EMBO reports

scientific report

ARGONAUTE 1 homeostasis invokes the coordinate action of the microRNA and siRNA pathways

Allison C. Mallory & Hervé Vaucheret[+]
Laboratoire de Biologie Cellulaire, Institut Jean-Pierre Bourgin, INRA, Versailles, France

ARGONAUTE 1 (AGO1) slices endogenous messenger RNAs (mRNAs) during both microRNA (miRNA)- and short interfering RNA (siRNA)-guided post-transcriptional silencing. We have previously reported that AGO1 homeostasis is maintained through the repressive action of miR168 on *AGO1* mRNA and the stabilizing effect of AGO1 protein on miR168, but siRNA-mediated *AGO1* regulation has not been reported. Here, we show that *AGO1*-derived siRNAs trigger RNA DEPENDENT RNA POLYMERASE 6 (RDR6)-, SUPPRESSOR OF GENE SILENCING 3 (SGS3)- and SILENCING DEFECTIVE 5 (SDE5)-dependent *AGO1* silencing, which also requires DICER-LIKE 2 (DCL2) and DCL4. By varying the efficacy of miR168-guided *AGO1* mRNA cleavage, we show that siRNA-mediated *AGO1* silencing depends on correct miRNA targeting, pointing to coordinated regulatory actions of the miRNA and siRNA pathways during the maintenance of AGO1 homeostasis. Finally, our results reveal that *dcl2*, *dcl3* and *dcl4* mutations similarly affect post-transcriptional gene silencing (PTGS) mediated by a sense transgene and PTGS mediated by inverted repeats, validating the branched pathway model proposed previously.

Keywords: ARGONAUTE; cosuppression; PTGS; miRNA; RNAi
EMBO reports (2009) **10**, 521–526. doi:10.1038/embor.2009.32

INTRODUCTION

ARGONAUTE 1 (AGO1), one of 10 AGO proteins in *Arabidopsis*, is the main AGO protein mediating short interfering RNA (siRNA)-directed post-transcriptional gene silencing (PTGS) and microRNA (miRNA)-directed regulation (Vaucheret, 2008). miRNAs are processed from partially self-complementary miRNA transcripts whereas siRNA production from sense transgenes, viruses and endogenous trans-acting siRNA (TAS) loci entails stabilization of their non-self-complementary transcripts by SUPPRESSOR OF GENE SILENCING 3 (SGS3) followed by RNA DEPENDENT RNA POLYMERASE 6 (RDR6)-mediated conversion to double-stranded RNA (dsRNA; Chen, 2005; Jones-Rhoades *et al*, 2006; Mallory *et al*, 2008). SILENCING DEFECTIVE 5 (SDE5), a homologue of a human messenger RNA (mRNA) export factor, also influences the production of siRNA through an unidentified mechanism (Hernandez-Pinzon *et al*, 2007). Once mature, miRNAs and siRNAs load onto AGO1, which shows a preference for small RNAs beginning with 5′-uridine (Montgomery *et al*, 2008a; Mi *et al*, 2008; Takeda *et al*, 2008), and guide AGO1-mediated cleavage and translational repression of complementary RNAs (Baumberger & Baulcombe, 2005; Qi *et al*, 2005; Brodersen *et al*, 2008).

AGO1 mRNA is regulated by the miRNA miR168 in an AGO1-dependent manner (Rhoades *et al*, 2002; Vaucheret *et al*, 2004). Plants expressing a miR168-resistant version of *AGO1* (*4m-AGO1*), which contains silent mutations that increase the number of mismatches between miR168 and *AGO1* mRNA without altering AGO1 protein sequence, show developmental defects that can be rescued on expression of a compensatory miRNA (4m-miR168) that restores nucleotide pairing (Vaucheret *et al*, 2004). In addition to this regulatory loop, two additional mechanisms—transcriptional co-regulation of *MIR168* and *AGO1*, and preferential stabilization of miR168 by AGO1—contribute to AGO1 homeostasis (Vaucheret *et al*, 2006).

Although AGO1 interacts with numerous siRNAs and mediates siRNA-guided PTGS, siRNA-mediated *AGO1* regulation has not been reported. Several deep-sequencing analyses have shown that *AGO1* transcripts give rise to both sense and antisense 21-nucleotide siRNAs that map downstream from, and generally in phase with, the miR168 cleavage site, a feature atypical of most miRNA targets (Lu *et al*, 2005; Axtell *et al*, 2006; Rajagopalan *et al*, 2006; Kasschau *et al*, 2007). This uncharacteristic production of siRNAs prompted us to test whether, in addition to miR168-directed regulation, *AGO1* mRNA might be sensitive to siRNA-directed regulation. Here, we show that *AGO1*-derived siRNAs trigger *AGO1* silencing in an RDR6-, SDE5- and SGS3-dependent manner, and that production of *AGO1*-derived siRNAs requires the action of DICER-LIKE 2 (DCL2) and DCL4, similar to viruses (Bouche *et al*, 2006; Deleris *et al*, 2006) and inverted repeat (IR) transgenes (Dunoyer *et al*, 2005, 2007; Fusaro *et al*, 2006). miR168-directed regulation of *AGO1* is necessary for robust siRNA-directed *AGO1* silencing, indicating that both the miRNA and siRNA pathways are involved in the maintenance of AGO1 homeostasis.

RESULTS AND DISCUSSION

AGO1 is hyper-susceptible to cosuppression

Plants showing typical *ago1* mutant developmental defects, including serrated leaves, phyllotaxy defects, floral organ defects

Laboratoire de Biologie Cellulaire, Institut Jean-Pierre Bourgin, INRA, Route de Saint-Cyr, 78026 Versailles, France
[+]Corresponding author. Tel: +33 130833170; Fax: +33 130833099;
E-mail: herve.vaucheret@versailles.inra.fr

Received 5 December 2008; revised 29 January 2009; accepted 9 February 2009; published online 3 April 2009

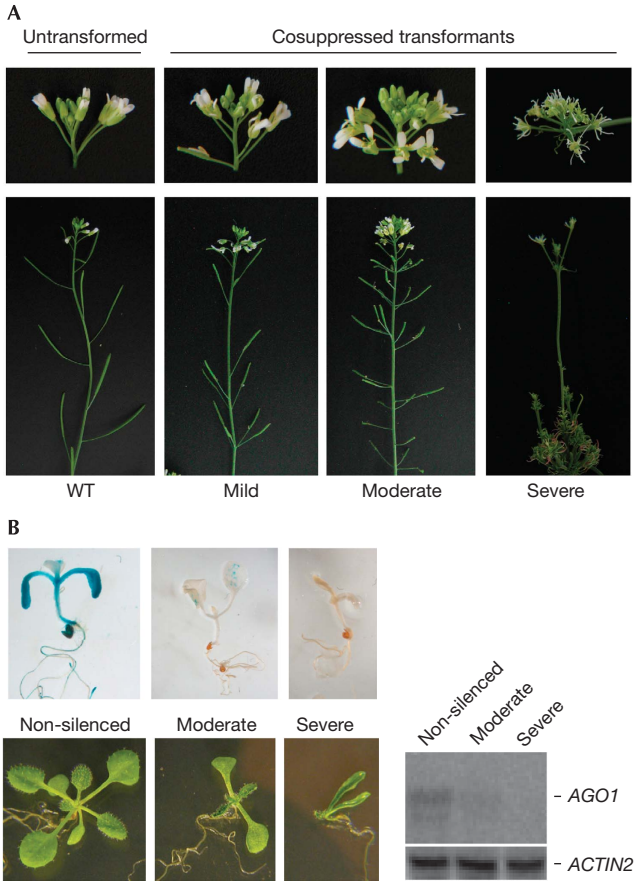

Fig 1 | Ectopic *AGO1* copies trigger cosuppression. (**A**) Varying degrees of developmental defects resulting from *AGO1* cosuppression, including spoon-shaped cotyledons, serrated leaves, altered floral phyllotaxis, floral organ defects and reduced fertility. Constructs that potentially encode a full-length AGO1 protein triggered mild and moderate phenotypes whereas constructs that encode a truncated AGO1 protein fused to Beta-glucuronidase (GUS) triggered moderate and severe phenotypes. (**B**) GUS staining and *AGO1* messenger RNA (mRNA) analysis in non-silenced, moderate and severe cosuppressed *pAGO1:168-AGO1-GUS* transformants show that both the *AGO1-GUS* transgene and the endogenous *AGO1* gene are cosuppressed. GUS intensity and the levels of *AGO1* mRNA show that the severity of developmental defects correlates with increased cosuppression. The blot was hybridized for ACTIN2 as a control. AGO, ARGONAUTE; WT, wild type.

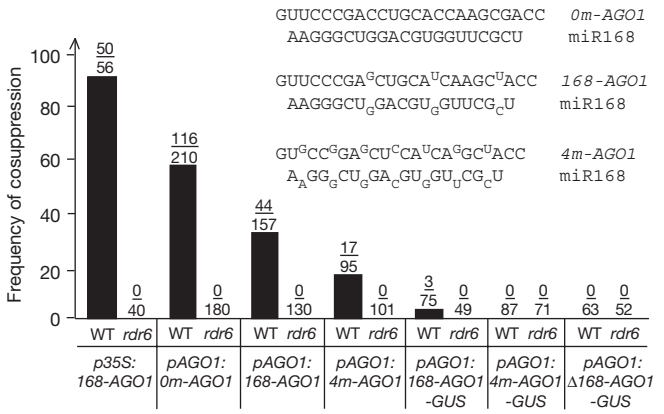

Fig 2 | miR168-directed *AGO1* cleavage facilitates AGO1 cosuppression. Frequencies of *AGO1* cosuppression observed after the transformation of wild-type (WT) plants and *rna dependent rna polymerase 6* (*rdr6*) mutants with the indicated constructs. The fraction of plants showing the cosuppression phenotypes of *AGO1* is indicated above each bar. The pairing between miR168 and WT and mutant *AGO1* messenger RNAs is shown. AGO, ARGONAUTE.

and reduced fertility (Fig 1), were obtained by the transformation of wild-type *Arabidopsis* with either an 8-kb *AGO1* genomic fragment that contains all of the upstream and downstream regulatory elements required for the function of *AGO1* (*pAGO1:168-AGO1*; Vaucheret *et al*, 2004), an *AGO1* cDNA expressed under the control of the strong cauliflower mosaic virus *35S* promoter (*p35S:168-AGO1*), a promoterless *AGO1* cDNA (Δ*p:168-AGO1*), or the 5′ 557 nt of the *AGO1* mRNA translationally fused to *Beta-glucuronidase* (*GUS*) and expressed from the *AGO1* promoter (*pAGO1:168-AGO1-GUS*). Developmental defects of *ago1* mutants were not observed when these constructs were introduced in the PTGS-deficient *rdr6* mutant (Fig 2), suggesting that these defects result from *AGO1* cosuppression.

The cosuppression of *AGO1* follows the previously established traits of cosuppression. For example, *AGO1* cosuppression efficiency was high using the strong 35S promoter (*p35S:168-AGO1*; Fig 2), which is consistent with reports showing that cosuppression efficiency depends on the strength of the transgene promoter (Van Blockland *et al*, 1994; Vaucheret *et al*, 1995; Que *et al*, 1997; Schubert *et al*, 2004). In addition, *AGO1* cosuppression frequency using the *pAGO1:168-AGO1-GUS* construct, which shares 557 nt of homology with the endogenous *AGO1* mRNA, was lower than that using the *pAGO1:168-AGO1*, *p35S:168-AGO1* and Δ*p:168-AGO1* constructs, which all share more than 3 kb of homology with the endogenous *AGO1* mRNA (Fig 2), which is consistent with reports showing that cosuppression efficiency decreases when shortening the length of homology between the transgene and the endogenous gene (Vaucheret *et al*, 1997; Crete & Vaucheret, 1999). Furthermore, the developmental defects of cosuppressed plants were more pronounced in *pAGO1:168-AGO1-GUS* plants than in *pAGO1:168-AGO1*, *p35S:168-AGO1* and Δ*p:168-AGO1* plants (Fig 1), probably because the *pAGO1:168-AGO1-GUS* transgene, unlike the three other transgenes, does not encode a full-length functional AGO1 protein. Indeed, cosuppression degrades only a fraction of the target mRNA; therefore, it is easier to reach low levels of the target protein when only the endogenous mRNA encodes a functional protein than when both the endogenous gene and homologous transgene are contributing to the final pool of functional protein. Consistently, reduction of *AGO1* mRNA accumulation below detectable levels was observed only in *pAGO1:168-AGO1-GUS* cosuppressed transformants (Fig 1; data not shown).

Cosuppression efficiency has been reported to vary greatly from gene to gene for reasons that are not fully understood. The frequency of *AGO1* cosuppression triggered by the *p35S:168-AGO1* construct (89%) was higher than the cosuppression

frequencies generally reported for 35S-driven transgenes (usually 20–40%), although it was not as high as that triggered by the *p35S-NIA2* construct (99%; Elmayan *et al*, 1998). Furthermore, the frequency of *AGO1* cosuppression triggered by the *pAGO1:168-AGO1* and *Δp:168-AGO1* constructs was higher than that reported for other genomic or promoterless transgenes, which probably trigger cosuppression only when inserted into highly transcribed areas of the genome (Van Blockland *et al*, 1994; Vaucheret *et al*, 1995). The high frequency of *AGO1* cosuppression suggests that, in addition to high transcription levels, determinants unique to AGO1 promote the triggering of AGO1 cosuppression.

miR168 complementarity modulates AGO1 cosuppression

AGO1 mRNA contains a miR168 complementarity site that is cleaved through the slicer activity of the AGO1 protein (Vaucheret *et al*, 2004; Baumberger & Baulcombe, 2005; Qi *et al*, 2005). As transgene-derived uncapped and unpolyadenylated RNAs have been shown to activate cosuppression (Gazzani *et al*, 2004; Luo & Chen, 2007), it is possible that miR168-guided cleavage of *AGO1* mRNA, which produces uncapped and unpolyadenylated cleavage fragments, contributes to the hyper-susceptibility of *AGO1* to cosuppression. To test this possibility, we engineered two constructs: *pAGO1:4m-AGO1-GUS* and *pAGO1:Δ168-AGO1-GUS*. The *pAGO1:4m-AGO1-GUS* construct contains the same sequence as *pAGO1:168-AGO1-GUS* but has silent mutations that create four additional mismatches between *AGO1* mRNA and miR168, and render *AGO1* resistant to miR168 regulation (Vaucheret *et al*, 2004). The *pAGO1:Δ168-AGO1-GUS* construct is expressed from the 1.5 kb *AGO1* promoter and consists of the 5′ 387 nt of *AGO1* mRNA translationally fused to *GUS*, but lacks the miR168 complementary site. Unlike the *pAGO1:168-AGO1-GUS* construct, the *pAGO1:4m-AGO1-GUS* and *pAGO1: Δ168-AGO1-GUS* constructs, both of which lack the miR168 complementary site, did not trigger *AGO1* cosuppression (Fig 2), suggesting that miR168-directed *AGO1* cleavage facilitates *AGO1* cosuppression.

To assess whether the rate of miR168-guided *AGO1* mRNA cleavage influences *AGO1* cosuppression efficiency, constructs producing full-length *AGO1* mRNAs that are either resistant or hyper-susceptible to miR168-guided cleavage were introduced into wild-type *Arabidopsis* plants and *rdr6* mutants. The *pAGO1:4m-AGO1* construct is similar to the *pAGO1:168-AGO1* construct but contains silent mutations that create four additional mismatches between the *AGO1* mRNA and miR168 and render *AGO1* resistant to miR168 regulation (Vaucheret *et al*, 2004). By contrast, the *pAGO1:0m-AGO1* construct repairs the three natural mismatches between *AGO1* mRNA and miR168, producing an *AGO1* mRNA that has an increased cleavage rate (Vaucheret *et al*, 2006). The frequency of *AGO1* cosuppression was 50% lower in *pAGO1:4m-AGO1* plants than in *pAGO1:168-AGO1* transgenic plants (Fig 2). Although the frequency of cosuppression was reduced in *pAGO1:4m-AGO1* plants, it was not abolished, suggesting that miR168-guided cleavage of *AGO1* mRNAs arising from the native *AGO1* gene is sufficient to trigger *AGO1* cosuppression. Confirming the positive role of miR168-guided *AGO1* mRNA cleavage during *AGO1* cosuppression, the frequency of cosuppression was nearly 30% higher in *pAGO1:0m-AGO1* plants than in *pAGO1:168-AGO1* plants (Fig 2). In all cases, cosuppression was observed only in wild-type plants and never in *rdr6* mutants.

AGO1 cosuppression correlates with AGO1 siRNA

miR168-guided *AGO1* mRNA cleavage results in the production of uncapped and unpolyadenylated fragments, which could contribute to *AGO1* cosuppression. However, other miRNA targets seem to be no more susceptible to cosuppression than non-targeted RNAs, suggesting that, in addition to miRNA-guided mRNA cleavage, other determinants promote the triggering of *AGO1* cosuppression. In contrast to other protein-coding mRNAs targeted by miRNAs, *AGO1* gives rise to 21-nt siRNAs that map downstream from, and generally in phase with, the *AGO1* miR168 cleavage site (Lu *et al*, 2005; Axtell *et al*, 2006; Rajagopalan *et al*, 2006; Kasschau *et al*, 2007). More than 70% of the cloned siRNAs that are complementary to *AGO1* mRNA begin with a 5′ uridine, a characteristic shared by small RNAs that associate preferentially with and function through AGO1. The production of siRNA following miR168-guided cleavage of *AGO1* mRNA is reminiscent of the production of trans-acting short interfering RNA (tasiRNA) following miRNA-guided cleavage of *TAS* transcripts (Mallory & Bouche, 2008). The production of AGO1 siRNAs and the heightened susceptibility of *AGO1* to cosuppression suggest that *AGO1* is naturally subjected to siRNA-mediated self-regulation in addition to miR168 regulation, and that heightened *AGO1* siRNA production in transgenic plants containing ectopic *AGO1* copies could be responsible for *AGO1* cosuppression. Consistent with their low cloning frequencies, we were unable to detect *AGO1* siRNAs in untransformed wild-type plants by RNA gel blot analyses (Fig 3). However, both sense and antisense *AGO1* siRNAs corresponding to sequences downstream from the miR168 complementary site were detected in *p35S:168-AGO1* transgenic plants undergoing cosuppression (Fig 3; data not shown). *AGO1* cosuppression was observed after the transformation of wild-type plants and *ago7* mutants, but was never observed in the PTGS-deficient mutants *rdr6*, *sgs3* or *sde5* (Fig 3).

DCL4 and DCL2 are required for AGO1 cosuppression

The mutants *ago1*, *hua enhancer 1* (*hen1*), *rdr6* and *sgs3* have been isolated in genetic screens for mutants defective in sense transgene-mediated PTGS (Mourrain *et al*, 2000; Morel *et al*, 2002; Boutet *et al*, 2003) whereas *nuclear rna polymerase d1a* (*nrpd1a*), *rdr2*, *rdr6*, *sde3*, *sde5* and *sgs3* have been recovered in genetic screens for mutants defective in transgene/virus (amplicon)-based PTGS (Dalmay *et al*, 2000, 2001; Herr *et al*, 2005; Hernandez-Pinzon *et al*, 2007), indicating that neither of these two systems retrieved the entire set of PTGS components. Indeed, *dcl* mutants have not been recovered from these two screens. However, it has been shown that DCL2 and DCL4 act redundantly in the production of endogenous tasiRNAs, some viral siRNAs and IR transgene-derived siRNAs (Gasciolli *et al*, 2005; Xie *et al*, 2005; Blevins *et al*, 2006; Bouche *et al*, 2006; Deleris *et al*, 2006; Fusaro *et al*, 2006; Dunoyer *et al*, 2007). To determine the DCL that is responsible for AGO1 siRNA production, we introduced the *p35S:168-AGO1* construct into *dcl* mutants and assayed *AGO1* cosuppression frequencies and siRNA accumulation. *AGO1* cosuppression was observed at high frequencies in wild-type plants and in *dcl2*, *dcl3* and *dcl4* single mutants, and *AGO1* siRNAs were detectable in these mutants (Fig 3). By

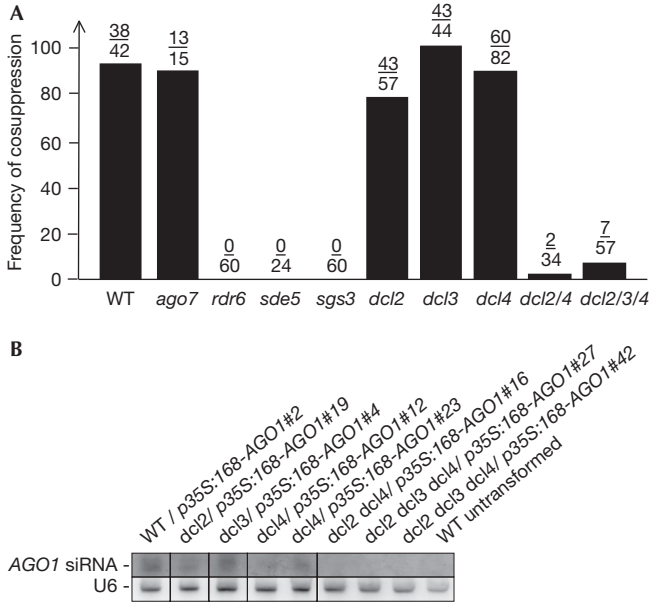

Fig 3 | *AGO1* cosuppression requires SGS3, SDE5, RDR6, DCL2 and DCL4. (**A**) Frequencies of *AGO1* cosuppression observed after the transformation of wild-type (WT) plants and the indicated mutants with *p35S:168-AGO1*. The fraction of plants showing the cosuppression phenotypes of *AGO1* is indicated above each bar. (**B**) RNA gel blot analysis of *AGO1* siRNAs in WT untransformed and *p35S:168-AGO1*-transformed WT and *dcl* mutant plants showing strong cosuppression of developmental defects. The accumulation of AGO1 siRNA depends on both DCL2 and DCL4. For both *dcl4* and *dcl2 dcl3 dcl4*, two independent transformants are presented. The blot was hybridized for U6 snRNA as a control. AGO, ARGONAUTE; DCL, DICER-LIKE; RDR6, RNA DEPENDENT RNA POLYMERASE 6; SDE5, SILENCING DEFECTIVE 5; SGS3, SUPPRESSOR OF GENE SILENCING 3; siRNA, short interfering RNA; snRNA, small nuclear RNA.

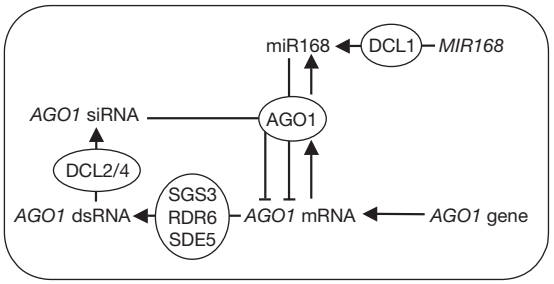

Fig 4 | Model for the coordinated regulatory action of both the miRNA and siRNA pathways during the maintenance of AGO1 homeostasis. DCL1 processes *MIR168* transcripts to produce the mature miR168, which associates with AGO1 protein and mediates cleavage of *AGO1* messenger RNA (mRNA). AGO1 protein preferentially stabilizes miR168, which helps keep the levels of *AGO1* mRNA, and thus the miRNA pathway, in check. In addition to miR168-directed regulation of *AGO1*, *AGO1* mRNA also gives rise to transcripts that are substrates for SGS3, SDE5 and RDR6 and to the production of dsRNA, which is processed by DCL2 and DCL4 to produce siRNAs that regulate the level of *AGO1* mRNA. AGO, ARGONAUTE; DCL, DICER-LIKE; dsRNA, double-stranded RNA; miRNA, microRNA; RDR6, RNA DEPENDENT RNA POLYMERASE 6; SDE5, SILENCING DEFECTIVE 5; SGS3, SUPPRESSOR OF GENE SILENCING 3; siRNA, short interfering RNA.

contrast, the frequency of *AGO1* cosuppression and accumulation of *AGO1* siRNAs were reduced in the *dcl2 dcl4* double mutant and in the *dcl2 dcl3 dcl4* triple mutant, indicating a redundant role for DCL2 and DCL4 during *AGO1* siRNA production and *AGO1* cosuppression, similar to that observed for tasiRNAs, viral siRNAs and IR transgene siRNAs (Gasciolli *et al*, 2005; Xie *et al*, 2005; Bouche *et al*, 2006; Deleris *et al*, 2006; Fusaro *et al*, 2006; Dunoyer *et al*, 2007). It is noted that the impact of *AGO1* cosuppression was slightly more pronounced in *dcl3* and *dcl2 dcl3 dcl4* than in wild-type plants and *dcl2 dcl4* mutants, respectively, suggesting an antagonistic role for DCL3 in cosuppression, similar to that reported for DCL3 in the *pSUC-PDS* IR-PTGS system (Smith *et al*, 2007).

Conclusions

Our results show that siRNAs arising from *AGO1* mRNA have the capacity to modulate the levels of *AGO1* mRNA. This siRNA-mediated *AGO1* mRNA degradation is miR168 cleavage dependent and requires SGS3, RDR6, SDE5 and DCL2/DCL4. Such regulation distinguishes *AGO1* from other protein-coding miRNA targets, which undergo RNA degradation through the EXORIBONUCLEASE (XRN) and exosome pathways following miRNA-guided cleavage (Shen & Goodman, 2004; Souret *et al*, 2004; Gy *et al*, 2007). It remains to be tested whether the production of *AGO1* siRNAs downstream from the miR168 complementary site is a special feature of miR168-directed *AGO1* regulation, similar to the specific requirement for miR173- and miR390-directed regulation of *TAS* transcripts for the production of tasiRNAs (Montgomery *et al*, 2008a,b). Together with AGO1-catalysed miR168-guided *AGO1* mRNA cleavage (Vaucheret *et al*, 2004) and AGO1-mediated preferential stabilization of miR168 (Vaucheret *et al*, 2006), *AGO1* siRNA-guided *AGO1* mRNA cleavage helps AGO1 levels to be kept in check, indicating that the miRNA and siRNA pathways coordinately regulate the maintenance of AGO1 homeostasis (Fig 4).

METHODS

Plant material. All plants are in the Columbia ecotype. *rdr6* (*sgs2-1*), *sgs3-1*, *ago7* (*zip-1*), *dcl2-1*, *dcl3-1* and *dcl4-2* mutant alleles have been described previously (Elmayan *et al*, 1998; Mourrain *et al*, 2000; Hunter *et al*, 2003; Xie *et al*, 2004, 2005). *sde5-3* corresponds to the T-DNA insertion line WiscDsLox429G09. Plants were transformed using the floral dip method (Clough & Bent, 1998). All plants were grown in standard long-day conditions (16 h light–8 h dark) at 22 °C. Constructs were prepared as described in the supplementary information online.

Molecular analyses. High molecular and low molecular RNA gel blot analyses were performed as described previously (Gy *et al*, 2007). All RNAs were extracted from floral inflorescence at the same developmental stage. *AGO1* mRNA probe was described previously (Vaucheret *et al*, 2006). GUS histochemical staining was performed as described previously (Elmayan *et al*, 2005).

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org)

ACKNOWLEDGEMENTS

We thank David Bartel for the use of his lab facilities at the beginning of this project and for helpful discussions, the Nottingham Arabidopsis Stock Centre for the distribution of mutant seeds, and Bruno Letarnec and Herve Ferry for plant care. This study was supported by the Agence Nationale de la Recherche (ANR-06-BLAN-0203 and ANR-06-POGM-007 to H.V., and ANR-08-BLAN-0082-01 to A.C.M.).

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

REFERENCES

Axtell MJ, Jan C, Rajagopalan R, Bartel DP (2006) A two-hit trigger for siRNA biogenesis in plants. *Cell* **127:** 565–577

Baumberger N, Baulcombe DC (2005) *Arabidopsis* ARGONAUTE1 is an RNA slicer that selectively recruits microRNAs and short interfering RNAs. *Proc Natl Acad Sci USA* **102:** 11928–11933

Blevins T *et al* (2006) Four plant Dicers mediate viral small RNA biogenesis and DNA virus induced silencing. *Nucleic Acids Res* **34:** 6233–6246

Bouche N, Lauressergues D, Gasciolli V, 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

Boutet S, Vazquez F, Liu J, Béclin C, Fagard M, Gratias A, Morel JB, Crété P, Chen X, 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

Brodersen P, Sakvarelidze-Achard L, Bruun-Rasmussen M, Dunoyer P, Yamamoto YY, Sieburth L, Voinnet O (2008) Widespread translational inhibition by plant miRNAs and siRNAs. *Science* **320:** 1185–1190

Chen X (2005) MicroRNA biogenesis and function in plants. *FEBS Lett* **579:** 5923–5931

Clough SJ, Bent AF (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J* **16:** 735–743

Crete P, Vaucheret H (1999) Expression and sequence requirements for nitrite reductase co-suppression. *Plant Mol Biol* **41:** 105–114

Dalmay T, Hamilton A, Rudd S, Angell S, Baulcombe DC (2000) An RNA-dependent RNA polymerase gene in *Arabidopsis* is required for posttranscriptional gene silencing mediated by a transgene but not by a virus. *Cell* **101:** 543–553

Dalmay T, Horsefield R, Braunstein TH, Baulcombe DC (2001) SDE3 encodes an RNA helicase required for post-transcriptional gene silencing in *Arabidopsis*. *EMBO J* **20:** 2069–2078

Deleris A, Gallego-Bartolome J, Bao J, Kasschau KD, Carrington JC, Voinnet O (2006) Hierarchical action and inhibition of plant Dicer-like proteins in antiviral defense. *Science* **313:** 68–71

Dunoyer P, Himber C, Voinnet O (2005) DICER-LIKE 4 is required for RNA interference and produces the 21-nucleotide small interfering RNA component of the plant cell-to-cell silencing signal. *Nat Genet* **37:** 1356–1360

Dunoyer P, Himber C, Ruiz-Ferrer V, Alioua A, Voinnet O (2007) Intra- and intercellular RNA interference in *Arabidopsis thaliana* requires components of the microRNA and heterochromatic silencing pathways. *Nat Genet* **39:** 848–856

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

Elmayan T, Proux F, Vaucheret H (2005) *Arabidopsis* RPA2: a genetic link among transcriptional gene silencing, DNA repair, and DNA replication. *Curr Biol* **15:** 1919–1925

Fusaro AF *et al* (2006) RNA interference-inducing hairpin RNAs in plants act through the viral defence pathway. *EMBO Rep* **7:** 1168–1175

Gasciolli V, Mallory AC, Bartel DP, Vaucheret H (2005) Partially redundant functions of *Arabidopsis* DICER-like enzymes and a role for DCL4 in producing trans-acting siRNAs. *Curr Biol* **15:** 1494–1500

Gazzani S, Lawrenson T, Woodward C, Headon D, Sablowski R (2004) A link between mRNA turnover and RNA interference in *Arabidopsis*. *Science* **306:** 1046–1048

Gy I, Gasciolli V, Lauressergues D, Morel JB, Gombert J, Proux F, Proux C, Vaucheret H, Mallory AC (2007) *Arabidopsis* FIERY1, XRN2, and XRN3 are endogenous RNA silencing suppressors. *Plant Cell* **19:** 3451–3461

Hernandez-Pinzon I, Yelina NE, Schwach F, Studholme DJ, Baulcombe D, Dalmay T (2007) SDE5, the putative homologue of a human mRNA export factor, is required for transgene silencing and accumulation of trans-acting endogenous siRNA. *Plant J* **50:** 140–148

Herr AJ, Jensen MB, Dalmay T, Baulcombe DC (2005) RNA polymerase IV directs silencing of endogenous DNA. *Science* **308:** 118–120

Hunter C, Sun H, Poethig RS (2003) The *Arabidopsis* heterochronic gene ZIPPY is an ARGONAUTE family member. *Curr Biol* **13:** 1734–1739

Jones-Rhoades MW, Bartel DP, Bartel B (2006) MicroRNAs and their regulatory roles in plants. *Annu Rev Plant Biol* **57:** 19–53

Kasschau KD, Fahlgren N, Chapman EJ, Sullivan CM, Cumbie JS, Givan SA, Carrington JC (2007) Genome-wide profiling and analysis of *Arabidopsis* siRNAs. *PLoS Biol* **5:** e57

Lu C, Tej SS, Luo S, Haudenschild CD, Meyers BC, Green PJ (2005) Elucidation of the small RNA component of the transcriptome. *Science* **309:** 1567–1569

Luo Z, Chen Z (2007) Improperly terminated, unpolyadenylated mRNA of sense transgenes is targeted by RDR6-mediated RNA silencing in *Arabidopsis*. *Plant Cell* **19:** 943–958

Mallory AC, Bouche N (2008) MicroRNA-directed regulation: to cleave or not to cleave. *Trends Plant Sci* **13:** 359–367

Mallory AC, Elmayan T, Vaucheret H (2008) MicroRNA maturation and action—the expanding roles of ARGONAUTEs. *Curr Opin Plant Biol* **11:** 560–566

Mi S *et al* (2008) Sorting of small RNAs into *Arabidopsis* argonaute complexes is directed by the 5′ terminal nucleotide. *Cell* **133:** 116–127

Montgomery TA, Howell MD, Cuperus JT, Li D, Hansen JE, Alexander AL, Chapman EJ, Fahlgren N, Allen E, Carrington JC (2008a) Specificity of ARGONAUTE7–miR390 interaction and dual functionality in TAS3 trans-acting siRNA formation. *Cell* **133:** 128–141

Montgomery TA *et al* (2008b) AGO1-miR173 complex initiates phased siRNA formation in plants. *Proc Natl Acad Sci USA* **105:** 20055–20062

Morel JB, Godon C, Mourrain P, Béclin C, Boutet S, Feuerbach F, Proux F, Vaucheret H (2002) Fertile hypomorphic ARGONAUTE (ago1) mutants impaired in post-transcriptional gene silencing and virus resistance. *Plant Cell* **14:** 629–639

Mourrain P *et al* (2000) *Arabidopsis* SGS2 and SGS3 genes are required for posttranscriptional gene silencing and natural virus resistance. *Cell* **101:** 533–542

Qi Y, Denli AM, Hannon GJ (2005) Biochemical specialization within *Arabidopsis* RNA silencing pathways. *Mol Cell* **19:** 421–428

Que Q, Wang HY, English JJ, Jorgensen RA (1997) The frequency and degree of cosuppression by sense chalcone synthase transgenes are dependent on transgene promoter strength and are reduced by premature nonsense codons in the transgene coding sequence. *Plant Cell* **9:** 1357–1368

Rajagopalan R, Vaucheret H, Trejo J, Bartel DP (2006) A diverse and evolutionarily fluid set of microRNAs in *Arabidopsis thaliana*. *Genes Dev* **20:** 3407–3425

Rhoades MW, Reinhart BJ, Lim LP, Burge CB, Bartel B, Bartel DP (2002) Prediction of plant microRNA targets. *Cell* **110:** 513–520

Schubert D, Lechtenberg B, Forsbach A, Gils M, Bahadur S, Schmidt R (2004) Silencing in *Arabidopsis* T-DNA transformants: the predominant role of a gene-specific RNA sensing mechanism versus position effects. *Plant Cell* **16:** 2561–2572

Shen B, Goodman HM (2004) Uridine addition after microRNA-directed cleavage. *Science* **306:** 997

Smith LM, Pontes O, Searle I, Yelina N, Yousafzai FK, Herr AJ, Pikaard CS, Baulcombe DC (2007) An SNF2 protein associated with nuclear RNA silencing and the spread of a silencing signal between cells in *Arabidopsis*. *Plant Cell* **19:** 1507–1521

Souret FF, Kastenmayer JP, Green PJ (2004) AtXRN4 degrades mRNA in *Arabidopsis* and its substrates include selected miRNA targets. *Mol Cell* **15:** 173–183

Takeda A, Iwasaki S, Watanabe T, Utsumi M, Watanabe Y (2008) The mechanism selecting the guide strand from small RNA duplexes is different among argonaute proteins. *Plant Cell Physiol* **49:** 493–500

Van Blokland R, Van der Geest N, Mol JNM, Kooter JM (1994) Transgene-mediated suppression of chalcone synthase expression in *Petunia hybrida* results from an increase in RNA turnover. *Plant J* **6:** 861–877

Vaucheret H (2008) Plant ARGONAUTES. *Trends Plant Sci* **13:** 350–358

Vaucheret H, Palauqui JC, Elmayan T, Moffatt B (1995) Molecular and genetic analysis of nitrite reductase co-suppression in transgenic tobacco plants. *Mol Gen Genet* **248:** 311–317

Vaucheret H, Nussaume L, Palauqui JC, Quillere I, Elmayan T (1997) A transcriptionally active state is required for post-transcriptional silencing (cosuppression) of nitrate reductase host genes and transgenes. *Plant Cell* **9:** 1495–1504

Vaucheret H, Vazquez F, Crete P, Bartel DP (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

Vaucheret H, Mallory AC, Bartel DP (2006) AGO1 homeostasis entails coexpression of MIR168 and AGO1 and preferential stabilization of miR168 by AGO1. *Mol Cell* **22:** 129–136

Xie Z, Johansen LK, Gustafson AM, Kasschau KD, Lellis AD, Zilberman D, Jacobsen SE, Carrington JC (2004) Genetic and functional diversification of small RNA pathways in plants. *PLoS Biol* **2:** E104

Xie Z, Allen E, Wilken A, Carrington JC (2005) DICER-LIKE 4 functions in trans-acting small interfering RNA biogenesis and vegetative phase change in *Arabidopsis thaliana*. *Proc Natl Acad Sci USA* **102:** 12984–12989