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The Conserved RNA Trafficking Proteins HPR1 and TEX1 Are Involved in the Production of Endogenous and Exogenous Small Interfering RNA in *Arabidopsis* [□]_W

Vincent Jauvion, Taline Elmayer, and Hervé Vaucheret¹

Institut Jean-Pierre Bourgin, Institut National de la Recherche Agronomique, 78026 Versailles Cedex, France

We previously identified *Arabidopsis thaliana* mutants defective in sense transgene posttranscriptional gene silencing (S-PTGS) that defined six loci; here, we describe mutants that define nine additional loci, including *HYPER RECOMBINATION1* (*HPR1*), *SILENCING DEFECTIVE3* (*SDE3*), and *SDE5*. Our analyses extend previous findings by showing that the requirement for the putative RNA helicase *SDE3* is inversely proportional to the strength of the PTGS inducer and that the putative RNA trafficking protein *SDE5* is an essential component of the trans-acting small interfering RNA (tasiRNA) pathway and is required for S-PTGS but not inverted repeat transgene-mediated PTGS (IR-PTGS). Our screen also identified *HPR1* as a PTGS actor. We show that *hpr1* mutations negatively impact S-PTGS, IR-PTGS, and tasiRNA pathways, resulting in increased accumulation of siRNA precursors and decreased accumulation of mature siRNA. In animals, *HPR1/THO1* is a member of the conserved RNA trafficking THO/TREX complex, which also includes *TEX1/THO3*. We show that *tex1* mutants, like *hpr1* mutants, impact *TAS* precursor and mature tasiRNA levels, suggesting that a THO/TREX complex exists in plants and that this complex is important for the integrity of the tasiRNA pathway. We propose that both *HPR1* and *TEX1* participate in the trafficking of siRNA precursors to the ARGONAUTE catalytic center.

INTRODUCTION

RNA silencing regulates gene expression through the action of small RNAs and serves as a eukaryotic defense response that thwarts invading RNA deriving from transposons, viruses, and transgenes (Mallory and Vaucheret, 2006; Ding and Voinnet, 2007; Kloc and Martienssen, 2008; Voinnet, 2009). In plants, 24-nucleotide small interfering RNAs (siRNAs) mediate transcriptional gene silencing through DNA methylation and chromatin modifications while 21-nucleotide siRNA and microRNA (miRNA) mediate posttranscriptional gene silencing (PTGS) through RNA cleavage and translational inhibition (Mallory and Vaucheret, 2006; Ding and Voinnet, 2007; Kloc and Martienssen, 2008; Voinnet, 2009). Transgene-based forward genetic screens have identified mutants defective for S-PTGS (sense transgene-mediated PTGS) and IR-PTGS (inverted repeat transgene-mediated PTGS) and have served to decipher endogenous PTGS pathways.

During S-PTGS, transgenes produce double-stranded RNA (dsRNA) that is processed into siRNA duplexes by the type III RNase DICER-LIKE2 (*DCL2*) (Mlotshwa et al., 2008). siRNAs are methylated at their 3' end by the methyltransferase HUA EN-

CHANCER1 (*HEN1*) (Boutet et al., 2003; Li et al., 2005; Yu et al., 2005) and then bound by ARGONAUTE1 (*AGO1*), which cleaves complementary target RNA (Morel et al., 2002; Baumberger and Baulcombe, 2005). *AGO1*-mediated cleavage generates RNA fragments that escape degradation by 5' → 3' (*XRN*) and 3' → 5' (*EXO*) exoribonucleases (Gazzani et al., 2004; Souret et al., 2004; Gy et al., 2007), due to the protecting activity of SUPPRESSOR OF GENE SILENCING3 (*SGS3*), and are transformed into dsRNA by RNA-DEPENDENT RNA POLYMERASE6 (*RDR6*) (Dalmay et al., 2000; Mourrain et al., 2000; Yoshikawa et al., 2005; Elmayer et al., 2009). These dsRNAs are processed into siRNA duplexes by *DCL4* to produce secondary siRNA (Dunoyer et al., 2005; Blevins et al., 2006; Bouche et al., 2006; Deleris et al., 2006; Fusaro et al., 2006) that are bound by *AGO1* and guide cleavage of target RNA, establishing an amplification loop that reinforces silencing and contributes to the systemic propagation of S-PTGS from cell to cell (short-distance signaling) and through the vasculature (long-distance signaling) (Palauqui et al., 1997; Voinnet et al., 1998; Brosnan et al., 2007; Dunoyer et al., 2010a; Molnar et al., 2010).

During IR-PTGS, the transcription of inverted repeat transgenes produces self-complementary transcripts that are processed into siRNA duplexes by *DCL4* without the need of an RNA-dependent RNA polymerase (Smith et al., 2000). S-PTGS and IR-PTGS pathways are *DCL4*, *HEN1*, and *AGO1* dependent (Dunoyer et al., 2005, 2007). Forward genetic screens based on an IR trigger expressed specifically in the phloem implicated components of the endogenous 24-nucleotide siRNA pathway (*CLASSY1* [*CLSY1*], *NUCLEAR RNA POLYMERASE D1a* (*NRPD1a*), and *RDR2*) but not components of the endogenous 21-nucleotide siRNA pathway in short-distance signaling of

¹ Address correspondence to herve.vaucheret@versailles.inra.fr.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantcell.org) is: Hervé Vaucheret (herve.vaucheret@versailles.inra.fr).

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IR-PTGS (Dunoyer et al., 2005, 2007; Smith et al., 2007). By contrast, grafting experiments implicated components of both the 24-nucleotide siRNA pathway (NRPD1a and RDR2) and the endogenous 21-nucleotide siRNA pathway (RDR6) in long-distance signaling of IR-PTGS triggered by a constitutive promoter (Brosnan et al., 2007), suggesting distinct mechanisms for short-distance and long-distance signaling (Dunoyer and Voinnet, 2008).

Forward genetic screens based on the line *L1*, which carries a posttranscriptionally silent *p35S:β-glucuronidase (GUS)* sense transgene, and the line *2a3*, which carries a *p35S:NIA2* sense transgene that triggers cosuppression of the *NITRATE REDUCTASE1 (NIA1)* and *NIA2* endogenous genes, identified a series of S-PTGS-deficient mutants called *sgs*. Previous analyses defined *sgs1* (one allele), *sgs2/sde1/rdr6* (26 alleles), *sgs3/sde2* (10 alleles), *sgs4/ago1* (14 alleles), *sgs5/hen1* (one allele), and *sgs6/met1* (two alleles), but 27 mutants remained unclassified (Elmayan et al., 1998; Fagard et al., 2000; Morel et al., 2000, 2002; Mourrain et al., 2000; Boutet et al., 2003; Adenot et al., 2006). Here, we define nine additional *sgs* loci, and we describe *sgs7/sde5*, *sgs9/hpr1*, and *sgs13/sde3* mutants. SDE3 encodes a putative RNA helicase that was previously identified in an amplicon-based genetic screen together with *rdr6/sde1*, *sgs3/sde2*, *nrdp1a/sde4*, and *sde5* (Dalmay et al., 2000; Herr et al., 2005; Hernandez-Pinzon et al., 2007), but SDE3 still awaits an endogenous function. Both *SDE5* and *HPR1* encode putative RNA trafficking proteins. Similar to *rdr6* and *sgs3*, *sde5* mutants impair S-PTGS and the endogenous trans-acting siRNA (tasiRNA) pathway but not IR-PTGS, suggesting that SDE5 acts specifically in the first two pathways. By contrast, *hpr1* mutants compromise S-PTGS, IR-PTGS, and the endogenous tasiRNA pathway, indicating that HPR1 acts at a step common to these three pathways. HPR1/THO1 is homologous to a member of the conserved THO/TREX complex (Reed and Cheng, 2005), and we show that a mutation in *TEX1/THO3*, which is homologous to another member of this complex, also impaired the production of tasiRNA. We propose that a THO/TREX complex also exists in plants and that this complex participates in the trafficking of siRNA precursors.

RESULTS

Forward Genetic Screens Based on the *L1* and *2a3* Lines Identify at Least 15 PTGS Loci

The 60 mutants recovered from the *L1* screen were classified in three groups based on the degree to which they affected PTGS. The first group consisted of 44 mutants producing 100% PTGS-deficient progeny. This group included one *sgs1* allele, 24 *sgs2/rdr6* alleles, seven *sgs3* alleles, 11 *sgs4/ago1* alleles, and one *sgs5/hen1* allele (Elmayan et al., 1998, 2009; Fagard et al., 2000; Mourrain et al., 2000; Morel et al., 2002; Boutet et al., 2003; Adenot et al., 2006). The second group consisted of 11 mutants producing a consistent number of both PTGS-deficient and PTGS-efficient progeny at each generation, indicating that the mutations partially impaired S-PTGS. This group included three *sgs4/ago1* hypomorphic alleles, two *sgs6/met1* alleles, and six unclassified mutants (Morel et al., 2002; Boutet et al., 2003;

Mallory et al., 2009). The third group contained five mutants that showed a delay in the onset of S-PTGS but eventually triggered S-PTGS in 100% of the progeny. The 21 mutants recovered from the *2a3* screen also affected PTGS to varying degrees and thus were classified according to the same criteria. The first group consisted of six mutants, including two *sgs2/rdr6* alleles and three *sgs3* alleles (Adenot et al., 2006; Elmayan et al., 2009). The second and third groups consisted of 12 and three unclassified mutants, respectively.

Complementation analyses were performed on six unclassified mutants derived from the *L1* screen and six unclassified mutants derived from the *2a3* screen. The six *L1*-derived mutants defined six novel complementation groups (*sgs7* to *sgs12*). One *2a3*-derived mutant was a hypomorphic *rdr6* allele (*rdr6-8*, which exhibited a T→A nucleotide change that resulted in a Y→N amino acid change at protein position 228), while the five other *2a3*-derived mutants defined three complementation groups. To determine the overlap between these two screens, the *L1* locus was introduced into *2a3*-derived mutants, and the *2a3* locus was introduced into *L1*-derived mutants. All *L1*-derived mutants (*sgs1* to *sgs12*) protected *2a3* against S-PTGS generally better than they protected *L1* against S-PTGS. By contrast, the three novel *2a3*-derived mutants delayed *L1* S-PTGS, although their effect on *2a3* was more pronounced. Complementation tests revealed that these three mutants defined three unique loci (hereafter, referred to as *sgs13* to *sgs15*). Altogether, these results show that at least 15 loci control S-PTGS in *Arabidopsis thaliana*.

SGS13/SDE3 Is Required for S-PTGS Triggered by Weak Silencers Only

The *sgs13* mutation was mapped to an 888-bp deletion that caused a 202-amino acid truncation at the C terminus of the *SDE3/At1g05460* gene (Figure 1A). *SDE3* encodes a putative RNA helicase required for S-PTGS triggered by the *GxA* amplicon (Dalmay et al., 2001). The *sgs13* mutation, hereafter referred to as *sde3-6*, impaired *2a3* S-PTGS and slightly delayed *L1* S-PTGS. At 11 d after germination (DAG), *NIA* siRNAs were undetectable and *NIA* mRNA accumulated in *2a3/sde3-6* plants to a level comparable to *2a3/rdr6* plants (Figure 1B). At 40 DAG, none of the plants ($n = 100$) showed signs of *NIA* cosuppression, indicating that *sde3* prevents *2a3* S-PTGS. By contrast, at 11 DAG, GUS activity in *L1/sde3-6* plants was higher than *L1* controls, although not as high as in *L1/rdr6* plants, and GUS siRNAs were detectable, although the level was consistently lower than in *L1* controls (Figure 1B). Confirming that the effect of *sde3-6* on *L1* was only partial, analyses of GUS activity, GUS mRNA, and GUS siRNA accumulation at 40 DAG indicated that *L1/sde3-6* was as silenced as control *L1* plants (Figure 1B). To decipher whether the limited effect of *sde3-6* on *L1* S-PTGS was due to the residual activity of the remaining first 800 amino acids of the protein or if SDE3 was simply a nonessential component of S-PTGS, we crossed *L1* to *sde3-1*, which has a 14-bp deletion in exon 4, causing a frameshift that results in the truncation of the protein at amino acid 491 (Dalmay et al., 2001; Figure 1A). Because the *sde3-1* allele originally was recovered in the C24 ecotype, we backcrossed it twice to *L1* (in the Columbia-0 [Col-0]

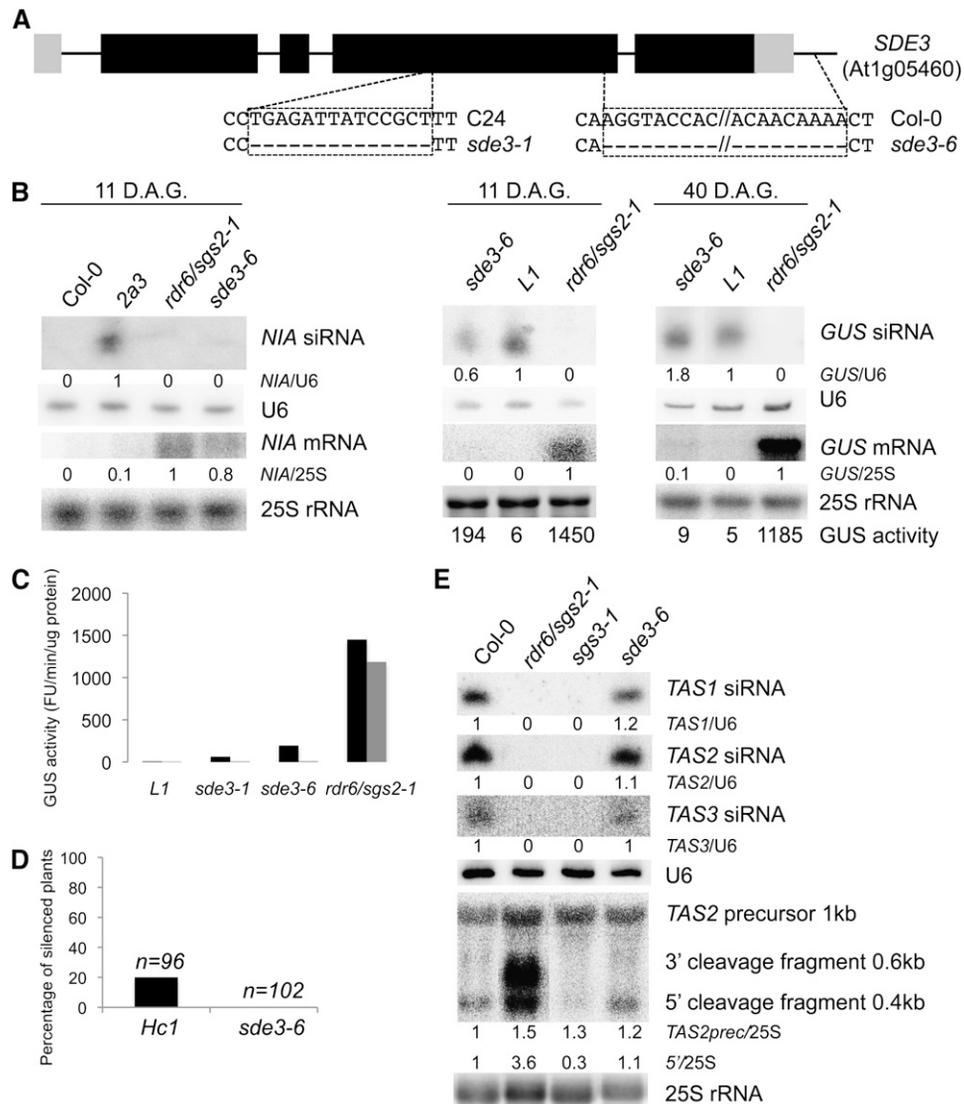


Figure 1. Analysis of S-PTGS in *sde3* Mutants.

(A) Schematic representation of the *SDE3* gene with the position and sequence changes introduced by the *sde3-1* and *sde3-6* mutations indicated. Black boxes represent exons, gray boxes untranslated regions, and thin lines introns and intergenic regions. Dash lines represent the sequences deleted in the *sde3* mutants.

(B) Low molecular weight and high molecular weight (LMW and HMW, respectively) RNA gel blots of aerial parts of 11-d-old seedlings and mature rosette leaves (40 DAG) of the indicated plant lines were probed with an RNA *GUS* or DNA *NIA2* probe. 25S rRNA and *U6* snRNA hybridizations served as loading controls for HMW and LMW blots, respectively. Normalized values are indicated below each lane. Mean *GUS* activity is indicated in fluorescence units per min per μg of total protein (FU/min/ μg protein).

(C) *GUS* activity (in FU/min/ μg protein) of the indicated genotypes at 11 (black) and 40 DAG (gray).

(D) Percentage of *Hc1* silenced plants at 40 DAG in the indicated genotypes. *n* = number of tested plants.

(E) LMW and HMW RNA gel blots of mature rosette leaves of the indicated genotypes were probed with DNA oligonucleotides complementary to *TAS1* [si-480(+)], *TAS2* (si-F), *TAS3* (tasi-ARF), and DNA complementary to *TAS2* precursor, respectively. The expected migration positions of primary *TAS* RNA precursors (pri) and the 5' and 3' cleavage products generated after miR173-guided cleavage are indicated. 25S rRNA and *U6* snRNA hybridizations served as loading controls for HMW and LMW blots, respectively. Normalized values with the Col control set to 1 are indicated.

ecotype) before comparing its effect on *L1* to *sde3-6*. The *sde3-1* mutation, like the *sde3-6* mutation, had only a minor effect on *L1* PTGS at 11 DAG and was fully silenced at 40 DAG (Figure 1C), confirming that *sde3* mutations delay the onset of *L1* S-PTGS but do not prevent its establishment.

sde3 mutations prevented S-PTGS triggered by two-component systems (*GxA* and *2a3*) but only slightly delayed S-PTGS triggered by a single-component system (*L1*). To confirm this effect, we tested the effect of *sde3* on the single-component system *Hc1*. The *Hc1* line carries the same *p35S:GUS* transgene as *L1*

but only triggers S-PTGS in 20% of the plants, whereas *L1* triggers S-PTGS in 100% of the plants (Elmayan et al., 1998; Gy et al., 2007). *sde3-6* abolished *Hc1* S-PTGS (Figure 1D), indicating that SDE3 is not specific to two-component systems. Rather, it suggests that SDE3 is required for weak S-PTGS reporters such as *Hc1*, *2a3*, and *GxA* but is dispensable for the strongly silenced *L1* reporter.

The fact that *sde3* has an effect on *L1* (although weaker than that on *Hc1*, *2a3*, and *GxA*) prompted us to further investigate the effect of *sde3* mutations on the endogenous tasiRNA pathway, which requires many of the same components as *L1* PTGS. Consistent with previous analyses of *sde3-4* and *sde3-5* (Vazquez et al., 2004; Dunoyer et al., 2010b), mature tasiRNA accumulated at wild-type levels in *sde3-6* (Figure 1E). *TAS* precursors and cleavage products also accumulated at wild-type levels in *sde3-6* (Figure 1E), confirming that SDE3 is not required for the tasiRNA pathway.

SGS7/SDE5 Is Required for S-PTGS Triggered by Strong Silencers

The *sgs7* mutation was mapped to a G→A nucleotide change at the acceptor site of the second intron of *SDE5/At3g15390* (Figure 2A). *SDE5* encodes a putative RNA export protein required for *GxA* S-PTGS (Hernandez-Pinzon et al., 2007). The *sgs7* mutation, hereafter referred to as *sde5-4*, resulted in decreased accumulation of *SDE5* mRNA (Figure 2B). *sde5-4* impaired both *L1* S-PTGS (Figure 2C) and *2a3* S-PTGS (Figure 2D). At 11 DAG, *GUS* siRNAs were undetectable and *GUS* mRNA accumulated at high levels in *sde5-4*, similar to a null *rdm6/sgs2-1* mutant (Figure 2C). However, the effect of *sde5-4* on *L1* silencing was not as strong as that of null *rdm6/sgs2-1* or *sgs3* mutations, which completely impair *L1* silencing because at 40 DAG, 20% ($n = 80$) of *L1/sde5-4* exhibited low *GUS* activity and accumulated *GUS* siRNA similar to silenced *L1* controls (Figure 2E). These results suggest either that SDE5 is not as essential as RDR6 and SGS3 for *L1* S-PTGS or that the *sde5-4* mutation could be a partial loss of function. To test this hypothesis, *L1* was introduced in the T-DNA insertion mutant *sde5-3* (Mallory and Vaucheret, 2009; Figure 2A), which likely is a null allele (Figure 2B). Both *sde5-3* and *sde5-4* had similar effects on *L1* S-PTGS at 11 DAG (i.e., high *GUS* activity, high *GUS* mRNA level, and undetectable *GUS* siRNA; Figure 2C), but, in contrast with the *sde5-4* mutant, *sde5-3* protected *L1* against S-PTGS with 100% efficiency, indicating that *sde5-4* is a partial loss of function and that SDE5 is required for S-PTGS.

SDE5 Is a Core Component of the tasiRNA Pathway

SDE5 was previously implicated as having a limited but nonessential role in the tasiRNA pathway. Indeed, similar to our *sde5-4* allele, the previously characterized *sde5-2* allele (Hernandez-Pinzon et al., 2007) did not exhibit the accelerated phase change zippy phenotype typical of *dcl4-2*, *rdm6/sgs2-1*, and *sgs3-1* null mutants impaired in the tasiRNA pathway (Peragine et al., 2004; Yoshikawa et al., 2005; Figure 3A). By contrast, *sde5-3* exhibited a zippy phenotype (Figure 3A), suggesting that like *sde5-4*, *sde5-2* could be a partial loss-of-function allele, prompting us to

reexamine the contribution of SDE5 to the tasiRNA pathway. Mature tasiRNAs were undetectable in *sde5-3*, similar to *rdm6/sgs2-1* and *sgs3-1* null alleles (Figure 3B), whereas they accumulated at 30% of wild-type levels in *sde5-2* (Hernandez-Pinzon et al., 2007; Dunoyer et al., 2010b) confirming that *sde5-2* is a partial loss-of-function mutant. RNA gel blot analysis of *TAS* precursors and cleavage products revealed an overaccumulation of *TAS* cleavage products in the *sde5-3* null allele (Figure 3B). This increase in *TAS* cleavage products was not as strong as that observed in the null *rdm6/sgs2-1*, but it was stronger than in the partial loss-of-function *rdm6-8* (Figures 2C and 3B) and the neomorphic *sgs3-3* allele (Figure 3B), which overprotects *TAS* cleavage products from degradation (Elmayan et al., 2009). Although the exact role of SDE5 in the tasiRNA pathway remains elusive, these data indicate that the contribution of SDE5 to the production of mature tasiRNA is more central than originally reported.

SGS9 Encodes the Putative Ortholog of HPR1, a Component of the Conserved THO/TREX Complex

The *sgs9* mutation was mapped to a G→A nucleotide change that introduces a stop codon in exon14 of *At5g09860* (Figure 4A). *At5g09860* encodes a protein that shares homology with HPR1/THO1, one of the eight components of the RNA trafficking THO/TREX complex conserved among fungi, invertebrates, and mammals (Reed and Cheng, 2005). The *sgs9* mutation, hereafter referred to as *hpr1-1*, suppresses *L1* S-PTGS with 80% efficiency. Analysis of a bulk of plants at 11 DAG revealed that *GUS* siRNAs were below detectable levels and that *GUS* mRNA accumulated to high levels (Figure 4B). Consistent with the *GUS* mRNA levels, *GUS* activity in *hpr1-1* was lower than in the null *sgs3-1*. At 40 DAG, 80% of the plants exhibited high *GUS* activity, while 20% exhibited low *GUS* activity similar to silenced *L1* controls. This high *GUS* activity correlated with increased *GUS* mRNA levels and undetectable *GUS* siRNA, whereas low *GUS* activity correlated with low *GUS* mRNA levels and detectable *GUS* siRNA (Figure 4B). Confirmation that *hpr1-1* is the cause of *L1* reactivation was shown by the analysis of a second allele, *hpr1-2*, which carries a T-DNA inserted in the eighth intron of *HPR1* (Figure 4A) that compromises the production of a functional *HPR1* mRNA (Figure 4C). Homozygous *hpr1-1*, *hpr1-2*, and F1 hybrids between *hpr1-1* and *hpr1-2* exhibited identical developmental defects (Figures 4D and 4E). Moreover, F1 hybrids between *L1/hpr1-1* and *hpr1-2* showed impaired *L1* S-PTGS (Figure 4F), confirming that *hpr1* mutations compromise S-PTGS.

The Putative Orthologs of HPR1 and TEX1, Two Members of the Conserved THO/TREX Complex, Contribute to the tasiRNA Pathway

All S-PTGS mutants identified so far in the *L1* screen also impair the endogenous tasiRNA pathway. Indeed, *ago1*, *hen1*, *rdm6*, *sde5*, and *sgs3* lack tasiRNA (Peragine et al., 2004; Vazquez et al., 2004; Adenot et al., 2006; Figure 3B). *TAS1*, *TAS2*, and *TAS3* tasiRNA levels were reduced in *hpr1-1* mutant seedlings (Figure 5A). Similarly, the *hpr1-2* allele exhibited a reduction in

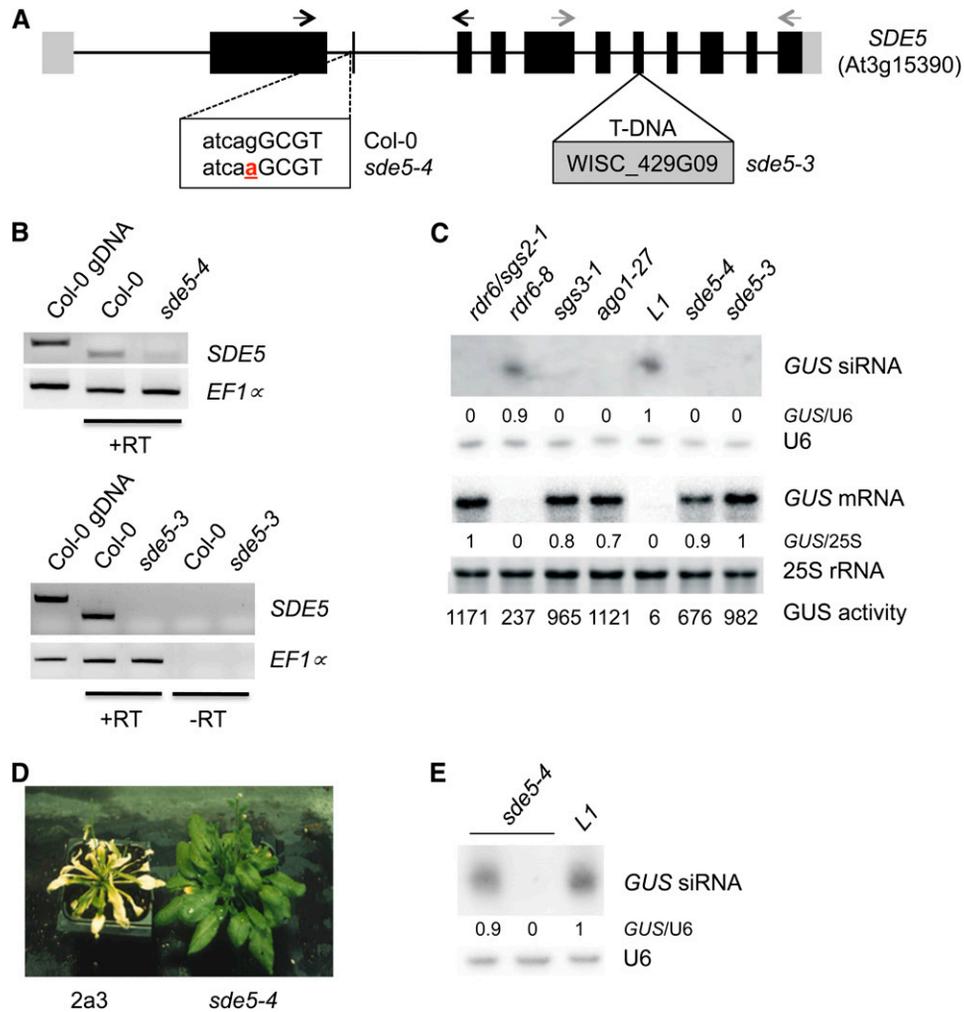


Figure 2. Analysis of S-PTGS in *sde5*, *rdr6*, and *sgs3* Mutants.

(A) Schematic representation of the *SDE5* gene with the position and sequence changes introduced by the *sde5-3* and *sde5-4* mutations indicated. Black boxes represent exons (sequence in capital letters), gray boxes untranslated regions, and thin lines introns (sequence in lowercase). Black and gray arrows represent the position of the primers used for RT-PCR of *sde5-4* and *sde5-3*, respectively.

(B) RT-PCR analyses of the *sde5-4* and *sde5-3* mutants. *EF1α* served as a standard for RT-PCR.

(C) LMW and HMW RNA gel blot analyses of aerial parts of 11-d-old seedlings of the indicated mutant plants were probed with DNA complementary to *GUS* mRNA. 25S rRNA and *U6* snRNA hybridizations served as loading controls for HMW and LMW blots, respectively. Normalized values are indicated. *GUS* activity is reported in FU/min/μg protein.

(D) Cosuppressed 2a3 line (left) and cosuppression-resistant 2a3/*sde5-4* mutant (right). *NIA* cosuppression is visualized by chlorosis.

(E) LMW RNA gel blot of mature rosette leaves (40 DAG) of silenced (left) and nonsilenced (right) *sde5-4* mutants and *L1* control plants were probed with an RNA *GUS* probe. *U6* snRNA hybridizations served as loading controls.

[See online article for color version of this figure.]

TAS2 tasiRNA accumulation (Figure 5B), implicating HPR1 in the tasiRNA pathway. Because HPR1/THO1 is part of the conserved, multicomponent THO/TREX complex in fungi, invertebrates, and mammals, we searched for additional mutants impaired in the putative orthologs of other THO/TREX components to determine if they also are necessary for the integrity of the tasiRNA pathway. We could not find homozygous plants in the progeny of *tho2/THO2* heterozygous plants, suggesting that *THO2* is an essential gene. By contrast, homozygous mutants

impaired in the putative ortholog of *TEX1/THO3* were viable and exhibited developmental defects similar to *hpr1* (Figure 5E), as well as a reduction in tasiRNA accumulation (Figure 5C). These data indicate that the putative orthologs of two THO/TREX components are needed for proper tasiRNA accumulation and suggest that a similar THO/TREX complex exists in plants. To determine at which step the production of tasiRNA is compromised in *hpr1* and *tex1* mutants, we analyzed the accumulation of *TAS2* precursors and cleavage products. Both *hpr1* and *tex1*

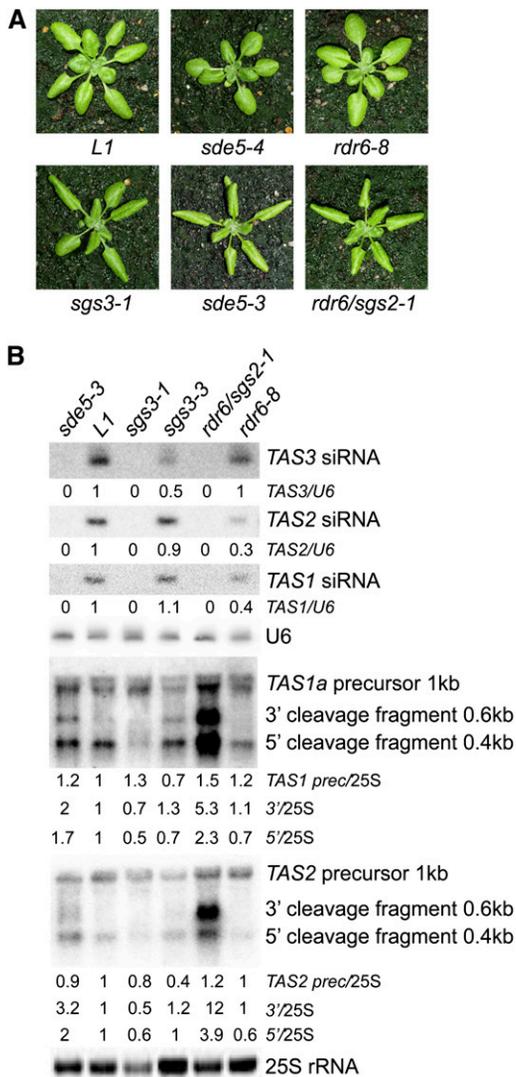


Figure 3. Analysis of the tasiRNA Pathway in *sde5*, *rdr6*, and *sgs3* Mutants.

(A) Pictures of the indicated mutant and *L1* control plants.

(B) LMW and HMW RNA gel blot analyses of mature rosette leaves of the indicated mutant and *L1* control plants. LMW RNA gel blots were probed with DNA oligonucleotides complementary to *TAS1* [si-480(+)], *TAS2* (F), and *TAS3* (tasiARF). HMW RNA gel blots were probed with DNA complementary to the *TAS1a* and *TAS2* precursors. The expected migration positions of primary *TAS* RNA precursors (*pri*) and the 5' and 3' cleavage products generated after miR173-guided cleavage are indicated. 25S rRNA and *U6* snRNA hybridizations served as loading controls for HMW and LMW blots, respectively, and normalized values with the *L1* control set to 1 are indicated.

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mutants overaccumulated *TAS2* precursors (Figure 5D). The increase in *TAS2* precursor accumulation was much stronger in *tex1* than in *hpr1*, consistent with a stronger reduction of mature tasiRNA accumulation in *tex1* (Figure 5C). In addition, the contribution of HPR1 to the tasiRNA pathway depended on the

tissue or developmental stage analyzed because the decrease in mature tasiRNA accumulation was stronger in *hpr1* seedlings than in *hpr1* rosette leaves (cf. Figures 5A and 5C, respectively).

HPR1 but Not SDE5 Is Involved in the IR-PTGS Pathway

Because SDE5, HPR1, and TEX1 encode putative RNA trafficking proteins involved in S-PTGS, we examined if they also contribute to IR-PTGS triggered by the *JAP3* construct expressing *PDS* dsRNA under the control of the phloem-specific *SUC2* promoter (Smith et al., 2007). Previous analyses indicated that *sde5-2* is not impaired in *PDS* IR-PTGS (Hernandez-Pinzon et al., 2007). However, the elucidation of *sde5-2* as a hypomorphic allele called for a reexamination of the role of SDE5 using a null allele. Introduction of the *JAP3* IR-PTGS locus in *sde5-3* did not impact the *PDS* IR-PTGS phenotype (Figure 6A). *PDS* IR precursors remained undetectable, similar to the *JAP3* control (Figures 6B), and *PDS* siRNA accumulation was unchanged (Figures 6C), indicating that SDE5 is dispensable for IR-PTGS, similar to RDR6 and SGS3 (Beclin et al., 2002; Dunoyer et al., 2005). By contrast, introduction of the *JAP3* IR-PTGS locus in *hpr1-1* resulted in a reduced *PDS* IR-PTGS phenotype (Figure 6A), increased *PDS* IR precursor accumulation (Figure 6B), and reduced *PDS* siRNA accumulation (Figure 6C), indicating that HPR1 is involved in IR-PTGS.

DISCUSSION

SDE3 Requirement in S-PTGS Is Inversely Proportional to the Silencer Strength

Due to the enormous interest in PTGS in the last 15 years, several forward genetic screens have been performed in plants, including four S-PTGS screens (Elmayan et al., 1998; Dalmay et al., 2000; Adenot et al., 2006; Herr et al., 2006), two IR-PTGS screens (Dunoyer et al., 2005; Smith et al., 2007), two miRNA screens (Brodersen et al., 2008; Yu et al., 2010), and two tasiRNA screens (Peragine et al., 2004; Cuperus et al., 2009). When combined with results from reverse genetic approaches, our knowledge of the PTGS pathways in plants has expanded to include AGO1, CLSY1, DCL2, DCL4, FCA, FPA, FLD, FVE, FY, HEN1, NRPD1a, NRPD2, and RDR2 in IR-PTGS (Dunoyer et al., 2005, 2007; Baurle et al., 2007; Smith et al., 2007; Manzano et al., 2009) as well as AGO1, AGO10, DCL2, DCL4, DDM1, ESP1, ESP3, ESP4, ESP5, FRY1, HEN1, MET1, RDR6, SDE3, SDE5, SGS3, XRN2, XRN3, and XRN4 in S-PTGS (Dalmay et al., 2000, 2001; Fagard et al., 2000; Morel et al., 2000, 2002; Mourrain et al., 2000; Boutet et al., 2003; Gazzani et al., 2004; Herr et al., 2006; Gy et al., 2007; Hernandez-Pinzon et al., 2007; Mlotshwa et al., 2008; Mallory et al., 2009; Mallory and Vaucheret, 2009). Our results clearly indicate that there are a larger number of proteins that play a role in S-PTGS and that these proteins have varying degrees of importance in the different silencing systems. Among three of the S-PTGS systems used to screen for PTGS-deficient mutants, one, *L1*, is a strong silencer, whereas two, *2a3* and *GxA*, are more weakly silenced. Indeed, *L1* was the line showing the most precocious onset of S-PTGS among the different lines carrying

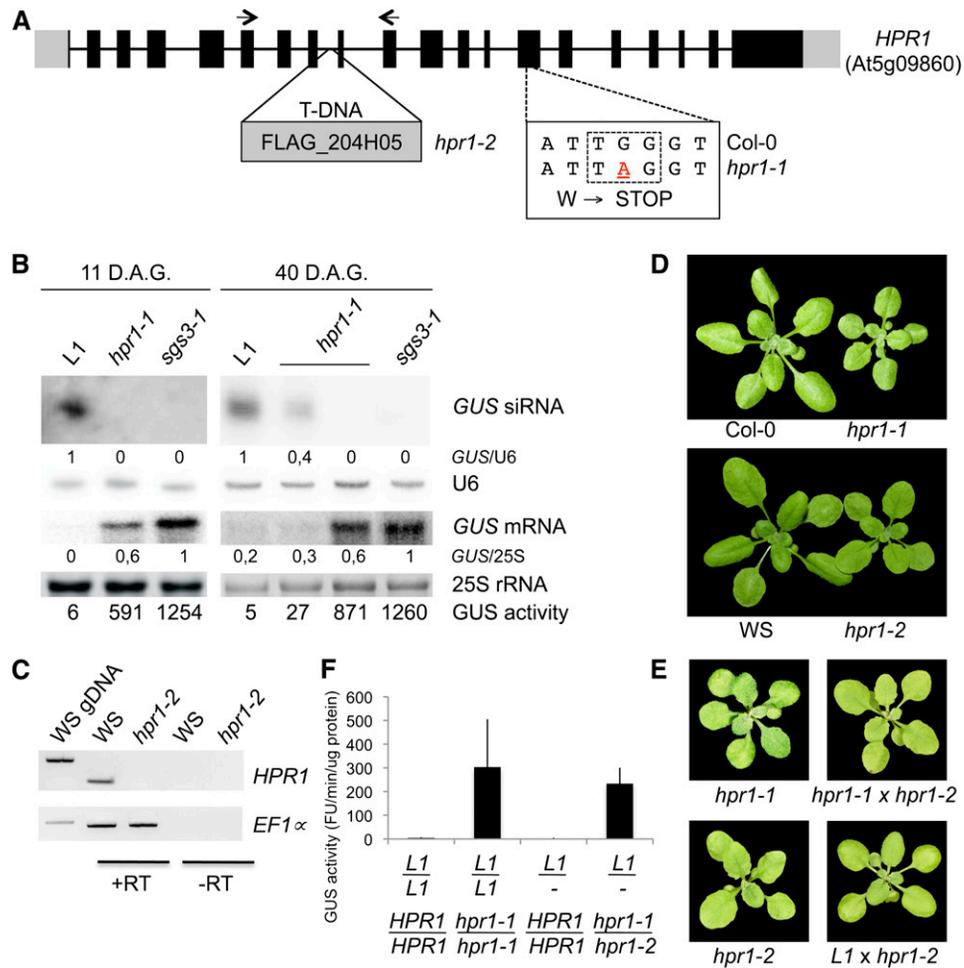


Figure 4. Analysis of S-PTGS in *hpr1* Mutants.

(A) Schematic representation of the *HPR1* gene with the position and sequence changes introduced by the *hpr1-1* and *hpr1-2* mutations indicated. Black boxes represent exons, gray boxes untranslated regions, and thin lines introns. Black arrows represent the position of the primers used for RT-PCR.

(B) LMW and HMW RNA gel blots of aerial parts of 11-d-old seedlings and mature rosette leaves (40 DAG) of silenced (left) and nonsilenced (right) *hpr1-1* mutants and *L1* control plants were probed with an RNA *GUS* probe and a DNA *GUS* probe, respectively. *25S* rRNA and *U6* snRNA hybridizations served as loading controls for HMW and LMW blots, respectively, and normalized values are indicated. GUS activity is indicated in FU/min/μg protein.

(C) RT-PCR analysis of the *hpr1-2* mutant. *EF1α* served as a quantification standard for RT-PCR.

(D) Pictures of the indicated mutant and wild-type control plants.

(E) *hpr1-1*, *hpr1-2*, an F1 plant of the cross *hpr1-1* × *hpr1-2*, and an F1 plant of the control cross *L1* × *hpr1-2*.

(F) GUS activity is indicated in FU/min/μg protein in rosette leaves of 40-d-old plants for each indicated genotype. Number of tested plants, $n = 10$. Standard deviations are indicated.

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the *p35S:GUS* transgene (Elmayan et al., 1998). By contrast, because early cosuppression of the *p35S:NIA2* transgene and *NIA1* and *NIA2* endogenous genes is lethal, only transgenic lines such as *2a3*, which triggers S-PTGS later in development, could be propagated (Elmayan et al., 1998). Consistent with *L1* being a strong silencer, *GUS* siRNAs appear earlier in development in the *L1* line than *NIA* siRNAs, which appear in the *2a3* line (V. Jauvion and H. Vaucheret, unpublished data). Moreover, all mutants recovered from the *L1* screen impaired *2a3* S-PTGS, whereas

some mutants recovered from the *2a3* screen have only modest effects on *L1* S-PTGS. This last group of mutants includes *sde3*, which was also recovered from the *GxA* screen (Dalmay et al., 2000). The *GxA* amplicon system also can be considered a weak silencer. Indeed, the *p35S:GFP* transgene (*G*) by itself is not silenced, and only in the presence of the *p35S:PVX-GFP* amplicon (*A*) is *GxA* PTGS efficient. The *sde3* mutations did not alleviate *GxA* PTGS as efficiently as *sde1/sgs2/rdr6*, *sde2/sgs3*, and *sde5/sgs7* (Dalmay et al., 2000; Hernandez-Pinzon et al.,

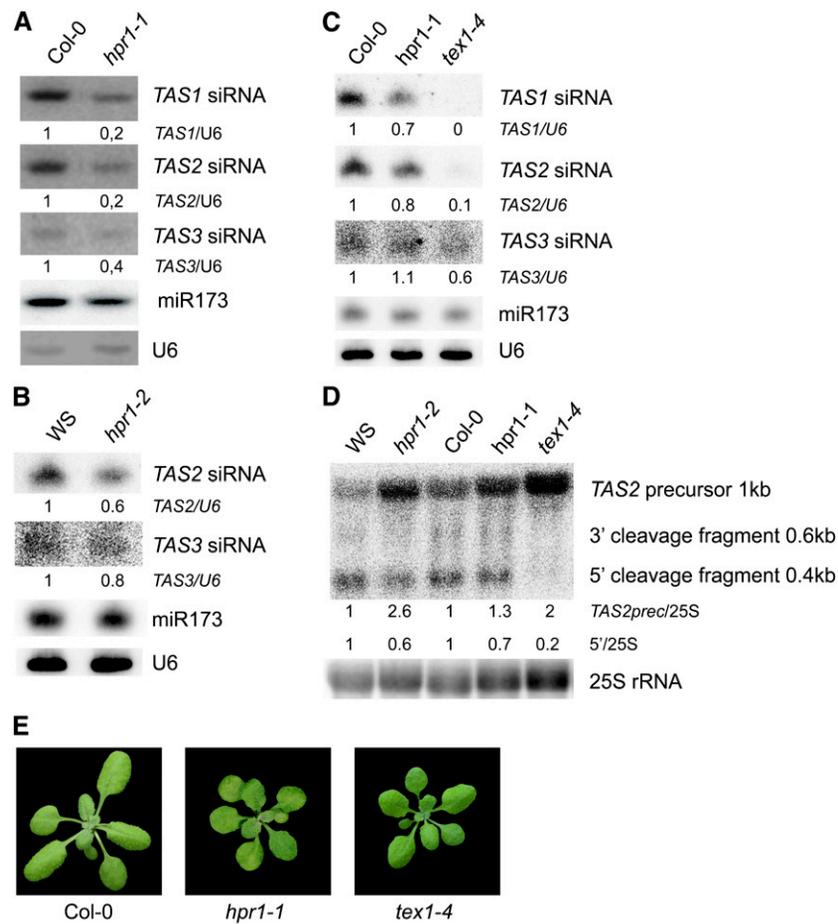


Figure 5. Analysis of the tasiRNA Pathway in *hpr1* and *tex1* Mutants.

(A) LMW RNA gel blot of seedlings of *hpr1-1* mutant and Col-0 control plants was probed with DNA oligonucleotides complementary to miR173, *TAS1* [si-480(+)], *TAS2* (si-F), and *TAS3* (tasi-ARF).

(B) LMW RNA gel blot of mature rosette leaves of *hpr1-2* and Col-0 control plants. LMW RNA gel blot was probed with DNA oligonucleotides complementary to *TAS2* (si-F), *TAS3* (tasi-ARF), and miR173.

(C) LMW RNA gel blot of mature rosette leaves of *hpr1* and *tex1* mutants and the Col-0 control was probed with DNA oligonucleotides complementary to miR173, *TAS1* [si-480(+)], *TAS2* (si-F), and *TAS3* (tasi-ARF).

(D) HMW RNA gel blot of mature rosette leaves of the two *hpr1* alleles and *tex1* mutant plants. HMW RNA gel blot was probed with DNA complementary to *TAS2* precursor. The expected migration positions of primary *TAS* RNA precursor (pri) and the 5' and 3' cleavage products generated after miR173-guided cleavage are indicated. *U6* snRNA hybridizations and 25S rRNA served as loading controls for all LMW and HMW blots, respectively, and normalized values with Col-0 and WS control set to 1 are indicated.

(E) Developmental phenotypes of 16-d-old *hpr1* and *tex1* mutants compared with Col-0.

[See online article for color version of this figure.]

2007), suggesting that the strength of the *GxA* system is between that of *L1* and *2a3*. We ruled out the possibility that *SDE3* could be specific to two-component systems (*2a3* and *GxA*) by showing that *sde3* mutations abolish S-PTGS of the *Hc1* line, which carries the same transgene as the *L1* line but triggers S-PTGS less efficiently than *L1* (Elmayan et al., 1998; Gy et al., 2007). Because the tasiRNA pathway requires *RDR6*, *SDE5*, and *SGS3* but not *SDE3*, the *L1* S-PTGS reporter system serves as a good mimic of the tasiRNA pathway. At present, although *SDE3* is involved in *Hc1*, *2a3*, and *GxA* S-PTGS and possesses a putative RNA helicase domain, it still awaits assignment of an endogenous function.

SDE5 Is a Core Component of S-PTGS and tasiRNA Pathways

Previous characterization of *SDE5* was based on hypomorphic *sde5* alleles and suggested a limited role of *SDE5* in silencing pathways (Hernandez-Pinzon et al., 2007). Our analysis of a null *sde5* allele shows that *SDE5* plays a more crucial role in the S-PTGS and tasiRNA pathways than previously appreciated. We show that a *sde5* null allele is not impaired in IR-PTGS but is deficient for S-PTGS and tasiRNA production, indicating that, like *RDR6* and *SGS3*, *SDE5* is a core component of the S-PTGS and tasiRNA pathways but is dispensable for IR-PTGS. It

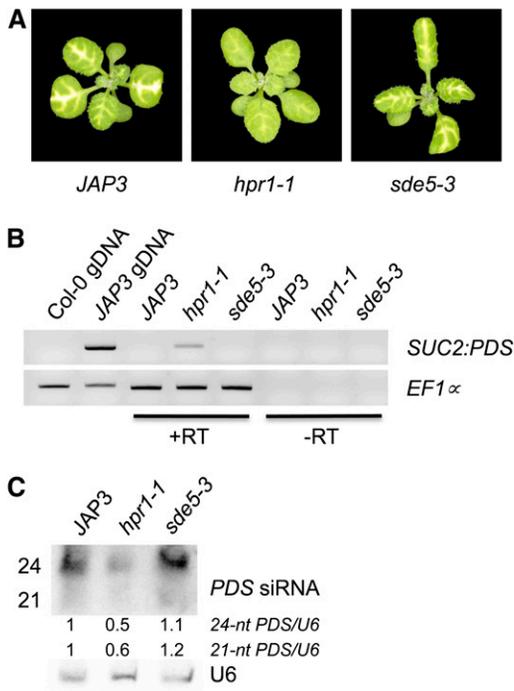


Figure 6. Analysis of IR-PTGS in *sde5* and *hpr1* Mutants.

(A) Progression of JAP3 silencing resulting from the spreading of *PDS* siRNAs from the companion cells of the phloem is evidenced by photobleaching in *hpr1-1* and *sde5-3* mutants compared with the *JAP3* control.

(B) RT-PCR of *SUC2:PDS* transgene-derived RNA in the indicated mutants and control *JAP3* line. gDNA indicates genomic DNA controls. *EF1 α* served as a quantification standard for RT-PCR.

(C) LMW RNA gel blot of mature rosette leaves of the indicated mutant plants was probed with a DNA probe complementary to *PDS*. *PDS* siRNA size is indicated on the left. *U6* snRNA hybridization served as a loading control. Normalized values with the *JAP3* control set to 1 are indicated.

remains unclear if the tasiRNA pathway takes place in the nucleus, the cytoplasm, or both. RDR6 and SGS3 likely localize in the cytoplasm, whereas DCL4 is nuclear (Hiraguri et al., 2005; Glick et al., 2008; Elmayan et al., 2009; Kumakura et al., 2009). However, caution should be taken because all localization experiments were done using constructs expressed under the control of the 35S promoter, which can affect protein localization. Indeed, RDR6 was observed in the nucleus in another report (Luo and Chen, 2007). A plausible scenario would be that *TAS* cleavage products are exported from the nucleus to the cytoplasm where they are protected against degradation by SGS3 and converted to dsRNA by RDR6 before reentering the nucleus to be processed into siRNA by DCL4. Whether SDE5 participates in the export of these RNAs from the nucleus to the cytoplasm or their import from the cytoplasm to the nucleus remains unknown. *TAS* cleavage products are detected in *sde5* null alleles, indicating that the absence of SDE5 does not prevent *TAS* precursors from being cleaved. If SDE5 participates in the export of *TAS* cleavage products from the nucleus to the cytoplasm, in *sde5* mutants, nuclear-retained *TAS* cleavage products would not

be transformed into dsRNA by RDR6 but also would not be protected by SGS3. The overaccumulation of *TAS* cleavage products observed in *sde5* is consistent with this hypothesis. However, it also is consistent with the hypothesis that SDE5 imports *TAS* dsRNA from the cytoplasm to the nucleus because *TAS* dsRNA that could not be processed into siRNA by DCL4 would also overaccumulate in the cytoplasm.

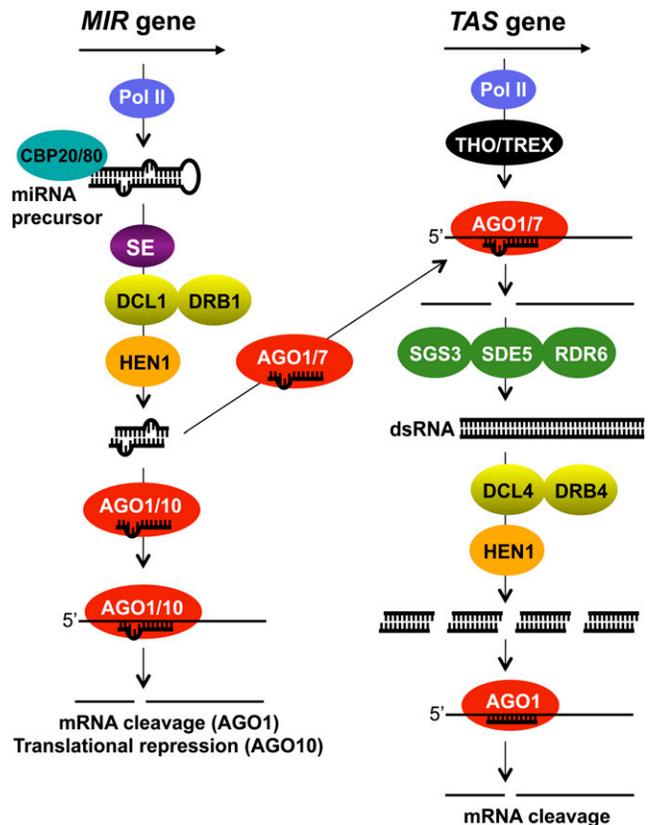


Figure 7. Model for the Putative Action Site of the THO/TREX Complex in the tasiRNA Pathway.

miRNA precursors are transcribed by PolII from *MIRNA* genes. CAP BINDING PROTEIN20 (CBP20) and CBP80 are recruited on the cap of miRNA precursors that are subsequently processed into small RNA duplexes by the type III RNase DCL1, which is aided by the DOUBLE-STRANDED RNA BINDING PROTEIN1 (DRB1/HYL1). SERRATE (SE), a C2H2 zinc finger protein, colocalizes in the D-bodies where DCL1 dicing takes place. Small RNA duplexes are methylated at their 3' end by HEN1 to protect them from degradation. Finally, one strand of the duplex is loaded by an AGO protein, which mediates either mRNA cleavage (AGO1), translational repression (AGO1 and AGO10/ZLL), or DNA methylation (AGO4). The tasiRNA pathway requires the miRNA pathway. *TAS* mRNAs produced by PolIII are exported/trafficked by the THO/TREX complex and then targeted by AGO1 and AGO7 loaded with a complementary miRNA. Cleavage fragments are protected from degradation and transformed into dsRNA by SGS3, SDE5, and RDR6. This dsRNA is processed by DCL4, assisted by DRB4, to produce tasiRNA duplexes that are methylated by HEN1. tasiRNAs are loaded on AGO1 and guide mRNA cleavage both in trans and cis.

[See online article for color version of this figure.]

HPR1 Acts in IR-PTGS, S-PTGS, and tasiRNA Pathways

In contrast with the S-PTGS-specific SDE3, SDE5 is required for both S-PTGS and the tasiRNA pathways, but not the IR-PTGS pathway. On the other hand, HPR1 plays a role in S-PTGS, tasiRNA, and IR-PTGS pathways, indicating that it is involved in a step common to these three pathways. In yeast, *Drosophila melanogaster*, and human, HPR1/THO1 is a member of the THO/TREX RNA trafficking complex and is required for the regulation of a subset of endogenous mRNA (Reed and Cheng, 2005). *Arabidopsis hpr1* mutants exhibit mild developmental defects, which cannot be attributed solely to the partial impairment of the tasiRNA pathway. Rather, these defects are likely explained by a general, but limited, role of HPR1 in the trafficking of a subset of endogenous RNA, similar to the role that it plays in yeast, *Drosophila*, and human (Reed and Cheng, 2005). Although the exact role of HPR1 in the trafficking of endogenous mRNA remains to be elucidated, the implication of HPR1 in S-PTGS, IR-PTGS, and tasiRNA pathways reinforces the idea that proteins of the endogenous RNA metabolism pathways are shared among the different RNA silencing machineries. Indeed, the Cap binding proteins CBP20 and CBP80 are involved in the miRNA pathway (Gregory et al., 2008; Laubinger et al., 2008), and the putative splicing factor ESP3, the RNA 3' end formation factors ESP1, ESP4, and ESP5, and the 5' → 3' exoribonucleases XRN2, XRN3, and XRN4 are involved in the S-PTGS pathway (Gazzani et al., 2004; Herr et al., 2006; Gy et al., 2007), while the 3' RNA processing proteins FCA, FPA, and FY are involved in IR-PTGS (Baurle et al., 2007; Manzano et al., 2009).

HPR1/THO1 is part of a multicomponent THO/TREX complex that is conserved among fungi, invertebrates, and mammals. Three lines of evidence suggest the existence of a plant THO/TREX complex that includes, at least, the putative orthologs of HPR1/THO1 and TEX1/THO3: (1) *Arabidopsis* HPR1 and TEX1 are homologous to their animal counterparts. *Arabidopsis* HPR1 is 29 and 28% identical to *Drosophila* and human HPR1, respectively, and *Arabidopsis* TEX1 is 50 and 48% to *Drosophila* and human TEX1, respectively. (2) *Arabidopsis hpr1* and *tex1* mutants display overlapping developmental defects, and (3) both *Arabidopsis hpr1* and *tex1* mutants show increased accumulation of uncleaved *TAS* precursors and decreased accumulation of mature tasiRNA. This overaccumulation of uncleaved *TAS* precursors is similar to what has been observed in *dcl1* mutants, which lack miRNAs that specifically initiate the tasiRNA pathway by cleaving *TAS* precursors (Allen et al., 2005; Yoshikawa et al., 2005). Because of these similarities, and because neither *hpr1* nor *tex1* affects miR173 accumulation, we propose that the THO/TREX complex acts in an upstream step of tasiRNA biogenesis that is downstream of miRNA production and, thus, likely acts at the level of miRNA-guided AGO-mediated cleavage of *TAS* precursors (Figure 7). Because S-PTGS and IR-PTGS involve AGO1-mediated cleavage of siRNA precursors, we propose that the THO/TREX complex generally participates in the trafficking of siRNA precursors to AGO catalytic centers. However, *tex1* and *hpr1* had stronger effects on *TAS1* and *TAS2*, which depends on AGO1, than on *TAS3*, which depends on AGO7, suggesting that the implication of the THO/TREX complex toward different RNA may depend on their localization and/or association with other proteins.

The abrogation of HPR1/THO1, THO2, and TEX1/THO3 had different consequences, suggesting that if a plant THO/TREX complex exists, it is a dynamic structure in which each member plays a distinct and maybe tissue-specific role. Indeed, homozygous *tho2* mutants could not be obtained, indicating that THO2 is essential. By contrast, homozygous *tex1* and *hpr1* mutants were viable and exhibited overlapping mild developmental phenotypes, indicating that TEX1 and HPR1 are not as essential as THO2. Finally, *tex1* had a stronger effect on the tasiRNA pathway than *hpr1*, although the contribution of HPR1 appeared to depend on the tissue or developmental stage.

Because S-PTGS and IR-PTGS involve the propagation of short-distance and long-distance silencing signals, it is also tempting to speculate that the RNA trafficking proteins HPR1 and TEX1 play a role in the trafficking of these systemic signals. Supporting this hypothesis, *hpr1* mutations did not abolish S-PTGS or IR-PTGS but delayed their establishment and/or impacted their efficiency. Grafting experiments paired with high-throughput sequencing technology using *hpr1* and *tex1* mutants will provide a platform to ascertain the contribution of these proteins to the systemic aspects of S-PTGS and IR-PTGS.

METHODS

Plant Material

The *L1*, *2a3*, and *JAP3* lines and the *rdl6/sgs2-1*, *sde3-1*, *sde5-3*, *sgs3-1*, and *sgs3-3* mutants have been described before (Elmayan et al., 1998, 2009; Mourrain et al., 2000; Dalmay et al., 2001; Smith et al., 2007; Mallory and Vaucheret, 2009). The *hpr1-1*, *rdl6-8*, *sde3-6*, and *sde5-4* mutants were isolated in the *L1* and *2a3* screens and are described in the results section. The T-DNA insertion mutant *hpr1-2* (FLAG_204H05) derives from the Institut National de la Recherche Agronomique (INRA) T-DNA collection (Samson et al., 2002) and was obtained from INRA. The T-DNA insertion mutants *tho2-1* (SALK_072011) and *tex1-4* (SALK_100012) derive from the SALK T-DNA collection (Alonso et al., 2003) and were obtained from the Nottingham Arabidopsis Stock Centre (NASC). Analyses were performed either on 11-d-old seedlings (aerial parts except for Figure 5A for which entire seedlings were taken) grown on Boutourage media (Duchefa) or rosettes leaves of 40-d-old plants grown on Boutourage media for 14 d and transferred to soil in controlled growth chambers. All plants were grown in standard long-day conditions (16 h of light/8 h of dark) at 21°C.

Molecular Analyses

DNA sequencing was performed as described before (Gy et al., 2007; Mallory and Vaucheret, 2009). RNA extraction, gel blot analyses, and quantification of GUS activity were performed as described before (Gy et al., 2007; Mallory and Vaucheret, 2009). GUS activity was quantified by measuring the quantity of 4-methylumbelliferone product generated from the substrate 4-methylumbelliferyl-β-D-glucuronide (Duchefa) on a fluorometer (Fluoroscan II; Thermo Scientific), and fluorescence values were normalized to total protein extracted, which was quantified using an absorbance microplate reader (Elisa Elx 808; Avanteq) and the Bradford protein assay. All RNA gel blot analyses were performed using 10 μg of total RNA, except for PDS siRNA analysis (Figure 6C), which was performed using 20 μg of total RNA. *GUS*, *PDS*, *TAS*, *U6*, miR173, and *25S* probes have been described before (Gy et al., 2007; Smith et al., 2007; Elmayan et al., 2009; Mallory and Vaucheret, 2009). *TAS1a* and *TAS2* probes allowed detection of precursors as well as both 5' and 3'

cleavage products (Elmayan et al., 2009). Hybridization signals were quantified using a Fuji phosphor imager and normalized to a *U6* oligonucleotide probe, and a *25S* DNA probe, for LMW and HMW gel blot analyses, respectively. All RNA gel blots were performed twice using biological replicates. For cDNA synthesis, RNAs were extracted with the Plant RNeasy kit (Qiagen), treated with DNaseI (Invitrogen), and 2 μ g of DNA-free RNA was reverse transcribed with oligo-dT (Invitrogen). PCR was performed using Taq DNA polymerase (Invitrogen) according to the manufacturer's protocol. Each reaction was performed on 5 μ L of 1:60 dilution of the cDNA and synthesized in a 25- μ L total reaction. Specific oligonucleotide pairs are listed in Supplemental Table 1 online. The number of PCR cycles for each DNA product was determined using the minimum number of cycles in which a detectable DNA fragment was amplified but not saturated. Ethidium bromide-stained DNA gels were imaged using a Gel Doc XR (Bio-Rad). Forty cycles of amplification were used at a 54°C annealing temperature for *sde5-4*, 34 cycles of amplification were used at a 57°C annealing temperature for *sde5-3*, 34 cycles of amplification were used at a 59°C annealing temperature for *HPR1*, 40 cycles of amplification were used at a 58°C annealing temperature for the *JAP3* transgene, and 24 cycles of amplification at a 62°C annealing temperature were used for *EF1 α* . The results were standardized to the expression level of *EF1 α* . Protein alignments were performed with MUSCLE (Edgar, 2004), and the similarity between alignments was calculated using the Prodist module of the PHYLP package (Felsenstein, 1989).

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: *AGO1* (At1g48410), *RDR6/SGS2* (At3g49500), *SDE3* (At1g05460), *SDE5* (At3g15390), *SGS3* (At5g23570), *HPR1/THO1* (At5g09860), *TEX1/THO3* (At5g56130), *THO2* (At1g24706), *MIR173* (At3g23125), *NIA1* (At1g77760), *NIA2* (At1g37130), *TAS1a* (At2g27400), *TAS2* (At2g39681), *TAS3a* (At3g17185), and the multigene family *EF1 α* (At1g07920, At1g07930, and At1g07940).

Supplemental Data

The following material is available in the online version of this article.

Supplemental Table 1. List of DNA Oligonucleotides Used in This Study.

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REFERENCES

Adenot, X., Elmayan, T., Laressergues, D., Boutet, S., Bouche, N., Gascioli, V., and Vaucheret, H. (2006). DRB4-dependent TAS3 trans-acting siRNAs control leaf morphology through AGO7. *Curr. Biol.* **16**: 927–932.

Allen, E., Xie, Z., Gustafson, A.M., and Carrington, J.C. (2005). MicroRNA-directed phasing during trans-acting siRNA biogenesis in plants. *Cell* **121**: 207–221.

Alonso, J.M., et al. (2003). Genome-wide insertional mutagenesis of *Arabidopsis thaliana*. *Science* **301**: 653–657.

Baumberger, N., and Baulcombe, D.C. (2005). Arabidopsis ARGONAUTE1 is an RNA Slicer that selectively recruits microRNAs and short interfering RNAs. *Proc. Natl. Acad. Sci. USA* **102**: 11928–11933.

Baurle, I., Smith, L., Baulcombe, D.C., and Dean, C. (2007). Widespread role for the flowering-time regulators FCA and FPA in RNA-mediated chromatin silencing. *Science* **318**: 109–112.

Beclin, C., Boutet, S., Waterhouse, P., and Vaucheret, H. (2002). A branched pathway for transgene-induced RNA silencing in plants. *Curr. Biol.* **12**: 684–688.

Blevins, T., Rajeswaran, R., Shivaprasad, P.V., Beknazariants, D., Si-Ammour, A., Park, H.S., Vazquez, F., Robertson, D., Meins, F., Jr., Hohn, T., and Pooggin, M.M. (2006). Four plant Dicers mediate viral small RNA biogenesis and DNA virus induced silencing. *Nucleic Acids Res.* **34**: 6233–6246.

Bouche, N., Laressergues, D., Gascioli, V., and Vaucheret, H. (2006). An antagonistic function for Arabidopsis DCL2 in development and a new function for DCL4 in generating viral siRNAs. *EMBO J.* **25**: 3347–3356.

Boutet, S., Vazquez, F., Liu, J., Beclin, C., Fagard, M., Gratias, A., Morel, J.B., Crete, P., Chen, X., and Vaucheret, H. (2003). Arabidopsis HEN1: A genetic link between endogenous miRNA controlling development and siRNA controlling transgene silencing and virus resistance. *Curr. Biol.* **13**: 843–848.

Brodersen, P., Sakvarelidze-Achard, L., Bruun-Rasmussen, M., Dunoyer, P., Yamamoto, Y.Y., Sieburth, L., and Voinnet, O. (2008). Widespread translational inhibition by plant miRNAs and siRNAs. *Science* **320**: 1185–1190.

Brosnan, C.A., Mitter, N., Christie, M., Smith, N.A., Waterhouse, P.M., and Carroll, B.J. (2007). Nuclear gene silencing directs reception of long-distance mRNA silencing in Arabidopsis. *Proc. Natl. Acad. Sci. USA* **104**: 14741–14746.

Cuperus, J.T., Montgomery, T.A., Fahlgren, N., Burke, R.T., Townsend, T., Sullivan, C.M., and Carrington, J.C. (2009). Identification of MIR390a precursor processing-defective mutants in Arabidopsis by direct genome sequencing. *Proc. Natl. Acad. Sci. USA* **107**: 466–471.

Dalmay, T., Hamilton, A., Rudd, S., Angell, S., and Baulcombe, D.C. (2000). An RNA-dependent RNA polymerase gene in Arabidopsis is required for posttranscriptional gene silencing mediated by a transgene but not by a virus. *Cell* **101**: 543–553.

Dalmay, T., Horsefield, R., Braunstein, T.H., and Baulcombe, D.C. (2001). SDE3 encodes an RNA helicase required for post-transcriptional gene silencing in Arabidopsis. *EMBO J.* **20**: 2069–2078.

Deleris, A., Gallego-Bartolome, J., Bao, J., Kasschau, K.D., Carrington, J.C., and Voinnet, O. (2006). Hierarchical action and inhibition of plant Dicer-like proteins in antiviral defense. *Science* **313**: 68–71.

Ding, S.W., and Voinnet, O. (2007). Antiviral immunity directed by small RNAs. *Cell* **130**: 413–426.

Dunoyer, P., Brosnan, C.A., Schott, G., Wang, Y., Jay, F., Alioua, A., Himber, C., and Voinnet, O. (2010b). An endogenous, systemic RNAi pathway in plants. *EMBO J.* **29**: 1699–1712.

Dunoyer, P., Himber, C., Ruiz-Ferrer, V., Alioua, A., and Voinnet, O. (2007). Intra- and intercellular RNA interference in *Arabidopsis thaliana* requires components of the microRNA and heterochromatic silencing pathways. *Nat. Genet.* **39**: 848–856.

Dunoyer, P., Himber, C., and Voinnet, O. (2005). DICER-LIKE 4 is required for RNA interference and produces the 21-nucleotide small

- interfering RNA component of the plant cell-to-cell silencing signal. *Nat. Genet.* **37**: 1356–1360.
- Dunoyer, P., Schott, G., Himber, C., Meyer, D., Takeda, A., Carrington, J.C., and Voinnet, O.** (2010a). Small RNA duplexes function as mobile silencing signals between plant cells. *Science* **328**: 912–916.
- Dunoyer, P., and Voinnet, O.** (2008). Mixing and matching: The essence of plant systemic silencing? *Trends Genet.* **24**: 151–154.
- Edgar, R.C.** (2004). MUSCLE: Multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res.* **32**: 1792–1797.
- Elmayan, T., Adenot, X., Gissot, L., Lauressergues, D., Gy, I., and Vaucheret, H.** (2009). A neomorphic *sgs3* allele stabilizing miRNA cleavage products reveals that SGS3 acts as a homodimer. *FEBS J.* **276**: 835–844.
- Elmayan, T., Balzergue, S., Beon, F., Bourdon, V., Daubremet, J., Guenet, Y., Mourrain, P., Palauqui, J.C., Vernhettes, S., Vialle, T., Wostrikoff, K., and Vaucheret, H.** (1998). *Arabidopsis* mutants impaired in cosuppression. *Plant Cell* **10**: 1747–1758.
- Fagard, M., Boutet, S., Morel, J.B., Bellini, C., and Vaucheret, H.** (2000). AGO1, QDE-2, and RDE-1 are related proteins required for post-transcriptional gene silencing in plants, quelling in fungi, and RNA interference in animals. *Proc. Natl. Acad. Sci. USA* **97**: 11650–11654.
- Felsenstein, J.** (1989). PHYLIP (Phylogeny Inference Package). *Cladistics* **5**: 164–166.
- Fusaro, A.F., Matthew, L., Smith, N.A., Curtin, S.J., Dedic-Hagan, J., Ellacott, G.A., Watson, J.M., Wang, M.B., Brosnan, C., Carroll, B.J., and Waterhouse, P.M.** (2006). RNA interference-inducing hairpin RNAs in plants act through the viral defence pathway. *EMBO Rep.* **7**: 1168–1175.
- Gazzani, S., Lawrenson, T., Woodward, C., Headon, D., and Sablowski, R.** (2004). A link between mRNA turnover and RNA interference in *Arabidopsis*. *Science* **306**: 1046–1048.
- Glick, E., Zrachya, A., Levy, Y., Mett, A., Gidoni, D., Belausov, E., Citovsky, V., and Gafni, Y.** (2008). Interaction with host SGS3 is required for suppression of RNA silencing by tomato yellow leaf curl virus V2 protein. *Proc. Natl. Acad. Sci. USA* **105**: 157–161.
- Gregory, B.D., O'Malley, R.C., Lister, R., Urich, M.A., Tonti-Filippini, J., Chen, H., Millar, A.H., and Ecker, J.R.** (2008). A link between RNA metabolism and silencing affecting *Arabidopsis* development. *Dev. Cell* **14**: 854–866.
- Gy, I., Gascioli, V., Lauressergues, D., Morel, J.B., Gombert, J., Proux, F., Proux, C., Vaucheret, H., and Mallory, A.C.** (2007). *Arabidopsis* FIERY1, XRN2, and XRN3 are endogenous RNA silencing suppressors. *Plant Cell* **19**: 3451–3461.
- Hernandez-Pinzon, I., Yelina, N.E., Schwach, F., Studholme, D.J., Baulcombe, D., and Dalmay, T.** (2007). SDE5, the putative homologue of a human mRNA export factor, is required for transgene silencing and accumulation of trans-acting endogenous siRNA. *Plant J.* **50**: 140–148.
- Herr, A.J., Jensen, M.B., Dalmay, T., and Baulcombe, D.C.** (2005). RNA polymerase IV directs silencing of endogenous DNA. *Science* **308**: 118–120.
- Herr, A.J., Molnar, A., Jones, A., and Baulcombe, D.C.** (2006). Defective RNA processing enhances RNA silencing and influences flowering of *Arabidopsis*. *Proc. Natl. Acad. Sci. USA* **103**: 14994–15001.
- Hiraguri, A., Itoh, R., Kondo, N., Nomura, Y., Aizawa, D., Murai, Y., Koiwa, H., Seki, M., Shinozaki, K., and Fukuhara, T.** (2005). Specific interactions between Dicer-like proteins and HYL1/DRB-family dsRNA-binding proteins in *Arabidopsis thaliana*. *Plant Mol. Biol.* **57**: 173–188.
- Kloc, A., and Martienssen, R.** (2008). RNAi, heterochromatin and the cell cycle. *Trends Genet.* **24**: 511–517.
- Kumakura, N., Takeda, A., Fujioka, Y., Motose, H., Takano, R., and Watanabe, Y.** (2009). SGS3 and RDR6 interact and colocalize in cytoplasmic SGS3/RDR6-bodies. *FEBS Lett.* **583**: 1261–1266.
- Laubinger, S., Sachsenberg, T., Zeller, G., Busch, W., Lohmann, J.U., Ratsch, G., and Weigel, D.** (2008). Dual roles of the nuclear cap-binding complex and SERRATE in pre-mRNA splicing and micro-RNA processing in *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci. USA* **105**: 8795–8800.
- Li, J., Yang, Z., Yu, B., Liu, J., and Chen, X.** (2005). Methylation protects miRNAs and siRNAs from a 3'-end uridylation activity in *Arabidopsis*. *Curr. Biol.* **15**: 1501–1507.
- Luo, Z., and Chen, Z.** (2007). Improperly terminated, nonpolyadenylated mRNA of sense transgenes is targeted by RDR6-mediated RNA silencing in *Arabidopsis*. *Plant Cell* **19**: 943–958.
- Mallory, A.C., Hinze, A., Tucker, M.R., Bouche, N., Gascioli, V., Elmayan, T., Lauressergues, D., Jauvion, V., Vaucheret, H., and Laux, T.** (2009). Redundant and specific roles of the ARGONAUTE proteins AGO1 and ZLL in development and small RNA-directed gene silencing. *PLoS Genet.* **5**: e1000646.
- Mallory, A.C., and Vaucheret, H.** (2006). Functions of microRNAs and related small RNAs in plants. *Nat. Genet.* **38**(Suppl): S31–S36.
- Mallory, A.C., and Vaucheret, H.** (2009). ARGONAUTE 1 homeostasis invokes the coordinate action of the microRNA and siRNA pathways. *EMBO Rep.* **10**: 521–526.
- Manzano, D., Marquardt, S., Jones, A.M., Baurle, I., Liu, F., and Dean, C.** (2009). Altered interactions within FY/AtCPSF complexes required for *Arabidopsis* FCA-mediated chromatin silencing. *Proc. Natl. Acad. Sci. USA* **106**: 8772–8777.
- Mlotshwa, S., Pruss, G.J., Peragine, A., Endres, M.W., Li, J., Chen, X., Poethig, R.S., Bowman, L.H., and Vance, V.** (2008). DICER-LIKE2 plays a primary role in transitive silencing of transgenes in *Arabidopsis*. *PLoS One* **3**: e1755.
- Molnar, A., Melnyk, C.W., Bassett, A., Hardcastle, T.J., Dunn, R., and Baulcombe, D.C.** (2010). Small silencing RNAs in plants are mobile and direct epigenetic modification in recipient cells. *Science* **328**: 872–875.
- Morel, J.B., Godon, C., Mourrain, P., Beclin, C., Boutet, S., Feuerbach, F., Proux, F., and Vaucheret, H.** (2002). Fertile hypomorphic ARGONAUTE (*ago1*) mutants impaired in post-transcriptional gene silencing and virus resistance. *Plant Cell* **14**: 629–639.
- Morel, J.B., Mourrain, P., Beclin, C., and Vaucheret, H.** (2000). DNA methylation and chromatin structure affect transcriptional and post-transcriptional transgene silencing in *Arabidopsis*. *Curr. Biol.* **10**: 1591–1594.
- Mourrain, P., et al.** (2000). *Arabidopsis* SGS2 and SGS3 genes are required for posttranscriptional gene silencing and natural virus resistance. *Cell* **101**: 533–542.
- Palauqui, J.C., Elmayan, T., Pollien, J.M., and Vaucheret, H.** (1997). Systemic acquired silencing: transgene-specific post-transcriptional silencing is transmitted by grafting from silenced stocks to non-silenced scions. *EMBO J.* **16**: 4738–4745.
- Peragine, A., Yoshikawa, M., Wu, G., Albrecht, H.L., and Poethig, R.S.** (2004). SGS3 and SGS2/SDE1/RDR6 are required for juvenile development and the production of trans-acting siRNAs in *Arabidopsis*. *Genes Dev.* **18**: 2368–2379.
- Reed, R., and Cheng, H.** (2005). TREX, SR proteins and export of mRNA. *Curr. Opin. Cell Biol.* **17**: 269–273.
- Samson, F., Brunaud, V., Balzergue, S., Dubreucq, B., Lepiniec, L., Pelletier, G., Caboche, M., and Lecharny, A.** (2002). FLAGdb/FST: A database of mapped flanking insertion sites (FSTs) of *Arabidopsis thaliana* T-DNA transformants. *Nucleic Acids Res.* **30**: 94–97.
- Smith, L.M., Pontes, O., Searle, I., Yelina, N., Yousafzai, F.K., Herr, A.J., Pikaard, C.S., and Baulcombe, D.C.** (2007). An SNF2

- protein associated with nuclear RNA silencing and the spread of a silencing signal between cells in *Arabidopsis*. *Plant Cell* **19**: 1507–1521.
- Smith, N.A., Singh, S.P., Wang, M.B., Stoutjesdijk, P.A., Green, A.G., and Waterhouse, P.M.** (2000). Total silencing by intron-spliced hairpin RNAs. *Nature* **407**: 319–320.
- Souret, F.F., Kastenmayer, J.P., and Green, P.J.** (2004). AtXRN4 degrades mRNA in *Arabidopsis* and its substrates include selected miRNA targets. *Mol. Cell* **15**: 173–183.
- Vazquez, F., Vaucheret, H., Rajagopalan, R., Lepers, C., Gascioli, V., Mallory, A.C., Hilbert, J.L., Bartel, D.P., and Crete, P.** (2004). Endogenous trans-acting siRNAs regulate the accumulation of *Arabidopsis* mRNAs. *Mol. Cell* **16**: 69–79.
- Voinnet, O.** (2009). Origin, biogenesis, and activity of plant microRNAs. *Cell* **136**: 669–687.
- Voinnet, O., Vain, P., Angell, S., and Baulcombe, D.C.** (1998). Systemic spread of sequence-specific transgene RNA degradation in plants is initiated by localized introduction of ectopic promoterless DNA. *Cell* **95**: 177–187.
- Yoshikawa, M., Peragine, A., Park, M.Y., and Poethig, R.S.** (2005). A pathway for the biogenesis of trans-acting siRNAs in *Arabidopsis*. *Genes Dev.* **19**: 2164–2175.
- Yu, B., Bi, L., Zhai, J., Agarwal, M., Li, S., Wu, Q., Ding, S.W., Meyers, B. C., Vaucheret, H., and Chen, X.** (2010). siRNAs compete with miRNAs for methylation by HEN1 in *Arabidopsis*. *Nucleic Acids Res.*, in press.
- Yu, B., Yang, Z., Li, J., Minakhina, S., Yang, M., Padgett, R.W., Steward, R., and Chen, X.** (2005). Methylation as a crucial step in plant microRNA biogenesis. *Science* **307**: 932–935.

NOTE ADDED IN PROOF

When this work was in press, Yelina et al. (2010) recovered *tex1* mutants in a screen for IR-PTGS deficiency and showed that TEX1 is required for the production of endogenous siRNAs.

Yelina, N.E., Smith, L.M., Jones, A.M., Patel, K., Kelly, K.A., and Baulcombe, D.C. (2010). Putative *Arabidopsis* THO/TREX mRNA export complex is involved in transgene and endogenous siRNA biosynthesis. *Proc. Natl. Acad. Sci. USA* **107**: 13948–13953.

Supplemental Table 1. Oligonucleotide Sequences**Oligonucleotides used for genotyping mutant lines**

Mutant	Oligonucleotide Name	Oligonucleotide Sequence	Enzyme (if applicable)
<i>hpr1-1</i>	24-4 dCAPS Fw	AAGGGCACCAGGGAAGAACG	(<i>AluI</i>)
	24-4 dCAPS <i>AluI</i> bis Rv	GGTAAGAGAGAAAGCAGCACACATAGC	
<i>hpr1-2</i>	204H05 Fw	GGTAAGAAGGGTTTCTCGTGC	
	204H05 Rv	GCGGAAGTAAGAGAGCGGG	
	TailB	CGGCTATTGGTAATAGGACACTGG	
<i>tex1-1</i>	100012 Fw	GAATTCGAATGGAACAAAGC	
	100012 Rv	ACAAGATCTGAATGTTTGGG	
<i>tho2-1</i>	072011 Fw	ACAATTGTTGCAGAAGGTGC	
	072011 Rv	AAGGAGACTCATCACTTCCC	
<i>rdr6-8</i>	F7-10 CAPS Fw	GTATTACACTTGAGATTGGG	(<i>RsaI</i>)
	F7-10 CAPS Rv	AAGGCTCATTACGGATACGG	
<i>sde3-1</i>	sde3-1 F	CAACGAGTTTGTAAACGAGTC	
	sde3-1 R	CAGGCTGAATGCAGCTACTC	
<i>sde3-6</i>	sde3-2900F	TTAATGTCGCCATAACCCG	
	T25N20-1R	CTCAGCGGATCGGGCAACGG	
<i>sde5-3</i>	429G09LB	TCTCCCTCCAGCAGCTTTAC	
	429G09RB	AGGTGCGGAAAGATATGTGG	
	P745	CGTCCGCAATGTGTTATTAAG	
<i>sde5-4</i>	dCAPS 14-5 Fw	CTTGGAGAAGGTTTCAAGC	(<i>HaeIII</i>)
	dCAPS 14-5 Rv	TTACCTTCTTCACATCATATCCGCATAGGC	

Oligonucleotides used for RT-PCR

Gene	Oligonucleotide Name	Oligonucleotide Sequence
<i>SUC2:PDS</i>	PDS right	TCAGCGGCCGCTTTGTATGCCAGTAGTGGATCATA
	JAP 3' end of transcript	CTAGTCTAGAATCGATCGGAAG
<i>EF1a</i>	EF1a F	TCACATCAACATTGTGGTCATTGGC
	EF1a R	TTGATCTGGTCAAGAGCCTCAAG
<i>HPRI</i>	204H05 RT-2 Fw	CATGTTGAGGACTTGCAACC
	204H05 Rv	GCGGAAGTAAGAGAGCGGG
<i>SDE5</i>	14-5 exon1 Fw	ATATGGAGTGAAGAAATGCG
(for <i>sde5-4</i>)	14-5 exon3 Rv	CTTGGTATCAGAAAAGTCAAGC
<i>SDE5</i>	429G09LB	TCTCCCTCCAGCAGCTTTAC
(for <i>sde5-3</i>)	429G09RB	AGGTGCGGAAAGATATGTGG

Oligonucleotides for Low and High Molecular Weight RNA gel blot analysis

Gene	Oligonucleotide Name	Oligonucleotide Sequence
<i>GUS</i>	GUS-T3-Fw-central-AntiSens	GATGCATTAACCCCTCACTAAAGGGATGATGTCAGCGTTGAACTGC
	GUS-T7-Rv-central-Sens	GATGCTAATACGACTCACTATAGGGGTGCGCTTGCTGAGTTCC
<i>NIA</i>	NIA2-T3-Fw-Central-AntiSens	GATGCATTAACCCCTCACTAAAGGGAGGTTGAGGTTTTGGACTGC
	NIA2-T7-Rv-Central-Sens	GATGCTAATACGACTCACTATAGGGCATCTTCTTGGCTTTATCCG

Oligonucleotide sequences are listed in the 5' to 3' orientation