Arabidopsis HEN1: A Genetic Link between Endogenous miRNA Controlling Development and siRNA Controlling Transgene Silencing and Virus Resistance

Stéphanie Boutet[1,4], **Franck Vazquez**[1,2], **Jun Liu**[3], **Christophe Béclin**[1,5], **Mathilde Fagard**[1,6], **Ariane Gratias**[1,7], **Jean-Benoit Morel**[1,8], **Patrice Crété**[2], **Xuemei Chen**[3], and **Hervé Vaucheret**[1,*]

[1]Laboratoire de Biologie Cellulaire, INRA, 78026 Versailles Cedex

[2]Laboratoire de Physiologie de la Différenciation Végétale, USTL, 59650 Villeneuve d'Ascq, France

[3]Waksman Institute, Rutgers University, 190 Frelinghuysen Road, Piscataway, New Jersey 08854

Summary

In animals, double-stranded short interfering RNA (siRNA) and single-stranded microRNA (miRNA) regulate gene expression by targeting homologous mRNA for cleavage or by interfering with their translation, respectively [1–3]. siRNAs are processed from injected or transgene-derived, long, perfect double-stranded RNA (dsRNA), while miRNAs are processed from short, imperfect dsRNA precursors transcribed from endogenous intergenic regions [4–9]. In plants, both siRNAs and miRNAs activate cleavage of homologous RNA targets [10–12], but little is known about the genes controlling their production or action. The SGS2/SDE1 protein contributes to produce transgene siRNA [10], while DCL1 and HEN1 contribute to endogenous miRNA accumulation [8, 9]. Here, we show that: i) SGS2, SGS3 [13], AGO1 [14, 15], and HEN1 contribute to produce transgene siRNA involved in sense post-transcriptional gene silencing (S-PTGS); ii) HEN1, but not SGS2, SGS3, or AGO1, contributes to the accumulation of the endogenous *miR171* miRNA and to the cleavage of Scarecrow target mRNA by *miR171* [11]; iii) SGS2, SGS3, AGO1, and HEN1 contribute to resistance against cucumber mosaic virus [13, 15], but not to siRNA and IR-PTGS triggered by hairpin transgenes directly producing perfect dsRNA [16]; and iv) the actions of HEN1 in miRNA/development and siRNA/S-PTGS can be uncoupled by single-point mutations at different positions in the protein.

[*]Correspondence: herve.vaucheret@versailles.inra.fr.
[4]Present address: Nutrition Azotée des Plantes, INRA, 78026 Versailles Cedex, France.
[5]Present address: IMVT, Institut de Biologie du Développement de Marseille, Campus de Luminy, Case 907, 13288 Marseille Cedex 09, France.
[6]Present address: UMR Physiologie et Biotechnologie Végétales, INRA Centre de Bordeaux, BP 81, 33883 Villenave d'Ornon Cedex, France.
[7]Present address: Laboratoire de Génétique Moléculaire, CNRS UMR 8541, ENS, 46 Rue d'Ulm, 75005 Paris, France.
[8]Present address: CIRAD, UMR BGPI 385, TA 40/02, Avenue d'Agropolis, 34398 Montpellier Cedex 05, France.

Results and Discussion

Through a forward genetic screen, we previously identified 30 *Arabidopsis* mutants deficient in S-PTGS triggered by the *35S-GUS* sense transgene carried at the L1 locus [13–15, 17]. These 30 mutants were classified into 4 complementation groups: *sgs1* (1 allele), *sgs2* (18 alleles), *sgs3* (5 alleles), and *ago1* (6 alleles). SGS2 (also known as SDE1 [10]) is a putative RNA-dependent RNA polymerase (RdRp) [10, 13] sharing sequence similarity with QDE-1, which controls quelling in *Neurospora* [18], and RRF-1, which controls RNAi in *C. elegans* [19]. SGS3 is a coiled-coil protein of unknown function that has no obvious homolog in animals or fungi [13]. AGO1 belongs to the paz piwi domain (PPD) protein family of unknown function [14, 15] and shares sequence similarity with QDE-2, which controls quelling in *Neurospora* [18], AGO-2 and PIWI, which control RNAi in *Drosophila* [20, 21], and RDE-1, which controls RNAi in *C. elegans* [22]. Using a reverse genetics approach, we also reported that the *ddm1* and *met1* mutations in a SWI2/SNF2-like chromatin remodeling protein and a Dnmt1-like DNA methyltransferase controlling TGS also impaired S-PTGS at the L1 locus, although to a lower extent [23]. We pursued our forward genetic screen of the L1 EMS library and identified 14 additional mutants. These mutants were genetically classified by crossing with representative *sgs1, sgs2, sgs3, ago1, ddm1*, and *met1* mutants. Together, the 44 EMS mutants deriving from line L1 belong to 6 complementation groups: *sgs1* (1 allele), *sgs2* (23 alleles), *sgs3* (6 alleles), *ago1* (12 alleles), *met1* (2 alleles), plus a new mutant, called 23-2, defining a novel group that we describe in this paper.

Previous analysis of the sensitivity of *sgs2*, *sgs3*, and *ago1* mutants to infection by different viruses revealed that mutants impaired in transgene S-PTGS are hypersensitive to infection by cucumber mosaic virus (CMV) due to a 5- to 6-fold overaccumulation of CMV RNA [13, 15]. This finding suggests that transgenes undergoing S-PTGS encode particular forms of RNA that share com mon features with viral RNAs targeted by the cellular PTGS machinery. Infection of 23-2 with CMV also revealed hypersensitivity and 5-fold overaccumulation of CMV RNA (Figure 1). This result therefore indicates that, with respect to CMV hypersensitivity, the 23-2 mutant behaves as the other mutants impaired in transgene S-PTGS that were identified through the same screen; this finding reinforces the mechanistic similarity between S-PTGS directed against sense transgenes and PTGS directed against CMV.

GUS mRNA accumulation increased 14-fold in 23-2 plants compared with L1 plants (Figure 2) and leads to a 1000-fold increase in GUS activity (data available in the Supplemental Data available with this article online). However, the level of *GUS* mRNA accumulation was slightly lower in 23-2 compared with *sgs2-1*, *sgs3-1*, and *ago1-27*, which showed a 19-, 18-, and 17-fold increase in *GUS* mRNA accumulation, respectively (Figure 2), and a 3500-fold average increase in GUS activity compared with L1 plants (data available in the Supplemental Data). In addition, GUS activity in the 23-2 mutant was more variable from plant to plant and from one part of the plant to another than it was in the other mutants. The absence of full reactivation of the *35S-GUS* transgene at the L1 locus and the variability of GUS expression could be attributed to epigenetic changes that have occurred at the L1 locus during the mutagenesis in addition to the impairment in PTGS that was demonstrated by CMV hypersensitivity and CMV RNA overaccumulation. However, the mutation in the 23-2

plants also partially impaired both S-PTGS of the *35S-GUS* transgene carried by the L2 locus [17] and cosuppression of the endogenous *NIA* genes and the *35S-NIA2* transgene triggered by the 2a3 locus [17] (data available in the Supplemental Data). This finding suggests that the 23-2 mutation only partially impairs transgene-mediated S-PTGS and cosuppression. To confirm that there was residual PTGS activity in 23-2, we infected 23-2 plants with turnip mosaic virus (TuMV) or cucumber mosaic virus (CMV) expressing HC-Pro and 2b proteins that counteract PTGS, but not TGS, in *Arabidopsis* and tobacco [13, 16, 24–26]. Full transgene expression was observed in infected 23-2 plants (data available in the Supplemental Data), suggesting that the lack of full transgene expression in the 23-2 mutant is due to limited residual PTGS activity.

S-PTGS in L1 plants correlated with the accumulation of *GUS* siRNA of both sense and antisense polarities, which correspond to various parts of the *GUS* coding sequence (Figure 2). As shown previously in the 6b5 tobacco line carrying the same *35S-GUS* construct silenced by S-PTGS [27], ~21–22 nt long siRNAs were observed, but no ~25 nt siRNAs were visualized. No *GUS* siRNA signal could be detected in *sgs2-1*, *sgs3-1*, and *ago1-27* mutants by using probes corresponding to the different parts of the transgene, even after long exposure. Furthermore, no sense or antisense siRNA could be detected in the 23-2 mutant by using a probe corresponding to the central part of the *GUS* coding sequence, but siRNAs of both polarities were still detectable, although at a level lower than in the L1 line, by using probes corresponding to the 5′ or 3′ region of the *GUS* coding sequence. This could reflect a differential effect of the protein impaired in the 23-2 mutant on the production or stabilization of different siRNA populations. Alternatively, some siRNAs could remain in the 23-2 mutant because they are not functional and therefore cannot act in the RISC complex.

Although they are impaired in S-PTGS and cosuppression triggered by various sense transgenes (L1, L2, 2a3), the *sgs2*, *sgs3*, and *ago1* mutants are not affected in IR-PTGS triggered by hairpin constructs directly producing double-stranded RNA. This finding suggests that the SGS2, SGS3, and AGO1 proteins act upstream of dsRNA formation in transgenic plants carrying sense transgenes [16]. This hypothesis was confirmed by the complete disappearance of siRNA in *sgs2-1*, *sgs3-1*, and *ago1-27* mutants (Figure 2), and this disappearance rules out the possibility that AGO1 could play a role similar to that of its homologs QDE-2 in *Neuropora* and AGO-2 in *Drosophila*, which are required for mRNA degradation by siRNA in the RISC complex [18–20]. Rather, AGO1 could play a role similar to that of its homolog PIWI in *Drosophila* and can participate in an early step of cosuppression [21]. Indeed, both *ago1* and *piwi* mutants no longer accumulated siRNA, whereas *qde-2* mutants still accumulated siRNA. Because *GUS* siRNAs are not totally absent in the 23-2 mutant, the corresponding protein could play a role downstream of dsRNA formation. To test this hypothesis, we introduced hairpin constructs directed against *AP1*, *AG*, or *CLV3* endogenous genes in the 23-2 mutant. Transformants exhibiting a strong *ap1*, *ag*, or *clv3* phenotype were obtained at a frequency similar (ca. 65%) to that observed in wild-type plants and in *sgs2-1*, *sgs3-1*, and *ago1-27* mutants [16]. We also introduced the 306-0-1 locus carrying a *35S- GUS-SUG* hairpin construct directly producing dsRNA [16] by crossing into a L1-depleted 23-2 plant and found that siRNA accumulated at the same level in wild-type and mutant plants (data not shown). In addition, silencing of the target

35S-GUS transgene carried at the 6b4 locus [16] by the 306-0-1 locus was as efficient in wild-type plants and in 23-2 (data available in the Supplemental Data). These results indicate that, like SGS2, SGS3, and AGO1, the protein impaired in the 23-2 mutant does not contribute to siRNA and IR-PTGS triggered by hairpin constructs directly producing long, perfect dsRNA.

Whereas *sgs2* and *sgs3* mutants have no obvious phenotypes [13], *ago1* mutants exhibited developmental abnormalities, ranging from leaf serration and reduced fertility in hypomorphic alleles to complete sterility and eventual death in null alleles [14, 15]. The 23-2 mutant also exhibited developmental abnormalities, including narrowing leaves, late flowering, and reduced fertility (Figure 1), but this phenotype did not resemble that of *ago1* mutants. Segregation analyses of 750 F2 plants derived from a cross between 23-2 and L1 showed that this developmental phenotype cosegregates with the release of S-PTGS and suggests that both effects result from a single mutation. This mutation was mapped to a 60-kb interval between markers CER442391 and CER442404 on chromosome 4 (data available in the Supplemental Data). This region covered by BAC T13K14 contains the *HEN1* gene previously identified through a screen for mutations that enhance the *hua1-1* and *hua2-1* mutations, which cause floral abnormalities [28], and was subsequently shown to control the accumulation of endogenous miRNA [8]. Crosses performed between 23-2 and *hen1-1, hen1-2,* or *hen1-3* alleles ([28]; X.C., unpublished data) yielded F1 progenies exhibiting the mutant phenotype and undergoing S-PTGS of *GUS* (data not shown). This finding indicates that 23-2 is a *hen1* allele, but that *hen1-1, hen1-2,* and *hen1-3* alleles are not impaired in S-PTGS and act in a dominant manner over the 23-2 allele with regard to S-PTGS. Sequencing of the *HEN1* gene in 23-2 revealed a G-to-A transition at position 25911 of BAC T13K14 (position 2412 of the predicted cDNA At4g20910), resulting in a Glu-to-Lys change in the C terminus of the protein. Introduction of the wild-type *HEN1* gene in the 23-2 plants completely restored both a wild-type phenotype and S-PTGS (data available in the Supplemental Data), demonstrating the involvement of HEN1 in both processes. The 23-2 mutant was therefore renamed *hen1-4*.

Previous analyses revealed that the *hen1-1* mutation affects the accumulation of endogenous miRNA, indicating that HEN1 acts in miRNA metabolism [8]. However, the effect on the accumulation of the corresponding mRNA target has not yet been investigated. Analysis of the accumulation of the endogenous *miR171* miRNA revealed that it accumulates at similar levels in wild-type (L1) plants and in *sgs2, sgs3,* and *ago1* mutants but cannot be detected in the 23-2/*hen1-4* mutant (Figure 3A). Correspondingly, the Scarecrow SCL6-III (At3g60630) mRNA that is targeted for cleavage by *miR171* [11] accumulated at similar levels in wild-type (L1) plants and in *sgs2, sgs3,* and *ago1* mutants but was increased 4-fold in the 23-2/*hen1-4* mutant (Figure 3B). These results therefore clearly demonstrate that the *hen1-4* mutation affects both endogenous miRNA (*miR171*) or transgene siRNA (*GUS*) accumulation, resulting in reduced cleavage of the corresponding mRNA targets.

The dual effect of the *hen1-4* mutation on the accumulation of both siRNA and miRNA and on the cleavage of the corresponding mRNA target suggests common features between these two pathways in plants. This is the first report of a mutation affecting both pathways in plants. The *dcr-1* mutation affecting the double-stranded specific RNase III DICER has been

shown to affect siRNA and miRNA only in *C. elegans* [29], whereas the *caf* mutation in the *Arabidopsis DCL1* gene affects miRNA, but not siRNA [30]. However, the actions of HEN1 in miRNA/development and siRNA/S-PTGS can be uncoupled by single-point mutations at different positions in the protein; this finding suggests that they may represent two partially overlapping activities or two distinct activities that have been combined on a single molecule. The fact that the *hen1-4* mutant is impaired in miRNA/development and in siRNA/S-PTGS (triggered by sense transgenes), but not in siRNA/IR-PTGS (triggered by hairpin transgenes), also suggests the existence of additional steps in the two former pathways. In animals, it is known that miRNAs derive from short RNA precursors (ca. 70-nt long) that fold into partial dsRNA molecules [4–6]. Conversely, hairpin transgenes triggering IR-PTGS produce long, perfect dsRNA molecules with a loop. Thus, HEN1 could play a role in the stabilization or processing of imperfectly folded dsRNA molecules. How sense transgenes triggering S-PTGS actually produce dsRNA is still not known, but it requires the action of a putative RdRP encoded by the *SGS2/SDE1* gene [10, 13]. This enzyme is assumed to synthesize antisense molecules complementary to the sense mRNA transcribed from the transgene. It is therefore reasonable to think that it could elongate partially folded dsRNA molecules to produce long, perfect dsRNA molecules. The HEN1 protein could participate in the initiation of this reaction by stabilizing partially folded dsRNA molecules corresponding to sense transgenes. If HEN1 actually contributes to the stabilization or processing of imperfect dsRNA, its requirement should not be all or nothing, but conversely should depend on the relative stability of the partially folded dsRNA. This hypothesis is consistent with the fact that some miRNAs are not totally eliminated in *hen1* mutants [8]. Alternatively, or in addition to this latter role, HEN1 could also play a role in the stabilization of some siRNA or miRNA after processing by a DICER-like enzyme. Indeed, siRNAs produced by the central part of the *GUS* coding sequence are totally absent in the *hen1-4* mutant, whereas siRNAs produced by the 5′ and 3′ parts are only reduced (Figure 2), suggesting that not all siRNA have the same action or stability. The residual siRNA and PTGS activity observed in the *hen1-4* mutant could also result from the presence of an active (transcribed) gene besides *HEN1* on BAC T13K14 that putatively encodes a protein (At4g20920) sharing 66% identity and 75% similarity with HEN1 (At4g20910). Because there is only 3 kb between *HEN1* and this gene, it will be very difficult to obtain a double mutant to see if it is totally impaired in siRNA accumulation and S-PTGS.

Experimental Procedures

Plant Material

L1, L2, 6b4, 306-1, and 2a3 lines as well as *sgs2-1, sgs3-1*, and *ago1-27* mutants used in this study were previously described [13, 15–17]. Details for all genetic analyses, loci introgression, and mutation mapping are available in the Supplemental Data.

Molecular Biology

Proteins were extracted and GUS activity was analyzed as described before [13]. All RNAs (mRNA, siRNA, and miRNA) were extracted and analyzed as described by Mallory et al. [27]. Northern blots were quantified by using a phosphorimager apparatus. Real-time PCR for quantification of the SCL6-III transcripts (At3g60630) was done as follows: PolydT

cDNAs were made by using the Invitrogen cDNA first strand synthesis system, and 5 μg total RNA was extracted with Tri-reagent (MRC). Quantifications were performed on a Biorad IQcycler apparatus with the Quantitech SYBR green kit (Qiagen) upon recommendations of the manufacturer. PCR was carried out in 96-well optical reaction plates heated to 95°C for 10 min to activate hotstartTaq DNA polymerase, followed by 50 cycles of denaturation at 95°C for 30 s and annealing-extension at 60°C for 45 s. Target quantifications were performed with specific primer pairs designed for each side of the cleavage site by using Beacon Designer from Biosoft. The primers used for SCL6-III (At3g60630) are 5′-ACCAAGACCAGTCAGCGGTAATC-3′ and 5′-AGTGTCGTCGTTGTTGTTGTTAAGG-3′. Results are normalized with actine2 (At3g18780) by using 5′-GCACCCTGTTCTTCTTACCG-3′ and 5′-AACCCTCGTAGATTGGCACA-3′. Each quantification was made in triplicate. For each couple of primers, conditions were, as recommended, 1 E 0.85 and r^2 0.985, where E is the PCR efficiency and r^2 corresponds to the correlation coefficient obtained with the standard curve.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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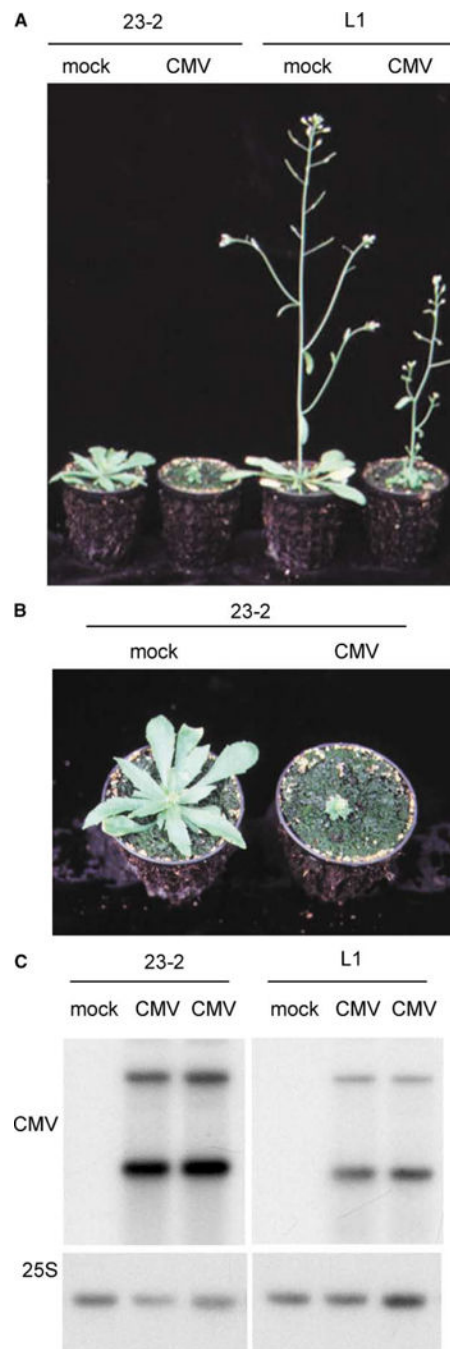
Figure 1. Phenotype and Virus Sensitivity of Line L1 and the 23-2 Mutant
(A) Phenotype of line L1 and of the 23-2 mutant 2 weeks after mock infection or infection by CMV. All plants are the same age, indicating the delay in flowering of the 23-2 mutant.
(B) A close-up of (A) showing the leaf phenotype of the 23-2 mutant.
(C) CMV RNA accumulation in line L1 and in the 23-2 mutant, mock infected or infected by CMV. Standardization with a 25S rDNA probe is shown below.
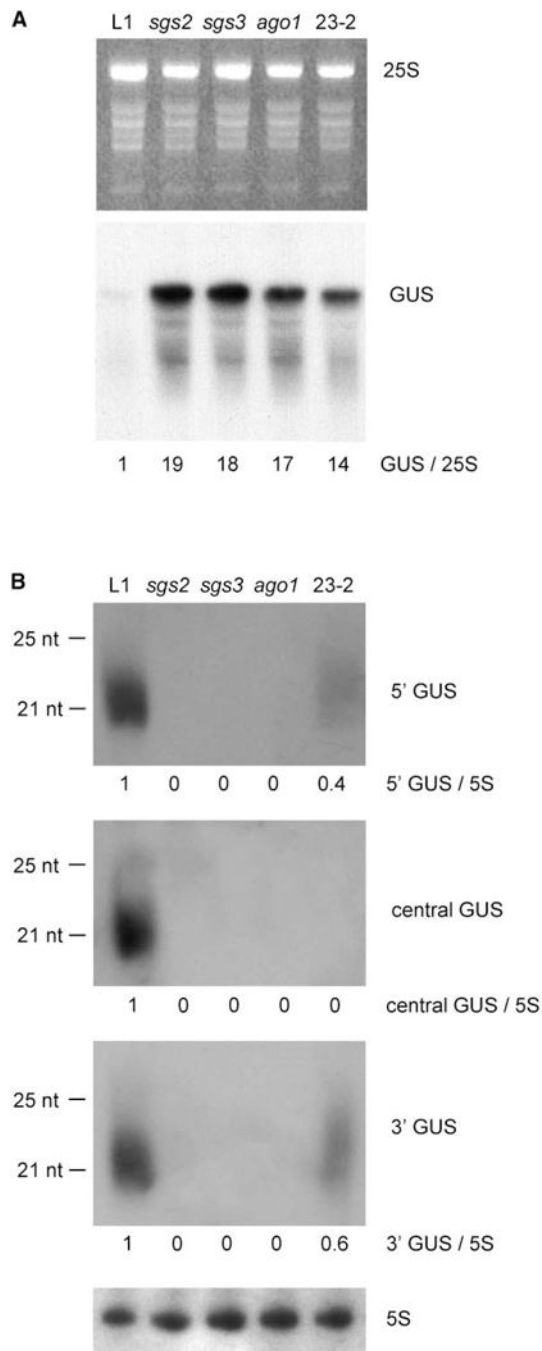
Figure 2. *GUS* mRNA and siRNA Accumulation in Wild-Type and Mutant Plants

(A) mRNA extracted from leaves was hybridized with a *GUS* DNA probe. An ethidium bromide-stained gel is shown for standardization. The ratio between GUS and 25S signals is indicated below.

(B) siRNA extracted from leaves was hybridized with antisense RNA probes corresponding to the 5′ part (position 1–558), central part (position 558–789), or 3′ part (position 789–1865) of the *GUS* coding sequence. Similar results were obtained with sense probes.

Hybridization with 5S RNA is shown for standardization. The ratio between GUS and 5S signals is indicated below.
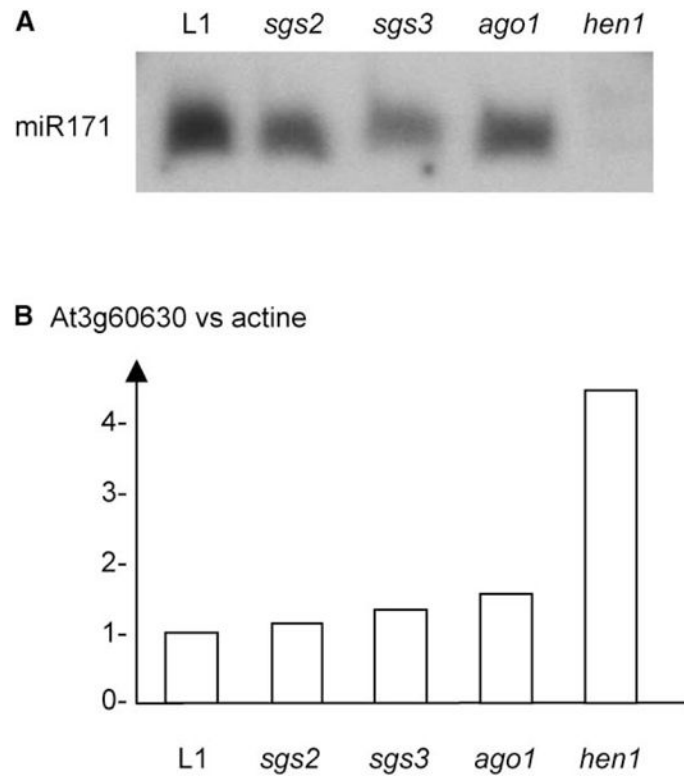
Figure 3. *miR171* miRNA and *SCL* mRNA Accumulation in Wild-Type and Mutant Plants

(A) Small RNA extracted from wild-type and mutant flowers was hybridized with a probe complementary to *miR171*.

(B) Total RNA extracted from wild-type and mutant flowers was quantified for SCL6-III mRNA relative accumulation by real-time PCR by using primers surrounding the cleavage site. Quantifications are normalized with actine2 transcript. The wild-type value is 1. AU, arbitrary unit.