR165/166. This miRNA targets the mRNA of three class III homeodomain leucine zipper (HD-ZIP III) transcription factors, PHABULOSA (PHB), PHAVOLUTA (PHV), and REVOLUTA (REV), for cleavage (10, 29, 30). Restricted expression of these genes in the shoot apex plays a critical role in patterning adaxial-abaxial polarity in lateral organs. Gain-of-function mutations of *PHB*, *PHV*, and *REV*, which cause adaxialization of leaves and vascular systems, have been mapped mostly to the target sites of miR165/166 and have been found to prevent the miRNA-mediated degradation of their mRNAs (10, 21, 23, 30). Notably, similar phenotypes have also been frequently observed with transgenic plants expressing virus-encoded silencing suppressors and occasionally with virus-infected plants (2, 3, 9, 11, 22), supporting the argument that siRNA- and miRNA-mediated pathways are closely related.

The plant RNA silencing pathway can be mechanistically divided into two stages, initiation and maintenance (5, 36). The initiation stage is characterized by its dependence on the trigger dsRNA and siRNAs directly derived from the trigger. The maintenance stage is independent of the trigger and is responsible for the persistent silencing, even after the inducer dsRNA is cleared from the cells. At this stage, RNA silencing is maintained through secondary synthesis of dsRNA by a cellular RNA-dependent RNA polymerase (RdRP), using the siRNA-complementary target RNA as a template.

The *Arabidopsis* spp. SDE1/SGS2/RDR6 (RDR6 hereafter) RdRP is a putative RdRP originally identified as required for RNA silencing of transgenes (6, 32). Additional evidence since then has established that it is essential for the maintenance of RNA silencing. RDR6 has been shown to be necessary for the continued silencing of a transgene after the complete elimination of inducer RNA, the cell-to-cell movement of the RNA silencing signal, and the spread of silencing along the target RNA to sequences beyond the region that is homologous to the trigger molecule (7, 17, 24, 40).

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Virus-induced silencing differs from transgene-mediated silencing at the initiation stage because the primary dsRNA inducer is generally thought to be the viral double-stranded replication intermediates, hence circumventing the requirement of a host RdRP. However, virus-induced silencing is similar to transgene-mediated silencing at the maintenance stage in that both require siRNA amplification and intercellular silencing signaling. Indeed, several studies have shown that plant viral silencing suppressors function primarily by preventing the movement of silencing signals out of the initially infected cells (14, 34, 35, 42). Therefore, plants encoding defective RDR6 would be expected to become more susceptible to virus infections. It was hence unexpected that mutant *Arabidopsis* plants lacking a functional *RDR6* gene (*sde1* and *sgs2* plants) failed to show increased susceptibility to most of the RNA plant viruses tested (6, 32).

This inconsistency between the role of RDR6 in RNA silencing maintenance and apparent lack of heightened viral susceptibility in its absence led us to speculate that the effect of RDR6 could be masked by specific experimental conditions that affect the efficacy of RNA silencing. Consistent with this speculation, higher plant growth temperature has recently been found to enhance RNA silencing (39). In this report, we have evaluated the role of a *Nicotiana benthamiana* homolog of RDR6, NbRDR6, in plant antiviral defense under different temperature conditions. Our results demonstrate that NbRDR6 is actively involved in defending both differentiated and apical plant tissues from invasion by several different RNA plant viruses, including members of the genera *Potexvirus*, *Carmovirus*, and *Tobamovirus*. We show that the consequence of NbRDR6 down-regulation depends on both the plant growth temperature and the nature of the invading virus, reflecting the delicate balance between the efficacy of the host RNA silencing and the ability of the invading virus to counteract this process.

MATERIALS AND METHODS

Cloning of full-length cDNA of NbRDR6. The tomato-expressed sequence tag EST360431 was identified by BLAST searches of The Institute of Genome Research (TIGR) database as being most closely related to *Arabidopsis* RDR6. Because the corresponding genes of tomato and *N. benthamiana* share high sequence homology at the nucleotide level (27), the sequence of EST360431 was used to design primers for successfully amplifying a cDNA fragment from *N. benthamiana* by reverse transcription coupled with PCR. The sequence of the amplified fragment served as the basis for further efforts in obtaining full-length cDNA of NbRDR6 by use of the procedure of rapid amplification of cDNA ends.

Virus-induced gene silencing (VIGS) of NbRDR6. The modified potato virus X (PVX) vector used in our work was kindly provided by Vicki Vance and colleagues (33). The EcoRV and NotI cloning sites were used to insert an 1,112-bp NbRDR6 fragment (nt 167 to 1288 of its cDNA) to produce PVX-NbRDR6. Infectious transcripts of the construct were used to infect *N. benthamiana* plant leaves, which were collected at 5 days postinoculation (dpi) and used for further infection.

Virus stocks and inoculations. Turnip crinkle virus (TCV) was propagated from infectious transcripts of pT1d1 (16). Capped transcripts of the PVX vector were used as the inoculum to propagate PVX. Tobacco mosaic virus (TMV) inoculum was propagated from infectious transcripts of the cDNA clone of a green fluorescent protein (GFP)-tagged strain of TMV (TMV-GFP) (38). Groups containing at least four infected plants each were reared under the conditions described in Results, and the experiments were repeated at least three times.

Generation of transgenic *N. benthamiana* plants expressing dsRNA targeting NbRDR6. Two fragments of NbRDR6 cDNA, 630 bp (nt 2970 to 3600) and 1,133 bp (nt 3811 to 2678) in length, were cloned into the vector pRTL2 (1) downstream of the cauliflower mosaic virus 35S promoter, with the 630-bp fragment in the sense orientation and the 1,133-bp fragment in the antisense orientation. The larger fragment contained the whole sequence of the smaller one, so that transcription in plants would produce NbRDR6-specific dsRNA. The complete cassette, including the 35S promoter and terminator sandwiching the insert, was then subcloned to the binary vector pPZP212. The resulting construct, pPZP-dsRDR6, was brought into *Agrobacterium tumefaciens* strain C58C1, which was used to transform *N. benthamiana* leaf disks (18).

RNA blot hybridizations. *N. benthamiana* plants with five to six true leaves were inoculated on the first two true leaves, and the infected plants were subject to RNA extraction 2 to 3 weeks after infection. To ensure the data generated between different treatments are comparable, we counted as the first systemic leaf the topmost emerging leaf with the length of the main vein being at least 1 cm and, except for some rare cases specifically noted in Results, picked the third systemic leaves of the infected plants for RNA extraction. To detect mRNA of NbRDR1 (a previously identified RdRP from *N. benthamiana* [45]), NbRDR6, NbPHV (the *N. benthamiana* homolog of PHV), and NbACT (*N. benthamiana* actin), 5 μg of total RNA of each sample was subjected to RNA blot hybridization with in vitro-transcribed RNA probes of approximately 800 nt long. The NbPHV-specific probe was synthesized from an 800-bp fragment of NbPHV cDNA containing a T7 promoter at its 5′ end. This sequence is highly homologous to that of *Nicotiana sylvestris* PHV (30). The NbACT probe was generated by reverse transcription-PCR using primers based on the tobacco actin sequence. The hybridizations were carried out at 68°C in UltraHyb buffer (Ambion, Austin, TX). For detection of miR165, low-molecular-weight RNA was enriched from total RNA samples and subjected to hybridization with the end-labeled complementary oligonucleotide at 35°C in UltraHyb-Oligo buffer (Ambion, Austin, TX). The membranes were washed twice at 42°C for 30 min each with a buffer containing 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 0.5% sodium dodecyl sulfate before exposure to X-ray films.

Microscopy. The uppermost (2-mm) regions of plant apices were detached and examined with an Olympus FluoView 500 confocal laser scanning microscope.

Nucleotide sequence accession number. The cDNA sequence of NbRDR6 has been deposited into GenBank with accession number AY722008.

RESULTS

Characterization of NbRDR6. *N. benthamiana* was chosen as the experimental host to investigate the effect of temperature on the antiviral role of RDR6 because it grows well at a relatively wide range of temperatures (at least between 15 and 33°C) and it is susceptible to a broad spectrum of well-characterized plant viruses. Importantly, the RNA silencing process has been shown to be robust in *N. benthamiana* by numerous previous studies. The sequence of full-length NbRDR6 cDNA was resolved (see Materials and Methods), revealing its amino acid sequence to be 62% identical and 76% similar to *Arabidopsis* RDR6 but only 37% identical and 53% similar to NbRDR1. In addition, it is only 34% identical and 52% similar to NbRDR2 (GenBank accession number AY722009), another *N. benthamiana* RdRP we have characterized (data not shown). Taken together, these data strongly suggest that NbRDR6 is the homolog of *Arabidopsis* RDR6.

We next demonstrated, by examining the impact of VIGS-based NbRDR6 down-regulation on transgene silencing, that NbRDR6 also functioned similarly to *Arabidopsis* RDR6 in RNA silencing maintenance. We took advantage of the GFP 16c line of *N. benthamiana* plants, which express GFP at high levels but which could be systemically silenced by transiently overexpressing GFP in very young plants (42). Upon completion of systemic silencing of GFP in the 16c plants, which were monitored using a handheld long-wavelength UV lamp (Fig. 1A), we infected these same plants with PVX-NbRDR6, designed to down-regulate NbRDR6 expression through VIGS. Infection with PVX-NbRDR6 led to reexpression of the silenced GFP transgene (Fig. 1A, bottom), whereas control

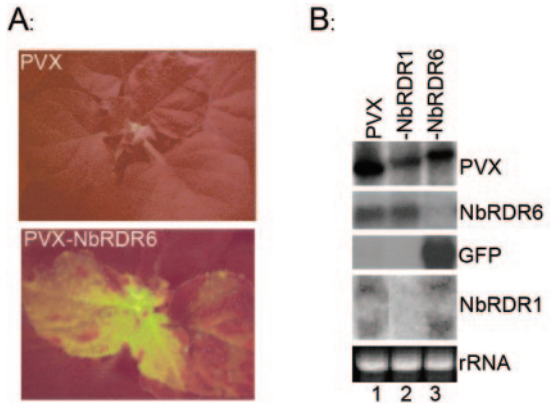

FIG. 1. PVX-mediated VIGS of NbRDR6 disrupts silencing of a GFP transgene in *N. benthamiana* 16c plants. (A) Images of systemically silenced 16c plants infected with the PVX vector (top) and PVX-NbRDR6 (bottom), showing that the silenced status of GFP was disrupted by VIGS of NbRDR6. (B) Total RNA extracted from plants infected with either the PVX vector alone or the vector containing the sequence of either NbRDR1 or NbRDR6 were subjected to RNA blot hybridization using the probes indicated to the right of the panels. The bottom panel shows the ethidium bromide-stained gel, serving as the loading control.

plants infected with PVX vector alone remained silenced (Fig. 1A, top). As an additional control, infection with a PVX derivative containing a portion of the NbRDR1 sequence (PVX-NbRDR1) also failed to disrupt the silencing of the GFP transgene (data not shown). RNA blot hybridization with a PVX-specific probe revealed the accumulation levels of genomic RNAs of respective VIGS constructs (Fig. 1B, top). Rehybridization of the same RNA samples with an NbRDR6 probe showed that NbRDR6 mRNA was reduced to below the level of detection by PVX-NbRDR6 infection (Fig. 1B, panel 2, lane 3) but not by either PVX or PVX-NbRDR1 infection (Fig. 1B, panel 2, lanes 1 and 2). The reduction of the NbRDR6 mRNA level was accompanied by a corresponding increase of GFP expression (Fig. 1B, panel 3, lane 3).

Previous studies by others have shown that RDR1 plays an important role in antiviral defense with both tobacco and *Arabidopsis* but that it is nonfunctional in *N. benthamiana* due to the presence of two premature stop codons in the middle of its mRNA (44–46). Here we also hybridized the RNA samples with an NbRDR1 probe. The NbRDR1 mRNA level was reduced by infection with PVX-NbRDR1 but not by infection with PVX-NbRDR6 (Fig. 1B, panel 4), indicating that VIGS of NbRDR6 did not lead to nonspecific targeting of NbRDR1. The NbRDR1 mRNA, similarly to its tobacco counterpart, was detected as two distinct bands (Fig. 1B, panel 4) (44). However, its expression level is rather low even in the absence of VIGS and, as we will show later, highly variable, suggestive of it being an expressed pseudogene (45). Together, these results verify that NbRDR6 is indeed functionally homologous to *Arabidopsis* RDR6.

Transgenic plants expressing dsRNA targeting NbRDR6 display enhanced susceptibility to TCV in a temperature-dependent manner. After the role of NbRDR6 in the maintenance of transgene silencing was established, we initially used the same PVX-based VIGS approach to show that plants down-regulated for NbRDR6 expression were generally more susceptible to subsequent infection by both TCV and TMV (F. Qu and T. J. Morris, unpublished data). However, the synergy between PVX and the challenger viruses caused very severe necrosis, making molecular verification difficult. We hence decided to evaluate the role of NbRDR6 in antiviral silencing by using transgenic plants expressing a dsRNA construct targeting NbRDR6. A total of 36 lines of T1 plants were screened for decreased expression of NbRDR6 mRNA with RNA blot hybridization. This screen identified eight lines that showed dramatically lower expression of NbRDR6 mRNA with no visible developmental defects. We chose one of the lines (line 6) for further experimentation.

We infected the dsRDR6 plants with TCV and kept the infected plants at 21 and 27°C, respectively, to monitor for possible temperature-dependent effects. The temperature effect on TCV symptoms was evident as early as 7 dpi and was clearly visible at 14 dpi (Fig. 2A). Wild-type (WT) and dsRDR6 plants both showed equally severe symptoms at 21°C, while at 27°C the dsRDR6 infected plants were evidently more severely diseased and stunted than WT plants. The differences in symptom severity correlated well with levels of viral RNA in the respective plants (Fig. 2B, top). Note that the TCV genomic RNA accumulated to levels nearly equal to that of the 25S rRNA in both WT and dsRDR6 plants at 21°C (Fig. 2B, bottom, lanes 5 to 8). Importantly, at the higher temperature of 27°C, the level of the viral RNA was dramatically reduced in WT plants (Fig. 2B, top and bottom, compare lanes 5 and 6 to lanes 13 and 14) but less so in dsRDR6 plants (Fig. 2B, compare lanes 7 and 8 to lanes 15 and 16). The RNA samples were also hybridized with an NbRDR6 probe to verify that the NbRDR6 mRNA was consistently below the level of detection in all of the dsRDR6 plants at both temperatures (Fig. 2B, panel 2). We further subjected these same RNA samples to hybridization with an NbRDR1 probe to determine if the dsRNA transgene might also interfere with the expression of other RdRPs. The result (Fig. 2B, panel 3) revealed that the levels of NbRDR1 mRNA, while highly variable, do not correlate with the levels of NbRDR6. The variation in the NbRDR1 mRNA levels could reflect the previous finding that RDR1 in *N. benthamiana* is likely an expressed pseudogene (45). In conclusion, these data strongly suggest that NbRDR6 plays a significant role in antiviral defense in *N. benthamiana* at the higher temperature.

PVX viral RNA is more abundant in dsRDR6 plants than in WT plants at both low and high temperatures. We next tested infection with a second unrelated RNA plant virus, PVX, on the dsRDR6 plants to determine if the temperature effect was a more general feature of NbRDR6-mediated antiviral defense. The symptoms for PVX at 14 dpi were similar to those observed for TCV. As shown in Fig. 3A, the symptoms of infected dsRDR6 plants were more severe and plant growth was more stunted than for their WT counterparts at the higher incubation temperature (27°C). Interestingly, a significant increase in PVX genomic RNA accumulation was evident with the dsRDR6 plants compared to the WT plants at both temperatures, an indication that the temperature effect was less dramatic for the PVX infections. Again, viral RNA accumulation appeared to be slightly better in both types of plants at 21°C than at 27°C (Fig. 3B, top). These results add further

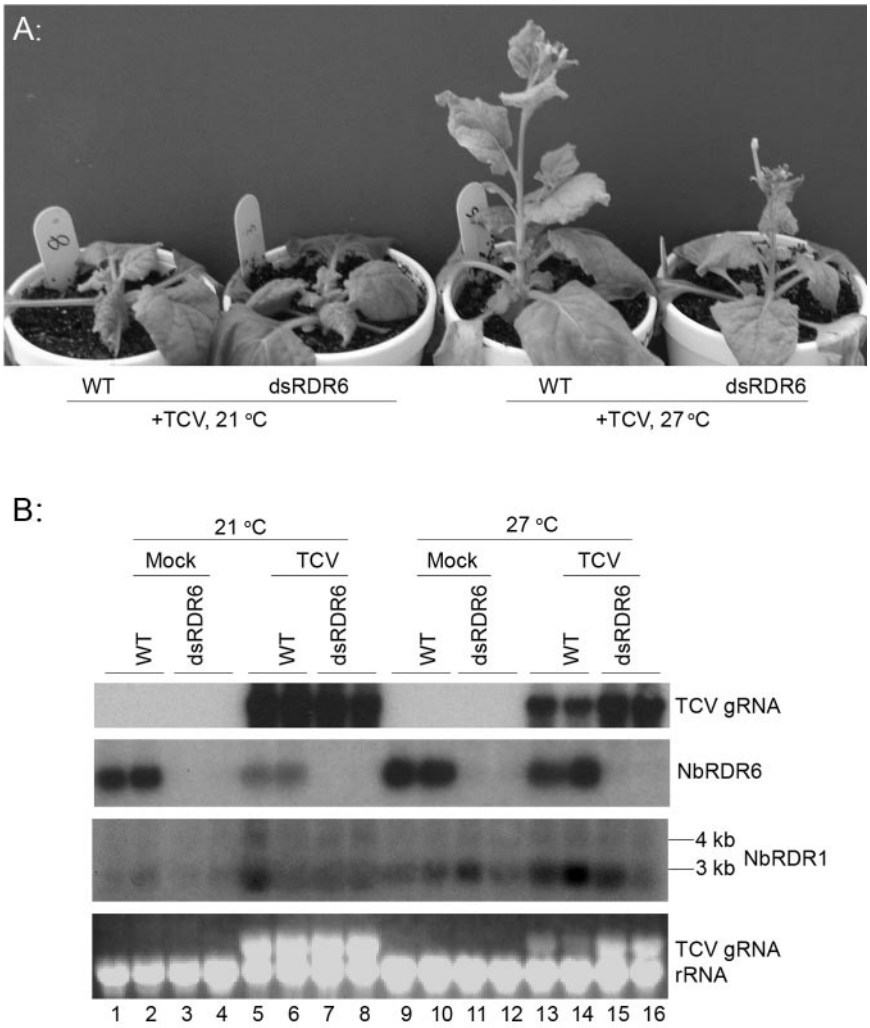

FIG. 2. Transgenic dsRDR6 *N. benthamiana* plants are more susceptible to TCV infection at higher growth temperature. (A) WT and dsRDR6 plants infected with TCV at 21 and 27°C at 14 dpi. Note the significantly greater symptom severity and stunting in the dsRDR6 plants kept at 27°C. (B) Total RNA samples extracted from both WT and dsRDR6 plants, mock infected or TCV infected, reared at 21 or 27°C, were subjected to RNA blot hybridization with the probes indicated to the right of the panels. Hybridization with the TCV probe (top) was performed using 0.5 μg of total RNA from each sample, showing a marked reduction of TCV RNA accumulation in the 27°C WT samples. In the second and third panels, RNA extracts of 5 μg each were hybridized with NbRDR6 and NbRDR1 probes, respectively. The NbRDR6 mRNA was consistently undetectable in dsRDR6 plants (panel 2). The lower level of NbRDR6 mRNA seen in lanes 5 and 6 likely reflects the reduced proportion of cellular RNA due to the very high levels of TCV RNA in the infected samples (panel 2). The NbRDR1 mRNA levels are highly variable and do not correlate with that of NbRDR6 mRNA. The bottom panel is an ethidium bromide-stained gel serving as the loading control. gRNA, genomic RNA.

support to the conclusion that NbRDR6 plays an important role in antiviral defense. In addition, these results suggest that PVX infection may behave more similarly to cucumber mosaic virus (CMV) infection, in that the impact of NbRDR6 down-regulation was detectable over a broader temperature range (32). Interestingly, both PVX and CMV are thought to have relatively weak suppressors of RNA silencing (12, 17, 42).

TMV-GFP infection caused more-severe plant stunting in NbRDR6 down-regulated plants grown at higher temperature. We next tested the susceptibility of the dsRDR6 plants to TMV-GFP at the two experimental temperatures (38) so that we could visually monitor the effects of temperature on virus spread in the infected plants. As was observed for the TCV and PVX infections, when kept at 21°C, both WT and dsRDR6 plants infected by TMV-GFP showed comparable symptoms 2 to 3 weeks after infection (Fig. 4A, top). Again, plants kept at 27°C consistently displayed milder symptoms than their 21°C counterparts. The differences between infected WT and dsRDR6 plants were more subtle and occurred later than for either the TCV- or the PVX-infected plants (Fig. 4A, bottom). Moreover, it was not consistently possible to correlate the levels of GFP fluorescence and viral RNA accumulation with the degrees of stunting of the infected plants. Based on the data we will present in the next section, we speculate that the difference in degree of stunting likely resulted from the more efficient apical colonization by TMV-GFP in the dsRDR6 plants.

Down-regulating the NbRDR6 expression promotes TMV invasion of shoot apices. As noted in the previous section, aside from the obvious stunting of plants held at 27°C, the WT

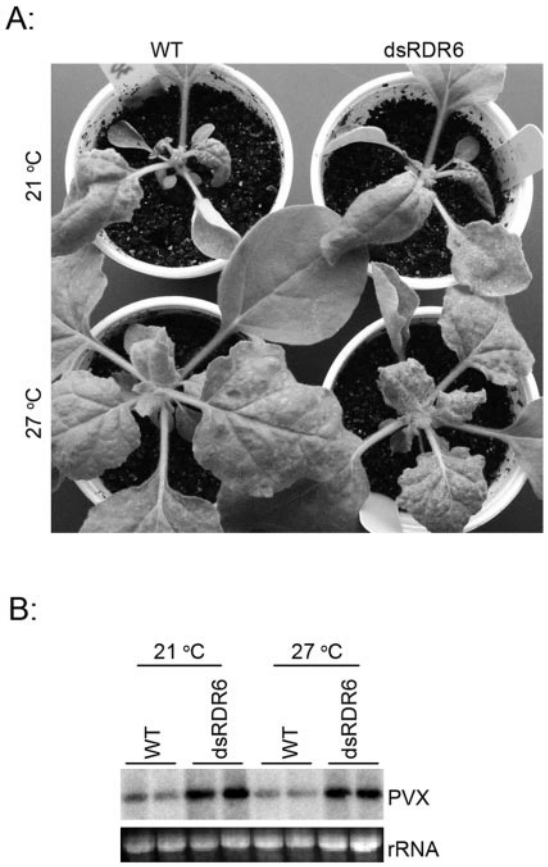

FIG. 3. Transgenic dsRDR6 plants permit higher levels of PVX replication than do WT plants at both temperatures tested. (A) PVX-infected plants at 14 dpi, showing slightly greater symptom severity in the dsRDR6 plants kept at 27°C. (B) RNA blot hybridization using a PVX-specific probe, showing that PVX viral RNA accumulated to higher levels at both 21 and 27°C in the dsRDR6 plants than in the WT plants.

and dsRDR6 plants infected with TMV-GFP displayed very similar symptoms when held for up to 3 weeks after infection. Intriguingly, when we continued to monitor the infected plants for a more extended period of time, we observed highly unusual leaf deformations in infected dsRDR6 plants kept at 21°C, beginning about 5 weeks after infection. As shown in Fig. 5A (panels 1 and 2), the leaves of TMV-GFP-infected dsRDR6 plants kept at 21°C had odd shapes, ranging from long rods without any blades and thick midveins with narrow and irregular blades to leaves with long petioles and short terminal blades which curled upwards to form cup-shaped structures. A majority of the newly emerging leaves on these plants were abnormal, giving the plants the crab-leg-like appearance quite distinct from the appearance of the WT plants under the same conditions (Fig. 5A, panel 1). Similarly deformed leaves were seen less frequently on infected dsRDR6 plants kept at 27°C (Fig. 5A, panels 3 and 4), very occasionally on infected WT plants, and never on mock-infected plants. Furthermore, plants kept at 27°C were beginning to flower at 5 weeks after infection and although they had notably fewer flowers than WT plants, the dsRDR6 infected plants had a much higher proportion of deformed filamentous flowers (Fig. 5A, panel 5). Again, the increased proportions of leaf and flower abnormalities could not be directly attributed to differences in viral accumulation levels in plant tissues at these advanced stages of infection (data not shown).

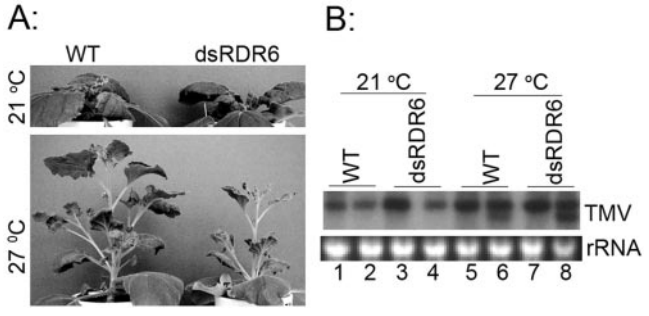

FIG. 4. Transgenic dsRDR6 plants infected with TMV-GFP show greater stunting than WT plants at 27°C. (A) TMV-GFP-infected plants at 21 dpi. (B) RNA blot hybridization using a TMV-specific probe, showing that viral RNA accumulation levels were similar for WT and dsRDR6 plants at both temperatures.

The leaf abnormalities described above closely resembled those documented for both *Arabidopsis* and *N. sylvestris* mutant plants with gain-of-function mutations in *PHB*, *PHV*, and *REV* genes (10, 29, 30) and for transgenic *Arabidopsis* as well as *N. benthamiana* plants expressing virus-encoded suppressors of RNA silencing (2, 9, 11). This suggested to us that in the dsRDR6 plants, the silencing suppressor encoded by TMV, the small subunit of TMV replicase (25), might be interfering with the miRNA-guided developmental regulation, as exemplified by the miR165-mediated degradation of *PHB*, *PHV*, and *REV* mRNAs. We therefore tried to evaluate the accumulation levels of both the mRNA of Nb*PHV* and miR165 in the apical tissues of the infected plants. For this purpose, we extracted RNA exclusively from the terminal 15 mm of stems and branches of uninfected and infected WT and dsRDR6 plants held at 21 and 27°C.

The RNA samples were first subjected to hybridization with a TMV probe to confirm the presence of TMV-GFP RNA in the apical tissues. The infected dsRDR6 plants accumulated TMV-specific RNA to only slightly higher levels in the apical tissues than did WT plants at both temperature conditions (Fig. 5B, panel I). This, however, may not completely reflect the difference in apical invasion because the true apical meristems constituted only a small portion of the tissues we collected for RNA extraction. The TMV genomic RNA band migrates slightly more quickly in the 27°C samples than in the 21°C samples, resulting from more frequent deletion of the GFP insert at the higher temperature (data not shown).

We then carried out RNA blot hybridization using an Nb*PHV* probe. The results presented in Fig. 5B, panel II, show significant differences in the levels of Nb*PHV* mRNA in these tissues. While the uninfected dsRDR6 plants expressed Nb*PHV* at a lower level than the WT plants (Fig. 5B, panel II, compare lanes 3 and 4 to lanes 1 and 2), this did not seem to visibly affect *N. benthamiana* development. However, the TMV-infected dsRDR6 plants accumulated Nb*PHV* mRNA to substantially higher levels than did WT plants (Fig. 5B, panel II, lanes 7 and 8 versus lanes 5 and 6). Moreover, the increase in the level of Nb*PHV* mRNA was most marked when the

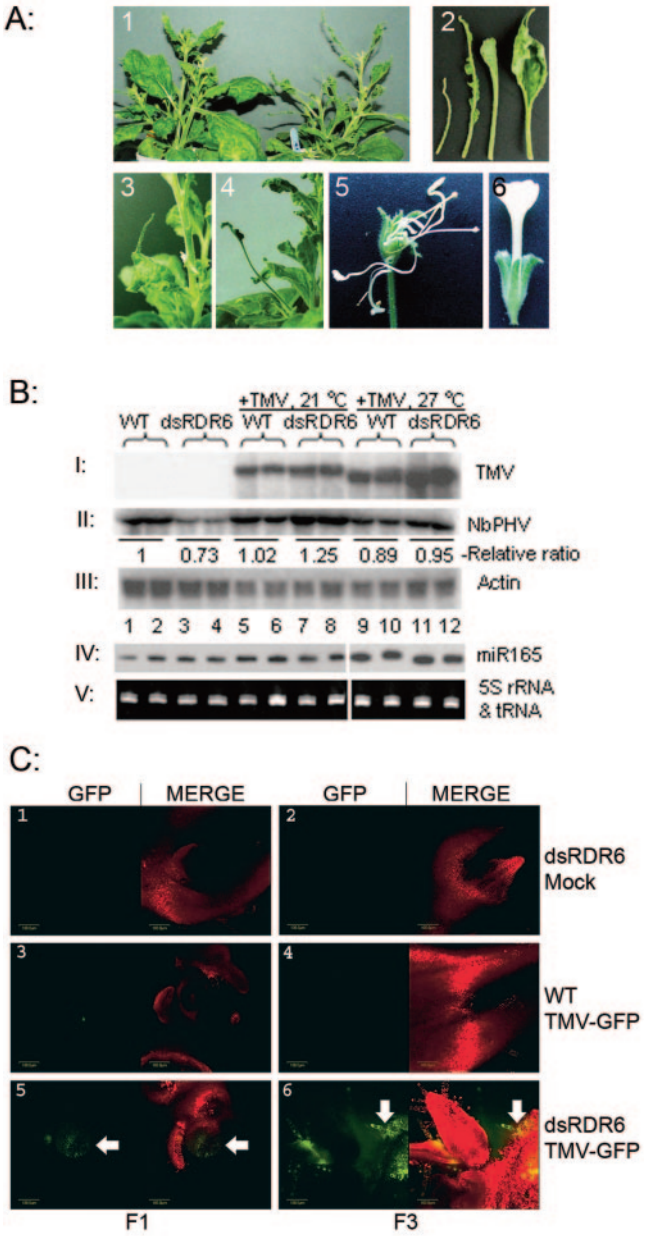

FIG. 5. TMV-GFP efficiently colonizes the shoot apices of transgenic dsRDR6 plants, causing developmental defects in leaves and flowers. (A) (Panel 1) TMV-GFP-infected WT (left) and dsRDR6 (right) plants kept at 21°C for 5 weeks. Note the distinctive crab-leg-like appearance of leaves on the dsRDR6 plants. (Panel 2) Characteristic deformations seen with selected leaves of the TMV-GFP-infected dsRDR6 plants kept at 21°C. (Panels 3 and 4) Similar leaf abnormalities seen with TMV-GFP-infected dsRDR6 plants kept at 27°C. (Panel 5) Typical flower abnormality observed with the TMV-GFP-infected dsRDR6 plants kept at 27°C. (Panel 6) Flower from a noninfected dsRDR6 plant kept at 27°C. (B) Total RNA was extracted from the apical tissues of the plants treated as described in Results and subjected to RNA blot hybridization with TMV (panel I), NbPHV (panel II), and actin (panel III) probes. The numbers underneath panel II show the relative levels of NbPHV mRNAs, determined by densitometry (Molecular Dynamics). The same RNA samples were also subjected to hybridization to illustrate the level of miR165 (panel IV). (C) Confocal microscopic images of plant shoot apices. Microscopic parameters, including laser settings, were the same for all images in the six panels. Bar = 100 μm. Each panel shows two different images, with the left image reflecting the GFP signal and the right image showing the result of merging the GFP signal with that of the chlorophyll autofluorescence which depicts the organ shape. (Panels 1 and 2) Mock-inoculated dsRDR6 plants. (Panels 3 and 4) TMV-GFP-infected WT plants. (Panels 5 and 6) TMV-GFP-infected dsRDR6 plants. Panels 1, 3, and 5 each depict an F1 floral primordium. Panels 2, 4, and 6 depict a more developed but unopened flower (F3), with the flower in panel 6 being manually opened. Arrows highlight GFP fluorescence.

infected dsRDR6 plant samples were compared to their uninfected counterparts (Fig. 5B, lanes 7 and 8 versus lanes 3 and 4). The difference was less dramatic for plants kept at the higher temperature (Fig. 5B, lanes 11 and 12 versus lanes 9 and 10), consistent with the less striking leaf abnormalities observed with these plants.

To ascertain that the difference in the Nb*PHV* mRNA levels was not caused by uneven loading of samples, we further subjected the RNA samples to hybridization with a probe that detects Nb*ACT* mRNA, which is not known to be targeted by miRNA. As shown in Fig. 5B, panel III, while minor variations in NbACT mRNA level are visible, they clearly do not account for the difference in the levels of Nb*PHV* mRNA.

These results collectively illustrate that the increased accumulation of Nb*PHV* mRNA in the apical tissues of TMV-GFP-infected dsRDR6 plants was highly correlated with the degree of abnormal leaf and floral development, further supporting the notion that the miRNA-mediated regulation of Nb*PHV* expression is most likely interfered with inside the apical tissues of the infected dsRDR6 plants. Interestingly, the accumulation levels of miR165 were unchanged for both healthy and infected WT and dsRDR6 plants (Fig. 5B, panel IV), suggesting that the TMV invasion interrupted the miR165-mediated targeting of Nb*PHV* mRNA rather than the miRNA production. This result is not unexpected given that viral silencing suppressors are known to act at similar steps on the miRNA- and siRNA-mediated pathways (2, 9) and given the finding that the silencing suppressor encoded by tomato mosaic virus, a virus closely related to TMV, blocks the utilization of siRNAs (25).

Together, these results prompted our speculation that the lack of NbRDR6-mediated defense permits TMV-GFP to invade the shoot apices more efficiently, allowing its silencing suppressor to interrupt the miRNA-mediated regulation of expression of these HD-ZIP III genes in leaf primordia. To determine whether TMV-GFP did indeed invade the shoot apices of dsRDR6 plants more readily, we repeated the infection experiments with TMV-GFP at 21°C and inspected the shoot apices of infected plants at 9 dpi, at which point systemic symptoms were evident. To ensure effective expression of GFP in the systemic leaves, we first checked these leaves for distribution of GFP fluorescence under long-wave UV light. For both dsRDR6 and WT, about half of the infected plants showed extensive vein-aligned networks of GFP fluorescence in the systemic leaves, suggesting that both types of plants supported systemic spread of TMV-GFP to similar levels. The uppermost shoot tissues, of about 2 mm in length, were detached from these plants and examined under a confocal laser scanning microscope (Fig. 5C). Usually, three developing flowers were visible on each shoot, with the first one (F1) being a

floral primordium and the third (F3) possessing all floral parts but remaining unopened. GFP fluorescence was never seen in the apical tissues of any of the four infected WT plants examined (see Fig. 5C, panels 3 and 4, for examples of F1 and F3, respectively), but it was clearly visible in all four of the infected dsRDR6 plant apices, within at least one of the developing flowers. Panel 5 of Fig. 5C shows a floral primordium with GFP florescence visible over the entire structure, whereas panel 6 shows a more developed F3 stage flower with even more evident GFP fluorescence. These results strongly suggest that the apical tissues of dsRDR6 plants are indeed more susceptible to sustained TMV-GFP infection than those of WT plants, clearly implicating NbRDR6 in the antiviral defense system operating in the shoot apices.

DISCUSSION

RDR6 is a putative RdRP that was originally identified as being required for the silencing of single-stranded RNA of transcribed transgenes in *Arabidopsis.* It has since been mechanistically implicated in the maintenance of RNA silencing (6, 7, 17, 32, 40). The possible role of RDR6 in the antiviral defense of the plant has been less firmly established, owing to the fact that only a few viruses have been shown to be more infectious in its absence (6, 32). In this report, we have characterized the *N. benthamiana* homolog of RDR6 and investigated the role of NbRDR6 in plant antiviral defense. A transgenic *N. benthamiana* line greatly diminished in NbRDR6 expression was used to test three RNA viruses belonging to distinct virus families for changes in susceptibility. Our results varied depending on the particular virus tested, but each virus displayed a definite increase in invasiveness that was affected by the temperature at which the plant was grown. We conclude, therefore, that the increased susceptibility in the absence of NbRDR6 is a consequence of interference with a step(s) in the RNA silencing pathway that is generally enhanced by higher growth temperature (39). Importantly, we have directly demonstrated, using GFP-tagged TMV, that NbRDR6 plays a critical role in defending shoot apices from virus invasion in *N. benthamiana* plants.

It has long been known that viruses do not commonly invade the shoot apices of infected plants. This is the basis for the agricultural practice of virus elimination by meristem tip culture (19). It is, however, not well understood why viruses are excluded from the meristematic tissues. Some recent evidence showing that viruses were found in the shoot apices of plants transformed with virus-encoded silencing suppressors suggests that meristem exclusion may be related to the RNA silencing-based defense, which is thought to be more robust in young developing tissues (4, 11). In this report, we have shown that incapacitating NbRDR6, a component of the RNA silencing pathway, enabled rigorous replication of TMV-GFP in flower meristems located at the shoot tips of *N. benthamiana* plants. This observation suggests that TMV-GFP is probably not physically barred from entering the shoot apices. Rather, it may be swiftly degraded by the highly active RNA silencing machinery upon its entry into the shoot tips. The fact that leaf and flower abnormalities also occur in TMV-GFP-infected WT plants (albeit much less frequently) argues against the presence of a physical barrier. Collectively, our results strongly support the idea that NbRDR6 is a critical component of the RNA silencing-based antiviral defense operating in the plant shoot apices.

Our observations of the whole infected plants demonstrate that NbRDR6 also plays an important role in the antiviral defense in differentiated leaf tissue for each of the three viruses tested, although there was evident variation in the extents of the effect and the degrees of influence by plant growth temperature. The NbRDR6-mediated antiviral defense seemed to be most effective against PVX, since it limited the PVX viral RNA accumulation even at the lower temperature of 21°C, at which point the RNA silencing-based defense has been shown to be less active in *N. benthamiana* (39). Interestingly, *Arabidopsis* RDR6-mediated antiviral defense was previously found to be effective against CMV but not against other viruses tested (6, 32). This may have been the result of conducting experiments at lower temperatures that are likely suboptimal for the RNA silencing function. It should also be noted that both PVX and CMV are thought to encode suppressors of RNA silencing that are relatively weak compared to those of other viruses in their respective genera (12, 41). This may in turn have contributed to the greater effectiveness of the RNA silencing-based antiviral defense in targeting these viruses, even though this defense mechanism had been weakened by the lower-temperature environment. These data led us to hypothesize that viral invasiveness and hence the outcome of a viral infection are critically influenced by the balance between the robustness of RNA silencing-based defense and the relative strength of the viral silencing suppressor. We recognize that there are many other components of the plant defense system besides silencing that affect viral invasion. However, our hypothesis provides a possible explanation for the role of temperature in this process. Succinctly stated, we suggest that viruses with stronger silencing suppressors are more likely to overcome RNA silencing-based defense weakened by lower growth temperatures, thus minimizing the impact of RNA silencing disruption (for example, in *rdr6* mutant plants) on virus infection at these temperatures. This may explain why most viruses, when tested at lower-temperature environments, replicated to similar levels in plants with or without a functional RDR6 (6, 32; this work). By extension, this hypothesis also predicts that, for viruses with stronger suppressors, the impact of RNA silencing disruption will be detectable when the plant growth temperature rises sufficiently to enable the enhanced silencing-based defense to overcome the virus-encoded suppressor in wild-type plants. Indeed, our results showing that dsRDR6 plants become more susceptible to TCV only at 27°C fit this scenario. It would be useful to test this idea further by comparing the levels of invasiveness of more closely related viruses that encode suppressors of different strengths, such as CMV and tomato aspermy virus (8).

In summary, we have demonstrated here that NbRDR6 is involved in host antiviral defense of both differentiated and meristematic tissues in a temperature-dependent manner. Our results demonstrate that the role of the NbRDR6-mediated RNA silencing pathway is a form of general antiviral defense directed against many more viruses than previously suspected (6, 32). Importantly, we also show that the functionality of the silencing defense pathway mediated by NbRDR6 can be masked by certain environmental conditions, suggesting that

the function of NbRDR6 may be closely related to the temperature sensitivity of the silencing pathway.

ACKNOWLEDGMENTS

We thank Vicki Vance and colleagues for providing us with the modified PVX vector.

This work is supported in part by grants from the U.S. Department of Energy (DE-FG03-98ER20315) and NIH (grant number P20 RR16469) as a part of the INBRE Program of the National Center for Research Resources.

ADDENDUM

A related publication by Schwach et al. (37) became available subsequent to submission of our manuscript for review. This paper confirms our conclusion that NbRDR6 plays an important role in mediating defense against viral invasion of the meristem and provides a model for its function. Importantly, our paper provides additional evidence for the broader involvement of RDR6 in antiviral defense, which is stimulated by higher temperature.

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