RDR2 Partially Antagonizes the Production of RDR6-Dependent siRNA in Sense Transgene-Mediated PTGS

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Abstract

**Background:** RNA-DEPENDENT RNA POLYMERASE6 (RDR6) and SUPPRESSOR of GENE SILENCING 3 (SGS3) are required for DNA methylation and post-transcriptional gene silencing (PTGS) mediated by 21-nt siRNAs produced by sense transgenes (S-PTGS). In contrast, RDR2, but not RDR6, is required for DNA methylation and TGS mediated by 24-nt siRNAs, and for cell-to-cell spreading of IR-PTGS mediated by 21-nt siRNAs produced by inverted repeat transgenes under the control of a phloem-specific promoter.

**Principal Findings:** In this study, we examined the role of RDR2 and RDR6 in S-PTGS. Unlike RDR6, RDR2 is not required for DNA methylation of transgenes subjected to S-PTGS. RDR6 is essential for the production of siRNAs by transgenes subjected to S-PTGS, but RDR2 also contributes to the production of transgene siRNAs when RDR6 is present because *rdr2* mutations reduce transgene siRNA accumulation. However, the siRNAs produced via RDR2 likely are counteractive in wildtype plants because impairment of RDR2 increases S-PTGS efficiency at a transgenic locus that triggers limited silencing, and accelerates S-PTGS at a transgenic locus that triggers efficient silencing.

**Conclusions/Significance:** These results suggest that RDR2 and RDR6 compete for RNA substrates produced by transgenes subjected to S-PTGS. RDR2 partially antagonizes RDR6 because RDR2 action likely results in the production of counteractive siRNA. As a result, S-PTGS efficiency is increased in *rdr2* mutants.

Introduction

Most Eukaryotic genomes produce small RNAs, 20 to 30 nucleotides (nt) in length, which regulate endogenous genes at either the transcriptional or posttranscriptional level [1,2,3]. Endogenous small RNA species fall into three major classes: microRNAs (miRNAs) and short-interfering RNAs (siRNAs), which are both produced by Dicer enzymes, and piwi-related RNAs (piRNAs), which are Dicer-independent. All classes of small RNAs associate with proteins of the Argonaute/Piwi family [4]. Exogenous siRNAs can also be produced in response to invasive DNA or RNA (transgenes, viruses, bacteria, etc). This *de novo* production of siRNAs relies on the existing cellular small RNA machineries [5]. In contrast to endogenous small RNAs that usually are specifically processed from their precursor RNAs by one or the other cellular machinery, exogenous precursor RNAs can be processed into various forms of siRNAs by the different cellular machineries. These different ways to process exogenous RNAs have different silencing outcomes. For instance, in plants, 21-nt and 22-nt siRNAs produced by DCL4 and DCL2, respectively, trigger posttranscriptional gene silencing (PTGS) when they are homologous to transcribed regions, either by guiding mRNA cleavage or translational repression [6]. In contrast, 24-nt siRNAs produced by DCL3 trigger transcriptional gene silencing (TGS) when they are homologous to promoter regions, either by guiding DNA methylation or histone modification [7].

What makes an exogenously derived double-stranded RNA (dsRNA) a particularly attractive substrate for one DCL or another remains unclear. When the exogenous dsRNA is directly transfected into the cell or produced in the cell in the form of a dsRNA, only its sequence and structure could account for DCL specificity. However, when the dsRNA derives from an exogenous single-stranded RNA (ssRNA) transformed into dsRNA by an endogenous RNA-dependent RNA polymerase (RDR), the DCL specificity could rely on the specific relationship existing between RDRs and DCLs. It is known that in wildtype plants, DCL4 processes endogenous RDR6-derived dsRNA [8], whereas DCL3 processes endogenous RDR2-derived dsRNA [9]. In contrast, DCL2 mostly processes RDR-independent dsRNA produced by endogenous inverted repeats [10]. DCL2, DCL3 and DCL4 can substitute to each other when one is missing [8] and only when DCL2, DCL3 and DCL4 are missing can DCL1 process some siRNAs in addition to miRNAs [11].

Although RDR6 and DCL4 normally function in PTGS while RDR2 and DCL3 function in TGS, several unexpected requirements have been observed during transgene silencing. First, only RDR2, but not RDR6, functions with DCL4 in cell-to-

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cell spreading of IR-PTGS, i.e. PTGS triggered by inverted repeat transgenes, when the primary transcript is expressed in a localized tissue-specific manner [12,13]. Secondly, RDR6 is required for DNA methylation of transcribed regions during S-PTGS i.e. PTGS triggered by sense transgenes [14]. In this report, we examine the roles of RDR2 and RDR6 in S-PTGS. We found that RDR2 is not required for DNA methylation of transcribed regions during S-PTGS, suggesting that this type of methylation is mostly triggered by RDR6-dependent 21-nt siRNAs. We also found that S-PTGS is more efficient in rdr2 mutants than in wildtype plants, suggesting that RDR2 partially antagonizes RDR6 during the triggering of S-PTGS.

Results

CLSY1, NRPD1 and RDR2 are not required for S-PTGS

Genetic screens were designed to identify cellular components involved in the initiation or spreading of IR-PTGS. Arabidopsis transgenic lines expressing IR-transgenes that generate dsRNA under the control of the SUCROSE (SUL) and PHITOENE DESATURASE (PDS), which, when silenced, lead to bleaching of the leaf tissue. These IR-PTGS lines exhibited silencing of the SUL and PDS targets in a layer of 10–15 cells around the vasculature, due to spreading of a mobile PTGS signal. Silencing in the pSUC2:SUL and pSUC2:PDS lines was not impaired by mutations in RDR6 and SGS3 [10,13,15]. Mutagenesis of the pSUC2:SUL and pSUC2:PDS lines retrieved mutants impaired in the chromatin-remodelling protein CLSY1, the largest (NRPD1) and second largest (NRPD2) subunits of PolIV and the RNA-dependent RNA polymerase RDR2 [10,12,13].

Neither clsy1, npd1 nor rdr2 mutants were recovered from the L1 forward genetic screen. Therefore, we examined the integrity of the S-PTGS pathway when CLSY1, NRPD1 and RDR2 are impaired by crossing L1 to null alleles of clsy1, npd1 and rdr2. F2 plants that are homozygous for both L1 and the clsy1, npd1 or rdr2 mutations were identified, and bulks of F3 plants were analyzed 11 days after germination (DAG). L1/rdr6 was used as a control for the absence of GUS S-PTGS. GUS mRNA and GUS activity in L1/clsy1, L1/npd1 and L1/rdr2 were low and comparable to control L1 plants (Figure 1), indicating that neither of these mutations delayed the onset of S-PTGS or compromised its establishment. Consistently, GUS siRNAs accumulated in L1/clsy1, L1/npd1 and L1/rdr2, although at levels lower than in L1 (Figure 1). These results indicate that CLSY1, NRPD1 and RDR2, which are required for the production of siRNAs associated to the spreading of IR-PTGS, are dispensable for S-PTGS.

CLSY1, NRPD1 and RDR2 are not required for DNA methylation in S-PTGS

CLSY1, NRPD1 and RDR2 are required for RNA-directed DNA methylation (RdDM) guided by endogenous 24-nt siRNAs [9,16,17]. As such, they are required for RdDM of the FW1 transgene and endogenous gene [16,18], and for RdDM of the endogenous PDS locus in pSUC2:PDS lines [19]. DNA methylation also is a hallmark of S-PTGS, as exemplified by GUS DNA methylation in the L1 line [20], which requires RDR6 and SGS3 [14]. Indeed, methylation at CG sites (monitored by HpaII digest) in the 3' end of GUS is abolished when S-PTGS is compromised (Figure 2B). However, it is not known if GUS DNA methylation occurs at CNG sites because RDR6 and SGS3 produce siRNA that directly guide DNA methylation at CNG sites or because RDR6 and SGS3 produce siRNA that are used by CLSY1, NRPD1 or RDR2 to produce secondary molecules that guide DNA methylation. Moreover, methylation at CG sites (monitored by HpaII digest) is strongly reduced but not abolished when S-PTGS is compromised (Figure 2B), suggesting that GUS DNA methylation at CG sites is partly independent of RDR6 and SGS3. Whether, RDR6-SGS3-independent DNA methylation at CG sites requires the CLSY1, NRPD1 and RDR2 components of RdDM is unknown.

To address the role of CLSY1, NRPD1 and RDR2 in GUS DNA methylation in the L1 line, DNA methylation of the GUS transgene was examined at 11 DAG in L1/clsy1, L1/npd1 and L1/rdr2 mutants. At CG sites, GUS DNA methylation in L1/clsy1, L1/npd1 and L1/rdr2 mutants was comparable to control L1 plants (Figure 2C), indicating that CLSY1, NRPD1 and RDR2 are dispensable for RDR6-SGS3-dependent DNA methylation in S-PTGS. At CG sites, we expected GUS DNA methylation in L1/clsy1, L1/npd1 and L1/rdr2 mutants to be reduced compared to control L1 plants if CLSY1, NRPD1 and RDR2 contributed to RDR6-SGS3-independent DNA methylation. However, GUS DNA methylation in L1/clsy1, L1/npd1 and L1/rdr2 mutants was not reduced compared to control L1 plants (Figure 2C), indicating that RDR6-SGS3-independent DNA methylation does not require CLSY1, NRPD1 and RDR2 either. The exact pathway contributing to RDR6-SGS3-independent GUS DNA methylation at CG sites is still not fully understood, but it likely requires MET1 because met1 mutants recovered from the L1 screen exhibit lower levels of GUS DNA methylation at CG sites than rdr6 and sgs3 mutants [21,22].

RDR2 counteracts the production of RDR6-dependent siRNA in S-PTGS

Examination of GUS siRNA levels in L1, L1/clsy1, L1/npd1 and L1/rdr2 revealed an important reduction of the level of GUS siRNAs in L1/rdr2 compared to L1 (Figure 1). Moreover, examination of DNA methylation revealed a slightly higher level of DNA methylation at CG sites in L1/rdr2 compared to L1 (visible as a slight reduction of the amount of unmethylated fragments on
Because *L1/clsy1* and *L1/nrpd1* did not show a lower level of *GUS* siRNAs or a higher level of DNA methylation than *L1*, it is unlikely that the impairment of the entire CLSY1/NRPD1/RDR2 pathway was responsible for these phenomena. Rather, we hypothesized that part of the *GUS* siRNAs detected in a wildtype plant was produced by RDR2,
compared with mRNA level and GUS activity at 8 DAG are higher in compromised but its establishment is delayed. As a result, rdr6-8 and functioning of S-PTGS in the GUS mRNA and GUS activity were intermediate between L1 and rdr2. mRNA level and GUS activity were much lower in L1/rdr2 plants, allowing to visualize differences in PTGS efficiency easily progressively decrease during the first weeks following germination (8 DAG). Indeed, GUS DNA methylation because the impairment of RDR2 activity does not compromise the establishment of S-PTGS, which therefore relies only on RDR6 derivatives of S-PTGS in wildtype plants. This counteracting effect of RDR2 during the triggering phase does not exclude that RDR2 could also counteract the amplification phase of S-PTGS.

Supporting this hypothesis, S-PTGS is totally abolished in rdr6-8 mutants, whereas GUS mRNA and GUS activity in L1 progressively decrease during the first weeks following germination [23], allowing to visualize differences in PTGS efficiency easily when L1 S-PTGS is not fully established yet. At 8 DAG, GUS mRNA level and GUS activity were much lower in L1/rdr2 compared with L1 (Figure 3).

We hypothesized that the higher S-PTGS efficiency in rdr2 mutants could be due to the exclusive synthesis of GUS dsRNA by RDR6 in rdr2 mutants, whereas GUS dsRNA are synthesized by both RDR6 and RDR2 in wildtype plants. This hypothesis implies that RDR2-derived GUS dsRNA do not play an active role in S-PTGS, which therefore relies only on RDR6-derived GUS dsRNA. Supporting this hypothesis, S-PTGS is totally abolished in rdr6 null alleles [14], indicating that RDR2 cannot compensate the absence of RDR6. To test further our hypothesis, we generated a double mutant between the rdr6-8 hypomorphic allele and an rdr2 null allele. The rdr6-8 hypomorphic allele corresponds to a T→A nucleotide change that results in a Y→N amino acid change at protein position 228 [24]. In this mutant, L1 S-PTGS is not compromised but its establishment is delayed. As a result, GUS mRNA level and GUS activity at 8 DAG are higher in L1/rdr6-8 compared with L1 (Figure 3). In the L1/rdr6-8/rdr2 double mutant, GUS mRNA and GUS activity were intermediate between L1/rdr6-8 and L1, indicating that RDR2 counteracts the optimum functioning of S-PTGS in the rdr6-8 hypomorphic allele.

**RDR2 counteracts the triggering of S-PTGS**

rdr2 mutations accelerate the establishment of L1 S-PTGS in wildtype plants or rdr6-8 mutants, suggesting that RDR2 counteracts S-PTGS during the triggering or the amplification phase of S-PTGS, or both. To further determine at which step RDR2 competes with RDR6, we introduced the Hc1 locus into a null rdr2 mutant. The Hc1 line carries the same β35S::GUS transgene as L1 but only triggers S-PTGS in 20% of the plants at each generation, whereas L1 triggers S-PTGS in 100% of the plants [20]. Introduction of the Hc1 locus into a mutant background therefore allows detecting an increase or a decrease in the triggering of S-PTGS [24,25]. If RDR2 competed with RDR6 during the triggering phase, we expected the percentage of silenced Hc1/rdr2 plants to be higher than in Hc1 plants. However, if RDR2 competed with RDR6 during the amplification phase, we expected S-PTGS to be established faster in Hc1/rdr2 plants compared with Hc1 plants, but the percentage of silenced plants to remain the same in Hc1 and Hc1/rdr2 plants. GUS activity was determined at the adult stage when silencing is fully established. As previously reported, 19% (18/96) of Hc1 plants were silenced. In contrast, PTGS affected 56% (107 out of 192) of Hc1/rdr2 plants (Figure 4), indicating that RDR2 counteracts the triggering of Hc1 S-PTGS in wildtype plants. This counteracting effect of RDR2 during the triggering phase does not exclude that RDR2 could also counteract the amplification phase of S-PTGS.

**Discussion**

Defense responses to invasive DNA or RNA (transgenes, viruses, bacteria, etc) rely on the existing cellular small RNA machineries. What makes an exogenously derived RNA a particularly attractive substrate for one or another machinery remains unclear. In the recent years, it has become clear that...
endogenous RNAs compete with each other for the accessibility to cellular machineries [9,11,26,27], and that exogenous RNAs also compete with endogenous RNAs [10,23]. Based on early genetic screens, RDR6 has been associated to 21-nt siRNA-related PTGS mediated by ambipolos (A-PTGS) or sense transgenes (S-PTGS) [14,26], and RDR2 to 24-nt siRNA-related DNA methylation and TGS [9,18]. However, subsequent genetic screens associated RDR2 to A-PTGS and to IR-PTGS mediated by inverted repeat transgenes [12,13,17]. During A-PTGS, RDR2 and RDR6 are partially redundant, at least in some tissues [17], whereas in IR-PTGS, RDR2, but not RDR6, is required for the production of 21-nt and 24-nt siRNAs involved in cell-to-cell spreading of PTGS [12,13]. Here, we examined the role of RDR2 and RDR6 in S-PTGS and found that both participate in the production of transgene siRNAs. However, the siRNAs produced via RDR2 appear unproductive because S-PTGS is more efficient in rdr6 mutants than in wildtype plants. These results suggest that RDR2 and RDR6 can compete for S-PTGS RNA substrates, and that RDR2 partially antagonizes the action of RDR6 during S-PTGS. This situation is inverse to that described for Gypsy-like transposons. Indeed, Gypsy-like dsRNA over-accumulate in rdr6 mutants, suggesting that RDR6 antagonizes the action of other RDR on these targets [27]. The cellular and/or molecular bases of the specificity of RDR proteins towards their RNA substrates remain to be determined.

Materials and Methods

Plant material

L1 and Hc1 lines, and clsy1-6, npd1a-6, rdr2-1, rdr2-5, rdr6-8 mutants have been described before [9,13,20,24].

Molecular analyses

DNA gel blot analysis, RNA gel blot analysis, and GUS fluorimetric assays were performed as described before, 3’ GUS, U6 and 258 probes have been described before [23,24,25].

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Author Contributions

Conceived and designed the experiments: VJ TE HV. Performed the experiments: VJ MR NB TE HV. Analyzed the data: VJ MR NB TE HV. Contributed reagents/materials/analysis tools: VJ MR NB TE HV. Wrote the paper: HV.

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