A pathway for the biogenesis of trans-acting siRNAs in Arabidopsis

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The Arabidopsis genes, TAS2 and TAS1a, produce structurally similar noncoding transcripts that are transformed into short (21-nucleotide [nt]) and long (24-nt) siRNAs by RNA silencing pathways. Some of these short siRNAs direct the cleavage of protein-coding transcripts, and thus function as trans-acting siRNAs (ta-siRNAs). Using genetic analysis, we defined the pathway by which ta-siRNAs and other short siRNAs are generated from these loci. This process is initiated by the miR173-directed cleavage of a primary poly[A] transcript. The 3' fragment is then transformed into short siRNAs by the sequential activity of SGS3, RDR6, and DCL4: SGS3 stabilizes the fragment, RDR6 produces a complementary strand, and DCL4 cleaves the resulting double-stranded molecule into short siRNAs, starting at the end with the miR173 cleavage site and proceeding in 21-nt increments from this point. The 5' cleavage fragment is also processed by this pathway, but less efficiently. The DCL3-dependent pathway that generates long siRNAs does not require miRNA-directed cleavage and plays a minor role in the silencing of these loci. Our results define the core components of a post-transcriptional gene silencing pathway in Arabidopsis and reveal some of the features that direct transcripts to this pathway.

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MicroRNAs (miRNAs) and small interfering RNAs (siRNAs) are 20–24-nucleotide [nt] noncoding RNAs that repress gene expression through sequence-specific interactions with RNA or DNA (Bartel 2004; Tomari and Zamore 2005). These small RNAs serve as guide molecules for protein complexes that mediate RNA degradation, translational repression, or heterochromatin production. miRNAs and siRNAs are distinguished by the structure of their precursors and by their targets. miRNAs are cleaved from the short, imperfectly paired stem of a much larger foldback transcript and regulate the expression of transcripts to which they may have limited similarity. siRNAs arise from a long, perfectly paired double-stranded RNA (dsRNA) and typically direct the cleavage of transcripts to which they are completely complementary, including the transcript from which they are derived. Recently, we [Peragine et al. 2004] and others [Vazquez et al. 2004b; Allen et al. 2005; Williams et al. 2005] described a novel class of endogenous siRNAs in Arabidopsis termed “trans-acting siRNAs” (ta-siRNAs). ta-siRNAs are endogenous siRNAs that direct the cleavage of nonidentical transcripts. ta-siRNAs are therefore functionally similar to miRNAs in that they regulate the expression of genes to which they have limited similarity.

miRNAs were first discovered as components of the heterochronic pathway in Caenorhabditis elegans (Lee et al. 1993; Reinhart et al. 2000) and have since been found in abundance in animals and plants [http://microrna.sanger.ac.uk/sequences], where they regulate a wide variety of developmental and physiological processes [Ambros 2004; Kidner and Martienssen 2005]. The normal function of endogenous siRNAs is less well understood. siRNAs derived from transposons and highly repeated centromeric sequences have been identified in several organisms and are thought to contribute to the transcriptional silencing of these sequences [Aravin et al. 2001, 2003; Dijkeng et al. 2001; Hamilton et al. 2002; Llave et al. 2002a; Rudenko et al. 2003; Sijen and Plasterk 2003; Chicas et al. 2004; Xie et al. 2004]. Endogenous siRNAs are likely to have a broader range of functions in C. elegans and Arabidopsis, however, as both of these organisms also produce siRNAs from single copy protein-coding and noncoding transcripts [Llave et al. 2002a; Reinhart et al. 2002, Ambros et al. 2003; Xie et al. 2004].

Plants produce two major size classes of siRNAs from endogenous transcripts and transgenes: ≈24-nt siRNAs [long siRNAs], and ≈21-nt siRNAs [short siRNAs] [Hamilton and Baulcombe 1999; Tang et al. 2003]. These types of siRNAs are produced by different pathways and have different functions. Genetic analysis in Arabidopsis indicates that long siRNAs are generated from transcripts produced by the RNA polymerase, POL IV [Herr
et al. 2005; Onodera et al. 2005; Yu et al. 2005], and require the RNA-dependent RNA polymerase (RdRp) RDR2, the Dicer-like protein DCL3 [Xie et al. 2004], the Argonaute protein AGO4 [Zilberman et al. 2003], and the methyl transferase HEN1 [Liu et al. 2004] for their biogenesis and/or stability. Long siRNAs mediate transcriptional silencing by promoting DNA and histone methylation [Hamilton and Baulcombe 1999; Zilberman et al. 2003; Liu et al. 2004; Xie et al. 2004]. Although most endogenous siRNAs are of this type, the biological function of this class of siRNAs is unknown because morphological or physiological phenotypes associated with mutations in the above-mentioned genes have not been described. ta-siRNAs and other short siRNAs are associated with post-transcriptional gene silencing (PTGS, also known as RNAi) and require the RdRp RDR6/SDE1/SGS2 [hereafter called RDR6] and novel (PTGS, also known as RNAi) and require the RdRp have been described. ta-siRNAs and other short siRNAs are mediate biogenesis and/or stability. Long siRNAs mediate transcriptional silencing by promoting DNA and histone methylation [Hamilton and Baulcombe 1999; Zilberman et al. 2003; Liu et al. 2004; Xie et al. 2004]. Although most endogenous siRNAs are of this type, the biological function of this class of siRNAs is unknown because morphological or physiological phenotypes associated with mutations in the above-mentioned genes have not been described. ta-siRNAs and other short siRNAs are associated with post-transcriptional gene silencing (PTGS, also known as RNAi) and require the RdRp RDR6/SDE1/SGS2 [hereafter called RDR6] and novel plant-specific protein SGS3 [Peragine et al. 2004; Vazquez et al. 2004b; Allen et al. 2005]. Mutations in RDR6 and SGS3 affect the timing of vegetative phase change and floral morphogenesis [Peragine et al. 2004], implying that at least some of the endogenous targets of small siRNAs have important biological functions.

Five loci have been shown, or predicted, to generate ta-siRNAs in Arabidopsis. Three of these [TAS1a, TAS1b, and TAS1c] produce identical or very closely related ta-siRNAs that target a group of related genes of unknown function [Peragine et al. 2004; Vazquez et al. 2004b]. TAS3 produces a ta-siRNA directed against the auxin-related transcription factors ARF2, ARF3, and ARF4 [Allen et al. 2005; Williams et al. 2005]. TAS2 has been predicted to produce ta-siRNAs that target the pentatrioside repeat [PPR] genes, At1g12770 and At1g63130 [Allen et al. 2005], although there is still no evidence for this function. Allen et al. (2005) showed that the production of siRNAs from TAS2 and TAS1a in a heterologous system requires miR173-directed cleavage of their primary transcripts and presented evidence that the biogenesis of ta-siRNAs from TAS3 may also be mediated by miRNA-directed cleavage.

Here we show that TAS2 and TAS1a produce ta-siRNAs directed against PPR genes and are likely to represent homologous loci. These genes produce structurally related noncoding transcripts that are processed into both short and long siRNAs. Genetic analysis of the biogenesis of short siRNAs demonstrated that this class of siRNAs is produced exclusively from the miRNA-generated cleavage fragments of TAS2 and TAS1a by a pathway involving SGS3, RDR6, and DCL4. Our results provide a comprehensive picture of this pathway and suggest the likely functions of these proteins in PTGS in plants.

**Results**

**At2g39680 (TAS2) is a source of ta-siRNAs**

At2g39680 (TAS2) was identified in a microarray analysis of RNA levels in zip-1, sgs3-11, and rdr6-11 [Peragine et al. 2004]. Among the genes identified in this study, TAS2 was unique in that it was elevated approximately fourfold in rdr6-11 but was not affected by zip-1 or sgs3-11. A search of the ASRP database [Gustafson et al. 2005] revealed that many siRNAs have been cloned from TAS2. BLAST searches conducted with these cloned siRNAs did not reveal obvious targets. However, searches conducted with the hypothetical TAS2 coding sequence indicated that it has four 20–30-nt regions with reverse complementarity to a family of PPR genes located on chromosome 1 [Fig. 1A; Lurin et al. 2004].

To determine if these PPR transcripts are cleaved at these complementary sites, we took advantage of an RNA ligase-mediated rapid amplification of 5` cDNA ends [RLM-5` RACE] protocol that has been widely used to identify cleavage sites in mRNA [Liu and Goro sky 1993]. For each of the three transcripts tested [At1g63130, At1g63230, and At1g62930], RLM-5` RACE yielded PCR products of the expected size with wild-type RNA [Fig. 1B]. Sequencing of these products revealed that each transcript is cleaved in the middle of the complementary region, at a site located 10 nt from a 3` A [Fig. 1A]; this is the pattern expected for cleavage mediated by a miRNA or siRNA [Elbashir et al. 2001; Llave et al. 2002b, Kasschau et al. 2003]. Three closely spaced cleavage sites were identified in At1g62930, suggesting that this gene is targeted by several related siRNAs [see below]. Blots of low-molecular-weight [LMW] RNA hybridized with oligonucleotide probes complementary to the TAS2 sequences in Figure 1A and to the cloned TAS2 siRNA, siR1511, revealed 21-nt siRNAs corresponding to these sequences in wild-type and zip-1 plants, but not in sgs3-11 and rdr6-11 [Fig. 1C]. Consistent with this result, cleavage products from At1g63130, At1g63230, and At1g62930 could not be detected by RLM-5` RACE in these mutants [Fig. 1B]; furthermore, quantitative RT-PCR performed with primers that flank the cleavage sites in At1g63130 and At1g62930 revealed that these transcripts are elevated two- to threefold in both mutants [Fig. 1D]. sgs3-11 and rdr6-11 had only a small effect on the accumulation of At1g63230, indicating that siRNAs play a minor role in the regulation of this gene. To confirm that these transcripts are targets of TAS2-derived siRNAs, we examined the phenotype of a T-DNA insertion in TAS2 [Fig. 2A] that reduces the production of siR1511 but does not affect the production of the TAS1a ta-siRNA, siR255 [Fig. 1E]. The abundance of At1g63130, At1g63230, and At1g62930 in plants homozygous for SALK_014168 was intermediate between the wild-type level and the level in sgs3-11 or rdr6-11 [Fig. 1D], consistent with the intermediate level of siR1511 in this mutant [Fig. 1E]. We conclude that TAS2 produces at least four distinct ta-siRNAs directed against PPR genes. SALK_014168 does not have an obvious effect on shoot morphology [data not shown], so the hyperaccumulation of PPR transcripts in sgs3-11 and rdr6-11 does not account for their precocious vegetative phenotype [Peragine et al. 2004].

**The primary transcripts of TAS2**

The transcripts produced from TAS2 were characterized in detail to determine how ta-siRNAs are generated from
nucleotide probes complementary to the TAS2 Amplification of the miR171 target, SCL6-IV sequenced (Fig. 2A). The predicted TAS2 this locus. Several existing TAS2 cDNAs were obtained from the Arabidopsis Biological Resource Center and re-sequenced (Fig. 2A). The predicted TAS2 open reading frame (ORF) is present in a 659-bp clone that has no poly[A] tail (GenBank BT011210). This ORF is unusually short and is not conserved in other plants, and so is unlikely to actually encode a protein. The other cDNAs from this region have a poly[A] tail and are antisense to this clone. The longest of these, M74P20STM (GenBank DQ000174), is 993 nt. A 602-nt clone, 205C13 (GenBank DQ000172), terminates in the middle of M74P20STM but has the same 3' end, suggesting that it represents a derivative of this longer transcript. Because most siRNAs from this locus correspond to the strand represented by these clones, we refer to this strand as the (+) strand, and refer to the strand that contains the ORF as the (−) strand.

The primary TAS2 transcripts were identified by performing 5' and 3' RACE with primers specific for (+) or (−) strands. Two (+) transcripts were identified by 3' RACE performed with a primer located near the 5' end of M74P20STM (Fig. 2A). Both transcripts have the same 3' end, but TAS2(+1) [GenBank DQ000170] contains a 569-nt intron that is spliced out of TAS2(Δ+) [GenBank DQ000171]. 5' RACE performed with a primer within this intron and a primer on the 3' side of the intron produced products with the same 5' end, suggesting TAS2(+) and TAS2(Δ+) are transcribed from the same promoter. This experiment also suggests that these transcripts have a 5' cap, since uncapped transcripts are not amplified by the standard 5' RACE protocol. The 5' end determined by this approach is located 62 nt upstream of the 5' end of the M74P20STM cDNA. Attempts to determine the 5' and 3' ends of the (−) transcript failed, probably because of the low abundance of this transcript [see below].

To identify cleaved TAS2(+1) transcripts, total floral RNA was subjected to RLM-5' RACE, using a primer located near the 3' end of TAS2(+1). This experiment yielded products that terminated at a single site within the intron in TAS2(+1). This site is identical to the 5' end of the cDNA 205C13 (Fig. 2A), indicating this cDNA corresponds to a 3' cleavage fragment of TAS2(+1). The corresponding 5' cleavage fragment was characterized by amplifying self-ligated transcripts by RT–PCR (Couttet et al. 1997). This experiment identified a transcript with a 5' end that matches the 5' end of the (+1) transcript, and a 3' end that terminates at the cleavage site identified by RLM-5' RACE. Three out of 12 self-ligated clones had one or two untemplated adenines at their 3' end. Untemplated uridines are added to the 3' end of the cleavage fragments of some miRNA-targeted transcripts in Arabidopsis (Shen and Goodman 2004), suggesting that the 5' fragment of TAS2(+1) may be processed in a similar fashion. We conclude that TAS2(+1) is cleaved into a 442-nt 5' fragment, TAS2(+5'), and a 613-nt 3' fragment, TAS2(+3'), by an RNAi-like mechanism (Fig. 2A).

The transcripts produced from TAS2 were further characterized by hybridizing blots of total and poly(A) RNA from wild-type Columbia (Col) and rdr6-11 mutants with a double-stranded probe [p1] that spans this cleavage site, as well as with short strand-specific probes [Fig. 2C]. All of the transcripts detected with the p1 probe hybridized to (+) strand-specific probes but not to (−) strand-specific probes, demonstrating that TAS2 is transcribed primarily in the (+) orientation [Fig. 2D], data

**Figure 1.** Atg39680 (TAS2) produces ta-siRNAs that target PPR genes. [A] Sequences in TAS2 that have reverse complementarity to miRNAs encoding PPR proteins. Fractions indicate the number of sequenced RLM-5' RACE clones that terminated at the indicated position. (B) sgs3-11 and rdr6-11 prevent the formation of RLM-5' RACE products from PPR transcripts targeted by siRNAs from TAS2. Amplification of the miR171 target, SCL6-IV, occurs normally in these mutants. (C) Blots of LMW RNA hybridized with oligo C+ (−) strands. Two (+) transcripts were identified by 3' RACE performed with primers specific for (+) or (−) strands. Three out of 12 self-ligated clones had one or two untemplated adenines at their 3' end. Untemplated uridines are added to the 3' end of the cleavage fragments of some miRNA-targeted transcripts in Arabidopsis (Shen and Goodman 2004), suggesting that the 5' fragment of TAS2(+1) may be processed in a similar fashion. We conclude that TAS2(+1) is cleaved into a 442-nt 5' fragment, TAS2(+5'), and a 613-nt 3' fragment, TAS2(+3'), by an RNAi-like mechanism (Fig. 2A).

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the number of RLM-5/H11032 accumulate to high levels in this mutant. (TAS2E)
transcript is not affected by Blots of total and poly(A)-selected RNA hybridized with the indicated
TAS2 transcript is targeted by TAS2 strand probes. (−) also present at very low levels because it was not de-
potentially pair with this sequence with one mismatch sequence of known miRNAs revealed that miR173 can
indicate that the cleavage fragments of single-stranded molecules in this mutant. These results
hybridization was detected with (−) strand probes, indicating that TAS2(+5′) and TAS2(+3′) accumulate as single-stranded molecules in this mutant. These results indicate that the cleavage fragments of TAS2(+)—but not TAS2(+) itself—are the targets of an RDR6-dependent si-lencing pathway. The 3′ fragment appears to be processed more efficiently than the 5′ fragment because it is present at much lower levels than the 5′ fragment in wild-type plants (Fig. 2D). The (−) transcript of TAS2 is also present at very low levels because it was not detected with (−) strand probes.

TAS2 is targeted by miR173 and is homologous to TAS1a, TAS1b, and TAS1c
A comparison of the cleavage site in TAS2(+) with the sequence of known miRNAs revealed that miR173 can potentially pair with this sequence with one mismatch and a single G-U pair (Fig. 2E). As is typical for miRNA-directed cleavage [Kasschau et al. 2003], the cleavage site in TAS2(+) is 10 nt from the 5′ end of miR173. Three previously identified sources of ta-siRNAs—TAS1a (At2g27440) and TAS1b (At1g50055) and TAS1c (At2g39675)—have a sequence that is nearly identical to this putative miR173 target sequence and are cleaved at the same position within this sequence as TAS2(+) [Fig. 2E]. TAS1a resembles TAS2 in that it produces a 1-kb primary transcript with a single ≈570-nt differentially spliced intron [Vazquez et al. 2004b]. The miR173 site is located within this intron in the same position as the miR173 site in TAS2 (Fig. 2A,B). Although the TAS1 loci do not have extended sequence similarity to TAS2, all three loci can theoretically pro-
duce siRNAs that target PPR (Fig. 3A,B). Indeed, the location of the cleavage sites in At1g12770 indicate that it is targeted by a ta-siRNA from TAS1a, as well as by a ta-siRNA from TAS2 (Fig. 3C). These observations, and the fact that TAS2 is immediately adjacent to TAS1c on chromosome 2, strongly suggest that TAS1a, TAS1b, TAS1c, and TAS2 are paralogous loci.

miRNA-directed cleavage is required for the biogenesis of short, but not long, siRNAs
Indirect evidence that miR173-directed cleavage is re-
required for the biogenesis of ta-siRNAs and other short
siRNAs from TAS2(+) and TAS1a(+), as well as from all three loci in these transcripts (Fig. 3A; Vazquez et al. 2004b; Allen et al. 2005). Most of the siRNAs that have been cloned from these loci, as well as all the hypothetical ta-siRNAs that target PPR genes, are spaced in near perfect 20–21-nt increments

Figure 2. TAS2(+) and TAS1(+), as well as all the hypothetical ta-siRNAs that target PPR genes, are spaced in near perfect 20–21-nt increments.
and are in perfect register with the miR173 cleavage site. Most significantly, the TAS2 ta-siRNAs that target PPR genes are located in the sixth, ninth, 11th, and 12th positions from this site (Fig. 3A). This spacing pattern is consistent with the evidence that Dicer cleaves double-stranded RNA sequentially from the end of the molecule [Elbashir et al. 2001; Ketting et al. 2001; Zhang et al. 2002; Siolas et al. 2004] and suggests that that miR173-targeted transcripts that are cleaved 10 nt 3’ of the miR173 cleavage site destabilizes portions of TAS2(+) because this event generates an end that is an appropriate substrate for DCL.

Loss-of-function mutations in DCL1, HYLI, and HST reduce or eliminate the accumulation of many miRNAs but have no effect on post-transcriptional transgene silencing [Park et al. 2002, 2005; Finnegan et al. 2003; Han et al. 2004, Vazquez et al. 2004a], suggesting that these genes are involved primarily, if not exclusively, in the processing of miRNAs. miR173 was absent in dcl1-7, reduced in hyl-2, and was not affected by hst-6 [Fig. 4A]. Consistent with the effect of these mutations on miR173, cleavage of TAS2(+) was completely abolished in dcl1-7 and reduced in hyl-2 but occurred normally in hst-6 [Fig. 4B]. The accumulation of siR1511 and siR255 was directly correlated the amount of transcript cleavage in these mutants: These siRNAs were missing in dcl1-7, present at reduced levels in hyl-2, and unaffected by hst-6 [Fig. 4A]. These results support the conclusion that the biogenesis of short siRNAs from TAS2 and TAS1a requires miRNA-directed cleavage of their primary transcripts.

There is evidence that miRNAs associate with their target transcripts during transcription [Bao et al. 2004], but when and where they direct the cleavage of these transcripts is still unknown. The effect of SALK_014168 on the accumulation of the 5’ cleavage fragment of TAS2(+) suggests that cleavage of this transcript occurs after transcription is completed. Because the SALK_014168 T-DNA is inserted 3’ of the miR173 cleavage site [Fig. 2A], it should not interfere with the production of the 5’ fragment if this fragment is generated during transcription. However, plants homozygous for this T-DNA produce reduced amounts of the 5’ fragment of TAS2(+) and also produce an array of large transcripts

![Figure 3](image1.png)

**Figure 3.** The position and predicted nucleotide sequence of TAS2 and TAS1 siRNAs. (A) siRNAs arising from the 3’ fragment of TAS2, TAS1a, TAS1c, and TAS1b are positioned in 21-nt increments relative to the miR173 cleavage site (arrow). The sequence of these siRNAs is available from http://asrp.cgrb.oregonstate.edu. siRNAs in boxes are within 2 nt of the site predicted by a 21-nt spacing pattern. (B) The sequence of hypothetical ta-siRNAs from TAS2 and TAS1a, and the PPR genes targeted by these ta-siRNAs. Transcripts that are cleaved 10 nt 3’ of the miR173 cleavage site in red. Sequences that are similar in TAS2 and TAS1a are indicated by a bracket. (C) At1g12770 has two closely spaced cleavage sites potentially targeted by ta-siRNAs from TAS2 and TAS1a.

![Figure 4](image2.png)

**Figure 4.** Production of ta-siRNAs from TAS2(+) and TAS1a(+) requires miR173-directed cleavage. Blots of LMW RNA (A,C) or total RNA (B,D) hybridized with the indicated probes. (A) Mutations that interfere with the biogenesis of miR173 have correlated effects on the accumulation of siR1511 and siR255. (B) Cleavage of TAS2(+) in dcl1-7, hyl1-2, and hst-6 is inversely correlated with the abundance of miR173 in these mutants. (C) A T-DNA inserted 3’ of the miR173 cleavage site destabilizes TAS2(+) and reduces the accumulation of the 5’ cleavage fragment. (D) The 5’ and 3’ portions of TAS2 and TAS1a produce different size classes of siRNAs. Production of 24-nt siRNAs requires DCL3 but not DCL1. (E) dcl2-1 and dcl3-1 have no effect on the accumulation of TAS2(+) and TAS1a(+) or their cleavage products. See Figure 2B for the location of the probes used in these experiments.
that probably correspond to partially degraded cleaved and uncleaved transcripts containing the T-DNA [Fig. 4C]. This result demonstrates that the presence of the T-DNA destabilizes $TAS2^{(+)}$ and suggests that the reduced accumulation of 5′ fragment can be attributed to the post-transcriptional instability of the mutant transcript.

To obtain a more complete picture of the processing of $TAS2$ and $TAS1a$, blots of LMW RNA and total RNA from various mutants were hybridized with long, double-stranded probes to the 5′ [p2] and 3′ [p3] parts of these transcripts, as well as with a probe [p1] that spans the miR173 cleavage site. Blots of LMW RNA revealed that the 5′ region of these transcripts produces predominantly long siRNAs, whereas the 3′ region produces both long and short siRNAs, with short siRNAs being the more abundant class [Fig. 4D]. $zip$-1 and $dcl2$-1 had no effect on the silencing of these loci. $sgs3$-11, $rdr6$-11, and $dcl1$-7 prevented the accumulation of short siRNAs, but had little or no effect on the accumulation of long siRNAs, whereas $dc1$-31 exclusively prevented the accumulation of long siRNAs. In contrast to $dc1$-7, $dcl$-2 and $dc3$-1 had no effect on the accumulation of the primary $TAS2$ and $TAS1a$ transcripts or their cleavage fragments [Fig. 4E]. These results demonstrate that the biogenesis of long siRNAs does not require miRNA-directed cleavage and also indicate that long siRNAs are generated by a pathway distinct from the one that produces small siRNAs. The long siRNA pathway does not appear to contribute significantly to the silencing of $TAS2^{(+)}$ and $TAS1a^{(+)}$ because the accumulation of these transcripts is not affected by $dc1$-31 [Fig. 4E].

SGS3 and RDR6 act sequentially on the $TAS2^{(+)}$ and $TAS1a^{(+)}$ cleavage fragments

It is evident from the elevated level of $TAS2^{(+3′)}$ and $TAS2^{(+5′)}$ in $rdr6$-11 that these fragments are silenced by the RDR6 pathway. Is the primary $TAS2^{(+)}$ transcript also silenced by this pathway? Although we did not observe an increase in $TAS2^{(+)}$ in $rdr6$-11 plants [Fig. 2D], this transcript may be protected by its association with miR173. Primed RNA-induced silencing complex (RISC).

To eliminate this possibility, we examined the accumulation of $TAS2^{(+)}$ in $dcl1$-7 $rdr6$-11 double mutants, in which miR173 is absent and $TAS2^{(+)}$ is not cleaved. Double mutants had the same amount of $TAS2^{(+)}$ as $dcl1$-7 [Fig. 5A], confirming that that this transcript is not a substrate for the RDR6 pathway.

SGS3 acts in same pathway as RDR6 [Beclin et al. 2002, Peragine et al. 2004, Vazquez et al. 2004b], but its specific function and the order in which these genes operate is still unknown. To address these questions, we examined the accumulation of $TAS2^{(+)}$ and $TAS1a^{(+)}$ in $sgs3$-11 and $sgs3$-11 $rdr6$-11 double mutants. In contrast to $rdr6$-11—which causes a significant increase in the level of the cleavage fragments of these transcripts—$sgs3$-11 reduced the accumulation of the 5′ fragment, and completely eliminated the 3′ fragment [Fig. 5B]. $sgs3$-11 $rdr6$-11 double mutants had the same low level of these fragments as $sgs3$-11, indicating that SGS3 is required for the accumulation of these cleavage fragments in $rdr6$-11 mutants, i.e., $sgs3$-11 is completely epistatic to $rdr6$-11. These results indicate that SGS3 directly or indirectly stabilizes these cleavage fragments and acts before RDR6.

The phenotype of $sgs3$-11 suggests that SGS3 somehow protects the cleavage fragments of $TAS2^{(+)}$ and $TAS1a^{(+)}$ from RNases that act on single-stranded RNA. One such RNase is XRN4. XRN4 is a cytoplasmically localized 5′-3′ exonuclease that degrades the 3′ cleavage fragments of some miRNA-targeted genes [Souret et al. 2004] and decapped mRNAs [Gazzani et al. 2004]. It is of particular interest because there is evidence that it competes with RDR6 [Gazzani et al. 2004]. However, $xrn4$-5 had no effect on the accumulation of $TAS2^{(+)}$ or its cleavage products, either as a single mutant, or in combination with $sgs3$-11 or $rdr6$-11 [Fig. 5C]. $xrn4$-5 also had no effect on the accumulation of $TAS2$ or $TAS1a$ siRNAs [Fig. 5C]. These results demonstrate that $TAS2$ is not a substrate of XRN4. The possibility that $TAS2$ is a substrate of the related exonucleases XRN2 or XRN3 [Souret et al. 2004] remains to be tested.

Small siRNAs are generated by DCL4

Screens of EMS-mutagenized plants produced a recessive mutation that is morphologically indistinguishable from $sgs3$-11 and $rdr6$-11 but complements both of these mutations [Fig. 6A]. This mutation was mapped by genetic crosses to a region on chromosome 5 containing DCL4 and was found to be associated with a point mutation in the helicase domain of DCL4 that converts glutamic acid...
at position 583 to lysine [Fig. 6B]. This nonconservative mutation (dcl4-2) is in the middle of a short amino acid sequence that is perfectly conserved in Dicers from Arabidopsis [At], Homo sapiens (Hs), C. elegans (Ce), Drosophila melanogaster (Dm), and S. pombe (Sp). (Hs), C. elegans (Ce), Drosophila melanogaster (Dm), and S. pombe (Sp). (dcl4-2) interferes with the biogenesis of siR1511 and siR255 but does not affect miR173. (D) dcl4-2 accumulates an array of small transcripts, some of which hybridize to an oligonucleotide probe specific for the (+) strand of TAS2(+). These small transcripts are not produced in rdr6-11 dcl4-2 double mutants.

Figure 6. DCL4 is a component of the SGS3/RDR6 pathway and acts downstream of RDR6. (A) Two-week-old wild-type and mutant plants. The vegetative phenotype of dcl4-2 is identical to that of sgs3-11 and rdr6-11. (B) The domain structure of the DCL4 protein (Schauer et al. 2002) and the location of the dcl4-2 mutation. dcl4-2 affects a glutamic acid that is completely conserved in Dicers from Arabidopsis [At], Homo sapiens (Hs), C. elegans (Ce), Drosophila melanogaster (Dm), and S. pombe (Sp). (C) dcl4-2 interferes with the biogenesis of siR1511 and siR255 but does not affect miR173. (D) dcl4-2 accumulates an array of small transcripts, some of which hybridize to an oligonucleotide probe specific for the (+) strand of TAS2(+).

dcl4-2 on the processing of the TAS2(+) cleavage fragments. Blots of total RNA hybridized with the p1 probe revealed that dcl4-2 accumulates an array of transcripts smaller than 400 nt [Fig. 6D]. These transcripts also hybridize to a probe for the (−) strand of the 3’ fragment [Fig. 6D], suggesting that at least some of these transcripts are aberrantly processed, double-stranded forms of the 3’ cleavage fragment. Consistent with this interpretation, rdr6-11 dcl4-2 double mutants lacked these small transcripts, and instead accumulated single-stranded, full-length 5’ and 3’ fragments of TAS2(+). That is, double mutants were indistinguishable from rdr6-11. This result demonstrates that RDR6 is required for the production of the small transcripts present in dcl4-2, and thus acts before DCL4. The observation that rdr6-11 blocks the production of (−) transcripts complementary to TAS2(−)’ is evidence that RDR6 functions as a RdRp. The epistatic relationship between rdr6-11 and dcl4-2 is also significant because it demonstrates that the substrate of DCL4 is double-stranded RNA.

Discussion

In this paper, we describe the pathway by which small siRNAs are generated from the TAS2 and TAS1a loci in Arabidopsis. Although these transcripts have the special feature of being cleaved by a miRNA, this pathway is unlikely to be restricted to this class of transcripts because two of its components, SGS3 and RDR6, are required for PTGS of sense transgenes and for resistance to some viruses (Dalmay et al. 2000b; Mourrain et al. 2000; Beclin et al. 2002; Himber et al. 2003; Muangsan et al. 2004). The lessons learned from this analysis of the processing of TAS2 and TAS1a may therefore have broad implications for the mechanism of PTGS in plants.

The SGS3/RDR6/DCL4 pathway

A model for the way in which small siRNAs are generated from TAS2 and TAS1a is illustrated in Figure 7. This process is initiated by the miR173-mediated cleavage of a capped, polyadenylated transcript. The resulting cleavage fragments are then bound by SGS3, or by proteins associated with SGS3, thus protecting them from degradation by enzymes that act on single-stranded RNA. RDR6 transforms the fragments into double-stranded molecules, and DCL4 subsequently cleaves these fragments into 21-nt siRNAs. Although both the 5′ and 3′ cleavage fragments of TAS2(+) and TAS1a(+) are processed by SGS3, RDR6, and DCL4, the 3′ fragment is processed more efficiently by this pathway than the 5′ fragment. This difference may be explained by the substrate specificity of Dicer. In vitro studies of siRNA biogenesis in animals indicate that Dicer cleaves siRNAs from the end of a dsRNA substrate and prefers molecules that have a 1- or 2-nt 3′ overhang (Elbashir et al. 2001; Zhang et al. 2002, 2004; Siolas et al. 2004). Because of polarity of RNA synthesis, RDR6-mediated transcription of the 3′ fragment will frequently terminate at the 5′
end of this fragment; that is, at the miR173 cleavage site. If RDR6 adds untemplated nucleotides to the 3’ end of the newly transcribed strand—as has been found in the case of a tomato RdRp (Schiebel et al. 1993)—this will generate a duplex end with a 3’ overhang and make this fragment an ideal substrate for DCL4 or a competing DCL. The observation that small siRNAs from the 3’ fragment are in exact register with the miR173 cleavage site provides strong support for this model. In contrast, initiation of transcription at random positions on the 5’ cleavage fragment will frequently yield molecules that have a single-stranded 3’ end, preventing processing of this end by Dicer; processing from the 5’ end is likely to be inhibited by the presence of the 5’ cap. These considerations suggest that processing of the 5’ fragment into ta-siRNAs (Allen et al. 2005) requires a special mechanism for directing RDR6 to the 3’ end of these fragments.

miRNA-directed cleavage cannot be the only factor that directs transcripts to this pathway, because most miRNA-targeted transcripts are not silenced by SGS3 and RDR6 (Peragine et al. 2004; Vazquez et al. 2004b). One factor that may account for the susceptibility of TAS2 is the presence of an antisense transcript. Although we were unable to detect this transcript on Northern blots, a cDNA representing the [−] strand of TAS2 has been identified. Hybridization between TAS2(+) and this transcript could yield primary siRNAs that serve as primers for RDR6. Because the [−] transcript overlaps extensively with the 3’ cleavage fragment, these primers would favor transcription of this fragment. Another factor that may direct the SGS3/RDR6/DCL4 pathway to TAS2(+) and TAS1a(+) is the presence of an unspliced intron. It is noteworthy that the miR173 site is located in this intron and that all of the ta-siRNAs generated from these transcripts are confined to this intron. Most Arabidopsis genes targeted by miRNAs have the miRNA target site in their coding region, and it is possible that the location of a miRNA target site in an intron, or in another post-transcriptionally processed part of a transcript (e.g., the 3’ UTR) somehow makes the transcript unusually susceptible to PTGS. This hypothesis is consistent with the observation that the miR390 target site in TAS3 is located in a predicted 3’ UTR (Allen et al. 2005). Nonsense-mediated decay of mRNAs is mediated by an interaction between the RNA splicing apparatus and the proteins involved in translation (Maquat 2004), and it may be that interactions between the RNA splicing apparatus and RISC are important for directing transcripts to the SGS3/RDR6/DCL4 pathway. The fact that TAS2(+) and TAS1a(+) are noncoding transcripts may also be important because there is evidence in mammals that untranslated miRNAs are much more susceptible to RNAi than translated mRNAs (Gu and Rossi 2005).

The functions of RDR6, SGS3, and DCL4

Beclin et al. [2002] showed that RDR6 and SGS3 are required for PTGS of sense transgenes but not hairpin transgenes, from which they concluded that these proteins are required for the production of duplex RNA from a single-stranded template. This conclusion is supported in the case of RDR6 by its similarity to a tomato RNA-dependent RNA polymerase (Dalmay et al. 2000b; Mourrain et al. 2000) and evidence that is required for the phenomenon of transitivity (Vaiistj et al. 2002) and for the amplification and spread of transgene silencing (Himber et al. 2003). Our observation that rdr6-11 blocks the production of the TAS2(−3’) transcripts in an dcl4-1 mutant background provides additional support for this conclusion and is the first direct evidence that RDR6 functions as an RdRp.

The biochemical function of SGS3 is less well defined. The effect of sgs3-11 on the accumulation of the cleavage fragments of TAS2(+) and TAS1a(+) demonstrates that SGS3 somehow stabilizes these single-stranded fragments, but whether it has a more direct role in the transformation of these transcripts into dsRNA is unknown. We suspect that SGS3 is actually required for the function of RDR6 because sgs3-11 eliminates the production of small siRNAs, and is completely epistatic to rdr6-11. If SGS3 merely has a protective function, one would imagine that sgs3-11 would have a less severe phenotype.

Biogenesis of ta-siRNAs in Arabidopsis
than rdr6-11 because transcripts that escape degradation in sgs3-11 mutants could be processed by RDR6. Whether SGS3 binds directly to RNA and, if so, the specific features that it recognizes, also remains to be determined. SGS3 encodes a coiled-coil protein with a zinc finger domain that could mediate RNA binding [Bateman 2002]. It also has a conserved domain of unknown function, termed the X domain. Other Arabidopsis proteins that have an X domain have a second conserved domain called the KH domain, and it has been suggested that SGS3 functions in collaboration with one or more proteins that have this domain [Bateman 2002].

The Dicer responsible for PTGS in Arabidopsis has remained a mystery because none of the three previously described DCLs are good candidates. DCL1 is required for the biogenesis of miRNAs [Park et al. 2002] but is not required for PTGS of a hairpin transgene [Finnegan et al. 2003]. DCL2 is required for the biogenesis of siRNAs from some viruses but has no apparent role in the generation of endogenous long or short siRNAs [Xie et al. 2004], and DCL3 is required exclusively for the production of long siRNAs [Xie et al. 2004], which have no apparent role in PTGS [Hamilton et al. 2002; Himber et al. 2003]. The observations that dcl4-2 reduces the abundance of sir1511 and sir255 and causes incomplete processing of cleavage fragments of TAS2(+) and TAS1a(+) suggests that DCL4 generates small siRNAs from these fragments. However, it is still unclear if it is the only DCL with this function because we have no evidence that dcl4-2 is a null mutation. The small siRNAs present in dcl4-2 could be the product of this mutant protein or could be generated by another DCL that also operates on these cleavage fragments. The best candidate is DCL1 because this enzyme generates 21–22-nt miRNAs. The presence of 24-nt forms of sir255 and sir1511 in dcl4-2 suggests that DCL3 is also capable of processing these fragments. This issue will be resolved by the phenotypes of other dcl4 alleles and the phenotypes of double mutants between dcl4 and mutations in other DCL genes. DCL4 is the only DCL in Arabidopsis that lacks a PAZ domain [Schauer et al. 2002] and resembles the Drosophila dicer DCR2 in this respect [Lee et al. 2004; DCR2 is required for the biogenesis of siRNAs [Lee et al. 2004] and participates with R2D2 in loading siRNAs into RISC [Liu et al. 2003; Denli et al. 2004; Pham et al. 2004; Tomari et al. 2004]. DCL4 may perform a similar function because its C-terminal domain has been shown to interact with the R2D2 homolog, DRB4 [Hiraguri et al. 2005].

The evolution and function of TAS1 and TAS2

TAS2 produces four different siRNAs directed against a recently expanded family of PPR genes, so it is not unreasonable to predict that TAS2 evolved from a member of this family. Two features that distinguish TAS2(+) from its PPR targets (aside from the fact that it is a non-coding transcript) are the presence of a miR173 target site and the fact that the region of similarity between TAS2 and its PPR targets lies in a large, differentially spliced intron. In contrast, the PPR genes targeted by TAS2 either have no introns or a few small ones: The target sites for TAS2 ta-siRNAs reside in coding regions of these genes. Two key events in the evolution of TAS2 may have been the acquisition of a poorly spliced intron and the acquisition of a miRNA target site within this intron. Interestingly, the PPR family from which TAS2 probably evolved is the same family that spawned the miRNA, miR161 [Allen et al. 2004]. This suggests that pressure to coordinately repress the expression of recently duplicated genes is an important factor in the evolution of gene silencing mechanisms in plants.

Although TAS2 has no significant similarity to the TAS1 loci outside the miR173 site, there is convincing evidence that these loci are evolutionarily related. In addition to the structural similarity between TAS2(+) and TAS1a(+) and the similarity in the way these transcripts are processed, all three TAS1 loci are theoretically capable of producing at least one siRNA that targets the same family of PPR genes targeted by TAS2. Furthermore, TAS2 and TAS1c are immediately adjacent to each other on chromosome 2 and are in the same orientation. This organization suggests that TAS2 and TAS1c arose by a direct duplication and that TAS1a and TAS1b arose from TAS1c after it had diverged from TAS2. If TAS2 and the TAS1 loci are indeed paralogs, the number of unique ta-siRNA loci that have been identified in Arabidopsis would be reduced to two: TAS1/TAS2 and TAS3.

The observation that T-DNA insertions in TAS2 and TAS1a [Vazquez et al. 2004b] do not have a major effect on shoot morphology indicates that these genes do not account for the developmental phenotype of mutations in SGS3, RDR6, and DCL4. TAS3 targets auxin-related genes known to be important in shoot morphogenesis, and it is possible that defects in the production of ta-siRNAs from this locus are responsible for this developmental phenotype. Defining the endogenous targets of the SGS3/RDR6/DCL4 pathway, and the biological functions of these targets, are important goals for future research.

Materials and methods

Genetic stocks and growth conditions

The stocks of zip-1, sgs3-11, rdr6-11, xrn4-5, dcl1-7, dcl2-1, dcl3-1 hst-6, and hy1l-2 used in this study were in a Columbia [Col] genetic background and have been described previously [Golden et al. 2002; Peragine et al. 2004; Souret et al. 2004; Vazquez et al. 2004a; Xie et al. 2004; Park et al. 2005]. Double mutants were identified in F2 families using the allele-specific PCR primers described in these studies and were propagated as homozygous lines, except in the case of sgs3-11 dcl1-7, which was maintained as a sgs3-11/sgs3-11 +/dcl1-7 stock. dcl4-2 was identified in a screen of M2 families from EMS-mutagenized Col seeds and was backcrossed to Col three times before characterization. dcl4-2 rdr6-11 double mutants were identified using the PCR assay for rdr6-11 described previously [Peragine et al. 2004] and a DCAPs marker for dcl4-2. Amplification with the dcl4CAP primers followed by digestion with MboII yields two fragments in wild-type DNA and a single fragment in dcl4-2.
Plants homozygous for the SALK_014168 T-DNA insertion were identified using the 014168-LP and 014168-RP primers. Plants were grown in soil in chambers under continuous fluorescent illumination (22°C). Sequences of the PCR primers and oligonucleotide probes used in this study are provided in Supplementary Tables 1 and 2.

Northern analysis

Total RNA was isolated from flowers as previously described (Peragine et al. 2004). Poly[A] mRNA was isolated from total RNA by two rounds of purification using the Oligotex mRNA Midi kit (Qiagen). LMW RNA was purified from total RNA according to Dalmay et al. (2000a). Total or poly[A] RNA were separated on agarose gels, blotted, and probed with randomly primed 32P-labeled probes (Stratagene) or with end-labeled oligonucleotide probes as previously described (Peragine et al. 2004). Double-stranded probes were hybridized overnight at 68°C in PerfectHyb Plus buffer (Sigma) according to the manufacturer’s instructions. Oligonucleotide probes were hybridized overnight at 55°C in ULTRAHyb-oligo buffer (Ambion). Following hybridization, blots were washed twice in 2× SSC and 0.5% SDS for 30 min at 55°C. LMW RNA was separated on 15% gels and transferred electrophoretically to Hybond N+ membranes. LMW blots were processed at 40°C for methylation of the template chromosome. RACE was performed by directly ligating this adaptor to untreated mRNA. Fragments were amplified with RACE1 primers, followed by RACE2 (Supplementary Table 1). Double-stranded RNA were generated from Col genomic DNA by PCR using the following primers: TAS2pr1 (TAS2pr1 and TAS2pr3), TAS2pr2 (TAS2pr1 and TAS2pr2), TAS2pr3 (TAS2pr3 and TAS2pr4), TAS1apr1 (TAS1apr1 and TAS1apr2), and TAS1apr2 (ASRP7225 and ASRP1927AS).

Transcript analysis

5′ and 3′ RACE were carried out using the GeneRacer Kit (Invitrogen). To identify the 5′ end of capped transcripts, the GeneRacer adaptor was ligated to mRNA [125 ng] previously treated with calf intestinal phosphatase (CIP) and tobacco acid phosphatase (TAP) according to the manufacturer’s instructions. RLM-5′RACE was performed by directly ligating this adaptor to untreated mRNA. Fragments were amplified with Platinum Pfx DNA polymerase (Invitrogen) or Taq DNA polymerase, purified, cloned into the TOPO [Invitrogen] or pGEM-T Easy (Promega) cloning vectors, and then sequenced. RNA was amplified by nested PCR, using the adaptor primers supplied by the manufacturer in combination with gene-specific primers. RLM-5′RACE of the PPR and TAS1[+] transcripts was performed using RACE1 primers, followed by RACE2 (Supplementary Table 1). 5′ RACE of TAS2[+] was performed using TAS2apr5, followed by TAS2apr6, TAS2apr7, or TAS2apr8. 3′ RACE of TAS2[+] was performed using the GeneRacer 3′ adaptor primers in combination with TAS2apr9 and TAS2apr10. Self-ligated transcripts were generated as previously described (Couttet et al. 1997). Briefly, total RNA [4 µg] from Col flowers was treated with RQI DNase (Promega), CIP, and TAP and incubated in a volume of 400 µL with T4 RNA ligase for 16 h at 16°C. cDNA synthesis was carried out using SuperScriptII reverse transcriptase [Invitrogen] and TAS2pr1 and 1, and the resulting products were amplified by PCR using the TAS2apr12 and pr13 primers. PCR fragments were gel-purified, cloned into the TOPO cloning vector, and then sequenced.

RT–PCR

Total RNA was purified after extraction using RNeasy and RNase-Free DNase (Qiagen). Further removal of genomic DNA was accomplished by treating samples with RQI DNase and then extracting RNA with acidic phenol/chloroform. Quantitative real-time RT–PCR was carried out using the primers 62930-F and 62930-R for At1g62930, 63130-F and 63130-R for At1g63130, 6230-R for At1g6230, SCL6-IV-F and SCL6-IV-R for SCL6-IV, and EIF4A-F and EIF4A-R for EIF4A.

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