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Endogenous *trans*-Acting siRNAs Regulate the Accumulation of *Arabidopsis* mRNAs

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Summary

Here we describe a set of endogenous short interfering RNAs (siRNAs) in *Arabidopsis*, some of which direct the cleavage of endogenous mRNAs. These siRNAs correspond to both sense and antisense strands of a noncoding RNA (At2g27400) that apparently is converted to double-stranded RNA and then processed in 21 nt increments. These siRNAs differ from previously described regulatory small RNAs in two respects. First, they require components of the cosuppression pathway (RDR6 and SGS3) and also components of the microRNA (miRNA) pathway (AGO1, DCL1, HEN1, and HYL1) but not components needed for heterochromatic siRNAs (DCL3 and RDR2), another class of endogenous plant siRNAs. Second, these siRNAs repress the expression of genes that have little overall resemblance to the genes from which they originate, a characteristic previously reported only for miRNAs. The identification of this silencing pathway provides yet another dimension to posttranscriptional mRNA regulation in plants.

Introduction

Endogenous noncoding small RNAs (20 to 25 nt long) are important regulators of gene expression in both plants and animals (Carrington and Ambros, 2003; Bartel, 2004; He and Hannon, 2004; Mallory and Vaucheret, 2004). Two types of endogenous small RNA regulators have been reported, microRNAs (miRNAs) and short interfering RNAs (siRNAs). miRNAs are processed from transcripts that can form local hairpin precursor structures, whereas siRNAs are processed from long bimolecular RNA duplexes or extended hairpins (Bartel and Bartel, 2003). miRNAs have been cloned from various organisms and are typically evolutionarily conserved (Bartel, 2004). Many of the genes that are regulated by miRNAs are essential for proper development in plants (Rhoades et al., 2002; Jones-Rhoades and Bartel, 2004), and the importance of various miRNA-mediated regulatory relationships has been demonstrated in plants and animals (Lee et al., 1993; Wightman et al., 1993; Moss et al., 1997; Reinhart et al., 2000; Aukerman and Sakai, 2003; Boutla et al., 2003; Brennecke et al., 2003; Emery et al., 2003; Johnston and Hobert, 2003; Palatnik et al., 2003; Xu et al., 2003; Chen, 2004; Mallory et al., 2004a, 2004b; Vaucheret et al., 2004).

In plants, proper miRNA accumulation depends on the activity of the nuclear proteins DCL1, HEN1, and HYL1 (Park et al., 2002; Reinhart et al., 2002; Boutet et al., 2003; Kasschau et al., 2003; Han et al., 2004; Sunkar and Zhu, 2004; Vazquez et al., 2004). *dcl1* null alleles are embryo-lethal, indicating that DCL1 is required for plant viability, at least during reproduction and/or at early stages of development (Schauer et al., 2002). Partial loss-of-function *dcl1* mutants with point mutations in the RNA helicase domain (*dcl1-7, dcl1-8*) or truncation of the second dsRNA binding domain (*dcl1-9*) are viable (Schauer et al., 2002) but show reduced miRNA accumulation and striking developmental defects including sterility, confirming the crucial role of DCL1 and presumably miRNAs during plant reproduction (Park et al., 2002; Reinhart et al., 2002). *hen1* and *hyl1* null alleles exhibit reduced miRNA levels and developmental defects that overlap with those of partial loss-of-function *dcl1* mutants. However, in contrast to *dcl1* null alleles, *hen1* and *hyl1* null alleles are viable. AGO1 also participates in the miRNA pathway (Kidner and Martienssen, 2004; Vaucheret et al., 2004). AGO1 is the founding member of the ARGONAUTE protein family (Bohmert et al., 1998). Hypomorphic *ago1* alleles exhibiting developmental defects overlapping with those of *hen1* and *hyl1* accumulate miRNAs but show increased accumulation of the corresponding target mRNAs, an observation that when combined with analogous roles of Argonaute proteins in animals (Carmell et al., 2002) suggests that AGO1 is part of the miRNA-programmed RNA-induced silencing complex (RISC) (Vaucheret et al., 2004). Most miRNAs have reduced accumulation in *ago1* null alleles, also suggesting that AGO1 stabilizes miRNAs (Vaucheret et al., 2004). A role for AGO1 in controlling the cellular localization of miRNAs has also been proposed (Kidner and Martienssen, 2004).

In plants, two types of siRNA pathways have been described. The first type involves siRNAs that trigger changes in the chromatin state of elements from which they derive (Hamilton et al., 2002; Finnegan and Matzke,

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2003; Zilberman et al., 2003; Xie et al., 2004). Accumulation of these siRNAs requires DCL3, one of the four *Arabidopsis* DCL proteins (Xie et al., 2004). In contrast to miRNAs, four chromatin-associated siRNAs described by Xie et al. (2004) require RDR2, one of multiple RNA-dependent RNA polymerases (RDR) in plants. A second pathway involves siRNAs deriving from and defending against exogenous RNA sequences such as viruses or sense transgene transcripts (S-PTGS). The production of siRNAs associated with S-PTGS requires another RDR protein, RDR6 (also known as SDE1/SGS2), and a protein of unknown function, SGS3 (Dalmay et al., 2000; Mourrain et al., 2000; Boutet et al., 2003). Thus far, no role for regulating endogenous RNAs has been assigned to RDR6 and SGS3.

Here, we identify an endogenous siRNA pathway that requires AGO1, DCL1, HEN1, HYL1, SGS3, and RDR6. This pathway differs from the heterochromatic siRNA pathway, which requires DCL3 and RDR2 but not SGS3 and RDR6, but resembles the miRNA pathway, which requires AGO1, DCL1, HEN1, and HYL1. Like miRNAs, these siRNAs direct cleavage of endogenous complementary mRNAs that have little overall resemblance to the genes from which the siRNAs originate; however, unlike miRNAs, their production requires RDR6 and SGS3. The identification of endogenous mRNAs that are regulated at the post-transcriptional level by these siRNAs raises the possibility that numerous endogenous genes may be regulated by a similar pathway.

Results

Identification of Endogenous RNAs that Are Deregulated in rdr6 *and* sgs3 *Mutants*

RDR6 and SGS3 are required for posttranscriptional gene silencing mediated by sense-transgene (S-PTGS) or DNA viruses but not inverted repeat transgenes (IR-PTGS) or RNA viruses (Dalmay et al., 2000; Mourrain et al., 2000; Beclin et al., 2002; Muangsan et al., 2004). To determine if RDR6 and SGS3 could also play a role in posttranscriptional regulation of endogenous mRNAs, RNA steady-state levels in wild-type plants, *sgs2(rdr6)* and *sgs3* mutants were compared by cDNA-amplified fragment length polymorphism (cDNA-AFLP). Out of the 256 possible pairs, 80 primer pairs were tested, representing approximately 4000 polyadenylated transcripts. Three cDNA-AFLP products reproducibly showed elevated levels in *rdr6* and *sgs3* mutants, and we analyzed one of these products in more detail (Figure 1).

This product corresponds to two genomic DNA regions on BAC F12K2 separated by a sequence of 572 nucleotides (Figure 1). Sequence analysis revealed cannonical donor and acceptor splice-site sequences, both at expected positions within the genomic sequences, suggesting that the cDNA-AFLP product results from the splicing of a single intron. RT-qPCR confirmed a 3- to 4-fold increase in the level of the At2g27400 transcript in *rdr6* and *sgs3* mutants, first indicated by our cDNA-AFLP analysis (data not shown). 5′-rapid amplification of cDNA ends (RACE) and 3′-RACE followed by sequencing revealed that the full-length 926 nt sequence of the transcript is composed of two exons of 164 and 190 nucleotides separated by an intron of 572 nucleotides. This transcript is polyadenylated and capped and originates from a nonannotated genomic region located between the annotated genes At2g27410 and At2g27395. Therefore, we named this transcript At2g27400. Because this transcript contains only one ORF, 21 amino acids in length, starting at the first AUG at position 55, relative to the 5′ end of the transcript, it is likely that RNA, not protein, is the final product of this gene. Two ESTs corresponding to At2g27400 were found in the *Arabidopsis* database. Both ESTs start at position 52, relative to the 5′ end of the transcript determined by 5′-RACE, and end within the intron. One is not polyadenylated and ends at position 674. The other is polyadenylated and ends at position 711, either due to the presence of a polyadenylation signal at position 714 or to oligo-dT mispriming in an A-rich region that follows this position. The absence of full-length spliced or unspliced EST in the *Arabidopsis* database suggests that the At2g27400 gene is not strongly transcribed or that the primary RNA and its spliced product are immediately subjected to further processing.

A Cluster of Small RNAs Originates from the Intron of the At2g27400 Transcript through RDR6 and SGS3 Activity

Because the At2g27400 transcript is a noncoding RNA, we looked for small RNAs deriving from the sequence of At2g27400 in sequenced libraries of small RNA databases from plants (Park et al., 2002; Reinhart et al., 2002; Sunkar and Zhu, 2004; Xie et al., 2004; R.R. and D.P.B., unpublished data). The At2g27400 unspliced transcript shares perfect identity with twelve independently cloned small RNAs within the portion of the intron that is common to both the unspliced transcript and the truncated EST (Figure 1). Ten small RNAs correspond to the At2g27400 transcript whereas two small RNAs are complementary to At2g27400 (Table 1). We refer to these RNAs by their position relative to the 5′ end of the At2g27400 transcription start (Figure 1) and the strand from which they originate (+ or −). Using an antisense At2g27400 RNA probe we detected 21 nt small RNAs of sense polarity in wild-type plants but not in *sgs2*(*rdr6*) and *sgs3* mutants (Figure 2A), indicating that the accumulation of these small RNAs depends on RDR6 and SGS3 activities. Small RNAs of antisense polarity were not detected using a sense At2g27400 RNA probe (data not shown), consistent with the smaller fraction of cloned small RNAs representing antisense polarity versus sense polarity (Table 1).

Nine of the twelve independently cloned small RNAs occur in perfect 21 nt increments, eight on the sense strand, one on the antisense strand (Table 1; Figure 1). Two of the others, siR521(+) and siR523(+), are variants of siR522(+) and deviate from perfect phasing by only one nucleotide at their 5′ termini. Only one, siR466(−) was processed in a different register, having a 10 nt overlap with siR477(−). The two 21 nt strands of each siRNA duplex produced by Dicer are not equally eligible for assembly into the cleavage complex (RISC); the strand with lowest base-pairing stability at its 5′ end preferentially enters into RISC (Khvorova et al., 2003; Schwarz et al., 2003). All eleven At2g27400-derived small RNAs that are in phase obey the asymmetry rule

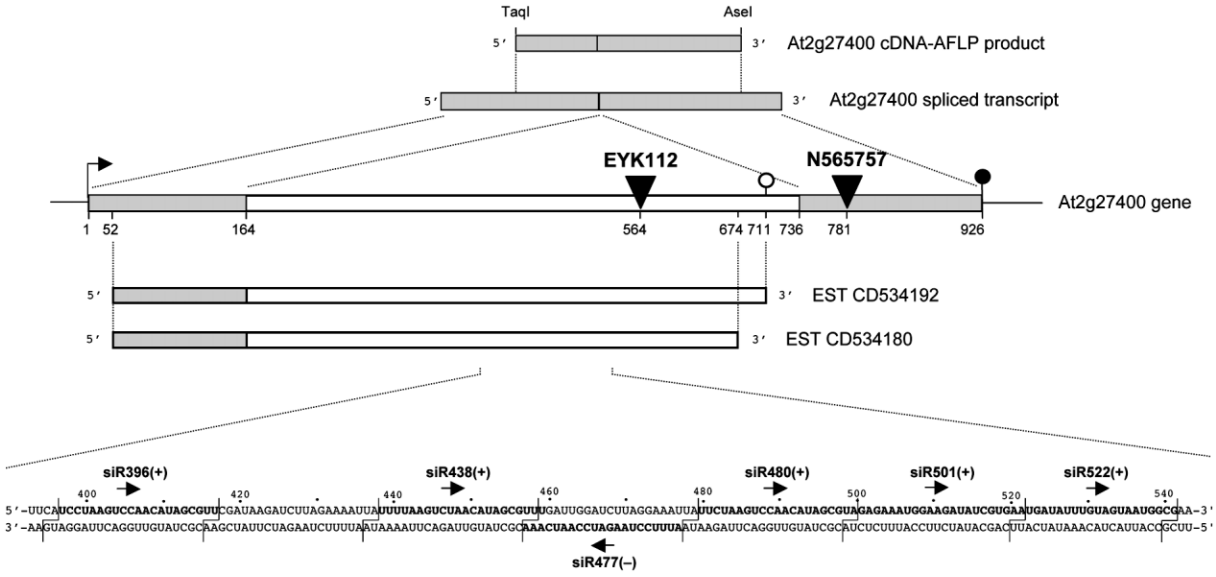

Figure 1. siRNAs Correspond to Both Sense and Antisense Strands of the At2g27400 Noncoding RNA

The diagrammatic representation of the At2g27400 gene is shown in the middle of the figure. The two exons are represented by gray boxes and the intron is represented in white. The cDNA-AFLP clone is a TaqI-AseI fragment internal to the spliced transcript. The 5′ and 3′ extensions of the transcript were obtained by 5′- and 3′-RACE. The arrow and filled circle indicate the position of transcription start and polyadenylation signal, respectively. The open circle indicates the position of the putative polyadenylation signal. Black triangles indicate the locations of the T-DNA insert within At2g27400 in the EYK112 and N565757 lines. The intron sequence corresponding to the cloned siRNAs is shown in the expanded region and the sequence of the cloned strand of the siRNA duplex is shown in bold. Numbers indicate positions on the full-length transcript, relative to the 5′ end.

that characterizes siRNAs and miRNAs in animals and plants, explaining the bias in polarity of the eleven cloned small RNAs and the difficulty in detecting siRNAs from the antisense strand on RNA blots (Figure 2A). This observation also implies that these small RNAs enter into a RISC-like complex (see below).

The requirement of RDR6 and SGS3 for small RNA accumulation (Figures 2A and 2B), the 21 nt phasing of small RNAs (Figure 1), and the presence of 21 nt clones from both the sense and antisense strands (Figure 1) suggest that these small RNAs likely derive from the successive Dicer-mediated cleavage of a long dsRNA formed by the RNA-dependent RNA polymerase activity of RDR6. Multiple small RNAs, ~21 nt in length, deriving from both strands of a long dsRNA precursor have the defining features of siRNAs (Elbashir et al., 2001) and thus we call these small RNAs the At2g27400 siRNAs.

Accumulation of At2g27400 siRNAs Requires AGO1, DCL1, HEN1, and HYL1 1 but not AGO7, DCL2, DCL3, RDR2, SDE3, and WEX

To determine the genetic requirements for At2g27400 siRNA production, their accumulation was assayed in silencing-related mutants (*ago1*, *ago7*, *dcl1*, *dcl2*, *dcl3*, *hen1*, *hyl1*, *rdr2*, *sde3*, and *wex*). At this stage of development, At2g27400 siRNAs were detected in wild-type

Table 1. Cloned siRNAs Deriving from the At2g27400 Transcript

At2g27400 siRNAs	Length (nt)	Phasing	# clones	alternative name
siR396(+)	21	Yes	2[a,b]	ASRP752[b], miR175c[d] miR389b.3[c], miR175d[d]
siR438(+)	21	Yes	1[c]	
siR466(−)	21	No	1[a]	
siR477(−)	21	Yes	1[a]	
siR480(+)	21	Yes	3[b,c,d]	ASRP255[b], miR389b.1[c], miR175a-2[d]
siR501(+)	21	Yes	1[a]	
siR521(+)	20	Yes[e]	1[b]	ASRP1745[b]
siR522(+)	21	Yes	1[b]	ASRP1852[b]
siR523(+)	22	Yes[e]	1[b]	ASRP1786[b]

(+) sense strand, (−) antisense strand
[a] this work
[b] Xie et al., 2004, http://cgrb.orst.edu/smallRNA/db/search_user_seq.html
[c] Sunkar and Zhu, 2004
[d] Park et al., 2002
[e] in phase on 3′ end, one nucleotide out of phase on 5′ end

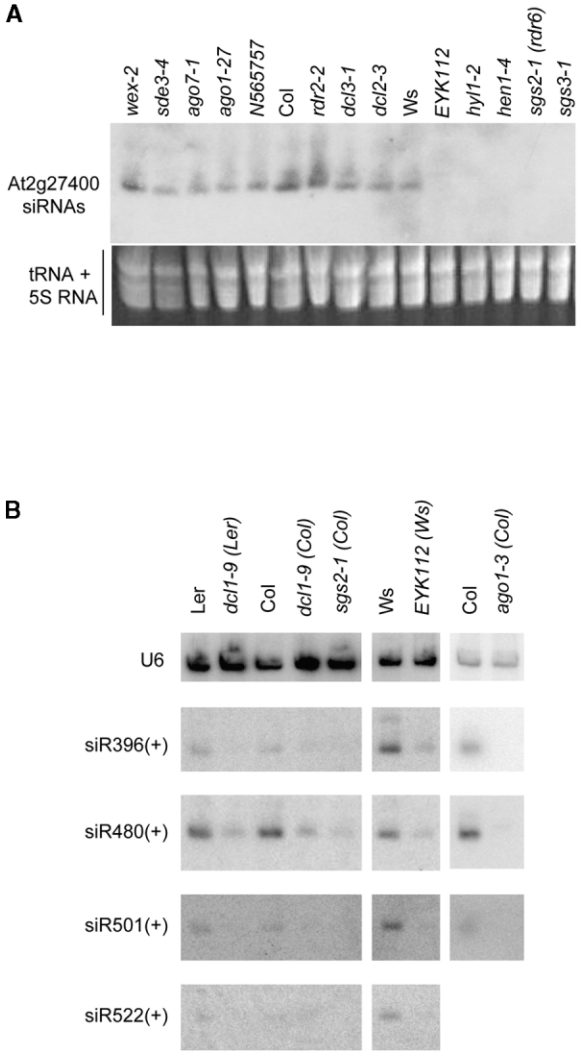

Figure 2. Accumulation of the At2g27400 siRNAs Requires AGO1, DCL1, HEN1, HYL1, RDR6, and SGS3

(A) RNA gel blot analysis of At2g27400 siRNA accumulation in wild-type plants (Col or Ws) and *wex-2*, *sde3-4*, *ago7-1*, *ago1-27*, *N565757*, *rdr2-2*, *dcl3-1*, *dcl2-3*, *EYK112*, *hyl1-2*, *hen1-4*, *sgs2-1 (rdr6)* and *sgs3-1* mutants (all in Col, except for EYK112 in Ws). 15 μg of total RNA was extracted from the aerial part of 4-6 leaf-old plants and hybridized to a full-length unspliced At2g27400 antisense RNA probe. EtBr staining of tRNA + 5S RNA is shown as a loading control.

(B) RNA gel blot analysis of siR396(+), siR480(+), siR501(+), and siR522(+) accumulation in wild-type plants (Col, Ler or Ws) and *dcl1-9*, *sgs2-1 (rdr6)*, *EYK112* and *ago1-3* mutants. 10 μg of total RNA was extracted from the aerial part of 4–6 leaf-old plants and successively hybridized to DNA oligo probes complementary to each siRNA. U6 is shown as a loading control.

plants, *ago7*, *dcl2*, *dcl3*, *rdr2*, *sde3*, and *wex* null mutants and in hypomorphic *ago1* mutants (Figure 2). In contrast, the accumulation of these siRNAs was reduced in the partial loss-of-function *dcl1* mutant and they were below detectable level in *ago1*, *hen1*, and *hyl1* null mutants (Figure 2), indicating that, like miRNAs, the accumulation of these siRNAs requires AGO1, DCL1, HEN1, and HYL1 but, unlike heterochromatic siRNAs, does not require DCL3 or RDR2.

siRNA Accumulation Is Decreased in At2g27400 Intron- but Not Exon2-Insertion Mutants

Among the siRNAs that correspond to At2g27400 on BAC F12K2, one, siR480(+), shows a perfect homology with two other intergenic regions located on BAC F2J10 and F17A14. Since the At2g27400 probe used in the previous experiment shares 80% nucleotide identity with F2J10 and 72% with F17A14, it is possible that some of the siRNAs detected with this probe could derive from one of these sequences. To determine if the siRNAs detected with the At2g27400 RNA probe derive from At2g27400, we analyzed small RNA accumulation in a mutant (*EYK112*) carrying a T-DNA inserted in the At2g27400 intron at position 568, relative to the 5′ end of the transcript determined by 5′-RACE, downstream of the location of the siRNAs, but within the portion of the intron that is common to both the unspliced transcript and the truncated EST (Figure 1). Using an antisense At2g27400 RNA probe, siRNAs were detected in wild-type plants but not in the *EYK112* mutant (Figure 2A), indicating that, at this stage of development, the siRNAs detected with this probe derive from BAC F12K2 (At2g27400) and not from BACs F2J10 or F17A14. Additional RNA blots were hybridized with DNA oligo probes complementary to individual siRNAs (Figure 2B). The accumulation of each of the siRNAs was strongly reduced in the *EYK112* mutant, although traces could still be detected. Since a chimeric transcript starting at the 5′ end of At2g27400 and ending in the T-DNA is present in *EYK112* homozygous plants (data not shown), it is possible that the residual amount of siRNAs is processed from this chimeric transcript or transcripts deriving from BACs F2J10 or F17A14. A second insertion mutant (*N565757*) was identified, carrying a T-DNA inserted in the At2g27400 exon2 at position 781, relative to the 5′ end of the transcript determined by 5′-RACE (Figure 1). Using an antisense At2g27400 RNA probe, siRNAs were detected at similar levels in wild-type plants and N565757 mutants (Figure 2A), suggesting that the T-DNA in N565757 is inserted beyond the 3′ end of the siRNA precursor transcript, and that siRNAs do not derive from the full-length unspliced transcript but rather from the spliced intron or a transcript corresponding to the truncated ESTs.

Targets of At2g27400 siRNAs Are Cleaved in Wild-Type Plants

In plants, miRNAs cleave endogenous mRNAs by binding to unique segments of near-perfect complementarity (Llave et al., 2002b, Kasschau et al., 2003; Tang et al., 2003). Ten out of eleven At2g27400-siRNAs obey the asymmetry rule that characterizes miRNAs and siRNAs (Khvorova et al., 2003; Schwarz et al., 2003), suggesting that they could enter a RISC-like complex to guide cleavage of complementary mRNAs. Indeed, two of the previously cloned siRNAs, siR438(+) and siR480(+), have been proposed to target At5g18040, At1g51670, At4g29760, and At4g29770 (Park et al., 2002; Sunkar and Zhu, 2004). siR396(+), which is nearly identical to siR438(+) and siR480(+), also has the potential to target the same genes. At5g18040 has only one mismatch with the three siRNAs, and At1g51670, At4g29760, and At4g29770 each have two mismatches with siR480(+)

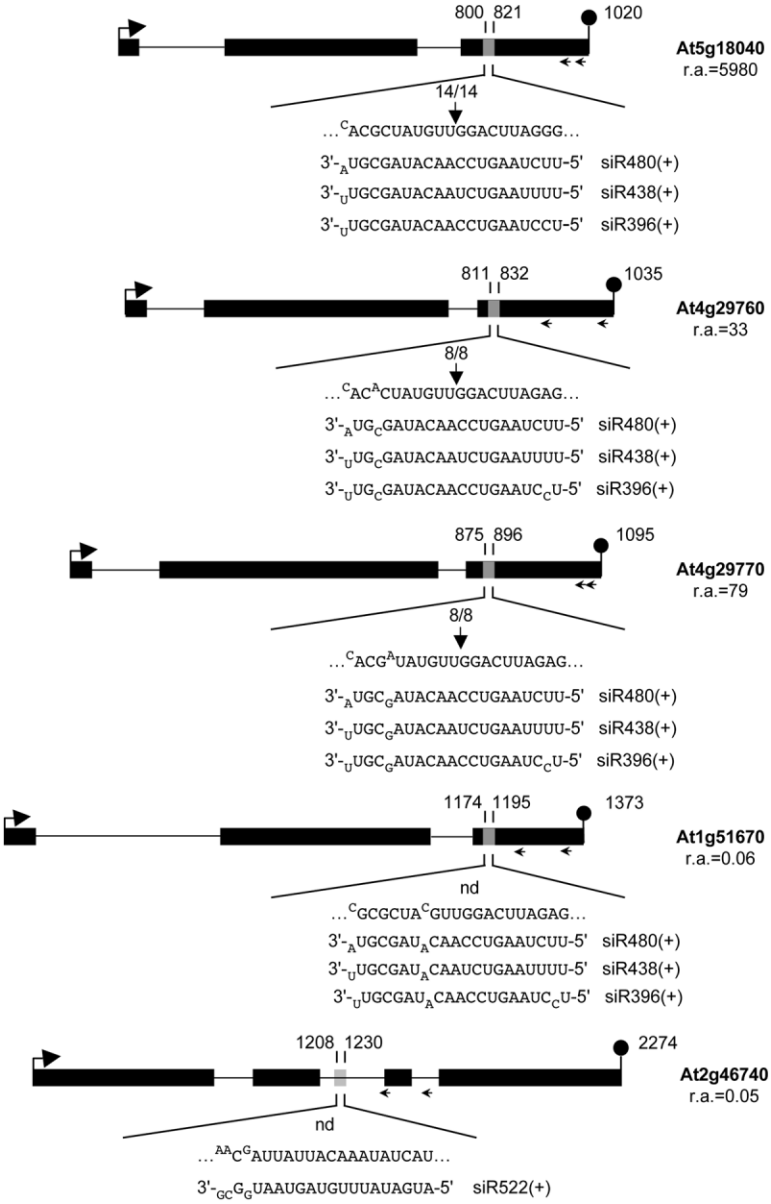

Figure 3. At2g27400 siRNAs Cleave Target mRNAs

Black boxes represent open reading frames. The arrows and filled circles represent transcription starts and polyadenylation signals, respectively. Gray boxes represent the siRNA complementary site, with the nucleotide positions relative to the transcription start indicated. The sequence of each complementary site and siRNA are shown in the expanded regions. The position of primers used for 5′-RACE is indicated by horizontal arrows. The frequency of 5′-RACE clones corresponding to each cleavage site (vertical arrows) is shown as a fraction. "nd" indicates that no cleavage product was detected. The relative abundance of mRNAs or pre-mRNAs (At2g46740) in wild-type Col plants versus *ACTIN2* is indicated (r.a.). The wild-type value for *ACTIN2* is arbitrarily fixed to 100,000.

and three mismatches with siR396(+) (Figure 3). These four genes encode related proteins of unknown function (data not shown). In a search for potential targets of the other siRNAs, we found that siR522(+) and its variants, siR521(+) and siR523(+), have three mismatches with the intron 2 of At2g46740, a gene encoding an FAD binding domain-containing protein.

To determine if At2g27400 siRNAs direct cleavage of complementary mRNAs, we performed 5′-RACE on these predicted targets, as previously done for miRNA targets (Llave et al., 2002b). Cleavage products were amplified for the three most abundant predicted targets (Figure 3). Sequencing of these 5′-RACE products mapped the 5′ ends to the position corresponding to the middle of the siRNA complementarity (Figure 3), indicating that At5g18040, At4g29760, and At4g29770 mRNAs are targeted for cleavage by siR396(+), siR438(+), and/or siR480(+). We did not detect cleavage products for At1g51670, which was predicted to also be targeted by siR396(+), siR438(+), and/or siR480(+), and for At2g46740, which also was predicted to be targeted by siR522(+). The absence of cleavage products from At2g46740 may be due to the location of the complementarity site, which is in an intron. Pre-messenger RNAs are often short-lived molecules. Consistent with this observation, we found that At2g46740 pre-messenger RNA is much less abundant than the other mRNA targets for which we detected 5′ cleavage products (Figure 3). The absence of At1g51670 cleavage products could be due to the low expression of At1g51670 in adult leaves and flowers that were used to make the 5′-RACE cDNA pool (Figure 3).

To further investigate if the five mRNAs are regulated by the At2g27400 derived siRNAs, we quantified the steady-state level of uncleaved target mRNAs in mutants impaired in At2g27400-derived siRNA accumula-

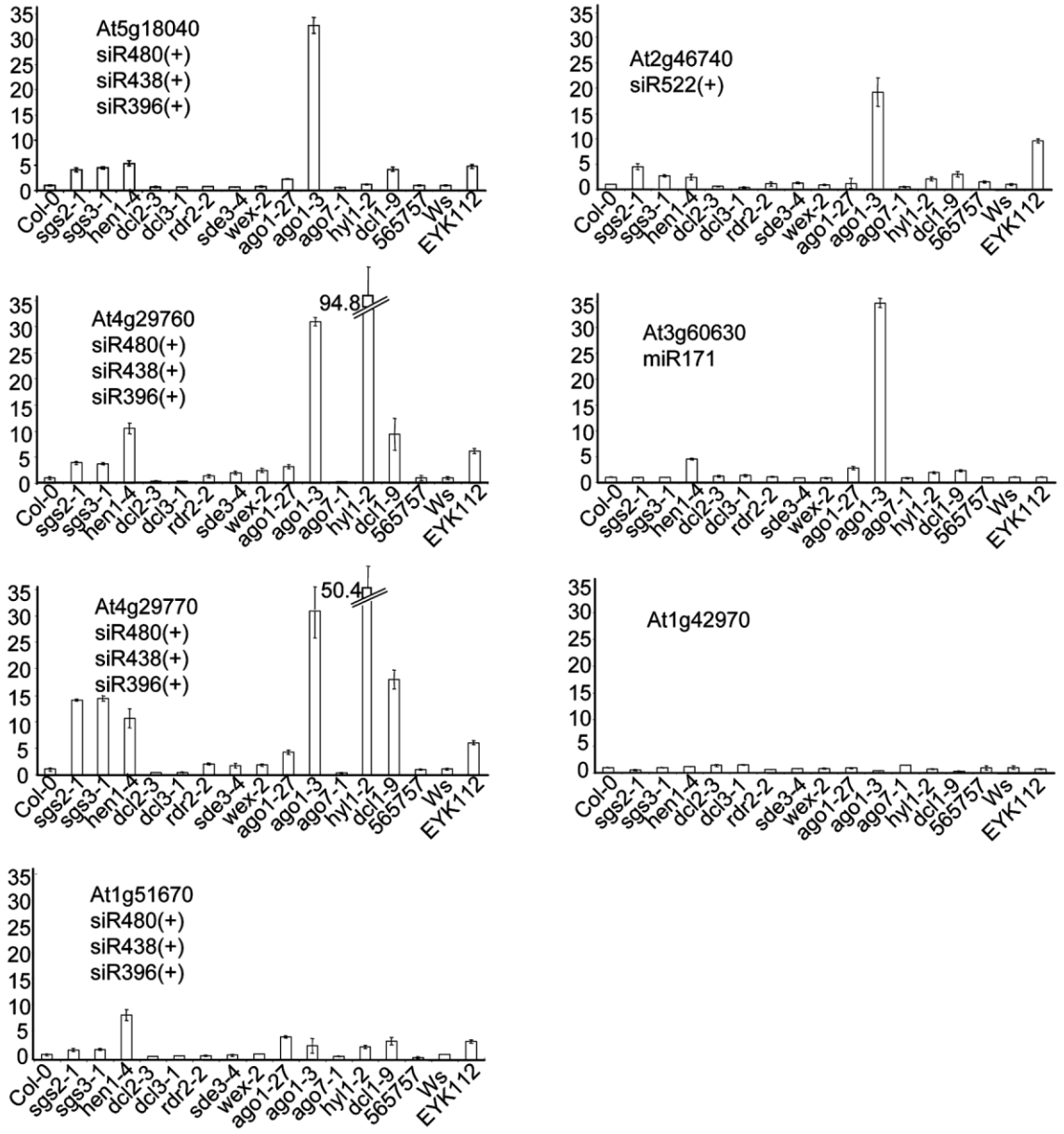

Figure 4. At2g27400 siRNAs Regulate the Expression of Target mRNAs

Steady-state levels of uncleaved target mRNAs was determined by RT-qPCR from total RNA extracted from the aerial part of 4-6 leaf-old plants using primers surrounding the siRNA complementary site. The At3g60630/*SCL6-III* miR171 target was used as a positive control and At1g42970/*GAPDH* as a negative, non-target, control. Quantifications were normalized to *ACTIN2*. The values in wild-type plants were arbitrarily fixed to 1. siRNA numbers and gene names corresponding to each pair are indicated at the left.

tion by RT-qPCR using primers surrounding the cleavage site. GAPDH (At1g42970) was used as a nontargeted mRNA control, while SCL6-III (At3g60630) was used as a miRNA target control. As expected for miRNA targets, uncleaved SCL6-III RNA levels increased in *dcl1*, *hen1*, *hyl1*, and *ago1* mutants but, importantly, not in *sgs2*(*rdr6*) and *sgs3* mutants (Figure 4). Uncleaved mRNA levels of At2g27400-derived siRNA targets were increased in *ago1, dcl1, hen1, and hyl1* mutants, as well as *rdr6, sgs3,* and the *EYK112* insertion mutant. Conversely, the accumulation of uncleaved mRNA was unchanged in *ago7*, *dcl2*, *dcl3*, *rdr2*, *sde3*, *wex*, and the *N565757* insertion mutant. These data are consistent with the change we observed in At2g27400-derived siRNAs accumulation (Figure 2) and indicate that At2g27400-derived siRNAs act to regulate endogenous mRNAs. As explained in the Discussion, we refer to this class of siRNAs as endogenous *trans*-acting siRNAs.

RDR6 and SGS3 Are Required for the At2g27400 siRNA Pathway but Have Limited or No Impact on the miRNA or Chromatin siRNA Pathways

The steady-state level of uncleaved SCL6-III mRNA that is a target of miR171 is unchanged in seedlings of *sgs2*(*rdr6*) and *sgs3* mutants (Figure 4), suggesting that SGS3 and RDR6 may be dispensable for miRNA-

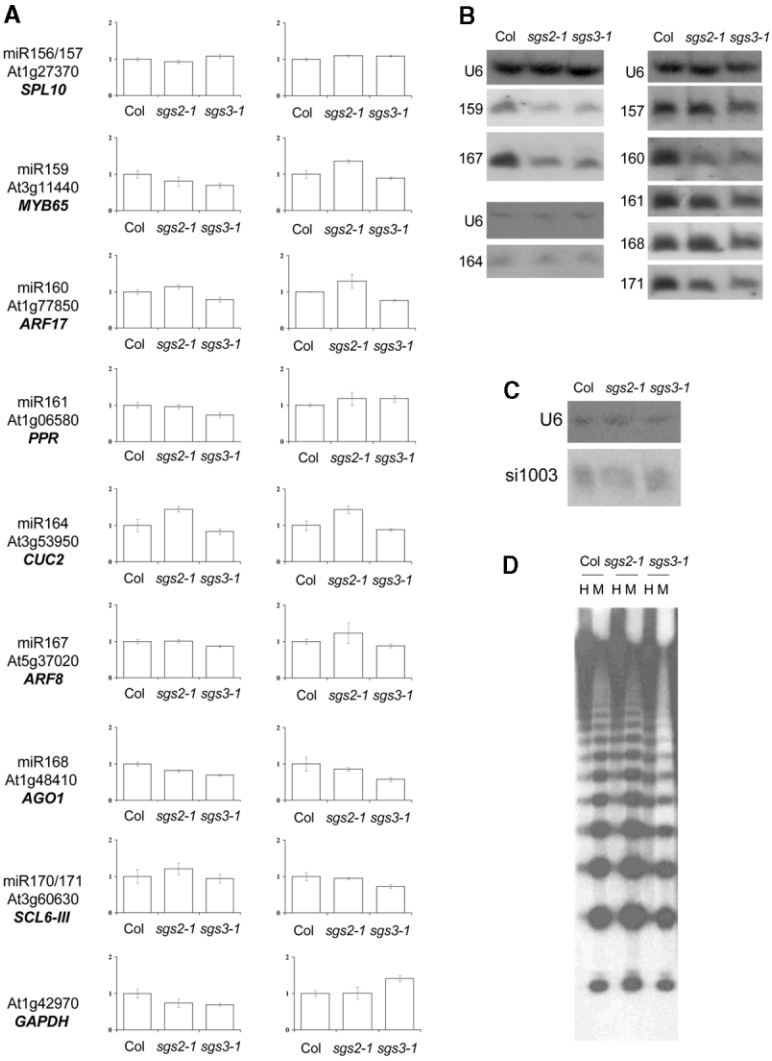

Figure 5. Analysis of miRNAs and Chromatin siRNAs in *rdr6* and *sgs*3 Mutants

(A) *SPL10, MYB65, ARF17, PPR, CUC2, ARF8, AGO1*, and *SCL6-III* mRNA relative accumulation was determined in RNA extracted from inflorescences of wild-type plants (Col) and *sgs2*(*rdr6*) and *sgs*3 mutants by RT-qPCR using primers surrounding the cleavage sites. RT-qPCR values determined for two independent cDNA synthesis are shown. Quantifications were normalized to *ACTIN2*. The wild-type Col value was arbitrarily fixed to 1. miRNA and target gene names are indicated on the left.

(B) RNA gel blots of 10 μg of RNA extracted from inflorescences of wild-type plants, *sgs2*(*rdr6*), and *sgs3* mutants were successively hybridized to specific miRNA probes, and finally hybridized to U6 as a loading control.

(C) RNA gel blot of 10 μg of RNA extracted from inflorescences of wild-type plants and *sgs2*(*rdr6*) and *sgs3* mutants was hybridized to an siRNA probe (ASRP1003) corresponding to 5S rDNA gene arrays and reprobed to U6 as a loading control.

(D) DNA gel blot analysis of DNA extracted from wild-type plants, *sgs2*(*rdr6*), *and sgs3* mutants. Genomic DNA was digested with the methylation-sensitive enzyme HpaII (H) or MspI (M) and hybridized to a 5S DNA probe.

directed cleavage. To test whether this applies at a different stage of development and for other miRNA targets, RT-qPCR was performed using RNAs extracted from inflorescences where most miRNA targets are abundantly expressed. No significant change in mRNA steady-state levels was observed between wild-type Col plants and *sgs2*(*rdr6*) or *sgs3* mutants (Figure 5A), indicating that RDR6 and SGS3 are indeed dispensable for miRNA-directed cleavage. Given this result, we expected miRNAs to accumulate in *sgs2*(*rdr6*) and *sgs3* mutants. Indeed, miRNAs accumulated in the mutants, although sometimes at a reduced level (Figure 5B), suggesting that this level is sufficient for proper miRNA-directed cleavage of the analyzed targets.

A previous report indicated that RDR6 and SGS3 are not required for the accumulation of 24 nt siRNAs derived from the retroposon AtSN1 or for DNA methylation of AtSN1 elements (Hamilton et al., 2002). To further analyze if mutations in RDR6 or SGS3 could impact the heterochromatic siRNA pathway, we looked at the accumulation of the 21 to 24 nt siRNAs deriving from tandem arrays of 5S rDNA genes, and at the methylation status of 5S DNA, which both require DCL3 and RDR2 (Xie et al., 2004). No change in 5S siRNA accumulation or 5S DNA methylation was observed in *sgs2*(*rdr6*) or *sgs3* mutants (Figures 5C and 5D), indicating that RDR6 and SGS3 are not required for proper functioning of the heterochromatic siRNA pathway and suggesting that the AGO1-DCL1-HEN1-HYL1-SGS3-RDR6-dependent siRNA pathway defined here is an endogenous posttranscriptional pathway.

Discussion

We identified a class of endogenous 21 nt regulatory siRNAs in plants that have similarities to but also differences from the previously characterized small silencing RNAs of plants. As is typical of plant miRNAs (Bartel, 2004), they are endogenous 21 nt regulatory RNAs, appear to derive from short double-stranded products of Dicer-like enzymes, appear to obey the asymmetry rules that determine which of the two strands of each short duplex enters RISC and accumulates, regulate endogenous target mRNAs in *trans* by directing cleavage in the middle of a single complementary site, target multiple members within a single gene family, and require AGO1,

DCL1, HEN1, and HYL1 for their biogenesis, accumulation, or function. In contrast to miRNAs, they derive from long double-stranded RNA, and their accumulation depends on RDR6 and SGS3. In these two respects, they resemble the siRNA mediators of S-PTGS, triggered by transgenes, and thus their characterization reveals the first endogenous regulatory role for RDR6 and SGS3. They differ from heterochromatic siRNAs, the other class of endogenous siRNAs that have been characterized in plants, in their posttranscriptional mode of silencing and the proteins required for their biogenesis, accumulation, or function. They differ from all siRNAs that have been described in plants in that they target genes that bear little resemblance to the genes from which the siRNAs derive, a function sometimes called "hetero-silencing" (Bartel, 2004), previously attributed only to miRNAs.

The siRNAs we describe here also differ from all endogenous siRNAs that have been described in animals, which like those previously found in plants, are known or suspected to mediate "auto-silencing," i.e., the silencing of the same locus (or very similar loci) from which they originate. For example, endogenous siRNAs perform important posttranscriptional regulation in *Drosophila*, where male fertility depends on the silencing of *Stellate* by endogenous siRNAs from the *Su(Ste)* repeats (Aravin et al., 2001). As a host gene for siRNAs, the *Su(Ste)* repeats differ from the source of the siRNAs we describe here in that the *Su(Ste)* repeats have extensive homology (greater than 1 kb of 90% identity) with their regulatory target, and their dsRNA product is generated by convergent transcription. Candidates for endogenous siRNAs that might mediate posttranscriptional silencing have also been reported in nematodes (Lau et al., 2001; Ambros et al., 2003; Lim et al., 2003). These are cloned ~21 nt RNAs that have perfect antisense complementarity to mRNAs of *C. elegans*. Although characterization of their biogenesis and potential functions has not been reported, it is reasonable to assume that these putative siRNAs ultimately derive from the same locus as their presumed targets, either through antisense transcription or an RdRp activity, because they generally match only a single locus in the genome.

Because of their unusual role in hetero-silencing, we refer to the new class of siRNAs identified here as endogenous "*trans*-acting siRNAs." Of course, all siRNAs are *trans*-acting in the sense that they are not part of the silenced molecule but are instead part of a ribonucleoprotein complex that recognizes the *cis*-acting siRNA complementary site. The newly identified siRNAs get their name because they are *trans*-acting in an additional sense—they direct hetero-silencing, repressing the expression of genes that bear little resemblence to the genes from which they derive. In this sense, other endogenous siRNAs that have been characterized are *cis*-acting, performing auto-silencing to repress the expression of genes that are the same or very similar to the loci from which they derive.

The endogenous *trans*-acting siRNAs analyzed in this paper derive from the At2g27400 gene, which likely produces RNA as its final product, in that it has no known or deduced function other than to serve as a host gene for the production of siRNAs. Among the small RNAs deriving from the sense strand, two nearly identical ones, siR480(+) and siR438(+), were previously reported as miRNAs (Table 1), annotated as miR175a-2/miR389b.1 and miR389b.3, respectively (Park et al., 2002; Sunkar and Zhu, 2004). Furthermore, siR396(+) was predicted to be a miRNA based on its similarity to siR480(+) (Park et al., 2002; Sunkar and Zhu, 2004). A predicted fold of the At2g27400 transcript does place siR396(+) in a hairpin with some resemblance to those of the known miRNAs (see Supplemental Figure S1 at http://www.molecell.org/cgi/content/full/16/1/69/DC1/), and in different suboptimal predicted folds, the segment that pairs to siR396(+) would presumably pair to siR438(+) and siR480(+), the two other clones that have been previously annotated as miRNAs. However, these potential stem loops differ subtly from those of the typical plant miRNAs. For the potential stem loops involving siR396(+), siR438(+), and siR480(+) pairing ends abruptly at both termini of the cloned RNA, whereas for plant miRNAs pairing typically extends, without interruption, beyond either the 5′ or 3′ terminus of the mature miRNA (Jones-Rhoades and Bartel, 2004). More convincing evidence comes when considering the siR396(+), siR438(+), and siR480(+) clones in the context of all the other clones that have now been found from the At2g27400 region. These three clones occur in 21 nt phasing with nearly all of the other At2g27400 siRNAs that have been found in wild-type plants (Table 1; Figure 1), including an siRNA from the antisense strand of the transcript, and like the other siRNAs, they do not accumulate in *rdr6* and *sgs3* mutants (Figure 2). They have the hallmarks of deriving from the processing of a long dsRNA, likely formed by the RNA-dependent RNA polymerase activity of RDR6, into multiple small RNA duplexes. siRNAs are produced by the Dicer-like processing of a long double-stranded precursor into multiple small RNA duplexes, whereas miRNAs are produced by the Dicer-like processing of each stem loop precursor into a single miRNA duplex (Bartel, 2004). Accordingly, we classified siR396(+), siR438(+), and siR480(+) as siRNAs, and attribute the location of siR396(+) within a predicted stem loop (Supplemental Figure S1) as a chance coincidence, perhaps not even an improbable one, given the observation that nearly 400,000 intergenic *Arabidopsis* 20-mers fall within miRNA-like stem loops (Jones-Rhoades and Bartel, 2004).

A mutant carrying a T-DNA inserted within the At2g27400 intron no longer accumulates the At2g27400 siRNAs (Figure 2). The coordinate reduction of siR396(+) with the other siRNAs further supports the hypothesis that all the cloned 21 nt RNAs derive from a common double-stranded RNA precursor. This insertion, which lies downstream of all the At2g27400 siRNAs (Figure 1), is not expected to influence the formation of stem loops (Supplemental Figure S1) but does truncate the 3′ sequence of the transcript (data not shown), perhaps compromising the RDR6-dependent conversion of the transcript into dsRNA. In contrast, siRNAs accumulate to wild-type levels in a mutant carrying a T-DNA inserted in exon2, suggesting that the elements needed for RDR6 recognition might reside at the 3′ end of either the spliced intron or a truncated unspliced transcript terminating at an alternative polyadenylation site located upstream of the 3′ end of the intron (Figure 1). The identification of an endogenous transcript that serves as a

template for RDR6-mediated production of dsRNA as well as the T-DNA lesion that might prevent RDR6 recognition could provide reagents for exploring the molecular basis of posttranscriptional gene silencing mediated by sense transgenes (S-PTGS/cosuppression). S-PTGS differs from IR-PTGS mediated by hairpin transgenes in that it requires the conversion of transgene-derived RNA into dsRNA (Beclin et al., 2002). It has been suggested that S-PTGS is triggered by "aberrant" RNAs transcribed from certain transgene loci (Dalmay et al., 2000; Mourrain et al., 2000); however, the features that define the transcripts as aberrant and therefore substrates for the production of dsRNA have been a mystery.

At2g27400 siR396(+), siR438(+), and/or siR480(+) regulate the expression of at least four genes encoding related proteins (Figure 4), all of which have siRNA complementary sites within their mature transcripts (Figure 3; Park et al., 2002, Sunkar and Zhu, 2004). In contrast, At2g27400 siR522(+) exhibits near-complementarity to only one target (At2g46740) and the siRNA complementary site is located within an intron. The overaccumulation of both At2g46740 spliced and unspliced transcripts in *rdr6* and *sgs3* mutants and in the *EYK112* insertion mutant (Figure 4; data not shown), which all have reduced At2g27400 siR522(+) accumulation (Figure 2), hints at the idea that siRNAs could guide cleavage within the introns of pre-mRNAs. Consistent with the intron-targeting hypothesis, the level of both At2g27400 unspliced and spliced transcripts was elevated in *rdr6* and *sgs3* mutants (data not shown), suggesting that antisense siRNAs derived from At2g27400 guide cleavage of the unspliced At2g27400 transcript, providing a possible feedback regulatory loop for the production of these siRNAs. However, this intriguing possibility of posttranscriptional regulation in the nucleus could not be corroborated by 5′-RACE experiments, perhaps because of the low abundance of the pre-mRNAs (Figure 3). Evidence for siRNA/miRNA targeting of intronic sequence within pre-mRNA has been reported only once in animals (Bosher et al., 1999), perhaps because efficiently spliced messages are not suitable targets for this type of regulation. Nuclear targeting of partially spliced *Arabidopsis DCL1* RNA by miR162 has been suggested (Xie et al., 2003), making our hypothesis of targeting of nuclear RNAs by siRNAs more plausible.

It is possible that additional *trans*-acting siRNAs exist in *Arabidopsis*, as suggested by other small RNA clones with clustered loci in the genome (Llave et al., 2002a; Reinhart et al., 2002). This raises the possibility that many endogenous mRNAs could be regulated posttranscriptionally by this class of siRNAs. The role of endogenous genes regulated by *trans*-acting siRNAs is yet unclear, whereas most of the genes regulated by miRNAs play important roles in plant development (Rhoades et al., 2002; Jones-Rhoades and Bartel, 2004). Indeed, *ago1*, *dcl1*, *hen1*, and *hyl1* mutants exhibit dramatic developmental defects (Bohmert et al., 1998; Jacobsen et al., 1999; Lu and Fedoroff, 2000; Chen et al., 2002), some of which have been confirmed to be due to the lack of miRNA regulation (Aukerman and Sakai 2003; Emery et al. 2003; Palatnik et al. 2003; Chen 2004; Mallory et al., 2004a, 2004b; Vaucheret et al., 2004). In contrast, *rdr6* and *sgs3* mutants exhibit a much milder phenotype, mostly characterized by downward curling of the leaf margin (Figure 6). This phenotype is very similar to that of *ago7* (*zippy*) mutants (Figure 6), which exhibit an early transition into the adult phase of vegetative development (Hunter et al., 2003); however, it is unlikely that misregulation of the At2g27400 siRNAs contribute to the phenotype of *ago7*, *rdr6*, and *sgs3* mutants. Indeed, the T-DNA insertion mutant EYK112 (Figure 1) that exhibits a strong reduction in the accumulation of these siRNAs (Figure 2) and increased accumulation of uncleaved target mRNAs (Figure 4) has no obvious phenotype (Figure 6). In addition, both siRNA accumulation and target mRNA accumulation are not affected by mutations in AGO7 (Figures 2 and 4), indicating that AGO7 is not required for this pathway, at least at this stage of development, and suggesting that the developmental defects of *ago7*, *rdr6*, and *sgs3* mutants could be due to the impairment of yet another siRNA pathway that remains to be discovered. Given the slight decrease in the accumulation of some miRNAs in *rdr6* and *sgs3* mutants (Figure 5), the developmental defects of these mutants could also be due to misregulation of miRNA targets not examined in our analysis.

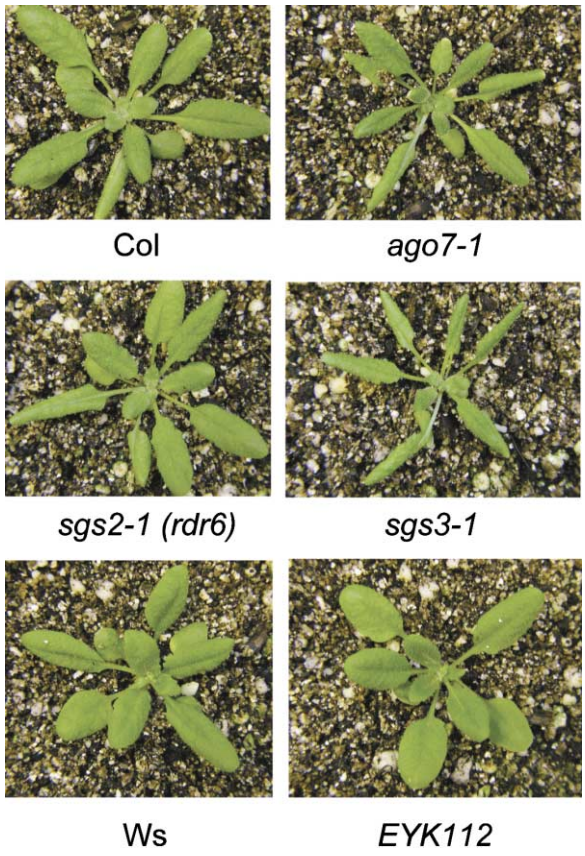

Figure 6. *rdr6* and *sgs3* Mutants Exhibit Developmental Defects Similar to *ago7*

Wild-type plants (Col or Ws), *sgs2-1*(*rdr6*), *sgs3-1*, and *ago7-1(zip)* mutants (in Col) and the *EYK112* mutant (in Ws) were grown in soil for 25 days. *sgs2-1*, *sgs3-1*, and *ago7-1* mutants but not *EYK112* exhibit similar downward curling of the leaf margin.

Is the AGO1-DCL1-HEN1-HYL1-SGS3-RDR6-dependent pathway, which produces endogenous *trans*-acting siRNAs, an ancient pathway from which the miRNA

pathway derived, or is it a more recent pathway? Whereas most miRNA/target pairs are conserved between *Arabidopsis* and rice (Rhoades et al., 2002; Jones-Rhoades and Bartel, 2004), we have not detected such conservation among the siRNA/target pairs described here. In addition, we do not find any matches to the At2g27400 siRNAs (0–2 substitutions) or At2g27400 transcript in EST databases of other plant species, including rice and *brassica*. This suggests that the At2g27400 siRNAs have emerged quite recently, and that the pathway as a whole might have recent origins. However, a few known miRNAs also appear to be of very recent origin (Jones-Rhoades and Bartel, 2004), and thus a more comprehensive list of AGO1-DCL1-HEN1-HYL1-SGS3-RDR6-dependent siRNAs will be needed to address the questions of which pathway might be more ancient and the degree to which the two pathways, which have such similar regulatory outcomes, might have overlapping origins.

Experimental Procedures

Plant Material

ago1-3, ago1-27, dcl1-9, dcl3-1, hen1-4, sgs2-1(rdr6) and *sgs3-1* mutants (all in the Col-0 ecotype) have been previously described (Bohmert et al., 1998; Elmayan et al., 1998; Jacobsen et al., 1999; Mourrain et al., 2000; Morel et al., 2002; Boutet et al., 2003; Vazquez et al., 2004; Xie et al., 2004). The *dcl1-9* mutant in Col was obtained by five backcrosses of the original *dcl1-9* mutant (Jacobsen et al., 1999) to Col. *ago7-1* (SALK_037458), *dcl2-3* (SALK_095069), *rdr2-2* (SALK_059661), *sde3-4* (SALK_092019), *wex-2* (SALK_003278) and the mutant carrying a T-DNA inserted in exon2 of the At2g27400 gene (SALK_065757) were identified in the collection of SALK mutants (all in the Col-0 ecotype) available from the *Arabidopsis* Stock Center (Alonso et al., 2003). The EYK112 mutant carrying a T-DNA inserted in the intron of the At2g27400 gene (FLAGDB_356B08) was identified in the INRA Versailles T-DNA collection of mutants (in the Ws ecotype) (Samson et al., 2002).

The molecular characterization of plant material, the methods for cDNA-AFLP profiling, real-time quantitative RT-PCR, RNA extraction and hybridization, modified 5′RLM-RACE for cloning of cleavage products, and 3′-RACE and 5′ RLM-RACE for cloning of full-length At2g27400 transcript are described in the Supplemental Data.

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