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

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Supplemental Results and Discussion

The 23-2/*hen1-4* Mutation Partially Suppresses S-PTGS and Cosuppression

The level of GUS activity produced by the L1 locus was 1000-fold higher in 23-2/*hen1-4* than in L1 plants, indicating that S-PTGS is strongly released. However, it was 3-fold lower in 23-2/*hen1-4* than in *sgs2-1*, *sgs3-1*, and *ago1-27* mutants that are totally impaired in S-PTGS (Figure S1A). To ensure that the reduced activity in 23-2 was due to the *trans* effect of an unlinked mutation that only partially releases S-PTGS and not to an epigenetic change at the L1 locus itself, we tested the effect of the *sgs2-1*, *sgs3-1*, *ago1-27*, and 23-2/*hen1-4* mutations on the *35S-GUS* sense transgene carried at the L2 locus and triggering S-PTGS and on the *35S-NIA2* transgene carried at the 2a3 locus and triggering cosuppression of endogenous *NIA* genes [S1]. Plants homozygous for the different mutations and homozygous for the L2 or 2a3 loci were identified as follows by crossing *sgs2-1*/L1, *sgs3-1*/L1, *ago1-27*/L1, and 23-2 (*hen1-4*/L1) mutants to a wild-type plant and then selfing the progeny. F2 plants homozygous for the mutations and hemizygous for the L1 locus were identified as segregating 75% of kanamycin-resistant GUS+ F3 plants and 25% of kanamycin-sensitive GUS− F3 plants. F3 plants homozygous for the mutations and lacking the L1 locus were identified as GUS− plants after sowing on a medium without kanamycin and were crossed to the homozygous lines L2 or 2a3. F1 hybrids were selfed. F2 plants homozygous for the mutations and homozygous for the L2 or 2a3 locus were identified as giving 100% kanamycin-resistant GUS+ F3 plants (L2) or hygromycin-resistant NIA+ F3 plants (2a3). Similar to what has been observed with the L1 locus, the level of GUS activity produced by the L2 locus was lower in plants carrying the 23-2/*hen1-4* mutation compared with plants carrying the *sgs2-1*, *sgs3-1*, and *ago1-27* mutations (Figure S1B). In addition, plants carrying the 23-2 mutation and the 2a3 locus exhibited a yellowing phenotype intermediate between that of *sgs2-1*, *sgs3-1*, and *ago1-27* mutants (green) and that of the cosuppressed line 2a3 (bleaching). The presence of this phenotype intermediate indicates that, unlike the *sgs2-1*, *sgs3-1*, and *ago1-27* mutations totally impairing cosuppression triggered by the 2a3 locus [S2, S3], the 23-2/*hen1-4* mutation only partially released cosuppression. Together, these results indicate that the 23-2/*hen1-4* mutation acts in *trans* but has a weaker impact on S-PTGS and cosuppression than the other mutations.

Residual PTGS Activity in the 23-2/*hen1-4* Mutant Is Counteracted by Viruses

To determine if the reduced expression of the *35S-GUS* sense transgenes in 23-2 was due to incomplete TGS or residual PTGS activity, we infected 23-2 with turnip mosaic virus (TuMV), which totally counteracts S-PTGS and IR-PTGS, but not TGS in *Arabidopsis* ([S2, S4]; C.B. and H.V., unpublished data). Full transgene expression was reached after infection of 23-2 by TuMV (Figure S1C), suggesting that the reduced activity in 23-2 was due to residual PTGS activity. Similarly, infection of 23-2 by cucumber mosaic virus (CMV), which partially counteracts S-PTGS and IR-PTGS in *Arabidopsis* [S2, S4], led to full transgene expression (Figure S1C), confirming that there is residual PTGS activity in 23-2 and indicating that this residual PTGS activity is totally counteracted by viruses.

The 23-2/*hen1-4* Mutation Does Not Suppress IR-PTGS

The 23-2/*hen1-4* mutation did not affect the accumulation of siRNA produced by the 306-0-1 locus carrying a *35S-ΔGUS-SUG* hairpin construct directly producing dsRNA. We therefore expected that the nonsilenced *35S-GUS* transgene carried at the 6b4 locus [S4] would still be silenced by IR-PTGS when brought into the presence of the 306-0-1 locus in the 23-2/*hen1-4* mutant. Plants homozygous for the 6b4 locus were crossed with a wild-type plant or with a plant homozygous for the 306-0-1 locus. As shown before [S4], 6b4 × wild-type plants expressed GUS at a high level, whereas 6b4 × 306-0-1 plants were totally silenced (Figure S1D). 6b4 × 306-0-1 hybrids were subsequently crossed with a homozygous *hen1-4* mutant depleted of the L1 locus as described above. F1 plants were sown on a medium supplemented with kanamycin and basta to select for the presence of the 6b4 and 306-0-1 loci, respectively, and were selfed. GUS activity was measured in wild-type (*HEN1*/*HEN1* or *HEN1*/*hen1-4*) and mutant (*hen1-4*/*hen1-4)* F2 siblings selected on a medium supplemented with kanamycin and basta (Figure 1D). The 6b4 locus was totally silenced by the 306-0-1 locus irrespective of the status (homozygous or hemizygous) of the two loci (as checked further by scoring the percentage of kanamycin- and basta-resistant plants in the F3 self progenies) and irrespective of the presence or absence of a functional *HEN1* gene. This result therefore indicates that HEN1 is not required for the production of siRNA by hairpin constructs and for IR-PTGS triggered by this type of construct.

Mapping of the 23-2/*hen1-4* Mutation

The 23-2 mutant (in Col) was crossed to a wild-type Ler plant, and the F1 progeny was allowed to self-fertilize. DNA was extracted from 478 Ler/23-2 F2 plants exhibiting developmental abnormalities and was PCR tested for linkage with polymorphic markers that discriminate between Col and Ler all along the five *Arabidopsis* chromosomes. The mutation in 23-2 was mapped in a 60-kb interval located between markers CER442391 and CER442404 ([S5], http://www.*Arabidopsis*.org/cereon/).

A Wild-Type Copy of the *HEN1* Gene Restores Development and S-PTGS in the 23-2/*hen1-4* Mutant

The 23-2 mutant (*hen1-4*/*hen1-4*, L1/L1 in Col) was crossed to the *hen1-1* mutant in which development has been restored by a *HEN1* genomic clone inserted at an ectopic locus (*hen1-1*/*hen1-1*, *gHEN1*/*gHEN1* in Ler) [S6]. The F1 progeny (*hen1-4*/*hen1-1*, *gHEN1*/-, L1/-) was allowed to self-fertilize. DNA was extracted from 100 F2 plants (77 plants with a wild-type phenotype and 23 plants exhibiting *hen1* developmental abnormalities) and was PCR tested for polymorphic markers that discriminate between Col and Ler at the L1 locus (nga172) and the *HEN1* locus (CER442404). A total of 27 plants homozygous for the *hen1-4* mutation (in Col) were identified; among these, 7 exhibited developmental abnormalities and 20 exhibited a wild-type phenotype due to the presence of the ectopic *gHEN1* locus. Five out of the seven plants exhibiting developmental abnormalities carried the L1 locus and showed high GUS activity similar to that observed in the original 23-2 mutant. A total of 16 out of the 20 plants with a wild-type phenotype carried the L1 locus. These 16 plants all undergo silencing of the *35S-GUS* transgene (i.e., they express GUS at a level similar to that observed in L1 plants), indicating that the ectopic *gHEN1* copy restores both development and S-PTGS in the *hen1-4* mutant.

Supplemental References

S1. Elmayan, T., Balzergue, S., Beon, F., Bourdon, V., Daubremet, J., Guenet, Y., Mourrain, P., Palauqui, J.C., Vernhettes, S., Vialle,

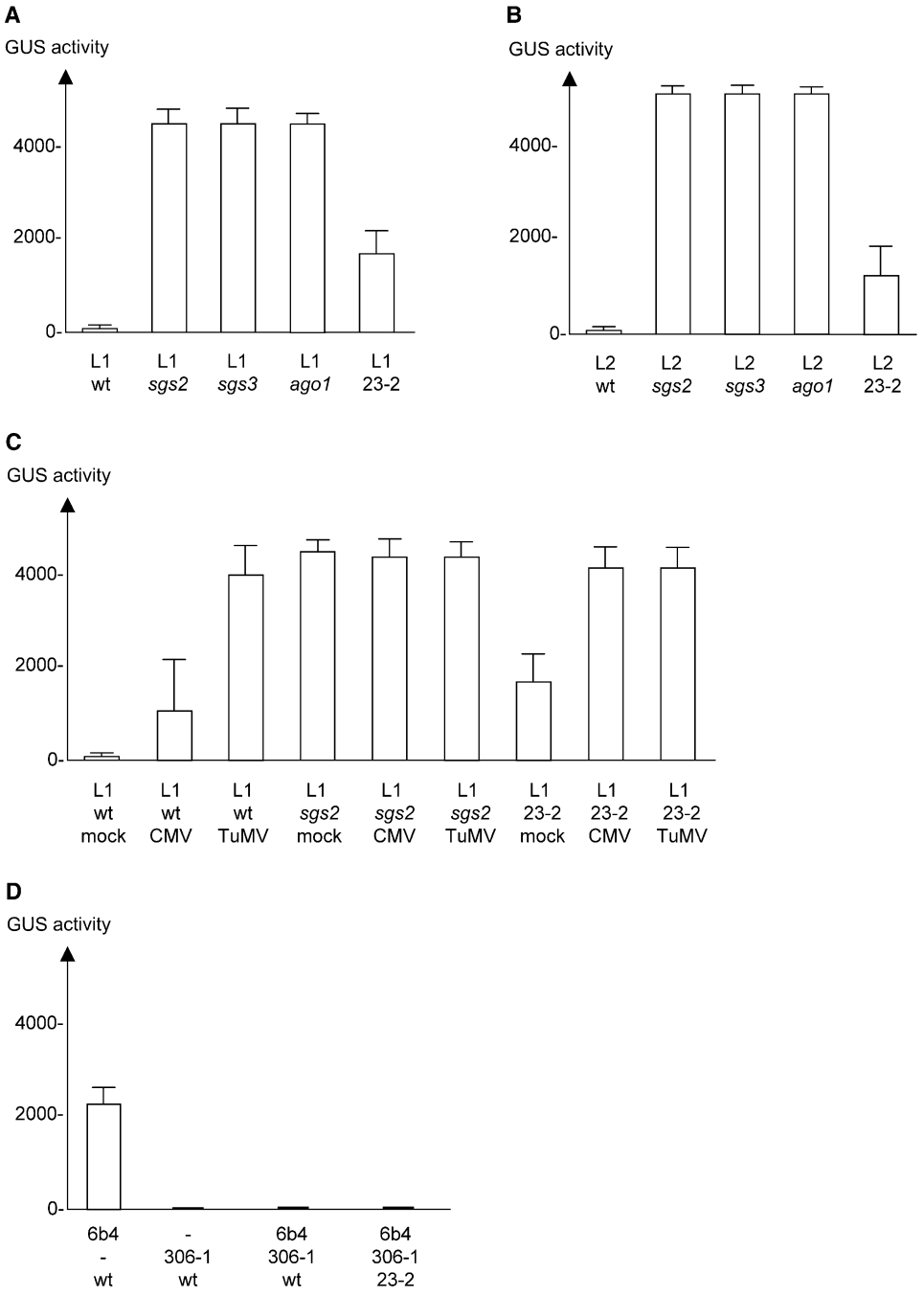

Figure S1. GUS Activity in Wild-Type Plants and *sgs2-1, sgs3-1, ago1-27*, and *hen1-4* Mutants

(A) GUS activity in L1 and mutants homozygous for the L1 locus triggering S-PTGS.
(B) GUS activity in L2 and mutants homozygous for the L2 locus triggering S-PTGS.
(C) GUS activity in L1 and mutants mock infected or infected by CMV or TuMV.
(D) GUS activity in wild-type and mutants carrying the 306-0-1 locus triggering IR-PTGS of the target 6b4 locus.
The average activity of ten measurements in ten independent plants is given. Deviation bars correspond to standard errors.

T., et al. (1998). *Arabidopsis* mutants impaired in cosuppression. Plant Cell *10*, 1747–1758.

S2. Mourrain, P., Beclin, C., Elmayan, T., Feuerbach, F., Godon, C., Morel, J.B., Jouette, D., Lacombe, A.M., Nikic, S., Picault, N., et al. (2000). *Arabidopsis* SGS2 and SGS3 genes are required for posttranscriptional gene silencing and natural virus resistance. Cell *101*, 533–542.

S3. Morel, J.-B., Godon, C., Mourrain, P., Béclin, C., Boutet, S., Feuerbach, F., Proux, F., and Vaucheret, H. (2002). Fertile hypomorphic *ARGONAUTE (ago1)* mutants impaired in post-tran-

scriptional gene silencing and virus resistance. Plant Cell *14*, 629–639.

S4. Beclin, C., Boutet, S., Waterhouse, P., and Vaucheret, H. (2002). A branched pathway for transgene-induced RNA silencing in plants. Curr. Biol. *12*, 684–688.

S5. Jander, G., Norris, S.R., Rounsley, S.D., Bush, D.F., Levin, I.M., and Last, R.L. (2002). *Arabidopsis* map-based cloning in the post-genome era. Plant Physiol. *129*, 440–450.

S6. Chen, X., Liu, J., Cheng, Y., and Jia, D. (2002). *HEN1* functions pleiotropically in *Arabidopsis* development and acts in C function in the flower. Development *129*, 1085–1094.