SGS3 and *SGS2/SDE1/RDR6* are required for juvenile development and the production of *trans*-acting siRNAs in *Arabidopsis*

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Higher plants undergo a transition from a juvenile to an adult phase of vegetative development prior to flowering. Screens for mutants that undergo this transition precociously produced alleles of two genes required for posttranscriptional gene silencing (PTGS)—*SUPPRESSOR OF GENE SILENCING3* (*SGS3*) and *SUPPRESSOR OF GENE SILENCING2(SGS2)/SILENCING DEFECTIVE1 (SDE1)/RNA-DEPENDENT POLYMERASE6* (*RDR6*). Loss-of-function mutations in these genes have a phenotype similar to that of mutations in the Argonaute gene *ZIPPY* (*ZIP*). Epistasis analysis suggests that *ZIP*, *SGS3*, *SGS2/SDE1/RDR6*, and the putative miRNA export receptor, *HASTY* (*HST*), operate in the same pathway(s). Microarray analysis revealed a small number of genes whose mRNA is increased in *ZIP*, *SGS3*, and *SGS2/SDE1/RDR6* mutants, as well as genes that are up-regulated in *SGS3* and *SGS2/SDE1/RDR6* mutants, but not in *ZIP* mutants. One of these latter genes (At5g18040) is silenced posttranscriptionally in *trans* by the sRNA255 family of endogenous, noncoding, small interfering RNAs (siRNAs). The increase in At5g18040 mRNA in *SGS3* and *SGS2/SDE1/RDR6* mutants is attributable to the absence of sRNA255-like siRNAs in these mutants. These results demonstrate a role for endogenous siRNAs in the regulation of gene expression, and suggest that PTGS plays a central role in the temporal control of shoot development in plants.

[*Keywords*: siRNA; miRNA; vegetative phase change; posttranscriptional gene silencing; RNAi; heterochrony]

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The anatomy and morphology of a plant shoot changes over the course of its development. Although many traits (e.g., leaf size) vary gradually, other traits change abruptly at predictable times in shoot development (Hackett and Murray 1993; Greenwood 1995; Poethig 2003). Two such changes occur during the postembryonic growth of the shoot. The juvenile-to-adult transition (vegetative phase change) occurs early in the life of the shoot, and is marked by differences in the anatomy, morphology, and chemistry of leaves and internodes produced before and after this transition. The second transition (reproductive phase change) occurs during the adult phase, and results in the production of flowers and flower-bearing branches in place of vegetative shoots. Genetic analysis of floral induction in *Arabidopsis* has produced many genes affecting the transition from vegetative to reproductive growth, and significant progress has been made in understanding the molecular mechanism of this transition (Mouradov et al. 2002). In contrast, we are just beginning to understand the mechanism of vegetative phase change.

Most of the genes known to affect vegetative phase change in *Arabidopsis* have pleiotropic mutant phenotypes, indicating that they are involved in many different processes. This class includes *SERRATE* (Clarke et al. 1999; Prigge and Wagner 2001), *SQUINT* (*SQN*) (Berardini et al. 2001), and *HASTY* (*HST*) (Telfer and Poethig 1998; Bollman et al. 2003). In contrast, loss-of-function mutations of *ZIPPY* (*ZIP*) have a much more specific effect on vegetative phase change (Hunter et al. 2003). Mutations in this Argonaute-like gene (*AGO7*) (Fagard et al. 2000) accelerate vegetative phase change and produce slightly abnormal flowers, but have no other obvious effects on shoot morphology (Hunter et al. 2003). This specific phenotype, and the possibility that ZIP might be involved in a regulatory pathway involving microRNAs (miRNAs) and/or small interfering RNAs (siRNAs), encouraged us to search for other mutations that have this same vegetative phenotype.

Screens for mutations with a *zip*-like phenotype produced alleles of two genes—*SUPPRESSOR OF GENE SILENCING3* (*SGS3*) and *SUPPRESSOR OF GENE*

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SILENCING2(SGS2)/SILENCING DEFECTIVE1(SDE1)/ RNA-DEPENDENT POLYMERASE6 (*RDR6*)—required for posttranscriptional silencing (PTGS; also known as RNA interference, RNAi) of transgenes. Genes subject to PTGS are targeted for destruction by complementary 21–22-nt siRNAs (Elbashir et al. 2001; Tang et al. 2003). In plants, PTGS can be initiated by both single-stranded (ssRNA) and double-stranded RNA (dsRNA). *SGS3* encodes a novel plant-specific protein and *SGS2/SDE1/ RDR6* encodes an RNA-dependent RNA polymerase (RdRP) required for silencing ssRNA. Both of these genes are believed to be involved in transforming ssRNA into the dsRNA precursors of siRNAs (Dalmay et al. 2000; Mourrain et al. 2000). Mutations in these genes confer sensitivity to cucumber mosaic virus (Beclin et al. 2002) and cabbage leaf curl virus (Muangsan et al. 2004), indicating that one of their normal functions is to provide resistance to viral infection. Two other RdRPs have been described in *Arabidopsis*. RNA-DEPENDENT RNA POLYMERASE2 (RDR2) is required for the production of siRNAs from endogenous transcripts, but is not required for virus resistance. RNA-DEPENDENT RNA POLYMERASE1 (RDR1) is induced upon viral infection and limits virus replication, but has no apparent role in the silencing of endogenous transcripts (Yu et al. 2003; Xie et al. 2004). RdRPs that participate in PTGS/RNAi have also been described in *Caenorhabditis elegans* (Smardon et al. 2000; Sijen et al. 2001; Simmer et al. 2002), *Neurospora crassa* (Cogoni and Macino 1999; Catalanotto et al. 2002; Shiu and Metzenberg 2002), *Schizosaccharomyces pombe* (Volpe et al. 2002, 2003) and *Dictyostelium discoideum* (Martens et al. 2002), and are required for a variety of different functions in the normal biology of these organisms. For example, *ego-1* is required for germ-line development in *C. elegans* (Smardon et al. 2000), *Rdp1* is necessary for centromere function in *S. pombe* (Volpe et al. 2003), and *Sad-1* silences unpaired DNA during meiosis in *N. crassa* (Shiu and Metzenberg 2002).

Here we show that *SGS3* and *SGS2/SDE1/RDR6* act in the same pathway(s) as the Argonaute protein, ZIP, and the putative miRNA nuclear export receptor, HST, to regulate vegetative phase change and floral development in *Arabidopsis*. Expression profiling revealed that only a few genes are affected in common by mutations in *ZIP*, *SGS3*, and *SGS2/SDE1/RDR6*; these genes are excellent candidates for genes involved in vegetative phase change. In addition, we identified genes that are affected by mutations in *SGS3* and *SGS2/SDE1/RDR6*, but not by a mutation in *ZIP*. One of the latter genes is silenced posttranscriptionally in *trans* by endogenous siRNAs derived from noncoding transcripts. These results reveal a developmental function for SGS3 and SGS2/SDE1/ RDR6, and suggest possible targets of their regulation.

Results

Identification of zip*-like mutants*

In *Arabidopsis*, leaves produced at different times during shoot development can be distinguished from each other by their size, shape, and pattern of trichome distribution (Chien and Sussex 1996; Telfer et al. 1997; Tsukaya et al. 2000; Hunter et al. 2003). The first two leaves are small, round, relatively flat, have a smooth margin, and are incapable of producing trichomes on their abaxial surface. Subsequent leaves are larger, elliptical in shape, curl downward, have a serrated margin, and have the capacity to produce abaxial trichomes, although these are not usually produced until leaf 5 or 6.

Loss-of-function alleles of *ZIP* accelerate the expression of adult-phase vegetative traits, but have relatively little effect on other aspects of shoot development (Hunter et al. 2003). The phenotype of these mutations is most apparent in the first two leaves of the rosette, which are elongated and curl downward instead of being round and flat. In addition to this effect on leaf shape, *zip* mutations produce a forward shift in abaxial trichome production. *zip* does not affect the rate of leaf initiation or flowering time, but does have a weak effect on flower morphology. Mutant carpels typically have a split septum that produces ectopic stigmatic tissue, and flowers have a variably penetrant defect in stamen and carpel elongation, leading to poor seed set.

Screens for EMS, fast-neutron, and T-DNA-induced mutations that have a *zip*-like phenotype produced a number of phenotypically similar, recessive mutations in two complementation groups (Figs. 1, 2). We mapped a mutation in the first complementation group near *SGS2/SDE1/RDR6* and a mutation in the second complementation group near *SGS3*. To confirm the identity of these new mutations, we performed complementation tests with *sgs2-1* and *sgs3-1*, the alleles originally described by Mourrain et al. (2000). *sgs2-1* and *sgs3-1* were found to have a *zippy*-like phenotype that was not complemented by the mutations identified in our screen. The polymorphisms associated with these new alleles of *SGS2/SDE1/RDR6* and *SGS3* are described in Table 1. Two T-DNA insertions in *SGS3* (SALK_001394 and

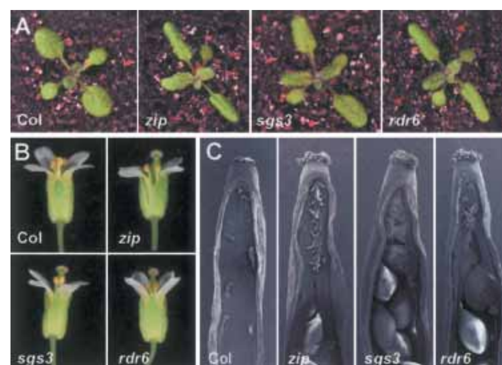

Figure 1. The morphology of wild-type Columbia (Col), *zip-1*, *sgs3-11*, and *rdr6-11*. (*A*) Thirteen-day-old plants. The first few leaves of mutant plants are elongated and curl downward. (*B*) Flowers. Mutant plants have variable seed sets because stamens frequently fail to contact the stigma. (*C*) Scanning electron micrographs of carpels with one valve and seeds removed. Mutant carpels have a split septum and produce stigmatic tissue in the middle of the septum at the apical end of the carpels.

Table 1. *New alleles of* RDR6 *and* SGS3

Allele	Mutagen	Mutation[a]
rdr6-11	T-DNA	C → T (805), nonsense
rdr6-12	Fast neutron	7 bp del. (997–1003), frameshift
rdr6-13	EMS	G → A (2597), missense
rdr6-14	EMS	G → A (3539), nonsense
sgs3-11	EMS	G → A (2283), splice site
sgs3-12	EMS	G → A (719), nonsense
sgs3-13	T-DNA	SALK_039005, insertion in exon 1
sgs3-14	T-DNA	SALK_001394, insertion in exon 1

[a]Numbered according to the genomic sequence of At3g49500 (*RDR6*) and At5g23570 (*SGS3*) as described in TAIR (http://www.arabidopsis.org).

SALK_039005) (Alonso et al. 2003) that have a morphological phenotype similar to these new alleles are also listed in this table. To simplify naming new alleles of *SGS2/SDE1/RDR6* we have chosen to use the nomenclature (*RDR6*) suggested by Xie et al. (2004) for *RdRP* genes in *Arabidopsis*.

sgs3-11 *and* rdr6-11 *prevent PTGS*

The results presented here were obtained using *sgs3-11* and *rdr6-11*. To determine whether these new mutations have an effect on transgene silencing like that of *sgs3-1* and *sgs2-1*, we determined their effect on the expression of L1, a *35S::GUS* transgene that is silenced posttranscriptionally in fully expanded leaves (Elmayan et al. 1998). Consistent with the phenotype of *sgs3-1* and *sgs2-1*, we found that *sgs3-11* and *rdr6-11* prevented silencing of L1 in all leaves of the shoot (Fig. 3). Silencing of L1 occurred normally in *zip-1*, as previously observed for the related L2 transgene (Hunter et al. 2003).

The phenotype of rdr6-11 *and* sgs3-11

An analysis of 25–35 plants of each genotype revealed that *sgs3-11* and *rdr6-11* have essentially the same phenotype as *zip-1*. All three mutants have elongated, downwardly curled leaves (Figs. 1A, 2) and produce abaxial trichomes precociously (Fig. 4A). Abaxial trichomes appear at the base of leaf 3 in *zip-1* and *rdr6-11* and at the base of leaf 4 in *sgs3-11*, and gradually increase in extent over the next few leaves. This gradual increase in abaxial trichome production resembles the pattern in wild-type plants, and implies that these mutations accelerate an otherwise normal transition to the adult phase. Mutant and wild-type plants do not exhibit a major difference in either total rosette leaf number (Fig. 4B) or in flowering time (Fig. 4C). The carpels of *zip-1*, *sgs3-11*, and *rdr6-11* typically have a split septum and produce stigmatic tissue at the center of the septum (Fig. 1C), although the expressivity of this floral phenotype varied among individual plants and along the length of the shoot. In addition, mutant plants often display a lack of coordination between stamen and carpel elongation, resulting in low seed set (Fig. 1B).

SGS3 and RDR6 act in the same pathway as ZIP

To determine whether these proteins act in the same pathway, we examined the phenotype of plants homozygous for different combinations of *zip-1*, *sgs3-11*, and *rdr6-11*. We found that the seedling and floral morphology (Figs. 2, 5), abaxial trichome production, total leaf number, flowering time, and seed set (Fig. 4) of single, double, and triple mutants were not dramatically different. Double- and triple-mutant plants produced slightly fewer leaves and flowered earlier than single mutants, but these differences—although statistically significant—were quite small and are probably attributable to differences in the age or vigor of the seed stocks used in this experiment, because the flowering time of triple-mutant plants was identical to wild-type. These results support the hypothesis that ZIP, SGS3, and RDR6 act in the same pathway(s).

Additional evidence for this conclusion is provided by the interaction between these mutations and *hst-1* and *sqn-1*. *HST* is the ortholog of the pre-miRNA nuclear export receptor exportin 5 (Bollman et al. 2003; Yi et al. 2003; Bohnsack et al. 2004; Lund et al. 2004), whereas *SQN* is the ortholog of cyclophilin 40 (Berardini et al. 2001). Loss-of-function mutations in these genes resemble *zip-1*, *sgs3-11*, and *rdr6-11* in that they accelerate the juvenile-to-adult transition. However, *hst* and *sqn* mutations have a much stronger effect on the expression of phase-specific traits and are more pleiotropic than these three mutations. Previously, we found that *zip-1* interacts additively with *sqn-1*, and that *hst-1* is epistatic to *zip-1* (Hunter et al. 2003). *sgs3-11* and *rdr6-11* interact with *sqn-1* and *hst-1* in the same fashion. *sgs3-11 hst-1* and *rdr6-11 hst-1* were morphologically indistinguishable from *hst-1* (Figs. 2, 5) and had the same pattern of trichome production as *hst-1* plants (Fig. 4A). Although *rdr6-11 hst-1* plants had two more leaves (Fig. 4B) and flowered 2 d earlier than *hst-1* (Fig. 4C), we do not believe that this difference is biologically meaningful, because this difference is the opposite of the normal relationship between leaf number and flowering time. Furthermore, the expressivity of these two traits varies significantly in *hst-1* even under fairly uniform conditions (Telfer and Poethig 1998). In contrast, *sgs3-11 sqn-1* and *rdr6-11 sqn-1* plants had an additive phenotype. The first two leaves of these double mutants were intermediate in morphology (Figs. 2, 5) and produced abaxial trichomes (Fig. 4A); abaxial trichomes were never seen earlier than leaf 3 in single mutants. Furthermore,

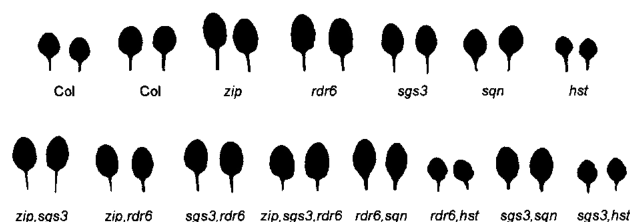

Figure 2. The morphology of the first two rosette leaves of wild-type (Col) and mutant plants.

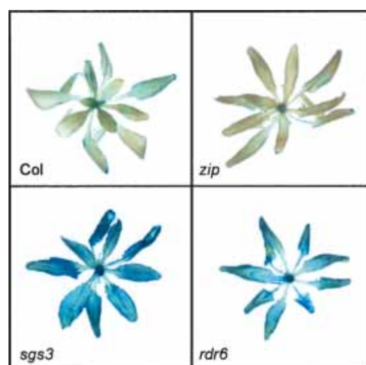

Figure 3. The effect of *zip-1*, *sgs3-11*, and *rdr6-11* on posttranscriptional silencing of the L1 (*35S::GUS*) transgene. *zip-1* does not interfere with the silencing of L1, but *sgs3-11* and *rdr6-11* prevent silencing in all fully expanded rosette leaves.

the siliques of double mutants had multiple carpels, completely lacked a septum, and had a very reduced seed set (Fig. 4D). These results support the conclusion that RDR6 and SGS3 are functionally related to ZIP, and that all three of these proteins probably operate in the same pathway (or on the same substrates) as HST. The additive interaction between these mutations and *sqn-1* may mean that SQN operates in a completely different regulatory pathway, or that SQN and these three proteins have related but nonoverlapping functions.

Genes regulated by ZIP, SGS3, and RDR6

In order to identify genes regulated by ZIP, SGS3, and RDR6, we performed a microarray analysis of gene expression in wild-type and mutant plants. RNA was isolated from shoot apices of 2-week-old plants grown under short days (10 h light:14 h dark) in order to delay flowering and thus limit the analysis to genes involved in vegetative development. Hybridizations to the Affymetrix ATH1 array (22,800 genes) were performed in duplicate, using RNA isolated from two sets of plants grown at different times in the same growth chamber.

This analysis produced 17 genes whose expression was increased in all three mutants in both replicates by an average of 1.3-fold or greater, but no genes that were down-regulated in a similar fashion (Table 2). We chose nine genes from this group for further analysis based on their level of expression or potential role in phase change. Northern analysis of RNA isolated from additional samples confirmed these microarray results in the case of *ARF3/ETTIN*, *ARF4*, and *SPL3* (Fig. 6A,B). However, we were unable to confirm these microarray results for six other genes, implying that most of the 17 genes listed in Table 2 are false positives.

SPL3 is a potential target of miR156 (Rhoades et al. 2002). To determine whether the increase in SPL3 mRNA could be attributed to a defect in miRNA biogenesis, we examined the accumulation of miR156 and two additional miRNAs in 2-week-old mutant and wild-type plants. Little or no difference in the levels of these miRNAs was observed (Fig. 6C), confirming the results obtained for miR171 (Boutet et al. 2003). This result suggests that ZIP, SGS3, and RDR6 are not involved in miRNA biogenesis. To test the hypothesis that ZIP, SGS3, and RDR6 are required for PTGS of *ARF3/ETTIN*, *ARF4*, and *SPL3*, we searched for siRNAs derived from these genes. Blots of low-molecular-weight RNA were

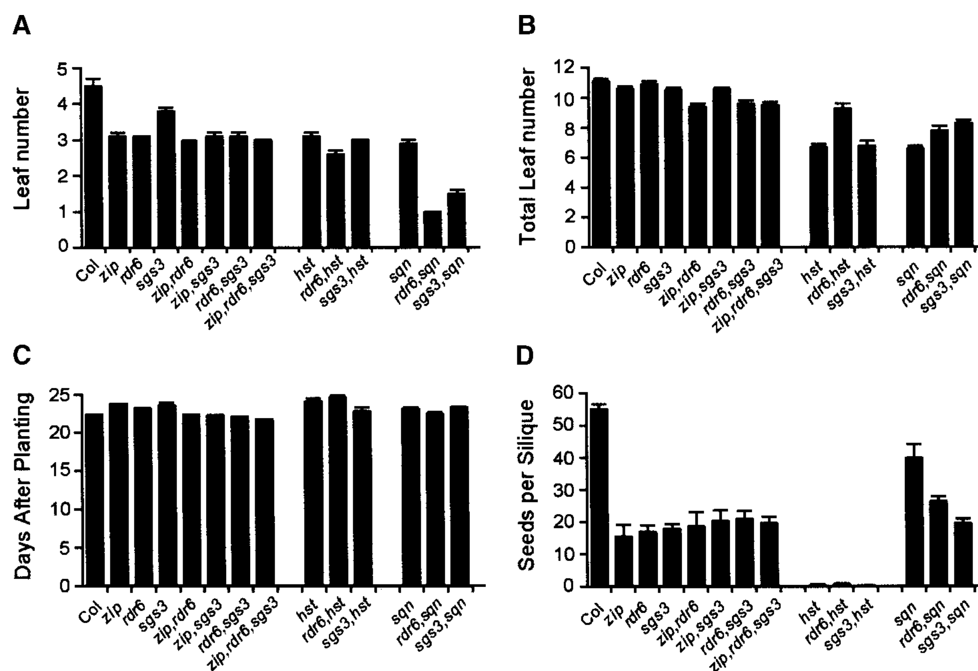

Figure 4. The phenotype of wild-type (Col) and mutant plants. (*A*) First leaf with abaxial trichomes. (*B*) Total number of leaves in the rosette. (*C*) Time to production of the first open flower. (*D*) Number of seeds per silique; 25–35 plants were measured for each genotype. Error bars indicate standard error of the mean.

Figure 5. The phenotype of *zip-1*, *sgs3-11*, *rdr6-11*, *hst-1*, and *sqn-1*, and plants homozygous for combinations of these mutations (13-day-old plants).

hybridized with cDNAs complementary to the coding region of these genes; however, we were unable to detect hybridization to these probes. We detected a weak signal with a probe for the SPL3 3′ UTR but not with a 3′ UTR probe containing seven mutations in the miR156 cognate site, indicating that this signal probably represents cross-hybridization with miR156. The basis for the accumulation of ARF3/ETTIN, ARF4, and SPL3 mRNA in these mutants therefore remains to be determined.

In addition to miRNAs, *Arabidopsis* possesses many 20–26-nt RNAs (i.e., small RNAs, sRNAs) derived from transposons and intergenic sequences (Llave et al. 2002a; Xie et al. 2004). sRNAs derived from several of these sequences require the RdRP, RDR2, and the Dicer-like protein, DCL3, for their biogenesis (Xie et al. 2004). To determine whether ZIP, SGS3, and RDR6 play a role in the biogenesis of these sRNAs, we examined the effect of *zip-1*, *sgs3-11*, and *rdr6-11* on the accumulation of sRNAs derived from the SINE retroelement *AtSN1*, and the *sRNA02* and *Cluster02* loci, all of which are 24 nt in size. Previous investigators have reported that *rdr6* and *sgs3* mutations have no effect on the accumulation of *AtSN1* (Hamilton et al. 2002). We confirmed this result and found that these mutations also do not affect the accumulation of *sRNA02* and *Cluster02* sRNAs (Fig. 6D).

SGS3 and RDR6 are required for the biogenesis of trans*-acting siRNAs*

Along with genes whose mRNA accumulates in all three mutants, we identified two genes (At1g63130 and At5g18040) whose mRNA accumulates in *sgs3-11* and *rdr6-11*, but not in *zip-1* (Fig. 7A). At1g63130 encodes a pentratricopeptide repeat (PPR) protein, whereas At5g18040 encodes a protein of unknown function. A BLAST search of the Small RNA Database (http://cgrb.orst.edu/smallRNA) (Xie et al. 2004) revealed that several endogenous 21-nt sRNAs are partially complementary to a site in the coding region of At5g18040 (Fig. 7B). Using a modified 5′ RACE protocol (Llave et al. 2002b), we found that At5g18040 mRNA is cleaved in the middle of this complementary sequence (Fig. 7B) and that the 3′ product of this cleavage reaction is reduced or absent in *rdr6-11* and *sgs3-11* plants (Fig. 7C). To determine the basis for this effect, we examined the expression of four sRNAs that are complementary to this site (sRNA255, sRNA752, sRNA289, and sRNA850) in 2-week-old plants mutant for *ZIP*, *SGS3*, *RDR6*, or

Table 2. *Microarray analysis of genes up-regulated in 2-week-old* zip-1, sgs3-11, *and* rdr6-11 *plants grown in short days*

Gene	Annotation	Fold increase[a]			Conf.[b]
		zip	*sgs3*	*rdr6*	
At2g33860	auxin response transcription factor 3 (ETTIN/ARF3)	3.1	3.6	4.2	yes
At5g60450	auxin response transcription factor (ARF4)	2.7	2.8	2.5	yes
At1g08830	copper/zinc superoxidase dismutase (CSD1)	2.0	2.5	2.3	no
At1g62480	vacuolar calcium-binding protein-related	2.2	2.1	1.7	no
At4g35770	senescence-associated protein (SEN1)	2.0	1.7	1.9	n.d.
At1g64660	methionine/cystathionine gamma lyase, putative	2.0	1.7	1.6	n.d.
At1g62500	lipid-transfer protein family	1.6	1.9	1.8	n.d.
At1g73500	MAP kinase, putative	1.7	1.8	1.8	no
At5g14780	formate dehydrogenase	1.8	1.9	1.4	n.d.
At3g05900	similar to anion exchange proteins	1.9	1.8	1.4	n.d.
At5g14920	gibberellin-regulated protein family	1.7	1.6	1.5	no
At1g12780	uridine diphosphate glucose epimerase	1.7	1.6	1.5	no
At4g15800	rapid alkalinization factor (RALF) family protein	1.5	1.6	1.4	no
At2g38530	lipid-transfer protein 2 (LTP2)	1.4	1.6	1.6	n.d.
At3g23030	auxin-inducible gene (IAA2)	1.4	1.4	1.7	n.d.
At2g33810	squamosa-promoter binding protein (SPL3)	1.3	1.5	1.5	yes
At1g11260	glucose transporter	1.4	1.4	1.3	n.d.

[a]Relative to wild-type Columbia.
[b]Confirmed by Northern analysis of mRNA prepared from a separately harvested sample. (n.d.) Not determined.

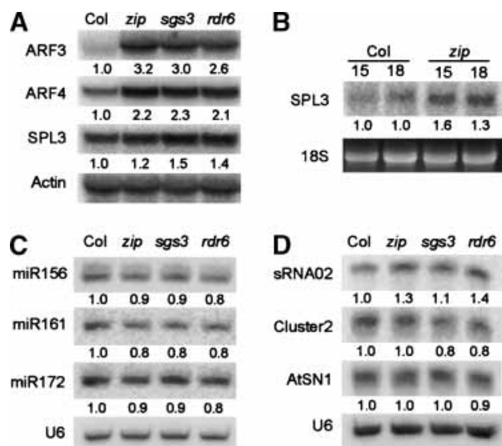

Figure 6. The effect of *zip-1*, *sgs3-11*, and *rdr6-11* on the accumulation of mRNAs, miRNAs, and siRNAs. (*A*) mRNA of *ARF3/ETTIN*, *ARF4*, and *SPL3* accumulates in *zip*, *sgs3-11*, and *rdr6-11*. Total RNA from 2-week-old rosettes of plants grown in short days was hybridized with probes to the indicated genes. Actin was used as a loading control. (*B*) *SPL3* mRNA is elevated in *zip-1* at 15 and 18 d after planting. Blot of total RNA from shoot apices of wild-type and *zip-1* rosettes hybridized with a probe to SPL3. 18S ribosomal RNA was used as a loading control. (*C*) The accumulation of miRNAs in *zip-1*, *sgs3-11*, and *rdr6-11*. Blots of low-molecular-weight RNA from 2-week-old mutant and wild-type plants hybridized with probes complementary to the functional strand of the indicated miRNAs. (*D*) The effect of *zip-1*, *sgs3-11*, and *rdr6-11* on the level of endogenous sRNAs. Blots of low-molecular-weight RNA from 2-week-old mutant and wild-type plants hybridized with probes to the indicated sRNAs. U6 was used as a loading control. The intensity of each signal was measured using NIH Image; the ratio of this signal relative to wild-type is indicated *below* each image.

DCL1. We were unable to detect expression of sRNA850 (data not shown). However, Northern analysis revealed that sRNA255, sRNA289, and sRNA752 are present in wild-type and *zip-1* 2-week-old rosettes, but are absent in *rdr6-11*, *sgs3-11*, and *dcl1-7* (Fig. 7D). It should be noted that because of their sequence similarity we cannot rule out the possibility that most, if not all, of the hybridization observed in these experiments corresponds to a single one of these sRNAs. These observations suggest that the increase in At5g18040 mRNA in *rdr6-11* and *sgs3-11* is attributable to a reduction in mRNA cleavage resulting from a defect in the biogenesis of one or more of these sRNAs.

sRNA255, sRNA752, sRNA289, and sRNA850 sRNAs are found at three sites in the genome (clusters 24, 64, and 68) in association with a number of other sRNAs (Table 3; Fig. 7E) (http://cgrb.orst.edu/smallRNA). Because sRNA255 is present at all three sites, we refer to this class of sRNAs as sRNA255-like sRNAs. The RNAs in cluster 28 are contained within an ~900-nt clone isolated from a root cDNA library (RAFL14-47-C13; GenBank AU226586 and AI997465), whereas cluster 64 sRNAs are present in an ~650-nt clone isolated from a cDNA library of senescing leaves of Landsberg *erecta* (Ler) (GenBank CD534180, CD534192). The sequences of sRNA255 and sRNA1745 are conserved in this Ler sequence, but sRNA752 and sRNA1927 are polymorphic between Ler and Col. The existence of these cDNAs suggests that the sRNAs in cluster 28 and cluster 64 derive from single transcripts. The precursor(s) of cluster 68 has not been identified, but the proximity of these sRNAs strongly suggests that they arise from a common transcript as well. sRNAs complementary to sRNA289 in cluster 28 and to sRNA28/850/1413 in cluster 68 have been identified, suggesting that these molecules derive from a dsRNA precursor (Table 3; Fig. 7E). This prediction is further supported by the observation that these complementary pairs of sRNAs (sRNA289/sRNA1796 and sRNA1413/sRNA1946) are predicted to form dsRNAs that have one or two 3′ unpaired nucleotides at each end—a feature characteristic of the products of Dicer-mediated cleavage (Elbashir et al. 2001). Based on these observations, and the fact the production of sRNA255-like sRNAs depends on SGS3 and RDR6, we conclude that the sRNAs in these clusters are siRNAs. Biogenesis of sRNA255-like sRNAs also requires the Dicer-like protein, DCL1, as these sRNAs are absent in *dcl1-7* mutants (Fig. 7D). DCL1 is required for miRNA biogenesis (Park et al. 2002; Reinhart et al. 2002), but is not required for PTGS initiated by a hairpin transgene (Finnegan et al. 2003) or for the production of endogenous 24-nt sRNAs (Xie et al. 2004). This result was therefore unexpected, and may reveal that DCL1 has a broader array of substrates than has previously been suspected.

Discussion

The mechanism of PTGS and its function in plant biology are only beginning to be deciphered. In plants, PTGS is involved in transgene silencing, virus resistance, the production of siRNAs from endogenous genes, and the suppression of gene expression by miRNAs. *SGS3* and *RDR6* are known to be involved in two of these processes: transgene silencing and virus resistance (Dalmay et al. 2000; Mourrain et al. 2000; Beclin et al. 2002; Vaistij et al. 2002; Himber et al. 2003; Muangsan et al. 2004). Here we demonstrate that *SGS3* and *RDR6* also play a role in normal development, and show that these genes are required for the production of endogenous siRNAs that act in *trans* to repress gene expression by an RNAi-like mechanism.

The function of SGS3 *and* RDR6

We found that loss-of-function mutations of *SGS3* and *RDR6* have a precocious vegetative phenotype and floral phenotype nearly identical to that of mutations in the Argonaute-like gene *ZIP*. In addition, we found that plants homozygous for any combination of these three mutations are not significantly different from single mutant plants, strongly suggesting that these genes operate in the same genetic pathway(s). The observation that

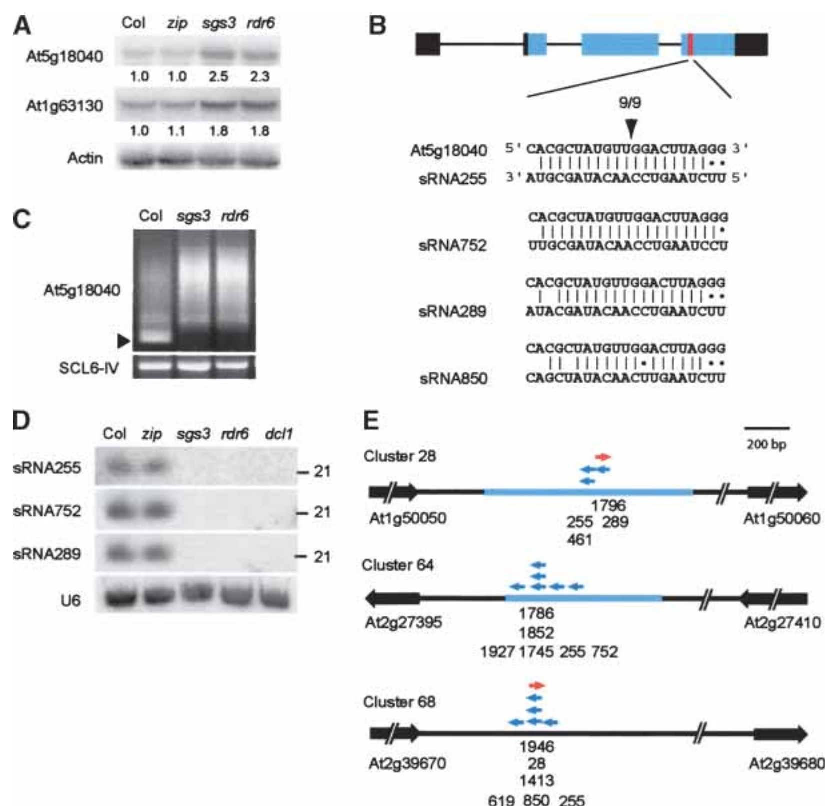

Figure 7. *SGS3* and *RDR6* repress gene expression by promoting the production of *trans*-acting siRNAs. (*A*) Genes affected by *sgs3-11* and *rdr6-11*, but not by *zip-1*. Total RNA from 2-week-old rosettes of plants grown in short days hybridized with probes to the indicated genes. Actin was used as a loading control. The intensity of each signal was measured using NIH Image; the ratio of this signal relative to wild-type is indicated *below* each image. (*B*) The genomic structure of At5g18040 and the location and sequence of the cognate site of sRNA255, sRNA752 and sRNA289, and sRNA850. UTRs are black, the coding region is blue, and the sRNA255 cognate sequence is red. Sequencing of nine 5′ RACE clones revealed that At5g18040 is cleaved in the middle of this cognate site. (*C*) 5′RACE products from Col, *sgs3-11*, and *rdr6-11* 2-week-old rosettes. This reaction gives one major product in Col (arrowhead) that is missing in *sgs3-11* and *rdr6-11*. 5′RACE of SCL6-IV, which is cleaved by miR171, was used as a control. (*D*) *sgs3-11*, *rdr6-11*, and *dcl1-7* block the production of sRNA255-like RNAs. Blot of low-molecular-weight RNA from 2-week-old plants hybridized with probes complementary to the indicated sRNAs. (*E*) *sRNA255*-like RNAs are found in three clusters on two different chromosomes. Arrows indicate the 5′-to-3′ orientation of the sRNA sequence. Red arrows are in direct orientation and blue arrows are in reverse orientation to the genomic DNA sequence.

sgs3 and *rdr6* mutants have a defect in transgene silencing that is not shared by *zip* implies that these proteins have distinct functions. Argonaute proteins are associated with siRNAs and miRNAs in the RNA-induced silencing complex in animals (Hammond et al. 2001; Mourelatos et al. 2002; Hutvagner et al. 2004; Shi et al. 2004) and fungi (Catalanotto et al. 2002), and are required for the activity of siRNAs and miRNAs in fungi, plants, and animals (Carmell et al. 2002). *SGS3* and *RDR6* are required for the synthesis of 21-nt siRNAs from transgenes that produce single-stranded transcripts (Dalmay et al. 2000; Mourrain et al. 2000; Beclin et al. 2002; Boutet et al. 2003; Himber et al. 2003), but are not required for PTGS initiated by hairpin transgenes (Beclin et al. 2002).

ZIP, SGS3, and RDR6 could regulate the biogenesis or function of endogenous small RNAs in several ways. One possibility is that ZIP is required for the activity of miRNAs or siRNAs that initiate transitive PTGS. In this scenario, ZIP would initiate miRNA-directed or siRNA-directed mRNA cleavage, and SGS3 and RDR6 would amplify this effect by producing dsRNA from untargeted regions of the mRNA, as RDR6 has been shown to do in the case of transgenes (Vaistij et al. 2002; Himber et al. 2003). Although there is as yet no evidence that miRNAs initiate transitive gene silencing, we believe it is reasonable to entertain this possibility because of the way in which these mutations interact with *hst*. Plants doubly mutant for any of these genes and *hst* are phenotypically identical to *hst*, implying that HST operates in the same pathway(s) as these genes. HST is the *Arabidopsis* ortholog of the miRNA export receptor, exportin 5 (Bollman et al. 2003; Yi et al. 2003; Lund et al. 2004), and mutations in *HST* reduce the levels of most miRNAs in *Arabidopsis* (M.-Y. Park and R.S. Poethig, unpubl.). The interaction between these mutations therefore suggests that miRNAs play important roles in the pathway(s) in which ZIP, SGS3, and RDR6 operate. This hypothesis also receives some support from the identity of the genes whose mRNA accumulates in *zip*, *sgs3*, and *rdr6* mutants. We identified three such genes by microarray analysis: *ARF3/ETTIN*, *ARF4*, and *SPL3*. *ARF3/ETTIN* and *ARF4* encode members of a large family of auxin-regulated transcription factors, several of which are potential targets of miRNAs (Park et al. 2002; Rhoades et al. 2002); whether ARF3/ETTIN and ARF4 are subject to miRNA regulation is unknown. *SPL3* encodes a member of the Squamosa Promoter Binding family of transcription factors (Cardon et al. 1999), 10 of which possess a cognate site for miR156 in either their coding region or 3′ UTR (Rhoades et al. 2002). *SPL3* is of particular interest because its mRNA increases during vegetative development (Cardon et al. 1997, 1999), making it an excellent candidate for a gene involved in vegetative phase change. Furthermore, overexpression of *SPL3* in transgenic plants causes early flowering (Cardon et al. 1997).

Table 3. *sRNAs in Cluster 28, Cluster 64, and Cluster 68*[a]

Cluster	sRNA	Sequence	Location	Length	Chromosome
28	255	UUCUAAGUCCAACAUAGCGUA	18553232–18553212	21	1
	461	UUCUAAGUCCAACAUAGCGUAA	18553232–18553213	22	1
	289	UUCUAAGUCCAACAUAGCAUA	18553253–18553233	21	1
	1796	UGCUAUGUUGGACUUAGAAU	18553235–18553254	20	1
64	1927	UUUCUUUAAAAUUGUUUCCGUGUA	11728892–11728869	24	2
	1786	UGAUAUUUGUAGUAAUGGCG	11729020–11729001	20	2
	1852	AUGAUAUUUGUAGUAAUGGCG	11729021–11729001	21	2
	1745	AAUGAUAUUUGUAGUAAUGGCG	11729022–11729001	22	2
	255	UUCUAAGUCCAACAUAGCGUA	11729063–11729043	21	2
	752	UCCUAAGUCCAACAUAGCGUU	11729147–11729127	21	2
68	619	AUAUUCCAGGAUAUGCAAAAG	16544872–16544852	21	2
	1946	UCGAUAUGUUGAACUUAGAAUA	16544895–16544916	22	2
	28	UUCUAAGUUCAACAUAUCGA	16544914–16544895	20	2
	850	UUCUAAGUUCAACAUAUCGAC	16544914–16544894	21	2
	1413	UUCUAAGUUCAACAUAUCGACG	16544914–16544893	22	2
	255	UUCUAAGUCCAACAUAGCGUA	16544935–16544915	21	2

[a]Sequences and nomenclature according to the Small RNA Database (http://cgrb.orst.edu/smallRNA/db).

We were unable to detect siRNAs derived from the coding region of these genes by Northern analysis, and thus have no evidence that these genes are actually subject to transitive PTGS. However, the possibility that these siRNAs may be present at too low a level to be detected by this approach has not been eliminated.

Although we are intrigued by the hypothesis that SGS3 and RDR6 are involved in miRNA-initiated transitive PTGS, Tang et al. (2003) noted that this phenomenon is inconsistent with the observation that mutations in miRNA cognate sites are typically dominant to the wild-type allele (McConnell et al. 2001; Palatnik et al. 2003; Vaucheret et al. 2004). If miRNAs are capable of initiating secondary siRNA production from untargeted regions of a transcript, the wild-type allele would be a source of such siRNAs in heterozygous plants, and these siRNAs should be capable of silencing both the wild-type and mutant alleles. Thus, if SGS3 and RDR6 are indeed involved in miRNA-initiated transitivity, it is likely that this process only occurs in a few cases. Another possibility is that the function that SGS3 and RDR6 perform in miRNA-directed gene silencing does not involve dsRNA synthesis, and thus is different from the function they perform in transgene silencing. For example, these genes may be required for miRNA-directed cleavage of target mRNAs. This function is suggested by the observation that the *C. elegans* RdRP, *rrf-1*, is likely to have a function beyond its role in dsRNA synthesis (Sijen et al. 2001).

A third hypothesis is that SGS3 and RDR6 are involved in the biogenesis of endogenous siRNAs that mediate PTGS in association with ZIP or another Argonaute protein. This hypothesis is suggested by our observation that SGS3 and RDR6 are required for the accumulation of a family of siRNAs that mediate the cleavage of At5g18040 mRNA. Although we have no evidence that ZIP is required for this particular cleavage event, it is not unreasonable to believe that it could be involved in the biogenesis or function of other SGS3/RDR6-dependent siRNAs that silence genes involved in vegetative phase change.

The function of PTGS in vegetative phase change

What is the role of *ZIP*, *SGS3*, and *RDR6* in vegetative phase change? The precocious loss-of-function phenotype of these genes demonstrates that they normally promote the expression of the juvenile phase, presumably by repressing the expression of adult-promoting genes during this phase. Therefore, one way in which phase change might be regulated is by a decrease in the activity of these genes late in shoot development. The available information about the expression of these genes does not support this hypothesis, however. *ZIP* mRNA actually increases, rather than decreases, during shoot development (Hunter et al. 2003). Although the expression pattern of *SGS3* and *RDR6* has not been characterized, we found that mutations in these genes interfere with PTGS throughout shoot development, implying that they are expressed constitutively. These data suggest that the juvenile-to-adult transition is regulated by a change in the transcription of regulatory RNAs whose subsequent processing or function is dependent on ZIP, SGS3, and RDR6, rather than by a change in the abundance or activity of these three proteins.

Although there is no evidence that temporal changes in the expression of *ZIP*, *SGS3*, and *RDR6* contribute to vegetative phase change, this may not be true for other components of gene-silencing pathways. Temporal variation in gene silencing has been described for transposons (Banks et al. 1988; Fedoroff and Banks 1988; Martienssen et al. 1990) and the *Pl-Bh* mutation (Cocciolone and Cone 1993; Hoekenga et al. 2000) in maize, as well as for several transgenes in *Arabidopsis* (Elmayan et al. 1998; Glazov et al. 2003; Vaucheret et al. 2004). As a rule, silencing increases along the length of the shoot; genes subject to silencing are typically expressed at a

higher level in organs produced early in development (cotyledons, juvenile leaves) than in organs produced later in shoot development (adult leaves, flowers). In maize, this temporal decrease in gene expression or transposon activity has been associated with increased DNA methylation (Fedoroff and Banks 1988; Hoekenga et al. 2000; Martienssen et al. 1990). This phenomenon suggests that temporal variation in gene-silencing pathways not only regulates vegetative phase change, but may also be controlled by this developmental transition. Determining the endogenous targets of these gene-silencing pathways is an important objective for future research.

The function of endogenous small RNAs

The first endogenous small RNAs to be described in animals (Lee et al. 1993; Lau et al. 2001; Lee and Ambros 2001; Lagos-Quintana et al. 2002) and plants (Llave et al. 2002a; Park et al. 2002; Rhoades et al. 2002) were miRNAs. These 20–22-nt RNAs are derived from hairpin precursors and repress the expression of mRNAs that have complementary sequences by directing the cleavage of these mRNAs, or repressing their translation. Other types of small (20–26-nt) noncoding RNAs have since been described in *C. elegans* (Ambros et al. 2003b) and *Arabidopsis* (Llave et al. 2002a; Xie et al. 2004). In *C. elegans*, these RNAs are called "tiny noncoding RNAs" (tncRNAs) (Ambros et al. 2003b). Some of these sRNAs and tncRNAs are fragments of mRNAs, whereas others arise from intergenic regions of the genome. tncRNAs and sRNAs do not appear to derive from hairpin precursors, although most require Dicer for their biogenesis and thus probably arise from dsRNA precursors. Several *Arabidopsis* sRNAs have been shown to promote heterochromatin formation (Zilberman et al. 2003; Xie et al. 2004). The function of tncRNAs in *C. elegans* is unknown.

The evidence presented here suggests that some sRNAs in *Arabidopsis* act in *trans* to cleave mRNAs. This conclusion is based on the effect of *sgs3-11* and *rdr6-11* on the level of sRNA255-like sRNAs and their putative target, At5g18040. We found that At5g18040 is cleaved in the middle of a sequence that is complementary to sRNA255-like sRNAs, and that this process is defective in *sgs3-11* and *rdr6-11* because sRNA255-like sRNAs are absent in these mutants. sRNA255-like sRNAs arise from noncoding transcripts. Although the precursor of sRNAs in cluster 64 can be folded to produce a hairpin containing sRNA752, several observations indicate that these sRNAs are siRNAs, not miRNAs. The most compelling evidence for this conclusion is the observation that RDR6 is required for their biogenesis. By definition, miRNAs arise from precursor molecules that are capable of forming dsRNA by intramolecular pairing (Ambros et al. 2003a). There is no obvious reason why an RdRP should be required for miRNA biogenesis and, indeed, we found no evidence that *rdr6-11* has a major effect on the accumulation of miRNAs. It is also significant that sRNA255-like sRNAs are produced from precursors that are the source of multiple sRNAs, some of which are complementary to each other. This observation suggests that sRNA255-like sRNAs arise from long, perfectly paired dsRNA precursors produced by SGS3/RDR6-mediated reverse transcription. One argument against this hypothesis is that the biogenesis of sRNA255-like sRNAs also requires DCL1, a protein that was shown to be required for miRNA biogenesis (Park et al. 2002; Reinhart et al. 2002), but not for the biogenesis of endogenous siRNAs (Xie et al. 2004). This result may reveal a role for DCL1 in the generation of a subset of endogenous siRNAs (see below). An alternative possibility is that sRNA255 precursors are cleaved once by DCL1 (because they resemble pre-miRNAs?) and are then transformed into siRNAs by a mechanism that is dependent on SGS3, RDR6, and another Dicer-like protein.

Two types of siRNAs are produced from transgenes undergoing PTGS: short (21–22-nt) siRNAs and long (23–26-nt) siRNAs (Hamilton et al. 2002; Klahre et al. 2002; Himber et al. 2003; Tang et al. 2003). Short siRNAs are associated with sequence-specific mRNA degradation, whereas long siRNAs are correlated with systemic silencing and DNA methylation (Hamilton et al. 2002). Endogenous sRNAs in *Arabidopsis* can be grouped into similar size and functional categories. *Arabidopsis* sRNAs range in size from 20–26 nt, with long (23–26-nt) sRNAs being the predominant class (Hamilton et al. 2002; Xie et al. 2004). Long sRNAs (e.g., AtSN1, Cluster02, and sRNA02) require RDR2 and DCL3 for their biogenesis and direct heterochromatin formation (Xie et al. 2004). sRNA255-like sRNAs are short (20–22-nt) sRNAs. These sRNAs resemble miRNAs and transgene-derived siRNAs in size, in their requirement for, respectively, DCL1 and RDR6, and in their ability to direct mRNA cleavage (Hamilton et al. 2002; Llave et al. 2002a,b; Park et al. 2002; Reinhart et al. 2002; Himber et al. 2003; Kasschau et al. 2003). This observation suggests that miRNAs and short sRNAs/siRNAs share components of their biosynthetic pathways and have similar regulatory functions. Hundreds of short sRNAs have been identified in *Arabidopsis* (Llave et al. 2002a; Xie et al. 2004). Even if only a small fraction of these short sRNAs target coding transcripts, their regulatory potential could be enormous.

Materials and methods

Plant material and growth conditions

All genetic stocks used in this study were in a Columbia (Col) genetic background. Sequence-indexed T-DNA insertions in *sgs3* (SALK_001394 and SALK_039005) were generated by Jose Alonso and Joe Ecker (Salk Institute) (Alonso et al. 2003) and were obtained from the *Arabidopsis* Biological Resource Center. Seeds of the L1 line, *sgs2-1*, and *sgs3-1* were provided by H. Vaucheret (INRA). Seeds were grown on Metromix 200 (Scotts) and placed at 4°C for 2 d before transfer to growth chambers (Conviron). For phenotypic analysis, plants were grown in 96-well flats under constant fluorescent light (120 µE/min/m^2; Sylvania VHO) at 22°C. High humidity was maintained during

germination and early growth by covering flats with transparent plastic lids. The lids were removed after about 10 d.

Abaxial trichomes were scored 2–3 wk after planting with the aid of a stereomicroscope. After flowering, leaves were removed, attached to cardboard with double-sided tape, and then scanned in a digital scanner. Scanning electron microscopy was performed on siliques that had been fixed in formalin-acetic acid, dehydrated in ethanol, dried, and coated with palladium.

Genetic analysis

New *zip*-like mutations were mapped using an F2 population from a cross to Ler. Once their identity had been determined, PCR markers useful for the following mutant alleles in genetic crosses were generated by taking advantage of the nucleotide changes corresponding to these mutations. *sgs3-11* was identified using a PCR primer pair (5′-CAAAAAACCTGTGGTG GTCTGCA-3′ and 5′-ACAACCTTGGCACGTTCCTGC-3′) that incorporate a PstI polymorphism at the site of the mutation. *rdr6-11* was identified by amplifying DNA with the primers 5′-TACTGTCCCTGGCGATCTCT-3′ and 5′-CCACCTCA CACGTTCCTCTT-3′, followed by cleavage with Taq1, and *zip-1* was identified by performing PCR with 5′-CTGTACTTT GACAGCGGAAACC-3′ and 5′-ACTGGCTTGGACTTTCT ACTAGGTTC-3′, followed by digestion with BsaI.

Double-mutant plants were identified in the F2 progeny from intercrosses between *zip-1*, *sgs3-11*, and *rdr6-11* using the PCR assays described above. Plants homozygous for all three mutations were identified in the F2 progeny of a cross between *zip-1 sgs3-11* and *rdr6-11*. Plants homozygous for these mutations and the L1 transgene were generated by backcrossing F1 progeny to the L1 line. Progeny of this backcross were allowed to self-pollinate, and these families were then screened on kanamycin to identify families homozygous for L1 (kanR/kanR) and segregating for the *zip*-like mutation. Mutant plants in these families were stained for GUS activity as described (Hunter et al. 2003).

Microarray analysis

RNA was harvested from shoot apices of 2-week-old Col, *zip-1*, *sgs3-11*, and *rdr6-11* plants grown under short-day conditions (10 h light:14 h dark) at 22°C. All leaves greater than 5 mm in length were removed prior to freezing the samples in liquid nitrogen. Total RNA was extracted with Trizol reagent (Invitrogen) from frozen tissue disrupted in a bead-beater. The extracted RNA was further purified with RNAeasy columns (QIAGEN). Synthesis of biotin-labeled cRNA, hybridization to the Affymetrix ATH1 Genome Array, and scanning was carried out by the University of Pennsylvania Microarray Facility. Microarray Suite 5.0 was used for image analysis.

Northern analysis

For Northern analysis, total RNA (20 µg) extracted from 2-week-old seedlings with Trizol reagent as described above was transferred to Hybond-N+ membranes (Amersham Biosciences). Probes were generated from Col genomic DNA or cDNA by PCR using the following primers: ARF3/ETTIN (5′-AGAGGC CGCTTCAAAGGAACAGAA-3′ and 5′-TGCATAGATGTCC CTTCCTTGCGA-3′), ARF4 (5′-GTTTCCAAGGGTCTTGCAA GGTCA-3′ and 5′-TTTGCTCGAGCTTTGCGGCTTAGA-3′), and SPL3 (5′-ACGAGAGAAGGCGGAAAAGCACAA-3′ and 5′-CGGGATCCCTAAGTCTCAATGCATTTAT-3′), and amplified fragments were sequenced after purification with the QIAquick Gel Extraction kit (QIAGEN). Hybridization was carried out at 68°C using PerfectHyb Plus buffer (Sigma). Probes were labeled with ^{32}P-dCTP using a Prime-it II Random Primer Labeling kit (Stratagene). Blots were washed once in 2× SSC and 0.1% SDS for 5 min at room temperature, twice in 0.5× SSC and 0.1% SDS for 20 min at 68°C, and once in 0.1× SSC 0.1% SDS for 20 min at 68°C. The hybridization signal was detected with a Storm 860 (Molecular Dynamics), and contrast was adjusted with Photoshop 7.

Low-molecular-weight RNA was purified from total RNA of 2-week-old seedlings or floral buds, and 10-µg or 20-µg aliquots were then resolved on 15% denaturing gel and electrically transferred to Hybond-N+ membranes following Dalmay et al. (2000). Oligonucleotide probes whose sequences are complementary to individual miRNAs were ^{32}P-labeled with T4 polynucleotide kinase (New England Biolabs). Probes to sRNA02 and Cluster02 sRNA were made as described (Xie et al. 2004). The AtSN1 probe was prepared as described by Zilberman et al. (2003), except that its fragment was cloned onto pGEM-T Easy vector (Promega) and SP6 RNA polymerase (Invitrogen) was used for in vitro transcription. Hybridization was carried out at 38°C using ULTRAhyb-oligo hybridization buffer (Ambion). Blots were washed twice in 2× SSC and 0.5% SDS for 30 min at 38°C. The hybridization signal was detected with a Storm 860, and contrast was adjusted with Photoshop 7.

5′ RACE

Total RNA was extracted from 2-week-old rosettes as described above. Poly(A)$^+$ mRNA was purified from total RNA using the Oligotex mRNA Mini kit (QIAGEN). 5′ RACE was carried out using the GeneRacer Kit (Invitrogen). The GeneRacer RNA Oligo adapter was directly ligated to mRNA (100 ng) without calf intestinal phosphatase and tobacco acid pyrophosphatase treatment. The GeneRacer oligo dT primer was used for cDNA synthesis. Initial PCR was carried out using the GeneRacer 5′ Primer and At5g18040 RACE1 (5′-GTGGGATACAGAAGTC AACAAGCAGACC-3′) or SCL6-IV-1152R, as described by Llave et al. (2002b). Nested PCR was carried out using 1 µL of the initial PCR reaction, GeneRace 5′ nested primer, and At5g18040 RACE2 (5′-GTGGGATACAGAAGTCAACAAGC AGACC-3′). RACE fragments were cloned and sequenced after gel purification.

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