Analysis of the Involvement of an Inducible *Arabidopsis* RNA-Dependent RNA Polymerase in Antiviral Defense

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RNA-dependent RNA polymerases (RdRPs) have been implicated in posttranscriptional gene silencing (PTGS) and antiviral defense. An *Arabidopsis* RdRP (*SDE1/SGS2*) has been previously shown to be required for transgene-induced PTGS but has no general role in antiviral defense. On the other hand, we have recently shown that transgenic tobacco deficient in an inducible RdRP (*NtRdRP1*) activity became more susceptible to both *Tobacco mosaic virus* and *Potato virus X*. Thus, different RdRPs may have distinct roles in closely related PTGS and antiviral defense. In the present study, we analyzed roles of a newly identified *Arabidopsis* RdRP gene (*AtRdRP1*) in plant antiviral defense. *AtRdRP1* encodes an RdRP closely related structurally to *NtRdRP1* and is also induced by salicylic acid treatment and virus infection. A T-DNA insertion mutant for *AtRdRP1* has been isolated and analyzed for possible alterations in response to viral infection. When infected by a tobamovirus and a tobnavirus, the knockout mutant accumulated higher and more persistent levels of viral RNAs in both the lower, inoculated and in upper, systemically infected leaves than did wild-type plants. These results suggest that the inducible *AtRdRP1* is the *Arabidopsis* ortholog of *NtRdRP1* and plays a role in antiviral defense. Examination of short viral RNAs and silencing studies using a viral vector harboring an endogenous plant gene suggest that, while not required for virus-induced PTGS, *AtRdRP1* can apparently promote turnover of viral RNAs in infected plants.

The activity of RNA-dependent RNA polymerases (RdRPs) was first reported in Chinese cabbage three decades ago (Astier-Manificier and Cornuet 1971). Since then, RdRPs have been found in a number of plants, including tobacco, tomato, cowpea, and cucumber (Astier-Manificier and Cornuet 1978; Dorssers et al. 1982; Duda 1979, 1973; Takanami and Fraenkel-Conrat 1982). A 128-kDa RdRP from tomato leaf tissue has been purified, and its corresponding gene has been cloned (Schiebel et al. 1993a, 1993b; Schiebel et al. 1998). The tomato RdRP catalyzes in vitro both primed and unprimed synthesis of short complementary RNA (cRNA) using single-

stranded (ss) RNA and DNA but not double-stranded (ds) nucleic acids as templates (Schiebel et al. 1993b). Differences in transcription efficiency were found to depend on nucleotide sequence of templates (Schiebel et al. 1993b). In several studies, an elevated RdRP activity was found in virus-infected plants (Dorssers et al. 1982; Duda 1979, 1973; Khan et al. 1986; Romaine and Zaitlin 1978). Sequences homologous to plant RdRPs have also been found in *Schizosaccharomyces pombe*, *Neurospora crassa*, and *Caenorhabditis elegans* (Cogoni and Macino 1999; Schiebel et al. 1998; Smardon et al. 2000).

The biological functions of RdRPs have been elusive. Based on early observations that RdRP activities were elevated in virus-infected plants, it was speculated that the RdRP activity might be one of the host factors involved in virus replication (Dorssers et al. 1982; Khan et al. 1986; Romaine and Zaitlin 1978). In addition, RdRPs have been proposed to play an important role in posttranscriptional gene silencing (PTGS) (Schiebel et al. 1998). Consistent with their proposed role in PTGS, mutations in the genes encoding proteins similar to plant RdRPs abolish gene quelling in *N. crassa* and RNA interference (RNAi) in *C. elegans* (Cogoni and Macino 1999; Smardon et al. 2000). An RdRP gene from *Arabidopsis* (*SDE1/SGS2*) has also been recently shown to be required for PTGS (Dalmay et al. 2000; Mourrain et al. 2000). *SDE1/SGS2* is also required for spreading of transgene-targeted RNA silencing from the initial regions into the adjacent 5' and 3' regions of the target gene, suggesting that this RdRP may be involved in producing dsRNA using the target RNA as a template (Vaistij et al. 2002).

Because of the close connection between PTGS and antiviral defense, the *Arabidopsis sde1/sgs2* mutants were also tested for virus resistance. Surprisingly, these mutants exhibited enhanced susceptibility to only *Cucumber mosaic virus* but not to several other viruses and were normal in virus-induced PTGS (VIGS) (Dalmay et al. 2000; Mourrain et al. 2000). Further studies using recombinant *Tobacco rattle virus* (TRV) indicated that the *sde1* mutant is normal in the initiation but is compromised in the maintenance of VIGS (Dalmay et al. 2000, 2001). Based on these observations, it has been proposed that, while plant RdRPs are important for transgene-induced PTGS, they are not required for the initiation of VIGS, since viruses contain their own replicases capable of synthesizing dsRNA molecules (Dalmay et al. 2000). However, maintenance (persistence) of VIGS does require *SDE1/SGS2*, possibly for synthesis of transgene-specific dsRNA that are involved in spreading of RNA targeting, leading to more long-lived degradation of transgene mRNA (Dalmay et al. 2001). It has been argued that, in contrast to other viruses, *Cucumber mosaic virus* replication produces aberrant RNAs that are similar

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to those postulated to be produced by transcription of transgenes (Voinnet 2001). SDE1/SGS2 would not be important in plant defense against the viruses that do not produce the putative aberrant RNAs required for persistent VIGS or the viruses that produce strong suppressors of PTGS. These results argue against a general role of SDE1/SGS2 in antiviral defense (Dalmay et al. 2000).

During the studies of salicylic acid (SA)-induced resistance to *Tobacco mosaic virus* (TMV), we have recently discovered that TMV infection and SA treatment enhance the activity of a tobacco RdRP (NtRdRP1) and induce its gene expression (Xie et al. 2001). This observation suggests that induction of this plant RdRP may be part of plant defense response to viral pathogens. Tobacco lines deficient in the inducible RDRP

activity were obtained by expressing antisense RNA for the *NtRDRP1* gene in transgenic plants (Xie et al. 2001). When infected by TMV, these transgenic plants accumulated significantly higher levels of viral RNAs and developed more severe disease symptoms than wild-type plants (Xie et al. 2001). After infection by a strain of *Potato virus X* (PVX) that does not spread in wild-type tobacco plants, the transgenic *NtRDRP1* antisense plants accumulated virus and developed symptoms not only locally in inoculated leaves but also systemically in upper uninoculated leaves (Xie et al. 2001). These results strongly suggest that the inducible RdRP activity plays a role in plant antiviral defense (Xie et al. 2001).

In *Arabidopsis*, there are six genes encoding proteins similar to RdRPs (Dalmay et al. 2000). This raises the possibility that

Atr1: 1	MGKTIQVFGFPNGVSAEEVKKFLERLTGSGTVYAIKVRQPKKGGPRVYAIVOFTSERHTR	60
Ntr1: 1	MGKTIQVFGFPYLLSAEAVKKFLENHTGNGTVYALEVKQ-SKGGRRAFKQVQFANNKSAE	59
Atr1: 61	LIITAAERLYYGRSYLKAFEEVQDIVPKPRASLHTISGLKMFEGCQVSTKKFLTILWSAQ	120
Ntr1: 60	FIIDLASKGLYYGPSYLKAWEMKTDIV-QPRTYVEHMDGVTLNFGCQISDKKFAVLGS-K	117
Atr1: 121	DVCVSFGIGMRKLHFSFSWYQKDYRLSESYENIWIDLHSPQGRSSKFLVIQVIGAPKIF	180
Ntr1: 118	DVSIKFGIGLKKIYFFLSHASADYKLOLSYENIWQVVLHRPYGONAOFLLLQLFQAPRIY	177
Atr1: 181	EKEDQPINLLFGIMDFYSDGSDQWIRRTDFTSSSCIGOSTAFCLELPVHLNVPDFRENF	240
Ntr1: 178	KRLED-----SCYSFFKETPDQWVRTDFTPS-WIGLSSSLCLEFRNGVQLPNFSESEF	230
Atr1: 241	ANYAEHRASSFLIESGSSYS-SNANTLVVVDPPGFSLEFEILFKLNTLVONACLSGPA	299
Ntr1: 231	FYYKESM-NQFILOTGFTFSFSQKLALVPIVHLPEGIELBYKILFKISSLIQHGCFPGLA	289
Atr1: 300	LDLDFYRLNQQKYDRALIDHCLEKLFHLGECCEPAHWLRDEYKKWISKGKLPLSPTIS	359
Ntr1: 290	LNFNFQFLVDPRRRNIACTIEHALEKLYLKECCYDVRWLTEQYDEYLGKROLPKSPPIIT	349
Atr1: 360	LDDGLVYMYRVQVTPARVYFSGPEVNVSNRVLRHYSKYINNFLRVSFVDEDELEKVRSMDL	419
Ntr1: 350	LDDGLVYVRRVVVTPCKVYFCGPEVNVSNRVLRYSEDINNFLRVSFVDEEWEKIHSTDIL	409
Atr1: 420	SPRSSTQR--RTKLYDRIYSVLRDGIIVIGDKKFEFLAFSSSQLRRENSAWMFAPIDRITAA	477
Ntr1: 410	LPRASTGNGTRTDIYERILSTLRNGFIIGDKRFEFLAFSSSQLRDINSVMMFASRPGLTAN	469
Atr1: 478	HIRAWMGDFDHIRNVAKYAARLGQSFSSSRETLNVRSDIEVIPDVEIISLGTRYVVFSDG	537
Ntr1: 470	DIRTWMGDFRQIRNVAKYAARLGQSEFGSSRETLNVRSDIEVIPDVACSLHGTNYIFSDG	529
Atr1: 538	IGKISAEFARKVARKCGLTEFSPSAFOIRYGGYKGVVAVDPNSSKKLSLRKSMSKFESEN	597
Ntr1: 530	IGKISADFAHRVTIKCGL-QYTPSSFOIRYGGYKGVVAVDPYSSMKLSLRKSMLKYESNN	588
Atr1: 598	TKLDVLAWSKYQPCYMNROLITLLSTLGVTDVFEKKQREVVDRDLDAILTHPLEAHEALG	657
Ntr1: 589	IKLDVLGWSKYQPCYLNROLVTLSTLGVTDVLEQKQNEAVDQDLDAILDHSLKAQEALE	648
Atr1: 658	LMAPGENTNILKALILCGYKPDAPFLSMMLQNFRAKLLLELRTKTRIFISGGRSMMGCCL	717
Ntr1: 649	LMSPGENTNILKEMLNCGYMPDAPFLSMMLQTFRAKLLDLRTKTRIFIPNGRTMMGCCL	708
Atr1: 718	DETRTLEYGOVVQYSDPMRPGRR-----FIITGPVVVAKNPCLHSG	759
Ntr1: 709	DESRTLEYGOVFVQFSGA---GRRQFFESHPPFNDSGSANCDFILKGNVVVAKNPCLHPG	765
Atr1: 760	DVRVLQAVNVPALNHMVDCVVFPOKGLRPHNECSGSDLDGDIYFVCWDQELVPPRTSEP	819
Ntr1: 766	DIRVLRADVVPALHHMVDCVVFPOKGRPHNECSGSDLDGDIYFVCWDPDLIPPRQVQS	825
Atr1: 820	MDYTPEPTQILDHDVTIEVEEYFANYIVNDSLGIANAHTAFADKEPLKAFSDPCIELA	879
Ntr1: 826	MDYTAPATTQLDHDVTIEVEEYFTNYIIVNDSLGIANAHVVFADREPDMAMSDPCQOLA	885
Atr1: 880	KKFSTAVDFPKTGVAIVIPQHLVYKEYPDFMEKPKDPTYESKNVIGKLFREVKERAPPLI	939
Ntr1: 886	QLFSIAVDFPKTGVPAAEIPSQLRPEYPDFMEKPKDPTYSEKVNIGKLFQKVKNKTPQAS	945
Atr1: 940	SIKSFTLDVASKSYDKDMEVDGFEEYVDEAFYOKANYDFKLGNLMDYYGIKTEAEILSGG	999
Ntr1: 946	SIATFTTRDVARKSYDSDMIVDGFEDYIDEAFYKSEYDNKLGNLMDYYGIKTEAEILSGG	1005
Atr1: 1000	IMRMSKSFTRRRDAESIGRAVRLRKEITLSIFNASEEEENESAKASAWYHVITYHSSYWGL	1059
Ntr1: 1006	IMKASKTFDRRKDAEAIGVAVRCLRKEARAWFKRRSDIDMLAKASAWYHVITYHHTYWGL	1065
Atr1: 1060	YNEGLNRDHFLSFAWCVDKLVRIKKTNLGRRQROETLERLDHVLRFQ	1107
Ntr1: 1066	YNEGLKRDHFISFPWCVDOLIQIKKAKARKRPVPHLSSSLGSQLSRKLVIV	1116

Fig. 1. Sequence alignment of AtRdRP1 with NtRdRP1. Black boxes are identical residues; dashed lines indicated gaps.

different plant RdRP proteins many have different biological functions. *SDE1/SGS2* is required for transgene-induced PTGS but does not play a general role in plant antiviral response (Dalmay et al. 2000; Mourrain et al. 2000). On the other hand, transgenic antisense tobacco plants deficient in the inducible NtRdRP1 exhibited enhanced susceptibility to both TMV and PVX. Thus, certain members of the RdRP gene family may have an important role in transgene-induced PTGS, while others are more involved in antiviral defense. Interestingly, sequence analysis indicated that among the six RdRP genes in *Arabidopsis*, *SGS2/SDE1* shares only a limited sequence similarity to *NtRdRP1*, but another RdRP gene (*AtRdRP1*), located on BAC F10B6, is structurally more closely related to *NtRdRP1* (Xie et al. 2001). In the present study, we report that, like *NtRdRP1*, *AtRdRP1* is induced by SA treatment and virus infection. To study its function directly, we have isolated a T-DNA insertion mutant for *AtRdRP1* and found that the mutant is also more susceptible to viruses. These results indicated that *AtRdRP1* is a functional analog of *NtRdRP1* and plays a role in antiviral defense.

RESULTS

Isolation and expression of *AtRdRP1*.

To isolate *AtRdRP1* cDNA clones, we screened an *Arabidopsis* cDNA library with an *AtRdRP1* DNA fragment probe amplified from *Arabidopsis* genomic DNA with two *AtRdRP1*-specific primers. Of several positive cDNA clones isolated, the longest one was sequenced, and the amino acid sequence of the encoded protein was deduced. As shown in Figure 1, the isolated cDNA clone encodes a protein of 1,107 amino acid residues. *AtRdRP1* shares 60% identical amino acid residues with

NtRdRP1 but is only 34% identical with *SDE1/SGS2*. Phylogenetic analysis demonstrated that the previously isolated tomato RdRP1 (*LeRdRP1*) (Schiebel et al. 1998) is highly homologous to NtRdRP1 and that *AtRdRP1* is more closely related to NtRdRP1 and *LeRdRP1* than to any of the other five *Arabidopsis* RdRPs, including *SDE1/SGS2* (Fig. 2). *SDE1/SGS2* appears to be most distantly related to *LeRdRP1* and NtRdRP1 (Fig. 2).

NtRdRP1 is induced by SA and virus infection (Xie et al. 2001), whereas *LeRdRP1* is induced by viroid infection (Schiebel et al. 1998). To determine whether expression of *AtRdRP1* is also inducible, we analyzed the levels of its transcript in *Arabidopsis* plants after SA treatment or infection by a crucifer strain of tobamovirus, TMV-cg, that infects *Arabidopsis* (Ishikawa et al. 1991). As shown in Figure 3A, little transcript for *AtRdRP1* was detected in untreated plants. Between 2 to 4 h after SA treatment, a transcript of *AtRdRP1* was induced, and the elevated levels were observed throughout the 24 h of the experiments (Fig. 3A). In TMV-cg-infected plants, elevated levels of the *AtRdRP1* transcript were first detected 2 days postinoculation (dpi) and were maintained at 3 to 4 dpi, followed by a substantial decline at 5 dpi (Fig. 3B). On the other hand, expression of *SDE1/SGS2* is constitutive with no significant change in SA-treated or virus-infected plants (data not shown). Thus, *AtRdRP1* shares not only a similar structure but also a similar expression pattern with *NtRdRP1*.

Isolation of an *AtRdRP1* T-DNA insertion mutant.

To study the functions of *AtRdRP1* directly, we attempted to isolate knockout mutants for the gene from the T-DNA transformed populations available at the University of Wisconsin (Sussman et al. 2000). Using a polymerase chain reaction (PCR) screen, we identified a line that contains a T-DNA insertion in the last exon (2,252 bp from the translation start) of the *AtRdRP1* gene (Fig. 4A). Seedlings from the mutant line were grown, and the segregation ratio of antibiotic resistance (20 resistant to 7 susceptible) indicated a single T-DNA insertion in the mutant. Homozygous mutant plants were identified by PCR with *AtRdRP1*- and T-DNA-specific primers. Thus, when using a combination of a T-DNA-specific primer and a primer corresponding to the 3' region of the insertion site, a DNA fragment of the expected size was amplified from homozygous knockout plants but not from the wild-type plants (Fig. 4B). Conversely, when using primers corresponding to the sequences flanking the insertion site, a fragment of the *AtRdRP1* gene was amplified from wild-type plants but not from the homozygous knockout mutant plants (Fig. 4C). To verify that the homozygous line is a null allele, we performed Northern blotting analysis, which confirmed the absence of a normal, full-length *AtRdRP1* transcript in SA-treated mutant plants (Fig. 4D). To ensure that any altered phenotype in the *AtRdRP1* knockout plants resulted from the T-DNA insertion into the gene, we isolated a full-length *AtRdRP1* genomic clone and transformed it into the mutant plants. Northern blotting analysis revealed that these transformed lines, just like the wild-type plants, produced the full-length *AtRdRP1* transcript after SA treatment (Fig. 4E). The *atrdp1* mutant grew and flowered at the same rates as did the wild-type plants and exhibited no significant alteration in morphology.

Role of *AtRdRP1* in antiviral defense.

Seeds from the homozygous knockout plants were harvested and progeny plants were grown for analysis of antiviral defense. For assays of response to TMV-cg, two fully expanded leaves of 3.5-week-old *atrdp1* mutant and wild-type plants were inoculated with the virus. In the inoculated leaves, the full-length viral genomic RNA and the three subgenomic RNAs

Fig. 2. Phylogenetic analysis of the relationship between the six *Arabidopsis* RdRP proteins and the inducible tobacco NtRdRP1 and tomato RdRP1. GenBank accession numbers are as follows: LeRdRP1, Y10403; NtRdRP1, AJ011576; AtRdRP1, AY148431; SDE1, AF268093; AtRdRP2, At4g11130; AtRdRP3, At2g19910; AtRdRP4, At2g19920; and AtRdRP5, At2g19930. Branch lengths represent weighted amino acid substitutions (a 0.1 branch length represents 10% weighted sequence divergence). Sequence alignment, distance calculation, and tree construction were performed with the use of the CLUSTAL W program (Thompson et al. 1997).

were accumulated at similar levels in wild-type and *atrdrp1* mutant plants during the first 4 dpi (Fig. 5A). At 5 dpi, the level of the full-length viral genomic RNA in the inoculated leaves was substantially reduced in wild-type plants but continued to increase in the *atrdrp1* mutant plants (Fig. 5A). From 5 to 7 dpi, the *atrdrp1* mutant accumulated 10 to 15 times more full-length viral genomic RNAs than did the wild-type plants in the inoculated leaves (Fig. 5A). Levels of the subgenomic RNAs in the inoculated leaves increased at steady but similar levels in wild-type and *atrdrp1* mutant plants (Fig. 5A).

In upper, systemically infected leaves, viral RNAs were detectable 4 dpi in both wild-type and *atrdrp1* mutant plants (Fig. 5B). By 5 dpi, the full-length viral RNA has reached the highest levels, while the three subgenomic RNAs continued to accumulate in the upper, uninoculated leaves of wild-type plants (Fig. 5B). In the mutant plants, not only the three subgenomic RNAs but also the full-length viral genomic RNAs continued to accumulate after 5 dpi in the upper, uninoculated leaves (Fig. 5B). Thus, by 7 dpi, the *atrdrp1* mutant accumulated approximately four times more full-length viral genomic RNAs than did the wild-type plants (Fig. 5B). The *atrdrp1* mutant plants transformed with the *AtRdRP1* genomic clone accumulated the same levels of viral genomic and subgenomic RNAs as wild-type plants after TMV-cg infection (data not shown). Thus, in both lower, inoculated and in upper, systemically infected leaves, the mutant plants appeared to enhance the accumulation of the full-length viral genomic RNA of TMV-cg. Interestingly, transgenic tobacco antisense plants deficient in the inducible NtRdRP1 also accumulated higher levels of the full-length viral genomic RNA of TMV than did wild-type tobacco plants after infection of the virus (Xie et al. 2001). Thus, the *Arabidopsis atrdrp1* mutant shared a striking similarity to the transgenic tobacco *NtRdRP1* antisense plants in the accumulation of viral RNAs after infection by a tobamovirus.

We also compared responses of wild-type plants and the *atrdrp1* mutant to TRV. In the inoculated leaves of wild-type plants, accumulation of RNA1, RNA2, and two subgenomic RNAs of RNA1 occurred substantially by 6 dpi, peaked at 7 dpi, followed by steady decline over the remaining four days of the experiments (Fig. 6A). In the *atrdrp1* mutants, accumulation of these viral RNAs in the inoculated leaves during the early stage (up to 7 dpi) was similar to that of wild-type plants (Fig. 6A). After 7 dpi, however, the viral RNAs continued to accumulate in the inoculated leaves of the mutant plants, and their levels were 5 to 10 times higher than those in the wild-type plants (Fig. 7A). In the upper, uninoculated leaves, wild-type plants accumulated only low levels of full-length RNA1 or RNA2, although the two subgenomic RNAs of RNAs were present at relatively high levels (Fig. 6B). In the *atrdrp1* mutant plants, accumulation of these viral RNAs was similar to that of wild-type plants during the first 8 dpi (Fig. 6B). After 8 dpi, substantial levels of both full-length RNA1 and RNA2 were observed in the mutant plants but not in the wild-type plants (Fig. 6B). After about 11 dpi, the two subgenomic RNAs also accumulated to higher levels in the mutant plants than in the wild-type plants (Fig. 6B). As with TMV-cg infection, the *atrdrp1* mutant plants transformed with the *AtRdRP1* genomic clone exhibited the same patterns of viral genomic and subgenomic RNAs as did wild-type plants after TRV infection (data not shown). Thus, the *atrdrp1* mutant plants were also more susceptible to TRV.

Possible role of AtRdRP1 in VIGS.

SDE1/SGS2 is required for transgene-induced PTGS but not for VIGS (Dalmay et al. 2000; Mourrain et al. 2000). Since AtRdRP1 plays a role in antiviral defense, it could be involved in VIGS. To test this possibility, we examined in the *atrdrp1* mutant the ability of a TRV vector construct to silence expression of the endogenous phytoene desaturase (PDS) gene. Infec-

Fig. 3. Induced expression of AtRdRP1 by **A**, salicylic acid (SA) and **B**, TMV-cg. Four-week old *Arabidopsis* plants (ecotype, Ws) were sprayed with SA (2 mM) or inoculated with TMV-cg (5 µg/ml). Total RNA was isolated at indicated times after the treatment or inoculation and was subjected to Northern blotting analysis using ³²P-labeled *AtRdRP1* cDNA as probes. The ethidium bromide staining of rRNA is shown for each lane to allow assessment of equal loading.

tion of wild-type plants with TRV-PDS caused a photobleaching symptom resulting from silencing of the endogenous PDS gene and suppression of photoprotective carotenoid production (Fig. 7). The same symptom was also observed on the *atrdrp1* mutant (Fig. 7). The more extensive photobleaching on the mutant plants was correlated with higher levels of viral RNA accumulation, as directly demonstrated by Northern blotting analysis (data not shown). No such photobleaching was observed when plants were infected with TRV or TRV-GFP (Dalmay et al. 2001).

To further study AtRdRP1-mediated antiviral defense, we also examined whether the *atrdrp1* mutant accumulated short viral RNAs after virus infection. Leaves of the *atrdrp1* mutant inoculated with either TMV-cg or TRV accumulated full-length viral RNAs to substantially higher levels than did those of the

wild-type plants (Figs. 5 and 6). This might be caused by compromised VIGS in the mutant and, consequently, lower rates of viral RNA degradation. Total RNA was isolated from virus-infected plants and was fractionated on 8% polyacrylamide gels. Fractionation of total RNA on the polyacrylamide gel allowed separation and transfer of small RNAs (<200 nt) from the gel to the Nylon membrane for efficient detection. Northern blot analyses were performed to determine the levels of small viral RNAs specific to the full-length genomic RNA of TMV-cg or to the full-length RNA1 of TRV. As shown in Figure 8, the analyses of both wild-type and mutant plants infected with either TMV-cg or TRV revealed the presence of approximately equal amounts of small, virus-specific RNAs, including those similar in size to the 25 nt siRNAs that are associated with PTGS. These observations demonstrated that the turnover of

Fig. 4. Isolation of a T-DNA insertion mutant for AtRdRP1. **A**, Diagram of the AtRdRP1 gene and the T-DNA insertion site in the *atrdrp1* knockout mutant. Also indicated are the three primers used in polymerase chain reaction (PCR) screens: primer a, 5'CATTTTATAATAACGCTGCGGACATCTAC; primer b, 5'ACTGCGTTGTGTTCGCCGAAAA; and primer c, 5'ATGATTTCTTGATTTGGAGTGTGTGAAT. **B**, Positive identification of a T-DNA insertion in the knockout plants by PCR using a T-DNA-specific primer (primer a) and an *AtRdRP1*-specific primer (primer b). The PCR amplified an expected 334-bp fragment from the knockout plants but not from the wild-type plants. **C**, Identification of homozygous knockout lines for *AtRdRP1* using PCR with a pair of primers (b and c) corresponding to *Arabidopsis* sequences flanking the insertion site. The PCR amplified an expected 1,361-bp fragment from the wild-type plants but not from the homozygous knockout lines. **D**, Northern blotting detection of the full-length transcript of *AtRdRP1*. The plants were treated with 2 mM SA for 12 h, and total RNA was isolated, separated, and probed with a 3' *AtRdRP1* cDNA fragment. The ethidium bromide staining of rRNA is shown for each lane to allow assessment of equal loading. **E**, Northern blotting detection of the full-length transcript of *AtRdRP1* in the *atrdrp1* mutant transformed with an *AtRdRP1* genomic clone. The *atrdrp1* mutant (lane 1) and transformant lines were treated with 2 mM SA for 12 h, and total RNA was isolated, separated, and probed with a 3' *AtRdRP1* cDNA fragment. The ethidium bromide staining of rRNA is shown for each lane to allow assessment of equal loading.

viral RNAs occurred in both wild-type and mutant plants and, if such turnover occurs via a VIGS-associated mechanism, it does not require AtRdRP1.

There was, however, a substantial difference in the ratios of full-length viral RNAs relative to small, virus-specific RNAs between wild-type and *atrdrp1* mutant plants. For example, starting from 5 dpi, TMV-cg-inoculated leaves of the *atrdrp1* mutant accumulated substantially higher levels of the full-length viral genomic RNA than did the wild-type plants (Fig. 5A), but the two groups of plants had similar levels of short viral RNAs (Fig. 8A). Likewise, the *atrdrp1* mutant accumulated higher levels of TRV RNA1 than did the wild-type plants (Fig. 6), but the levels of small RNAs specific to TRV RNA1 were similar in the two groups of plants (Fig. 8B). The different ratios of full-length viral RNAs relative to small RNAs most likely reflects a quantitative difference in the turnover of viral

RNAs between wild-type and *atrdrp1* mutant plants. Thus, although turnover of viral RNAs occurred in the *atrdrp1* mutant plants, it occurred at a substantially lower rate than in the wild-type plants.

PR1 expression and resistance to a bacterial pathogen.

Both *NtRdRP1* and *AtRdRP1* are induced by treatment with SA, a signal molecule that elicits defense mechanisms against a wide spectrum of microbial pathogens (Chen et al. 1995; Klessig et al. 2000). SA-induced resistance to viral pathogens may be mediated by an enhanced RNA-silencing antiviral defense mechanism (Ji and Ding 2001). Thus, increased susceptibility of the *atrdrp1* mutant plants to viral pathogens could reflect compromise of a general SA-mediated defense system in the mutant plants. To test this possibility, we analyzed SA-induced expression of *PR1*, a reliable molecular marker of SA-

Fig. 5. Accumulation of TMV-cg viral RNA in **A**, lower, inoculated and **B**, upper, uninoculated leaves of *Arabidopsis* plants. Two leaves of 3.5-week old *Arabidopsis* plants were inoculated with TMV-cg (5 µg/ml). The lower, inoculated leaves and the upper, systemic infected leaves of five plants were collected at each indicated day after inoculation for RNA isolation. Northern blotting analysis was performed with ³²P-labeled TMV-cg coat protein gene as probe. The ethidium bromide staining of rRNA is shown for each lane to allow assessment of equal loading. The experiments were repeated three times with similar results.

induced systemic acquired resistance (Maleck et al. 2000). Both wild-type plants and *atrdrp1* mutants were sprayed with 2 mM SA, and RNA was extracted from the plants 24 h after the treatment and was analyzed for the *PR1* transcript. As shown in Figure 9A, no expression of the defense gene was detected in untreated wild-type plants and the mutant, but high levels of *PR1* transcripts were detected in both the wild-type and mutant plants following treatment with SA (Fig. 9A). Thus, disruption of the *AtRdRP1* gene had no significant effect on SA-induced expression of the defense gene. In addition, we inoculated both untreated and SA-sprayed wild-type plants and *atrdrp1* mutant plants with a virulent strain of the bacterial pathogen *Pseudomo-*

nas syringae, and the growth of the pathogen was monitored three days later. As shown in Figure 9B, multiplication of the bacterial pathogen occurred to similar levels in both wild-type and mutant plants, and SA-induced resistance to the bacterium was unaffected by mutation of the *AtRdRP1* gene. Thus, the *atrdrp1* plants appeared to be normal in the general defense responses and were compromised specifically in antiviral defense.

DISCUSSION

Previously, we reported the identification of an RdRP from tobacco (NtRdRP1) that appears to be involved in antiviral de-

Fig. 6. Accumulation of *Tobacco rattle virus* (TRV) viral RNA in **A**, lower inoculated and **B**, upper uninoculated leaves of *Arabidopsis* plants. Two leaves of 3.5-week old *Arabidopsis* plants were inoculated with TRV freshly extracted from infected *Nicotiana benthamiana* (15 µg/g of soluble protein in 5 mM sodium phosphate, pH 7.5). The lower, inoculated leaves and the upper, systemic infected leaves of five plants were collected at each indicated day after inoculation for RNA isolation. Northern blotting analysis was performed with ³²P-labeled TRV RNA1 and RNA2 gene as probes. The ethidium bromide staining of rRNA is shown for each lane to allow assessment of equal loading. The experiments were repeated three times with similar results.

fense (Xie et al. 2001). NtRdRP is induced by SA treatment and virus infection. Transgenic plants deficient in the inducible NtRdRP1 activity accumulated more viral RNAs and developed more severe symptoms after infection by TMV and PVX. In the present study, we report identification of an *Arabidopsis* RdRP (AtRdRP1) that is highly homologous to NtRdRP1. Like NtRdRP1, AtRdRP1 is also induced by SA treatment and virus infection (Fig. 3). To study its function directly, we have isolated a T-DNA insertion mutant for the RdRP gene and discovered that the mutant plants also accumulate higher levels of viral RNAs after infection with TMV-cg and TRV (Figs. 5 and 6). However, the knockout mutant expressed the *PR1* gene and responded normally to a bacterial pathogen (Fig. 9), indicating that the mutant is not compromised in the general SA-mediated defense responses. These results indicate that AtRdRP1 and NtRdRP1 are functional analogs that play a specific role in antiviral defense. RdRPs in plants are encoded by a relatively small gene family with six members in *Arabidopsis*. Recently, one of the six RdRP genes, *SDE1/SGS2*, has been shown to be required for transgene-induced PTGS but does not appear to play a general role in antiviral defense, since the *sde1/sgs2* mutants responded normally to infection by a number of viruses (Dalmay et al. 2000; Mourrain et al. 2000). Thus, certain members of the RdRP gene family may have an important role in transgene-induced PTGS, while others could be involved more in antiviral defense.

How does the inducible AtRdRP1 mediate antiviral defense? Infection of the mutant with TRV-PDS resulted in photobleaching of leaves due to VIGS of the endogenous PDS gene (Fig. 7). In addition, in the TMV-cg- or TRV-infected *atrdrp1* mutant, we observed production of short viral RNAs, including those similar in size to the 25 nt siRNAs associated with PTGS (Fig. 8). Thus, AtRdRP1 is not required for VIGS, and therefore, it is unlikely that the inducible AtRdRP1 activates antiviral defense through initiating or triggering VIGS. On the other hand, there is a substantial difference in the ratio of degraded short viral RNAs relative to undegraded, full-length viral RNAs between the wild-type plants and the *atrdrp1* mutant (Figs. 5, 6, and 8). Within several days after virus inoculation, wild-type

plants contained lower levels of undegraded viral genomic RNAs than did the *atrdrp1* mutant but continued to accumulate similar, if not higher, levels of short viral RNAs than did the *atrdrp1* mutant. The difference in the relative amount of full-length viral RNAs versus short viral RNAs likely reflects a difference in the turnover of viral RNAs between the wild-type and the *atrdrp1* mutant plants. Apparently, the inducible RdRP reduces accumulation of viral RNAs at least partly by enhancing the turnover of viral RNAs.

AtRdRP1 may promote turnover of viral RNAs through a number of possible mechanisms. Purified tomato RdRP is highly homologous to AtRdRP1 and has been shown to catalyze in vitro both primed and unprimed synthesis of short cRNA, using ssRNA and ssDNA as templates (Schiebel et al. 1993b). Differences in transcription efficiency were found to depend on the nucleotide sequence of the templates (Schiebel et al. 1993b). The sequence specificity may explain why the inducible RdRP can preferentially block accumulation of some but not all viral genomic or subgenomic RNAs. For example, in TMV-cg-infected plants, the full-length genomic RNA but not the three subgenomic RNAs preferentially accumulated at higher levels in the *atrdrp1* mutant (Fig. 5). The inducible RdRP may recognize certain sequences present only in the full-length viral RNAs for synthesis of dsRNAs and directly activate PTGS-mediated degradation of full-length viral RNAs. AtRdRP1 may also participate in certain rate-limiting steps of VIGS triggered by dsRNAs generated from viral RNA replication. In this case, viral RNA replicases may generate the dsRNAs that are degraded to short siRNAs to target viral RNAs for degradation. Biochemical studies have recently shown that RNA degradation targeted by homologous siRNAs involves annealing and extension of siRNAs by an RdRP (Lipardi et al. 2001; Nishikura 2001; Sijen et al. 2001). The inducible AtRdRP1 could play a role in the extension of viral siRNAs, thereby sustaining the production of siRNAs and degradation of newly synthesized viral RNAs. Furthermore, AtRdRP1 may recognize certain sequences of full-length viral RNAs as templates for synthesis of cRNAs that could interfere with viral RNA replication, translation, or both leading to en-

Fig. 7. Virus-induced PTGS of the phytoene desaturase (PDS) gene in *Arabidopsis*. The wild-type plants and the *atrdrp1* mutant (4 weeks old) were inoculated with *Tobacco rattle virus*-PDS freshly extracted from infected *Nicotiana benthamiana* (15 µg/g of soluble protein in 5 mM sodium phosphate, pH 7.5). The pictures were taken 25 days postinoculation.

hanced production of small viral RNAs relative to full-length genomic RNAs. The synthesized cRNAs may also negatively influence the viral RNA replication cycle by interfering with the synthesis of a specific strand (+ or -) of viral RNAs and reducing overall accumulation of viral RNAs. Extensive further experiments will be necessary to distinguish among these possible mechanisms.

Mechanistic studies have shown that VIGS can be divided into two phases (Ruiz et al. 1998). In the first (initiation) phase, viral RNA replication leads to production of dsRNA that can directly trigger PTGS. This initiation phase of VIGS is virus-dependent but host RdRP-independent. In the second (maintenance) stage, certain aberrant viral RNAs may serve as

templates by host SDE1/SGS2 to synthesize dsRNAs for persistence of VIGS. A number of studies have shown that the apparent lack of a general role of SDE1/SGS2 in antiviral defense is caused by the various mechanisms that viruses have evolved to evade or suppress SDE1/SGS2-dependent PTGS (Dalmay et al. 2000, 2001; Mourrain et al. 2000). Some viruses may have lost the ability to produce aberrant ssRNA that serve as templates for SDE1/SGS2-dependent PTGS (Dalmay et al. 2001). Some viruses may still produce aberrant ssRNAs but hide them somehow in the cells, so they are prevented from serving as templates for SDE1/SGS2 (Dalmay et al. 2001). Other viruses produce strong suppressors for SDE1/SGS2-dependent PTGS (Dalmay et al. 2001). Since the *atrdrp1* mu-

Fig. 8. Accumulation of short RNAs in *Arabidopsis* plants after infection with **A**, TMV-cg or **B**, Tobacco rattle virus (TRV). Two leaves of 3.5-week-old *Arabidopsis* plants were inoculated with TMV-cg or TRV. The inoculated leaves of five plants were collected at each indicated day after inoculation for RNA isolation. The blot from TMV-cg-infected plants was probed with a ^{32}P -labeled DNA fragment corresponding to the 2.3-kb 5' region of the full-length genomic RNA of TMV-cg. The blot from TRV-infected plants was probed with a ^{32}P -labeled DNA fragment corresponding to TRV RNA1. The position of a 25-nt oligonucleotide used as a size marker is indicated on the gels.

tant is compromised in response to viruses to which the *sde1/sgs2* mutant responds normally, AtRdRP1-mediated antiviral defense must be different in key mechanistic aspects from SDE1/SGS2-mediated PTGS. For example, the action of AtRdRP1 may not be dependent on aberrant RNAs that may be required for SDE1/SGS2-dependent PTGS. In addition, the suppressors of SDE1/SGS2-dependent PTGS may not be functional for RdRP1-dependent antiviral defense. It is possible that an AtRdRP1-dependent antiviral mechanism may have evolved as a counter mechanism in response to various counter defense strategies developed by many viral pathogens.

MATERIALS AND METHODS

Materials.

Plants of *Arabidopsis thaliana* Heyn, ecotype Wassilewskijia (Ws) used throughout the experiments were grown at 24°C in a 14 h light cycle. Infectious clones of TRV were described before (MacFarlane and Popovich 2000; Liu et al. 2002). TRV-PDS was constructed by amplifying a 640-bp fragment of the *Arabidopsis* PDS gene using primers 463 (5'TACGCCATGGCGCTTTGATTCTCCGAAGC) and 464 (5'CGAGGTACCTGTGTTCTTCGTTTTCTG). The PDS fragment was inserted in sense orientation in place of the GFP gene from clone pTRV-GFPc. Viral transcripts were amplified by inoculation to *Nicotiana benthamiana* prior to reinoculation in infected leaf extract to *Arabidopsis* plants. TMV-cg and TRV were mechanically inoculated on whole leaves of plants by rubbing on the virus (5 µg per ml of solution in 5 mM sodium phosphate, pH 7.5) mixed with carborundum. Mock inoculation was performed with the phosphate buffer only.

Isolation of AtRdRP1 cDNA and genomic clones.

An *Arabidopsis* cDNA library (in ZAP Express lambda vector; Stratagene, La Jolla, CA, U.S.A.) of 10⁶ phages was screened using a ³²P-labeled *AtRdRP1* gene fragment amplified by PCR, using two *AtRdRP1*-specific primers (5'CAGAGGGTTTGTAGGCAAAG-3' and 5'ACACTGCAACTTCTTGTCTGA-3'). The hybridization was performed as described (Du and Chen 2000).

Genomic clones for *AtRdRP1* were isolated from an *Arabidopsis* genomic library (Clontech, Palo Alto, CA, U.S.A.), using the full-length *AtRdRP1* cDNA as a probe. A 6.8-kb *SpeI/XbaI* fragment of the *AtRdRP1* gene was isolated from the genomic clone and subcloned into the *Arabidopsis* transformation vector pOCA28 for complementation assays (Du and Chen 2000).

Isolation of *atrdp1* T-DNA insertion mutant.

PCR screens of the T-DNA insertion lines of the transformed *Arabidopsis* populations from the University of Wisconsin (Madison, WI, U.S.A.) were performed as described previously (Sussman et al. 2000). Seeds of the positive pool after the first two rounds of screens were requested, and screens for positive individual plants were performed through PCR amplification and direct sequencing of amplified DNA fragments as described previously (Sussman et al. 2000).

Northern blotting.

For Northern blot analysis of plant gene expression or viral RNA accumulation, 3.5-week-old *Arabidopsis* plants were treated or infected, and two fully expanded leaves from each of four to five plants were harvested at each timepoint for total RNA isolation using the TRIZOL reagent (BRL Life Technologies, Rockville, MD, U.S.A.). The RNA was separated on agarose (1.2%)-formaldehyde gels and was blotted onto nylon membrane. Hybridization was performed using random-primed

³²P-labeled DNA probes in PerfectHyb plus hybridization buffer (Sigma, St. Louis)) overnight at 68°C. The membranes were washed at 68°C once in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 0.1% sodium dodecyl sulfate (SDS) for 5 min, twice in 0.5× SSC and 0.1% SDS, and twice in 0.1× SSC and 0.1% SDS.

Detection of short RNAs.

Total RNAs were isolated from plants using the Trizol agent according the manufacturer's instruction. The RNA was separated on an 8% polyacrylamide gel containing 8.3 M urea and 1% SDS in 1× TBE running buffer (0.09 Tris-borate, 0.002 M ethylenediamine-tetraacetate, pH 8.0). After separation, the gel was soaked in 20× SSC solution for 15 min, and RNA was blotted to a positive-charged Nylon membrane. Only small RNAs (<200 bp) were resolved on the polyacrylamide gels and transferred to the blot with a high efficiency. Large RNA molecules (>200 bp) were concentrated on the top of the gel and transferred to the Nylon membrane with a very low efficiency. Hybridization was performed with ³²P-labeled DNA probes in PerfectHyb plus

Fig. 9. Expression of *PR1* gene and disease resistance. **A**, 4-week-old plants were sprayed with water or 2 mM salicylic acid (SA), and total RNA was isolated 12 h after the treatment and was probed with a ³²P-labeled *Arabidopsis* *PR1* gene probe. **B**, Five plants (4 weeks old) were sprayed with water or 2 mM SA. After 12 h, the plants were inoculated with *P. syringae* pv. *tomato* DC300 (OD₆₀₀ = 0.001), and samples were taken three days after infection. The experiments were repeated three times with similar results. cfu = colony-forming units.

hybridization buffer overnight at 40°C. The membrane was washed twice in 2× SSC and 0.1% SDS for 10 min.

Plant inoculation.

Pathogen inoculations were performed by infiltration of leaves of at least six plants for each treatment with *P. syringae* pv. *tomato* DC3000 strain (OD₆₀₀ = 0.001 in 10 mM MgCl₂). Inoculated leaves were harvested three days after infiltration and homogenized in 10 mM MgCl₂. Diluted leaf extracts were plated on King's B medium supplemented with rifampicin (100 µg per ml) and kanamycin (25 µg per ml) and were incubated at 25°C for two days before counting the colonies.

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