Doctoral Thesis (Abridged)

博士論文（要約）

Biochemical analysis of RNA-DEPENDENT RNA POLYMERASE6 in post-transcriptional gene silencing

(転写後遺伝子サイレンシングにおける RNA 依存性 RNA ポリメラーゼ６の生化学的解析)

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General introduction

RNA silencing

RNA silencing is a small RNA mediated mechanism for the regulation of gene expression and is conserved in eukaryotic organisms. About twenty nucleotides (nt) small RNAs including microRNAs (miRNAs) or small interfering RNAs (siRNAs), which are derived from genome or double-stranded RNAs (dsRNAs), are incorporated into Argonaute proteins (AGOs) to form RNA-induced silencing complex (RISC) (Lagos-Quintana *et al*., 2001; Hammond *et al*., 2000; Hutvagner and Zamore, 2002; Mourelatos *et al*., 2002). RISC cleaves the complementary target RNAs or represses translation (Hutvagner and Zamore, 2002; Martinez *et al*., 2002; Pillai *et al*., 2005) (Figure 1).

Unlike mammals, plants have RNA-dependent RNA polymerase (RdRP or RDR) mediated gene silencing pathways called post-transcriptional gene silencing (PTGS) and transcriptional gene silencing (TGS). In these pathways, RdRPs convert single-stranded RNAs (ssRNAs) into dsRNAs, which trigger siRNA-mediated gene silencing.

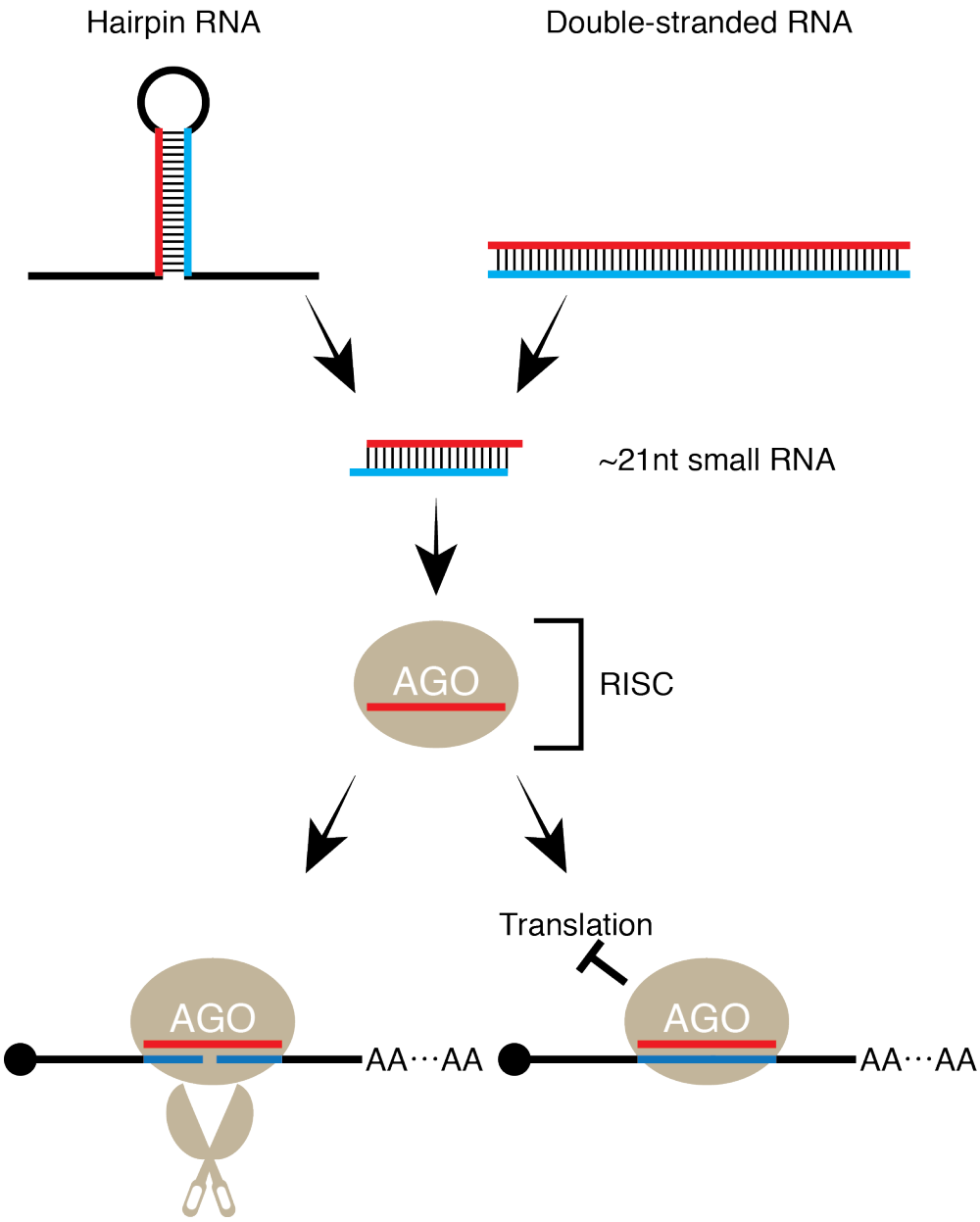

Figure 1. Biogenesis and function of small RNAs.

Small RNAs derived from hairpin RNAs or long dsRNAs assemble with AGO proteins into RISC. RISC cleaves the complementary target RNAs or represses translation.

RNA-dependent RNA polymerases in plants

In *Arabidopsis thaliana* (*A. thaliana*), there are six RdRPs (Wassenegger and Krczal, 2006; Zong *et al*., 2009; Willmann *et al*., 2011). Among them, RDR1, RDR2 and RDR6 are required for gene silencing pathways (Willmann *et al*., 2011). In contrast, it is unclear whether RDR3, RDR4 or RDR5 participates in gene silencing. Given that canonical endogenous mRNAs are not repressed by RdRP-mediated PTGS in normal condition (Lu *et al*., 2005a), RdRPs should be tightly regulated to avoid induction of self PTGS.

RDR1

RDR1 converts viral RNAs into dsRNAs, which trigger PTGS (Diaz-Pendon *et al*., 2007; Donaire *et al*., 2008; Qi *et al*., 2009; Wang *et al*., 2010). Interestingly, RDR1 expression is induced by viral infection or treatment with salicylic acid, which is an endogenous hormone for antiviral defense (Malamy *et al*., 1990; Xie *et al*., 2001; Yu *et al*., 2003). However, the mechanistic detail of RDR1 mediated PTGS is still unclear.

RDR2

RDR2 involves in small RNA-mediated DNA methylation, which triggers TGS (Xie *et al*., 2004; Herr *et al*., 2005). RDR2 is physically associated with RNA polymerase IV (Pol IV) and converts the nascent transcripts of Pol IV into dsRNAs (Law *et al*., 2011; Haag *et al*., 2012). RDR2 has three enzymatic activities, primer-dependent RdRP activity, primer-independent RdRP activity and terminal

nucleotidyl-transferase (TNTase) activity *in vitro* (Blevins *et al*., 2015; Devert *et al*., 2015).

RDR6

RDR6 is the most characterized RdRP in plants. Similar to RDR2, RDR6 also possesses primer-dependent RdRP activity, primer-independent RdRP activity and TNTase activity *in vitro* (Curaba and Chen, 2008; Fukunaga and Doundna, 2009; Devert *et al*., 2015). Unlike RDR1 and RDR2, RDR6 participates in sense transgene-induced PTGS (S-PTGS) and endogenous secondary siRNA biogenesis (Dalmay *et al*., 2000; Mourrain *et al*., 2000; Allen *et al*., 2005; Yoshikawa *et al*., 2005).

Sense transgene-induced post-transcriptional gene silencing

S-PTGS has been discovered from co-suppression phenomenon. When Chalcone synthase (CHS), an important factor in flavonoid biosynthesis, was overexpressed in violet petunia, the overexpression resulted in white petunia (Napoli *et al*., 1990; van der Krol *et al*., 1990). Thus, endogenous CHS mRNAs were co-suppressed with transgene-derived CHS mRNAs, which have the same sequences with endogenous CHS mRNAs (Napoli *et al*., 1990; van der Krol *et al*., 1990). How does the overexpression of transgene trigger PTGS? After transgene-derived RNAs are converted into dsRNAs by RDR6, DICER-LIKE2 or 4 (DCL2 or 4) processes dsRNAs into 22- or 21-nt siRNAs, which are incorporated into AGO1 to form RISC. Then the RISCs cleave the endogenous CHS mRNAs as well as transgene-derived mRNAs resulting in silencing of CHS gene and the production of white petunia (Dalmay *et al*., 2000; Fagard *et al*., 2000; Mourrain *et al*., 2000; Deleris *et al*., 2006) (Figure 2). In addition to the proteins described above, SUPPRESSOR OF GENE SILENCING3 (SGS3) and SILENCING DEFECTIVE5 (SDE5) have been identified as crucial proteins in S-PTGS (Dalmay *et al*., 2000; Mourrain *et al*., 2000; Hernandez-Pinzon *et al*., 2007).

SGS3

SGS3 cooperates with RDR6 for dsRNA synthesis by stabilizing the RNAs, which are template RNAs of RDR6 (Mourrain *et al*., 2000; Muangsan *et al*., 2004; Yoshikawa *et al*., 2005; Yoshikawa *et al*., 2013). Moreover, SGS3 co-localizes with

RDR6 to form siRNA body *in vivo* (Kumakura *et al*., 2009; Jouannet *et al*., 2012). However, SGS3 does not affect recombinant RDR6 activity *in vitro* (Fukunaga and Doundna, 2009). It was reported that SGS3 is 5′ overhang dsRNA binding protein (Fukunaga and Doundna, 2009). Moreover, SGS3 interacts with the 22-nt miRNA loaded AGO1-RISCs and target RNAs, but not 21-nt miRNA loaded AGO1-RISC (Yoshikawa *et al*., 2013). However, how SGS3 participates in S-PTGS is still unclear.

SDE5

SDE5 functions in the downstream of RDR6 and upstream of SGS3 in PTGS (Yoshikawa *et al*., 2016). Moreover, it was reported that SDE5 is a putative RNA transport protein (Hernandez-Pinzon *et al*., 2007). However, the molecular function of SDE5 is unclear.

As I mentioned above, several questions remain unclear in S-PTGS mechanism. Among them, one of the most important questions is how transgene-derived RNAs, but not endogenous mRNAs, specifically trigger PTGS.

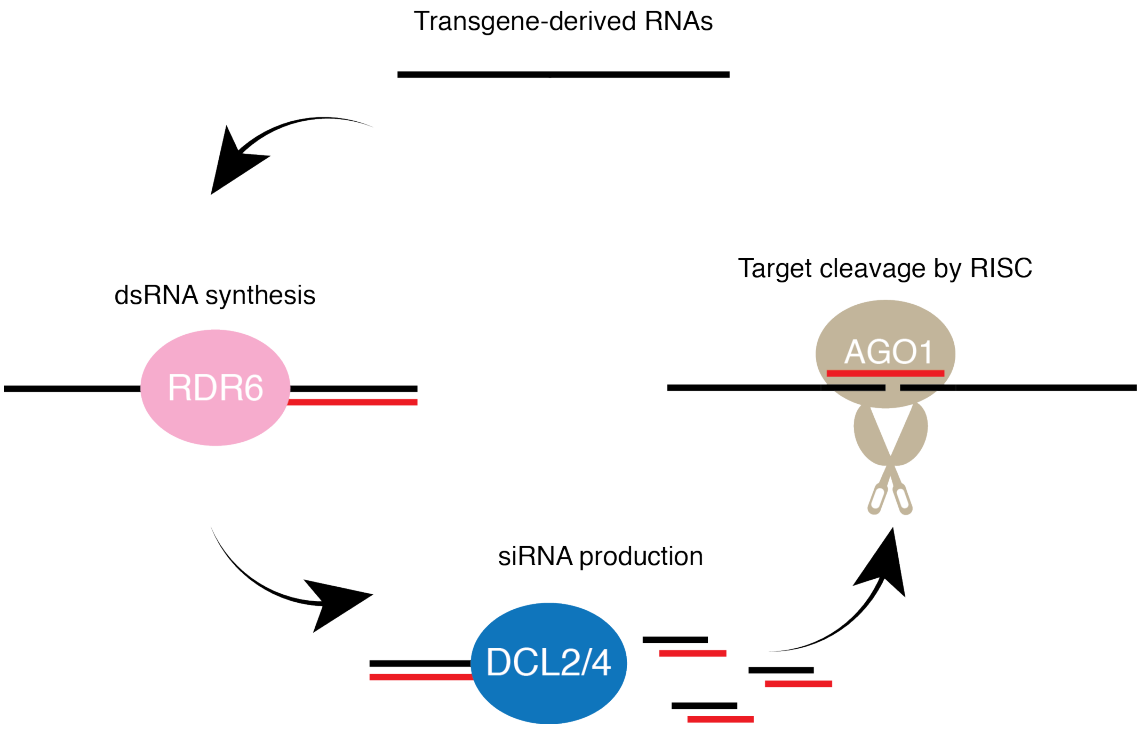

Figure 2. S-PTGS mechanism.

RDR6 converts transgene-dervied RNAs into dsRNAs. Then, DCL2 or 4 processes the dsRNAs into siRNAs, which are incorporated new AGO proteins form RISC. These RISCs are cleaved the transgene RNAs.

Endogenous secondary siRNA biogenesis

Plants have several endogenous RNAs (PHAS or TAS RNAs), which produce secondary siRNAs, called phased secondary siRNAs (phasiRNAs) or trans-acting siRNAs (tasiRNAs) (Peragine *et al*., 2004; Vazquez *et al*., 2004; Allen *et al*., 2005; Yoshikawa *et al*., 2005, Fei *et al*., 2013). The PHAS and TAS RNAs have miRNA target site, which is important for the production of the secondary siRNAs (Allen *et al*., 2005; Yoshikawa *et al*., 2005; Axtell *et al*., 2006; Fei *et al*., 2013). Interestingly, phasiRNA and tasiRNA biogenesis pathway shares the proteins involved in S-PTGS pathway (Peragine *et al*., 2004; Vazquez *et al*., 2004; Allen *et al*., 2005; Yoshikawa *et al*., 2005; Hernandez-Pinzon *et al*., 2007; Montgomery *et al*., 2008a). There are two models for phasiRNA and tasiRNA biogenesis, called "one-hit" model and "two-hit" model.

One-hit model

TAS1 or TAS2 RNAs are well known "one-hit" RNAs, which have one miR173 target site (Allen *et al*., 2005; Yoshikawa *et al*., 2005). TAS1- or TAS2-derived tasiRNAs biogenesis is initiated by the cleavage of TAS1 or TAS2 mRNAs by miR173-AGO1-RISC (Montgomery *et al*., 2008a). Then, SGS3 interacts with AGO1-RISC and the TAS1 or TAS2 RNAs, which form 5′ overhang dsRNA between the RNAs and 22-nt miR173 (Yoshikawa *et al*., 2013). After target cleavage, RDR6 converts the 3′ fragments of TAS1 or TAS2 RNAs into dsRNAs that are processed into siRNAs by DCL4 (Allen *et al*., 2005; Yoshikawa *et al*., 2005; Xie *et al*., 2005) (Figure

3A). Interestingly, it was reported that the introduction of 22-nt miRNA-AGO1-RISC target site into reporter RNAs is sufficient to trigger secondary siRNA biogenesis (Felippes and Weigel, 2009; Chen *et al*., 2010; Cuperus *et al*., 2010).

Two-hit model

TAS3 RNAs are well known "two-hit" RNAs, which have two miR390-AGO7-RISC target sites (Adenot *et al*., 2006; Axtell *et al*., 2006; Montgomery *et al*., 2008b). While the 3′ proximal target site is cleaved by miR390-AGO7-RISC, the 5′ proximal target site is not cleaved due to central mismatches between miR390 and the target site (Axtell *et al*., 2006; Montgomery *et al*., 2008b). After target cleavage, RDR6 converts 5′ fragments—but not 3′ fragments—into dsRNAs, which trigger PTGS (Axtell *et al*., 2006; Montgomery *et al*., 2008b) (Figure 3B). The generated tasiRNAs are assembled into AGO1 to target AUXIN RESPONSE FACTOR3 and 4, resulting in proper development (Allen *et al*., 2005; Adenot *et al*., 2006; Axtell *et al*., 2006).

Interestingly, most of the endogenous precursor RNAs of the secondary siRNAs has two or more small RNA target sites (Axtell *et al*., 2006; Howell *et al*., 2007). Moreover, it was proposed that "one-hit" transits to "two-hit" by the secondary siRNAs, which are produced from "one-hit" target RNAs and cleave it in cis (Rajeswaran *et al*., 2012). The two small RNA target sites are important to produce secondary siRNA biogenesis, but it is unclear how "two-hit" facilitate phasiRNA or tasiRNA biogenesis.

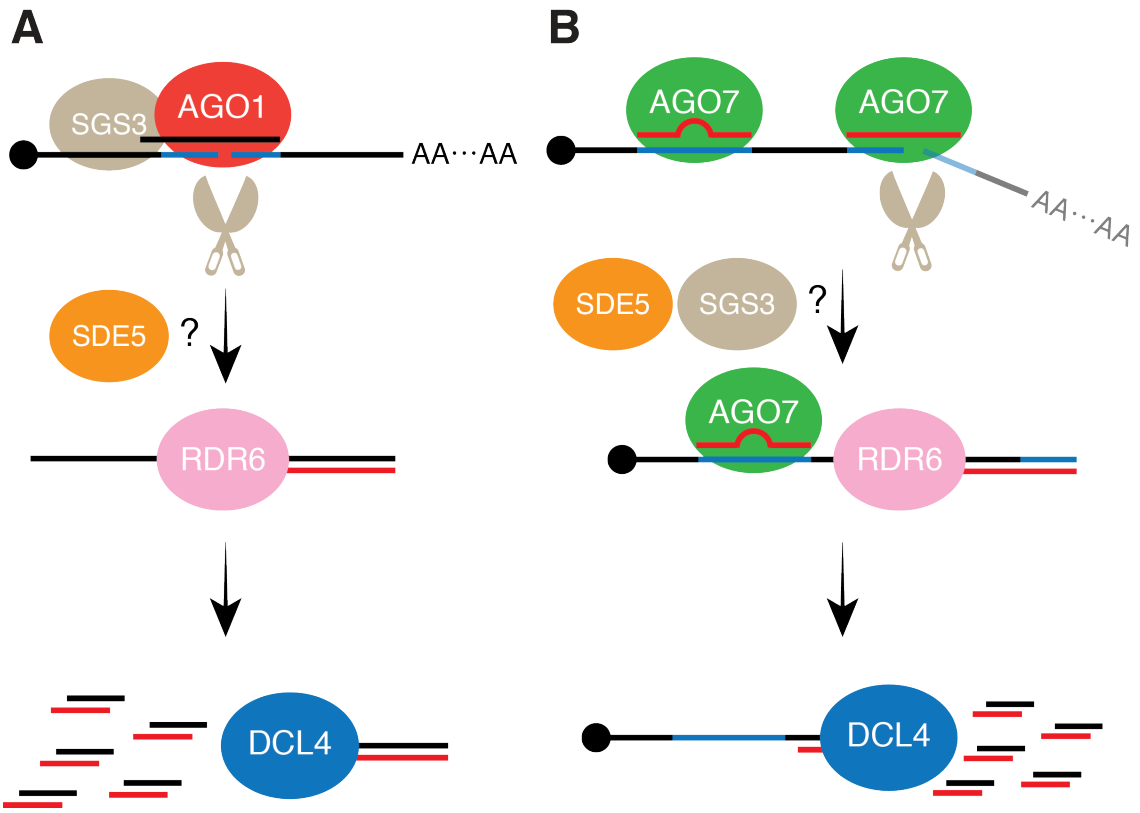

Figure 3. Endogenous secondary siRNA biogenesis.

In TAS1 or TAS2-tasiRNA biogenesis, miR173-AGO1 cleaves target RNAs. Then, RDR6 converts the 3′ fragments into dsRNAs, which are processed into 21-nt siRNAs by DCL4. In TAS3-tasiRNA biogenesis, miR390-AGO7 binds to 5′ proximal target site and cleaves 3′ proximal target site. Then, RDR6 converts the 5′ fragments into dsRNAs, which are processed into 21-nt siRNAs by DCL4.

Questions

In this dissertation, I focused on two questions about PTGS. First, how do plants discriminate transgene-derived RNAs from endogenous RNAs? If endogenous mRNAs trigger PTGS *in vivo*, it should be lethal. Thus, plants should have "discriminator", which specifically recognizes transgene-derived RNAs. I propose the answer about this question in the Part I in this dissertation. Second, what is the function of "two-hit" by RISC for endogenous secondary siRNA biogenesis? Although it is known that two miRNA target sites of PTGS target transcripts play important roles for the siRNA production, it remains unclear why two miRNA target sites are important. I propose the answer about this question in the Part II in this dissertation.

Part I: Template specificity of RDR6

Introduction

Plants have a defense mechanism called PTGS, which protects against invasion by exogenous genes such as viruses or transgenes (Wingard, 1928; Napoli *et al*., 1990; van der Krol *et al*., 1990; Baulcombe and English, 1996; Ratcliff *et al*., 1997). PTGS is initiated by RDR6-mediated dsRNA synthesis, which is followed by the dsRNA processing into 22- or 21-nt siRNAs by DCL2 or 4 (Dalmay *et al*., 2000; Mourrain *et al*., 2000; Deleris *et al*., 2006). The generated secondary siRNAs are incorporated into AGO proteins to form RISC, which cleave target RNAs (Baumberger and Baulcombe, 2005; Qi *et al*., 2005).

How do plants specifically silence exogenous RNAs, but not endogenous RNAs? It has been proposed that transgene-derived RNAs contain aberrant RNAs and these aberrant RNAs might trigger gene silencing (Baulcombe and English, 1996; English *et al*., 1996). This hypothesis was supported by the observation that in the 5′ to 3′ and/or 3′ to 5′ RNA decay pathway mutant plants, PTGS was triggered from endogenous aberrant RNAs, which should be degraded by the decay pathways in wild type plants (Branscheid *et al*., 2015; Martinez de Alba *et al*., 2015; Zhang *et al*., 2015). Supporting the aberrant RNA model, it was reported that PTGS was triggered by the accumulated poly(A)-less RNAs, which are improperly transcription-terminated RNAs derived from transgenes (Luo and Chen, 2007). Given that dsRNA synthesis by RDR6 initiates PTGS, an attractive hypothesis is that RDR6 discriminates aberrant poly(A)-less RNAs from self RNAs with poly(A) tail. However, there is no evidence that RDR6 prefers poly(A)-less RNAs to polyadenylated RNAs as its templates.

It is difficult to characterize the enzymatic activity of protein *in vivo* due to the effect of other factors. To precisely characterize RDR6 activity, I thought that biochemical approach is the best strategy to validate the hypothesis.

Results

RDR6 cannot convert polyadenylated RNAs into dsRNAs

In order to characterize RDR6 activity, I tried to overexpress wild-type and catalytic mutant RDR6 (D867A) (Curaba and Chen, 2008) with a N-terminal FLAG tag followed by SUMOstar protease cleavage site into *Drosophila* S2 sells (Schneider, 1972). The recombinant proteins were immunoprecipiated with anti-FLAG antibody and then eluted with SUMOstar protease (Figure 4A). I had successfully prepared highly purified wild-type and catalytic mutant recombinant RDR6 (Figure 4B). Given that poly(A)-less RNAs triggered PTGS (Luo and Chen, 2007), I prepared the reporter RNAs with 3′ poly(A) tail (N_{100}-A_{60}) or without 3′ poly(A) tail (N_{100}-A_0) *in vitro* (Figure 4C). I incubated recombinant RDR6 and N_{100}-A_0 RNAs or N_{100}-A_{60} RNAs in the presence of NTP and [α-^{32}P]-UTP. After deproteinization and RNA purification, the RNAs were resolved on denaturing gel. Then, I tried to detect the radiolabeled RNAs, which were synthesized by RDR6. When the catalytic mutant RDR6 was incubated with the reporter RNAs, no signal was detected (Figure 4D). In contrast, when the wild-type RDR6 was incubated with the reporter RNAs, strong signals were detected regardless of the presence or absence of the 3′ poly(A) tail, suggesting that RDR6 has ability to synthesize complementary RNAs from the polyadenylated template RNAs (Figure 4D). However, in addition to the RNA-dependent RNA polymerase (RdRP) activity, RDR6 is known to possess 3′ terminal nucleotidyl-transferase (TNTase) activity that adds nucleotides at the 3′ end of RNAs (Curaba and Chen, 2008). Thus, in order to selectively observe RdRP products, I removed TNTase products by the treatment of a

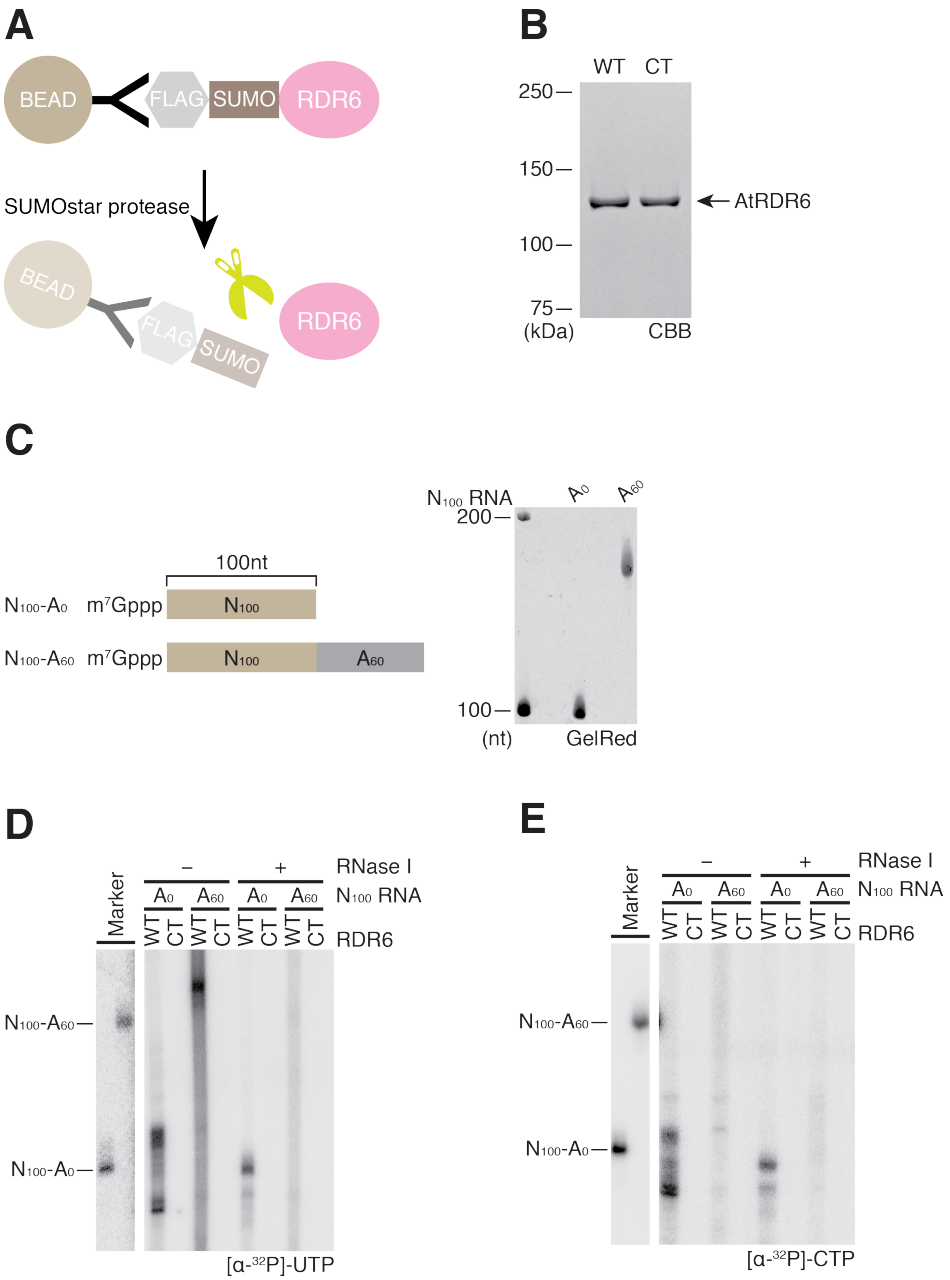

Figure 4. RDR6 specifically converts poly(A)-less RNAs.

(A) A scheme for the purification of RDR6. (B) Coomassie brilliant blue (CBB) staining of wild-type (WT) and catalytic mutant (CT) recombinant AtRDR6. (C) A scheme for *in vitro* transcribed RNAs with or without poly(A) tail. (D) RdRP assay using the reporter RNAs as shown in (C) as templates in the presence of [α-^{32}P]-UTP. The products were resolved on an 8 % polyacrylamide urea gel and and visualized by phosphorimaging. The radiolabeled synthetic RNAs were used as the markers. (E). RdRP assay with using the reporter RNAs as shown in (C) as templates in the presence of [α-^{32}P]-CTP. Denaturing PAGE was performed essentially as in (D). The radiolabeled synthetic RNAs were used as the markers.

single-stranded RNA specific ribonuclease, RNase I after the reaction. When I performed RdRP assay on N_{100}-A_0 RNAs, specific signals were detected after RNase I treatment (Figure 4D). In contrast, no signal for N_{100}-A_{60} RNAs was detected after RNase I treatment, suggesting that the signals for N_{100}-A_{60} RNAs were TNTase products (Figure 4D). Given that TNTase activity of RDR6 preferentially adds uridine at the 3′ end of template RNAs (Curaba and Chen), I performed RdRP assay using [α-^{32}P]-CTP instead of [α-^{32}P]-UTP. As expected, RdRP products were detected with N_{100}-A_0 RNAs, but not N_{100}-A_{60} RNAs regardless of RNase I treatment (Figure 4E). Taken together, I concluded that RDR6 preferentially converts poly(A)-less RNAs into dsRNAs.

8-nt poly(A) tail is sufficient to block RdRP activity of RDR6

Next, in order to determine the length of the poly(A) tail that can block complementary RNA synthesis by RDR6, I prepared four synthetic RNAs which have 32-nt non-G sequence with 0-, 4-, 8- or 16-nt poly(A) tail (H_{32}-A_0, H_{32}-A_4, H_{32}-A_8, or H_{32}-A_{16}). (Figure 5A). As expected, H_{32}-A_0 RNAs were converted into dsRNAs (Figure 5B). Weak signals were detected with H_{32}-A_4 RNAs (Figure 5B). In contrast, no clear band was detected with H_{32}-A_8 and H_{32}-A_{16} RNAs (Figure 5B), although smeared signals were detected with H_{32}-A_8 RNAs (Figure 5B). This result indicates that an 8-nt poly(A) tail can block dsRNA conversion by RDR6.

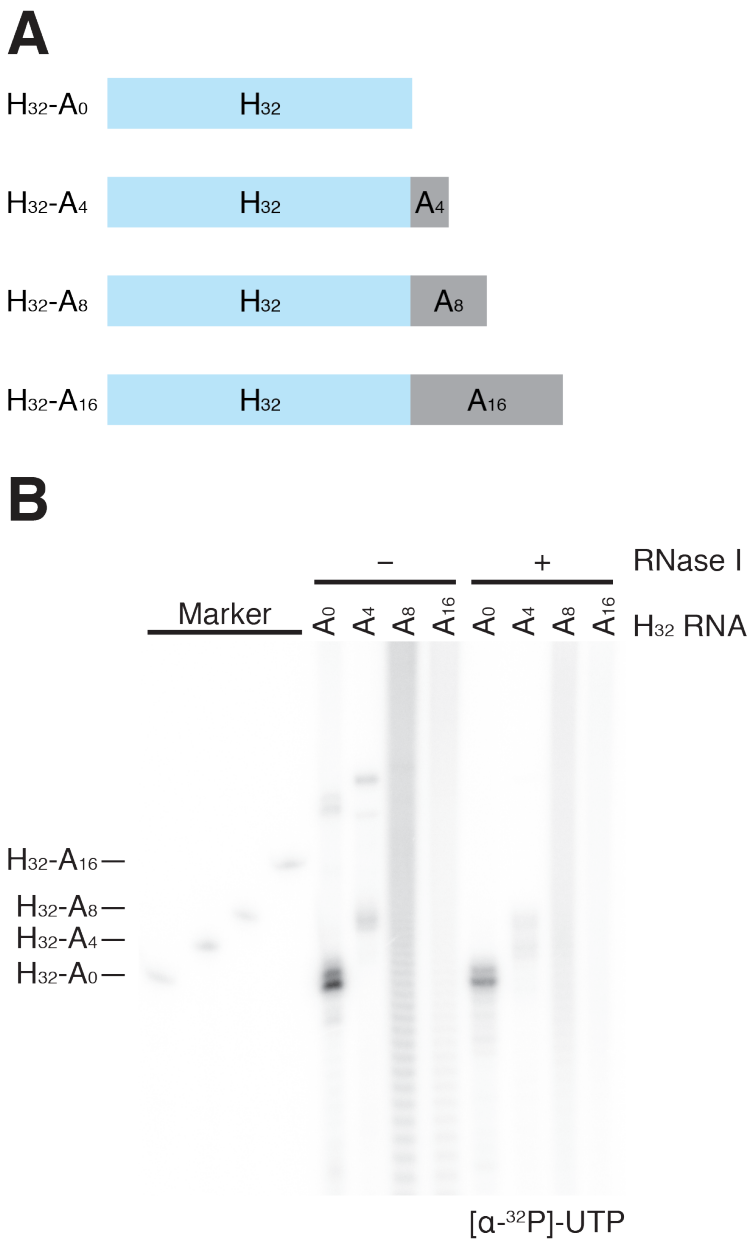

Figure 5. 8-nt poly(A) tail blocks dsRNA conversion by RDR6.

(A) A schematic representation of synthetic RNAs with 0-, 4-, 8- 16-nt poly(A) tail. (B) RdRP assay was performed using the RNAs shown in (A), in the presence of [α-^{32}P]-UTP. The products were resolved on an 15 % polyacrylamide urea gel and visualized by phosphorimaging. The radiolabeled synthetic RNAs were used as the markers.

Homo-polyribonucleotides except for poly(A) cannot block the RdRP activity of RDR6

I showed that RDR6 abhors polyadenylated RNAs as its templates (Figure 4D, 4E and 5B), but whether RDR6 abhors poly(A) tail or homo-polyribonucleotides remains unclear. To check whether other homo-polyribonucleotides can block the RdRP activity of RDR6 like the poly(A) tail, I prepared five synthetic RNAs, which have 32-nt non-G sequence with 8-nt random sequence, poly(A), poly(U), poly(G) or poly(C) at the 3′ end of the RNAs (H_{32}-N_8, H_{32}-A_8, H_{32}-U_8, H_{32}-G_8 or H_{32}-C_8) (Figure 6A). Guanine was omitted in H32 sequence to exclude the formation of G-quadruplex in the H_{32}-G_8. Except for H_{32}-A_8 RNAs, the clear bands were detected with H_{32}-N_8, H_{32}-U_8, H_{32}-G_8, and H_{32}-C_8 RNAs (Figure 6B). I concluded that the poly(A) tail specifically blocks dsRNA synthesis by RDR6. I also performed RdRP assay using five synthetic RNAs, which have 28-nt sequence with 11-nt unrelated sequence, 12-nt poly(A), poly(U), poly(G) or poly(C) at the 3′ end of the RNAs (N_{28}-N_{11}, N_{28}-A_{12}, N_{28}-U_{12}, N_{28}-G_{12} or N_{28}-C_{12}, respectively) (Figure 6C). As expected, the signals for the complementary RNAs were detected with N_{28}-N_{11}, N_{28}-U_{12} or N_{28}-C_{12} (Figure 6D). However, similar to N_{28}-A_{12}, no signals were detected with N_{28}-G_{12} (Figure 6D), suggesting that G-quadruplex formed at the 3′ end of RNAs blocks dsRNA conversion by RDR6.

RDR6 abhors RNAs with secondary structure at the 3′ end as its templates

To validate whether the secondary structure of RNAs at the 3′-end affect RDR6 activity like G-quadruplex, I prepared the synthetic RNAs, N_{28}-N_{11} and N_{28}-N_{22}

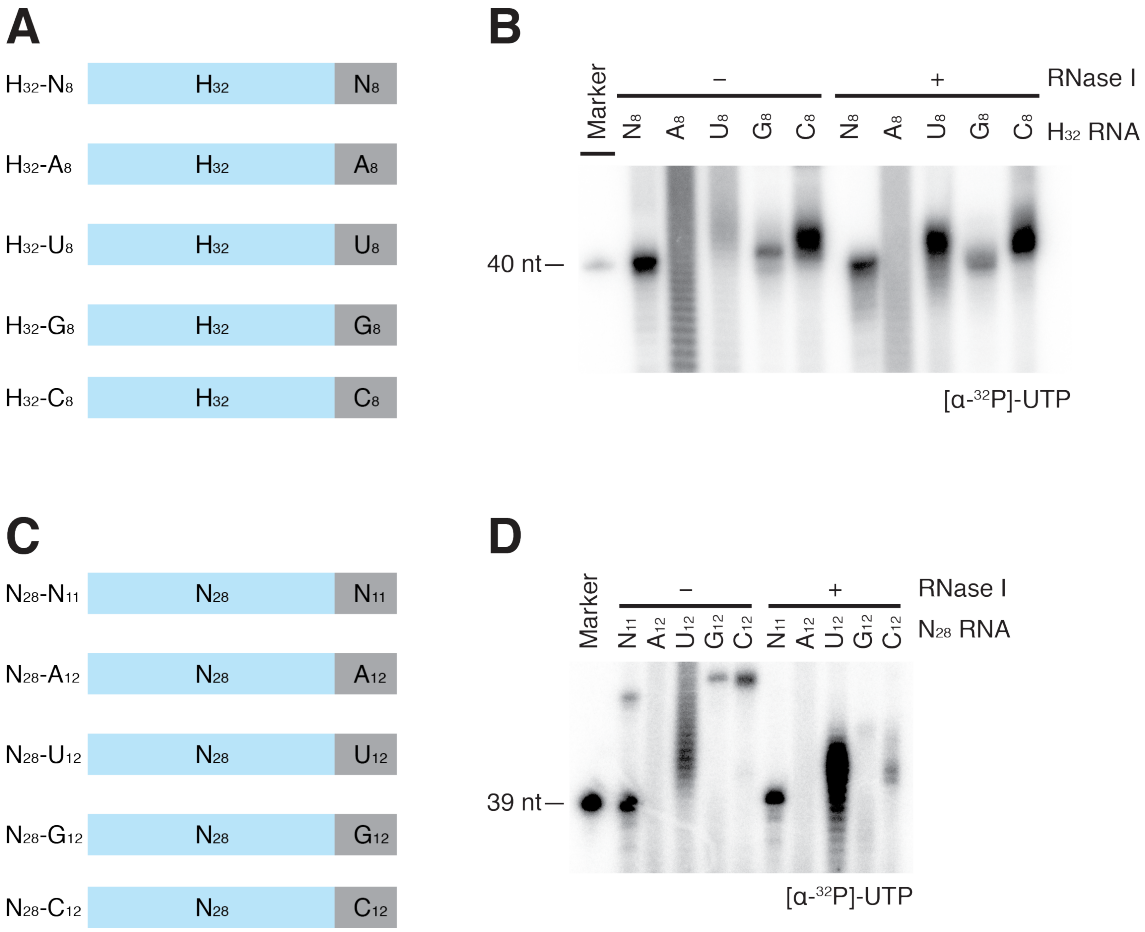

Figure 6. Poly(A) tail and G-quadruplex blocks dsRNA conversion by RDR6.

(A) A schematic representation of synthetic RNAs with 8-nt poly(A), poly(U), poly(G), poly(C) and random sequence. (B) RdRP assay was performed essentially as in Figure 5B except that the template RNAs shown in (A) were used. The radiolabeled H_{32}-A_8 RNA was used as the marker. (C) A schematic representation of synthetic RNAs with 11-nt unrelated sequence or 12-nt poly(A), poly(U), poly(G) and poly(C). (D) RdRP assay was performed essentially as in Figure 4B except that the template RNAs shown in (C) were used. The products were resolved on a 10 % polyacrylamide urea gel. The radiolabeled N_{28}-N_{11} RNA was used as the marker.

RNAs (Figure 7A). The RNA secondary structure was predicted at the central region of N_{39} RNA. In contrast, the RNA secondary structures were predicted at 3′ end and central region of N_{50} RNA with RNA fold software (Figure 7A). While the clear signals for the complementary RNAs were detected with N_{39} RNAs, only the faint signals were detected with N_{50} RNAs (Figure 7B). This result suggests that RDR6 abhors structured RNAs at the 3′ end as its templates.

3′ poly(A) tail blocks the initiation of dsRNA synthesis by RDR6

I showed that the poly(A) tail blocked RDR6 activity, but it remains unclear which step of dsRNA synthesis by RDR6 is inhibited by the poly(A) tail. To this end, I prepared five *in vitro* transcribed reporter RNAs, which have a 100-nt sequence with N_{60}, A_{60}, U_{60}, $N_{30}A_{30}$ or $A_{30}N_{30}$ at the 3′ end (Figure 8A). As expected, the signals for the complementary RNAs were not detected with N_{100}-A_{60} and N_{100}-$N_{30}A_{30}$ RNAs (Figure 8B). In contrast, the signals for the complementary RNAs were detected with N_{100}-$A_{30}N_{30}$ RNAs as well as the positive controls, N_{100}-N_{60} and N_{100}-U_{60} RNAs (Figure 8B), suggesting that internalized poly(A) sequence does not affect the elongation step of complementary strand synthesis by RDR6. This result also suggests that the 3′ poly(A) tail blocks the initiation step of dsRNA synthesis by RDR6.

The 3′-monophosphoryl terminus of RNA blocks RDR6 activity

I showed that RDR6 does not prefer polyadenylated RNAs as its template (Figure 4D, 4E, 5B, 6B, 6D and 8B). Considering the template preference of RDR6,

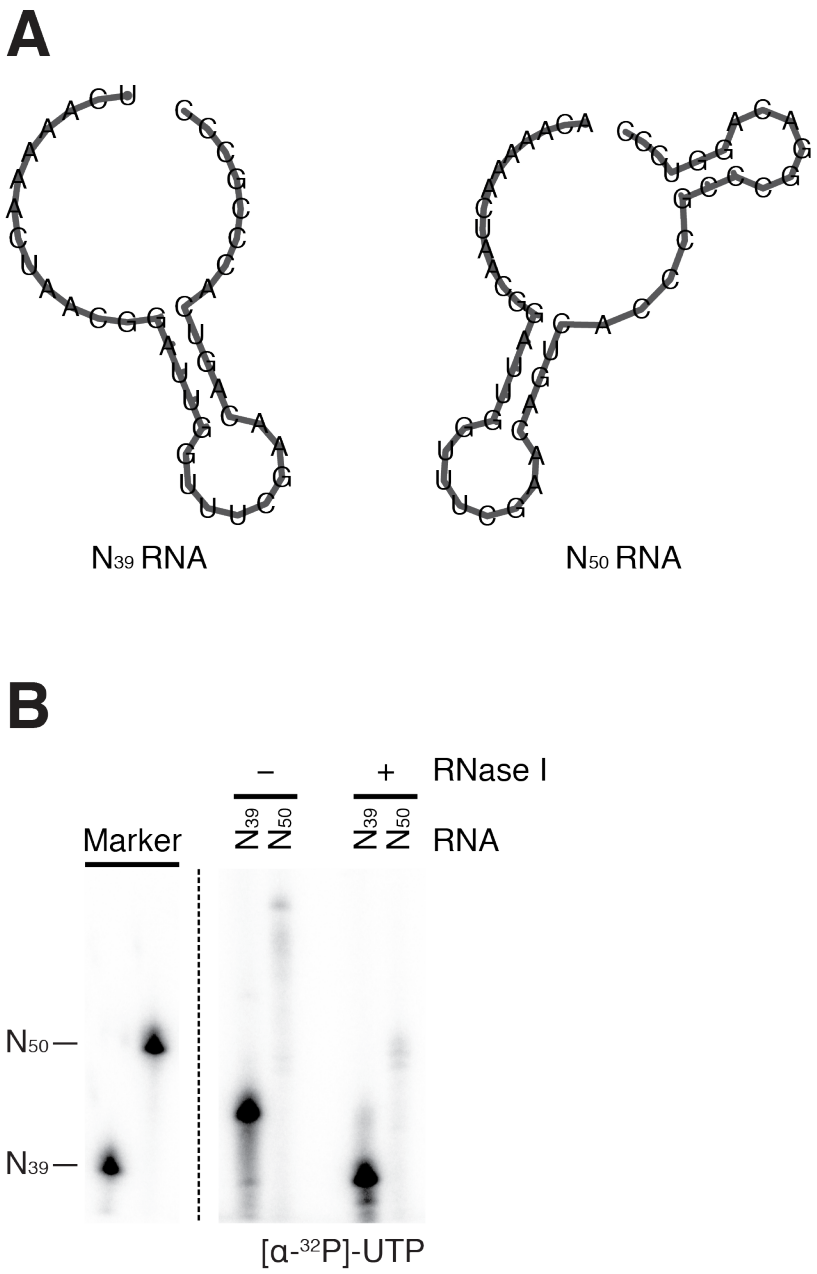

Figure 7. RDR6 abhors the RNAs with secondary structure at 3′ end.

(A) A prediction of secondary structures of N_{39} and N_{50} RNAs with RNA fold software. (B) RdRP assay was performed essentially as in Figure 6E except that template RNAs shown in (A). The radiolabeled N_{39} and N_{50} RNA were used as the markers.

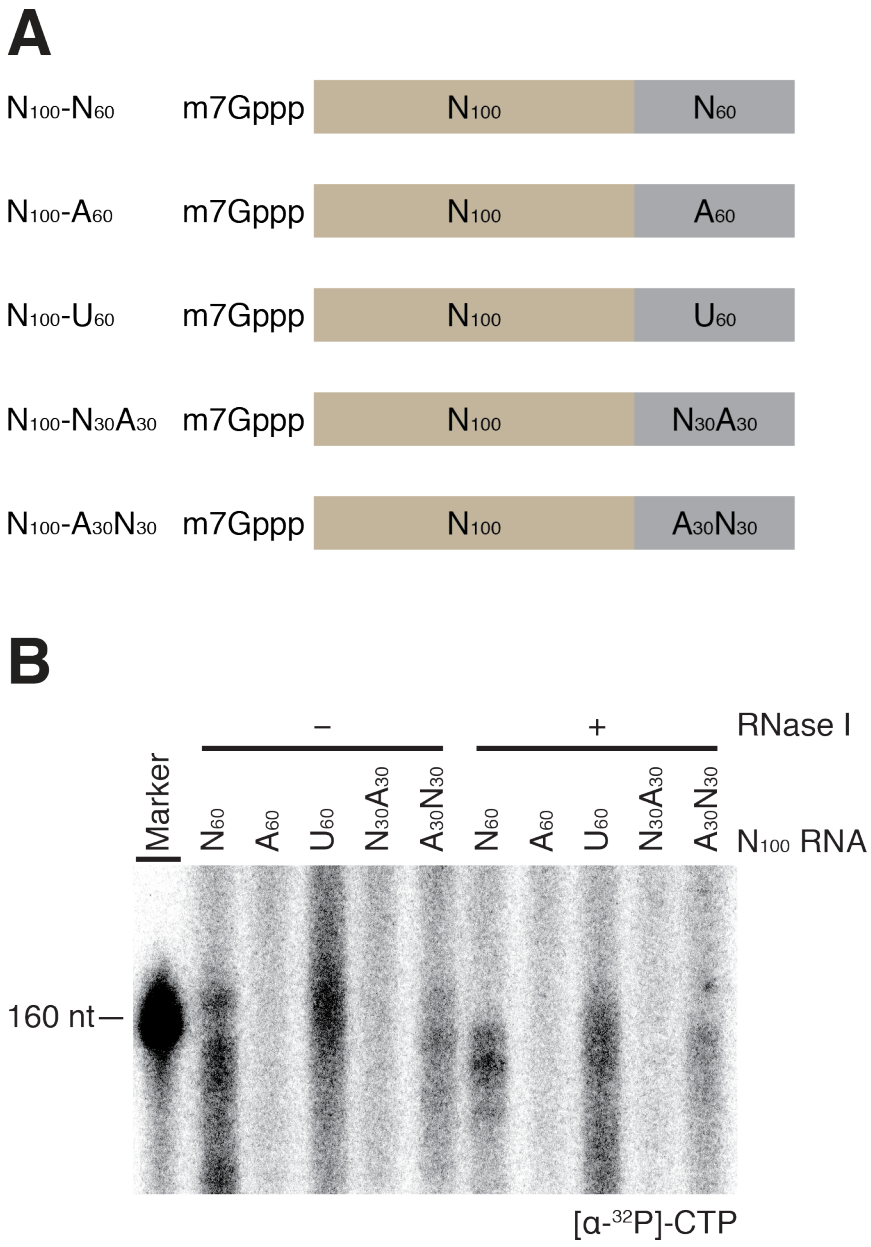

Figure 8. 3′ poly(A) tail block the initiation step of dsRNA synthesis by RDR6.

(A) A schematic representation of *in vitro* transcribed 100-nt RNAs with N60, A60, U60, N30A30 and A30N30 sequence at the 3′ end. (B) RdRP assay was performed essentially as in Figure 4E, except that the template RNAs shown in (A) and [α-^{32}P]-CTP were used. The radiolabeled N_{100}-A_{60} reporter RNAs were used as the marker.

RDR6 can recognize the 3′ end of RNAs. Next, to check whether RDR6 can discriminates between the 3′-hydroxyl and 3′-monophosphoryl termini of RNAs, I prepared the RNAs with 3′-monophosphoryl terminus using β-elimination (Figure 9A). When the RNAs were treated with $NaIO_4$, the vicinal hydroxyl groups of RNAs were oxidized and then the 3′-monophosphoryl termini were generated by the β-elimination reaction at high pH (Alefelder *et al*., 1998) (Figure 9A). I performed RdRP assay using the RNAs with a 3′-hydroxyl or 3′-monophosphoryl terminus (Figure 9B). Interestingly, no signal was detected with the 3′-monophosphoryl RNAs (Figure 9B), suggesting that 3′-monophosphoryl termini of RNAs block TNTase activity and RdRP activity of RDR6. This result also suggests that RDR6 can distinguish between 3′-hydroxyl RNAs and 3′-monophosphoryl RNAs.

The RNA recognition motif of RDR6 does not affect the template specificity of RDR6

Finally, I asked how RDR6 discriminates between poly(A)-less RNAs and polyadenylated RNAs. Considering that RDR6 has an RNA recognition motif (RRM) at its N-terminal, I hypothesized that the RRM of RDR6 discriminates between poly(A)-less RNAs and polyadenylated RNAs. To test this hypothesis, I prepared the wild-type and catalytic mutant recombinant ΔRRM RDR6 (ΔNWT and ΔNCT, respectively) using S2 cell expression system (Figure 10A and B). Then, I performed RdRP assay with the recombinant proteins using N_{100}-U_{60} or N_{100}-A_{60} RNAs (Figure 10C). Unexpectedly, deletion of RRM did not affect the enzymatic activity or template

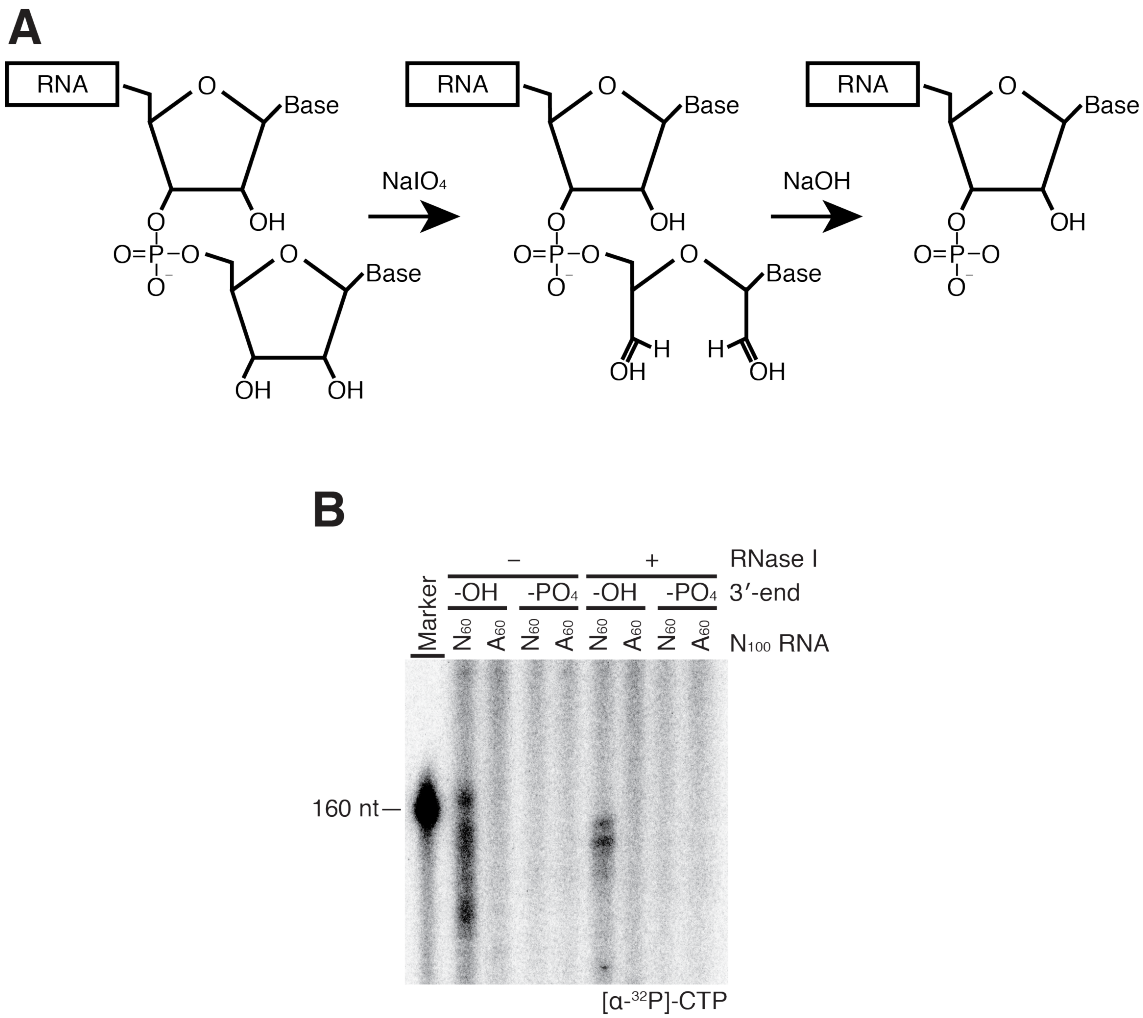

Figure 9. 3′-PO_4 of RNAs blocks RDR6 activity.

(A) A scheme for preparation of the RNAs with 3′-monophosphoryl terminus. (B) RdRP assay was performed essentially as in Figure 4E, except that the N_{100}-N_{60} or N_{100}-A_{60} RNAs with 3′-hydroxyl or 3′-monophosphoryl terminus and [α-^{32}P]-CTP were used. The radiolabeled N_{100}-A_{60} reporter RNAs were used as the marker.

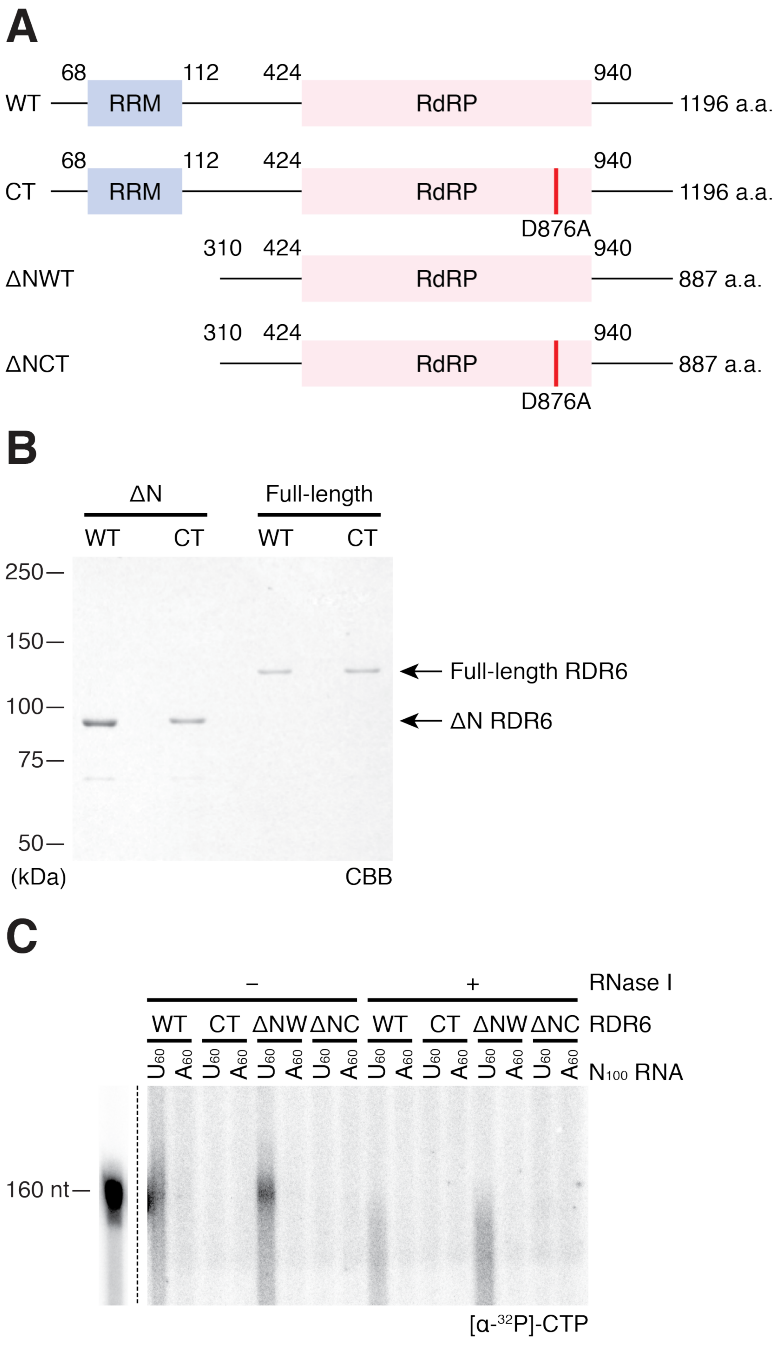

Figure 10. RRM of RDR6 does not affect the template specificity of RDR6.

(A) A schematic representation of recombinant RDR6 and its mutant. (B) CBB staining of purified recombinant proteins. (C) RdRP assay was performed essentially as in Figure 4E, except that the N_{100}-U_{60} or N_{100}-A_{60} RNAs and [α-^{32}P]-CTP were used. The radiolabeled N_{100}-U_{60} reporter RNAs were used as the marker.

specificity of RDR6 (Figure 10C). This result suggests that the C-terminal RdRP domain of RDR6 discriminates poly(A)-less RNAs from polyadenylated RNAs.

Discussion

Template specificity of RDR6

Here, I demonstrated that RDR6 prefers aberrant poly(A)-less RNAs to canonical polyadenylated RNAs as its templates at the initiation step of dsRNA synthesis. The template specificity of RDR6 can explain why the transgene-derived aberrant poly(A)-less RNAs triggered PTGS in vivo (Luo and Chen, 2007), and why self mRNAs are protected from PTGS (Figure 11).

A previous report showed that RDR6 cannot discriminate between poly(A)-less RNAs and polyadenylated RNAs *in vitro* (Curaba and Chen, 2008). In contrast, I showed the opposite conclusion that RDR6 can distinguish poly(A)-less RNAs from polyadenylated RNAs. One difference between these two studies is the protein expression system. While RDR6 was purified from RDR6 overexpressed tobacco in the previous report, RDR6 was purified from S2 cells in my study. However, because RDR6 from BY-2 tobacco cell lysate has the template specificity (Figure 12), it is difficult to explain the opposite conclusion with the difference of protein expression system. Moreover, the buffer condition for RdRP assay is almost identical in these two studies. The major difference is RNase I treatment for removing TNTase products. In this study, all RDR6 products were treated with RNase I. In contrast, the previous report barely utilized RNase I in almost RdRP assays. Given that TNTase activity of RDR6 is strong in the RdRP assay using [α-^{32}P]-UTP (Figure 4D), the previous study might not have discriminated

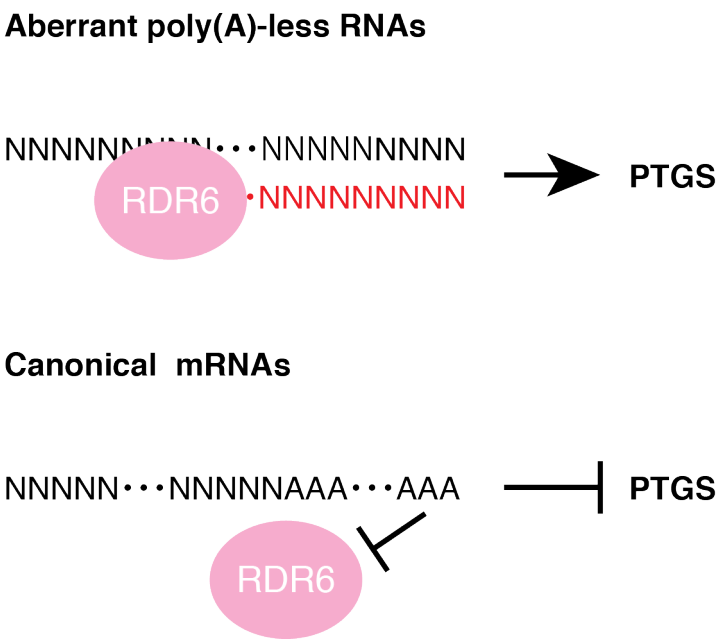

Figure 11. A model for the template specificity of RDR6.

RDR6 selectively converts aberrant poly(A)-less RNAs into dsRNAs, which trigger PTGS. However, canonical mRNAs blocks dsRNA synthesis by RDR6, thus plants protect self-mRNAs from PTGS.

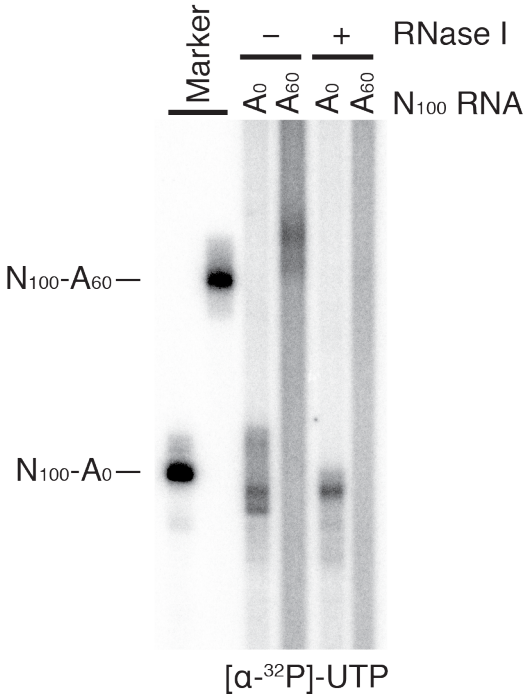

Figure 12. Recombinant RDR6 from tobacco cell lysate specifically converts poly(A)-less RNAs into dsRNAs. RdRP assay was performed using N_{100}-A_0 and N_{100}-A_{60} reporter RNAs with recombinant RDR6 from tobacco cell lysate in the presence of [α-^{32}P]-UTP. Denaturing PAGE was performed essentially as in Figure 4E. The radiolabeled N_{100}-A_0 and N_{100}-A_{60} reporter RNAs were used as the markers.

between the RdRP activity and TNTase activity of RDR6.

In this study, I showed that not only 3′ poly(A) tail but also secondary RNA structures block dsRNA conversion by RDR6 (Figure 8B). This property of RDR6 might explain why rRNAs and tRNAs, which possess stable RNA secondary structures, do not trigger PTGS *in vivo* even though the RNAs lack a poly(A) tail at the 3′ end.

The mechanism of template selection by RDR6

How does RDR6 discriminates between poly(A)-less RNAs and polyadenylated RNAs? The observation that the 3′ poly(A) tail inhibits the initiation step of complementary strand synthesis by RDR6 suggests that RDR6 can recognize the 3′ end of RNAs in the initiation step (Figure 8B). However, it remains unclear whether the template specificity of RDR6 is exerted at initial step of complementary synthesis or at RNA binding. If polyadenylated RNAs blocks dsRNA synthesis at initial step, the template specificity might be derived from RdRP domain. In contrast, if RDR6 preferentially interacts with poly(A)-less RNAs, this property might be derived from RRM, which functions as RNA binding. However, the template specificity of RDR6 was not influenced by the presence or absence of RRM (Figure 10C), suggesting that the RdRP domain, but not RRM, might exert the template specificity of RDR6. However, it cannot be excluded that the RdRP domain of RDR6 preferentially interacts with poly(A)-less RNAs. Thus, it is suggested that the template specificity of RDR6 is derived from RdRP domain, but whether this property is exerted at initial step of complementary synthesis or at RNA binding remains unclear.

TNTase activity of RDR6

In this study, I showed that RDR6 has TNTase activity *in vitro*. The fact that catalytic mutant RDR6 does not have TNTase activity as well as RdRP activity suggests that TNTase activity shares catalytic active site with RdRP activity. Although previous studies also showed that RDR6 has TNTase activity *in vitro* (Curaba and Chen, 2008; Devert *et al*., 2015), there has been no report about the physiological function of the TNTase activity of RDR6 *in vivo*. What is the physiological function of the TNTase activity of RDR6? One possibility is that TNTase activity links with RdRP activity. A previous study showed that the TNTase activity of Nodavirus replicase is required for its RdRP activity (Wang *et al*., 2013). Thus, the TNTase activity of RDR6 might play crucial roles in the complementary RNA synthesis of RDR6. Indeed, TNTase activity of RDR6 produced internalized polyadenylated template RNAs, which potentially serve as templates for RDR6, by adding [α-^{32}P]-UTPs at the 3′ end of poly(A) tail (Figure 8B). However, I could not detect any signals for dsRNAs derived from TNTase products of polyadenylated RNAs (Figure 4D). This is probably attributed to the low RdRP and TNTase activities of RDR6 in our assay condition, which make such two-step reaction products below the delectable level. At this point, the actual in vivo reaction efficiency of RDR6 is unclear. However, given that aberrant mRNAs are selectively silenced by PTGS in plants, I speculate that the frequency of non-A addition to the poly(A) tail by TNTase activity of RDR6 is quite limited compared to the total pool of mRNAs in vivo. Further study will be needed to clarify the physiological function of TNTase activity of RDR6.

Materials and methods

Cell culture

Nicotinana tabacum BY-2 suspension cells (RIKEN BRC Japan) were incubated at 130 rpm by shaking in a dark at 27 °C. BY-2 cells were cultured in 100 ml the medium (KOH, pH 5.8) containing 0.46 g Murashige and Skoog Plant Salt Mixture (Wako), 3 g sucrose, 0.1 mg Thiamine hydrochloride, 10 mg myo-Inositol, 20 mg KH_2PO_4 and 0.2 g 2, 4-Dichlorophenoxyacetic Acid. *Drosophila melanogaster* S2 cells were cultured in Schneider's Drosophila medium (Thermo Fisher Scientific) added with 10% fetal bovine serum (Thermo Fisher Scientific) and 1×Antibiotic-Antimycotic (Thermo Fisher Scientific) at 27 °C.

Plasmid construction

pBYL-RDR6

The ORF of *Arabidopsis thaliana* RDR6 (AT3G49500) were cloned into pBYL2 vector (Mine *et al*., 2010), which was linearized by the restriction enzyme AscI (New England Biolabs), by In-Fusion HD cloning kit (Takara).

pBYL-3×HA-RDR6

The ORF of RDR6 was amplified from *pBYL-RDR6* using oligo 1 (5′-GATTACGCTGCTCATGGCGGAGGGTCAGAGG-3′) and oligo 2 (5′-TCAAGCTGGCGCGCCTTAGAGACGCTG-3′). 3×HA fragment was amplified from pAHW vector (Drosophila gateway vector collection) using oligo 3

(5′-CCAAGCTGGCGCGCCATGTACCCATACG-3′) and oligo 4 (5′-CTCTGACCCTCCGCCATGAGCAGCG-3′). These two PCR fragments were cloned into pBYL2 vector, which was linearized by AscI , by In-Fusion HD cloning kit.

pAFW-SUMO-RDR6

The ORF of RDR6 was amplified from *pBYL-RDR6* using oligo 5 (5′-AACAGATTGGAGGTATGGGGTCAGAGGGAAATATGAAG-3′) and oligo 6 (5′-CTGGGTCGGCGCGCCTTAGAGACGCTGAGC-3′). SUMOstar cleavage site sequence was amplified from pBYL-3×FLAG-SUMO-AtAGO1 plasmid (Iwakawa and Tomari, 2013) using oligo 7 (5′-AAGCAGGCTCCGCGGCCATGGGGTCCCTGCAGGACTCAGA-3′) and oligo 8 (5′-CTTCATATTTCCCTCTGACCCCATACCTCCAATCTGTT-3′). Overlap PCR was performed with these two fragments using oligo 6 and oligo 7. The PCR product was cloned into pAFW vector (Drosophila gateway vector collection), which was linearized by SacII (Takara) and AscI, by In-Fusion HD cloning kit.

pAFW-SUMO-RDR6-CT

To construct RDR6 catalytic mutant of which the 876th aspartate was substituted by alanine (Curaba and Chen, 2008), two fragments of RDR6 were amplified from *pAFW-SUMO-RDR6* using the primer pairs, oligo 7 and oligo 9 (5′-CCCAAGCCACAAAGTACAGGGCCCCGTCAAGGTCACTGCC-3′), and oligo 6 and oligo 10 (5′-GGCAGTGACCTTGACGGGGCCCTGTACTTTGTGGCTTGGG-3′).

Overlap PCR was performed with these two fragments using oligo 6 and oligo 7. The PCR product was cloned into pAFW vector (Drosophila gateway vector collection), which was linearized by SacII and AscI, by In-Fusion HD cloning kit.

pASW-SUMO-Drosophila melanogaster codon optimized RDR6

Drosophila melanogaster codon optimized RDR6 sequence was amplified from pUC57-Drosophila melanogaster codon optimized RDR6 (GenScript) using oligo 11 (5′-ACCGCGAACAGATTGGAGGTATGGGCAGTGAGGGCAATAT-3′) and oligo 12 (5′-AAAGATCCTGCTAGCTTACAGGCGCTGGGC-3′). SUMOstar cleavage site sequence was amplified from *pAFW-SUMO-RDR6* using oligo 13 (5′-GGCCAGCGGGAGCCCATGGGGTCCCTGCAG-3′) and oligo 14 (5′-ATATTGCCCTCACTGCCCATACCTCCAATCTGTTCGCGGT-3′). Overlap PCR was performed with these two PCR fragments using oligo 12 and oligo 13. The PCR product was cloned into the linearized pASW vector (Iwasaki *et al*., 2010), which was amplified using oligo 15 (5′-GGGCTCCCGCTGGCCC-3′) and oligo 16 (5′-GCTAGCAGGATCTTTGTGAAGG-3′), by In-Fusion HD cloning kit.

pASW-SUMO-Drosophila melanogaster codon optimized RDR6-CT

To construct *Drosophila melanogaster* codon optimized RDR6 catalytic mutant, two fragments of RDR6 were amplified from *pASW-SUMO-Drosophila melanogaster codon optimized RDR6* using the primer pairs, oligo 13 and oligo 17 (5′-CCCAGGCCACGAAGTACAGGGCGCCATCCAGGTCCGATCC-3′), and oligo

12 and oligo 18

(5′-GGATCGGACCTGGATGGCGCCCTGTACTTCGTGGCCTGGG-3′). Overlap PCR was performed with these two fragments using oligo 12 and oligo 13. The PCR product was cloned into the linearized pASW vector, which was amplified using oligo 15 and oligo 16, by In-Fusion HD cloning kit.

pASW-SUMO- Drosophila melanogaster codon optimized ΔNRDR6

To construct RRM deletion *Drosophila melanogaster* codon optimized RDR6, RRM deletion RDR6 sequence was amplified from *pASW-SUMO-Drosophila melanogaster codon optimized RDR6* using oligo 12 and oligo 19 (5′-GAACAGATTGGAGGTGGAGAGCCGGTGTCCG-3′). The PCR product was cloned into the linearized pASW-SUMO, which was amplified from *pASW-SUMO-Drosophila melanogaster codon optimized RDR6* using oligo 16 and oligo 20 (5′-ACCTCCAATCTGTTCGCGGT-3′), by In-Fusion HD cloning kit.

pASW-SUMO- Drosophila melanogaster codon optimized ΔN-RDR6-CT

To construct RRM deletion *Drosophila melanogaster* codon optimized RDR6 catalytic mutant, RRM deletion RDR6 catalytic mutant sequence was amplified from *pASW-SUMO-Drosophila melanogaster codon optimized RDR6-CT* using oligo 12 and oligo 19. The PCR product was cloned into the linearized pASW-SUMO, which was amplified from *pASW-SUMO-Drosophila melanogaster codon optimized RDR6* using oligo 16 and oligo 20, by In-Fusion HD cloning kit.

Preparation of reporter RNAs

N_{100} reporter RNAs were *in vitro* transcribed using T7-Scribe Standard RNA IVT Kit (CELLSCRIPT) with the PCR fragments described below. The in vitro transcribed RNAs were gel purified and then capped with ScriptCap m7G Capping System (CELLSCRIPT) except when non-capped RNAs were used as the templates. The synthetic RNA oligos, which are H_{32}, N_{28}, N_{39}, or N_{50} reporter RNAs (GeneDesign), are listed in the Supplementary table 1.

PCR fragments for in vitro transcription

N_{100}-A_0, N_{100}-A_{60}, N_{100}-N_{60}, N_{100}-U_{60}, N_{100}-$N_{30}A_{30}$ and N_{100}-$A_{30}N_{30}$ PCR fragments were amplified from pGL3-basic vector (Promega) using the primer pairs, oligo 21 (5′-GTACTTAATACGACTCACTATAGGTAAAGCCACCATGGAAGAC-3′) and oligo 22 (5′-GAGCCTTATGCAGTTGCTCTCC-3′), oligo 21 and oligo 23 (5′-TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT TTTTTGAGCCTTATGCAGTTGCTCTCC-3′), oligo 21 and oligo 24 (5′-CCACCTCGATATGTGCATCTGT-3′), oligo 21 and oligo 25 (5′-AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA AAAAAAAAAAAAGAGCCTTATGCAGTTGCTCTCC-3′), oligo 21 and oligo 26 (5′-TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTGTGTTCCAGGAACCAGGGCG-3′), oligo 21 and oligo 27

(5′-CCACCTCGATATGTGCATCTGTAAAAGCAATTTTTTTTTTTTTTTTTTTTTTTTTTTTTTGAGCCTTATGCAGTTGCTCTCC-3′), respectively

β-elimination

One micromolar N_{100}-N_{60} and N_{100}-A_{60} RNAs were incubated with 100 µl of $NaIO_4$ buffer (25 mM NaIO4 and 1×borate buffer (29.6 mM borax and 29.6 mM boric acid, pH 8.6)) at 25 °C for 30 minutes in a dark. After the reaction, the mixtures were supplemented with 10 µl of glycerol and 0.5 M NaOH and then incubated at 45 °C for 90 minutes. These RNAs were precipitated with 100% EtOH and washed with 70% thoroughly.

Preparation of BY-2 lysate

The BY-2 lysate was prepared as described previously (Tomari and Iwakawa, 2017). Briefly, protoplasts were prepared from three-day-old BY-2 cells treated with enzyme solution (2% (w/v) Cellulase Onozuka RS (Yakult), 0.2% (w/v) Pectolyase Y-23 (Wako), 0.4 M Mannitol and 20 mM $MgCl_2$) at 25 °C for 3 hours. After washed with Mannitol buffer (0.4 M Mannitol, 20 mM $MgCl_2$), the protoplasts were loaded onto 10 to 40% linear gradient Percoll (GE) solution containing 5 mM HEPES-KOH (pH 7.4), 20 mM $MgCl_2$ and 0.7 M Mannitol overlaid onto 70% Percoll solution, and then centrifuged at 11,000 × g at 25 °C for 90 minutes (Beckman). After centrifugation, evaculolated protoplast were collected, and then washed with Mannitol buffer. The cells were suspended with TR buffer (30 mM HEPES-KOH (pH7.4), 100 mM KOAc, 2 mM

$Mg(OAc)_2$, 1 × EDTA-free protease inhibitor cocktail) and disrupted with homogenizer. The disrupted protoplasts were centrifuged at 17,000 × g at 4 °C for 10 minutes. After centrifugation, the supernatant were transferred into new tube and stored at −80 °C.

Preparation of recombinant RDR6 from plant lysate

First, 3×HA-RDR6 mRNA was transcribed from linearized *pBYL-3×HA-RDR6*, which was cleaved by NotI (Takara) using T7-Scribe Standard RNA IVT Kit. And, *in vitro* transcribed RNAs were capped and polyadenylated using ScriptCap m7G Capping System and A-Plus Poly(A) Polymerase Tailing Kit (CELLSCRIPT), respectively. Fifty microliters of 1 μM 3×HA-RDR6 mRNAs were incubated with 250 μl BY-2 lysate and 125 μl of substrate mixture (3 mM ATP, 0.4 mM GTP, 100 mM creatine phosphate, 200 μM each of 20 amino acids, 320 μM spermine and 0.4 U/μl creatine phosphokinase (Calbiochem)). Recombinant protein was immunopurified with Dynabeads protein G (Invitrogen) coated with anti-HA antibodies (#ab130275, Abcam) at 4 °C for 1 hour. The beads were washed twice with 1×lysis buffer (30 mM HEPES-KOH pH 7.4, 100 mM KOAc, 2 mM $Mg(OAc)_2$) containing 800 mM NaCl and 1% Triton X-100 and once with 1 × lysis buffer and resuspended in 20 μl of 1 × lysis buffer containing 1 mM DTT and 20% glycerol.

Plasmid transfection in S2 cells

S2 cells (1.5×10^6 cells/ml) of 10 ml were transfected with 10 μg of the plasmids (*pAFW-SUMO-RDR6*, *pAFW-SUMO-RDR6-CT*, *pASW-SUMO-Drosophila*

melanogaster codon optimized RDR6, *pASW-SUMO-Drosophila melanogaster codon optimized RDR6-CT*, *pASW-SUMO- Drosophila melanogaster codon optimized ΔNRDR6* or *pASW-SUMO- Drosophila melanogaster codon optimized ΔN-RDR6-CT*) by X-tremeGENE HP DNA Transfection Reagent (Roche) and incubated 3 days.

Preparation of recombinant RDR6 from S2 cells

RDR6-transfected S2 cells were centrifuged and washed with PBS. The pellets were resuspended with the equal volume of hypotonic lysis buffer (10 mM HEPES-KOH pH 7.4, 10 mM KCl, 1.5 mM $Mg(OAc)_2$, 5mM DTT, 1 × EDTA-free protease inhibitor cocktail), incubated on the ice for 15 minutes, vortexed for 30 seconds, and centrifuged at 17,000 × g for 20 minutes. The supernatant was immunoprecipitated with anti-FLAG antibodies (#F1804, Sigma) coated Dyna beads protein G (Invitrogen) or Streptoavidin sepharose HP (GE) at 4 °C for 1 hour. After washed two times with 1 × lysis buffer containing 1% Triton X-100 and one time with 1 × lysis buffer, the beads were supplemented with 1 × lysis buffer containing 1 mM DTT, 20% glycerol, 0.05 U/μl SUMOstar protease (LifeSensors) and incubated at 4 °C for 20 minutes to elute RDR6. The concentrations of RDR6 were measured by SDS-PAGE with Bovine Serum Albumin (BSA) for calibration curve. Gels were stained with Coomassie brilliant blue. The gel images were acquired by LAS-3000 (Fujifilm) or Amersham Imager 600 (Amersham).

RNA-dependent RNA polymerase assay

RNA-dependent RNA polymerase assays were performed as described previously (Makeyev and Bamford, 2002; Curaba and Chen, 2008) with some modifications. Two microliters of 100–200 nM RDR6 from S2 cells or 4 μl slurry of RDR6-coated Dynabeads protein G (Invitrogen), which is prepared from plant lysate, were assayed in 20 μl reaction mixtures containing 50 mM HEPES-KOH (pH 7.6), 20 mM NH_4OAc, 8 mM $MgCl_2$, 0.1 mM EDTA, 2% PEG 4000, 0.1 mM each of ATP, UTP, GTP, CTP, 0.8 U/μl RNasin and 0.165 μM [α-^{32}P]-UTP or CTP (~3000 Ci/mmol) with 1 μl of 1 μM template RNAs at 25 °C for 2 hours. When the radiolabeled RNAs were used as template RNAs, 0.165 μM UTP was used instead of [α-^{32}P]-UTP or CTP. Ten microliters of the reaction mixtures were treated with Proteinase K for 65 °C for 20 minutes. The RNAs in these mixtures were precipitated with ethanol, resuspended with formamide dye, and then resolved on 8% (N_{100} RNAs), 10% (Figure 16) or 15% (synthetic RNA oligos except for Figure 16) polyacrylamide denaturing gel. The gel images were acquired by Typhoon FLA 7000 IP (GE).

RNase I protection assay

RNase I protection assays were performed as described (Curaba and Chen, 2008) with some modification. Ten microliters of RNA-dependent RNA polymerase reaction mixtures or 10 μl of 100 nM RNAs were supplemented with 10 μl of RNase I buffer containing 50 mM Tris-HCl (pH 8.0), 300 mM NaCl, 15 mM $MgCl_2$, and 50 Units (N_{100} RNAs) or 25 Units (synthetic RNA oligos) of RNase I (Ambion) at 37 °C

for 20 minutes. After the reactions, the mixtures were treated with Proteinase K and precipitated with ethanol as described above.

Part II: Two-hit of target RNA enhances dsRNA synthesis in tasiRNA biogenesis

“Part II” cannot be disclosed because the contents of “Part II” will be published in academic journal within five years.

General discussion

Conclusion

Since co-suppression was discovered in 1990, how plants discriminate exogenous RNAs from endogenous RNAs has been a long-standing question (Napoli *et al*., 1990; van der Krol *et al*., 1990). In this dissertation, I demonstrated that RDR6 specifically converts aberrant poly(A)-less RNAs into dsRNAs. Given that transgene-derived RNAs lack poly(A) tail (Luo and Chen, 2007), transgene-derived aberrant poly(A)-less RNAs trigger PTGS, which silences endogenous mRNAs, which have the same sequence with transgene RNAs. The property of RDR6 may answer the long-standing question.

Although a number of endogenous PTGS target RNAs have two miRNA target sites, which play crucial roles for secondary siRNA biogenesis (Axtell *et al*., 2006; Howell *et al*., 2007), how "two-hit" by RISC triggers secondary siRNAs production was remain unclear. In this dissertation, I demonstrated that "two-hit" by RISC enhances dsRNA synthesis of target RNAs, resulting in the efficient secondary siRNA production.

I suggest that plants specifically silence non-self aberrant poly(A)-less RNAs using the template specificity of RDR6. Moreover, I suggests that when plants produce endogenous secondary siRNAs, plants eliminate poly(A) tail of RNAs, resulting in the best templates for dsRNA synthesis by RDR6 (Figure 20).

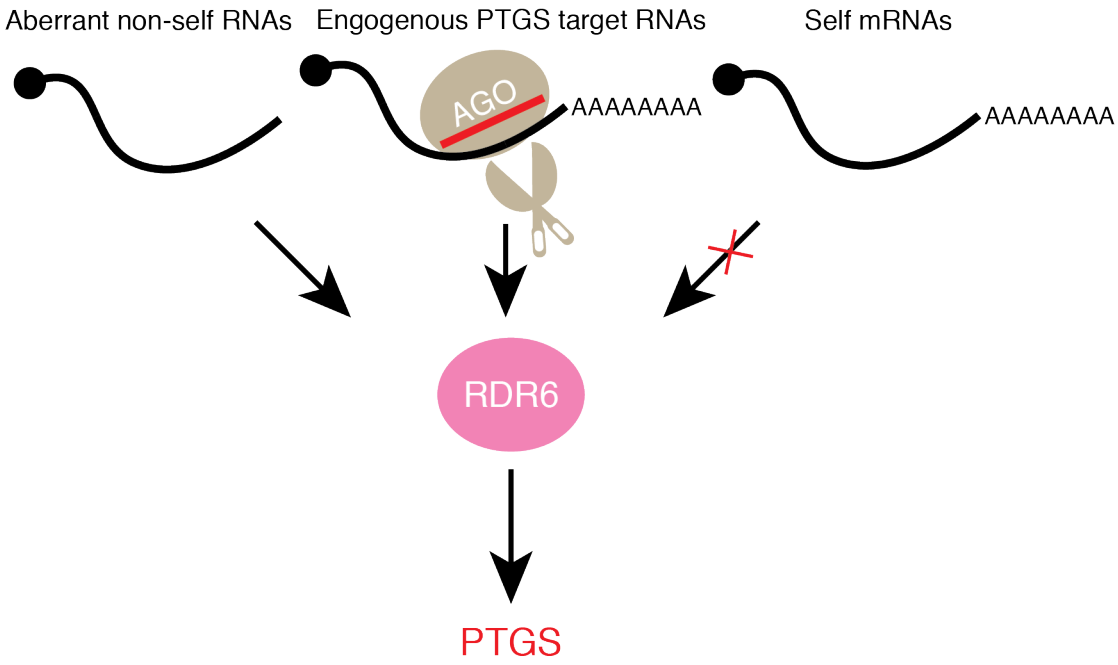

Figure 20. A Model for the template specificity of RDR6 in S-PTGS and endogenous secondary siRNA biogenesis. Using the template specificity of RDR6, plants selectively silence aberrant non-self RNAs and efficiently produce endogenous secondary siRNAs by eliminating poly(A) tail of self RNAs.

RDR6 function in vivo

"*RDR6 function in vivo*" cannot be disclosed because the contents of "*RDR6 function in vivo*" will be published in academic journal within five years.

Eukaryotic RdRPs

Eukaryotic RdRPs are conserved in several eukaryotic organisms (Cogoni and Macino, 1999; Dalmay *et al*., 2000; Mourrain *et al*., 2000; Smardon *et al*., 2000; Zong *et al*., 2009; Lewis *et al*., 2017). *Neurospora crassa* QDE-1, a well-known RdRP, which converted transgene-derived RNAs into dsRNAs, triggering RNA silencing (Cogoni and Macino, 1997; Cogoni and Macino, 1999). QDE-1 is a double-barrel polymerase and binds to magnesium ion at its catalytic active site (Salgado *et al*., 2006). Considering the similarity of RdRP domain between QDE-1 and other eukaryotic RdRPs, the RdRPs might be a double-barrel polymerase.

Similar to RDR6, other species RdRPs have TNTase activity and RdRP activity *in vitro* (Makeyev and Bamford, 2002; Aoki *et al*., 2007; Aalto *et al*., 2010). Unlike RDR6, QDE-1 and *Caenorhabditis elegans* RRF-1 synthesize the antisense RNAs from the internal region of template RNAs (Aoki *et al*., 2007; Aalto *et al*., 2010). Given that RNA silencing by QDE-1 is depleted in the mutant of QDE-2, a fungi AGO (Cogoni and Macino, 1997; Fagard *et al*., 2000) and RRF-1 converts target RNAs of PRG-1, a nematode AGO, into dsRNAs (Batista *et al*., 2008; Gu *et al*., 2009), QDE-1 and RRF-1 might convert RISC target transcripts, but not other mRNAs.

A previous phylogenetic analysis proposed that eukaryotic RdRPs might be evolved in eukaryotic organisms independently, resulting in the divergence of the RdRP function *in vivo* (Zong *et al*., 2009). Perhaps, eukaryotic RdRPs might have evolved not to convert self RNAs into dsRNAs by interacting with other factors or by having a template preference about aberrant RNAs.

Similarity between S-PTGS and endogenous secondary siRNA biogenesis

Previous studies identified that AGO1, SGS3, RDR6 and SDE5 are the key proteins in S-PTGS (Dalmay *et al*., 2000; Mourrain *et al*., 2000; Hernandez-Pinzon *et al*., 2007). Interestingly, AGO1, SGS3, RDR6 and SDE5 also participate in tasiRNA biogenesis pathway (Peragine *et al*., 2004; Vazquez *et al*., 2004; Allen *et al*., 2005; Yoshikawa *et al*., 2005; Xie *et al*., 2005). Given that AGO1 identified as S-PTGS deficient mutant (Dalmay *et al*., 2000; Mourrain *et al*., 2000; Hernandez-Pinzon *et al*., 2007), AGO1 should participate in S-PTGS. Supporting this, *ago1* mutant transgenic plants do not affect inverted repeat-triggered PTGS mechanism, which omits dsRNA synthesis in PTGS, suggesting that AGO1 functions in the upstream of dsRNA synthesis in S-PTGS (Beclin *et al*., 2002; Muangsan *et al*., 2004). Moreover, SGS3 interacts with 22-nt AGO1-RISC and the target RNAs (Yoshikawa *et al*., 2013) and SGS3 is also required for S-PTGS (Dalmay *et al*., 2000; Mourrain *et al*., 2000). Thus, the introduced transgenes in previous reports might have 22-nt miRNA-target site, triggering S-PTGS (Figure 21). The other possibility is that RDR6 converts transgene-derived RNAs into dsRNAs, which are processed into 22-nt siRNAs, which trigger secondary siRNA amplification by AGO1 and SGS3 (Figure 21). Future study might confirm the possibility by performing secondary siRNA biogenesis assay in AGO1-, SDE5- and DCL2-overexpressed lysate in the presence of reporter RNAs without poly(A) tail and miRNA target site.

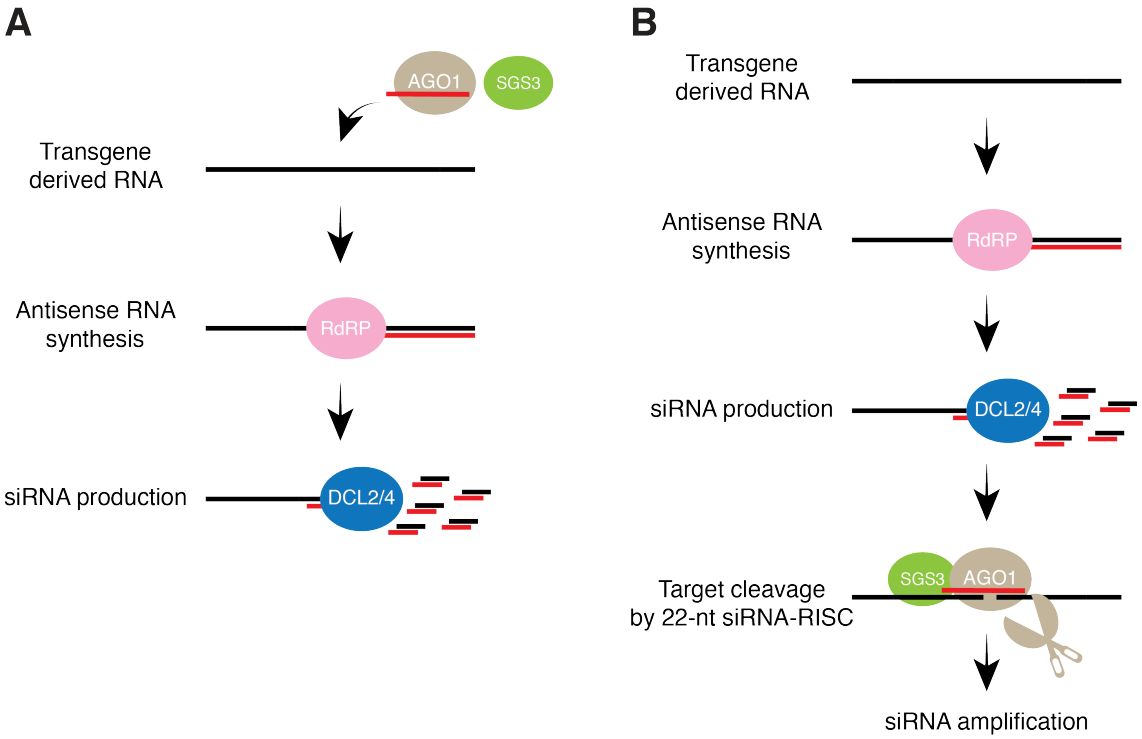

Figure 21. Two possibilities for a function of AGO1 in S-PTGS.

(A) If transgene RNAs have 22-nt miRNA-AGO1-RISC target site, RISC cleaves the transgene RNAs. Then, RDR6 is recruited onto the cleaved fragments and converts them to their dsRNA forms, which are processed into siRNAs by DCL2 or 4. (B) If transgene RNAs do not have miRNA target site, RDR6 converts aberrant transgene-derived RNAs into dsRNAs, which are processed into 21- or 22-nt siRNAs by DCL4 or 2. The 22-nt siRNAs are assembled into AGO1 to form RISC. 22-nt siRNA-AGO1 triggers secondary siRNA biogenesis.

Perspective

In this study, in order to understand the mechanisms for S-PTGS and endogenous secondary siRNA biogenesis, I focused on the template specificity of RDR6. I proved that the template specificity of RDR6 is utilized for defense against exogenous gene and endogenous secondary siRNA biogenesis in plants. However, there are several questions about the template specificity of RDR6 and tasiRNA biogenesis. First, it still remain unclear the mechanism for the template specificity of RDR6. To figure out the mechanism, future study might require an approach with structural insight. Second question is about the direction of dsRNA processing by DCL4. While DCL4 processes dsRNA into siRNAs with 3′ to 5′ direction of 5′ cleaved fragment RNAs in the case of "two-hit", DCL4 produces siRNAs with 5′ to 3′ direction of 3′ cleaved fragment RNAs in the case of "one-hit" (Allen *et al*., 2005). However, how the direction of dsRNA processing is determined remains unclear. Future study might focus on the direction of DCL4 activity (Figure 22). Finally, it was reported that TAS transcripts have short open reading frame (ORF) and the miRNA target site on TAS transcripts are placed into ORF or near their stop codon, resulting in ribosome stalling (Zhang *et al*., 2012; Hou *et al*., 2016; Yoshikawa *et al*., 2016). However, it remains unclear how ribosome stalling up-regulates tasiRNA production. Future study should find the relationship between ribosome stalling and tasiRNA biogenesis.

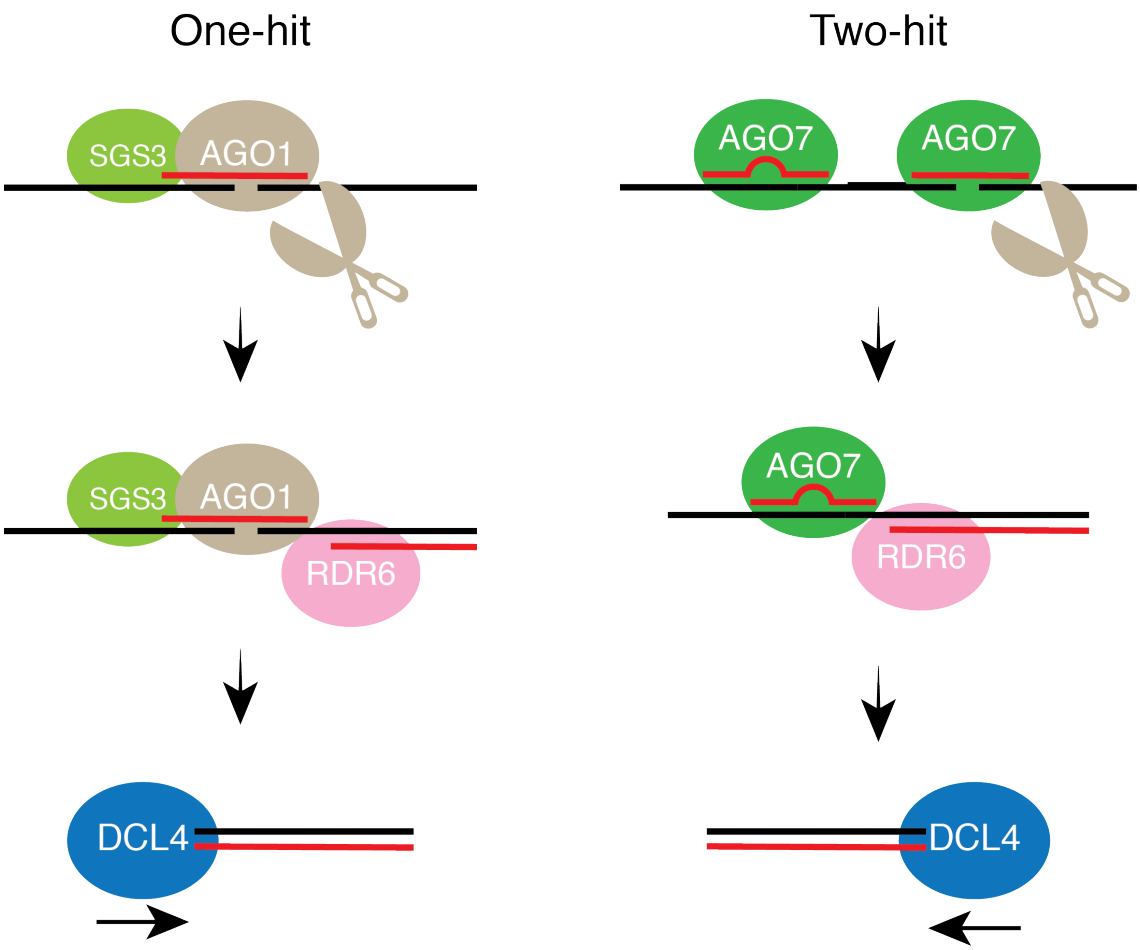

Figure 22. A scheme for a direction of DCL4 activity in endogenous secondary siRNA biogenesis.

In one-hit model, DCL4 processes dsRNAs into siRNAs with 5′ to 3′ direction of 3′ fragments. In contrast, in two-hit model, the dsRNAs of 5′ fragments are processed into siRNAs with 3′ to 5′ direction.

Supplementary tables

Supplementary table 1. Synthetic RNA oligos for RdRP assay

Name	Sequence (5′-to-3′)
H_{32}-A_0	UCAAAAACUAACAAAUUAAUUUCAAACAAUCU
H_{32}-A_4	UCAAAAACUAACAAAUUAAUUUCAAACAAUCUAAAA
H_{32}-A_{16}	UCAAAAACUAACAAAUUAAUUUCAAACAAUCUAAAAAAAA
H_{32}-A_{16}	UCAAAAACUAACAAAUUAAUUUCAAACAAUCUAAAAAAAAAAAAAAAA
H_{32}-N_8	UCAAAAACUAACAAAUUAAUUUCAAACAAUCUNNNNNNNN
H_{32}-U_8	UCAAAAACUAACAAAUUAAUUUCAAACAAUCUUUUUUUUU
H_{32}-G_8	UCAAAAACUAACAAAUUAAUUUCAAACAAUCUGGGGGGGG
H_{32}-C_8	UCAAAAACUAACAAAUUAAUUUCAAACAAUCUCCCCCCCC
N_{28} -A_{12}	UCAAAAACUAACGGAUUGGUUUCGAACAAAAAAAAAAAA
N_{28} -U_{12}	UCAAAAACUAACGGAUUGGUUUCGAACUUUUUUUUUUUU
N_{28} -G_{12}	UCAAAAACUAACGGAUUGGUUUCGAACGGGGGGGGGGGG
N_{28} -C_{12}	UCAAAAACUAACGGAUUGGUUUCGAACCCCCCCCCCCCC
N_{39} (N_{28}-N_{11})	UCAAAAACUAACGGAUUGGUUUCGAACAGUCACCCGCCC
N_{50}	ACAAAAACUAACGGAUUGGUUUCGAACAGUCACCCGCCCGGACAGGUCCC

Supplementary table 2. microRNAs for tasiRNA biogenesis assay

Name	Sequence (5′-to-3′)
miR390 5p	AAGCUCAGGAGGGAUAGCGCC(M)
miR390 3p	CGCUAUCCAUCCUGAGUUUCA(M)
5′ U miR390 5p	UAGCUCAGGAGGGAUAGCGCC(M)
5′ U miR390 3p	CGCUAUCCAUCCUGAGUUACA(M)
5′ U 22-nt miR390 5p	UAGCUCAGGAGGGAUAGCGCCG(M)
5′ U 22-nt miR390 3p	GCGCUAUCCAUCCUGAGUUACA(M)

N(M) denotes 2'-O-methylated ribonucleotide.

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