

Fertile Hypomorphic *ARGONAUTE (ago1)* Mutants Impaired in Post-Transcriptional Gene Silencing and Virus Resistance

Jean-Benoit Morel,¹ Christian Godon,¹ Philippe Mourrain, Christophe Béclin, Stéphanie Boutet, Frank Feuerbach, Florence Proux, and Hervé Vaucheret²

Laboratoire de Biologie Cellulaire, Institut National de la Recherche Agronomique, 78026 Versailles Cedex, France

Transgene-induced post-transcriptional gene silencing (PTGS) results from specific degradation of RNAs that are homologous with the transgene transcribed sequence. This phenomenon, also known as cosuppression in plants and quelling in fungi, resembles RNA interference (RNAi) in animals. Indeed, cosuppression/quelling/RNAi require related PAZ/PIWI proteins (AGO1/QDE-2/RDE-1), indicating that these mechanisms are related. Unlike *Neurospora crassa qde-2* and *Caenorhabditis elegans rde-1* mutants, which are morphologically normal, the 24 known *Arabidopsis ago1* mutants display severe developmental abnormalities and are sterile. Here, we report the isolation of hypomorphic *ago1* mutants, including fertile ones. We show that these hypomorphic *ago1* mutants are defective for PTGS, like null *sgs2*, *sgs3*, and *ago1* mutants, suggesting that PTGS is more sensitive than development to perturbations in AGO1. Conversely, a mutation in *ZWILLE/PINHEAD*, another member of the *Arabidopsis AGO1* gene family, affects development but not PTGS. Similarly, mutations in *ALG-1* and *ALG-2*, two members of the *C. elegans RDE-1* gene family, affect development but not RNAi, indicating that the control of PTGS/RNAi and development by PAZ/PIWI proteins can be uncoupled. Finally, we show that hypomorphic *ago1* mutants are hypersensitive to virus infection, confirming the hypothesis that in plants PTGS is a mechanism of defense against viruses.

INTRODUCTION

In plants, transgenes can be silenced through transcriptional gene silencing (transcriptional gene silencing) or through the specific degradation of transgene RNA (post-transcriptional gene silencing [PTGS]) (review in Kooter et al., 1999; Wolffe and Matzke, 1999; Fagard and Vaucheret, 2000; Matzke et al., 2001; Vaucheret and Fagard, 2001). PTGS transgenes can trigger the degradation of homologous endogenous RNAs, a phenomenon referred to as cosuppression in plants (Napoli et al., 1990; Smith et al., 1990; van der Krol et al., 1990) and quelling in the fungus *Neurospora crassa* (Romano and Macino, 1992; Cogoni et al., 1996). Cosuppression and quelling strongly resemble RNA interference (RNAi), a PTGS phenomenon mediated by the introduction of double-stranded RNA (dsRNA) in animals (Fire et al., 1998; Fire, 1999). Indeed, genetic dissection of cosuppression in *Arabidopsis*, quelling in *N. crassa*, and RNAi in *Caenorhabditis elegans* led to the isolation of two sets of similar proteins: (1) *SGS2*, *QDE-1*, and *EGO-1* (Cogoni and Macino, 1999a; Mourrain et al., 2000; Smardon et al., 2000), which are simi-

lar to the tomato RNA-dependent RNA polymerase (RdRP); and (2) *AGO1*, *QDE-2*, and *RDE-1* (Tabara et al., 1999; Catalanotto et al., 2000; Fagard et al., 2000), which contain PAZ and PIWI domains (Cerutti et al., 2000). Given these findings, it was suggested that PTGS, quelling, and RNAi could be related phenomena deriving from an ancestral mechanism directed against invading nucleic acids (Catalanotto et al., 2000; Cogoni and Macino, 2000; Fagard et al., 2000). Such a role is supported by the finding that in *Arabidopsis*, PTGS-deficient *sgs2*, *sgs3*, and *sde3* mutants are hypersusceptible to viral infection (Mourrain et al., 2000; Dalmay et al., 2001).

Models for PTGS/quelling/RNAi predict that dsRNA molecules are intermediates leading to specific RNA degradation (Bass, 2000). These dsRNAs can be supplied exogenously (RNAi) or produced by sense transgenes (cosuppression, quelling) when they are arranged as an inverted repeat (Waterhouse et al., 1998; Chuang and Meyerowitz, 2000; Smith et al., 2000) or when they produce aberrant RNA that serves as a matrix for the RdRP encoded by the *SGS2/SDE1* and *QDE-1* genes (Cogoni and Macino, 1999a; Dalmay et al., 2000; Mourrain et al., 2000). During RNAi in *Drosophila melanogaster*, the dsRNAs are degraded by an RNase III-like dsRNA endonuclease (DICER) to generate short sense, antisense, and dsRNA pieces of 21 or 22 nucleotides (Bernstein et al., 2001), which also are found in PTGS plants (Hamilton

¹ These two authors contributed equally to this work

² To whom correspondence should be addressed. E-mail herve.vaucheret@versailles.inra.fr; fax 33-1-30-83-30-99.

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and Baulcombe, 1999). These short RNA pieces and mRNA are thought to participate in an RNA degradation complex called RISC in *D. melanogaster* (Bernstein et al., 2001), leading to complete RNA degradation. Interestingly, a protein similar to tomato RdRP also is required for RNAi in *C. elegans* (Smardon et al., 2000), indicating that, in worms as in plants and fungi, RdRP could participate not only in the synthesis of complementary RNA (and consequently in the formation of dsRNA) but also in the amplification/regeneration of a systemic signal, allowing silencing to spread throughout the organism (Palauqui et al., 1997; Fire et al., 1998; Voinnet et al., 1998).

In addition to RdRPs (Cogoni and Macino, 1999a; Mourrain et al., 2000; Smardon et al., 2000), RNases (Ketting et al., 1999; Bernstein et al., 2001), and RNA helicases (Domeier et al., 2000; Wu-Scharf et al., 2000; Dalmay et al., 2001), RNAi/quelling/cosuppression also require a set of related proteins (RDE-1 [Tabara et al., 1999], QDE-2 [Catalanotto et al., 2000], and AGO-1 [Fagard et al., 2000]) collectively designated PAZ/PIWI proteins (Cerutti et al., 2000). The role of PAZ/PIWI proteins in RNAi/quelling/cosuppression is unclear. RDE-1 was shown to be required for the formation of the interfering agent but not for interference thereafter (Grishok et al., 2000). Conversely, an additional member of this PAZ/PIWI family, DmAGO2, was identified as part of the RISC complex in *D. melanogaster* (Hammond et al., 2001). The role of PAZ/PIWI proteins in the control of development also is unclear. In *C. elegans*, the role of three related PAZ/PIWI proteins, RDE-1, ALG-1, and ALG-2, has been investigated. *rde-1* mutants are defective for RNAi but are morphologically normal (Tabara et al., 1999), whereas *alg-1* and *alg-2* mutants are impaired in the regulation of small temporal RNAs that control developmental timing but are not defective in RNAi (Grishok et al., 2001). In *N. crassa*, *qde-2* mutants are defective for quelling but are morphologically normal (Catalanotto et al., 2000). In Arabidopsis, the 24 known *ago1* mutants show severe developmental abnormalities, including complete sterility (Bohmert et al., 1998; Camus, 1999; Fagard et al., 2000). Because all other Arabidopsis PTGS-deficient mutants (*sgs1* to *sgs3* and *sde1* to *sde4*) (Elmayan et al., 1998; Dalmay et al., 2000; Mourrain et al., 2000) show no significant defect in development, the possible link between PTGS and development in plants is unclear. Whether endogenous genes that control development are regulated by part of the PTGS machinery (including AGO1 and additional steps) or AGO1 is a protein that participates independently in PTGS and development pathways is unknown.

Here, we describe new *ago1* mutants that are impaired in PTGS even though they are fertile, suggesting differential sensitivity of PTGS and development to AGO1 defects. We also show that *zll/pnh* mutants are affected in development but not in PTGS, indicating that not all members of the AGO family participate in PTGS, as is the case for the RDE-1 family in *C. elegans* (Grishok et al., 2001). In addition, we show that, like the *sgs2*, *sgs3*, and *sde3* mutants (Mourrain et al.,

2000; Dalmay et al., 2001), *ago1* mutants are hypersensitive to virus infection, indicating that all of the components of PTGS identified to date in plants are involved in virus resistance.

RESULTS

Identification of New PTGS-Deficient Mutants Showing Original Developmental Alterations

We reported previously the isolation of four classes of PTGS-deficient mutants after mutagenesis of the silenced 35S-*uidA* line L1 with ethyl methanesulfonate. Mutants called *sgs1*, *sgs2*, and *sgs3* (for suppressor of gene silencing) showed no significant changes in development (Elmayan et al., 1998; Mourrain et al., 2000), whereas mutants called *ago1* showed severe alterations of development (unexpanded cotyledons, small rosettes with narrow leaves, unique short stems with no leaves, and sterile flowers) (Bohmert et al., 1998; Fagard et al., 2000) (Figure 1). After screening for PTGS-deficient mutants, we identified three mutants (called 19-3, 33-2, and 69-4) exhibiting β -glucuronidase (GUS) activity at levels similar to that of other *sgs* mutants and showing developmental defects differing from those of the 24 known *ago1* mutants (Figure 1). Mutant 33-2 developed a small rosette with dark green and serrated leaves, initiated flowering 10 to 15 days after the wild type, and was almost totally sterile, although occasionally it produced short siliques containing two to five seed (a maximum of 50 seed could be obtained per plant). Mutants 19-3 and 69-4 developed rosettes with dark green and serrated leaves. Rosette size was intermediate between that of mutant 33-2 and wild-type plants. These mutants initiated flowering 7 to 12 days after the wild type and were fertile: they produced 45 to 60% of the amount of seed of the wild type (depending on growth conditions).

Mutants 19-3, 33-2, and 69-4 Are Alleles

Backcrosses of [GUS+] (nonsilenced) mutants 19-3, 33-2, and 69-4 to [GUS-] (silenced) line L1 yielded [GUS-] F1 plants with a wild-type phenotype. Selfing these F1 hybrids yielded 75% (70 of 96, 75 of 96, and 74 of 96, respectively) [GUS-] plants with a wild-type phenotype and 25% [GUS+] plants with the original mutant phenotype, suggesting that in each mutant a single recessive mutation was responsible for the developmental defects and for the deficiency in PTGS. Reciprocal crosses (see Methods) between these three mutants yielded [GUS+] plants with a mutant phenotype. Furthermore, no wild-type plant was found in the F2 progeny among 500 plants analyzed for each reciprocal cross, indicating that these mutants carry allelic mutations. Interestingly, F1 hybrids between 69-4 or 19-3 (which are



Figure 1. Vegetative and Reproductive Developmental Defects in PTGS-Deficient Mutants.

Newly isolated PTGS mutants (69-4/*ago1-27*, 19-3/*ago1-25*, and 33-2/*ago1-26*) compared with the L1 (wild type [wt]) line, from which the mutants were isolated after ethyl methanesulfonate mutagenesis, and with a representative of the original *ago1* alleles (60-1/*ago1-24*). Photographs show plants after 12, 20, and 35 days of growth in vitro in short days and 15, 30, 34, and 40 days after their transfer to a greenhouse for further growth in long days. The newly isolated mutants had dark and highly serrated leaves. The mutants 69-4/*ago1-27* and 19-3/*ago1-25* were fertile, in contrast to mutants 33-2/*ago1-26* and 60-1/*ago1-24*. The previously identified 60-1/*ago1-24* mutant showed similar but stronger developmental defects and eventually died in soil (fifth and sixth photographs from the top in the right column). Only in vitro did this mutant develop a stem bearing sterile flowers (bottom photograph in the right column).

both fertile and larger than 33-2) and 33-2 (which is sterile) were fertile and had the phenotype of 69-4 or 19-3, indicating that the weaker alleles (69-4 and 19-3) act dominantly over the stronger allele (33-2).

Mutants 19-3, 33-2, and 69-4 are Hypomorphic ago1 Mutants

To map the corresponding locus, we analyzed 800 recombinant chromosomes derived from a cross between mutant 69-4 (in ecotype Columbia) and the polymorphic ecotype Landsberg *erecta*. The mutation was mapped on chromosome 1 between markers GAPB and nga280 (Figure 2A). Subsequent analysis indicated that the mutation was lo-

cated between markers mi291 and F1114t7. Because this 300-kb region also carried the *AGO1* gene, we used a cleaved-amplified polymorphic sequence (CAPS) marker (CER462285) located 6 kb upstream of the *AGO1* gene and found that there was no recombination point between this marker and the mutation in the 800 recombinant chromosomes, strongly suggesting that the mutation was located in the *AGO1* gene. Crosses between parents heterozygous for the *ago1-24* mutation (the homozygous *ago1-24* mutant is sterile; Figure 1) (Fagard et al., 2000) and fertile mutants 19-3 and 69-4 yielded 50% (28 of 52 and 35 of 74, respectively) [GUS⁻] plants with a wild-type phenotype and 50% [GUS⁺] plants with the 19-3 or 69-4 (fertile) phenotype, indicating that mutants 19-3 and 69-4 are *ago1* alleles. Because *ago1-24* mutants are sterile and show stronger developmental de-

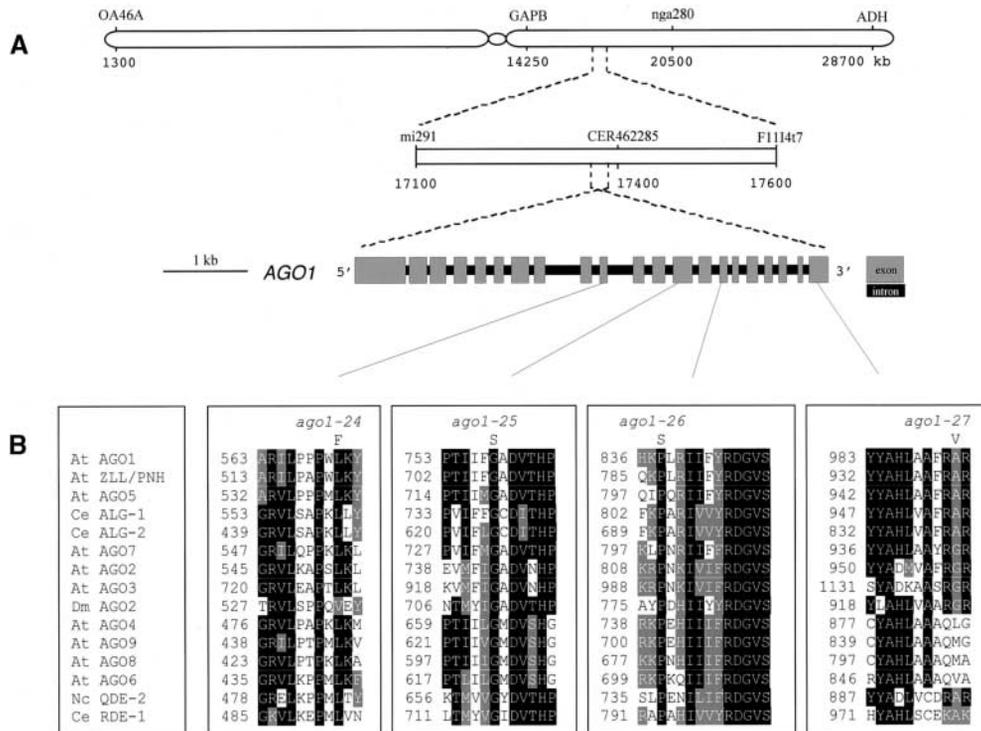


Figure 2. Mapping and Sequences of the *ago1-25*, *ago1-26*, and *ago1-27* Mutations.

(A) Physical map of the chromosomal area carrying the 69-4 (*ago1-27*) mutation. The *ago1-27* mutation was mapped between the CAPS marker GAPB and the microsatellite nga280 on chromosome 1 and subsequently between the restriction fragment length polymorphism marker mi291 and the CAPS marker F1114t7 (<http://www.Arabidopsis.org/>). No recombination point was found between the CAPS marker CER462285 (<http://www.Arabidopsis.org/cereon/>) and the *ago1-27* mutation in the 800 recombinant chromosomes tested.

(B) Sequencing of the 69-4, 19-3, and 33-2 mutant lines indicated that they all carry mutations in the C-terminal part of the AGO1 protein. The AGO1 protein (At1g48410) is aligned with the nine other members of the Arabidopsis (At) PAZ/PIWI family: AGO2 (At1g31280), AGO3 (At1g31290), AGO4 (At2g27040), AGO5 (At2g27880), AGO6 (At2g32940), AGO7 (At1g69440), AGO8 (At5g21030), AGO9 (At5g21150), and ZLL/PNH (At5g43810); with three members of the *C. elegans* (Ce) PAZ/PIWI family: RDE1, ALG-1, and ALG-2; and with *N. crassa* (Nc) QDE-2 and *D. melanogaster* (Dm) AGO2 proteins. Sequence identities are indicated by closed boxes, and conservative changes are shaded. Amino acid positions are indicated at left. Dots indicate gaps introduced by the MultiAlign algorithm to maximize alignment (<http://prodes.toulouse.inra.fr/multalin/multalin.html>). The alignment was processed by BOXSHADE version 3.21 (http://www.ch.embnet.org/software/BOX_form.html).

fects than 19-3 and 69-4, this further confirmed that the weaker allele acted dominantly. Similarly, crosses between parents heterozygous for the *ago1-24* mutation and parents heterozygous for the 33-2 mutation (the homozygous 33-2 mutant is sterile) yielded 75% (27 of 37) [GUS⁻] plants with a wild-type phenotype and 25% [GUS⁺] plants with the 33-2 (sterile) phenotype, indicating that mutant 33-2 also is an *ago1* allele. Selfing the fertile 19-3 × *ago1-24* and 69-4 × *ago1-24* F1 hybrids yielded 75% (381 of 500 and 368 of 500, respectively) plants with the 19-3 or 69-4 phenotype and 25% plants with the *ago1-24* phenotype. Thus, the absence of wild-type plants in the F2 population confirmed that 19-3 and 69-4 are *ago1* alleles. Similar results were obtained after crossing mutants 19-3, 33-2, and 69-4 with other known *ago1* mutants (data not shown), suggesting that these new mutants are hypomorphic *ago1* alleles according to the developmental defects and act as dominant alleles over null *ago1* alleles.

We identified the mutations by sequencing the *AGO1* gene in mutants 19-3, 33-2, and 69-4. Single nucleotide transitions were found in the 3' portion of the gene, leading to the following substitutions: Gly-758 → Ser (19-3), Pro-838 → Ser (33-2), and Ala-992 → Val (69-4) (Figure 2B). These new hypomorphic *ago1* alleles were named *ago1-25*, *ago1-26*, and *ago1-27*, respectively. Alignment of *AGO1* with RDE-1 and QDE-2 (which are required for RNAi and quelling) revealed that the essential Gly-758, Pro-838, and Ala-992 also are present in RDE-1 and QDE-2 within conserved motifs (Figure 2B), suggesting that these amino acids also could participate in the silencing function of these two proteins. Interestingly, they are present as well in ALG-1 and ALG-2, which are required for development but not RNAi, but not in DmAGO2, which is part of the RISC complex (Figure 2B).

Hypomorphic Mutant 69-4 (*ago1-27*) Is Deficient for PTGS like Other Null PTGS Mutants

We determined that hypomorphic *ago1* mutants, like *sgs* mutants and null *ago1* mutants, were deficient for PTGS. Nuclei, RNA, and DNA extracted from mutant 69-4 (*ago1-27*) were analyzed by run-on RNA gel blot analysis (Figures 3A and 3B) and DNA gel blot analysis (Figure 4) and compared with line L1 and *sgs2-1* and *sgs3-1* mutants. The 35S-*uidA* transgene was transcribed at a high level in L1, *sgs2-1*, *sgs3-1*, and *ago1-27* (Figure 3A), whereas *uidA* mRNA accumulated only in *sgs2-1*, *sgs3-1*, and *ago1-27* mutants (Figure 3B). The ratio between transcription and mRNA accumulation was similar in *sgs2-1*, *sgs3-1*, and *ago1-27* mutants, indicating that PTGS was affected similarly in these three mutants. In addition, methylation of the *uidA* coding sequence was reduced similarly in the three mutants (Figure 4A), as in mutant *ago1-24* (Fagard et al., 2000). To confirm that the hypomorphic mutant *ago1-27* (69-4) was generally impaired in the process of PTGS, we crossed mutant *ago1-27*

with line 2a3. This line carries a 35S-*NIA2* transgene that triggers cosuppression of endogenous *NIA* genes with 100% efficiency in a wild-type background (Elmayan et al., 1998). F2 plants that are homozygous for both the *ago1-27* mutation and the 2a3 locus were identified, and their progeny were analyzed. We observed that cosuppression was abolished completely (0 of 500 plants died when grown on nitrate as a sole source of nitrogen) and that methylation of the 35S-*NIA2* transgene was reduced strongly in mutant *ago1-27*, as in mutants *sgs2-1* and *sgs3-1* (Figure 4B). These results indicate that the silencing properties of the *ago1-27* allele could not be distinguished from that of null *ago1* and *sgs* mutants, suggesting that this hypomorphic *ago1* allele is completely deficient for PTGS.

Hypomorphic *ago1-26* and *ago1-27* Mutants Are Hypersusceptible to Infection by Cucumber Mosaic Virus

We reported previously that the PTGS-deficient *sgs2* and *sgs3* mutants are hypersusceptible to infection by cucumber mosaic virus (CMV) (Mourrain et al., 2000), a member of the cucumovirus family that infects more than 800 plant species. To determine if PTGS as a whole is a defense mechanism that helps to limit virus infection, we tested whether PTGS-deficient *ago1* mutants also were hypersusceptible to CMV. The identification of hypomorphic *ago1* mutants allowed us to perform such experiments. As was the case for *sgs2* and *sgs3* mutants, disease symptoms were much more pronounced in CMV-infected *ago1-26* and *ago1-27* mutants than in CMV-infected wild-type plants (Figure 5A and data not shown). When infected, both *ago1-26* and *ago1-27* mutants had very small stems, were completely sterile, and eventually died. In contrast, infected wild-type plants developed stems with elongated internodes and were able to form seed. We confirmed that the difference in symptom severity caused by CMV infection was attributable to an average fivefold to sixfold overaccumulation of viral RNA in *ago1-26* and *ago1-27*, as in *sgs2* and *sgs3* mutants (Figure 5B).

A Mutation in the ZWILLE/PINHEAD Gene Affects Development but Not PTGS

The *AGO1* gene belongs to a multigene family of 10 members (Figure 2B). Mutants affected in a member called ZWILLE or PINHEAD (ZLL or PNH) show developmental defects resembling that of *ago1* mutants, suggesting that the functions of *AGO1* and ZLL/PNH partially overlap (Moussian et al., 1998; Lynn et al., 1999). To determine if ZLL/PNH also participates in PTGS, we crossed line L1 to the *zll-3* mutant, which shows the strongest developmental defects (Moussian et al., 1998) and lacks a functional protein (T. Laux, personal

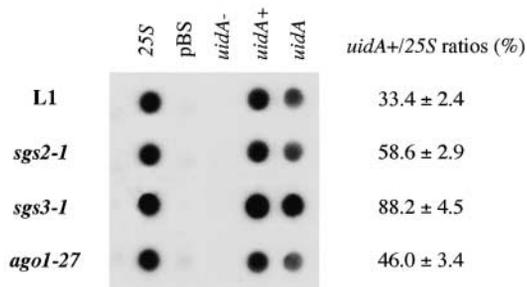
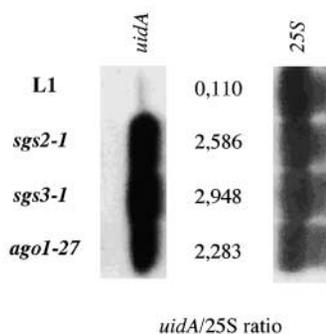
A Transcription rate**B** *uidA* and 25S RNAs

Figure 3. PTGS Is Abolished Completely in the *ago1-27* Mutant as in the *sgs2* and *sgs3* Mutants.

The *ago1-27* mutant was compared with the L1 line, which shows PTGS of the 35S-*uidA* transgene, and with representative alleles of the *sgs2* and *sgs3* mutants deficient for PTGS (Mourrain et al., 2000).

(A) Transcript levels were determined using run-on analysis as described by Elmayan et al. (1998). Radiolabeled RNA extracted from nuclei from adult plants were hybridized to 2 μ g of double-stranded DNA (pBS, empty pBluescript KS- vector; 25S, rDNA cloned in pBS; *uidA*, GUS gene cloned in pBS) and 2 μ g of single-stranded DNA (sense, *uidA*-; antisense, *uidA*+). The signals of three independent experiments were quantified, and the ratios of *uidA*+ to 25S are indicated with standard deviations.

(B) RNA accumulation was measured by RNA gel blot analysis using 10 μ g of total RNA. Note the faint band visible in the L1 lane with the *uidA* GUS probe. The signals were quantified, and the ratios of *uidA* to 25S are indicated.

communication). Plants homozygous for the *zll-3* mutation and homozygous for the L1 locus were identified in the F2 progeny of the cross and selfed. GUS activity was measured in rosette and cauline leaves and in stems of 50 F3 plants derived from two independent F2 plants. GUS activity was similar to that found in L1 plants (Table 1), indicating that the *ZLL/PNH* gene is not required for PTGS at the L1 locus (at least in these tissues).

DISCUSSION

By a combination of genetic and biochemical approaches, many components of RNAi, quelling, and cosuppression have been identified (Matzke et al., 2001). The involvement of proteins similar to DNA helicase (Cogoni and Macino, 1999b) and of proteins involved in chromatin remodeling and DNA methylation (Morel et al., 2000) indicates that quelling and PTGS have nuclear determinants. These components probably are involved in the primary production and/or in the maintenance of production of aberrant RNA that triggers the RNA degradation process. In addition, proteins similar to RdRP (Cogoni and Macino, 1999a; Mourrain et al., 2000; Smardon et al., 2000), 3' to 5' exonuclease RNase D (Ketting et al., 1999), dsRNA endonuclease RNase III (Bernstein et al., 2001), and RNA helicase (Domeier et al., 2000; Wu-Scharf et al., 2000; Dalmay et al., 2001) are involved in sequence-specific and systemic RNA degradation (Cogoni et al., 1996; Palauqui et al., 1997; Fire et al., 1998). Most of the RNAi/quelling/cosuppression mutants do not show developmental defects in standard conditions of growth. Indeed, Arabidopsis mutants such as *sgs1*, *sgs2*, *sgs3*, *sde1*, *sde2*, *sde3*, and *sde4* are viable (Elmayan et al., 1998; Dalmay et al., 2000; Mourrain et al., 2000), as are *N. crassa qde-1*, *qde-2*, and *qde-3* and *C. elegans rde-1* mutants (Cogoni and Macino, 1997; Tabara et al., 1999), suggesting that PTGS, quelling, and RNAi as a whole are dispensable for development. In contrast, the 24 reported mutations in the Arabidopsis *AGO1* gene have pleiotropic effects on development and fertility that strongly compromise life and reproduction in standard conditions of growth (Bohmert et al., 1998; Camus, 1999; Fagard et al., 2000). Similarly, *mut-7* and *ego-1* mutants impaired in RNAi also show gametogenesis defects and sterility in *C. elegans* (Ketting et al., 1999; Smardon et al., 2000). Thus, whether PTGS/RNAi regulate some step(s) of the expression of endogenous genes during development or simply share a common enzyme(s) or pathway(s) with development was undetermined.

Here, we report the identification and characterization of hypomorphic Arabidopsis *ago1* mutants that are as impaired in PTGS as the previously identified strong *ago1* mutants (Fagard et al., 2000), although they show limited developmental abnormalities (Figure 1). The identification of these hypomorphic mutants allows us to define three classes of *ago1* mutants. The first class corresponds to PTGS-deficient *ago1* mutants showing very strong developmental defects that strongly compromise their growth in soil and that are completely sterile in vitro or in soil (*ago1-1* to *ago1-24*) (Bohmert et al., 1998; Camus, 1999; Fagard et al., 2000). The second class corresponds to PTGS-deficient *ago1* mutants that develop vigorously in soil although they are smaller than wild-type plants and that are almost completely sterile (*ago1-26*) (this work). Finally, the third class corresponds to PTGS-deficient *ago1* mutants that are fertile and that show limited developmental defects (*ago1-25* and

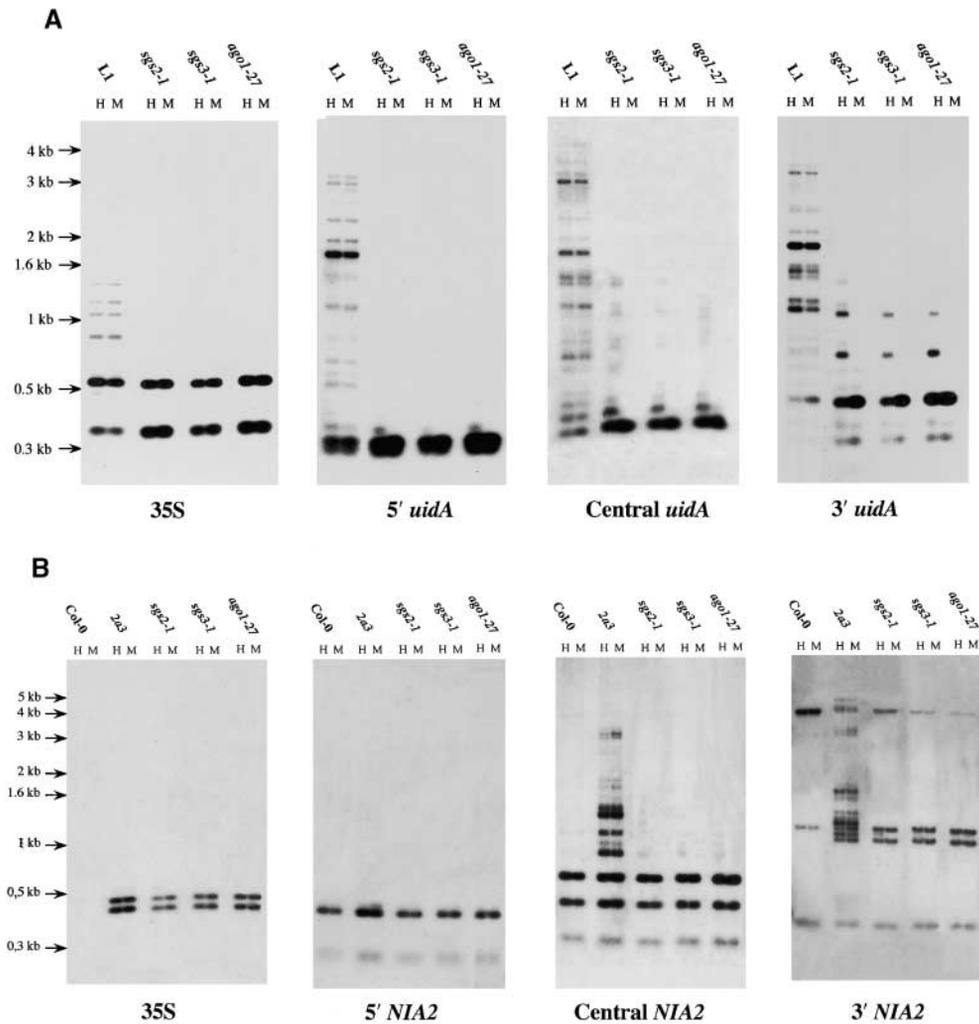


Figure 4. Methylation of the *uidA* and *NIA2* Transgenes Is Affected Similarly in the *ago1-27* Mutant and in the *sgs2* and *sgs3* Mutants.

Methylation of the 35S-*uidA* transgene (**A**) and of the 35S-*NIA2* transgene (**B**) was measured using the sensitive enzymes HpaI (H) and MspI (M) and probes corresponding to the transgene promoter (35S) and to the 5', central, and 3' regions of the transgenes, as described by Elmayan et al. (1998). In the case of the 35S-*NIA2* transgene, wild-type Columbia (Col-0) DNA was included to distinguish between the endogenous and transgenic copies of the *NIA2* genes. Although the silenced transgene loci were hypermethylated in a wild-type background (as shown by the high-molecular-mass bands), the *sgs2-1*, *sgs3-1*, and *ago1-27* mutants exhibited reduced methylation of the transgenes.

ago1-27) (this work). The partial uncoupling of PTGS defects and developmental defects in hypomorphic *ago1* mutants indicates that PTGS is much more sensitive than development to perturbations in AGO1.

Whether AGO1 regulates development via the regulation of endogenous genes by PTGS or acts independently in PTGS and development is not known. However, it is unlikely that PTGS as a whole could regulate development because *sgs* and *sde* PTGS-deficient mutants (including null alleles) do not exhibit developmental defects comparable to those of *ago1* mutants. Nevertheless, part of the PTGS machinery,

including AGO1 and proteins that regulate subsequent RNA degradation steps, could participate in the regulation of some endogenous genes. The partial uncoupling between silencing and developmental defects in hypomorphic *ago1* mutants could be related to the differences in transcription levels of the target transgenes and endogenes. Indeed, transgenes are expressed more strongly than endogenous genes because they are expressed under the control of the 35S promoter. Therefore, mutations in the hypomorphic *ago1* mutants could trigger little perturbations in the amount and/or activity of the AGO1 protein that could have dramatic

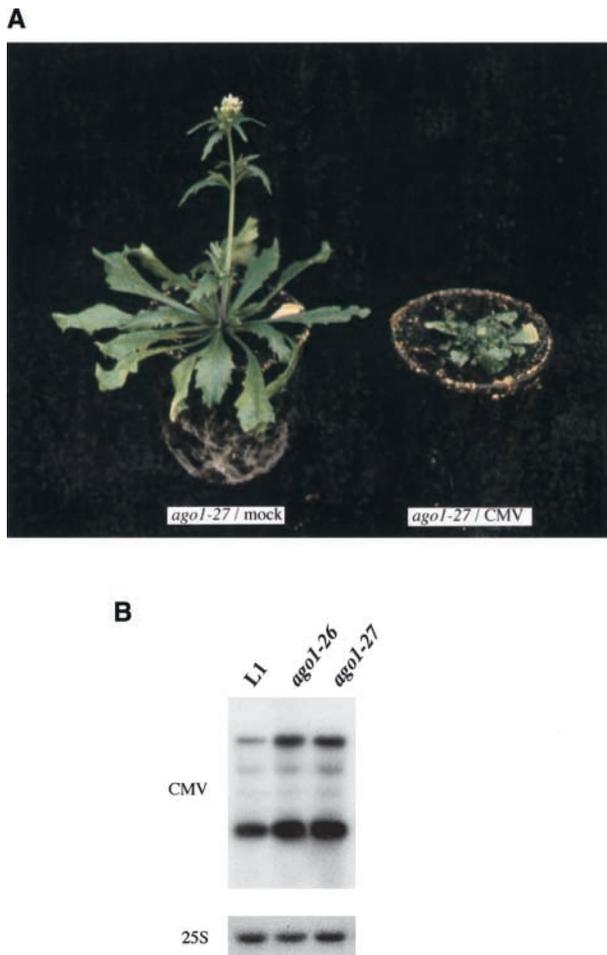


Figure 5. The *ago1* Mutants Are Hypersusceptible to CMV.

Plants were inoculated with CMV as described by Mourrain et al. (2000). Symptoms of mock-infected and CMV-infected *ago1-27* mutants (**A**) and virus accumulation in CMV-infected L1 plants and *ago1-26* and *ago1-27* mutants (**B**) are shown 3 weeks after infection. Five micrograms of total RNA was tested with full-length CMV and constitutive 25S probes. As in the *sgs2* and *sgs3* mutants (Mourrain et al., 2000), CMV accumulation was fivefold to sixfold higher in the *ago1* mutants compared with the L1 line as measured by signal quantification on a phosphorimager. The accumulation correlated with increased symptoms, and the infected mutants eventually died, whereas CMV-infected L1 plants produced seed. The experiment was performed three times, and the results of one representative experiment are shown.

consequences in the case of transgenes because they are strongly expressed, whereas endogenous mRNA targets, which are likely to be present at lower levels, could be processed correctly with less AGO1 activity.

Alternatively, AGO1 could be a protein acting independently in PTGS and development, and the structural require-

ments for a functional AGO1 protein may be different in the two pathways. Mutations that result from T-DNA insertions that impede the production of a full-length protein (Bohmert et al., 1998; C. Bellini, personal communication) as well as a single change affecting Leu-571 in the *ago1-24* mutant (Fagard et al., 2000) are responsible for both PTGS deficiency and very strong developmental defects. Conversely, point mutations at the 3' end of the gene (changing Gly-758, Pro-838, and Ala-992) strongly affect PTGS but trigger weaker developmental defects (this work). Therefore, the C terminus of the protein may be much more specialized in the AGO1 silencing function. Nevertheless, the entire protein is required to produce a functional protein and allow normal development of the plant. Interestingly, mutations in AGO1 that completely impair PTGS affect amino acids that also are found in QDE-2 and RDE-1 (which are required for quelling and RNAi) in highly conserved motifs (Figure 2B). Neither *N. crassa qde-2* nor *C. elegans rde-1* null mutants show developmental defects (Tabara et al., 1999; Catalanotto et al., 2000). Conversely, *C. elegans alg-1* and *alg-2* mutants (which are defective in the production of two RDE-1 homologs) are impaired in the regulation of small temporal RNAs that control developmental timing but are not defective in RNAi (Grishok et al., 2001). Therefore, AGO1 could combine RDE-1 silencing functions and ALG-1/ALG-2 developmental functions on a single molecule, an hypothesis that is in agreement with the fact that mutations in AGO1 that are responsible for developmental abnormalities affect amino acids that also are found in ALG-1 and ALG-2 (Figure 2B). Conversely, two of the four mutations in AGO1 that impair PTGS in Arabidopsis affect amino acids that are not found in DmAGO2, which participates in the RISC complex in *D. melanogaster* (Figure 2B). Together, these results suggest that AGO1 functions may be more similar to those of ALG-1, ALG-2, and RDE-1 (the latter being involved in the production of the interfering agent) than those of DmAGO2 (which is involved in interference thereafter), a result that is in agreement with the fact that silencing induced by panhandle transgenes that constitutively produce dsRNA occurs as efficiently in wild-type plants as in *ago1* mutants (C. Béclin, S. Boutet, and H. Vaucheret, unpublished results).

As proposed above, differences in the structural requirements for a functional AGO1 protein in PTGS and development could explain the partial uncoupling of PTGS defects and developmental defects in hypomorphic *ago1* mutants. Alternatively, other members of the AGO family in Arabidopsis may compensate for (part of) the defects of AGO1 developmental functions but not AGO1 silencing functions. Indeed, AGO1 belongs to a family of 10 members in Arabidopsis (Figure 2B). Except for two members for which mutants with developmental defects have been isolated (*AGO1* and *ZLL/PNH*), the roles of the other members of the family are unknown (Bohmert et al., 1998; Moussian et al., 1998; Lynn et al., 1999). *AGO1* clearly participates in silencing because both PTGS of an exogenous 35S-*uidA* transgene and cosuppression of *NIA* transgenes and endogenous genes

Table 1. GUS Activity Conferred by the L1 Locus in Leaves and Stems of Wild-Type Plants and *zll-3* or *ago1-27* Mutants

Tissue	Genotype		
	L1/L1 <i>ZLL/ZLL</i> <i>AGO1/AGO1</i>	L1/L1 <i>zll-3/zll-3</i> <i>AGO1/AGO1</i>	L1/L1 <i>ZLL/ZLL</i> <i>ago1-27/ago1-27</i>
Rosette leaves	5.8 ± 4.9	4.7 ± 3.9	4850 ± 237
Cauline leaves	5.7 ± 3.9	6.2 ± 5.2	5063 ± 351
Stems	8.9 ± 4.5	11.1 ± 7.5	4756 ± 189

GUS activity (nmol methylumbelliferone min⁻¹ μg⁻¹ protein) is the average measurement (±SD) in 50 plants (see Methods for details on the obtainment of the L1/L1 *zll-3/zll-3* plants).

are abolished in *ago1* mutants. Among four sequenced PTGS-deficient *ago1* mutants (Fagard et al., 2000; this work), three show point mutations changing amino acids that are conserved among the 10 members (Leu-571, Gly-758, and Pro-838), a result that is not informative on the role of the other genes. However, the fourth mutation affects an amino acid (Ala-992) that is absent in seven members (*AGO2*, *AGO3*, *AGO4*, *AGO6*, *AGO7*, *AGO8*, and *AGO9*) and present in only three closely related members (*AGO1*, *ZLL/PNH*, and *AGO5*), suggesting that only these three also could participate in silencing. To answer this question, we determined whether PTGS could occur in the *zll-3* mutant showing the strongest developmental defects and lacking a functional protein (T. Laux, personal communication). We found that PTGS occurs efficiently in the tissues we tested (leaves and stems). Because PTGS is inhibited in *ago1* mutants but not in *zll/pnh* mutants, *AGO1* could be the only member having a function in PTGS, and the other members could participate in (partially) redundant developmental processes, as has been shown for *ZLL/PNH* (Moussian et al., 1998; Lynn et al., 1999). Whether PTGS and/or development are affected in *ago5* mutants needs to be determined to answer this question definitively.

Because *sgs* and *sde* PTGS-deficient mutants do not exhibit developmental defects comparable to those of *ago1* mutants, the role of PTGS in regulating the expression of endogenous genes and subsequently the development of the plants is still a matter of debate. Conversely, a natural role of the PTGS machinery seems to be in limiting virus infection. Indeed, we showed that PTGS-deficient *sgs2* and *sgs3* mutants are hypersusceptible to infection by CMV, a single-stranded RNA cucumovirus (Mourrain et al., 2000). Subsequently, it was shown that *sde3* mutants also are hypersusceptible to infection by CMV (Dalmy et al., 2001). Here, we showed that hypomorphic PTGS-deficient *ago1* mutants also are hypersusceptible to infection by CMV and overaccumulate CMV RNA, like *sgs2*, *sgs3*, and *sde3* mutants, indicating that *AGO1* also participates in protecting

plants against some viruses. Therefore, *AGO1*, *SGS2*, *SGS3*, and *SDE3* act as components of a phenomenon that is naturally targeted against viruses and that can be activated against (trans)gene RNA under circumstances that remain to be elucidated.

METHODS

Plant Material and Growth Conditions

After seed sterilization, plants of *Arabidopsis thaliana* ecotype Columbia, from which were derived the silenced transgenic lines L1 (35S-*uidA*) and 2a3 (35S-*NIA2*) (Elmayan et al., 1998), the *ago1-24* mutant (Fagard et al., 2000), and the *zll-3* mutant (Moussian et al., 1998), were grown in sterile medium under a short day (8 hr of light/16 hr of dark) regimen at 100 μmol m⁻² sec⁻¹. Plants were transferred to soil after 6 weeks and grown under a long day regimen (16 hr of light/8 hr of dark).

Mutant Selection and Genetic Analysis

Thirty thousand plants derived from the self-progeny of 1500 ethyl methanesulfonate-mutagenized seed of the silenced 35S-*uidA* transgenic line L1 were grown in the greenhouse. After 4 weeks of growth, β-glucuronidase (GUS) activity (nmol 4-methylumbelliferone min⁻¹ μg⁻¹ total protein) was measured in the leaves as described previously (Elmayan et al., 1998), and the [GUS+] plants were retained for genetic analysis. Mutants with a wild-type phenotype (*sgs1*, *sgs2*, and *sgs3*) and mutants showing the phenotype of null *ago1* mutants were described previously (Elmayan et al., 1998; Fagard et al., 2000; Mourrain et al., 2000). The remaining [GUS+] plants showing a different phenotype were crossed to *sgs1-1*, *sgs2-1*, and *sgs3-1* mutants (Elmayan et al., 1998; Mourrain et al., 2000) and to parents heterozygous for the *ago1-24* mutation (Fagard et al., 2000). Recovery of 100% [GUS-] F1 plants with a wild-type phenotype in crosses with *sgs* mutants and of 50% [GUS+] F1 plants with the mutant phenotype in crosses with the heterozygous parent of *ago1-24* was indicative of allelism between the tested mutation and *ago1*. Further analyses with the newly identified hypomorphic *ago1* mutants were performed after two backcrosses with the L1 line.

Introgression of the 2a3 locus into the *ago1-27* background was performed as follows. The *ago1-27* mutant (*ago1-27/ago1-27*, L1/L1, -/-) was crossed to line 2a3 (*AGO1/AGO1*, -/-, 2a3/2a3). F1 hybrids were selfed, and their progeny were sown on medium supplemented with hygromycin (to which resistance is conferred by the 2a3 locus). Hygromycin-resistant F2 plants exhibiting the *ago1* phenotype were transferred to soil, and their progeny were harvested. Each batch of F3 seed was sown on medium supplemented with kanamycin (to which resistance is conferred by the L1 locus) or hygromycin, allowing the identification of mutants homozygous for the 2a3 locus only (*ago1-27/ago1-27*, -/-, 2a3/2a3) and their progeny, which are 100% kanamycin sensitive and 100% hygromycin resistant.

Introgression of the L1 locus into the *zll-3* background was performed as follows. The *zll-3* mutant (*zll-3/zll-3*, -/-) was crossed to line L1 (*ZLL/ZLL*, L1/L1). F1 hybrids were selfed, and their progeny were sown on medium supplemented with kanamycin (to which resistance is conferred by the L1 locus). Kanamycin-resistant F2 plants

exhibiting the *zll* phenotype were transferred to soil, and their progeny were harvested. Each batch of F3 seed was sown on medium supplemented with kanamycin, allowing identification of mutants homozygous for the L1 locus (*zll-3/zll-3*, L1/L1) and their progeny, which are 100% kanamycin resistant.

Molecular Characterization

Genomic DNA was extracted by the standard cetyl-trimethyl-ammonium bromide extraction method (Mourrain et al., 2000). The cleaved-amplified polymorphic sequence marker CER462285 located 6 kb upstream of the *AGO1* gene on bacterial artificial chromosome F11A17 was derived from the Cereon database (<http://www.Arabidopsis.org/cereon/>). The primers used were GTGTGTGTGGTGTGTTGCAG and CGTGAAGTCCATCCAGAAGCC. The 0.8-kb amplified fragment was cut by *Accl* to reveal the published polymorphism between ecotypes Columbia (L1 background) and Landsberg *erecta*. Sequencing of the *ago1* alleles was performed on several independent polymerase chain reaction products and on both strands to confirm point mutations. Methylation analysis by DNA gel blotting was performed using the methylation-sensitive *HpaII* and *MspI* restriction enzymes as described previously (Mourrain et al., 2000). RNA and run-on signals were quantified using a phosphorimager.

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Fertile Hypomorphic *ARGONAUTE (ago1)* Mutants Impaired in Post-Transcriptional Gene Silencing and Virus Resistance

Jean-Benoit Morel, Christian Godon, Philippe Mourrain, Christophe Béclin, Stéphanie Boutet, Frank Feuerbach, Florence Proux and Hervé Vaucheret
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