Published online 3 August 2011 *Nucleic Acids Research, 2011, Vol. 39, No. 21* ***9339–9344***
doi:10.1093/nar/gkr590

The miRNA pathway limits AGO1 availability during siRNA-mediated PTGS defense against exogenous RNA

Angel Emilio Martínez de Alba, Vincent Jauvion, Allison C. Mallory, Nathalie Bouteiller and Hervé Vaucheret*

Institut Jean-Pierre Bourgin, UMR1318, INRA, 78026 Versailles Cedex, France

Received June 10, 2011; Revised and Accepted July 1, 2011

ABSTRACT

In plants, most microRNAs (miRNAs) and several endogenous small interfering RNAs (siRNAs) bind to ARGONAUTE1 (AGO1) to regulate the expression of endogenous genes through post-transcriptional gene silencing (PTGS). AGO1 also participates in a siRNA-mediated PTGS defense response that thwarts exogenous RNA deriving from viruses and transgenes. Here, we reveal that plants supporting transgene PTGS exhibit increased levels of AGO1 protein. Moreover, increasing AGO1 levels either by mutating miRNA pathway components or, more specifically, by impairing miR168-directed regulation of *AGO1* mRNA leads to increased PTGS efficiency, indicating that the miRNA pathway dampens the efficiency of PTGS, likely by limiting the availability of AGO1. We propose that during the transgene PTGS initiation phase, transgene siRNAs and endogenous siRNAs and miRNA compete to bind to AGO1, leading to a transient reduction in AGO1–miR168 complexes and a decline in *AGO1* mRNA cleavage. The concomitant increase in AGO1 protein levels would facilitate the formation of AGO1–transgene siRNA complexes and the entry into the PTGS amplification phase. We suggest that the miRNA pathway imposes an important limitation on PTGS efficiency, which could help protect endogenous mRNAs from being routinely targeted by PTGS.

INTRODUCTION

Although a large portion of the plant genome is actively transcribed into RNA, only a small fraction encodes proteins. In many cases, non-protein coding RNAs produce small RNAs, 20–30 nt in length that direct transcriptional or post-transcriptional repression of genes with conserved cellular functions. Most small RNAs fall into one of two classes: microRNAs (miRNAs) or short-interfering RNAs (siRNAs). Although they are closely related biochemically, siRNAs and miRNAs differ functionally and in their mode of biogenesis (1,2). Primary miRNA transcripts (pri-miRNA) are transcribed from specific non-protein-coding *MIR* genes and fold to form imperfectly paired stem–loops. The maturation and processing of pri-miRNA requires the activity of several proteins, including the Cap-binding proteins CBP20 and CBP80/ABH1, the zinc finger protein SERRATE (SE), the double-stranded RNA-binding protein DRB1/HYL1, the RNAseIII protein DCL1 and the small RNA export protein HASTY (3). miRNAs bind to ARGONAUTE (AGO) proteins and act in *trans* to modulate the spatial and temporal expression of partially complementary mRNAs. In contrast, siRNAs are processed by DCL4/DCL2 from long perfectly dsRNA precursors produced by overlapping or converging transcription, or by the action of cellular or viral RNA-dependent RNA polymerases (RDRs) (1,2). Based upon their origin and function, plant endogenous siRNAs can be divided into four classes: *trans*-acting siRNAs (tasiRNAs), natural antisense transcript-derived siRNAs (natsiRNAs), endogenous inverted repeat-derived siRNA and POLYMERASE IV-dependent siRNAs (p4-siRNAs). Small RNAs bind to specific ARGONAUTE (AGO) family proteins (4), which function as the core of RNA-induced silencing complexes (RISCs), using small RNAs as sequence-specific guides to target complementary RNA. Small RNA–AGO complexes silence their target mRNAs post-transcriptionally through RNA cleavage and translational repression, or transcriptionally by directing chromatin modifications (5).

Arabidopsis encodes 10 AGO proteins (4). The central role of AGO1 in both miRNA and siRNA-mediated RNA silencing has been established through both genetic analysis and the identification of its associated small RNAs (6). Moreover, variations in AGO1 activity greatly affect *Arabidopsis* performance as revealed by the different

*To whom correspondence should be addressed. Tel: +33 130833170; Fax: +33 130833099; Email: herve.vaucheret@versailles.inra.fr

developmental defects exhibited by hypomorphic and null *ago1* alleles (7–10). Due to the wide-reaching importance of AGO1 in development, its expression is tightly regulated in both an AGO1- and a PNH/ZLL/AGO10-dependent manner by negative feedback loops involving miR168 and AGO1-derived siRNAs (11–14). AGO1 also is positively regulated by the cyclophylin protein SQUINT (SQN) (10) and negatively regulated by the F-box protein FBW2 (15).

To examine the crosstalk between the miRNA and siRNA pathways, we determined the effect of miRNA pathway mutants on sense transgene-mediated post-transcriptional gene silencing (S-PTGS) using the *Arabidopsis L1* and *Hc1* reporter lines, which carry a transgene consisting of the viral 35S promoter fused to the bacterial *uidA* gene-encoding β-glucuronidase (GUS) (16). We revealed that destabilization of the miRNA pathway leads to increased accumulation of AGO1 and positively influences S-PTGS efficiency. Overall, our results indicate that components of the miRNA pathway act as endogenous suppressors of S-PTGS likely by controlling AGO1 accessibility to the different RNA silencing pathways and suggest that a functional equilibrium between the miRNA and PTGS pathways serves as an important manager of gene repression.

MATERIALS AND METHODS

Plant material

All plants are in the Columbia accession. The *L1* and *Hc1* lines, the *cbp20-1, se-1, se-2, se-3, dcl1-7, dcl1-9, hyl1-1, hyl1-2, hst-6, sgs2-1, sgs3-1* and *sqn-1* mutants, and the *4mAGO1* construct have been described before (10,11,16–19). Lines *L1* and *Hc1* carry a *p35S-uidA* transgene. Line *L1* is systematically silenced by PTGS, whereas line *Hc1* triggers PTGS only in 20% of the plant population (16). The T-DNA insertion mutant *cbp80* (SAIL_1227_E07) was obtained from NASC. *Arabidopsis thaliana* seeds were surface-sterilized by immersion in a 0.8% (w/v) Bayrochlor (Bayrol France SA, Mundolscheim, France) in 80% ethanol for 10 min under strong agitation, rinsed once in 100% ethanol, and dried under a sterile airflow. Sterilized seeds were sown on Bouturage media (Duchefa) and were kept at 4°C for 72 h. After stratification, plants were grown in controlled growth chambers. Analyses were performed on flowers from 51 dag plants grown on Bouturage media (Duchefa) in 16 h light, 8 h dark at 22°C and transferred to soil at 14 dag and grown in controlled growth chambers in standard long-day conditions (16 h of light, 8 h of dark) at 21°C. The double mutants were obtained by cross-pollenizing either the *L1* or *Hc1* lines with each of the different mutants, and the homozygous lines were identified based on PCR assays.

RNA extraction, fractionation and RNA gel blot hybridization

For RNA gel blot analyses, frozen tissue was homogenized in a buffer containing 0.1 M NaCl, 2% SDS, 50 mM Tris–HCL (pH 9), 10 mM EDTA (pH 8) and 20 mM β-mercaptoethanol and RNAs were extracted two times with phenol and recovered by ethanol precipitation.

LMW were fractionated by denaturing PAGE on 15% gels, stained with ethidium bromide and transferred to nylon membrane (HybondNX, Amersham). Blots were hybridized with radioactively labeled riboprobes for detecting *GUS* plus and minus siRNAs, random-primed DNA probes for GUS mRNA, and an end-labeled oligonucleotide probe for U6 detection. Riboprobes were obtained by *in vitro* transcription under the control of the promoter of the T7 or the T3 RNA polymerase. Hybridization was at 35°C with PerfectHyb Plus hybridization buffer (Sigma). Hybridization signals were quantified using a Fuji phosphor imager and normalized to a U6 oligonucleotide probe for small RNA gel blot analyses.

Protein extraction, GUS activity quantification and immuno blotting

GUS activity was quantified by measuring the quantity of 4-methylumbelliferone product generated from the substrate 4-methylumbelliferyl-b-D-glucuronide (Duchefa) on a fluorometer (Fluoroscan II; Thermo Scientific), and fluorescence values were normalized to total protein extracted, which was quantified using an absorbance microplate reader (Elisa Elx 808; Avantec) and the Bradford protein assay. The same homogenized samples that were used to extract RNA were also used to extract protein for all analyses. Protein was extracted in buffer containing 20 mM Tris–HCl pH 7.5, 5 mM $MgCl_2$, 300 mM NaCl, 0.1% NP-40. Protein concentrations were determined using BioRad DC protein assay. Twenty micrograms of protein were resuspended in Laemmli buffer (20 mM Tris–HCl pH 6.8, 2% SDS, 5% glycerol, 40 mM DTT and 0.02% bromophenol blue), heated at 100°C for 5 min, and separated on an 8% SDS–PAGE gel. Proteins were transferred to PVDF membrane (BioRad) using a semi-dry electro blotter. For AGO1 detection, the membrane was blocked in 5% non-fat dry milk in 1× TBS, 0.1% Tween-20 (1× TBST) for 1 h at room temperature, rinsed for 5 min in 1× TBST, and incubated with a 1:5000 dilution of AGO1 primary polyclonal antibody (Agrisera) in 5% non-fat dry milk and 1× TBST for 1 h at room temperature. The membrane was then rinsed in 1× TBST for 20 min before incubation with a secondary peroxidase-conjugated anti-rabbit antibody in 5% non-fat dry milk in 1× TBST at room temperature for 1 h. AGO1 signal was revealed using the Immun-Star WesternC Kit (Bio-Rad) following the manufacturer's specifications.

RESULTS AND DISCUSSION

Mutants deficient in the miRNA pathway undergo PTGS

Forward and reverse genetic screens based on sense transgenes have revealed that AGO1, DCL2, DCL4, HEN1, HPR1, RDR6, SDE5 and SGS3 are required for S-PTGS (8,16,20–26). This set of proteins partially overlaps with the set of proteins required for the biogenesis and cleavage activity of the miRNA pathway (AGO1, CBP20, CBP80, DCL1, HEN1, HST, HYL1 and SE). To

further examine the interdependency of these two pathways, the *L1* locus, which carries a post-transcriptionally silent *p35S:GUS* transgene (16), was introgressed into *dcl1, hst, hyl1* and *se* mutants, and GUS activity was analyzed at 40 days after germination (dag) (Figure 1A). In contrast to *L1*/*rdr6, L1*/*sgs3* and *L1*/*ago1* mutants, which had high levels of GUS activity (25) and Figure 1A), *L1*/*dcl1, L1*/*hst, L1*/*hyl1* and *L1*/*se,* mutants had levels of GUS activity similar to *L1* silenced plants, indicating that *dcl1, hst, hyl1* and *se* mutations do not compromise *L1* S-PTGS.

Mutants deficient in the miRNA pathway exhibit increased PTGS efficiency

A kinetic analysis of GUS activity and *GUS* mRNA levels in *L1* plants at 5, 11 and 17 dag revealed that GUS activity and *GUS* mRNA levels progressively decreased from 5 to 17 dag, and then remained low (Figure 1B). To further examine the effect of mutations in the miRNA pathway on *L1* S-PTGS, GUS profiles were analyzed at 11 dag, before *L1* S-PTGS is fully established in wild-type plants. GUS activity and *GUS* mRNA levels were substantially lower (ranging between 20- and 2-fold lower) in *L1*/*hst, L1*/*hyl1* and *L1*/*se* mutants than in *L1* plants at 11 dag, suggesting that S-PTGS was improved in these mutants (Figure 1C).

To test if mutations in miRNA-specific components could enhance S-PTGS, we introduced the *Hc1* locus (16), which carries the same *p35S:GUS* transgene as the *L1* locus but triggers S-PTGS less efficiently, into *cbp20, cbp80, dcl1, hst, hyl1* and *se* mutants. *cbp20* and *cbp80* mutations were included in this analysis because they also disrupt miRNA production. In line *Hc1*, at each generation, S-PTGS consistently is restricted to only 20% of the plant population whereas S-PTGS occurs in 100% of the *L1* plant population (16). GUS activity and GUS mRNA analysis indicated that S-PTGS efficiency was elevated from 20% to 42, 75, 100, 100, 96 and 100% in *Hc1*/*cbp20, Hc1*/*cbp80, Hc1*/*dcl1, Hc1*/*hst, Hc1*/*hyl1* and *Hc1*/*se* plants (Figure 2A and B), respectively, indicating that CBP20, CBP80, DCL1, HST, HYL1 and SE act as endogenous suppressors of S-PTGS.

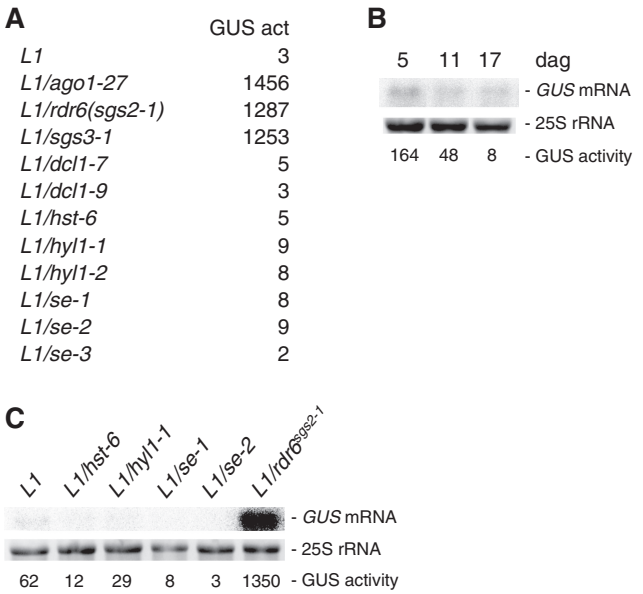

Figure 1. Mutants impaired in the miRNA pathway undergo efficient *L1* PTGS. GUS activity is indicated in fluorescence units per minute per milligram of total protein (FU/min/mg protein). High-molecular weight (HMW) RNA gel blots were hybridized with a GUS riboprobe. 25S RNA served as loading control. (**A**) GUS activity of the indicated lines was analyzed from mature rosette leaves 40 days after germination (dag). (**B**) GUS and RNA analyzed from aerial parts of *L1* line seedlings at the indicated age in dag. (**C**) GUS and RNA analyzed from aerial parts of seedlings from the indicated mutant and control lines at 11 dag.

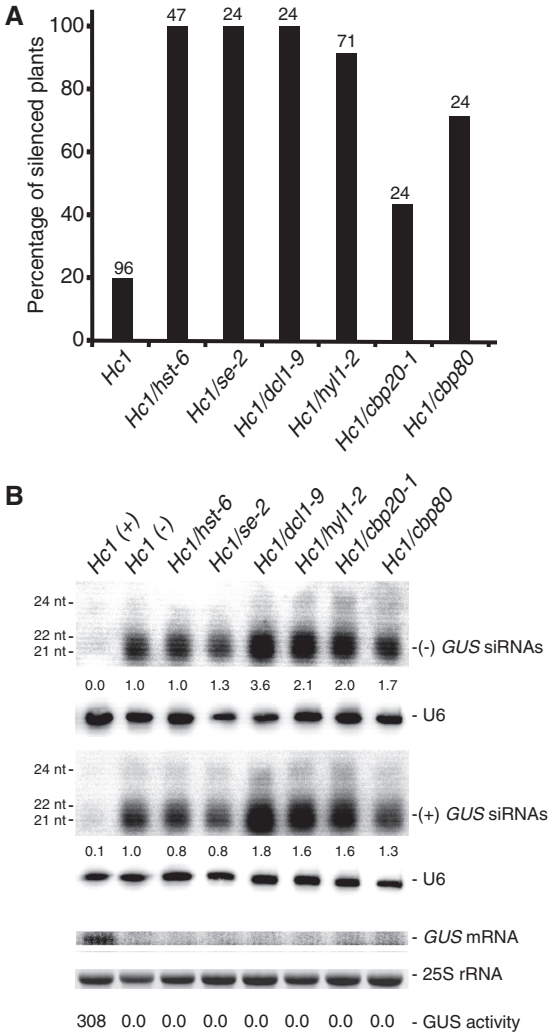

Figure 2. Mutants impaired in the miRNA pathway exhibit enhanced *Hc1* PTGS. (**A**) Percentages of silenced *Hc1* plants in indicated plant lines. The number of individual plants analyzed is indicated above each bar. (**B**) RNA gel blot analyses of the indicated mutant and control lines of *GUS* siRNAs of minus polarity (top), plus polarity (middle) and *GUS* mRNA (bottom), in flowers from plants 51 dag. For line *Hc1,* silenced and non-silenced plants were analyzed (− and +, respectively). 25S RNA and U6 snRNA hybridization served as loading controls. Values of siRNAs are normalized to *U6* RNA (with *Hc1* (−) control set at 1.0).

Increased PTGS efficiency in miRNA mutants correlates with increased AGO1 levels

Competition for a limiting factor shared between the miRNA and S-PTGS pathways could explain the increase of S-PTGS efficiency in mutants impaired in the miRNA pathway. HEN1 and AGO1 are shared components of the two pathways. However, it is improbable that HEN1 is limiting for S-PTGS. Indeed, HEN1 methylates and protects all types of small RNAs from degradation, including endogenous 24-nt siRNAs that represent about 90% of the small RNA population of a wild-type plant and miRNAs that represent about 10%. Elimination of the 24-nt siRNA population by mutating *RDR2* or *NRPD1* does not significantly enhance methylation of the remaining miRNAs (27). Therefore, it is unlikely that eliminating the miRNA population in *cbp20, cbp80, dcl1, hst, hyl1* and *se* mutants would significantly increase the methylation of PTGS-related siRNAs. In fact, unchanged levels of GUS siRNAs were observed in *cbp20, cbp80, dcl1, hst, hyl1* and *se* mutants (Figure 2B).

By contrast, AGO1 could be limiting for S-PTGS in wild-type plants. Owing to its crucial role in the miRNA pathway, the expression of AGO1 is tightly regulated. AGO1 homeostasis is partially achieved by a feedback regulatory loop that involves miR168, a miRNA that guides cleavage and translational repression of *AGO1* mRNA through the activity of the AGO1 protein (11,28). AGO1 primarily binds to miRNAs, but it can also bind 21-nt siRNAs, including endogenous siRNAs and transgene/virus PTGS-related siRNAs (4), and AGO1 homeostasis involves a second feedback regulatory loop that involves *AGO1* siRNAs deriving from *AGO1* mRNA following miR168-guided cleavage (13). Like miR168, these siRNAs have the potential to guide cleavage and translational repression of *AGO1* mRNA (14). Through these regulatory loops, AGO1 levels are adjusted to the amount of miRNAs and siRNAs present in the cell. It is likely that during the establishment of exogenous PTGS in wild-type plants, the transgene/virus siRNAs that are produced compete with miRNAs and endogenous siRNAs for AGO1. Indeed, transgenes that trigger PTGS commonly are driven by very strong promoters such as the *Cauliflower Mosaic Virus* 35S promoter. Moreover, during virus infection, viral siRNA can represent up to 64% of the total sRNA population (29). In this scenario, the competition between transgene or virus siRNAs and miR168, a miRNA that is not efficiently loaded on AGO1, could lead to a transient depletion of AGO1–miR168 complexes, which in turn would reduce the cleavage of *AGO1* mRNA, resulting in an increase in the amount of AGO1 protein and the incorporation of both transgene siRNAs and endogenous siRNAs and miRNAs in silencing complexes. Supporting this hypothesis, western blot analysis of silenced *L1* plants revealed that the amount of AGO1 protein in silenced *L1* plants was higher than that in wild-type plants (Figure 3A), while miR168 levels remained unchanged (Figure 3B). Moreover, analysis of *Hc1* plants revealed that the amount of AGO1 protein was higher in *Hc1* silenced than in non-silenced plants (Figure 3C). Finally, western blot analysis of *cbp20, cbp80, dcl1, hst, hyl* and *se* mutants revealed that the amount of AGO1 protein was higher in these mutants compared with non-silenced *Hc1* plants (Figure 3C), indicating that more AGO1 protein was available for transgene siRNA loading in these mutants, which, in turn, likely increases S-PTGS efficiency.

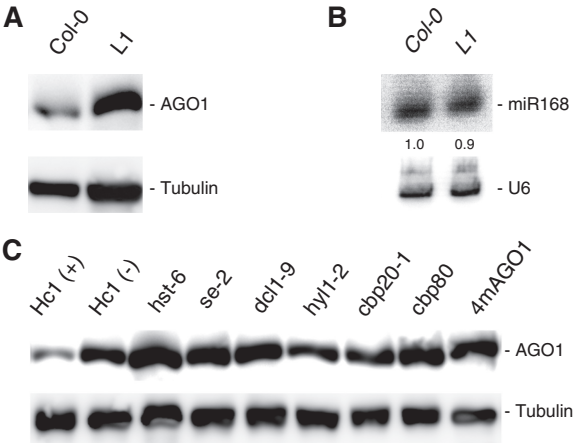

Figure 3. AGO1 overacumulates in silenced plants and in mutants impaired in the miRNA pathway. (**A** and **C**) Protein blot from flowers collected at 51 dag was probed with an anti-AGO1 antibody. Tubulin was used as a loading control. (**B**) RNA gel blot analysis of miR168 in flowers from 51 dag plants. U6 snRNA hybridization served as loading control. Values of miR168 are normalized to U6 snRNA (with Col-0 set at 1.0).

AGO1 is limiting for PTGS

To test if AGO1 availability is the limiting factor in S-PTGS effectiveness, we introduced the *Hc1* locus in *4mAGO1* plants, which express an *AGO1* mRNA that is resistant to cleavage by miR168, owing to additional mismatches between miR168 and the *AGO1* mRNA (11). In *4mAGO1* plants, *AGO1* mRNA levels are higher than those of wild-type plants (11), and western blot analysis revealed that AGO1 protein levels also are increased (Figure 3C). In contrast, globally miRNA levels are unchanged in *4mAGO1* plants, with the exception of miR168, which is increased (12). We reasoned that if AGO is limiting for S-PTGS effectiveness, *4mAGO1* expression should increase PTGS efficiency. Indeed, *Hc1* PTGS efficiency was elevated to 100% in *Hc1*/*4mAGO1* plants (Figure 4A and B). We also introduced the *Hc1* locus in *sqn* mutants. SQN encodes a cyclophylin protein that acts as a positive regulator of AGO1, likely at the level of its activity (10). According to our hypothesis, we anticipated that *sqn* mutants would be less prone to undergo PTGS owing to reduced AGO1 activity. Indeed, *Hc1* PTGS efficiency was reduced to 1% in *sqn* mutants (Figure 4A and B), confirming the direct correlation between AGO1 level/activity and the efficiency of S-PTGS.

CONCLUSIONS

Our results reveal that components of the miRNA pathway act as endogenous suppressors of transgene

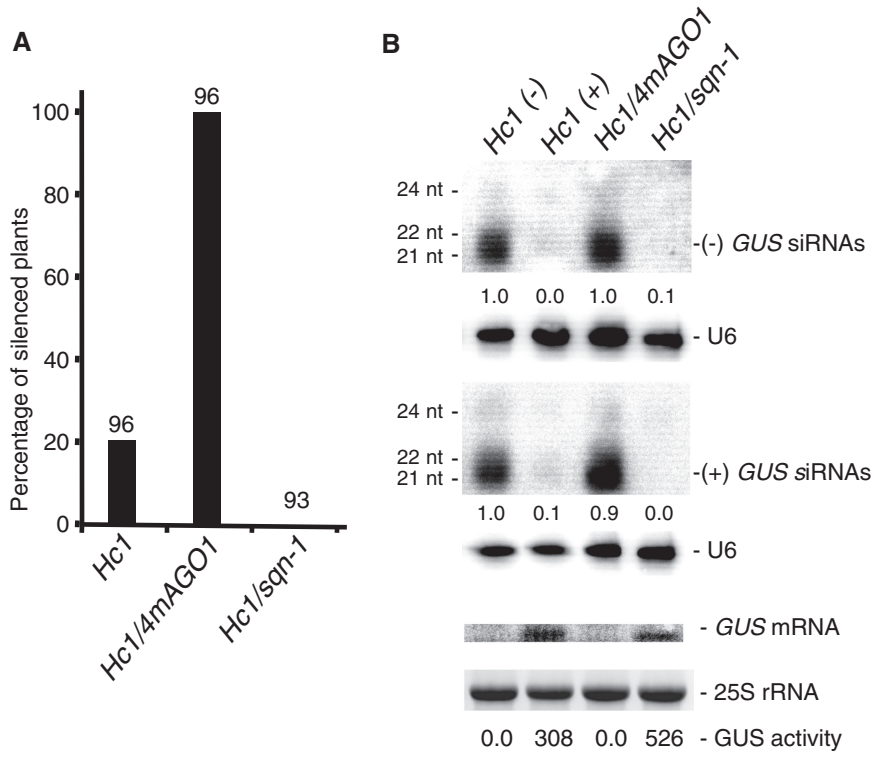

Figure 4. Mutants affecting AGO1 activity exert different effect on *Hc1* PTGS. (**A**) Percentages of silenced *Hc1* plants in the indicated lines. The number of individual plants analyzed is indicated above each bar. (**B**) RNA gel blot analysis of the indicated lines of *GUS* siRNAs of minus polarity (top), plus polarity (middle) and *GUS* mRNA (bottom), in flowers 51 dag. For line *Hc1,* silenced (−) and non-silenced (+) plants were analyzed. 25S RNA and U6 snRNA hybridization served as loading controls. Values of siRNAs are normalized to *U6* RNA (with *Hc1* (−) control set at 1.0).

PTGS, suggesting that the miRNA pathway antagonizes siRNA-mediated PTGS, likely by limiting the availability of AGO1. Recently, it was shown that increasing Ago2 expression during early Xenopus development increases RNAi efficiency, suggesting that Ago activity is limiting in other systems (30). We anticipate that the antagonistic effect of the miRNA pathway is not limited to transgene PTGS but extends to siRNA-mediated PTGS defense against invasive RNA such as RNA deriving from pathogens. Supporting this hypothesis, *dcl1* mutants exhibit increased resistance to *Red Clover Necrotic Mosaic Virus* (31), *Turnip Crinckle Virus* (32) and *Agrobacterium tumefaciens* (33).

We also show that plants supporting transgene PTGS accumulate higher levels of AGO1 protein than wild-type plants. Induction of *AGO1* mRNA accumulation was reported previously as a general response to viral infection (34–36). However, this induction is generally not accompanied by an increase in AGO1 protein or activity levels because viral suppressors of RNA silencing (VSR) encoded by most viruses counteract either small RNA production, small RNA stability, small RNA loading on AGO1, *AGO1* mRNA translation, AGO1 protein stability or AGO1 activity (37). Because the *L1* and *Hc1* transgenes do not encode suppressors of RNA silencing, both lines provide a means to analyze the consequences of an induction of siRNA-mediated PTGS defenses against exogenous RNA unencumbered by the effect of suppressor proteins. We propose that during transgene PTGS, AGO1 induction results from the competition of transgene siRNAs with endogenous siRNAs and miRNAs, including miR168, leading to a relative depletion of AGO1–miR168 complexes and, thus, reduced cleavage of *AGO1* mRNA and a concomitant increase in AGO1 protein amounts.

ACKNOWLEDGEMENTS

We thank Hervé Ferry, Phillipe Marechal and Fabrice Petitpas for plant care. We also thank C. Koncz for the *cbp20-1* mutant, M. Tsiantis for the *se-2* mutant, and S. Poethig for the *hst-6* and *sqn-1* mutants.

FUNDING

Agence Nationale de la Recherche ANR-10-BLAN-1707-03 (to H.V.); (ANR-08-BLAN-0082 to A.C.M.); Région Ile-de-France (a PhD fellowship to V.J.). Funding for open access charge: Agence Nationale de la Recherche ANR-10-BLAN-1707-03.

Conflict of interest statement. None declared.

REFERENCES

1. Brodersen,P. and Voinnet,O. (2006) The diversity of RNA silencing pathways in plants. *Trends Genet*., **22**, 268–280.

2. Vaucheret,H. (2006) Post-transcriptional small RNA pathways in plants: mechanisms and regulations. *Genes Dev.*, **20**, 759–771.
3. Xie,Z., Khanna,K. and Ruan,S. (2010) Expression of microRNAs and its regulation in plants. *Semin. Cell. Dev. Biol.*, **21**, 790–797.
4. Vaucheret,H. (2008) Plant ARGONAUTES. *Trends Plant. Sci.*, **13**, 350–358.
5. Fabian,M.R., Sonenberg,N. and Filipowicz,W. (2010) Regulation of mRNA translation and stability by microRNAs. *Annu. Rev. Biochem.*, **79**, 351–379.
6. Mallory,A.C. and Vaucheret,H. (2010) Form, function, and regulation of ARGONAUTE proteins. *Plant Cell*, **22**, 3879–3889.
7. Bohmert,K., Camus,I., Bellini,C., Bouchez,D., Caboche,M. and Benning,C. (1998) AGO1 defines a novel locus of Arabidopsis controlling leaf development. *EMBO J.*, **17**, 170–180.
8. Morel,J.B., Godon,C., Mourrain,P., Beclin,C., Boutet,S., Feuerbach,F., Proux,F. and Vaucheret,H. (2002) Fertile hypomorphic ARGONAUTE (ago1) mutants impaired in post-transcriptional gene silencing and virus resistance. *Plant Cell*, **14**, 629–639.
9. Kidner,C.A. and Martienssen,R.A. (2004) Spatially restricted microRNA directs leaf polarity through ARGONAUTE1. *Nature*, **428**, 81–84.
10. Smith,M.R., Willmann,M.R., Wu,G., Berardini,T.Z., Moller,B., Weijers,D. and Poethig,R.S. (2009) Cyclophilin 40 is required for microRNA activity in Arabidopsis. *Proc. Natl. Acad. Sci. USA*, **106**, 5424–5429.
11. Vaucheret,H., Vazquez,F., Crete,P. and Bartel,D.P. (2004) The action of ARGONAUTE1 in the miRNA pathway and its regulation by the miRNA pathway are crucial for plant development. *Genes Dev.*, **18**, 1187–1197.
12. Vaucheret,H., Mallory,A.C. and Bartel,D.P. (2006) AGO1 homeostasis entails coexpression of MIR168 and AGO1 and preferential stabilization of miR168 by AGO1. *Mol. Cell*, **22**, 129–136.
13. Mallory,A.C. and Vaucheret,H. (2009) ARGONAUTE 1 homeostasis invokes the coordinate action of the microRNA and siRNA pathways. *EMBO Rep.*, **10**, 521–526.
14. Mallory,A.C., Hinze,A., Tucker,M.R., Bouche,N., Gasciolli,V., Elmayan,T., Lauressergues,D., Jauvion,V., Vaucheret,H. and Laux,T. (2009) Redundant and specific roles of the ARGONAUTE proteins AGO1 and ZLL in development and small RNA-directed gene silencing. *PLoS Genet.*, **5**, e1000646.
15. Earley,K., Smith,M., Weber,R., Gregory,B. and Poethig,R. (2010) An endogenous F-box protein regulates ARGONAUTE1 in *Arabidopsis thaliana*. *Silence*, **1**, 15.
16. Elmayan,T., Balzergue,S., Beon,F., Bourdon,V., Daubremet,J., Guenet,Y., Mourrain,P., Palauqui,J.C., Vernhettes,S., Vialle,T. *et al.* (1998) Arabidopsis mutants impaired in cosuppression. *Plant Cell*, **10**, 1747–1758.
17. Grigg,S.P., Canales,C., Hay,A. and Tsiantis,M. (2005) SERRATE coordinates shoot meristem function and leaf axial patterning in Arabidopsis. *Nature*, **437**, 1022–1026.
18. Papp,I., Mur,L.A., Dalmadi,A., Dulai,S. and Koncz,C. (2004) A mutation in the Cap Binding Protein 20 gene confers drought tolerance to Arabidopsis. *Plant Mol. Biol.*, **55**, 679–686.
19. Park,M.Y., Wu,G., Gonzalez-Sulser,A., Vaucheret,H. and Poethig,R.S. (2005) Nuclear processing and export of microRNAs in Arabidopsis. *Proc. Natl. Acad. Sci. USA*, **102**, 3691–3696.
20. Adenot,X., Elmayan,T., Lauressergues,D., Boutet,S., Bouche,N., Gasciolli,V. and Vaucheret,H. (2006) DRB4-dependent TAS3 trans-acting siRNAs control leaf morphology through AGO7. *Curr. Biol.*, **16**, 927–932.
21. Bouche,N., Lauressergues,D., Gasciolli,V. and Vaucheret,H. (2006) An antagonistic function for Arabidopsis DCL2 in development and a new function for DCL4 in generating viral siRNAs. *EMBO J.*, **25**, 3347–3356.
22. Boutet,S., Vazquez,F., Liu,J., Beclin,C., Fagard,M., Gratias,A., Morel,J.B., Crete,P., Chen,X. and Vaucheret,H. (2003) Arabidopsis HEN1: a genetic link between endogenous miRNA controlling development and siRNA controlling transgene silencing and virus resistance. *Curr. Biol.*, **13**, 843–848.
23. Deleris,A., Gallego-Bartolome,J., Bao,J., Kasschau,K.D., Carrington,J.C. and Voinnet,O. (2006) Hierarchical action and inhibition of plant Dicer-like proteins in antiviral defense. *Science*, **313**, 68–71.
24. Elmayan,T., Adenot,X., Gissot,L., Lauressergues,D., Gy,I. and Vaucheret,H. (2009) A neomorphic sgs3 allele stabilizing miRNA cleavage products reveals that SGS3 acts as a homodimer. *FEBS J.*, **276**, 835–844.
25. Jauvion,V., Elmayan,T. and Vaucheret,H. (2010) The conserved RNA trafficking proteins HPR1 and TEX1 are involved in the production of endogenous and exogenous small interfering RNA in Arabidopsis. *Plant Cell*, **22**, 2697–2709.
26. 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.
27. Yu,B., Bi,L., Zhai,J., Agarwal,M., Li,S., Wu,Q., Ding,S.W., Meyers,B.C., Vaucheret,H. and Chen,X. (2010) siRNAs compete with miRNAs for methylation by HEN1 in Arabidopsis. *Nucleic Acids Res.*, **38**, 5844–5850.
28. Rhoades,M.W., Reinhart,B.J., Lim,L.P., Burge,C.B., Bartel,B. and Bartel,D.P. (2002) Prediction of plant microRNA targets. *Cell*, **110**, 513–520.
29. Ding,S.W. (2010) RNA-based antiviral immunity. *Nat. Rev. Immunol.*, **10**, 632–644.
30. Lund,E., Sheets,M.D., Imboden,S.B. and Dahlberg,J.E. (2011) Limiting Ago protein restricts RNAi and microRNA biogenesis during early development in Xenopus laevis. *Genes Dev.*, **25**, 1121–1131.
31. Takeda,A., Tsukuda,M., Mizumoto,H., Okamoto,K., Kaido,M., Mise,K. and Okuno,T. (2005) A plant RNA virus suppresses RNA silencing through viral RNA replication. *EMBO J.*, **24**, 3147–3157.
32. Qu,F., Ye,X. and Morris,T.J. (2008) Arabidopsis DRB4, AGO1, AGO7, and RDR6 participate in a DCL4-initiated antiviral RNA silencing pathway negatively regulated by DCL1. *Proc. Natl. Acad. Sci. USA*, **105**, 14732–14737.
33. Dunoyer,P., Himber,C. and Voinnet,O. (2006) Induction, suppression and requirement of RNA silencing pathways in virulent Agrobacterium tumefaciens infections. *Nat. Genet.*, **38**, 258–263.
34. Csorba,T., Bovi,A., Dalmay,T. and Burgyan,J. (2007) The p122 subunit of Tobacco Mosaic Virus replicase is a potent silencing suppressor and compromises both small interfering RNA- and microRNA-mediated pathways. *J. Virol.*, **81**, 11768–11780.
35. Havelda,Z., Varallyay,E., Valoczi,A. and Burgyan,J. (2008) Plant virus infection-induced persistent host gene downregulation in systemically infected leaves. *Plant J.*, **55**, 278–288.
36. Zhang,X., Yuan,Y.R., Pei,Y., Lin,S.S., Tuschl,T., Patel,D.J. and Chua,N.H. (2006) Cucumber mosaic virus-encoded 2b suppressor inhibits Arabidopsis Argonaute1 cleavage activity to counter plant defense. *Genes Dev.*, **20**, 3255–3268.
37. Burgyan,J. and Havelda,Z. (2011) Viral suppressors of RNA silencing. *Trends Plant Sci.*, **16**, 265–272.