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## Biochemical Activities of *Arabidopsis* RNA-dependent RNA Polymerase 6\*,<sup>□</sup>

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### Abstract

In *Arabidopsis*, genetic evidence demonstrates that RNA-dependent RNA polymerase 6 (RDR6) plays a fundamental role in at least four RNA silencing pathways whose functions range from defense against transgenes or viruses to endogene regulation in development and in stress responses. Despite its critical role in RNA silencing, the biochemical activities of RDR6 have yet to be characterized. In this study, we transiently expressed *Arabidopsis* RDR6 in *Nicotiana benthamiana* and investigated the biochemical activities of immunopurified RDR6 *in vitro*. We showed that RDR6 possesses terminal nucleotidyltransferase activity as well as primer-independent RNA polymerase activity on single-stranded RNAs. We found that RDR6 cannot distinguish RNAs with or without a cap or poly (A) tail. We also demonstrated that RDR6 has strong polymerase activity on single-stranded DNA. All these activities require the conserved catalytic Asp<sup>867</sup> residue. Our findings have important implications on the processes involving RDR6 *in vivo* and provide new biochemical insights into the mechanisms of RNA silencing in *Arabidopsis*.

RNA silencing is a universal regulatory mechanism that employs small interfering RNAs (siRNAs)<sup>2</sup> to provide specificity to protein effectors that act to repress transcription or cleave an mRNA target (1). siRNAs are produced from long double-stranded RNAs (dsRNAs), which in some organisms are generated from single-stranded RNA (ssRNA) templates by RNA-dependent RNA polymerases (RDRs). In *Caenorhabditis elegans*, fungi, and plants, genes encoding RDRs are required for RNA silencing (2–6). *Arabidopsis* has six genes that encode potential RDRs, and genetic evidence suggests that *RDR1*, *RDR2*, and *RDR6* act in different biological processes with *RDR6* being the major RDR acting in post-transcriptional gene silencing (PTGS) (3,4,6,7). It is thought that RDR6 initiates RNA silencing by recognizing “aberrant RNAs” and converting them to dsRNAs, which are then processed into siRNAs. In *Arabidopsis*, genetic evidence suggests that an RNA that lacks a cap or a poly(A) tail is deemed “aberrant” and is preferentially recognized by the silencing machinery (8–10). Most eukaryotic organisms also produce microRNAs that are similar to siRNAs in length but the biogenesis of microRNAs does not require RDRs (11).

Amplification of siRNA production constitutes a major step that enhances the efficiency of RNA silencing and this amplification requires RDRs (12). Secondary siRNAs produced by the amplification process can originate from regions of the target mRNA not targeted by the primary siRNAs. The production of siRNAs outside of the region targeted by the initial RNA

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<sup>□</sup>The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Figs. S1–S6.

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silencing trigger is known as transitivity (13–15). In *C. elegans*, the amplification process generates secondary siRNAs that bear a 5' triphosphate and that are predominantly in antisense orientation to the target mRNA, suggesting that they are directly synthesized by RDR through a primer-independent mechanism (16,17). In *Arabidopsis*, it was observed with a *GFP* transgene containing a modified miR171 binding site that small RNA-guided amplification can probably occur without cleavage of the target mRNA. This led to the hypothesis that an RDR uses the small RNA as a primer to copy the target mRNA to lead to 3' to 5' transitivity (18). However, the priming hypothesis cannot explain secondary siRNAs originating from regions 3' to the matching primary siRNAs in the target mRNA.

In *Arabidopsis*, the biogenesis of several types of siRNAs requires RDRs. Transcriptional gene silencing (TGS), mediated by 24-nucleotide siRNAs that guide histone and DNA methylation, requires RDR2 (6). PTGS initiated by a sense transgene (S-PTGS) or by infection with cucumber mosaic virus or cabbage leaf curl virus (CaLCuV), requires RDR6 (3,4,19). More recently, two other PTGS mechanisms, also RDR6-dependent, were found to regulate endogene expression through two new classes of siRNAs, the *trans*-acting siRNAs (ta-siRNAs) (20–22) and the natural *cis*-acting siRNAs (23,24). Whereas natural *cis*-acting siRNAs are involved in stress responses, ta-siRNAs control developmental phase transitions such that the absence of ta-siRNAs underlies the developmental defects of *rdr6* mutants (25). The biogenesis of both types of siRNAs occurs via a two-step process. First, a small RNA (an microRNA or an siRNA) guides the cleavage of a target mRNA. Then, the cleavage fragments are presumably copied into dsRNAs by RDR6 and the dsRNAs are processed into the ta-siRNAs or the natural *cis*-acting siRNAs.

Viral RDRs have been biochemically well characterized. Many viral RDRs use a *de novo* mechanism of initiation in which the first phosphodiester bond is formed between two nucleoside triphosphates, but some use a primer-dependent mechanism (26–29). A eukaryotic

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<sup>2</sup>The abbreviations used are:

<b>siRNA</b>	small interfering RNA
<b>dsRNA</b>	double-stranded RNA
<b>ssRNA</b>	single-stranded RNA
<b>RDR</b>	RNA-dependent RNA polymerase
<b>CaLCuV</b>	cabbage leaf curl virus
<b>VIGS</b>	virus-induced gene silencing
<b>TGS</b>	transcriptional gene silencing
<b>PTGS</b>	post-transcriptional gene silencing
<b>ta-siRNA</b>	<i>trans</i> -acting siRNA
<b>HA</b>	hemagglutinin
<b>MES</b>	4-morpholineethanesulfonic acid

protein with RDR activity was first isolated through biochemical means in tomato (30–32). RDR activities, however, have been detected in extracts from various plants such as Chinese cabbage, cauliflower, tobacco, and wheat (7,33–37). Except for the tomato RDR, only QDE-1 from *Neurospora crassa* and Rdp-1 from *Schizosaccharomyces pombe* are cellular RDRs that have been biochemically characterized (38,39). In the model plant *Arabidopsis*, genetic evidence demonstrates the requirement for RDR6 in a number of silencing pathways (3,4,19, 21,23,25), but the RNA-dependent RNA polymerase activity of RDR6 has been inferred based on sequence homology with the tomato enzyme and has yet to be confirmed and characterized biochemically.

Here we report *in vitro* enzymatic activities of *Arabidopsis* RDR6 that was overexpressed and purified from *Nicotiana benthamiana* leaves. We show that RDR6 can indeed copy various ssRNAs into dsRNAs but, contrary to expectations, the presence of a cap or poly(A) tail does not affect its activity. In addition, RDR6 possesses strong polymerase activities on ssDNA templates. These findings provide biochemical insights into the molecular mechanisms of RNA silencing in *Arabidopsis*.

## EXPERIMENTAL PROCEDURES

### Plasmid Construction

The full-length cDNA of *RDR6* (3.6 kb) was amplified from the ATG to the stop codon by PCR using the primers R6-1 (5'-ccggaattcgagaaatggggtcagagggaaata-3') and R6-2 (5'-ccgcccgcgcaacataaccttttagagactgagc-3'). The resulting amplicon was cloned into pENTR1A (Invitrogen) and the pENTR1A-RDR6 construct was verified by sequencing. The pENTR1A-RDR6 plasmid was amplified by PCR using two complementary primers R6-1m (5'-gaccttgacggggccctgtactttgtg-3') and R6-2m (5'-cacaagtacagggccccgtaaggc-3') to substitute the putative catalytic residue Asp located at amino acid 867 by an Ala residue. The pENTR1ARDR6m plasmid was verified by sequencing. Both RDR6 and RDR6m cDNAs were introduced into the pEG201 vector (40), which contains an HA tag for N-terminal fusion, and the reading frame was verified by sequencing.

### Plant Growth and Transformation

Plants were grown in soil in growth chambers at 22 °C under continuous illumination. *Arabidopsis* transgenic lines were obtained by *Agrobacterium*-mediated transformation using the floral dip procedure (41). *Agrobacteria* containing the plasmids pEG201-RDR6 or pEG201-RDR6m were used to transform both wild-type and *rdr6-11* (25) plants. Transformants were selected on Murashige and Skoog medium with 50 µg/ml kanamycin. For each construct, 20 independent transformants in both wild-type and *rdr6-11* backgrounds were transferred into soil. T2 plants were selected again on kanamycin-containing medium and transferred into soil for phenotypic analysis. Young flowers were harvested from 6 and 2 individual transgenic lines for 35S::HA-RDR6 and 35S::HA-RDR6m, respectively, quickly frozen in liquid nitrogen, and stored at -80 °C for Northern analysis.

### Small RNA Northern Blot Analysis

RNA isolation and Northern blotting were performed as described (42). Briefly, 40 µg of total RNA was resolved on a 15% polyacrylamide, 7 M urea gel, transferred to a Zeta-Probe GT membrane (Bio-Rad), and hybridized with an end-labeled oligonucleotide probe.

### Expression and Purification of HA-RDR6 and HA-RDR6m Proteins

Transient expression of HA-RdRp6 and HARdRp6m in *N. benthamiana* was based on the protocol established by Popescu *et al.* (43). Plasmids pEG201-RDR6, pEG201-RDR6m, and

p19 (a viral RNA silencing suppressor from the *Tomato bushy stunt virus*) were first transformed into *A. tumefaciens* GV3101. One transformed colony (verified by PCR) for each plasmid was cultured at 28 °C until the  $A_{600}$  reached 1 to 1.5. The bacteria were spun for 15 min at  $2,000 \times g$ , resuspended in infiltration media (10 mM MES, 10 mM  $MgCl_2$ , 200  $\mu$ M acetosyringone) to a final  $A_{600}$  of 1, and incubated for 3 to 4 h at room temperature. Bacteria were then washed with 1 volume of fresh infiltration media, and mixed at a 1:1 ratio between *Agrobacteria* containing pEG201-RDR6 or pEG201-RDR6m and those containing p19. The infiltration of bacterial mixtures was performed with a 1-ml syringe on the abaxial side of leaves of 3-week-old *N. benthamiana* plants. After 48 h, infiltrated leaves were harvested, ground in liquid nitrogen, and stored at -80 °C.

For protein extraction, 1 volume of tissue powder was mixed with 2 volumes of protein lysis buffer (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 5 mM  $MgCl_2$ , 0.1% (v/v) Nonidet P-40, 10% (v/v) glycerol, 1 mM phenylmethylsulfonyl fluoride, 2 mM dithiothreitol, 1 protease inhibitor mixture tablet/20 ml (Roche)), and incubated for 20 min at 4 °C. Lysates were centrifuged one time for 30 min at  $1,000 \times g$  at 4 °C, then three times for 30 min each at  $16,000 \times g$  at 4 °C. 1/50 volume of anti-HA affinity matrix (Roche) was added to the supernatant and the mixture was incubated on a rotator (8 rpm) for 1 h at 4 °C. The beads were then washed once with 20 volumes of protein lysis buffer, 4 times with 20 volumes of the washing buffer (20 mM Tris-HCl, pH 7.6, 100 mM NaCl, 0.1 mM EDTA, 0.05% (v/v) Tween 20, 2 mM dithiothreitol, 1 protease inhibitor tablet/20 ml) and finally resuspended in 7 volumes of Storage Buffer (20 mM HEPES/KOH, pH 7.6, 20 mM NaCl, 0.2 mM EDTA, 20% (v/v) glycerol, 2 mM dithiothreitol, 1 protease inhibitor tablet/20 ml), and stored in aliquots at -80 °C.

### RNA Template Synthesis

Single-stranded RNA templates were synthesized by *in vitro* transcription of DNA fragments obtained by PCR amplification from a luciferase/nopaline synthase chimeric template (supplemental Fig. S2A). The PCR primers were designed to introduce the T7 RNA polymerase promoter sequence in the correct orientation (supplemental Fig. 2B). PCR fragments were gel purified and then used as templates for *in vitro* transcription using the MEGAscript kit (Ambion). Reactions were stopped by DNase treatment using 2 units of RQ1 (Promega) for 15 min at 37 °C. The RNA products (full-length and truncated forms) were resolved in a 6% polyacrylamide, 7 M urea gel. The bands corresponding to the full-length RNAs were excised, cut into small pieces, and incubated with 3 volumes of elution buffer (20 mM Tris-HCl, pH 7.6, 0.5 M NaOAc, 10 mM EDTA, 1% (w/v) SDS) for 1 h at 37 °C. After phenol/chloroform extraction, the RNAs were precipitated and resuspended in diethyl pyrocarbonate/water at a final concentration ranging between 0.1 and 0.5  $\mu$ g/ $\mu$ l. The purity of the RNAs was determined by analysis on a 6% polyacrylamide, 7 M urea gel.

Capped RNAs were generated from gel-purified *in vitro* transcripts using the ScriptCap™ m<sup>7</sup>G Capping System (Epicenter). The reactions were stopped by phenol/chloroform extraction and the RNAs were precipitated as previously described.

Polyadenylated RNAs (RNAs-A<sup>n</sup>) were synthesized using poly(A) polymerase (U. S. Biochemical Corp.). The reactions were carried out with 2  $\mu$ g of RNA, 1  $\times$  PAP (20 mM Tris-HCl pH 7.0, 0.6 mM  $MnCl_2$ , 0.02 mM EDTA, 0.2 mM dithiothreitol, 100  $\mu$ g/ml acetylated bovine serum albumin, 10% glycerol) reaction buffer, 1 mM ATP, 600 units of PAP enzyme in a 25- $\mu$ l final volume for 15 min at 37 °C. Reactions were stopped by incubation at 65 °C for 10 min and were directly loaded on a 6% polyacrylamide, 7 M urea gel and the polyadenylated RNAs were excised from the gel and isolated.

## RDR Activity Assays

RDR activity assays were performed according to Makeyev and Bamford (38) with some modifications. Briefly, assays were conducted in 30- $\mu$ l reaction mixtures containing 50 mM HEPES-KOH (pH 7.6), 20 mM NH<sub>4</sub> OAc, 2% (w/v) PEG4000, 8 mM MgCl<sub>2</sub>, 0.1 mM EDTA, 1 mM each of ATP, CTP, and GTP, 0.1 mM UTP, and 0.8 unit/ $\mu$ l of RNasin. The final quantity of the ssRNA templates was between 5 and 15 pmol. Unless indicated otherwise, the mixture was supplemented with 0.15 mCi/ml of [ $\alpha$ -<sup>32</sup>P]UTP (~3000 Ci/mmol). Reactions were initiated by adding 2  $\mu$ l of HA-RDR6 bound on anti-HA-agarose beads, and incubated on a rotator (8 rpm) for 2 h at room temperature (25–27 °C). Reactions were stopped by adding 50  $\mu$ l of buffer (20 mM Tris-HCl, pH 7.6, 20 mM MgCl<sub>2</sub>, 2% SDS), 90  $\mu$ g of proteinase K (Fermentas) in a final volume of 100  $\mu$ l, followed by incubation for 20 min at 65 °C. The reaction mixtures were extracted with phenol-chloroform and the RNAs were precipitated, resolved on 6% polyacrylamide, 7 M urea gels, and detected by autoradiography or with a PhosphorImager.

## Nuclease Treatments

For RNase I, RNase H, and RQ1 (DNase) treatments, the RDR reactions were supplemented with 14  $\mu$ l of the RNase I buffer (50 mM Tris-HCl, pH 8.0, 300 mM NaCl, 15 mM MgCl<sub>2</sub>), the RNase H buffer (50 mM Tris-HCl, pH 8.0, 250 mM KCl, 10 mM dithiothreitol), or the RQ1 buffer (20 mM Tris-HCl pH 8.0, 30 mM MgSO<sub>4</sub>, 30 mM CaCl<sub>2</sub>), respectively. Treatments were started by the addition of 50 units of RNase I (New England Biolabs), 10 units of RNase H (New England Biolabs), or 5 units of RQ1 (Promega) followed by incubation at 37 °C for 20 min. The reactions were stopped by proteinase K treatment as described previously.

## Assays for Priming Activity with an RNA Oligonucleotide

30 pmol of miR173\* (5'-rGrArUrUrCrUrCrUrGrUrGrUrArArGrCrGrArArG-3') was mixed with 15 pmol of the 246R template in a 10- $\mu$ l solution containing 50 mM HEPES-KOH, pH 7.4, and 100 mM KCl. The mixture was incubated for 3 min at 95 °C and then for 10 min at 42 °C, and then used for RDR assay as previously described.

## RESULTS

### A Conserved Catalytic Residue in Eukaryotic RDRs Is Critical for RDR6 Function in Vivo

To study the biochemical properties of *Arabidopsis* RDR6, we sought to immunopurify sufficient quantities of RDR6 from plants. To this end, full-length *RDR6* cDNA was cloned behind the cauliflower mosaic virus (CaMV) <sup>35</sup>S promoter and in-frame with an N-terminal HA tag. The *35S::HA-RDR6* construct was used to generate transgenic plants in the *rdp6-11* mutant background. *rdp6-11* plants showed downward curling of rosette leaves (Fig. 1A) (25). All 20 independent *35S::HA-RDR6 rdp6-11* transgenic lines analyzed showed a complete restoration of the wild-type seedling phenotype (Fig. 1A), suggesting that HA-RDR6 was functional. To further confirm this, an RNA blot analysis was performed to quantify the levels of two ta-siRNAs (ASRP255, ASRP1511) and a microRNA, miR173, using total RNAs extracted from 6 of 20 *35S::HA-RDR6 rdp6-11* transgenic lines (Fig. 1B). Both ta-siRNAs were present in wild-type plants and were not detected in *rdp6-11* mutant plants, whereas miR173, used as a loading control, was not affected by the *rdp6* mutation (Fig. 1B) (21,25). As expected, in the six *35S::HA-RDR6 rdp6-11* transgenic lines both ta-siRNAs were restored to wild-type levels (Fig. 1B).

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A previous study on two RDR6 homologs, QDE-1 (*N. crassa*) and Rdp-1 (*S. pombe*), showed that the conserved aspartate residue (located at amino acid 1011 in QDE1 and amino acid 903 in Rdp-1) is essential for RDR activity (38,39). We introduced a point mutation into the RDR6 cDNA to substitute the corresponding Asp (at position 867) with Ala. As expected, when the mutant construct (*35S::HA-RDR6m*) was introduced into *rdr6-11*, none of 20 independent transformants showed any rescue of the *rdr6* mutant phenotype (Fig. 1A). The ASRP255 and ASRP1511 ta-siRNAs were not detected in two *35S::HA-RDR6m rdr6-11* transgenic lines tested (Fig. 1B). These results indicated that the Asp<sup>867</sup> residue was essential for RDR6 activity. The HA-RDR6m protein served as a negative control in our biochemical analysis of RDR6 *in vitro*.

### RDR6 Has Two Distinguishable Activities in Vitro

To investigate the biochemical properties of RDR6, we needed to immunopurify relatively large amounts of RDR6 from plants. Although the HA-RDR6 protein could be immunopurified from *35S::HA-RDR6 rdr6-11* plants (supplemental Fig. S1), the amount of the protein was too little for the large numbers of assays we wished to perform. We sought to obtain larger quantities of the protein through transient expression of *35S::HA-RDR6* in *N. benthamiana* leaves. We found that on average 20-fold more HA-RDR6 was purified with anti-HA-agarose beads when we incubated 10-fold less tissue from infiltrated *N. benthamiana* leaves than from *Arabidopsis* transgenic seedlings (supplemental Fig. S1). Therefore, we used immuno-purified HA-RDR6 expressed in *N. benthamiana* for all our subsequent biochemical assays. The purity of our RDR6 preparation was evaluated by gel electrophoresis followed by SYPRO RUBY staining (supplemental Fig. S1). Although the preparation had very few contaminating proteins, it was essential that the RDR6m preparation was included in the activity assays as a control to ensure that the observed activity was due to RDR6 instead of a contaminating protein.

For RDR assays, we first synthesized ssRNAs of various sizes by *in vitro* transcription (Fig. 2A). Each ssRNA was named by its size in nucleotides followed by the letter F or R to indicate its forward or reverse orientation as compared with the DNA template (sequence in supplemental Fig. S2). The ssRNAs were then gel-purified and used as templates in RDR6 activity assays in the presence of [ $\alpha$ -<sup>32</sup>P]UTP as well as all four cold NTPs. The first assays using HA-RDR6 and HA-RDR6m were performed with an increasing quantity (from 1 to 27 pmol) of the 185F template. The reaction products, containing the radioisotope, were resolved on denaturing polyacrylamide gels and detected by phosphorimaging (Fig. 2B). Reactions performed with HA-RDR6 showed a major product, corresponding in size to the 185F template, and the quantity of the product was proportional to that of the template (Fig. 2B, lanes 1–4). In contrast, no products were observed in the reaction performed with HA-RDR6m (Fig. 2B, lane 5). This confirmed that the products obtained with HA-RDR6 were due to HA-RDR6 instead of a contaminating protein. Therefore, RDR6 was able to use a ssRNA as a substrate to generate an RNA product of a similar size. To stay within the linear range, all subsequent RDR assays were performed with a quantity of substrates between 5 and 15 pmol.

Some viral RDRs are known to have 3' nucleotidyltransferase activity that adds one or a few nucleotides to the 3' ends of the substrate RNA (44). To investigate whether the observed RDR6 products resulted from a polymerase activity that led to newly synthesized RNA complementary to the full-length template, or from a 3' nucleotidyltransferase activity that simply labeled the 3' end of the RNA template, we performed an assay with 15 pmol of the 246R template and [ $\alpha$ -<sup>32</sup>P]UTP with or without supplemented cold NTPs (Fig. 2C). In the absence of cold NTPs, a unique product was observed at the template size (lane 6), which became completely degraded after RNase I treatment (lane 7). The sensitivity to RNase I treatment indicated that the product was single stranded and likely resulted from the addition of one or a few nucleotides to the 3' end of the template RNA. The absence of the product when

the reaction was performed with HA-RDR6m (supplemental Fig. S3) confirmed that *Arabidopsis* RDR6 possessed a 3' nucleotidyltransferase activity. Moreover, UTP seemed to be preferred by RDR6 over the other three NTPs for this activity (supplemental Fig. S3). When the reaction was performed in the presence of both cold NTPs and radioactive UTP, the products were resistant to RNase I treatment, indicating that the products were in dsRNA forms (Fig. 2, lanes 8 and 9). Indeed, denaturation prior to RNase I treatment led to complete digestion of the RNAs (data not shown). These results showed that RDR6 possessed two different activities *in vitro*, a nucleotidyltransferase activity and a polymerase activity, but the polymerase activity was predominant in the presence of all four NTPs. The newly synthesized RNA from the polymerase activity stayed bound to the template RNA. The fact that no products larger than the template size were found (Fig. 2C, lanes 8 and 9) suggested that RDR6 did not have a back-priming activity as observed for QDE-1 (38). Because the nucleotidyltransferase activity was undetectable in the presence of all four NTPs, we performed all subsequent reactions under this condition to analyze the polymerase activity of RDR6.

We tested RDR6 activity on various ssRNA templates and found that it was able to act on RNA templates of various sizes and sequences (supplemental Fig. S4A). We also tested the effects of  $Mn^{2+}$  or  $Mg^{2+}$  on RDR6 activity. Maximum activity was observed at a concentration of 8 mM for each cation (supplemental Fig. S5). All subsequent reactions were performed with  $Mg^{2+}$  at this concentration.

### RDR6 Does Not Distinguish Capped Versus Non-capped RNAs or RNAs with or without Poly (A) Tails

Molecular genetic evidence suggests that RNAs lacking a cap or a poly(A) tail are preferential targets for silencing (8–10). This led to the hypothesis that aberrant RNAs are preferred substrates for RDR6. In the biogenesis of ta-siRNAs, genetic evidence suggests that RDR6 uses the 5' or 3' fragments resulting from microRNA-guided cleavage of an mRNA as substrates. These RNA fragments lack either a cap or a poly(A) tail. We evaluated whether structural features at the 5' and 3' ends of an RNA template affect its recognition by RDR6 *in vitro*.

We first tested RNAs with different 5' end features. *In vitro* transcription products possessed a 5' triphosphate. We used the ScriptCap™ m<sup>7</sup>G Capping System (Epicenter) to convert the 5' triphosphate of the 246R RNA into a 7-methylguanosine cap (CAP-246R). In parallel, the 246R RNA was also treated with phosphatase to generate a 246R RNA with 5' OH (OH-246R), which was further treated with T4 polynucleotide kinase to generate an RNA with a 5' monophosphate (P-246R). The same treatments were also performed on the capping reaction mixture followed by digestion with the 5' monophosphate-specific exonuclease TERMINATOR. This allowed the elimination of all leftover non-capped RNAs after the capping reaction (supplemental Fig. S6). RDR6 assays were performed with 246R RNAs with a 5' triphosphate, 5' monophosphate, 5' OH, and 5' cap. We found that these 5' end modifications did not affect RDR6 activity (Fig. 3A).

Next, we tested whether the presence of a poly(A) tail at the 3' end of the template affects RDR6 activity (Fig. 3B). For this, we compared the activity of RDR6 on a 206R ssRNA containing 40 additional adenosines at the 3' end (206R-A<sup>40</sup>) with that on a 246R ssRNA without the 3' As. No difference in RDR6 activity was seen for two different concentrations of the template RNAs (Fig. 3B). We also used yeast poly(A) polymerase to add a longer poly(A) tail onto the 185F RNA. RDR6 activity on this 185F-A<sup>n</sup> RNA was compared with that on a similarly sized 640F RNA. Again, no difference in RDR6 activity was detected (supplemental Fig. S4B).

To determine whether a capped and polyadenylated RNA can serve as a substrate for RDR6, we performed assays on 395R-A<sup>40</sup> RNAs with or without a 5' cap. No difference in RDR6 activity was detected for these two templates (supplemental Fig. S4).

### RDR6 Does Not Have Polymerase Activity on dsRNAs nor Does It Have Priming Activity

One potential model to explain the requirement for RDR6 in the amplification of RNA silencing is that RDR6 uses dsRNAs as templates to generate more dsRNAs. To test whether RDR6 was able to use a dsRNA as a template, we performed an *in vitro* assay with two complementary ssRNAs, 435F and 435R (Fig. 2A), which were annealed prior to the reactions. We annealed the same amount of the forward template and an increasing amount of the reverse template to increase the dsRNA/ssRNA ratio. In the presence of an equal quantity of both RNAs, when more dsRNA templates were presumably present (Fig. 4A, lane 3), the activity was greatly reduced compared with the reaction performed with only the forward RNA (lane 1) or with an excess of the reverse RNA (Fig. 4A, lane 4). The small amount of products when both templates were present at supposedly equal quantity (Fig. 4A, lane 3) could be due to the presence of residual ssRNAs or due to weak RDR6 activity on dsRNAs. To conclusively determine the activity of RDR6 on dsRNAs, we annealed equal amounts of 435F and 435R followed by digestion with RNase I to remove any residual ssRNAs. The treated dsRNAs were then used in an RDR6 assay with the corresponding ssRNAs as controls. As shown in Fig. 4B, no products were found in the reaction with dsRNAs, indicating that RDR6 was unable to use dsRNAs as templates.

Another model to explain the requirement for RDR6 in the amplification of RNA silencing is that RDR6 uses a primary siRNA as a primer and the target mRNA as the template to synthesize dsRNAs. To investigate whether RDR6 can use a small RNA as a primer to copy a template RNA, we performed an assay with the 246R template in the presence or absence of the 21-nucleotide miR173<sup>\*</sup>, which was completely complementary to a region of the template (Fig. 2A). If RDR6 were able to use the small RNA as a primer, it would be predicted to generate a product of ~80 nucleotides (indicated by the *black arrow*). However, no product of this size was detected (Fig. 4C, lane 2). To exclude the possibility that the priming activity might have been masked by the background smear (Fig. 4C, lane 2), we performed the assay with miR173<sup>\*</sup> labeled at the 5' end and cold NTPs so that only products resulting from the priming activity would be visualized. Again, no products were found (data not shown). RNase I treatment after the RDR6 reactions showed that miR173<sup>\*</sup> was protected from digestion, suggesting that it had been annealed to the template (data not shown). Therefore, the lack of priming activity was not due to lack of annealing of the primer to the template.

### RDR6 Can Use Single-stranded DNA as a Template

One of the first known functions of RDR6 is to mediate virus-induced gene silencing (VIGS). However, almost all ssRNA viruses tested induced VIGS in an RDR6-independent manner (3,4), whereas VIGS induced in response to infection by the CaLCuV, a ssDNA virus, was strongly inhibited in an *rdr6* mutant (19). These observations led us to test whether RDR6 could also use ssDNA as a template. We produced ssDNA using asymmetric PCR on the dsDNA fragment that was used initially to transcribe the 246R template. RDR6 assay was then performed in the presence of [ $\alpha$ -<sup>32</sup>P]UTP and all four cold NTPs (Fig. 5A). RDR6 was able to generate a product of the same size as the template (lane 1), whereas no activity was detected with RDR6m (lane 3). Interestingly, this activity appeared at least 10-fold higher than the one with the same amount of a ssRNA template (lanes 1 and 2), suggesting that ssDNA was a better template for RDR6 than ssRNA under our assay conditions. To rule out that the product was due to the 3' nucleotidyl activity that labeled the 3' end of the template DNA, we performed assays with or without cold NTPs and digested the products with RNase H and RQ1 (a DNase) to evaluate the properties of the products (Fig. 5B). When the reactions were performed in the



absence of cold NTPs, a product of the same size as the template was found (*lane 1*), suggesting that RDR6 had the nucleotidyltransferase activity even on ssDNA. The fact that the product was sensitive to RQ1 but insensitive to RNase H confirmed that the product was ssDNA (*lanes 2 and 3*). When the reaction was performed in the presence of cold NTPs, a product of the same size as the template was found (*lane 4*). This product, however, was sensitive to RNase H but insensitive to RQ1 (*lanes 5 and 6*). This indicated that the product was newly synthesized RNA (hence insensitivity to RQ1) that stayed paired with the template DNA (hence sensitivity to RNase H). Therefore, RDR6 was able to use ssDNA as template to synthesize a complementary RNA.

It is worth noting that RDR6 was unable to incorporate dNTPs with either DNA or RNA templates (data not shown). Furthermore, RDR6 was unable to use a dsDNA as template (Fig. 5C, *lanes 7 and 8*). Therefore, RDR6 was exclusively an RNA polymerase, but it could act on either ssRNA or ssDNA but not dsDNA.

## DISCUSSION

### Biochemical Properties of RDR6

Our studies demonstrate that RDR6 indeed possesses RNA-dependent RNA polymerase activity on ssRNAs. It makes a complete copy of its substrate RNA, and the newly synthesized RNA stays bound to the template RNA. Our results clearly show that RDR6 does not have the intrinsic capacity to distinguish capped *versus* non-capped RNAs, which was also observed with an RDR activity in wheat germ extracts (37), or RNAs with or without poly(A) tails, suggesting that the recognition of aberrant RNAs involves other proteins *in vivo*.

In the literature, conflicting observations were made regarding the ability of RDRs to use ssDNA templates. Studies with RDR preparations of various purities from cauliflower and tobacco showed little or no activities on dsDNA or ssDNA (34–36). A highly pure preparation of tomato RDR, however, had similar activity on ssRNA and ssDNA templates (30). With the help of the catalytically inactive HA-RDR6m as a control, we unequivocally demonstrated that *Arabidopsis* RDR6 acts on ssDNA but not dsDNA templates *in vitro*.

### RDR6 in the Amplification of Silencing

Amplification of RNA silencing can occur through a number of potential mechanisms that are not mutually exclusive. The first mechanism is that an RDR copies an aberrant RNA into dsRNA and then uses the newly synthesized RNAs as substrates in a second round of dsRNA synthesis. We showed that the newly synthesized RNAs stay bound to the template RNAs and that RDR6 cannot use dsRNA as substrates, which argue against this mechanism of amplification. However, *in vivo* the dsRNAs may be unwound by an RNA helicase to generate ssRNAs that can serve as substrates for RDR6. In fact, the production of secondary siRNAs in *Arabidopsis* requires SDE3, a protein with an RNA helicase signature (45,46). The second mechanism of amplification is that a primary siRNA serves as a primer for RDR to generate dsRNA from the target mRNA. This mechanism could explain the generation of secondary siRNAs outside the initial trigger region. A recent study with a GFP transgene targeted by a microRNA supports such a mechanism of transitivity (18). However, our biochemical analysis indicates that RDR6 is unable to use a 21-nucleotide small RNA as a primer. A third mechanism is that an RDR is recruited to its potential substrate by primary siRNAs and directly synthesizes small RNAs from the template. In *C. elegans*, the fact that secondary siRNAs contain a 5'-triphosphate group and are complementary to target mRNAs supports this hypothesis (16,17). *In vitro*, *Neurospora* QDE-1 is able to synthesize small RNAs from a long RNA template (38). We found that RDR6, unlike QDE-1, was unable to generate short RNA products *in vitro* (data not shown). A last mechanism of transitivity is inferred from the process of tasiRNA

biogenesis. In *Arabidopsis*, genetic evidence suggests that RDR6 uses mRNA fragments generated through microRNA-mediated cleavage as substrates in the biogenesis of ta-siRNAs (20–22,25). It is conceivable that in S-PTGS, a primary siRNA leads to the cleavage of a target mRNA and the cleavage fragments serve as templates for RDR6 in the generation of secondary siRNAs. We found that in *vitro* RDR6 does not have any preference for an RNA without a poly(A) tail or an RNA without the cap. Therefore, the recruitment of the cleavage fragments to RDR6 must involve others proteins *in vivo*.

### RDR6 and VIGS against Geminiviruses

The CaLCuV is a single-stranded DNA virus, which belongs to the Geminivirus family. Infection of *Arabidopsis* with CaLCuV is counteracted by VIGS through a mechanism dependent on *RDR6* and *SGS3* (19), which are also required for S-PTGS (3,4,47). However, Geminiviruses do not replicate through RNA intermediate forms and viral genes are expressed by the host RNA polymer-ase. How does RDR6 distinguish viral mRNAs from endogenous mRNAs? As it is suggested for S-PTGS, the overexpression of viral genes may be somehow “sensed” by the cell such that the mRNAs are channeled to RDR6. It is also possible that overlapping antisense transcripts from the viral genes initiate silencing through an RDR6-dependent process, similar to the silencing mechanism by natural *cis*-acting siRNAs (23,48). However, considering that RDR6 is able to use ssDNA as templates, it is tempting to suggest that instead of targeting viral RNA, the genomic ssDNA of CaLCuV is used by RDR6 to generate ssRNAs, which could then either base pair with the expressed viral mRNAs or be used by RDR6 as a template to generate dsRNAs.

VIGS against cucumber mosaic virus, a double-stranded DNA virus, also requires RDR6 (3, 4). Because RDR6 cannot act on dsDNA, it is likely that a ssDNA intermediate or transcripts from cucumber mosaic virus are targeted by RDR6.

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

### Acknowledgments

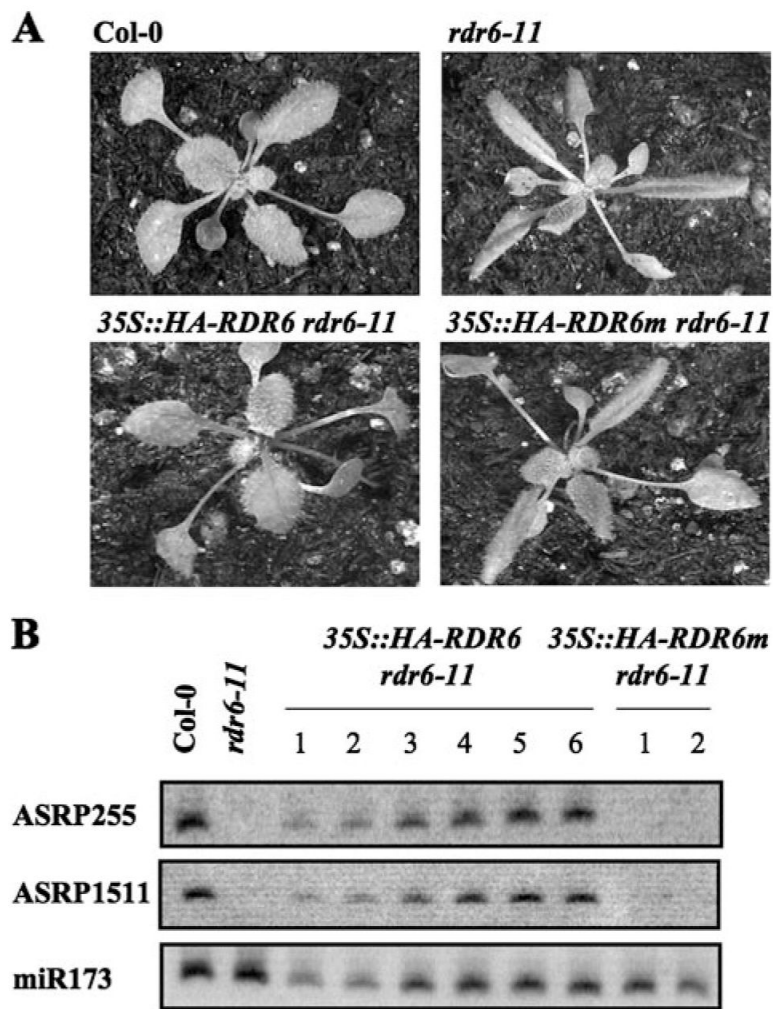
We thank YunJu Kim for a plasmid that contains the luciferase/NOS chimeric gene and the miR173\* sequence. We are grateful to Drs. Popescu and Dinesh-Kumar for suggestions on transient expression of proteins in *N. benthamiana*. We thank Theresa Dinh, Vanitharani Ramachandran, Bin Yu, and Binglian Zheng for comments on the manuscript.

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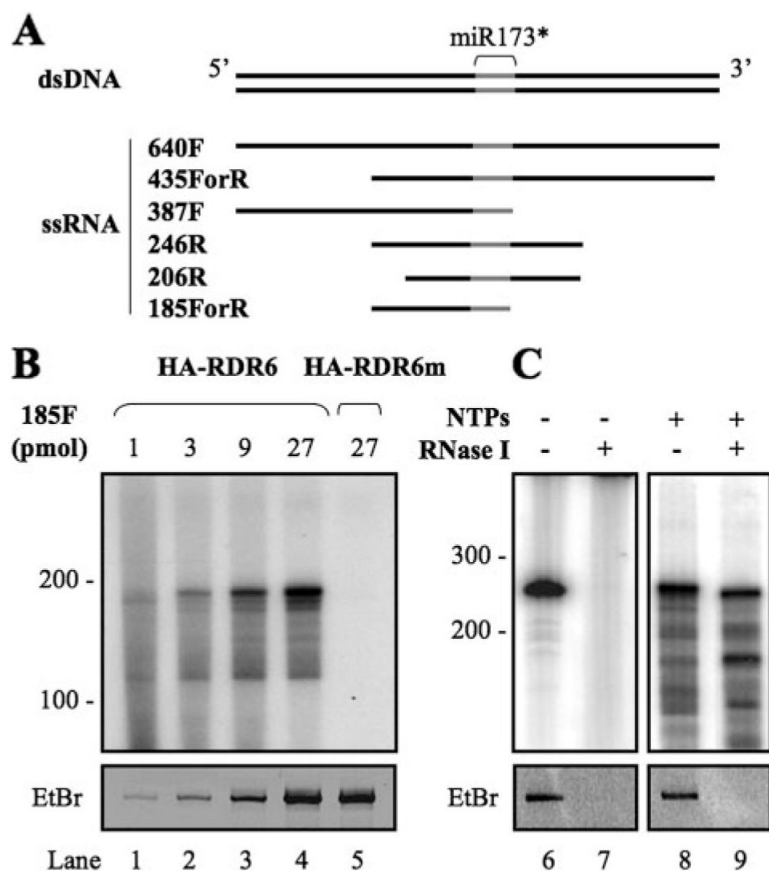
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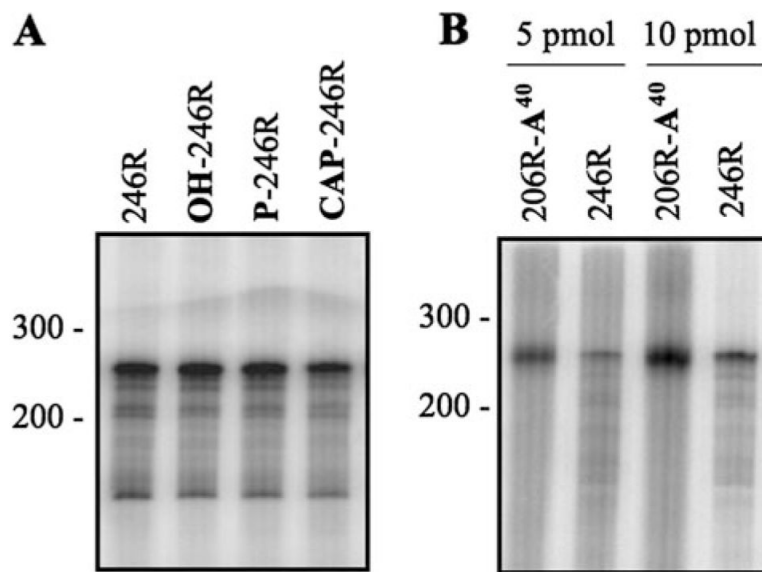
**FIGURE 1. Complementation of the *rdr6-11* mutation by the *35S::HA-RDR6* transgene**  
**A**, 23-day-old Columbia wild type (*Col-0*), *rdr6-11* mutant and two *rdr6-11* transgenic lines containing either *35S::HA-RDR6* or the *35S::HA-RDR6m* transgene. *rdr6-11* mutants showed downward curling of rosette leaves. This phenotype was rescued by *35S::HA-RDR6* but not *35S::HA-RDR6m*. **B**, Northern blot analysis of two ta-siRNAs (ASRP255 and ASRP1511). The analysis was performed with 40  $\mu$ g of total RNAs extracted from inflorescences of wild type (*Col-0*), *rdr6-11* mutants, and six independent *35S::HA-RDR6* *rdr6-11* and two independent *35S::HA-RDR6m* *rdr6-11* transgenic lines. The same membrane was used (after stripping) for the 3 hybridizations. *miR173*, a microRNA, served as a loading control.





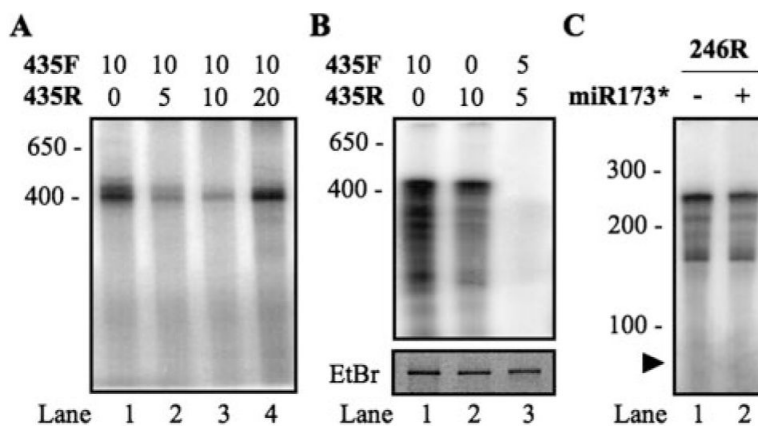
**FIGURE 2. RNA polymerase and nucleotidyltransferase activities of purified RDR6**

**A**, schematic representation of ssRNAs used as templates in various RDR assays. A dsDNA fragment (sequence in supplemental Fig. S2A) of a luciferase/nopaline synthase chimeric gene with an engineered miR173\* sequence was used as the template for PCR amplification of various segments, which in turn served as templates for *in vitro* transcription to generate the ssRNA transcripts. The name of each ssRNA referred to the size (in nucleotides) and orientation (*F*, forward; or *R*, reverse) compared with the DNA sequence. **B** and **C**, reaction products from RDR assays resolved on 6% polyacrylamide, 7 M urea gels and visualized by phosphorimaging. **B**, assays were performed with immunopurified HA-RDR6 or HA-RDR6m and different quantities of the 185F RNA template (1 to 27 pmol) in the presence of all four cold NTPs and [ $\alpha$ - $^{32}$ P]UTP. The image of the stained gel at the *bottom* illustrates the amounts of templates used. **C**, assays were performed with HA-RDR6, 15 pmol of the 246R template, and [ $\alpha$ - $^{32}$ P]UTP, in the presence (+) or absence (-) of the four cold NTPs. After the reaction, the products were treated (+) or not (-) with RNase I for 20 min at 37 °C. The *lower panels* were images from ethidium bromide (EtBr) staining of the PAGE gels before phosphorimaging. The fact that no RNAs were visible by EtBr staining in *lane 9* suggested that the amount of RDR6 products was very small.



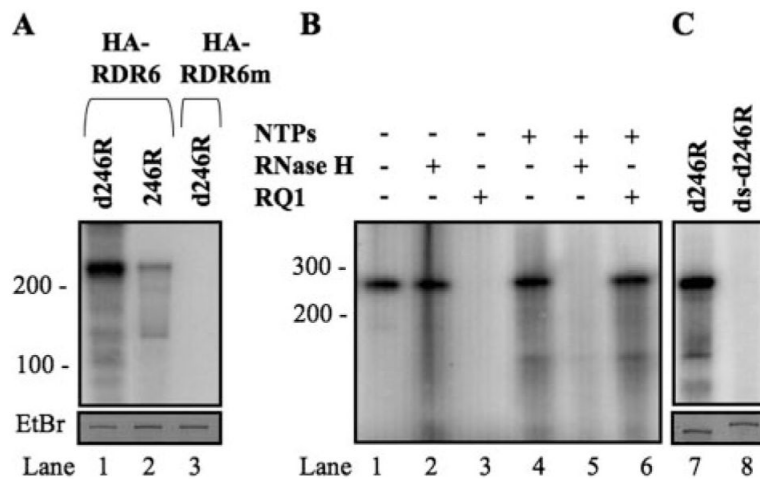
**FIGURE 3. RDR6 activity on RNA templates with different 5' and 3' end structures**

All enzymatic assays were performed with HA-RDR6 in the presence of [ $\alpha$ -<sup>32</sup>P]UTP and four cold NTPs. *A*, assays were performed with 15 pmol of four 246R templates possessing different structures at the 5' ends, a triphosphate (246R), a hydroxyl group (OH-246R), a monophosphate (P-246R), or a m<sup>7</sup>G cap (CAP-246R). *B*, assays were performed with 5 or 10 pmol of 206R-A<sup>40</sup>, a 206-nucleotide RNA with 40 additional adenosines at the 3' end. The 246R template lacked an A tail and was used as a control.



**FIGURE 4. RDR6 activity on double-stranded RNAs and the absence of a priming activity**

All enzymatic assays were performed with HA-RDR6 in the presence of [ $\alpha$ - $^{32}$ P]UTP and all four cold NTPs. *A*, assays were performed with 10 pmol of the 435F template that had been hybridized with an increasing quantity (0–20 pmol) of its complementary strand, 435R. *B*, assays were formed with ssRNAs (*lanes 1* and *2*) and equal amounts of 435F and 435R annealed together and treated with RNase I to remove any residual ssRNAs (*lane 3*). A portion of the RNA templates used for the reactions were resolved on a denaturing gel and stained with ethidium bromide to demonstrate the near equal amounts of templates used. *C*, priming activity was tested on 15 pmol of the 246R template in the presence (+) or absence (-) of 30 pmol of miR173\* as a primer. The expected product synthesized from the primer (~80 nucleotide) would migrate to the position indicated by the *arrowhead*.



**FIGURE 5. DNA-dependent RNA polymerase activity of RDR6**

A, all enzymatic assays were performed with HA-RDR6 or HA-RDR6m in the presence of  $\alpha$ - $^{32}$ P]UTP and all four cold NTPs. The same PCR product was used to synthesize the ssDNA, by asymmetric PCR, and the ssRNA, by *in vitro* transcription. 5 pmol of a ssDNA template (d246R) and the same amount of a ssRNA template (246R) were tested with HA-RDR6 (lanes 1–2) or HA-RDR6m (lane 3). B, assays were conducted with a ssDNA (d246R) template, HA-RDR6, and [ $\alpha$ - $^{32}$ P]UTP in the presence (+) or absence (-) of cold NTPs. After the RDR6 reactions, the reaction mixture was treated (+) or not (-) with RNase H or RQ1 (a DNase). C, assays were conducted with HA-RDR6, [ $\alpha$ - $^{32}$ P]UTP, and all four cold NTPs on a ssDNA (d246R) and a dsDNA (ds-d246R) template. The stained gel at the *bottom* illustrates near equal amounts of templates used. The larger size of the dsDNA template as compared with the ssRNA template is due to the presence of the T7 promoter sequence in the dsDNA.