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Post-transcriptional gene silencing triggered by sense transgenes involves uncapped antisense RNA and differs from silencing intentionally triggered by antisense transgenes

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ABSTRACT

Although post-transcriptional gene silencing (PTGS) has been studied for more than a decade, there is still a gap in our understanding of how *de novo* silencing is initiated against genetic elements that are not supposed to produce double-stranded (ds)RNA. Given the pervasive transcription occurring throughout eukaryote genomes, we tested the hypothesis that unintended transcription could produce antisense (as)RNA molecules that participate to the initiation of PTGS triggered by sense transgenes (S-PTGS). Our results reveal a higher level of asRNA in *Arabidopsis thaliana* lines that spontaneously trigger S-PTGS than in lines that do not. However, PTGS triggered by antisense transgenes (AS-PTGS) differs from S-PTGS. In particular, a hypomorphic *ago1* mutation that suppresses S-PTGS prevents the degradation of asRNA but not sense RNA during AS-PTGS, suggesting a different treatment of coding and non-coding RNA by AGO1, likely because of AGO1 association to polysomes. Moreover, the intended asRNA produced during AS-PTGS is capped whereas the asRNA produced during S-PTGS derives from 3′ maturation of a read-through transcript and is uncapped. Thus, we propose that uncapped asRNA corresponds to the aberrant RNA molecule that is converted to dsRNA by RNA-DEPENDENT RNA POLYMERASE 6 in siRNA-bodies to initiate S-PTGS, whereas capped asRNA must anneal with sense RNA to produce dsRNA that initiate AS-PTGS.

INTRODUCTION

Genome-wide surveys have revealed the abundance of natural-antisense transcripts (NATs) existing across eukaryotes (1–3). These molecules originate either from the transcription of the DNA strand opposite to a reference gene (*cis*) or from a distant locus (*trans*). The simultaneous production of both sense and antisense RNA has been reported to lead to a variety of outcomes but no common pattern seems to emerge from the different examples (3). On the other hand, it has long been known that convergent transcription of pericentromeric repeats in *Schizosaccaromices pombe* leads to the formation of heterochromatin and silencing (4,5). Converging transcript can indeed be used as a tool for transcriptional silencing not only in fission yeast but also zebrafish as well as mammalian cells (6–8). Also, compelling evidence in mouse oocytes has proven that antisense (as)RNA molecules can regulate genes and transposons at the post-transcriptional level both *in cis* and *in trans* (9,10). There is therefore an enormous regulation potential in asRNA that begs further investigation.

As in other eukaryotes, many complementary RNA pairs have been identified in plants (11–13). While some transcriptomic studies have detected negatively correlated expression patterns for RNA pairs in *Arabidopsis thaliana* (13,14) others have failed to detect any significant trend (15). The question therefore remains as to how these RNA molecules can regulate genes. In plants, the intentional expression of asRNA to interfere with a precise target sequence was commonly used even before the discovery of RNA interference (RNAi) (16,17). This technique was applied to different models with varying efficiencies but the underlying mechanism was never investigated at the genetic level. In contrast, many genetic screens

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have been set up to decipher the mechanisms of inverted repeat-triggered and sense-triggered post-transcriptional gene silencing (IR-PTGS and S-PTGS). Both involve an initiating double-stranded (ds)RNA being processed by the DICER-LIKE proteins (DCL) into small interfering (si)RNA molecules (18–20). These small molecules are bound by ARGONAUTE (AGO) proteins to form the core of the RNA-induced silencing complex (RISC) that interferes with complementary RNAs. In the case of IR transgenes, dsRNA is automatically formed from a single transcript folding back on itself making silencing highly efficient because the dsRNA molecule is directly processed by DCL2 and/or DCL4 into siRNA molecules (21–23). In the case of sense transgenes, it is assumed that aberrant RNAs are produced, and subsequently converted into dsRNA by RNA-DEPENDENT RNA POLYMERASE (RDR)6 (24,25). This process is thought to occur in cytoplasmic congregations of proteins called siRNA-bodies, which are distinct from P-bodies, and where RDR6 localizes (26).

So far, the reason why a sense transgenes inserted at a given locus triggers S-PTGS while the same transgene at another location does not has remained mysterious. Long ago, it was hypothesized that only some types of transgene arrangements at the insertion site allow enough converging transcription to trigger RNAi (27), but this hypothesis was not formally explored. It gained our interest when examples of endogenous *cis*-NATs were shown to trigger silencing. Indeed, some stresses induce convergent transcription at precise loci and cause PTGS of complementary transcripts that is necessary for proper response (11,13,28,29). Such a regulatory mechanism has also been reported to be associated with specific developmental phases in Arabidopsis (30) and barley (31). It therefore appears that asRNAs have an important functional role in plants. Importantly, these different studies have shown genetic requirements for this type of regulation that only partially overlap with those required for S-PTGS and IR-PTGS.

To investigate whether asRNAs play a role in the triggering of S-PTGS, well-established Arabidopsis lines carrying sense transgenes were analyzed. We found that asRNA molecules were indeed present at higher level in lines that spontaneously undergo S-PTGS than in lines that stably express the sense RNA. Then, we developed a two-components silencing system involving the independent expression of sense and antisense RNA to decipher the genetic requirements of antisense-triggered PTGS (AS-PTGS). We identified DCL2 and DCL4 as essential factors, while AGO1, RDR6 and SGS3 appear only required for the production of secondary siRNA. Our analyses also revealed a different contribution of AGO1 to the degradation of sense and antisense RNAs, highlighting the differences between S-PTGS and AS-PTGS. We eventually discovered that the unintended asRNA produced during S-PTGS is uncapped, which probably allows its conversion into dsRNA by RDR6, whereas the intended asRNA produced during AS-PTGS is capped and must anneal with the sense RNA to form dsRNA.

MATERIALS AND METHODS

Plant material

Transgenic lines *6b4*, *L1*, *L2*, *JAP3* and *SUC-SUL*, and *ago1-1*, *ago1-27*, *dcl2*$^{Kas\text{-}1}$, *dcl4-5*, *rdr6*$^{sgs2\text{–}1}$, *sgs3-1*, *xrn3-3* and *xrn4-5* mutants have been described previously (22,23,25,32–35). Seeds were sterilized with bleach and ethanol and grown on S-Medium from Duchefa (http://www.duchefa-biochemie.com) under 18 h of light and 6 h dark cycles with an average temperature of 22°C.

Cloning and transformation

To generate the *p35S:SUG-tCaMV* construct, the *GUS* coding sequence was extracted as an PstI–PstI fragment from plasmid pRAJ260 and cloned between the 35S promoter and terminator of plasmid pLBR19. Then, the *p35S:SUG-tCaMV* construct was extracted as a SstI-XbaI fragment and cloned into the binary vector pBiB-Hyg before transfer to Agrobacterium. Arabidopsis plants were transformed by floral dipping and transformants selected on medium supplemented with 30 μg/ml hygromycin.

RNA extraction, fractionation, RNA gel blot hybridization and sequencing

Shoots or whole plants were pooled 5, 11 or 17 days after germination on sterile S-medium and grinded into liquid nitrogen. Buffer containing 0.1 M NaCl, 2% SDS, 50 mM Tris–HCl (pH 9), 10 mM EDTA (pH 8) and 20 mM ß-mercaptoethanol was added to the frozen powder and RNA was extracted twice with phenol and recovered by ethanol precipitation. For small RNA analysis, total RNA was separated on a 15% denaturing polyacrylamide gel and stained with ethidium bromide. For sequencing, small RNA bands were cut out of the gel and sent to GATC Biotech (http://www.gatc-biotech.com) for library preparation and sequencing using HiSeq2000 sequencer. For blots, RNA was transferred to nylon membrane, hybridized in Sigma PerfectHyb buffer (https://www.sigmaaldrich.com/) and stripped with boiling 0.1% SDS solution.

To obtain high molecular weight RNA, total RNA was precipitated overnight in 2 M LiCl at 4°C and recovered by centrifugation. The samples were then separated on 1.5% agarose gel running in 20 mM HEPES, 1 mM EDTA and 0.7% formaldehyde. RNA was transferred to nylon membrane by capillarity and hybridized in Sigma PerfectHyb buffer.

Strand-specific qRT-PCR

Sterile plants were pooled 11 days after germination and grinded into liquid nitrogen. RNA was isolated using RNeasy Plant Mini Kit from QIAGEN (https://www.qiagen.com). cDNA were generated form 1 μg of total RNA using the first strand cDNA kit from Thermo Fisher Scientific (http://www.lifetechnologies.com) using the specific primer RT_ASGUS_Linker: **CGACTGGAGCACGAGGACACTGA**GACTGGCATGAACTTCGGTGAA (bold letters represent linker sequence). PCR amplification was

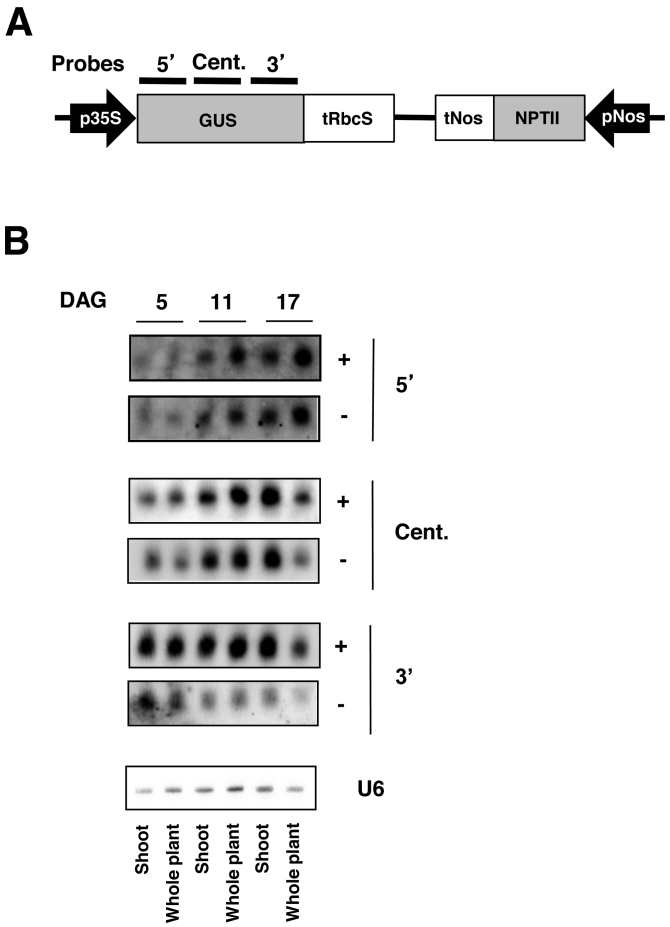

Figure 1. Silencing originates from the 3′ in *L1*. (**A**) Scheme of the T-DNA cassette in *6b4*, *L1* and *L2* lines. Regions covered by different probes are represented above the GUS coding sequence. (**B**) small RNA blots of either shoots or whole *L1* plants isolated at different days after germination (DAG) indicated above. The membranes were blotted with different RNA probes with different orientations indicated on the right. A U6 probe was used as loading control for all membranes, one representative blot is shown.

done using a primer containing the Linker sequence, LK: CGACTGGAGCACGAGGACACTGA, and a primer specific to the RbcS region, RbcS1Rev: TCACAGTTCGATAGCGAAAACCGA. SsoAdvanced Universal SYBR Green Supermix from Biorad (http://www.bio-rad.com/) was used in the Mastercycler® ep realplex from Eppendorf (www.eppendorf.com). *eIF1a* expression was used has amplification control since unspecific annealing of RT primer produces cDNA from highly abundant RNA (36).

Data processing

Datasets were cleaned using bioinformatic tools developed previously (37). Reads ranging from 20 to 25 nucleotides in length were selected using PRINSEQ (38) and aligned using standard Bowtie2 algorithm (39). Aligned reads were sorted with SAMtools (40) and distribution plots were generated using a script from the S-MART tools (37).

RESULTS

Production of siRNAs starts at the 3′ end of *GUS* in the S-PTGS line *L1*

A series of plants carrying a *p35S-GUS-tRbcS* transgene were previously characterized, including line *6b4* that stably expresses *GUS* and lines *L1* and *L2*, which spontaneously undergo S-PTGS (32). Probing *GUS* siRNA accumulation in *L1* using three large fragments covering the *GUS* coding sequence (Figure 1A) revealed that siRNAs come from 5′, central and 3′ parts of the gene (41). However, no siRNA were detected using a probe covering the first 300bp at the 5′ end of *GUS* (42). To determine if the absence of siRNAs at the 5′ end reflects an incomplete spreading of siRNAs from 3′ to 5′, the kinetics of siRNA accumulation was determined using the original 5′, central and 3′ probes (41). The kinetics was followed from day 5 to day 17 during which GUS activity decreases (32), indicating the progressive establishment of *L1* S-PTGS. Whereas siRNAs of both polarities accumulated at high level at the 3′ end as early as 5 days after germination (DAG), they accumulated at lower level in the central region and were barely detectable at the 5′ end (Figure 1B). From day 5 to day 17, the accumulation of siRNAs increased at the 5′ end, decreased at the 3′ end while it remained constant in the central part. Together, these results suggest that siRNAs spread from 3′ to 5′ during the establishment of *L1* S-PTGS.

Spontaneous triggering of S-PTGS correlates with the production of asRNA

So far, the reason why lines *L1* and *L2*, but not *6b4*, undergo PTGS has remained unexplained. Given the organization of the *p35S-GUS-tRbcS* and *pNos-NPTII-tNos* transgenes on the T-DNA (Figure 1A) and the weak efficiency of the *Nos* terminator (43), transcription read-through that bypasses the *Nos* terminator of the *NPTII* transgene could produce an RNA antisense to the 3′ end of *GUS* (27). Subsequent annealing of the asRNA to *GUS* RNA could form dsRNA that initiate PTGS, consistent with the first appearance of siRNAs at the 3′ end of *GUS*. Alternatively, the asRNA could be recognized as an aberrant RNA and transformed into dsRNA by RDR6 in siRNA-bodies (26). Therefore, the presence of such asRNA (hereafter referred to as *SUG*) was tested by qRT-PCR in *6b4*, *L1* and *L2*. To insure strand-specificity, a linker-oligonucleotide was used to prime the cDNA synthesis reaction (36). The level of *SUG* RNA detected in lines *L1* and *L2* was two-fold higher than in *6b4* (Figure 2A). However, because lines *L1* and *L2* undergo PTGS (32), *SUG* RNA is likely degraded together with *GUS* RNA, and therefore underestimated in this experiment. To circumvent this issue, *SUG* RNA levels were tested in the *rdr6* mutant background in which S-PTGS is blocked (25). The level of *SUG* RNA detected in lines *L1*/*rdr6* and *L2*/*rdr6* was 4- to 5-fold higher than in line *6b4*/*rdr6* (Figure 2B), highlighting a positive correlation between the presence of *SUG* RNA and the triggering of S-PTGS.

Remarkably, lines *L1*, *L1*/*rdr6*, *L2* and *L2*/*rdr6* tolerate kanamycin (the selection marker for the *NPTII* resistance gene) to a level as high as 150μg/ml, whereas lines *6b4*

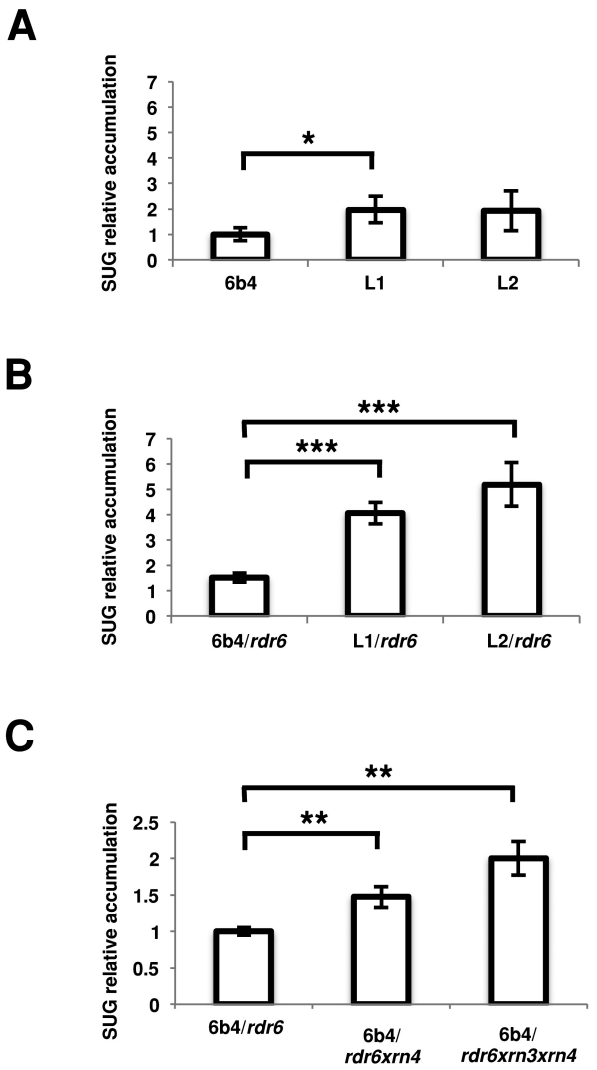

Figure 2. Antisense RNA is more abundant in lines that spontaneously trigger S-PTGS and is degraded by 5′->3′ RNA exonucleases. (**A**) Relative presence of *SUG* RNA in different lines in WT background. *SUG* ΔCt were calculated using *eIF1a* Ct and expressed as a fold change compared to *6b4* (*6b4* = 1). Error bars represent the standard deviation of the biological triplicate. (**B**) Relative presence of *SUG* RNA in different lines in *rdr6* background. *SUG* ΔCt were calculated using eIF1a expression as internal control and represented as a fold change compared to *6b4* (*6b4* = 1). Error bars represent the standard deviation of the biological triplicate. (**C**) Relative presence of *SUG* RNA in different *rdr6 xrn* backgrounds. *SUG* ΔCt were calculated using *eIF1a* Ct and expressed as a fold change compared to 6b4/*rdr6* (*6b4* = 1). Error bars represent the standard deviation of the biological triplicate. The values obtained were submitted to the Student's *t*-test to calculate the null hypothesis probability (*P*). The asterisks represent the different levels of confidence (*$P < 0.05$, **$P < 0.01$, ***$P < 0.005$).

and *6b4/rdr6* exhibit growth defects on 30μg/ml kanamycin and die on 50μg/ml kanamycin. At first, this result indicates that, unlike the *p35S-GUS-tRbcS* transgene, the *pNos-NPTII-tNos* transgene does not undergo silencing in lines *L1* and *L2*. Secondly, this result indicates that the *NPTII* gene is more efficiently transcribed in lines *L1* and *L2* than in line *6b4*. This likely explains why *SUG* RNA is more abundant in the silenced lines, assuming that the *SUG* RNA results from a read-through that bypasses the *Nos* terminator of the *pNos-NPTII-tNos* transgene. Together, these results suggest that the higher transcription occurs at the genomic location where the T-DNA integrates, the higher is the chance that sufficient amounts of *GUS* and *SUG* RNAs are produced to trigger S-PTGS.

Development of an AS-PTGS system

To determine if the production of an asRNA consistently triggers PTGS of a stable *GUS* reporter, a *p35S-SUG-tNos* transgene was generated and introduced into wild-type (WT) Arabidopsis plants. The resulting *SUG* lines were crossed with *6b4* to identify plants in which GUS is efficiently silenced. In three crosses, hybrid plants carrying the *6b4* and *SUG* transgenes showed significantly reduced GUS activity (Supplemental Figure S1). Hereafter, all experiments were performed with the *SUG6* line that showed a 3:1 Mendelian segregation indicating insertion at a single locus. At first, the accumulation of *SUG* long RNA and siRNA was analyzed in homozygous *SUG6* plants. Results indicate that the *SUG* RNA stably accumulates in line *SUG6*, and that no siRNA is spontaneously produced in this line (Figure 3). Then, plants homozygous for both *6b4* and *SUG6* were produced and analyzed in a similar manner. Results indicate that the *6b4/SUG6* line lacks both GUS and *SUG* long RNAs, while both sense and antisense siRNA accumulated (Figure 3). This result indicates that silencing in the *6b4/SUG6* line results from post-transcriptional degradation of *GUS* and *SUG* long RNAs and not from transcriptional interference between the *p35S-GUS-tRbcS* and *p35S-SUG-tNos* transgenes. Hereafter we refer to this system as antisense-triggered PTGS (AS-PTGS).

Genetic determinants of AS-PTGS

To decipher the genetic requirement for AS-PTGS, line *6b4/SUG6* was crossed to various RNA silencing mutants. Since the most straightforward explanation for AS-PTGS is the spontaneous pairing of *GUS* and *SUG* RNA to form dsRNA that is subsequently processed by DCL proteins, we first tested the effect of *dcl2* and *dcl4* mutations. Indeed, DCL2 and DCL4 have previously been identified as essential factors in both S-PTGS and IR-PTGS as well as in antiviral PTGS (21,23,44,45). We observed that *dcl2* and *dcl4* single mutations seemingly have no effect on *GUS/SUG* silencing, and that only the *dcl2dcl4* double mutant released AS-PTGS. Concordantly, *GUS* and *SUG* RNA accumulated in *6b4/SUG6/dcl2dcl4* plants at the level of *6b4* and *SUG6* individual plants, respectively, and lacked siRNAs (Figure 3). These results are similar to those obtained using the *6b4/306* (IR-PTGS) and *L1* (S-PTGS) systems (23,42), indicating that S-PTGS, IR-PTGS and AS-PTGS have the same requirements for DCL proteins.

The involvement of AGO1, a central protein in S-PTGS and IR-PTGS (46,47), was tested using the *ago1-1* null allele. Analysis of *6b4/SUG6/ago1-1* plants revealed partial release of AS-PTGS. Indeed, GUS and *SUG* RNAs accumulate, but at levels lower than those observed in *6b4/SUG6/dcl2dcl4* plants (Figure 3). Moreover, sense and antisense siRNAs are still detected, but again at levels lower

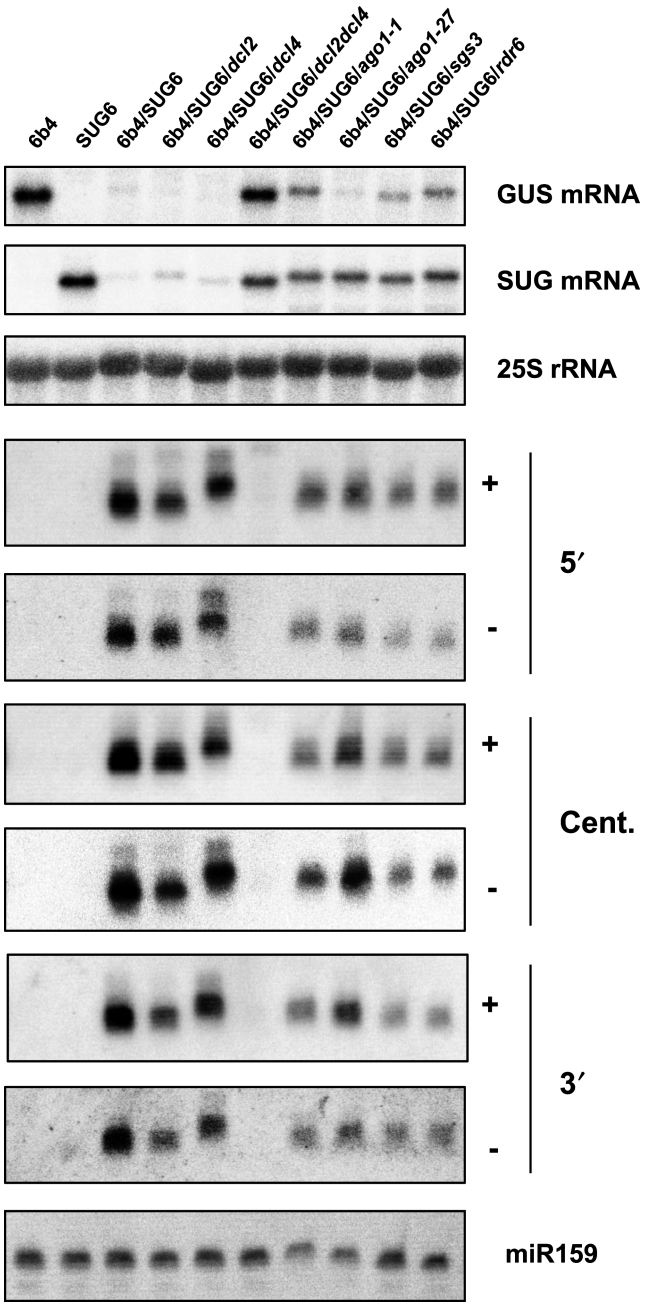

Figure 3. Genetic requirement of AS-PTGS. HMW and LMW RNA blots of whole 11 DAG plants in various backgrounds indicated on top. For *GUS* and *SUG* mRNA, the 3′ RNA probe was used in different orientation. Loading was controlled using a *25S* RNA probe. For small RNA, 5′, Central and 3′ RNA probes were used in different orientations as indicated on the right. The different membranes were probed with a miR159 oligonucleotide for loading and a representative blot is shown.

than those observed in *6b4/SUG6* plants (Figure 3). These results suggest that, in *6b4/SUG6/ago1–1* plants, AS-PTGS is limited to the processing of annealed *GUS/SUG* dsRNA by DCL2 and DCL4, and that the siRNAs that are detected correspond to primary siRNAs.

RDR6 and SGS3 are required for the production of secondary siRNAs, which are essential components of S-PTGS, but dispensable for IR-PTGS (24,25,34,47,48). Therefore, we tested the effect of *rdr6* and *sgs3* null alleles on AS-PTGS. Like *ago1-1*, *rdr6* and *sgs3* null mutations partially impaired *6b4/SUG6* silencing (Figure 3). Indeed, GUS mRNA accumulates at similar level in *6b4/SUG6/rdr6, 6b4/SUG6/sgs3* and *6b4/SUG6/ago1-1*, i.e. below the level observed in *6b4/SUG6/dcl2dcl4* plants. Moreover, both sense and antisense siRNA molecules are still detected in both cases, although at a level lower than in *6b4/SUG6*, indicating that partial PTGS is still operating (Figure 3). Analyses of the effect of *rdr6* and *sgs3* mutations on two other *SUG* lines (*SUG7* and *SUG9*) yielded similar results. Indeed, GUS activity in *6b4/SUG6/rdr6, 6b4/SUG6/sgs3, 6b4/SUG7/rdr6, 6b4/SUG7/sgs3, 6b4/SUG9/rdr6* and *6b4/SUG9/sgs3* plants was higher than in *6b4/SUG6*, *6b4/SUG7* and *6b4/SUG9* plants but lower than in *6b4/rdr6* and *6b4/sgs3* controls (Supplemental Figure S1). This confirms that the partial impairment of AS-PTGS by *rdr6* and *sgs3* mutations is not specific to the *SUG6* locus. These results therefore suggest that the production of secondary siRNAs through RDR6 and SGS3 is generally required to complete RNA degradation during AS-PTGS.

A hypomorphic *ago1* mutation uncouples the degradation of sense and antisense RNA

The *ago1-27* hypomorphic allele exhibits minor morphologic defects compared with the *ago1-1* null allele, indicating that it retains enough AGO1 activity to allow plants to go through their life cycle (33). Nevertheless, the *ago1-27* mutation suppresses S-PTGS as efficiently as *ago1* null alleles (33), indicating that S-PTGS is highly sensitive to AGO1 perturbation. In contrast, the *ago1-27* mutation does not affect IR-PTGS triggered by 35S-driven transgenes (48), and only partially suppresses IR-PTGS specifically triggered in the companion cells of the phloem, which leads to silencing in a layer of 15–20 cells surrounding the phloem (Supplemental Figure S2). Introduction of the *6b4/SUG6* system into *ago1-27* revealed that *GUS* mRNA is still degraded in *6b4/SUG6/ago1-27* plants (Figure 3), which contrasts with the complete abolition of S-PTGS in *L1/ago1-27* (33,41). Nevertheless, *ago1-27* did have an effect on AS-PTGS because *SUG* RNA accumulates at a level similar to that observed in *6b4/SUG6/ago1-1* (Figure 3). Moreover, siRNAs accumulated at lower levels in *6b4/SUG6/ago1-27* than in *6b4/SUG6* controls (Figure 3). Together, these results, suggest a different treatment of coding and non-coding RNA molecules by the mutant *ago1-27* protein during AS-PTGS, which may be due to the fact that AGO1 associates with polysomes (49).

Small RNA populations in silenced lines

The different effect of *ago1-27* on S-PTGS and AS-PTGS prompted us to take a closer look at the siRNA populations present in WT and mutants carrying the *6b4/SUG6* or *L1* transgenes. Small RNA eluted from acrylamide gels were used to generate libraries for short-reads RNA sequencing using Illumina HiSeq 2000. At first, the 20- to 25-nt reads matching the *GUS/SUG* transgenes were analyzed. The total aligned read count per million (RPM) is given in

Table 1. Number of *GUS/SUG* siRNA reads found in different backgrounds

	6b4/SUG6		L1	
	RPM	Ratio to WT	RPM	Ratio to WT
WT	15 605	1.00	48 865	1.00
dcl2	8251	0.53	23 525	0.48
dcl4	9630	0.62	57 253	1.17
dcl2dcl4	528	0.03	511	0.01
ago1–27	4523	0.29	426	0.01
ago1-1	3808	0.24	474	0.01
rdr6	4120	0.26	514	0.01

20 to 25 nucleotides long reads aligning to transgene sequences are reported in read per million (RPM). The ratio to the WT background sample of the different series (*6b4/SUG6* and *L1*) is given on the right.

Table 1, and the ratio to WT reveals the extent of the decreased siRNA levels in the different mutant backgrounds. Results with the *L1* locus are in line with published northern blot analyses. Indeed, as recently reported, the *dcl2* mutation reduces siRNA production and silencing efficiency, while the *dcl4* mutation stimulates siRNA production and silencing because of the role of DCL2 in transitivity, and only the *dcl2dcl4* double mutation abolishes siRNA production and PTGS (23). In addition to *dcl2dcl4, ago1-1, ago1-27* and *rdr6* abolish siRNA production and PTGS as previously reported (41). The amount of *GUS/SUG* siRNAs in *6b4/SUG6* was lower than that in *L1*, consistent with the reduced transcription level of *6b4* compared with *L1* (32). In *6b4/SUG6/dcl2dcl4*, *GUS/SUG* siRNAs were below detection levels, consistent with *GUS* and *SUG* long RNA accumulating at the level of *6b4* and *SUG6* plants taken individually (Figure 3). *GUS/SUG* siRNA levels were low but not absent in *6b4/SUG6/rdr6* and *6b4/SUG6/ago1-1,* in line with the partial impairment of silencing observed in these mutants. Consistent with northern blot analysis, *GUS/SUG* siRNA levels were higher in *6b4/SUG6/ago1–27* than in *6b4/SUG6/ago1-1*, but still lower than in *6b4/SUG* controls.

The distribution of aligned reads was then plotted on the transgene transcript sequences. Note that the *SUG* transgene carries the full *GUS* ORF but that its 3′UTR differs from that of the *GUS* transgene. Figure 4 shows the distribution of sense and antisense siRNAs along the *GUS* sequence and the *RbcS* 3′UTR in the various backgrounds. As seen in previous studies, discrete populations of small RNA molecules are overrepresented compared to others (50,51), suggesting a preferential production/accumulation of certain siRNAs. However, such hot-spots have been shown to often result from the preferential ligation of certain siRNAs by a given set of adaptors (52,53). Given that this ligation bias is the same in every backgrounds, the relative intensity of each peak can still be compared between in mutants and WT. Beside the lower level of *GUS/SUG* siRNAs in *6b4/SUG6* compared with *L1*, the major difference between these two systems consists in the presence of siRNA all over the *GUS* coding sequence in *6b4/SUG6* whereas they are absent at the 5′ end of the *GUS* gene in *L1* (Figure 4), consistent with a previous study (42). Given that *L1* produces more siRNAs than *6b4/SUG6* (Table 1), the absence of siRNAs in the 5′ region cannot be attributed to a lack of coverage. Moreover, siRNAs corresponding to this region are not reduced in *6b4/SUG6/rdr6* and *6b4/SUG6/ago1-1,* whereas siRNAs from the rest of the gene are reduced by *ago1-1* and *rdr6* (Figure 4), suggesting that this region only produces primary siRNAs.

AS-PTGS against endogenous mRNA

To test if the genetic requirements described for *6b4/SUG6* silencing are specific to this system or if it generally applies to AS-PTGS, the coding sequence of the *PHYTOENE DESATURASE* (*PDS*) gene was placed behind the *35S* promoter in inverted orientation to produce an antisense *SDP* RNA. *PDS* was selected as a target because the silencing of this gene produces an obvious visual phenotype (21,34). The *p35S-SDP-tNos* transgene was introduced into WT, *dcl2dcl4* and *rdr6* backgrounds. Primary transformants were visually inspected at 14 DAG and classified as severely or moderately silenced for the endogenous *PDS* gene (Table 2). Eighty three percent of the WT transformants showed a *pds* phenotype, confirming that antisense transgenes can efficiently silence certain endogenous genes. Given the results obtained with the *6b4/SUG6* system, and knowing that, unlike transgenes, endogenous genes are incapable of producing secondary siRNAs (54,55), we expected the *rdr6* mutation to very moderately affects *PDS* AS-PTGS. Indeed, the frequency of transformants exhibiting a *pds* phenotype was only slightly reduced in *rdr6* compared with WT (Table 2). In contrast, the percentage of silenced plants was dramatically reduced in the *dcl2dcl4* mutant. In particular, no *dcl2dcl4* transformant exhibited the severe *pds* phenotype that was observed in 42% of the WT transformants. Nevertheless, 22% of the *dcl2dcl4/p35S-SDP-tNos* transformants still exhibited a moderate *pds* phenotype, suggesting that DCL1 and/or DCL3 can partially substitute to DCL2 and DCL4 in the *dcl2dcl4* mutant, as previously shown for IR-PTGS (47).

Uncapped asRNA likely is the aberrant RNA molecule that is converted into dsRNA by RDR6 to initiate S-PTGS

The results presented above indicate that antisense transgenes can efficiently silence homologous sense transgenes or endogenous genes through the action of siRNAs processed by DCL2 or DCL4. However, these results do not prove that the asRNA detected in *L1* and *L2* lines during S-PTGS acts as the asRNA intentionally produced in AS-PTGS. Indeed, although both S-PTGS and AS-PTGS produce siRNAs and are suppressed in the *dcl2dcl4* double mutant, there are more differences than similarities between these

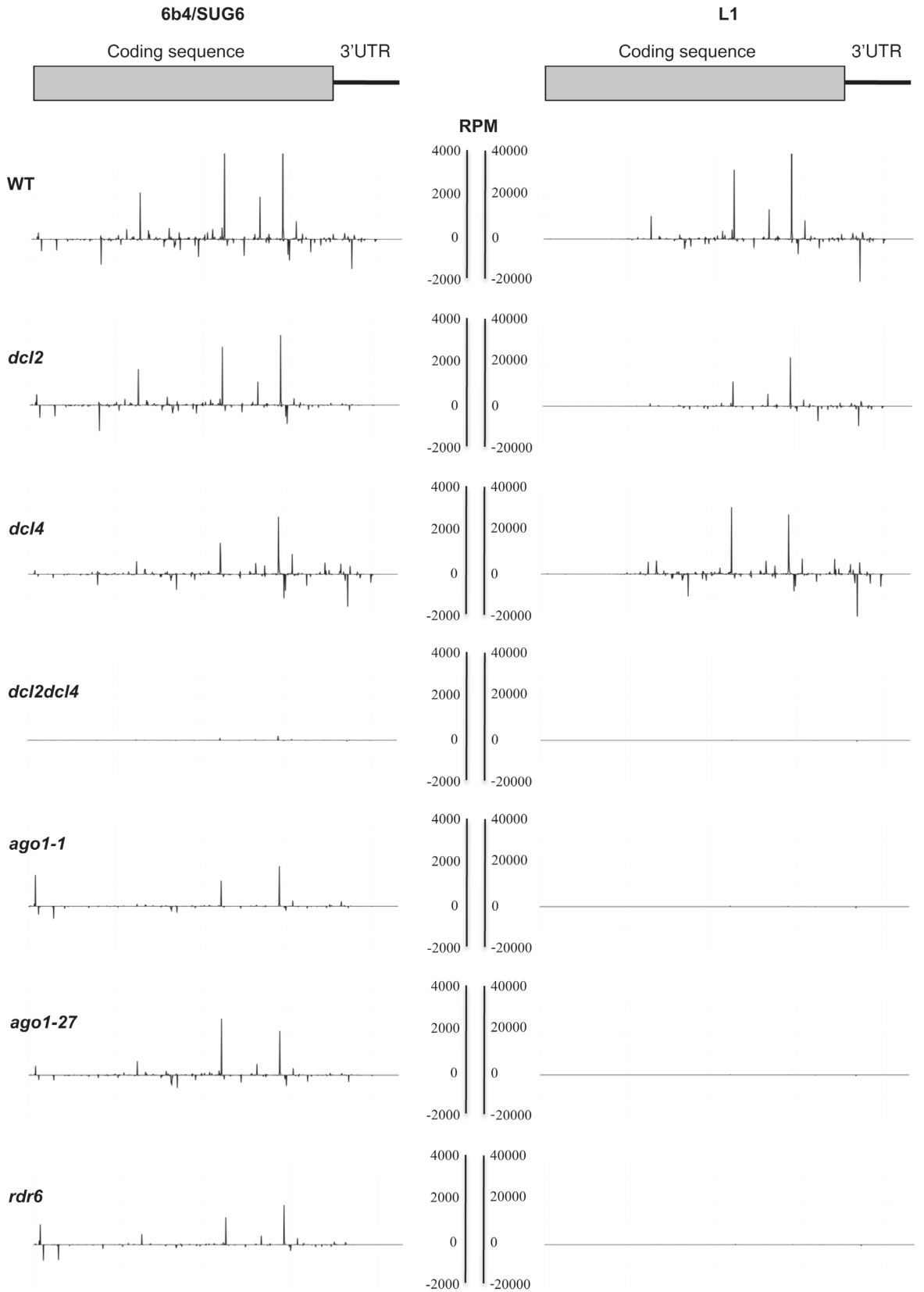

Figure 4. Distribution of *GUS* siRNA reads in WT and mutant backgrounds. Distribution of the normalized aligned reads along the *GUS* coding sequence and the *RbcS* 3′UTR for *6b4* (left column) and *L1* (right column). The background is indicated on the left and the ladder for the graphics in represented in the middle as RPM.

two pathways. First, *dcl2* and *dcl4* single mutations reduce and enhance S-PTGS respectively (23), whereas they have no effect on AS-PTGS (Figure 3). This is the consequence of DCL2 producing secondary siRNAs more efficiently than DCL4, but being obscured by DCL4 in WT plants. Secondly, *ago1*, *rdr6* and *sgs3* null mutations abolish S-PTGS but only reduce the efficiency of AS-PTGS. Thirdly, the hypomorphic *ago1-27* mutation suppresses S-PTGS as efficiently as the null *ago1-1* mutation whereas it does not restore *GUS* mRNA accumulation on AS-PTGS. These differences may be explained by the very little amount of asRNA produced during S-PTGS whereas larger amounts of asRNA are produced during AS-PTGS. Indeed, even in the *rdr6* background where PTGS is impaired, asRNA derived from *L1* can only be detected by RT-PCR (Figure 2), whereas the amount of asRNA produced in the *SUG6* line is detectable by northern blot (Figure 3). Using a DNA probe that detects *GUS* and *SUG* with equal efficiency reveals that the *SUG6* line produces less *SUG* than the *6b4* line produces *GUS* (Supplemental Figure S3). Nevertheless, the amount of *SUG* intentionally produced by the *p35S-SUG-tNos* transgene is clearly more abundant than what is accidentally produced due to the organization of the *p35S-GUS-tRbcS* and *pNos-NPTII-tNos* transgenes on the T-DNA in *L1*.

The higher abundance of *SUG* in the *6b4*/*SUG6* system compared with *L1* likely makes the amplification step more important, if not crucial, for S-PTGS. However, qualitative aspects of the asRNA may also play an important role. We detected *SUG* RNA in lines *L1* and *L2* (Figure 2A and B), which likely results from transcription read-through bypassing the weak *Nos* terminator of the *NPTII* transgene, resulting in a chimeric *NPTII-SUG* RNA. If this RNA were marked as aberrant and transformed into dsRNA by RDR6 in siRNA-bodies, this would cause silencing of both *GUS* and *NPTII*, resulting in the loss of kanamycin resistance. However, lines *L1* and *L2* are highly resistant to kanamycin, ruling out this hypothesis. Moreover, our qRT-PCR experiments succeeded at amplifying internal fragments within the *SUG* sequence but failed at amplifying an *NPTII-SUG* chimeric RNA. Therefore, we performed 5′ RACE experiments on *L1*/*rdr6* plants to characterize the 5′ end of the *SUG* RNAs. We identified five different uncapped 5′ ends, which are all located within the *RbcS* terminator sequence (Supplemental Figure S4). To explain this result, we propose that the 3′ end maturation of the *NPTII-SUG* chimeric RNA, which involves cleavage at the *tNos* cleavage/polyadenylation site followed by the addition of a poly(A) tail, results in the production of a capped and polyadenylated *NPTII* mRNA and of an uncapped *SUG* RNA. Translation of the capped and polyadenylated *NPTII* mRNA promotes high levels of kanamycin resistance, while the uncapped *SUG* RNA cleavage product likely is degraded by 5′-to-3′ exonucleases, resulting in a series of molecules which 5′ ends are located downstream the *tNos* cleavage/polyadenylation site.

The uncapped *SUG* RNA liberated by 3′ end maturation of the *NPTII* transcript makes a very good candidate for the long-searched aberrant RNA that upon transformation into dsRNA by RDR6 in siRNA-bodies initiates S-PTGS specifically against the *GUS* transgene. To test this hypothesis and further determine if the level of uncapped *SUG* RNA directly conditions the entry into S-PTGS, we examined the effect of the *xrn3* and *xrn4* mutations on the level of *SUG* RNA. Indeed, *xrn3* and *xrn4* mutants are compromised in 5′-to-3′ degradation of uncapped RNA in the nucleus and cytoplasm, respectively, and we previously reported that both mutations allow line *6b4* to trigger PTGS (35). Because *xrn3* only has a weak inducing effect on S-PTGS, we analyzed the effect of either *xrn4* alone or in combination with *xrn3,* which results in 100% triggering of *6b4* S-PTGS. Since both *GUS* and *SUG* RNA are degraded when S-PTGS is triggered (see Figure 2A vs B), the level of *SUG* RNA was examined in *6b4*/*xrn4rdr6* and *6b4*/*xrn3xrn4rdr6* plants and compared to that in *6b4*/*rdr6* plants in which *rdr6* prevents S-PTGS. Results presented in Figure 2C indicate that the amount of *SUG* RNA is the highest when both nuclear and cytoplasmic 5′-to-3′ degradation of uncapped RNA are prevented, thus supporting the hypothesis that the more uncapped *SUG* RNAs escape degradation, the more they have a chance to enter siRNA-bodies and trigger S-PTGS.

DISCUSSION

The overwhelming presence of antisense RNA (asRNA) in eukaryote cells raises important questions as to the biological implications of such molecules. In fission yeast, convergent transcripts processed by Dicer leads to the production of siRNA molecules that guide heterochromatin formation at pericentromeric regions (4,5). This mechanism however appears to extend beyond the centromere organization as it is also used to self-regulate the RNAi genes in *S. pombe* (56). In mouse oocytes, Dicer-dependent degradation of RNA pairs is thought to be essential for the proper regulation of female meiosis (9,10). Hence, this conserved RNAi-dependent mechanism can be used to artificially target silencing of genes in fission yeast and mammalian cells (7) as well as in zebrafish (8). It is therefore likely that the biological significance of asRNA molecules has not yet been fully unraveled.

In plants, transgene silencing phenomenon mediated by sense transgenes (S-PTGS) or inverted repeat transgenes

Table 2. Number of plants silenced for *PDS* gene

	WT-like	moderate	severe	total
WT	33 (23)	51 (36)	59 (41)	143 (100)
dcl2dcl4	146 (78)	42 (22)	0 (0)	188 (100)
rdr6	57 (32)	56 (32)	63 (36)	176 (100)

Transformed plants from different indicated backgrounds were scored for having severe, moderate or no silencing phenotype. Percentage of total plants for each category is indicated inside parenthesis.

(IR-PTGS) have been the subject of numerous studies that have shed light on antiviral and endogenous RNAi pathways (reviewed in (18–20)). However, little is known about PTGS triggered by transgenes that intentionally produce asRNA (AS-PTGS). To better understand how asRNA can trigger silencing, we set up two systems, one directed against an endogenous gene and one directed against a stably expressed sense transgene. Firstly, expressing an asRNA complementary to the endogenous *PDS* gene results in efficient *PDS* silencing (Table 2). AS-PTGS against *PDS* is strongly reduced by the combination of *dcl2* and *dcl4* mutations and not by the *rdr6* mutation (Table 2), similar to IR-PTGS directed against *PDS* or *SUL* (34,47). Secondly, crossing a line stably expressing an exogenous *GUS* mRNA with a line stably expressing *SUG*, an asRNA complementary to the *GUS* mRNA, causes the degradation of *GUS* and *SUG* RNAs with concomitant appearance of 21- and 22-nt siRNAs, indicating efficient AS-PTGS. Again, the combination of *dcl2* and *dcl4* mutations neutralizes AS-PTGS (Figure 3), just like it does for IR-PTGS against the *GUS* mRNA (23). However, whereas IR-PTGS against the *GUS* mRNA occurs normally in *sgs3* and *rdr6* mutants (48), AS-PTGS is partially impaired in *sgs3* and *rdr6* mutants (Figures 3 and 4). Likely, during AS-PTGS, DCL2/DCL4-mediated processing of annealed *GUS*/*SUG* RNAs generates primary siRNA molecules that direct the cleavage of unpaired *GUS* and *SUG* RNAs, similar to primary siRNA molecules generated from the hairpin RNA during IR-PTGS. However, the frequency of intermolecular annealing between *GUS* and *SUG* RNAs to form dsRNA during AS-PTGS is certainly lower than the frequency of intramolecular annealing of the inverted-repeat RNA produced during IR-PTGS. Therefore, we can speculate that the amount of primary siRNAs produced during AS-PTGS is insufficient to catalyze the cleavage of all unpaired *GUS* and *SUG* RNAs. This contrasts the amount of primary siRNAs produced during IR-PTGS, which is likely sufficient to catalyze the cleavage of all target RNAs. This makes IR-PTGS independent of RDR6 and SGS3. However, efficient AS-PTGS can only be completed if some cleaved products of *GUS*/*SUG* RNAs are protected by SGS3 and transformed into dsRNA by RDR6, allowing the production of secondary siRNAs by DCL2 and DCL4, which complete the degradation of all unpaired *GUS* and *SUG* RNAs.

The independence of IR-PTGS on SGS3 and RDR6, and the partial dependence of AS-PTGS on these two components contrast the complete impairment of S-PTGS in *sgs3* and *rdr6* mutants. It has been hypothesized that S-PTGS is triggered by an accidentally produced aberrant RNA that is somehow converted to dsRNA. Because the initial aberrant RNA trigger is not abundant, it leads to the production of very few primary siRNAs, thus making S-PTGS totally dependent on SGS3 and RDR6 for the production of secondary siRNAs that execute the cleavage *GUS* target mRNAs. However, little is known about this aberrant RNA trigger. A positive correlation has been observed between S-PTGS efficiency and transgene transcription rate and/or improperly terminated mRNA molecules (32,43,57) but the nature of the aberrant RNA trigger remains unsolved. The hypothesis that antisense transcription could provide the initial trigger for S-PTGS (26) has been much less explored than the other avenues and we sought to correct this.

The S-PTGS system that our group has been using has an intrinsic predisposition to produce asRNA given the converging orientation of the *GUS* and *NPTII* transgenes on the T-DNA (Figure 1A). In line with this, examination of small RNA blots in silenced lines revealed that siRNA molecules are first generated from the 3′ of the *GUS* mRNA and then spread 5′ (Figure 1B). However, silencing is not bidirectional, and the *NPTII* transgene does not undergo PTGS. Rather, lines exhibiting *GUS* S-PTGS are four to five times more resistant to kanamycin than non-silenced lines. Consistently, four to five times more *SUG* asRNA are found in silenced lines compared with non-silenced lines (Figure 2B). We therefore hypothesized that read-through transcription of the *NPTII* transgene reaches to the *GUS* sequence because the *Nos* terminator is a weak terminator in plants (43). Consequently, T-DNAs integrated at genomic location that do not promote high levels of transcription, would produce insufficient amount of *NPTII-SUG* to trigger *GUS* S-PTGS and only confer low levels of resistance to kanamycin. Inversely, T-DNAs integrated at genomic location that promote high levels of transcription would produce high amounts of *NPTII-SUG* thereby triggering *GUS* S-PTGS and conferring a high level of resistance to kanamycin. The opposite effects observed on *GUS* and *NPTII* rules out the possibility that the chimeric *NPTII-SUG* RNA is the aberrant RNA that enters into siRNA-bodies for conversion into dsRNA by RDR6 because this would result in both *GUS* and *NPTII* silencing. Moreover, our incapacity to detect the chimeric *NPTII-SUG* RNA suggests that it is very labile. Consistently, RACE experiments revealed *SUG* 5′ ends located downstream of the *NPTII* sequence, suggesting that the read-through *NPTII-SUG* transcript is cleaved at the *tNos* or *tRbcS* polyadenylation sites, resulting in the production of a capped and polyadenylated *NPTII* mRNA that confers kanamycin resistance and an uncapped *SUG* RNA. We propose that this uncapped *SUG* RNA is the long-searched aberrant RNA trigger that enters into siRNA-bodies where it is protected by SGS3 and transformed into dsRNA by RDR6, thus specifically initiating S-PTGS against the *GUS* transgene.

Despite the attractiveness of the above hypothesis, it remains possible that the read-through *NPTII-SUG* transcript is not detected because, upon annealing with the *GUS* mRNA, it forms dsRNA that is immediately processed into *GUS*/*SUG* siRNAs by DCL2 and DCL4, thus specifically initiating PTGS against the *GUS* transgene, and letting the *NPTII* transgene unsilenced. If it were the case, S-PTGS should in fact correspond to a form of AS-PTGS that strongly requires SGS3 and RDR6 because the amount of primary siRNAs is very low. However, S-PTGS is enhanced by *xrn3* and *xrn4* mutations, which impair 5′-to-3′ degradation of uncapped RNA in the nucleus and cytoplasm, respectively, indicating that the S-PTGS trigger likely is uncapped. Since the read-through *NPTII-SUG* transcript is capped, it should not be susceptible to degradation by XRN3 and XRN4. Therefore, S-PTGS efficiency should not be affected by *xrn3* and *xrn4* if the read-through *NPTII-SUG* transcript is the trigger. In contrast, the *SUG* RNA resulting from the maturation of the read-through *NPTII-*

SUG transcript is uncapped, and thus susceptible to degradation by XRN3 and XRN4, supporting the hypothesis that it is the actual S-PTGS trigger.

Remarkably, AS-PTGS and IR-PTGS are not abolished in *ago1* mutants. Indeed, neither the null *ago1-1* nor the hypomorphic *ago1-27* mutations suppress AS-PTGS (Figure 3) or IR-PTGS triggered by 35S-driven transgenes (48). Moreover, the hypomorphic *ago1-27* mutation only slightly reduces IR-PTGS specifically triggered around the phloem (Supplemental Figure S2), suggesting that another AGO is at play during AS-PTGS and IR-PTGS, at least when AGO1 is impaired. AGO2, which has been implicated in antiviral PTGS together with AGO1 (58), is a good candidate but its role in AS-PTGS and IR-PTGS remains to be tested. The impairment of S-PTGS in both the null *ago1-1* and the hypomorphic *ago1-27* mutants contrasts the mild or null effect of these mutations on AS-PTGS and IR-PTGS, and indicates an absolute dependence of S-PTGS on AGO1. Given that S-PTGS is also absolutely dependent on the production of secondary siRNAs, it follows that AGO1 is essential for the production or action of secondary siRNAs. To reconcile these results, we propose that not only the *ago1-27* mutation reduces AGO1 cleavage activity (59), but it also impairs AGO1 capacity to engage the production of secondary siRNAs after cleavage. The Arabidopsis *ago1-27* mutation affects the Alanine at position 994. In Arabidopsis, this Alanine is conserved in AGO5 and AGO10, but not in other AGOs. Given that AGO2, which carries a Glycine at the position equivalent to 994 in AGO1, is incapable to initiate the production of secondary siRNAs (60), it is possible that *ago1-27* cannot produce secondary siRNAs because it is mutated at position 994. Therefore, AS-PTGS and IR-PTGS still function, at least partially, in *ago1-27* because primary siRNAs are abundant enough to guide the cleavage of target mRNA through both the partially functioning *ago1-27* protein and through another AGO, which could be AGO2 (58,60). In contrast, S-PTGS does not function in *ago1-27* because primary siRNAs are scarce and secondary siRNAs are absent. Likely, the low abundance of SUG asRNA arising from read-through transcription of the NPTII transgene results in low amounts of primary siRNAs, which are incapable of significantly impacting the accumulation of the GUS target mRNA. Secondary siRNAs are therefore essential, but neither the mutant ago1-27 protein nor the other AGO at play is capable of producing them, resulting in S-PTGS impairment in *ago1-27*. Testing the effect of *ago2* and mutations in other AGO proteins will help clarifying how many AGOs are at play during S-PTGS, AS-PTGS and IR-PTGS.

RNA gel blots and small RNA sequencing revealed that during S-PTGS siRNAs are not produced from the 5′ end of the GUS mRNA ((42) and Figure 4), suggesting that the AGO1/RDR6 module is incapable of completing the spreading of siRNAs from the 3′ to the 5′ end. In contrast, siRNAs produced during AS-PTGS span the entire GUS/*SUG* sequence (Figure 4). Given that siRNAs at the 5′ end of *GUS* are not eliminated in *ago1–1* and *rdr6* plants whereas siRNAs from the rest of the gene are reduced in these mutants (Figure 4, Table 1 and Supplemental Table S1), it is tempting to speculate that siRNAs coming from the 5′ end of *GUS* during AS-PTGS are primary siRNAs deriving from DCL2/DCL4-mediated processing of annealed GUS/*SUG* dsRNA, and not secondary siRNAs produced by AGO1/RDR6. Of course, it is impossible to determine if secondary siRNAs come from *GUS* or *SUG*. However, knowing that the AGO1/RDR6 module is incapable to complete the spreading of secondary siRNAs from the 3′ to the 5′ end of GUS during S-PTGS, the presence of primary siRNAs at the 5′ end of *GUS* (3′ end of *SUG*) at similar level in *ago1-1* and *rdr6* and in WT plants during AS-PTGS strongly suggests that the AGO1/RDR6 module is incapable of initiating the production of secondary siRNAs from the 3′ of *SUG* during AS-PTGS. In contrast, the AGO1/RDR6 module is capable of producing secondary siRNAs from the 3′ of GUS during both S-PTGS and AS-PTGS because AGO1/RDR6-dependent secondary siRNAs coming from the 3′UTR of the GUS transgene are found in both *L1* and *6b4*/*SUG6* plants (Figure 4). What could explain the incapacity of the AGO1/RDR6 module to produce secondary siRNAs from the 3′ end of *SUG* during AS-PTGS? Contrary to *GUS* mRNA, *SUG* RNA does not have a proper reading frame and might therefore be treated differently. This may be because AGO1 is interacting with polysomes (49). Therefore, AGO1 bound to primary siRNAs originating from the *GUS*/*SUG* dsRNA during AS-PTGS would be recruited primarily to sense/coding *GUS* mRNAs but only marginally to antisense/non-coding *SUG* RNAs, leading to RDR6-dependent production of secondary siRNAs from *GUS* but not *SUG*. Eventually, AGO1 bound to secondary siRNAs would cleave both *GUS* and *SUG* RNAs in WT plants because of the abundance and dual polarity of secondary siRNAs. Supporting this hypothesis, the hypomorphic *ago1-27* mutation prevents *SUG* degradation but has little effect on *GUS* degradation during AS-PTGS (Figure 3), likely because the mutant ago1–27 protein still exhibit enough activity to cleave *GUS* but cannot produce secondary siRNAs that are required to complete the degradation of *SUG*.

Could complementary RNAs deriving from endogenous genes trigger S-PTGS or AS-PTGS? Natural antisense transcripts (NATs) can derive from opposite strands of the same locus (*cis*-NATs) or from separate loci (*trans*-NATs), and they can involve protein-coding genes as well as non-protein coding genes. Mining Arabidopsis RNAseq datasets revealed 4080 *cis*-NATs and 2491 *trans*-NATs, and of these 6571 loci, 5385 produce siRNAs (11). The number of NATs is probably underestimated because of limited knowledge on the possibility that genes transcribe 3′ extension under certain circumstances. Indeed, genome-wide analysis of Arabidopsis *fry1* mutants, which exhibit decreased XRN2, XRN3 and XRN4 activities, revealed read-through transcription at ∼2000 endogenous genes (61). Moreover, the number of NATs that are capable of producing siRNAs is probably underestimated because one of the two genes of the pair is generally expressed only under certain specific conditions, at least in the few cases analyzed (62). Indeed, despite the high number of NATs, very few examples of actual regulation involving nat-siRNAs have been documented (28,29). If NATs are capped and polyadenylated, nat-siRNA mediated regulation should resemble AS-PTGS. However, the processing of nat-siRNAs usually implicates DCL1, DCL2 or DCL3 but not DCL4 (11,13), suggesting

a different pathway than AS-PTGS. Moreover, our results suggest that if a NAT pair involves at least one read-through transcript, maturation at the cleavage/polyadenylation site liberates an uncapped 3′ extension RNA that has the capacity to trigger S-PTGS on complementary mRNA. Also, supporting a similarity with S-PTGS, siRNAs originating from outside of the overlapping regions are found in ∼80% of the *cis*-NAT pairs (13), suggesting that siRNAs originating from the overlapping region guide primary cleavage, and that at least one cleavage product is transformed into dsRNA by a cellular RDR to produce secondary siRNAs from outside of the overlapping region (13). Several models of NAT regulation have been evoked (62), however a case-by-case analysis is likely required to decipher the regulatory mechanism at each NAT. We anticipate that the transgene S-PTGS and AS-PTGS models developed here will help understanding the regulation of gene expression by endogenous asRNA.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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