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Report

DRB4-Dependent *TAS3 trans*-Acting siRNAs Control Leaf Morphology through AGO7

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Summary

***trans*-acting siRNAs (ta-siRNAs) are endogenous RNAs that direct the cleavage of complementary mRNA targets [1, 2]. *TAS* gene transcripts are cleaved by miRNAs; the cleavage products are protected against degradation by SGS3, copied into dsRNA by RDR6, and diced into ta-siRNAs by DCL4 [1–6]. We describe hypomorphic *rdr6* and *sgs3 Arabidopsis* mutants, which do not exhibit the leaf developmental defects observed in null mutants and which, like null alleles, are impaired in sense-transgene-induced posttranscriptional gene silencing and virus resistance. Null *rdr6* and *sgs3* mutants lack *TAS1*, *TAS2*, and *TAS3* ta-siRNAs and overaccumulate *ARF3/ETTIN* and *ARF4* mRNAs, which are *TAS3* ta-siRNA targets. A hypomorphic *rdr6* mutant accumulates wild-type *TAS3* ta-siRNA levels but not *TAS1* and *TAS2* ta-siRNAs, suggesting that *TAS3* is required for proper leaf development. Consistently, *tas3* but not *tas1* or *tas2* mutants exhibits leaf morphology defects, and *ago7/zip* and *drb4* mutants, which exhibit leaf morphology defects, lack *TAS3* but not *TAS1* and *TAS2* ta-siRNAs in leaves. These results indicate that the dsRNA binding protein DRB4 is required for proper ta-siRNA production, presumably by interacting with DCL4, an interaction analogous to that of HYL1 with DCL1 during miRNA production [7–9], and that *TAS3* ta-siRNAs are required for proper leaf development through the action of AGO7/ZIPPY.**

Results and Discussion

Identification of Novel *rdr6* and *sgs3* Alleles

Independent forward-genetic screens have identified overlapping sets of mutations impairing posttranscriptional gene silencing (PTGS) in *Arabidopsis*. *ago1*, *hen1*, *met1*, *rdr6 (sgs2)*, *sgs1*, and *sgs3* mutants were recovered from a screen based on the single-transgene system L1 (35S-GUS) [10–13]. *nrpd1a (sde4)*, *rdr2*, *rdr6 (sde1)*, *sde3*, and *sgs3 (sde2)* mutants were recovered from a screen based on the dual-transgene system GxA (35S-GFP + 35S-PVX-GFP) [14–16]. In addition, reverse-genetic experiments revealed the impact of *ddm1* on L1 PTGS [17], of *nrpd2a* on GxA PTGS [16], and of *wex* on another single-transgene system, 35S-GFP [18]. The partial overlap between these sets of mutants prompted us to screen for new mutations that disrupt silencing of a cosuppression system based on a transgene with homology to two *Arabidopsis* genes. We previously showed that the *Arabidopsis* line 2a3 carrying the *35S-NITRATE REDUCTASE 2* (*NIA2*) transgene exhibits cosuppression of the two endogenous *NIA* genes (*NIA1* and *NIA2*) with 100% efficiency, leading to early death when grown in selective conditions (i.e., when plants are grown 3 weeks in vitro prior to transfer to soil) [10]. Cosuppression efficiency is reduced when 2a3 plants are grown under permissive conditions (i.e., when seeds are germinated directly in soil), allowing approximately 10% of the plants to survive and set seeds. We took advantage of this system to perform a mutagenesis under permissive conditions and screened for plants that could survive when grown in selective conditions (see Supplemental Experimental Procedures in the Supplemental Data available online). Among 19 confirmed mutants, five showed a complete impairment of cosuppression (100% of the plants survived at each generation under selective growth conditions). The 14 other mutants showed partial impairment of cosuppression (the number of plants that survived under selective conditions varied between 5% and 90%, depending on the mutant, and remained constant at each generation). Among the five cosuppression-deficient mutants, four exhibited downward-curled leaf margins, a phenotype previously observed in *ago7 (zip)*, *dcl4*, *rdr6 (sgs2)*, and *sgs3* mutants recovered from a screen for early juvenile-to-adult vegetative transition [1, 6, 19]. Complementation analyses indicated that the four cosuppression-deficient mutants exhibiting downward-curled leaf margins corresponded to two novel *rdr6* alleles and two novel *sgs3* alleles, which are hereafter referred to as *rdr6-5*, *rdr6-6*, *sgs3-7*, and *sgs3-8*. The fifth cosuppression-deficient mutant recovered from the 2a3 screen was morphologically indistinguishable from wild-type Col (Figure 1), but genetic analyses revealed that this mutant belonged to the *sgs3* complementation group. Sequencing of the *SGS3* gene in this mutant (hereafter referred to as *sgs3-9*) revealed a single point mutation, which leads to a Glu-to-Ala amino acid substitution at position 500 of the protein, identical to the mutation found in the *sgs3-3* allele previously recovered from the L1 genetic screen [12]. The possibility of seed contamination was eliminated because the *sgs3-3* and *sgs3-9* mutants contained signatures of the L1 and 2a3 transgenes, respectively.

Hypomorphic *rdr6* and *sgs3* Alleles Are Defective in PTGS but Not in Leaf Development

The identification of an *sgs3* allele that exhibits a wild-type leaf phenotype prompted us to examine the phenotypic effect of the six *sgs3* and twenty-three *rdr6/sgs2* alleles previously identified in our screen for impaired

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Figure 1. Morphological Characterization of *ago7*, *dcl4*, *drb4*, *rdr6*, *sgs3*, *tas1*, *tas2*, and *tas3* Mutants

rdr6/sgs2-5, *rdr6/sgs2-18*, *sgs3-9*, *tas1b-1*, and *tas2-1* mutants do not display obvious vegetative defects, whereas *ago7-1*, *dcl4-2*, *drb4-1*, *rdr6/sgs2-1*, *sgs3-1*, and *tas3-1* mutants have downward-curled leaf margins. All plants are in the Col ecotype. Pictures were taken after 21 days of growth. The average length and width of the sixth rosette leaf of ten individual plants were measured after 26 days of growth, and the average length divided by width and standard deviations are reported under the photographs. Measurements were taken at the widest and longest point of each leaf. Mutants that developed a sixth leaf with an average length divided by width superior to three are highlighted in gray.

L1 silencing [10, 12, 13]. Indeed, among these 29 PTGS-deficient mutants, only *rdr6/sgs2-1*, *rdr6/sgs2-2*, and *sgs3-1* have been reported to have defects in leaf development, and the remaining 26 have not been examined for developmental defects [2, 4]. Overall, five of the six *sgs3* alleles led to downward curling of the leaf margins, but the *sgs3-3* mutant plants exhibited a wild-type leaf phenotype (data not shown), confirming that the mutation found in *sgs3-3* and *sgs3-9* alleles does not affect leaf development. Among the twenty-three *rdr6/sgs2* alleles previously identified, 21 led to downward curling of leaf margins, whereas the *rdr6/sgs2-18* mutant plants exhibited a wild-type leaf phenotype, and the *rdr6/sgs2-5* mutant plants had a near-wild-type phenotype (Figure 1). Sequencing of the RDR6 gene in these two alleles revealed point mutations that led to Pro-to-Leu and Asp-to-Asn substitutions at positions 611 and 825 of the protein, respectively. Because GUS mRNA levels are similar in null alleles with leaf defects and hypomorphic alleles with wild-type leaves (Figure 2A), these results indicate that PTGS and developmental defects can be uncoupled in hypomorphic *rdr6* and *sgs3* mutants, analogous to the uncoupling of PTGS and development observed in hypomorphic *ago1* mutants [11] and in hypomorphic *dcl4* mutants [20], confirming that PTGS is more sensitive than development to perturbations in the siRNA machinery.

Hypomorphic *rdr6* and *sgs3* Alleles, like Null *rdr6* and *sgs3* Alleles, Overaccumulate CMV RNA

We previously reported that null *hen1*, *rdr6*, and *sgs3* alleles, as well as hypomorphic *ago1* alleles recovered from the L1 screen, exhibited hypersusceptibility to cucumber mosaic virus (CMV) infection, a result that correlated with the overaccumulation of CMV RNA [11–13]. The fact that defects in PTGS and leaf phenotype can be uncoupled in hypomorphic *rdr6* and *sgs3* alleles prompted us to examine whether hypomorphic *rdr6* and *sgs3* alleles were hypersusceptible, like null *rdr6* and *sgs3* alleles, to CMV infection or tolerant, like wild-type plants, of CMV. Infection of L1, *rdr6/sgs2-1*, *rdr6/sgs2-5*, *rdr6/sgs2-18*, *sgs3-1*, *sgs3-3*, and *sgs3-9* with CMV revealed similar overaccumulations of CMV RNA in null alleles and hypomorphic alleles compared to wild-type L1 plants (Figure 2B). These results indicate that both transgene and virus PTGS are more sensitive than leaf development to perturbations in RDR6 and SGS3 functions, and they reinforce the hypothesis of mechanistic similarities between S-PTGS directed against sense transgenes and PTGS directed against CMV.

TAS3 ta-siRNAs Accumulate in Hypomorphic but Not in Null *rdr6* and *sgs3* Alleles

In addition to their role in PTGS, RDR6 and SGS3 are essential for the production of ta-siRNAs that direct the

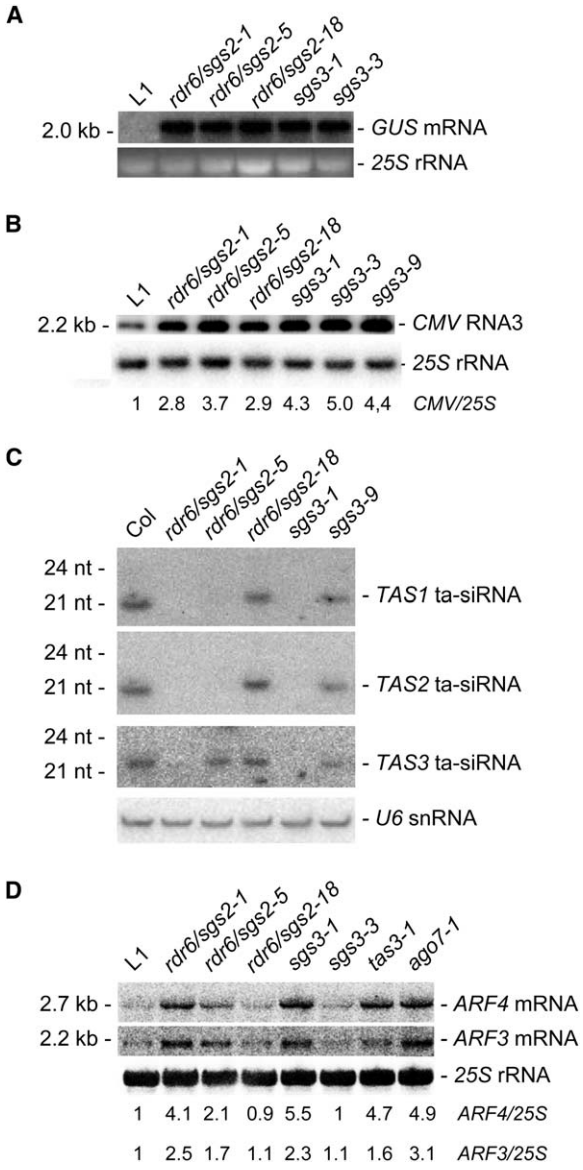

Figure 2. Molecular Characterization of Hypomorphic *rdr6*/*sgs2* and *sgs3* Mutants

(A) RNA gel-blot analysis of 5 μg total RNA prepared from mature leaves of wild-type L1 and mutant plants. The blot was probed with DNA complementary to GUS. 25S RNA served as a loading control.
(B) RNA gel-blot analysis of 0.5 μg total RNA prepared from mature leaves of wild-type L1 and mutant plants infected with CMV. The blot was successively probed with DNA complementary to the CP gene of CMV RNA3. 25S RNA served as a loading control.
(C) RNA gel-blot analysis of 10 μg total RNA prepared from mature leaves of wild-type L1 and mutant plants was successively probed with DNAs complementary to *TAS1*-, *TAS2*-, and *TAS3*-derived ta-siRNAs. U6 snRNA served as a loading control.
(D) RNA gel-blot analysis of 5 μg total RNA prepared from 17-day-old seedlings of L1 and mutant plants was successively probed with DNAs complementary to *ARF3* and *ARF4*. 25S RNA served as a loading control.

cleavage of several endogenous mRNAs [1–3, 6]. The identification of hypomorphic *rdr6* and *sgs3* mutants that do not exhibit leaf defects prompted us to analyze the accumulation of ta-siRNAs in these mutants. As reported previously, *TAS1*, *TAS2*, and *TAS3* ta-siRNAs were below detectable levels in the strong alleles *rdr6*/*sgs2-1* and *sgs3-1* (Figure 2C). In contrast, *TAS1*, *TAS2*, and *TAS3* ta-siRNAs were detected in the hypomorphic alleles *rdr6*/*sgs2-18* and *sgs3-9* (Figure 2C). *TAS3* but not *TAS1* or *TAS2* ta-siRNAs were detected in the hypomorphic mutants *rdr6*/*sgs2-5* (Figure 2C), indicating a hierarchy in the sensitivity of PTGS and ta-siRNAs to perturbations in the siRNA machinery. These results also suggest that *TAS3* but not *TAS1* or *TAS2* ta-siRNAs are required for proper leaf development.

ARF3/ETTIN and *ARF4* mRNAs Overaccumulate in Null but Not in Hypomorphic *rdr6* and *sgs3* Alleles

ARF3/*ETTIN* and *ARF4* were previously shown to be targets of *TAS3* ta-siRNAs [3] and to overaccumulate in *ago7*/*zip*, *rdr6*, and *sgs3* null alleles identified in a screen for precocious transition from juvenile to adult vegetative phase [1]. RNA gel-blot analysis of *ARF3*/*ETTIN* and *ARF4* in null and hypomorphic *rdr6* and *sgs3* alleles revealed a tight correlation between the level of *ARF3*/*ETTIN* and *ARF4* mRNA accumulation (Figure 2D) and the strength of leaf defects (Figure 1), suggesting that the upregulation of *ARF3*/*ETTIN* and *ARF4* may be responsible for leaf defects, either directly or indirectly. Consistent with this hypothesis, plants expressing a 35S-*ARF3*/*ETTIN* construct exhibit downward curling of leaf margins similar to that of *rdr6* and *sgs3* mutants [21]. Furthermore, because *TAS3* ta-siRNA levels are below detection and *ARF3*/*ETTIN* and *ARF4* mRNA levels are increased in null *rdr6* and *sgs3* alleles but not in hypomorphic alleles (Figures 2C and 2D), it is likely that the regulation of *ARF3*/*ETTIN* and *ARF4* by *TAS3* ta-siRNAs is essential for proper leaf development.

tas3 Mutants Exhibit Defects in Leaf Morphology Similar to Those Observed in Null *ago7/zip*, *rdr6*, and *sgs3* Alleles

We previously reported that the *tas1a-1* mutant seedlings carrying a T-DNA inserted downstream of the location from which the ta-siRNAs derive (Figure 3A) exhibited a dramatic reduction of the accumulation of *TAS1* ta-siRNAs [2], indicating that the *TAS1a* locus contributes more *TAS1* ta-siRNAs than the *TAS1b* and *TAS1c* loci at this stage of development. A dramatic reduction in the accumulation of *TAS1* ta-siRNAs was also observed in mature leaves of *tas1a-1* (Figure 3B), whereas the accumulation of *TAS1* ta-siRNAs was unchanged in *tas1b-1* (Figure 3B), a mutant carrying a T-DNA inserted within the location of ta-siRNAs in the *TAS1b* locus (Figure 3A), confirming the predominant expression of the *TAS1a* locus in vegetative tissues. The *tas1a-1* and *tas1b-1* mutants did not show obvious developmental defects [2], suggesting that the lack of *TAS1* ta-siRNAs was not responsible for the leaf developmental defects observed in null *rdr6* and *sgs3* mutants.

The *tas2-1* mutant carrying a T-DNA inserted downstream of the location from which the ta-siRNAs derive (Figure 3A) did not accumulate detectable *TAS2* ta-siRNA levels (Figure 3B) and did not show obvious leaf developmental defects (Figure 1), confirming that *TAS2* ta-siRNAs are not required for proper leaf development [6].

In contrast, the *tas3-1* mutant exhibited leaf developmental defects similar to those of null *ago7*/*zip*, *rdr6*,

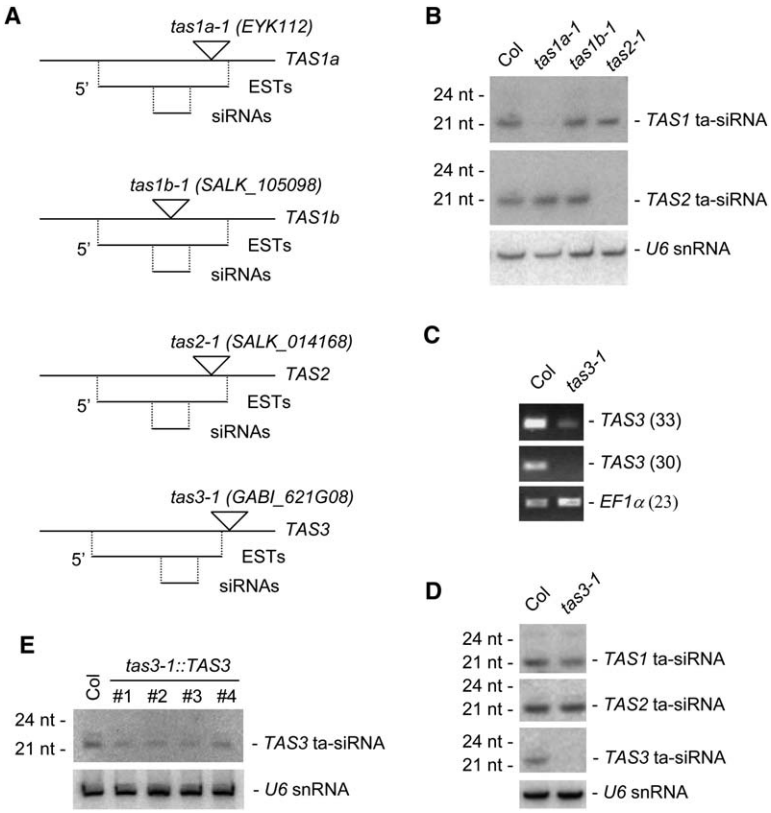

Figure 3. Molecular Characterization of *tas1*, *tas2*, and *tas3* Mutants

(A) Structure of the *TAS* loci in wild-type Col and *tas* mutants. The location of *TAS* ESTs and of the ta-siRNAs is indicated. The T-DNA inserts in *tas* mutants are indicated by triangles.

(B) RNA gel-blot analysis of 10 μg total RNA prepared from mature leaves of wild-type Col and *tas1* and *tas2* mutants. The blot was successively probed with DNAs complementary to *TAS1* and *TAS2* ta-siRNAs. U6 snRNA served as a loading control.

(C) RT-PCR analysis of 1 μg total RNA prepared from mature leaves of wild-type Col and *tas3* mutants. *TAS3* precursor RNA was amplified by using primers located on both sides of the miR390 complementarity site. *EF1α* was used as a control. The number of cycles of amplification is indicated in parentheses.

(D) RNA gel-blot analysis of 10 μg total RNA prepared from mature leaves of wild-type Col and *tas3* mutants. The blot was successively probed with DNAs complementary to *TAS1*, *TAS2*, and *TAS3* ta-siRNAs. U6 snRNA served as a loading control.

(E) RNA gel-blot analysis of 10 μg total RNA prepared from mature leaves of wild-type Col and four independent *tas3-1* transformants in which wild-type leaf development has been restored by introduction of *TAS3* wild-type genomic fragment.

and *sgs3* mutants (Figure 1). Although the T-DNA was inserted downstream of the location of the *TAS3* ESTs (Figure 3A), the *tas3-1* mutant exhibited a severe reduction in the accumulation of *TAS3* precursor RNA (Figure 3C), did not accumulate detectable *TAS3* ta-siRNAs (Figure 3D), and accumulated *ARF3*/*ETTIN* and *ARF4* mRNAs at levels similar to those of null *rdr6* and *sgs3* mutants (Figure 2D). We assume that the T-DNA in *tas3-1* negatively impacts the transcription of the TAS3 locus in *cis* because transformation of the *tas3-1* mutant with a genomic fragment carrying the wild-type *TAS3* locus restored wild-type leaf morphology (data not shown) and *TAS3* ta-siRNA accumulation (Figure 3E), confirming that *TAS3* ta-siRNAs are required for proper leaf development.

ago7/zip Mutants Lack *TAS3* but Not *TAS1* or *TAS2* ta-siRNAs

In many eukaryotes, at least one member of the Argonaute family associates with small RNAs and catalyzes mRNA cleavage [22]. In *Arabidopsis*, AGO1 associates with miRNAs and cleaves mRNA:miRNA duplexes [23, 24]. Although the association of AGO1 with miR173 and miR390 specifically has not been reported, it is likely that AGO1 cleaves *TAS* precursor RNAs paired with miR173 and miR390 as it cleaves other miRNA-targeted mRNAs. Consistent with this hypothesis, we could not detect *TAS1*, *TAS2*, and *TAS3* ta-siRNAs in leaves of the *ago1-1* null allele (Figure 4A). AGO1 also associates with the *TAS1*-derived ta-siRNA sRNA255/siR480(+) [24], suggesting that AGO1 could cleave mRNAs paired with ta-siRNAs. However, *ago7/zip* null alleles exhibit a phenotype similar to that of null *rdr6* and *sgs3* alleles [1, 2, 19], suggesting that AGO7/ZIPPY plays a role in the ta-siRNA pathway. In addition, *ago7/zip* mutants exhibited increased accumulation of the *TAS3* ta-siRNA target mRNAs *ARF3*/*ETTIN* and *ARF4* (Figure 2D), but not of *TAS1* and *TAS2* ta-siRNA target mRNAs [1–3, 6, 25], suggesting that AGO7/ZIPPY acts specifically during *TAS3*-derived ta-siRNA-mediated regulation. To test this hypothesis, we analyzed the accumulation of ta-siRNAs in *ago7/zip*. Unlike *ago1* mutants that lacked *TAS1*, *TAS2*, and *TAS3* ta-siRNAs, *ago7/zip* mutants accumulated *TAS1* and *TAS2* ta-siRNAs but lacked *TAS3* ta-siRNAs (Figure 4A), consistent with the similar phenotype of *ago7/zip* and *tas3* mutants (Figure 1) and the similar increase in *ARF3*/*ETTIN* and *ARF4* mRNA accumulation in *ago7/zip* and *tas3* mutants (Figure 2D). Conversely, *TAS3* precursor RNAs accumulated at low levels in *tas3* mutants (Figure 3C) but accumulated at similar levels in wild-type plants and *ago7/zip* mutants (Figure 4B), which rules out the hypothesis that AGO7/ZIPPY could associate with miR390 to cleave *TAS3* precursor RNAs. Rather, it is likely that AGO1 associates with miR390 like any other miRNA and cleaves *TAS3* precursor RNAs. After DCL4-mediated processing, AGO7/ZIPPY specifically associates with *TAS3* ta-siRNAs and cleaves their targets, including *ARF3*/*ETTIN* and *ARF4*. We propose that the specific association of *TAS3* ta-siRNAs with AGO7/ZIPPY may be related to the fact that miR390-guided AGO1-catalyzed cleavage of *TAS3* precursor RNA occurs downstream of the location of *TAS3* ta-siRNAs, whereas miR173-guided AGO1-catalyzed cleavage *TAS1* and *TAS2* precursor RNA occurs upstream of the location of *TAS1* and *TAS2* ta-siRNAs.

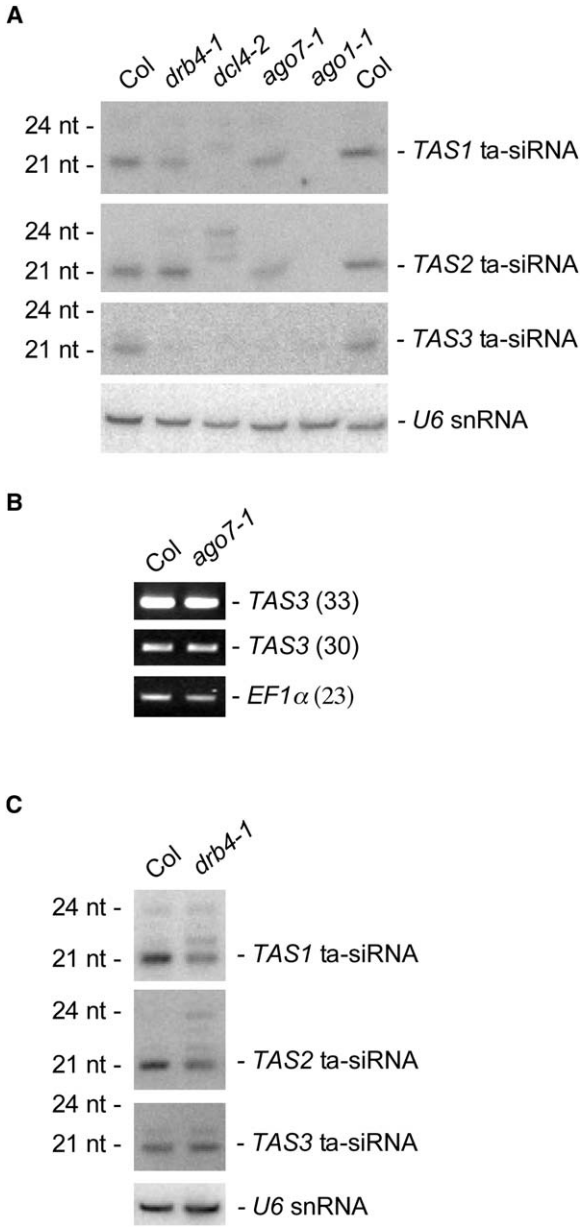

Figure 4. Molecular Characterization of *ago1*, *ago7*, *dcl4*, and *drb4* Mutants

(A) RNA gel-blot analysis of 10 μg total RNA prepared from mature leaves of wild-type Col and *ago1*, *ago7*, *dcl4*, and *drb4* mutants. The blot was successively probed with DNAs complementary to *TAS1*-, *TAS2*-, and *TAS3*-derived ta-siRNAs. U6 snRNA served as a loading control.

(B) RT-PCR analysis of 1 μg total RNA prepared from mature leaves of wild-type Col and *ago7* mutants. *TAS3* precursor RNA was amplified by using primers located on both sides of the miR390 complementarity site. *EF1α* was used as a control. The number of cycles of amplification in indicated in parentheses.

(C) RNA gel-blot analysis of 10 μg total RNA prepared from floral infloresences of wild-type Col and *drb4* mutants. The blot was successively probed with DNAs complementary to *TAS1*-, *TAS2*-, and *TAS3*-derived ta-siRNAs. U6 snRNA served as a loading control.

Production of ta-siRNAs Requires the HYL1-like DRB4 dsRNA Binding Protein

Distinct dsRNA binding proteins specifically associate with RNaseIII proteins of the Dicer and Drosha families to produce miRNAs and siRNAs. For example, in *Drosophila*, Pasha interacts with Drosha and Loq interacts with Dicer-1 to produce miRNAs, whereas R2D2 interacts with Dicer-2 to produce siRNAs [26]. In plants, HYL1 interacts with DCL1 in vitro [8], and *hyl1* mutants are impaired in miRNA production [7, 9]. Four HYL1-like dsRNA binding proteins, DRB2–DRB5, exist in the *Arabidopsis* genome, and DRB4 has been shown to interact with DCL4 in vitro [8]. We identified a mutant that carries a T-DNA inserted between the start of transcription and the first ATG of the *DRB4* gene and that lacks detectable *DRB4* mRNA (data not shown). The *drb4-1* mutant exhibited defects in leaf morphology similar to those of *ago7/zip*, *dcl4*, *rdr6*, *sgs3*, and *tas3* mutants (Figure 1), although there were subtle differences, which may result from the existence of partially redundant proteins as well as slight differences in the genetic background. Consistent with the defects in leaf morphology, *TAS3* ta-siRNAs were below detectable levels in *drb4-1* leaves (Figure 4A). *TAS1* and *TAS2* ta-siRNAs were detectable (Figure 4A), suggesting that DRB4 may be more stringently required for the production of *TAS3* ta-siRNAs than other ta-siRNAs in leaves. However, levels of *TAS3* ta-siRNA were unchanged in *drb4-1* flowers, whereas levels of *TAS1* and *TAS2* ta-siRNAs were reduced (Figure 4C). In addition, 22–24 nt *TAS1* and *TAS2* siRNAs, similar to those observed in *dcl4-1* [4] and *dcl4-2* [5], were detected in *drb4-1* flowers (Figure 4C). These results indicate that DRB4 is required for a proper production of *TAS1*, *TAS2*, and *TAS3* ta-siRNAs. They also suggest that DRB4 deficiency can be functionally compensated by distinct DRBs that allow efficient production of *TAS1* and *TAS2* or *TAS3* ta-siRNAs in leaves and flowers, respectively. The phenotypic and molecular similarities between *dcl4* and *drb4* confirm the interaction between DRB4 and DCL4 observed in vitro and suggest that in the absence of either DCL4 or DRB4, *TAS* dsRNA can be processed into siRNAs of different sizes by either other DCLs associated with DRB4 or DCL4 associated with other DRBs.

Conclusions

Hypomorphic *rdr6* and *sgs3* alleles were identified in a screen for S-PTGS-deficient mutants, reminiscent of the identification of hypomorphic *ago1* mutants in the same screen [11], and of hypomorphic *dcl4* mutants in a screen for IR-PTGS-deficient mutants [20]. Uncoupling transgene-induced PTGS from developmental defects in *ago1*, *dcl4*, *rdr6*, and *sgs3* hypomorphic alleles indicates that transgene-induced PTGS is generally more sensitive than development to small perturbations in the siRNA pathway. Impairment of the PTGS machinery in null *rdr6* and *sgs3* alleles allows high levels of recombinant proteins to be produced from strongly expressed transgenes, which often trigger S-PTGS in wild-type plants [10, 27]. We believe that hypomorphic *rdr6* and *sgs3* alleles will be useful to understand the role of a given gene through overexpression studies because they are impaired in PTGS as null alleles are but do not exhibit the obvious developmental defects of null alleles [1].

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures and are available with this article online at: http://www.current-biology.com/cgi/content/full/16/9/927/DC1/.

Acknowledgments

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