

# The action of *ARGONAUTE1* in the miRNA pathway and its regulation by the miRNA pathway are crucial for plant development

Hervé Vaucheret,<sup>1,2,6</sup> Franck Vazquez,<sup>1,3</sup> Patrice Crété,<sup>3,5</sup> and David P. Bartel<sup>2,4,7</sup>

<sup>1</sup>Laboratoire de Biologie Cellulaire, Institut Jean-Pierre Bourgin, INRA, 78026 Versailles Cedex, France; <sup>2</sup>Whitehead Institute for Biomedical Research, Cambridge, Massachusetts 02142, USA; <sup>3</sup>Laboratoire de physiologie de la différenciation végétale, UPRES-EA3569, IFR118, EPT1016, Université des Sciences et Technologies de Lille, 59650 Villeneuve d'Ascq Cedex, France; <sup>4</sup>Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, USA

MicroRNAs (miRNAs) are endogenous 21–24-nt RNAs that can down-regulate gene expression by pairing to the messages of protein-coding genes to specify mRNA cleavage or repression of productive translation. They act within the RNA-induced silencing complex (RISC), which in animals contains a member of the Argonaute family of proteins. In the present study, we show that *Arabidopsis ago1* mutants have increased accumulation of mRNAs known to be targeted for cleavage by miRNAs. In hypomorphic *ago1* alleles, this compromised miRNA function occurs without a substantial change in miRNA accumulation, whereas in null alleles it is accompanied by a drop in some of the miRNAs. Therefore, AGO1 acts within the *Arabidopsis* miRNA pathway, probably within the miRNA-programmed RISC, such that the absence of AGO1 destabilizes some of the miRNAs. We also show that targeting of *AGO1* mRNA by miR168 is needed for proper plant development, illustrating the importance of feedback control by this miRNA. Transgenic plants expressing a mutant *AGO1* mRNA with decreased complementarity to miR168 overaccumulate *AGO1* mRNA and exhibit developmental defects partially overlapping with those of *dcl1*, *hen1*, and *hyl1* mutants showing a decrease in miRNA accumulation. miRNA targets overaccumulate in miR168-resistant plants, suggesting that a large excess of AGO1 protein interferes with the function of RISC or sequesters miRNAs or other RISC components. Developmental defects induced by a miR168-resistant *AGO1* mRNA can be rescued by a compensatory miRNA that is complementary to the mutant *AGO1* mRNA, proving the regulatory relationship between miR168 and its target and opening the way for engineering artificial miRNAs in plants.

[*Keywords:* AGO1; miRNA; negative feedback regulation; posttranscriptional gene silencing; RNAi]

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Noncoding small RNAs (20–24 nt long) have recently been recognized as important regulators in both plants and animals, acting in the control of invading nucleic acids or in the regulation of gene expression (Hannon 2002; Zamore 2002; Ambros 2003; Bartel 2004). Small RNAs that derive from long double-stranded RNAs are called siRNAs. siRNAs can direct the cellular immune system to silence exogenous invading nucleic acids (viruses, transgenes) by RNA cleavage through phenomena

called posttranscriptional gene silencing (PTGS) in plants or RNA interference (RNAi) in animals (Hannon 2002; Zamore 2002). siRNAs can also trigger changes in the chromatin state that silence endogenous invading and/or repeated elements (transposons, centromeres) at the transcriptional level (Finnegan and Matzke 2003). Endogenous small RNAs that are processed from stem-loop precursor RNAs and regulate genes that are distinct from the genes from which they derive are called miRNAs (Lagos-Quintana et al. 2001; Lau et al. 2001; Lee and Ambros 2001; Bartel 2004). Such RNAs have been cloned from worm, fly, fish, mouse, human, and plant (Bartel 2004). Many of them are conserved between species, indicating an evolutionarily conserved role in gene regulation.

In animals, miRNAs are derived from long primary transcripts (pri-miRNAs) that are cleaved by the nuclear-localized RNase III Droscha into 70-nt precursors (pre-

Present addresses: <sup>5</sup>Laboratoire de Génétique et Biophysiques des Plantes, UMR 163 CEA-CNRS-Université de la Méditerranée, 13288 Marseille cedex 9, France.

Corresponding authors.

<sup>6</sup>E-MAIL [herve.vaucheret@versailles.inra.fr](mailto:herve.vaucheret@versailles.inra.fr); FAX 33-1-3083-3099.

<sup>7</sup>E-MAIL [dbartel@wi.mit.edu](mailto:dbartel@wi.mit.edu); FAX (617) 258-6768.

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miRNAs; Lee et al. 2003). These 70-nt precursors are actively exported out of the nucleus by Ran-GTP and the export receptor Exportin-5 (Yi et al. 2003; Bohnsack et al. 2004; Lund et al. 2004). Pre-miRNAs are subsequently cleaved by another RNase III with an RNA helicase domain called Dicer to generate short-lived double-stranded miRNA intermediates (Grishok et al. 2001; Hutvagner et al. 2001; Ketting et al. 2001; Lee et al. 2003). One strand of these duplex RNAs accumulates as the mature miRNA and down-regulates gene expression by pairing to the messages of protein-coding genes to specify repression of productive translation or mRNA cleavage (Lee et al. 1993; Wightman et al. 1993; Olsen and Ambros 1999; Bartel 2004; Yekta et al. 2004). The miRNAs act within a ribonucleoprotein complex that is similar if not identical to the RNA-induced silencing complex (RISC; Hutvagner and Zamore 2002; Mourelatos et al. 2002; Doench et al. 2003; Zeng et al. 2003). The RISC was first described as the ribonucleoprotein complex that mediates RNAi (Hammond et al. 2001). When purified from either fly or human cells, it contains a member of the ARGONAUTE protein family that is considered a core component of the complex (Hammond et al. 2001; Caudy et al. 2002, 2003; Hutvagner and Zamore 2002; Ishizuka et al. 2002; Martinez et al. 2002; Mourelatos et al. 2002). ARGONAUTE proteins (also known as PPD proteins because they carry PAZ and PIWI domains) have a  $\beta$ -barrel core that together with a side appendage weakly bind to single-stranded RNAs at least 5 nt in length and also to double-stranded RNA (Lingel et al. 2003; Song et al. 2003; Yan et al. 2003), suggesting that ARGONAUTE proteins could be directly associated with siRNAs/miRNAs before and after they recognize their mRNA targets. RISC-associated proteins also include the nuclease Tudor-SN, the putative RNA-binding VIG protein and the Fragile X-related protein (Hammond et al. 2001; Caudy et al. 2002, 2003; Hutvagner and Zamore 2002; Ishizuka et al. 2002; Martinez et al. 2002).

In plants, most knowledge of miRNA biogenesis comes from the analysis of mutants originally isolated during developmental screens, which turned out to be partially defective in the accumulation of miRNAs. Plant mutants that are totally defective for miRNA accumulation have not been reported yet, and it is highly probable that plants totally depleted in miRNAs are inviable. Indeed, mutants that lack the nuclear DICER-LIKE1 (DCL1) protein are embryo-lethal, indicating that DCL1 is required for plant viability, at least during reproduction and/or at early stages of development (Schauer et al. 2002). Partial loss-of-function *dcl1* mutants exhibiting point mutations in the RNA helicase domain (*dcl1-7*, *dcl1-8*) or truncation of the second dsRNA-binding domain (*dcl1-9*) are viable but show developmental defects including sterility (Schauer et al. 2002). Accompanying these defects are greatly reduced miRNA levels, suggesting the crucial role of miRNAs during plant development and reproduction (Park et al. 2002; Reinhart et al. 2002). The proper accumulation of miRNAs also depends on the activity of two other nuclear proteins: HEN1 and HYL1 (Park et al. 2002;

Boutet et al. 2003; Han et al. 2004; Vazquez et al. 2004). *hen1*- and *hyl1*-null alleles show reduced miRNA levels and developmental defects that overlap with that of partial loss-of-function *dcl1* mutants. However, in contrast to *dcl1*-null alleles, *hen1*- and *hyl1*-null alleles are viable. This suggests either that HEN1 and HYL1 do not have essential functions in the miRNA pathway or that other genes encode proteins with partially redundant functions.

Two other proteins have been proposed to play a role in the plant miRNA pathway: HASTY (HST) and ARGONAUTE 1 (AGO1). HST encodes a protein that is homologous to Exportin-5, and *hst* mutants have developmental phenotypes, suggesting that HST participates in the transport of miRNAs or pre-miRNAs from the nucleus to the cytoplasm (Bollman et al. 2003; Yi et al. 2003; Bohnsack et al. 2004; Lund et al. 2004). AGO1 is the founding member of the ARGONAUTE protein family, which comprises 10 members in *Arabidopsis* (Morel et al. 2002). The first *ago1*-null alleles were isolated in a screen for mutants exhibiting early developmental defects (Bohmert et al. 1998). Additional *ago1*-null or hypomorphic alleles were isolated during a screen for PTGS-deficient mutants (Fagard et al. 2000; Morel et al. 2002). AGO1 was subsequently shown to be specifically required for siRNA accumulation and DNA methylation triggered by sense transgenes (S-PTGS) but not inverted-repeat transgenes (IR-PTGS; Beclin et al. 2002; Boutet et al. 2003). These results prompted the early proposal that AGO1 is not part of RISC but rather is acting upstream from the mRNA degradation step in the S-PTGS pathway, and that S-PTGS and IR-PTGS are two branches of the PTGS pathway that converge toward a common RISC that contains other AGO proteins (Beclin et al. 2002). Indeed, some of the other proteins in the AGO family are associated with specific functions or processes, including AGO4, which is linked to transposon siRNAs as well as DNA and histone methylation (Zilberman et al. 2003), ZIP/AGO7, which controls vegetative phase changes (Hunter et al. 2003), and PNH/ZLL/AGO10, which controls meristem maintenance (Mousian et al. 1998, 2003; Lynn et al. 1999). Alternatively, it is possible that *ago1* mutants are specifically deficient for S-PTGS because AGO1 is part of the S-PTGS RISC but is absent or can be replaced by other family members in the IR-PTGS RISC.

Based on our knowledge of RISC in animals, one or multiple AGO proteins should be a component of the miRNA RISC that mediates cleavage of plant mRNAs. AGO1, ZIP/AGO7, and PNH/ZLL/AGO10 are good candidates because *ago1*, *zip/ago7*, and *pnh/zll/ago10* mutations affect development. Indeed, it is expected that mutants defective in the miRNA RISC would exhibit developmental defects overlapping those of *dcl1*, *hen1*, or *hyl1* mutants. AGO1 is a particularly attractive candidate to act in the miRNA RISC owing to the possible feedback regulation of AGO1 mRNA by miR168 (Rhoades et al. 2002). Such feedback regulation would be analogous to that proposed for DCL1 mRNA by miR162 (Xie et al. 2003). Among the 10 plant AGO mRNA homologs, only

AGO1 has extensive complementarity to miR168 or any of the other known miRNAs, suggesting that AGO1 might be the only member of the ARGONAUTE family that is regulated by an miRNA, just as DCL1, the Dicer family member known to be required for miRNA accumulation, is the only one of the four Dicer homologs known to be an miRNA target. 5'-RACE experiments revealed that AGO1 mRNA fragments that terminate precisely at the predicted site of miR168-directed cleavage accumulate in wild-type plants, showing that miR168 directs the cleavage of AGO1 mRNA (Vazquez et al. 2004). Furthermore, the steady-state level of uncleaved AGO1 mRNA increases in flowers of *hen1* mutants that show reduced accumulation of miR168, indicating that miR168-directed cleavage of AGO1 mRNA is important for AGO1 regulation (Vazquez et al. 2004).

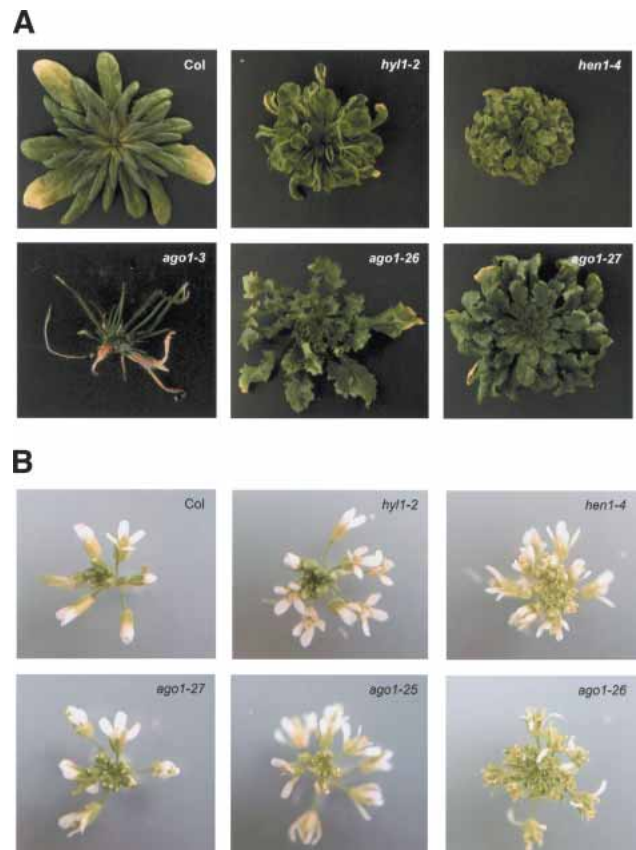
In this paper, we present evidence for a role of AGO1 in the miRNA pathway. We propose that AGO1 acts in RISC; however, we cannot rule out that other AGO proteins also participate in the miRNA RISC, together with AGO1 or in cells where AGO1 is not present. We also demonstrate that the feedback regulation of AGO1 mRNA by the miRNA pathway through the action of miR168 is crucial for proper plant development; decreasing the complementarity of AGO1 mRNA with miR168 results in increased accumulation of AGO1 mRNA and to developmental defects. Finally, we show that these defects can be rescued by expressing a compensatory miRNA that is complementary to the mutant AGO1 mRNA, proving the regulatory relationship between miR168 and its target and opening the way for the engineering of artificial miRNAs.

## Results

### *ago1* mutants exhibit elevated steady-state levels of miRNA targets and developmental defects overlapping with those of *dcl1*, *hen1*, and *hyl1* mutants

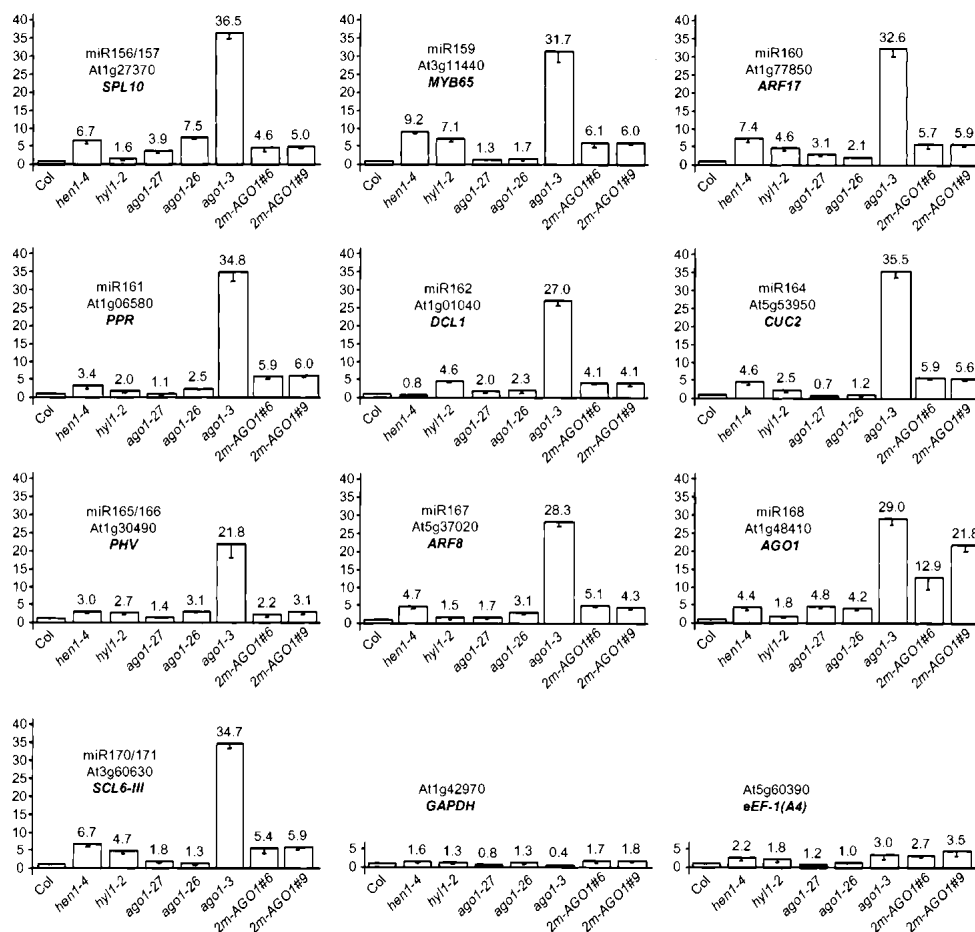
We previously reported the identification of an allelic series of *ago1* mutants through a screen for PTGS-deficient mutants (Fagard et al. 2000; Morel et al. 2002). Three of these alleles (*ago1-22*, *ago1-23*, *ago1-24*) exhibit a phenotype similar to that of the *ago1-3*-null allele previously identified through a phenotypic screen for developmental mutants (Fig. 1). Three other alleles (*ago1-25*, *ago1-26*, *ago1-27*) exhibit less dramatic developmental defects, although they are deficient for PTGS, suggesting that PTGS is more sensitive to perturbation in AGO1 than is development. Interestingly, *ago1* hypomorphic mutants exhibit plant stature, leaf shape, and flower phenotypes partially overlapping those of *dcl1*, *hen1*, or *hyl1* mutants, which are impaired in the accumulation of miRNAs (Fig. 1), suggesting that AGO1 could play a role in the miRNA pathway.

In *dcl1*, *hen1*, and *hyl1* mutants, the impairment of the miRNA pathway results in increased steady-state levels of uncleaved target mRNAs or decreased steady-state levels of cleavage products (Kasschau et al. 2003;



**Figure 1.** *ago1*, *hen1*, and *hyl1* mutants exhibit overlapping developmental defects. (A) Rosettes of plants grown under short-day conditions. (B) Flowers of plants grown under long-day conditions.

Vazquez et al. 2004), with the exception of *AP2* mRNA, for which regulation by miR172 relies on translational repression (Aukerman and Sakai 2003; Chen 2004). To determine if AGO1 also participates in the regulation of endogenous mRNAs by miRNAs, we quantified steady-state levels of 10 mRNAs targeted for cleavage by miRNAs of 10 different families. As controls we quantified the level of *GAPDH* and *eEF-1(A4)* mRNA, which are not thought to be targeted by miRNAs. Because it is not possible to extract enough RNA from flowers of *ago1*-null alleles, we analyzed mRNA steady-state levels in rosettes of representative *ago1* mutants: *ago1-27* (hypomorphic), *ago1-26* (intermediate), *ago1-3* (null), as well as *hen1-4*- and *hyl1-2*-null alleles (all in the Col ecotype) grown in short days. A limited increase in the accumulation of miRNA targets was observed in *hen1*, *hyl1*, and *ago1* hypomorphic mutants (Fig. 2), each target responding to a different extent in the different genetic backgrounds as previously reported in leaves or flowers of *dcl1*, *hen1*, and *hyl1* mutants (Kasschau et al. 2003; Vazquez et al. 2004). A consistent and stronger increase in the accumulation of miRNA targets but not of *GAPDH* and *eEF-1(A4)* mRNAs was observed in the *ago1-3*-null allele, which was confirmed on a second set of independent plants (data not shown). The observe in-



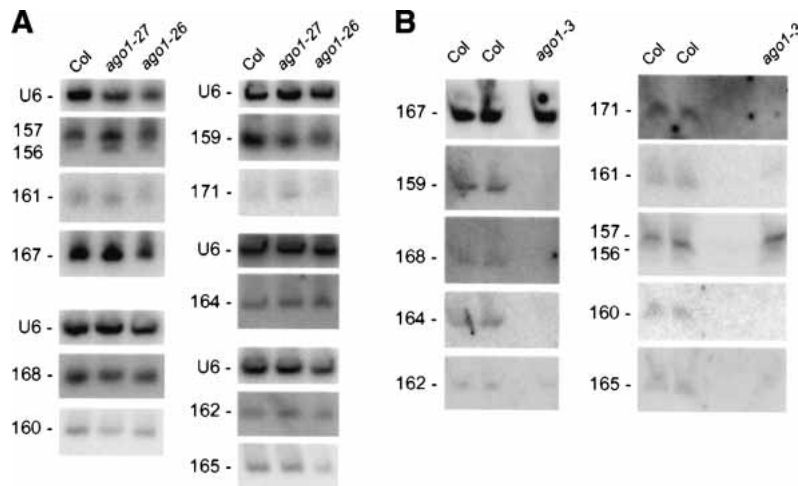
**Figure 2.** *ago1*, *hen1*, and *hyl1* mutants have increased steady-state levels of miRNA targets. RNA extracted from rosettes of isogenic wild-type or mutant siblings deriving from heterozygote parents and of untransformed plants or *2m-AGO1* transformants was quantified for the indicated mRNA by real-time quantitative PCR using primers surrounding the cleavage site. *GAPDH* and *eEF-1(A4)* were used as nontarget controls. Quantifications were normalized to that of *ACTIN2*, then to the value of the wild-type plants or wild-type siblings, which was arbitrarily fixed to 1.

crease in *ago1-3* should be interpreted with caution. Most of the miRNA targets analyzed are expressed at much higher levels in meristem than in leaves. Because *ago1*-null alleles develop smaller leaves than *hen1*, *hyl1*, and *ago1* hypomorphic mutants (Fig. 1A), the ratio between meristematic cells and leaf cells in a rosette is probably higher in *ago1*-null alleles, thus introducing a possible bias in the analysis. Nevertheless, the similar increase observed in *hen1*, *hyl1*, and *ago1* hypomorphic mutants, which all exhibit similar development, indicates that miRNA-directed mRNA cleavage involves AGO1.

#### miRNA accumulation in *ago1* mutants

The increased accumulation of target mRNAs observed in *dcl1*, *hen1*, and *hyl1* mutants is likely caused by reduced miRNA accumulation and subsequent reduced cleavage efficiency (Kasschau et al. 2003; Vazquez et al. 2004). To examine at which step *ago1* mutants are impaired in the miRNA pathway, we analyzed miRNA ac-

cumulation in rosettes of wild-type plants and *ago1* mutants using the same plant material used for the RT-qPCR experiments. We observed that miRNAs are present in *ago1-27* and *ago1-26* at levels more or less similar to those observed in wild-type plants (Fig. 3A), suggesting that AGO1 acts downstream from DCL1, HEN1, and HYL1 in the miRNA pathway to promote cleavage of target mRNAs. In the *ago1-3*-null allele, only miR156/157 and miR167 accumulated to a level similar to that of wild-type plants (Fig. 3B). For the eight other miRNAs examined, accumulation in *ago1-3* was reduced, in some cases to below the level of detection. We suggest that AGO1 is important for the stabilization of miRNAs, although we cannot rule out a role in miRNA production. It seems likely that *Arabidopsis* AGO1 functions similarly to *Drosophila* AGO2 or human eIF2C (Hammond et al. 2001; Mourelatos et al. 2002), which associate with miRNAs in the RISC, which, in turn, mediates the posttranscriptional regulation of target messages. Other possibilities exist, including the idea that AGO1 might even act before miRNA processing, as sug-



**Figure 3.** miRNA accumulation in *ago1* mutants. miRNA accumulation was determined by RNA gel blot analysis using 30  $\mu$ g (A) or 10  $\mu$ g (B) of the same RNA used for RT-qPCR analyses. Blots were successively hybridized to different probes complementary to miRNAs. (A) miRNA accumulation in the *ago1-26* and *ago1-27* hypomorphic alleles. (B) miRNA accumulation in the *ago1-3*-null allele.

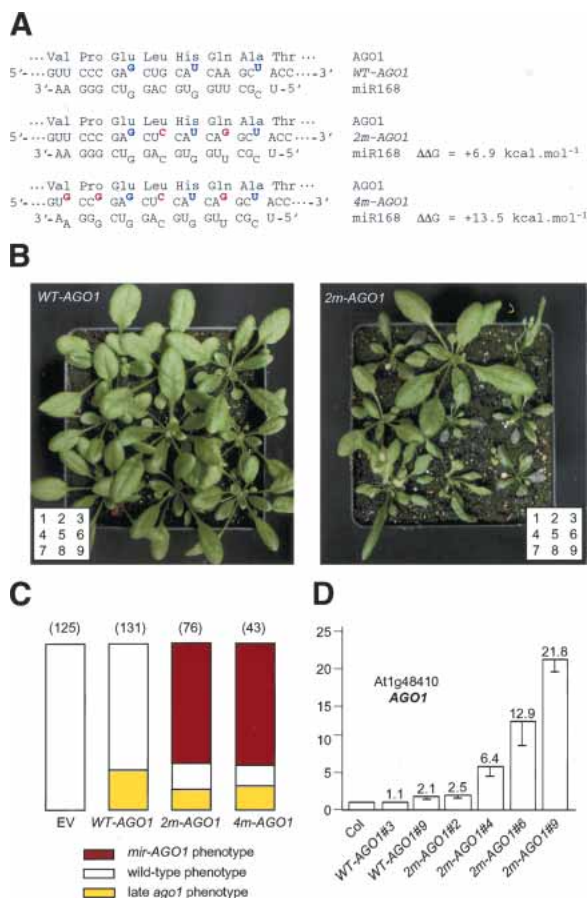
gested by the recent observation that the precursor of miR165 is mislocalized in *ago1* mutants (Kidner and Martienssen 2004).

*Plants impaired in miR168-directed cleavage of AGO1 exhibit developmental defects*

The results presented in Figure 2 show that the steady-state level of uncleaved *AGO1* mRNA is increased in *hen1*, *hyl1*, and *ago1* mutants, strongly supporting the idea that *AGO1* mRNA undergoes a negative feedback regulation by the miRNA pathway through the action of miR168. If the negative feedback regulation of *AGO1* mRNA by the miRNA pathway is essential to maintain a proper regulation of plant development by miRNAs, plants impaired in this feedback regulation but expressing functional AGO1, DCL1, HEN1, and HYL1 proteins should exhibit developmental defects. To test this hypothesis, we introduced silent mutations in the *AGO1* gene to decrease the complementarity between *AGO1* mRNA and miR168 without changing the AGO1 protein sequence, a strategy that has already proven to be successful to specifically reduce miRNA-directed cleavage of MYB, TCP, and REV mRNAs (Emery et al. 2003; Palatnik et al. 2003). In a first step, the wild-type *AGO1* gene was subcloned as an 8-kb fragment carrying the entire transcribed region plus 1.5 kb upstream of the transcription start and 0.5 kb downstream from the polyadenylation signal. Introduction of this construct (*WT-AGO1*) into the *ago1-27* hypomorphic allele or the *ago1-1*-null allele restored a wild-type phenotype in ~80% of the transformants, indicating that the *WT-AGO1* construct contains all the upstream and downstream regulatory elements required for wild-type function. Introduction of *WT-AGO1* into wild-type plants had no effect on plant development in 94 out of 131 transformants analyzed. The remaining 37 transformants looked normal at early stages of development but progressively exhibited the characteristics of *ago1* hypomorphic alleles, including late flowering, serrated leaves, fused flowers, and limited fertility (data not shown), suggesting that the

*WT-AGO1* construct had triggered late cosuppression of the endogenous *AGO1* gene. To test this hypothesis, we introduced the *WT-AGO1* construct into the cosuppression-deficient *sgs2* mutant (Mourrain et al. 2000). None of the 130 transformants analyzed showed an *ago1* hypomorphic phenotype, thus confirming that the phenotype observed in transformed wild-type plants resulted from cosuppression of the endogenous *AGO1* gene.

The wild-type *AGO1* gene encodes an mRNA that naturally contains three mismatches with miR168 (Rhoades et al. 2002). Two mutant constructs (*2m-AGO1* and *4m-AGO1*) were made by introducing two or four silent mutations into the *WT-AGO1* construct, thus adding two or four mismatches with miR168 that increase the predicted free energy of the miRNA/target duplex by 6.9 kcal/mole and 13.5 kcal/mole, respectively (Fig. 4A). When introduced into wild-type plants, the *2m-AGO1* and *4m-AGO1* constructs yielded 13%–18% of transformants with a cosuppressed phenotype similar to the 28% of cosuppressed transformants observed with the *WT-AGO1* construct (Fig. 4C). In addition, the *2m-AGO1* and *4m-AGO1* constructs yielded 63%–68% of transformants exhibiting developmental defects (referred to as the *miR-resistant AGO1* or *mir-AGO1* phenotype) that were not observed in plants carrying the *WT-AGO1* construct (Fig. 4B,C). When introduced into the cosuppression-deficient mutant *sgs2*, the *4m-AGO1* construct yielded 91% of transformants with the *mir-AGO1* phenotype and no transformants with the cosuppression phenotype (data not shown). In young seedlings, the *mir-AGO1* phenotype was mostly characterized by the emergence of curled leaves resembling those of *hen1* and *hyl1* mutants (Fig. 5A). Additional developmental defects were also observed, including abnormal cotyledons. As plants grew, developmental defects became more variable from plant to plant and from one leaf to another (Fig. 5A). Adult transformants exhibited a shorter stature, asymmetric rosette leaf formation, twisted or spoon-shaped leaves resembling those observed in *hyl1* and *dcl1* mutants, respectively, a disorganized phyllotaxy, and an accelerated senescence of leaves exemplified by



**Figure 4.** Silent mutations in the *miR168* complementary site of the *AGO1* mRNA induce developmental defects. (A) The *WT-AGO1* mRNA naturally contains three mismatches with *miR168* (in blue), including a G:U wobble pair. Silent mutations in *2m-AGO1* and *4m-AGO1* constructs introduce two and four additional mismatches (in red), reducing complementarity with *miR168*.  $\Delta\Delta G$  was calculated using mfold. (B) Representative sets of transformants carrying the *WT-AGO1* or *2m-AGO1* construct. (C) Proportion of transformants showing a wild-type phenotype (open bar), an *ago1* phenotype caused by late cosuppression (yellow bar), or an *mir-AGO1* phenotype caused by *AGO1* overexpression (red bar). Plants were transformed with either an empty vector (EV) or the *WT-AGO1*, *2m-AGO1*, or *4m-AGO1* constructs. The number of transformants analyzed is indicated in parentheses. (D) *AGO1* mRNA accumulation determined by real-time quantitative PCR in untransformed plants (Col) or plants transformed with the *WT-AGO1* or *2m-AGO1* constructs. Quantifications were normalized to that of *ACTIN2*. The value in Col was arbitrarily fixed to 1. Numbers (#) correspond to the plants shown in B.

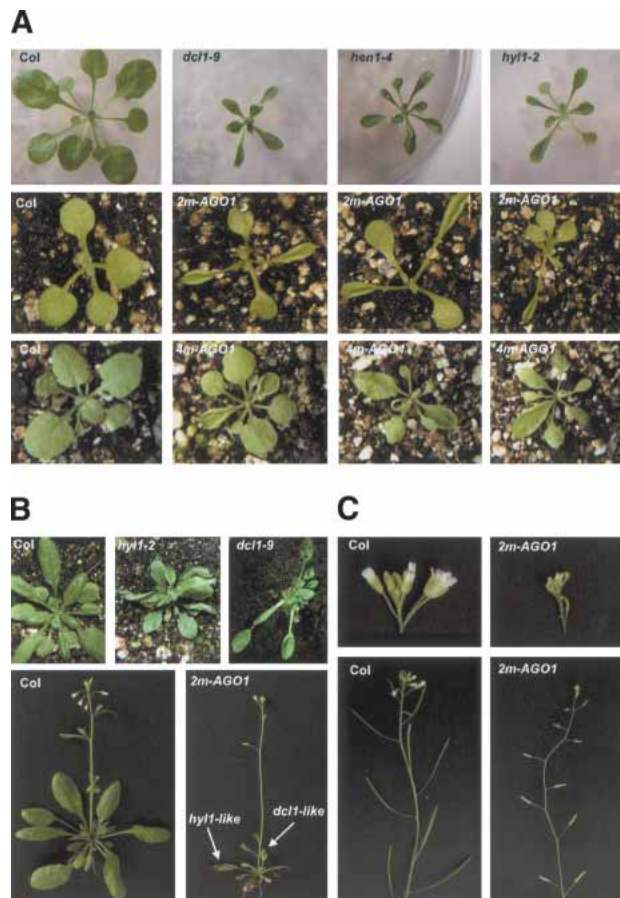
photobleaching spots on a highly anthocyaned background (Figs. 4B, 5B). In the most affected transformants, the shoot apical meristem aborted and the plants died before flowering. In less severely affected transformants, a short stem developed bearing degenerate flowers that were mostly sterile (Fig. 5C). The rest of the transformants produced seeds, the amount of which inversely correlated with the severity of the developmental defects observed in the vegetative phase.

In the progeny of fertile transformants exhibiting a weak phenotype, a 3:1 ratio of abnormal/wild-type plants was observed that is consistent with the expected dominant character of constructs triggering ectopic gene expression. This result also indicates that developmental changes induced by *2m-AGO1* or *4m-AGO1* constructs are reversible by segregation of the constructs at meiosis and do not induce inherited imprints, consistent with the posttranscriptional level of deregulation induced by the constructs. Transformants exhibiting more severe phenotypes produced small siliques containing either no seeds or very few seeds. In the progeny of such transformants, the ratio of abnormal/wild-type plants was less than 3:1 and in the case of the most affected fertile transformant declined to 1:5. This deficit in abnormal plants strongly suggests that expression of the *2m-AGO1* or *4m-AGO1* construct might compromise gamete or embryo viability. This hypothesis is consistent with the lower number of transformants generated with the *2m-AGO1* construct compared with the *WT-AGO1* construct and the even lower number obtained with the *4m-AGO1* construct (Fig. 4C). Because the T-DNA stably integrates into mature female gametes during the floral dipping procedure used to transform *Arabidopsis* (Bechtold et al. 2000, 2003), it is likely that only the transformed embryos that do not express the *2m-AGO1* or *4m-AGO1* construct at high levels can survive and develop into seeds.

*Plants expressing a miR168-resistant AGO1 mRNA exhibit elevated steady-state levels of miRNA targets and developmental defects similar to those of dcl1, hen1, and hyl1*

The accumulation of uncleaved *AGO1* mRNA was determined by RT-qPCR in rosettes of representative transformants (Fig. 4D). No difference or a slight increase of 2.1-fold was observed between wild-type plants and transformants carrying the *WT-AGO1* construct (*WT-AGO1* #3 and #9). A limited increase (2.5-fold and 6.4-fold) was observed in *2m-AGO1* transformants that did not exhibit a strong phenotype (*2m-AGO1* #2 and #4). A stronger increase (12.9-fold and 21.7-fold) was observed in *2m-AGO1* transformants exhibiting a strong phenotype (*2m-AGO1* #6 and #9). These results are consistent with RT-qPCR analyses showing that *AGO1* mRNA strongly overaccumulates in the *ago1-3* mutant, in which miRNA-mediated cleavage is generally impaired (Fig. 2), and confirm that *miR168* regulates the *AGO1* mRNA level through cleavage. They also indicate that plants can tolerate a limited increase in the amount of *AGO1* mRNA accumulation without dramatically affecting development, whereas a strong increase in the amount of *AGO1* mRNA triggers dramatic developmental defects.

Because *2m-AGO1* and *4m-AGO1* transformants exhibited spoon-shaped, curled or twisted leaves resembling those of *dcl1*, *hen1*, and *hyl1* mutants (Fig. 5A,B), we analyzed the steady-state level of mRNAs targeted for cleavage by miRNAs in rosettes of two *2m-AGO1*



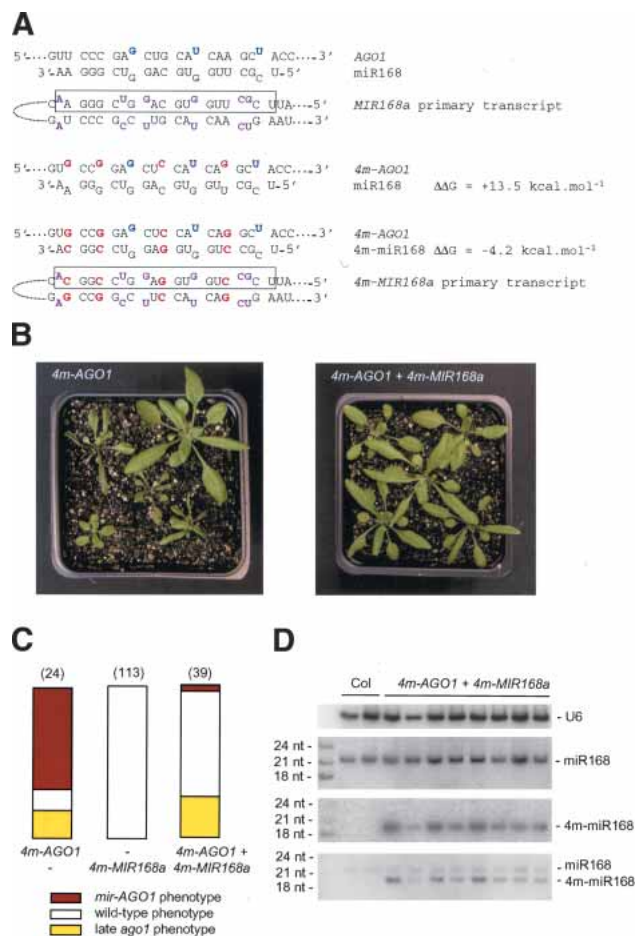
**Figure 5.** Developmental defects in *2m-AGO1* transformants. (A, top row) Wild-type plant (Col) and *dcl1*, *hen1*, and *hyl1* mutants. (Middle row) Wild-type plant (Col) and *2m-AGO1* transformants exhibiting curled leaves resembling those of *hen1* and *hyl1* mutants, at 10 d. Transformants with aberrant cotyledons were occasionally observed (right). (Bottom row) Wild-type plant (Col) and *4m-AGO1* transformants exhibiting a variety of developmental defects, including asymmetric rosette leaf formation and curled or twisted leaves, at 20 d. (B, top row) Wild-type plant (Col) and *dcl1* and *hyl1* mutants. (Bottom row) Adult wild-type plant (Col) and a representative *2m-AGO1* transformant exhibiting spoon-shaped or twisted anthocyaned leaves resembling those of *dcl1* and *hyl1* mutants. (C, top row) Inflorescence of a wild-type plant (Col) and of a representative *2m-AGO1* transformant. (Bottom row) Stems and siliques (seed pots) of the same plants. The wild-type Col plant is fertile, whereas the *2m-AGO1* transformant is sterile with aborted siliques.

transformants exhibiting a strong phenotype (*2m-AGO1* #6 and #9). For every miRNA target examined, the level in *2m-AGO1* transformants was slightly higher than that in wild-type plants (Fig. 2), suggesting that an increase in the amount of AGO1 perturbs the miRNA pathway. Different hypotheses can be proposed to explain this phenomenon. It is possible that the excess of AGO1 protein interferes with the formation or the functioning of RISC by displacing other AGO proteins. Alternatively, the excess of free AGO1 protein could independently titrate miRNAs and other RISC components

into separate incomplete complexes. Such a sequestration of miRNAs and/or other RISC components by free AGO1 protein could therefore mimic the effect of *dcl1*, *hen1*, or *hyl1* mutations that reduce the accumulation of miRNAs, thus leading to some of the same developmental defects.

*Phenotypic rescue of 4m-AGO1 plants with a compensatory 4m-MIR168 transgene*

To prove that the developmental abnormalities observed in *4m-AGO1* transformants result from the absence of regulation of *4m-AGO1* mRNA by miR168, we mutagenized the *MIR168a* gene to introduce compensatory mutations that would allow the production of an miRNA that can pair with the mutant *AGO1* mRNA transcribed from the *4m-AGO1* construct. miR168 is processed from an imperfect hairpin in which 15 of 21 miRNA residues are predicted to take part in Watson-Crick pairs involving residues on the other arm of the hairpin (Fig. 6A). When we altered the *AGO1* gene to introduce mutations that decrease the complementarity with miR168, we chose nucleotides that kept the amino acid sequence unchanged and also corresponded to paired nucleotides in the miRNA precursor. Therefore, compensatory mutations could be introduced on both sides of the stem of the dsRNA precursor encoded by the *MIR168a* gene to generate a new miRNA precursor with the same predicted folding geometry as the original precursor (Fig. 6A). We started with *MIR168a* gene because of the existence of a corresponding EST starting 20 bp upstream of the putative fold-back stem-loop and ending 180 bp downstream, thus indicating that this gene is transcribed. The wild-type *MIR168a* gene was subcloned as a 2.4 kb-fragment with 1.4 kb upstream of and 1.0 kb downstream from the *miR168* sequence so as to include regulatory sequences. The *miR168* and *miR168\** sequences were successively mutagenized to generate the *4m-MIR168a* construct (Fig. 6A). The predicted free energy of the 4m-miR168/4m-AGO1 duplex was slightly more favorable than that of the wild-type miR168/wt-AGO1 duplex (by 4.2 kcal/mole), suggesting that the newly generated miRNA would cleave the mutant *4m-AGO1* mRNA at least as well as the wild-type miRNA cleaves the wild-type *AGO1* mRNA (Fig. 6A). Wild-type plants were transformed with either *4m-AGO1* or *4m-MIR168a* or cotransformed with both constructs (each carried on a different vector with unique selectable markers). The number of transformants carrying the two constructs was low because the frequency of double transformation by independent bacteria is low. Nevertheless, it was higher than that observed with the *4m-AGO1* construct alone, suggesting that the deleterious effect of the *4m-AGO1* construct is abolished in the presence of the *4m-MIR168a* construct. Indeed, we observed that only one out of 39 transformants carrying both *4m-AGO1* and *4m-MIR168a* constructs displayed the *mir-AGO1* phenotype, whereas 17 out of 24 transformants carrying the *4m-AGO1* construct alone displayed this phenotype (Fig. 6B,C). To confirm the effi-



**Figure 6.** Compensatory mutations in the *MIR168a* gene rescue developmental defects induced by silent mutations in the miR168 complementary site of the *AGO1* mRNA. (A) The *MIR168a* gene encodes a primary transcript that is partially paired (unpaired nucleotides are purple). The miRNA is boxed. Compensatory mutations in the *4m-MIR168a* transgene (red) conserved the structure of the primary transcript and restored pairing with the *4m-AGO1* mRNA. Original mismatches (blue) were kept.  $\Delta\Delta G$  was calculated using mfold. (B) Representative sets of transformants carrying the *4m-AGO1* construct alone or the *4m-AGO1* and *4m-MIR168a* constructs together. (C) Proportion of transformants showing a wild-type phenotype (open bar), an *ago1* phenotype caused by late cosuppression (yellow bar), or a *mir-AGO1* phenotype caused by *AGO1* overexpression (red bar). Plants were transformed with the *4m-AGO1* or *4m-MIR168a* constructs or both. The number of transformants analyzed is indicated in parentheses. (D) Accumulation of the compensatory miRNA (m4-miR168) in double transformants carrying the *4m-AGO1* and *4m-MIR168a* constructs. RNA gel blot analysis was performed using 20  $\mu$ g of total RNA extracted from two nontransformed plants (Col) and eight independent double transformants. The blot was hybridized with a probe complementary to 4m-miR168, stripped, rehybridized with a probe complementary to miR168, stripped, and finally rehybridized with the two probes simultaneously.

ciency of the rescue, we transformed a larger number of wild-type plants with the two constructs. Only two out of 77 additional double transformants displayed the *mir-*

*AGO1* phenotype (data not shown). No phenotype was observed in plants transformed with the *4m-MIR168* construct alone (Fig. 6C). Double transformants that carry both *4m-AGO1* and *4m-MIR168a* constructs and exhibit a wild-type phenotype accumulated 4m-miR168, whereas no signal was visible in untransformed plants (Fig. 6D), indicating that the probe does not cross-hybridize with the endogenous miR168 and that the signal corresponds to bona fide 4m-miR168. Rehybridization of the blot with a probe complementary to miR168 revealed that the level of accumulation of miR168 was not affected by the expression of 4m-miR168. Interestingly, 4m-miR168 migrated faster than miR168, probably by 1 nt. These results show that the *4m-MIR168a* construct is functional and expresses sufficient 4m-miR168 to rescue the phenotype conferred by the *4m-AGO1* construct. However, the observation that 4m-miR168 is shorter than miR168 by 1 nt indicates that subtle changes in the duplex induced by changes in the primary sequence of the miRNA transcript might affect the boundaries of the cleavage.

## Discussion

In this paper, we show that *Arabidopsis ago1* mutants that exhibit a range of developmental defects (Fig. 1) also exhibit increased accumulation of mRNAs targeted by miRNAs (Fig. 2). miRNA accumulation is not affected in *ago1* hypomorphic alleles that retain a PAZ domain but is reduced in *ago1*-null alleles (Fig. 3), supporting a role for *AGO1* in the miRNA pathway. *AGO1* could participate in the distribution of miRNAs or miRNA precursor transcripts as recently suggested by Kidner and Martienssen (2004) or could function in RISC, similar to eIF2C2 in human, *AGO2* in *Drosophila*, or QDE-2 in *Neurospora* (Hammond et al. 2001; Catalanotto et al. 2002; Hutvagner and Zamore 2002; Martinez et al. 2002; Mourelatos et al. 2002). However, it is unlikely that *AGO1* is the only *AGO* protein that associates with miRNAs in RISC. Indeed, because *dcl1*-null alleles lacking the enzyme that processes miRNA precursors are not viable, it is expected that plants lacking a functional RISC are not viable either. However, *ago1*-null alleles are viable, although they exhibit dramatic developmental defects (Fig. 1). This viability is likely caused by the function of other *AGO* protein(s), in particular *PINHEAD/ZWILLE*, which is 75% similar to *AGO1* and has a pattern of expression overlapping with that of *AGO1*. Indeed, Lynn et al. (1999) reported that plants homozygous for a *pnh*-null mutation and heterozygous for an *ago1*-null mutation exhibit developmental defects stronger than those of single homozygous *pnh* mutants, whereas plants homozygous for an *ago1*-null mutation and heterozygous for a *pnh*-null mutation exhibit developmental defects stronger than those of single homozygous *ago1* mutants, clearly indicating the redundant functions. In addition, plants homozygous for both *ago1*-null and *pnh*-null mutations are embryo-lethal, pointing out the crucial roles of these two proteins in plant development (Lynn et al. 1999). Interestingly, *ago1* hypomorphic mutants strongly resemble transgenic plants expressing the viral suppressor of PTGS HC-Pro (Kass-



chau et al. 2003). The molecular phenotypes are also similar. Both *ago1* mutants and HC-Pro-expressing plants have accumulation of miRNA targets and in both miRNAs are still present and accumulate to similar or higher levels compared with wild-type plants (Fig. 3A; Kasschau et al. 2003). The overlapping physical and molecular phenotypes of *ago1* mutants and HC-Pro transgenic plants suggest that HC-Pro could alter the miRNA pathway by interfering with the action of AGO1.

We find that decreasing the complementarity of *AGO1* mRNA with miR168 increases the level of *AGO1* mRNA (Fig. 4) and has dramatic consequences on plant development and reproduction (Fig. 5). This observation, together with our finding that AGO1 is needed for proper miRNA function, supports the previous speculation that *AGO1* mRNA is subjected to negative feedback regulation through the action of miR168. In this scenario, if the amount of AGO1 activity in a wild-type cell decreases below a critical level, the efficiency of miR168-mediated cleavage of *AGO1* mRNA would also decrease, allowing more *AGO1* mRNA to be translated into AGO1 protein and restoring activity to the initial level. Of course, the miR162 regulation of *DCL1* mRNA (Xie et al. 2003) could also come into play, evoking more complex scenarios. For example, lowered AGO1 activity would decrease the amount of miR162-directed *DCL1* mRNA cleavage, which could increase the amount of miRNAs produced, potentially increasing the amount of *AGO1* mRNA cleavage and further lowering AGO1 activity. Thus, the outcome of a change in AGO1 activity could depend on many factors, including which of the components, *DCL1*, AGO1, miR162, and miR168, are limiting or in excess at the time of the perturbation. Another consideration is that a large increase in *AGO1* mRNA, and presumably AGO1 protein, results in an apparent decrease in RISC activity (Fig. 2, *2m-AGO1* plants). Whether such a large increase is attainable in a wild-type plant, or whether marginal increases in AGO1 are instead held in check by miR168 feedback repression, is not known. Clearly, in transgenic plants expressing an miR168-resistant *AGO1* mRNA, the system cannot return to the equilibrium because the mutant *AGO1* mRNA is insensitive to feedback regulation by miR168.

The expression of a mutant miRNA that is able to pair with the mutant *AGO1* mRNA can restore the feedback regulation and rescue the developmental defects induced by the mutant *AGO1* mRNA (Fig. 6). This phenotypic rescue proves the regulatory relationship between miR168 and its target. It also demonstrates the feasibility of engineering artificial miRNAs in plants. These have potential as tools for targeted silencing of a single gene or a gene family, as well as utility for exploring facets of miRNA maturation and function.

## Materials and methods

### Plant material and growth conditions

The *ago1-1*, *ago1-3*, *ago1-25*, *ago1-26*, *ago1-27*, *dcl1-9*, *hen1-4*, and *hyl1-2* mutants have been described previously (Bohmert et al. 1998; Morel et al. 2002; Boutet et al. 2003; Vazquez et al.

2004). Plants were grown under cool-white light in long days (16 h of light, 8 h of dark) at 23°C or short days (8 h of light, 16 h of dark) at 17°C.

### RNA analysis

Total RNA was extracted as described previously (Vazquez et al. 2004), separated by denaturing 15% polyacrylamide gel electrophoresis, and blotted to a nylon membrane (Genescreen Plus; PerkinElmer Inc.) as described (Lau et al. 2001). MicroRNA probes were prepared by end-labeling antisense oligonucleotides using T4 polynucleotide kinase (New England Biolabs). Blots were rehybridized with a probe complementary to U6.

### Real-time RT-PCR

RNA was extracted from mutant and wild-type siblings segregating from heterozygote parents grown in short days for 4 mo. Poly(dT) cDNAs were made by using the Invitrogen cDNA first-strand synthesis system. Quantifications were performed on a Bio-Rad 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 for 10 min to 95°C to activate hot start Taq DNA polymerase, followed by 50 cycles of denaturation for 30 sec at 95°C and annealing-extension for 45 sec at 60°C. Target quantifications were performed with specific primer pairs designed for each side of the cleavage site by using Beacon Designer from Biosoft. Primers used for At1g27370/*SPL10*, At3g11440/*MYB65*, At1g77850/*ARF17*, At1g06580/*PPR*, At5g53950/*CUC2*, At5g37020/*ARF8*, At1g48410/*AGO1*, At3g60630/*SCL6-III*, and At3g18780/*ACTIN2* have been described (Vazquez et al. 2004). The primers used to quantify additional mRNAs are At1g01040/*DCL1*, 5'-GATCCATTCCTAAGCGAAGTTTCAGAG-3' and 5'-GCCC GAGCAACATAAAGATCCATAG-3'; At1g30490/*PHV*, 5'-AG ACCTTGGCGGAGTTCCTTG-3' and 5'-GTTGCGTGAAA CAGCTACGATACC-3'; At1g42970/*GAPDH*, 5'-TCTTTCCC TGCTCAATGCTCCTC-3' and 5'-TTTCGCCACTGTCTCTC CTCTAAC-3'; At5g60390/*eEF-1(A4)*, 5'-CTGGAGTTTTGAG GCTGGTAT-3' and 5'-CCAAGGGTGAAAGCAAGAAGA-3'.

For each cDNA synthesis, quantifications were made in triplicate. For each quantification, conditions were, as recommended,  $1 \geq E \geq 0.85$  and  $r^2 \geq 0.985$ , where E is the PCR efficiency and  $r^2$  corresponds to the correlation coefficient obtained with the standard curve. For each quantification, a melt curve was realized at the end of the amplification experiment by steps of 0.5°C from 55°C to 95°C, to ensure that quantification was not caused by primer self-amplification but by a pure and common PCR product. Results were normalized to that of *ACTIN2*, then to the value of the isogenic wild-type sibling. For each mutant analyzed, results were considered as acceptable if the variation between the wild-type sibling and a true wild-type plant was <15%.

### Molecular cloning and plant transformation

A KpnI-SalI fragment carrying the 3'-half of the *AGO1* gene was subcloned from BAC F11A17 (position 12600–18030) into the binary vector pBin+. The 5'-half of the *AGO1* gene was amplified by PCR from BAC F11A17 using the following pair of primers: 5'-CTCGACTCTCGAGGTAGTATTAATTAACGAGTTCTAAGTTCTTCTCCGTTATGAG-3' and 5'-GGTTCTGGT ACCTGGGTAGGACTCACCTCAGACAGTGTAGGCTGAG AAGACACCGC-3', cut with XhoI and KpnI (at positions 10,050 and 12,600 on BAC F11A17) and cloned into the Bluescript vector pKS+. The 5'-primer introduced a PacI site downstream from the XhoI site so that the 5'-half of the *AGO1* gene can be mobilized as a PacI-KpnI fragment and cloned into the pBin+ vector containing the 3'-half of the gene to reconstitute a

complete *AGO1* gene (*WT-AGO1*). Silent mutations were introduced into the miR168 complementary site using the Quick Change Site-Directed Mutagenesis Kit (Stratagene) and the following pair of primers: 5'-CCACCGCAGAGACAATCAGTCCGGAGCTCCATCAGGCTACCTCACCTACTTATCAAGCG-3' and 5'-CGCTTGATAAGTAGGTGAGGTAGCCTGATGGAGCTCCGGCACTGATTGTCTCTGCGGTGG-3'. The *4m-AGO1* construct resulted from the perfect replacement of the wild-type sequence by the primer sequence, whereas the *2m-AGO1* construct resulted from partial replacement of the wild-type sequence by the primer sequence. The wild-type and mutagenized PacI-KpnI fragments were entirely sequenced to ensure that no other mutations have been introduced and transferred from pKS+ into the pBin+ vector containing the 3'-half of the gene to reconstitute a complete *AGO1* gene.

The *MIR168a* gene was subcloned from BAC T5K18 into the Bluescript vector pKS+ as a PstI-ClaI fragment (position 55287–57718 on T5K18). Compensatory mutations that restore complementarity to the *4m-AGO1* mRNA were introduced into the *MIR168a* gene using the Quick Change Site-Directed Mutagenesis Kit (Stratagene). The *miR168* sequence was first mutagenized using the following pair of primers: 5'-CACCA TCGGGCTCGGATTTCGCCTGGTGGAGGTCCGGCACCAA TTCGGCTGACACAGCC-3' and 5'-GGCTGTGTCAGCCGA ATTGGTGCCGGACCTCCACCAGGCGAATCCGAGCCC GATGGTG-3'. The *miR168\** sequence was subsequently mutagenized using the following pair of primers: 5'-TTGGTTT GTGAGCAGGGATTGGAGCCGGCCTTCCATCAGCTGAA TCGGATCCTCGAGGTGTA-3' and 5'-TACACCTCGAGGA TCCGATTGAGCTGATGGAAGGCCGGCTCCAATCCCTG CTCACAAACCAA-3'. The mutagenized PstI-ClaI fragment was sequenced to ensure that no other mutations have been introduced and then was transferred from pKS+ into the pCambia1200 binary vector.

The *WT-AGO1*, *2m-AGO1*, and *4m-AGO1* constructs (in pBin+) and the *4m-MIR168a* construct (in pCambia1200) were transferred from *Escherichia coli* to *Agrobacterium tumefaciens* by triparental mating. *Arabidopsis* plants were transformed by the flower-dipping method (Bechtold and Pelletier 1998; Clough and Bent 1998). Transformants were selected by sowing seeds onto a medium supplemented with kanamycin (pBin+) or hygromycin (pCambia1200).

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