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AGO1 defines a novel locus of *Arabidopsis* controlling leaf development

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An allelic series of the novel *argonaute* mutant (*ago1-1* to *ago1-6*) of the herbaceous plant *Arabidopsis thaliana* has been isolated. The *ago1* mutation pleotropically affects general plant architecture. The apical shoot meristem generates rosette leaves and a single stem, but axillary meristems rarely develop. Rosette leaves lack a leaf blade but still show adaxial/abaxial differentiation. Instead of cauline leaves, filamentous structures without adaxial/abaxial differentiation develop along the stem and an abnormal inflorescence bearing infertile flowers with filamentous organs is produced. Two independent T-DNA insertions into the *AGO1* locus led to the isolation of two corresponding genomic sequences as well as a complete cDNA. The *AGO1* locus was mapped close to the marker mi291a on chromosome 1. Antisense expression of the cDNA resulted in a partial mutant phenotype. Sense expression caused some transgenic lines to develop goblet-like leaves and petals. The cDNA encodes a putative 115 kDa protein with sequence similarity to translation products of a novel gene family present in nematodes as well as humans. No specific function has been assigned to these genes. Similar proteins are not encoded by the genomes of yeast or bacteria, suggesting that *AGO1* belongs to a novel class of genes with a function specific to multicellular organisms.

Keywords: *AGO1*/cell expansion/gene family/leaf development/multicellular organization

Introduction

One of the most successful approaches for investigating the underlying principles of plant development has been the analysis of morphological mutants of the small weed *Arabidopsis thaliana*. Positional cloning as well as T-DNA tagging techniques have permitted the isolation of genes that are only defined by the phenotype of the respective mutant. Prominent examples are the homeotic genes involved in flower development (Weigel and Meyerowitz, 1994). The development of floral organs (sepals, petals, stamens and carpels) and of vegetative leaves, the main photosynthetic organs of plants, has many common aspects and it is generally accepted that floral organs are modified leaves. Thus, a mutation in a single genetic locus often affects both organ types. This relatedness has been demonstrated in a striking experiment, in which floral organs were transformed into leaf-like organs by eliminating the activity of three floral regulatory genes (Bowman *et al.*, 1991).

In dicotyledonous plants the shoot system develops from a meristematic tissue at the tip of the shoot, the apical meristem. The basic unit of a shoot system, a phytomer, consists of a leaf, an internode and an axillary meristem from which a lateral shoot can develop. In general, leaf development of dicotyledonous plants begins with the formation of leaf primordia at the flanks of the shoot apical meristem and requires specific cell divisions as well as controlled cell expansion. Dissection studies revealed that leaf primordia and axillary buds are coordinately initiated. When leaf primordia are separated from their axillary meristem, the axillary meristem fails to develop into a bud (Steeves and Sussex, 1989). There seem to be no specifically localized meristematic tissues within the leave itself, because cell divisions occur in different areas during the expansion of the leaf (Poethig and Sussex, 1985a,b; Pyke *et al.*, 1991). The final leaf shape is the result of cell divisions followed by cell expansion along the longitudinal axis of the leaf and a shift in cell polarity to achieve the lateral growth of the leaf lamina. At the same time, periclinal cell divisions (parallel to the leaf surface) seem to be suppressed. The dorsoventrality of the leaf originates by differential growth of tissues in the dorsal (adaxial) and ventral (abaxial) portions of the leaf primordium.

Mutants affected in one or several aspects of leaf development have been described for many different plants (Marx, 1987; Smith and Hake, 1992; Tsukaya, 1995). Because leaf primordia develop from apical meristems, it is not surprising that mutants with more general lesions in the development of the shoot apical meristem produce abnormal leaves (Medford *et al.*, 1992). Furthermore, the overexpression of genes active in the shoot apical meristem can lead to abnormal leaf development as in the case of the *knotted1*-like (*KNAT1*) homeobox gene of *A.thaliana* (Lincoln *et al.*, 1994, Chuck *et al.*, 1996). The *revoluta* (*rev*) mutant of *A.thaliana* is an example in which the apical meristem, as well as the non-apical meristems is affected, resulting in a reduction in secondary shoot development and an increase in the size of leaves, stems and floral organs (Talbert *et al.*, 1995). The *lateral suppressor* mutant of tomato (Williams, 1960; Schumacher *et al.*, 1995) is defective for axillary meristem development. However, these defects do not lead to a modified leaf formation. Similarly, *A.thaliana* mutants with reduced apical dominance, such as the cytokinin-overproducing *amp1* mutant (Chaudhury *et al.*, 1993), generally show no alterations in leaf shape. In these mutants, the axillary meristems are still present, but their functioning is altered.

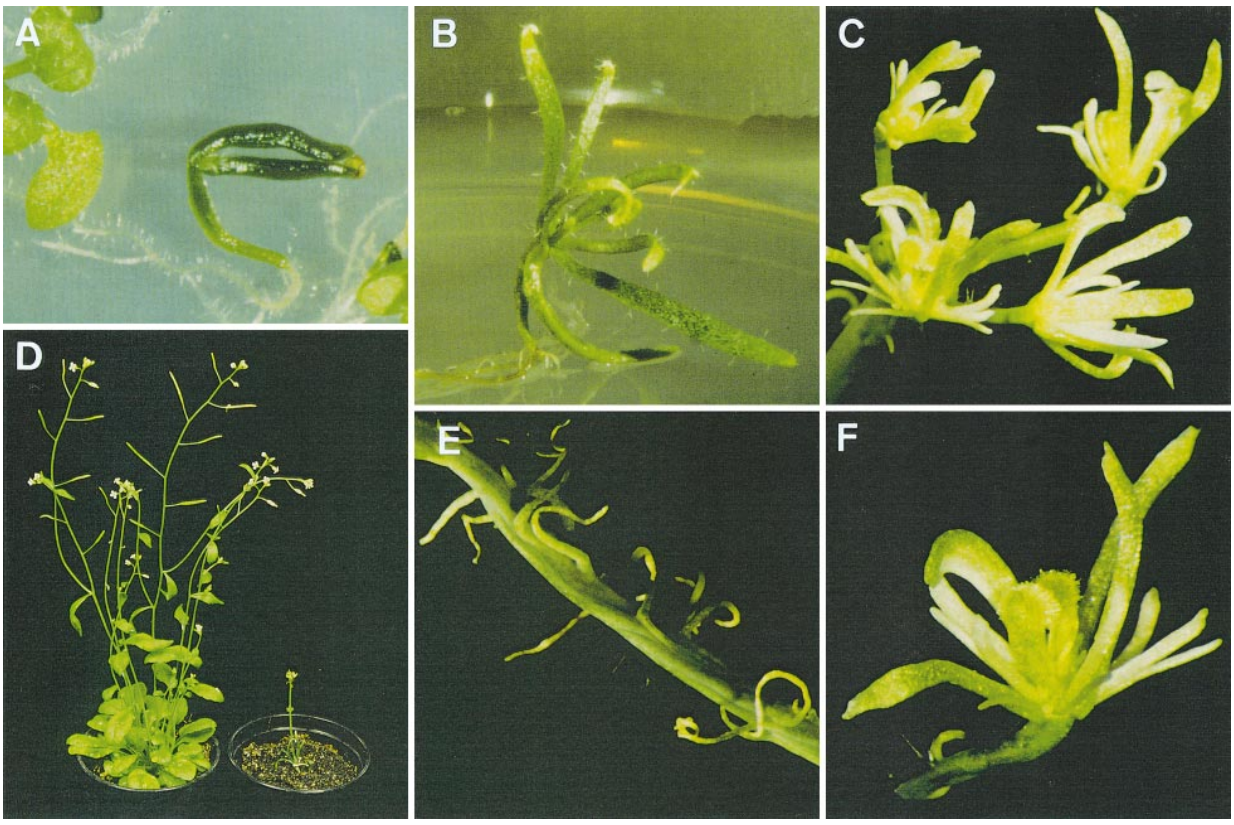

Fig. 1. Phenotype of the *ago1-1* mutant. (**A**) 11-day-old *ago1-1* seedling (center) and hygromycin B resistant heterozygous plant (upper left corner); (**B**) 35-day-old *ago1-1* seedling; (**C**) inflorescence of a 6-week-old *ago1-1* mutant; (**D**) 6-week-old wild-type (left) and *ago1-1* mutant (right); (**E**) cauline leaf-like structures on an *ago1-1* inflorescence stem (6-weeks-old); (**F**) *ago1-1* flower (6-weeks-old).

Brassinolide-deficient mutants are severely affected in general cell expansion (Kauschmann *et al.*, 1996). This leads to a >10-fold reduction in leaf size in extreme cases. However, the deficiency in cell expansion does not result in an altered leaf length-to-width ratio and the reduction in leaf blade expansion correlates with a reduced vascular system. As opposed to this deficiency in overall cell expansion, two leaf developmental genes are known in *A.thaliana*, *ANGUSTIFOLIA* (*AN*) and *ROTUNDIFOLIA* (*ROT*), which seem to control leaf blade expansion by regulating the polarity of leaf cell elongation (Tsuge *et al.*, 1996). In the *lam-1* mutant of *Nicotiana sylvestris* (tobacco), leaves grow to their normal length, but fail to expand laterally (McHale, 1992, 1993). Apparently, a change in the polarity of cell divisions, a prerequisite for lateral leaf expansion, does not occur in the mutant. An increased periclinal cell division activity in the *fat* mutant of *N.sylvestris* leads to an increase in leaf thickness (McHale, 1992). Similar to the *lam-1* mutant of *N.sylvestris*, leaves of the *phantastica* (*phan*) mutant of *Antirrhinum majus* (snap-dragon) lack a lamina. However, the dorsoventrality of leaves and floral organs is reduced to a large extent in the *phan* mutant and it is assumed that the *PHAN* gene product is required to establish dorsal cell identity in leaf primordia (Waites and Hudson, 1995). The genetic analysis of pea leaf development has shown that different genes contribute to compound leaf formation (Marx, 1987). The basic structure of the pea leaf is represented by the tendril, the only structure present in some mutants, and spatially and temporally controlled leaf lamina expansion gives rise to the final compound leaf structure.

While numerous leaf developmental mutants have been isolated and characterized at the histological level, our knowledge about the corresponding genes is mainly based on genetic, but not on molecular evidence. Here, we describe the genetic as well as the molecular characterization of a novel locus of *A.thaliana* which appears to be essential for the proper development of leaves and floral organs, as well as for the formation of axillary meristems in plants.

Results

Isolation of argonaute mutants

As part of our strategy to identify genetic loci controlling cell elongation in higher plants, we visually examined mutagenized *A.thaliana* populations for individuals with aberrant leaf morphology. In an M_2 population consisting of 673 seed batches derived from individual M_1-plants that were mutagenized with ethyl methanesulfonate (EMS), a particular class of mutants (e52, e436, e553 and e691) could be recognized easily by their unexpanded pointed cotyledons and their very narrow rosette leaves as shown in Figure 1. Because of their unusual appearance, which reminded us of a small squid, we named these mutants *argonaute*. A mutant (dlw2) with a very similar phenotype was identified among the T_2 progeny of one out of 12250 T-DNA lines of the Versailles T-DNA mutant collection (Bechtold *et al.* 1993). During a routine experiment, an

Table I. Origin of *argonaute* mutants

Line	Allele	Mutagen	Seed stock	Ecotype
nab1	*ago1-1*	T-DNA (*hygB*)	T_2	Columbia-2
dlw2	*ago1-2*	T-DNA (*nptII*)	T_2	Wassilewskija
e052	*ago1-3*	EMS	M_2	Columbia-0
e436	*ago1-4*	EMS	M_2	Columbia-0
e553	*ago1-5*	EMS	M_2	Columbia-0
e691	*ago1-6*	EMS	M_2	Columbia-0

Table II. Allelism test for the different mutant lines

Cross[a]	Phenotype[b]		
	$Ago1^+$	$Ago1^-$	χ^2
e052×e436	82	26	0.04
e436×e052	79	21	0.85
e553×e052	204	59	0.92
e052×e553	93	21	2.63
e436×e553	73	19	0.92
e553×e436	171	55	0.05
e691×052	37	15	0.41
e052×dlw2	71	24	0.00
e436×dlw2	181	61	0.00
e553×dlw2	193	54	1.29

[a]All parents (female×male) were heterozygous (*ago1/AGO1*)
[b]Calculated values χ^2 were based on an expected ratio of three $Ago1^+$ to one $Ago1^-$ plant. $P > 0.05$.

additional *argonaute* mutant was identified among the T_2 progeny of a transgenic line (nab1) following the *in planta* transformation of Col-2 wild-type with a T- DNA construct carrying a marker conferring resistance to hygromycin B. The origin and genetic background of the different *argonaute* mutant lines is summarized in Table I.

Following at least three backcrosses of the EMS lines, allelism tests were performed for all lines except nab1. For this purpose, plants assumed to carry the recessive mutant allele were crossed and the progeny analyzed. The result is shown in Table II. Approximately one-quarter of the daughter plants showed the mutant phenotype, as would be expected in the absence of complementation. Based on this result we concluded that the four EMS-mutant lines and the T-DNA line dlw2 carry mutant alleles of a locus designated *ago1*. Later, the molecular analysis of the *AGO1* locus revealed that the nab1 T-DNA line also harbors an *ago1* allele (see below). Heterozygous (*ago1/AGO1*) lines were indistinguishable from the wild-type and progeny of heterozygous plants segregated into Ago^+ and Ago^- plants with a ratio of approximately three-to-one, as shown for the *ago1-1* and *ago1-2* mutant lines in Table III. Taken together, these results are consistent with the inheritance of a monogenic, recessive trait conferring the Ago^- phenotype, and suggest that the affected gene is located on the nuclear genome.

Description of the Ago1⁻ phenotype

Plants homozygous for *ago1* are greatly disturbed in general body architecture. Following a seemingly normal embryo development (see below), emerging *ago1* mutant seedlings are characterized by their dark green hypocotyl and their unexpanded pointed cotyledons as shown in Figure 1A. Older plants are depicted in Figure 1B and

Table III. Segregation analysis for the *ago1-1* and *ago1-2* T-DNA mutant lines

Mutant line[b]	Phenotype[a]			
	$Ago1^+Antib^R$	$Ago1^-Antib^S$	$Ago1^+Antib^S$	$Ago1^-Antib^S$
ago1-1	1872	783	735	0
ago1-2	448	248	243	0

[a]$Antib^R$, antibiotic resistant (R) or sensitive (S). Hygromycin B resistance in case of *ago1-1* and kanamycin resistance in case of *ago1-2*.
[b]3390 offsprings of 27 heterzygous *ago1-1/AGO1-1* plants or 939 of a single heterozygous *ago1-2/AGO1-2* plant were analyzed.

1D. They carry narrow and almost succulent rosette leaves with trichomes. Flowering *ago1-1* mutant plants produce a single shoot bearing a terminal inflorescence (Figure 1C). Progenies of some allelic mutant lines, including *ago1-2* and *ago1-3*, developed side shoots from the axils of rosette leaves, but we never observed a branched inflorescence stem. In place of cauline leaves, filamentous structures can be found along the stem (Figure 1E). The microscopic analysis of cross sections of these structures reveals their radial symmetry and indicates a lack of adaxial/abaxial differentiation (Figure 2C). No axillary meristems develop at the base of these structures (data not shown), as would be the case for bracts of the wild-type. The stem is increased in thickness and partially fasciated. The examination of rosette leaf and cotyledon cross sections reveals their reduced, but still visible adaxial/abaxial differentiation (Figure 2). The increase in leaf thickness correlates with an increase in the number of mesophyll cell layers. Upon visual examination, no clear distinction can be made between the petiole and the leaf blade of rosette leaves. A typical inflorescence of an *ago1-1* mutant containing few flowers is shown in Figure 1C. Four petals and sepals are present as in the wild-type, but they are narrow and pointed (Figure 1F). Most flowers contain two carpels carrying a stigma, but their edges do not fuse to form a normal pistil. In some cases, more than two carpels are present in one flower. A false wall between the two carpels and ovules was never observed. Usually six stamens are present, but they lack anthers. Thus all the *ago1* mutant alleles investigated here, cause complete sterility of the homozygous plant.

Scanning electron micrographs of the surface of rosette leaves (data not shown) and of cotyledons (Figure 3) reveal that the epidermal cells of the mutant did not expand in the lateral direction. Thus, the overall shape of the *ago1* rosette leaf is reflected by the shape of an individual epidermal cell. The narrow and pointed leaves of *ago1* mutants are reminiscent of leaves of monocotyledonous plants. However, leaf veins of *ago1* mutants as shown in Figure 4 are branched and do not run parallel as would be typical for a leaf from a monocotyledonous plant. The branching pattern is similar to the wild-type except that branches running in the main direction of the leaf are extended.

To investigate the development of *ago1* mutants, we examined as many as 200 embryos of heterozygous plants for each developmental stage, from early globular to mature embryos. Without exception, all embryos were indistinguishable and appeared wild-type, suggesting that

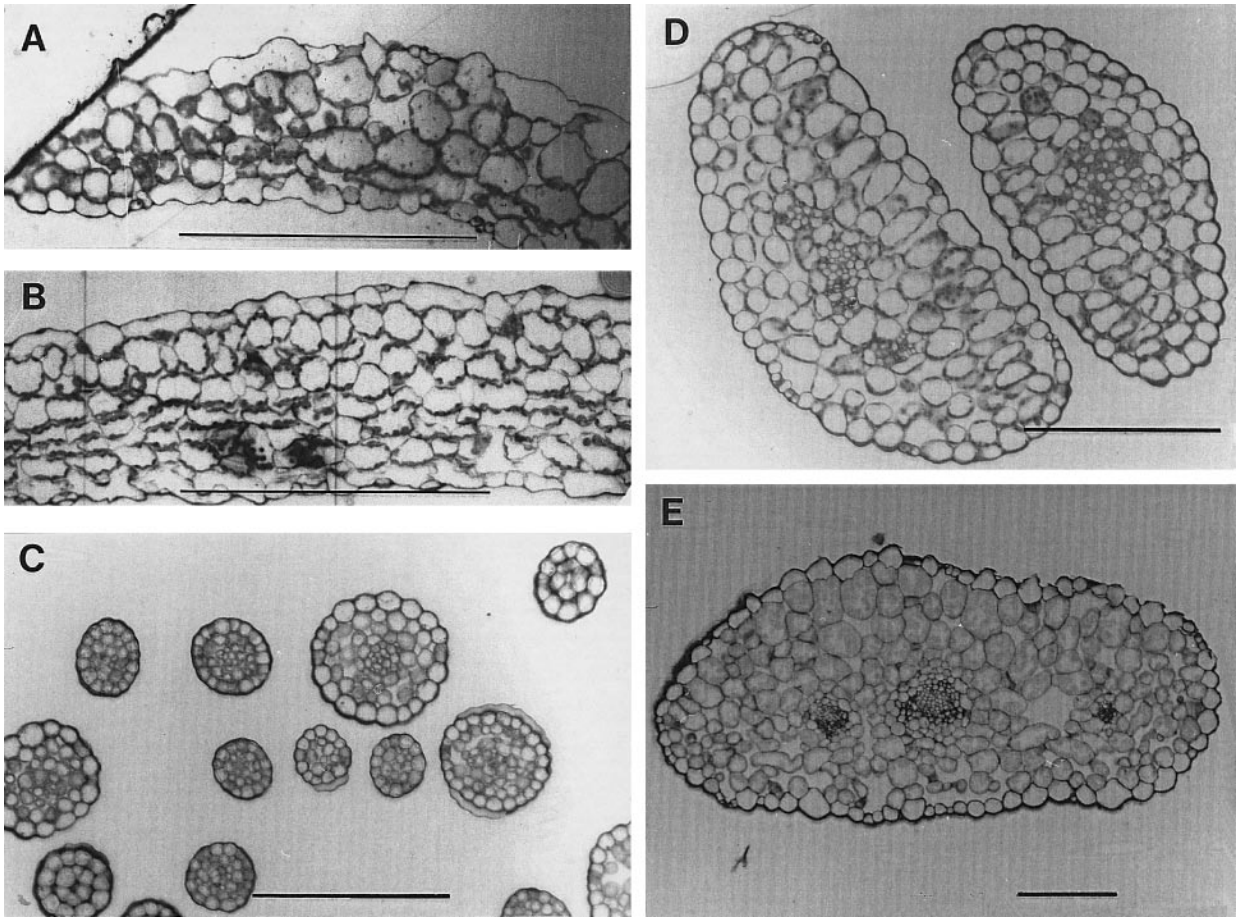

Fig. 2. Cross sections of different leaves of wild-type and *ago1-3* mutant. (**A**) wild-type cotyledon; (**B**) wild-type rosette leaf; (**C**) cauline leaf like structures of the mutant; (**D**) mutant cotyledons; (**E**) mutant rosette leaf. The bars represent 200 μm.

embryo development of *ago1* mutants was not affected, at least at the morphological level. With regard to germination, we observed some differences between alleles. In particular, *ago1-2/ago1-2* homozygous plants showed poor germination. Addition of gibberellic acid to the medium improved the germination of *ago1-2* seeds. Other differences between alleles concerned the formation of secondary leaves and the growth of roots. Thus 60% of *ago1-3/ago1-3* homozygous plants did not develop secondary leaves but showed increased root growth. The remaining 40% of homozygous plants developed secondary leaves with typical *ago1* morphology and showed normal root growth.

The ago1-1 and ago1-2 loci cosegregate with T-DNA markers

The two allelic mutant lines *ago1-1* and *ago1-2* were isolated from the T_2 progenies of T- DNA transformed parents. Thus, the possibility arose that the phenotype of these two lines was caused by T-DNA insertion into the *AGO1* locus permitting its isolation by T-DNA tagging. A prerequisite for this approach was the cosegregation of a single T-DNA with the *ago1* locus. Probing genomic DNA isolated from the T_3 progenies of the two mutant lines by Southern blot hybridization with T-DNA-derived DNA fragments, we determined that the genomes of both lines contain a single T-DNA copy (data not shown). This result was confirmed by segregation analysis of the T_3 progenies of the heterozygous (*ago1-1/AGO1-1*) $Ago1^+$/hygromycin B resistant and heterozygous (*ago1-2/AGO1-2*) $Ago1^+$/kanamycin resistant plants, as shown in Table III. For both lines, plants resistant to the respective antibiotic were present in a ratio of three-to-one as expected for a single dominant trait. Without exception, homozygous (*ago1/ago1*) $Ago1^-$ plants carried an antibiotic resistant marker indicating the cosegregation of the respective T-DNA and the *ago1* locus. Conversely, T_4 progenies of 100 $Ago1^+$/hygromycin B resistant plants (*ago1-1/Ago1-1*) segregated without exception into $Ago1^+$ and $Ago1^-$ plants with a ratio of three-to-one. Taken together, these data indicate very close genetic linkage between the T-DNA insertions and the *ago1* locus in both lines. Based on this analysis we assumed that the *ago1-1* as well as the *ago1-2* lines carry a single T-DNA insertion into the *AGO1* locus. Thus genomic DNA fragments flanking the respective T-DNAs were isolated for both T-DNA lines by inverse PCR and ligated into an *Escherichia coli* cloning vector.

The independent ago1-1 and ago1-2 lines carry different T-DNA insertions into the same gene

An ~1500 bp long DNA fragment flanking the T-DNA in the *ago1-1* line was used to probe the PRL2 λ cDNA library of *A.thaliana* (Newman *et al.*, 1994). Two positive cDNA clones with inserts of ~2.2 and 3.5 kb were isolated. The sequences of the T-DNA flanking genomic fragments

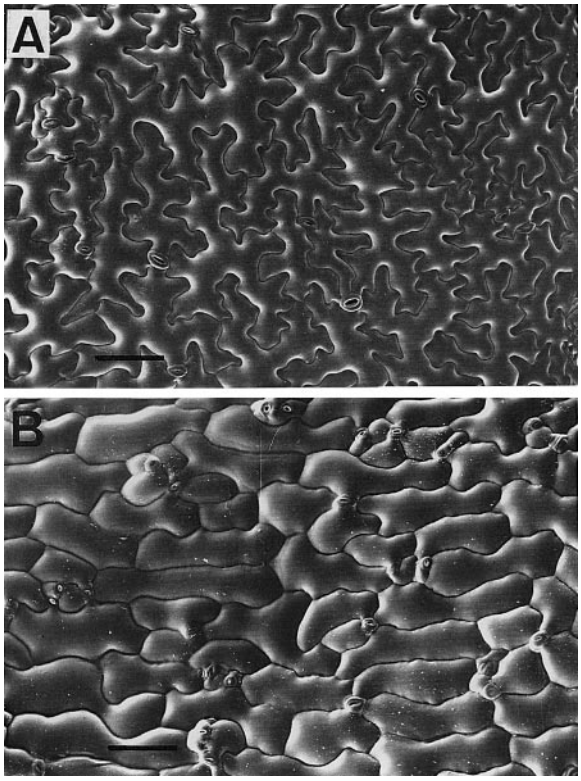

Fig. 3. Scanning electron micrographs of the wild-type and *ago1-3* mutant cotyledon adaxial epidermis. (**A**) wild-type; (**B**) mutant. The plantlets were 18 days old. The bars represent 100 μm. The epidermal surface is oriented such that the proximal–distal axis of the leaf is running horizontally from left to right through both panels.

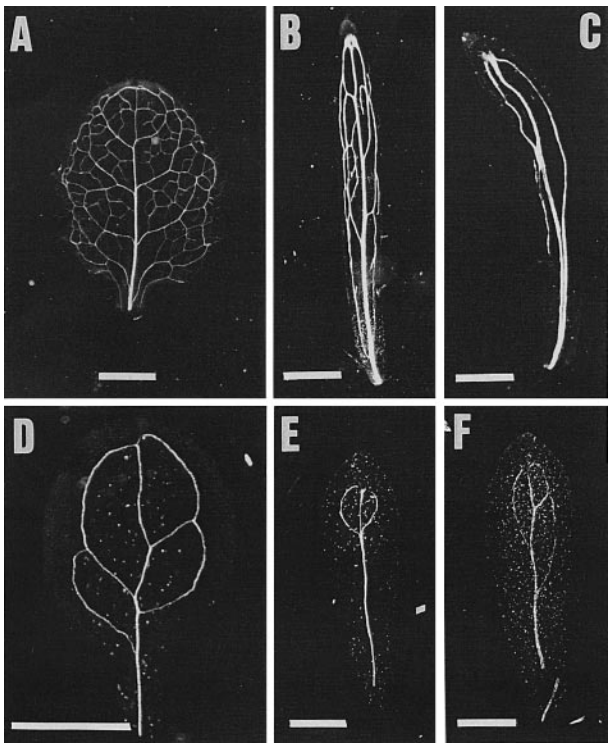

Fig. 4. Visualization of vascular bundles of wild-type and *ago1-3* mutant. (**A**) wild-type rosette leaf; (**B**) and (**C**) mutant rosette leaves; (**D**) wild-type cotyledon; (**E**) and (**F**) mutant cotyledons. The bars represent 1 mm.

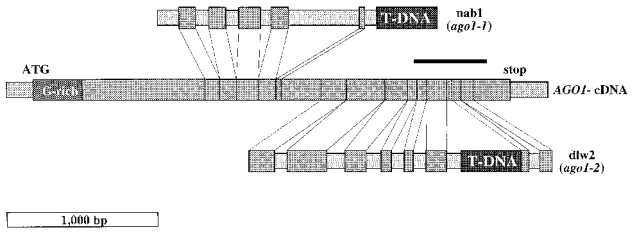

Fig. 5. Comparison of the *AGO1*-cDNA and T-DNA flanking genomic DNA. The coding sequence is indicated with wider dark grey shaded boxes. Exon–intron borders are indicated. The solid bar above the cDNA represents the highly conserved part of the sequence depicted in Figure 7.

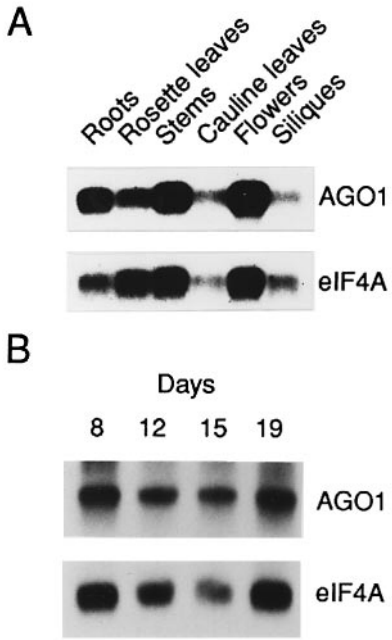

Fig. 6. Expression of *AGO1* in different wild-type tissues (A) and wild-type seedlings (B) of different age as indicated. Poly(A)$^+$ mRNA was isolated from tissues of soil grown plants (**A**) or plants grown on agar solidified medium (**B**). The blots were hybridized with the *AGO1* cDNA (upper panel) or with a partial cDNA encoding eIF4A for the purpose of normalization.

as well as that of the longest cDNA were determined and deposited at the DDBJ\EMBL\GenBank (U91995). A schematic representation of the cDNA and the positions of the T-DNAs and flanking genomic fragments is given in Figure 5. The 3473 bp long cDNA insert contains a 3147 bp open reading frame that is preceded in-frame by a stop codon. The coding sequence is followed by a stretch of 20 adenosine residues 3′ of the stop codon. Thus, it is presumed that the coding sequence of this cDNA is complete. The length of the transcript of ~3.5 kb was confirmed by a polyadenylated-mRNA Northern blot probed with the cDNA as shown in Figure 6. Only very weak signals were detected probing total-RNA blots, indicating a low abundance of the transcript. Comparing the DNA sequences of the genomic fragments flanking the T-DNAs with the cDNA sequence revealed the intron-exon structure of a portion of the gene and allowed the determination of the exact location of the T-DNA integrations as shown in Figure 5. The two different T-DNA integration points disrupting the same transcript in two independent *ago1* mutant lines with identical

Fig. 7. Multiple amino acid sequence alignment between AGO1 and similar proteins of selected species. Only the carboxy terminal part of the predicted AGO1 sequence (amino acids 840–994) is shown (top and bottom). The amino acid sequences were deduced from genomic DNA sequences (G) for *Caenorhabditis elegans (*Ce) or from cDNA (EST) sequences (E) for *Arabidopsis thaliana* (At), *Homo sapiens* (Hs), *Oryza sativa* (Os), *Rattus* sp. (Rs), *Schistosoma mansonii* (Sm), and *Zea mais (*Zm). DDBJ/EMBL/GenBank accession numbers and cosmid designations (*C.elegans*) are indicated for each sequence. For clarity, only a selection of the most significant alignments is shown. Insertions or deletions are not indicated.

phenotype provide strong evidence that the analyzed cDNA indeed corresponds to the *AGO1* gene of *A.thaliana*.

AGO1 belongs to a class of genes common to multicellular organisms

The open reading frame of the *AGO1* cDNA encodes a putative 115 kDa protein (AGO1). Sequence comparison, according to Altschul *et al.* (1990), between the predicted amino acid sequence of AGO1 and translations of DNA sequences deposited in public databases, revealed a large number of putative proteins from different animals and plants with high sequence similarity. A multiple amino acid sequence alignment between the carboxy terminal part of AGO1 (amino acids 840–994) and putative proteins from other species is shown in Figure 7. The depicted part of the sequence was chosen, because it showed a particularly high degree of similarity between AGO1 and putative proteins from plants or animals. The largest number of similar sequences was available from *Caenorhabditis elegans*. While two sequences (Z69661 and P34681; cf. Figure 7) showed an identity of 43% over the complete AGO1 sequence, additional sequences with a lower degree of similarity to AGO1 were present in the database. Thus these putative proteins of *C.elegans* are encoded by a large gene family. A similar conclusion with regard to AGO1-like sequences could be drawn for other animal and plant species, for which the respective data are available.

Interestingly, no similarity to any yeast or bacterial sequences was detected. Given the availability of complete genome sequences of several bacterial species and yeast, this striking observation would suggest that *AGO1*-like genes may have a specific function in multicellular organisms. The comparison with amino acid sequences of proteins with known functions did not produce any positive results. A distinctive feature of the amino acid sequence was a glycine-rich stretch close to the amino-terminus as indicated in Figure 5. The hydrophobicity analysis of AGO1 according to Kyte and Doolittle (1982) did not reveal any potential membrane spanning regions of the protein. Furthermore, no putative signal sequence cleavage site was detected using the program ‘sigcleave’ (von Heijne, 1986) in combination with the GCG-Wisconsin sequence analysis package (Devereux *et al.*, 1984). These results suggest that AGO1 is a soluble protein localized in the cytoplasm. However, a more specific role cannot be predicted based on sequence analysis.

AGO1 is expressed throughout the plant and development

In an attempt to link the molecular data on the *AGO1* locus and the phenotype of *ago1* mutants, we examined the expression of *AGO1* in different wild-type tissues and in wild-type seedlings of different age. For this purpose we prepared polyadenylated-mRNA Northern blots and probed these with the *AGO1* cDNA or with a control cDNA encoding the translation initiation factor eIF4A of *A.thaliana* (H36588) assumed to be uniformly expressed. As shown in Figure 6A , the 3.5 kb *AGO1* mRNA was detected in all tissues examined. Furthermore, during 10 days of seedling development from the cotyledon stage (Figure 6B, 8 days) until the development of 6–10 secondary leaves (Figure 6B, 19 days), no major changes in the expression of *AGO1* were detected. Quantification of the signals with a PhosphorImager and normalization of the *AGO1* signal to the signal for eIF4A suggested that differences in the expression of *AGO1* between all tissues and developmental stages were $<20\%$, except for the samples from rosette leaves and siliques that showed a 50% reduction in *AGO1* signal intensity compared with the other four tissues tested (Figure 6A).

AGO1 maps to chromosome 1

To determine the map position of the *AGO1* locus, specific primers based on the *AGO1* cDNA sequence were employed to screen the CIC YAC library by PCR (Creusot *et al.*, 1995). Clones CIC2A7, CIC7E6 and CIC12G3 gave positive signals. These clones are part of a YAC contig

that also contains the marker mi291a (chromosome 1; 122.4 cM). Independently, RFLP mapping on a set of 100 recombinant inbred lines was performed (Lister and Dean, 1993). The *AGO1*-cDNA probe detected a *Hpa*II polymorphism between the parents. The analysis of the segregation data employing the Mapmaker program (Lander *et al.* 1987) confirmed the localization of *AGO1* on chromosome 1 between RFLP markers GAPB and m213 located at map positions 105.5 and 129.3 cM on the most recently published version of the RFLP map of *A.thaliana* (Lister and Dean, 1996).

Ectopic expression of the AGO1-cDNA has drastic effects on the transgenic plants

To collect further evidence that the analyzed cDNA is indeed derived from the *AGO1* locus, we generated transgenic plants expressing the *AGO1*-cDNA in antisense orientation under the control of the 35S-CMV promoter. Furthermore, in an attempt to demonstrate genetic complementation and to begin to understand the function of *AGO1*, we transformed *AGO1-1/ago1-1* heterozygous plants as well as wild-type plants with a T-DNA carrying the *AGO1*-cDNA in sense orientation under the control of the 35S-CMV promoter. To be able to distinguish easily between the original *AGO1*-inactivating T-DNA conferring hygromycin B resistance and the superinfecting construct, the second T-DNA carried a gene conferring resistance to kanamycin. Figure 8B shows an antisense plant with a rosette leaf reminiscent of an *ago1* mutant leaf (compare Figure 1) suggesting that the Ago1$^-$ phenotype can be mimicked by antisense expression of the *AGO1*-cDNA. Interestingly, the presence of the sense construct led, in a few transgenic plants, to the formation of goblet-like rosette leaves (Figure 8C) and even tubular petals (Figure 8G). These deformations could be caused by excessive lateral growth of these organs in contrast to our observations for *ago1* mutants. Although we could not obtain sufficient material from individual transgenic plants to determine transcript amounts by Northern blot analysis, the comparison of the phenotypes suggests that up- and down-regulation of *AGO1* activity has precisely opposite effects on leaf development. However, it should be noted that while only few plants carrying the sense construct had goblet-like leaves or tubular petals, many transgenic plants showed the Ago1-like narrow leaves to some degree (for example Figure 8F), an effect that could be due to co-suppression of the endogenous *AGO1* gene by the T-DNA construct. Furthermore, many of the different transgenic plants, regardless of sense or antisense orientation of the *AGO1* cDNA, showed abnormal rosette leaves. Two examples are depicted in Figure 8D and 8E. Shoot-like structures along leaves were observed in many cases indicating ectopic meristematic activity. One example is shown in Figure 8B. Apparently, the development of *A.thaliana* is very sensitive to changes in the *AGO1* gene activity.

Transformation of AGO1-1/ago1-1 heterozygous plants with the 35S-CMV::AGO1 cDNA sense construct

In an attempt to show genetic complementation of *ago1-1*, *AGO1-1/ago1-1* heterozygous plants were transformed with the 35S-CMV::*AGO1* cDNA sense construct, kanamycin resistant transformants were recovered, and their T_2 progeny were tested for hygromycin B- resistant plants. Plants resistant to this antibiotic carry an insertion of the original T-DNA into the *AGO1* locus. Several T_1 plants derived from independent transformation events segregating hygromycin B-resistant T_2 plants were identified. The result of the segregation analysis for one of the heterozygous lines with regard to Ago1 mutant phenotype and hygromycin B resistance is shown in Table IV. In comparison with the segregation analysis of non-transformed lines (Table III), antibiotic resistant mutants (Ago1$^-$ Hyg^R, Table IV) were strikingly under-represented. Transfer of these 11 plants to agar plates containing kanamycin revealed that all were kanamycin sensitive. Thus these plants did not contain the 35S-CMV::*AGO1*-cDNA sense construct. This result is in agreement with the complementation of the *ago1-1/ago1-1* homozygous mutant by the 35S-CMV::*AGO1*-cDNA sense construct and provides additional evidence that the isolated *AGO1* cDNA indeed corresponds to the *AGO1* locus.

Discussion

Plant morphogenesis is brought about by the coordinated activity of the root and apical meristems giving rise to the underground or aerial parts of the plant body, respectively. In general, the vegetative development of many plant species can be viewed as the result of a non-determinate addition of successive units called phytomers (Evans and Grover, 1940). Each of these units consists of a leaf, an internode and an axillary meristem. According to this model, flower organs can be considered as derived from a basic leaf structure with superimposed modifications. If the phytomer model is correct, it should be possible to identify mutants that are perturbed in the organization of this repeated basic unit. The phenotype of the *ago1* mutant seems to support several aspects of the phytomer model. This mutant is mainly characterized by the absence of the lateral expansion of the leaf blade. The aptitude for lateral expansion is lost not only for rosette leaves, but also for cauline leaves and floral organs. No longitudinal differentiation of the leaf primordia into petiole and leaf proper is observed for rosette leaves of the *ago1* mutant, nor floral organs such as petals and stamens. Thus, this phenotype is not only consistent with the phytomer model, but also agrees well with current models on the role of floral identity genes suggesting that the leaf pathway is a developmental ground state (Coen and Meyerowitz, 1991). Concurrent to the abnormal development of the leaf blade, the formation or activity of axillary meristems is strongly reduced not only for rosette leaves, but also for cauline leaves along the stem axis. The concerted effects of a homozygous *ago1* mutation on leaves and axillary meristems suggest that a common regulatory process is involved in the formation of these two structures, or in the regulation of activity in the case of the axillary meristems, as assumed by the phytomer model. Detailed cytological experiments in the future should establish more precisely the reasons for a strong reduction in axillary meristem activity. At this time it remains unclear whether there is a defect in the establishment of the axillary meristem or in the activity of an already established axillary meristem.

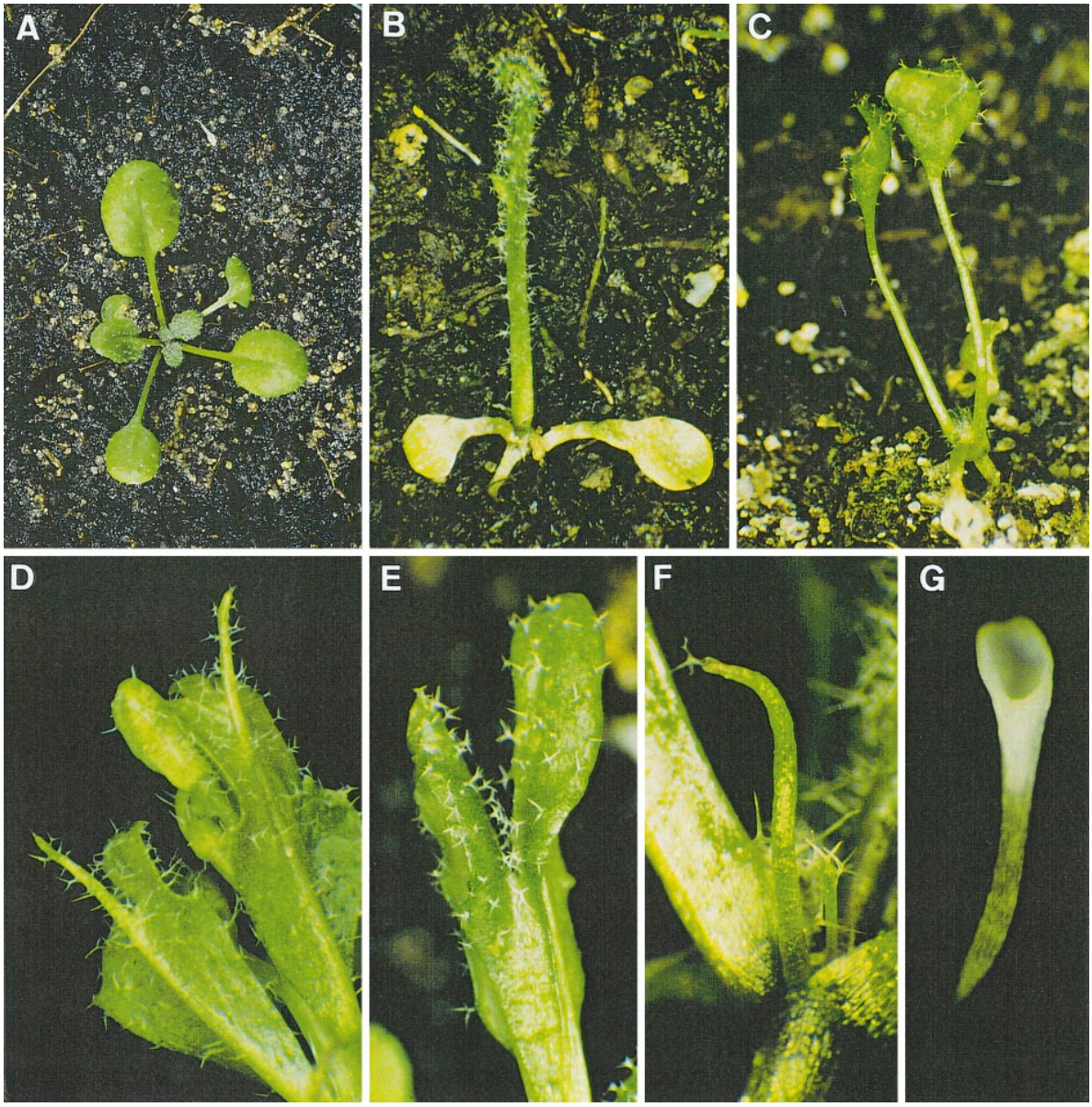

Fig. 8. Phenotype of transgenic wild-type and heterozygous *AGO1-1/ago1-1* lines transformed with sense- or antisense-35S-CMV::*AGO1*-cDNA constructs. (**A**) untransformed wild-type (4 weeks old); (**B**) antisense plant (5 weeks old); (**C**) sense plant (4 weeks old); (**D**) and (**E**) leaves of antisense plants; (**F**) leaves of a sense plant (4 weeks old); (**G**) petal of a sense plant.

Table IV. Segregation analysis of one T_2 plant derived from transformation of *AGO1-1/ago1-1* heterozygous plants with a 35S-AGO1-cDNA construct

Number of plants per phenotype class[a]					
$Ago1^{+}Hyg^{R}$	$Ago1^{-}Hyg^{R}$	$Ago1^{+}Hyg^{S}$	$Ago1^{-}Hyg^{S}$	no germ.	total
265	11	102	0	110	378

[a]T_3 seeds were germinated on hygomycin B containing plates to follow the presence of the T-DNA inserted into *AGO1*.

The defect in leaf blade formation is already visible for cotyledons of young *ago1* seedlings. Unlike all other aerial organs, cotyledons are generally not believed to be generated by the action of the apical meristem. Interestingly, *ago1* cotyledons also show alterations such as the lack of petioles and leaf blades typically observed for leaves of adult *ago1* plants. Although this observation is in agreement with the results of many cytological studies suggesting that cotyledons are closely related to leaves, it conflicts with the interpretation of previous genetic studies on *leafy* cotyledon mutants of *A.thaliana.* Based on the analysis of these mutants, it has been suggested that cotyledon formation is under the control of a morphogenetic program quite distinct from that of leaves (Meinke *et al.*, 1992). On the other hand, the phenotype of the *shoot meristemless* mutant of *A.thaliana* that is still able to generate leaf-like structures from the hypocotyl in the absence of a functional apical meristem (Barton and Poethig, 1993), would suggest that at least part of the morphogenetic program required for the generation of leaf primordia is not dependant on the presence of a functional meristem. Thus in conclusion, *AGO1* defines a factor required for the proper morphogenesis of leaves, but its activity may not necessarily depend on the presence of a functional apical meristem.

Three genes particularly involved in the process of leaf blade formation have been previously defined by genetic analysis of various dicotyledonous plants. The *fat* and *lam-1* mutants of *Nicotiana sylvestris* are characterized by their narrow leaves (McHale, 1992, 1993). Mesophyll cells of the *fat* mutant undergo anticlinal as well as periclinal divisions instead of preferential anticlinal divisions typical for the wild-type. This excess in periclinal divisions in the mutant leads to extra layers of mesophyll cells and reduced leaf blade expansion. The *lam-1* mutant seems to be more specifically affected in the initiation of the blade tissues from cells of the layers L2 and L3 of the apical meristem. This feature as well as the preservation of leaf dorsoventrality seem to be shared by the *lam-1* mutant of *N.sylvestris* and the *ago1* mutant of *A.thaliana*. However, contrary to the *ago1* mutant, the *lam-1* mutant is fertile, suggesting that the defect in leaf blade formation is not compromising proper flower organ development as in the *ago1* mutant. The third known leaf blade mutant, *phantastica* of *A.majus* is defective in the establishment of leaf dorsoventrality (Waites and Hudson, 1995). This occasionally results in needle-like leaves as observed for some of the cauline leaves of the *ago1* mutant. Contrary to the *phan* mutant leaves that all lack dorsoventrality, complete loss of dorsoventrality is only observed for the cauline leaves of the *ago1* mutants. Thus, the phenotypes of all three mutants differ to some degree from that of the *ago1* mutant. In pea, the *AFILIA* and *TL* genes control the transition from leaflets to tendrils, an extreme case of heterophylly (Meicenheimer *et al.*, 1983). The histological analysis of the *afila* mutants indicates that these genes also control the cell proliferation processes leading towards leaf blade formation. These genes seem to be specific to the morphogenesis of true leaves and affect neither cotyledon nor flower organ formation. Although it seems unlikely that all the genes discussed are functionally homologous, since the respective mutants show related, but not identical phenotypes, it may well be that AFILA, TL, LAM-1 and AGO1 are all members of the same developmental pathway leading towards leaf blade initiation or expansion. Isolation and combination of the respective mutations in a single species or heterologous expression of *AGO1* in the respective mutants may help to demonstrate relatedness and hierarchy of the different genes mentioned above.

In addition to *AGO1*, there are other genes of *A.thaliana* under investigation that affect different aspects of leaf development. The *angustifolia* (*an*) mutant with narrow leaves and selectively reduced lateral cell expansion (Tsukaya *et al.*, 1994) and the *rotundifolia* (*rot*) mutant with selectively reduced longitudinal cell expansion (Tsuge *et al.*, 1996) identify two independent processes of cell expansion in the leaf. Both mutant loci map to a different position than *ago1*. The analysis of *ago1* and *an* or *rot* double mutants should reveal, whether AN and ROT act downstream of AGO1 in the leaf developmental pathway, as would be expected because only cell expansion, but not cell division, is affected in these mutants. Similarly, the *acaulis* mutants (*acl-1* and *acl-2*, Tsukaya *et al.*, 1993, 1995) as well as many dwarf mutants, including brassinosteroid biosynthesis mutants, are defective in general cell expansion and may also be affected in basic processes of cell wall biosynthesis. Again, the respective gene products are proposed to act downstream of AGO1, once the developmental pattern has been established, but this has to be confirmed by double-mutant analysis. Furthermore, it will be interesting to know to what extent, if at all, the expression of these genes is affected in the *ago1* mutant.

The analysis of the AGO1 amino acid sequence represents a first step towards the identification of the exact role of the *ago1* gene and the elucidation of *ago1* gene product function. Although *AGO1* is a member of a large gene family conserved during evolution, not only in the plant but also the animal kingdoms, its precise function in other species is not known because no mutants other than *ago1* of *A.thaliana* are available. This is one of the few examples where a first indication for the role of a gene family has been obtained by gene disruption strategies employing higher plants. Interestingly, related genes with similar sequences are not present in unicellular organisms, including some for which the complete genome sequence is known, such as certain bacterial species or yeast. This is valuable information which suggests, but of course does not prove, that this type of gene is only useful in organisms with multicellular organization. Systematic sequencing programs involving additional species will reveal to what extent this observation can be generalized. Because the sequence analysis of AGO1 revealed no clues regarding its presumed function at this time, we are mainly left with the phenotype of the presumed loss-of-function *ago1-1* and *ago1-2* mutant alleles, the transgenic lines ectopically expressing the *AGO1*-cDNA, and the expression pattern to postulate a role for *AGO1*. Because a similar gene seems to be absent from unicellular organisms and because cell and gamete viability are not impaired in the loss-of-function mutants, the gene is apparently not essential for basic cellular processes. Rather, the function of AGO1 has to be sought in processes of intercellular communication, as a modified developmental pattern is clearly a main feature of *ago1* mutants and transgenic lines. The observation that *AGO1* is expressed at low levels in all organs and at all developmental stages examined explains the pleiotropic phenotype of the *ago1* mutants. Thus, the *ago1* mutants are affected in leaf blade development as well as axillary meristem formation or activity. These organs are generated by coordinated cell division events in the L2 and L3 layers of the apical meristem (Steeves and Sussex, 1989). The *AGO1* gene product may therefore play a role in these proliferation processes, but its activity is certainly not restricted to the apical meristem. Furthermore, as the dramatic effects of the ectopic expression of the *AGO1* cDNA in *A.thaliana* demonstrate, the expression of the gene must be tightly regulated, as would be expected for a gene involved in the regulation of developmental processes. Because ectopic meristems form in some of the transgenic lines, AGO1 may be involved in generating positional information required for proper organ formation and meristematic activity during development. The availability of the cDNA for this interesting and novel gene will provide the means to elucidate the function of AGO1 during plant development. Furthermore, we anticipate that the discovery of *AGO1* similar genes in animals will spark the interest to elucidate their general role in the development of multicellular organisms.

Materials and methods

Plant material and growth conditions

Plants of *Arabidopsis thaliana* Heyn, ecotypes Columbia (Col-0 and Col-2) and Wassilewskija (WS) were grown following seed sterilization on MS-medium (Murashige and Skoog, 1962) containing 0.8% (w/v) agarose, 1% (w/v) sucrose and, as required, hygromycin B (25 µg/ml) or kanamycin (62 µg/ml) under a 16 h light/8 h dark regime at 100 µmol/m/s. If necessary, plantlets were transferred to soil [Einheitserde Type P/Einheitserde Type T/sand (2:1:1); Gebrüder Patzer, Sinntal-Jossa, Germany] at the appropriate stage, depending on the mutant line, and grown under the same light regime as used for the *in vitro* culture.

Mutant isolation

The *argonaute* mutant *ago1-1* was identified by chance among the progeny of a transgenic Col-2 line transformed with a T-DNA construct carrying a hygromycin B resistance marker along with a storage protein antisense construct intended for a different purpose (K.Bohmert and C.Benning, unpublished). The mutant line *ago1-2* was identified in the progeny of T-DNA mutagenized lines (ecotype WS) currently produced at the Station de Génétique et Amélioration des Plantes in Versaille, France (Bechtold *et al.*, 1993). The mutant lines *ago1-3*, *ago1-4*, *ago1-5* and *ago1-6* were isolated from an ethyl methanesulfonate mutagenized Col-0 seed stock. Seeds were mutagenized essentially as described by Chory *et al.* (1989). Treated seeds (M_1) were sown in soil and M_2 seeds were harvested from individual M_1 plants following selfing. The mutants were identified by visual examination of seedlings germinated on agar plates either in the light or dark.

Genetic analysis

Because *ago1* mutants are infertile, all crosses were performed with heterozygous parents as indicated. Generally, the phenotype of 8- to 10-day-old plantlets grown on agar plates was scored by visual examination. RFLP mapping on 100 recombinant inbred lines was performed using standard procedures (Lister and Dean, 1993). PCR screening of the CIC YAC library was done according to Creusot *et al.* (1995).

Isolation of T-DNA flanking genomic fragments by inverse PCR

Genomic DNA flanking the T-DNAs inserted into the *ago1-1* and *ago1-2* alleles was isolated employing the inverse PCR (iPCR) technology. The procedure was essentially performed as described by Slightom *et al.* (1995). In case of the *ago1-1* 1500 bp genomic fragment flanking the right T-DNA border that was later used as a probe for the isolation of the cDNA, ~300 ng genomic DNA was digested with *Pst*I prior to ligation. Approximately 100 ng ligated DNA were used as template for the iPCR. The following two PCR oligonucleotide primers were employed : 5′-CGCAGCGATCGCATCCAT-3′ and 5′-GAAGTACTCGCCGAT-AGT-3′. The iPCR was performed with 30 cycles of denaturation at 95°C for 1 min/cycle (5 min for first cycle), annealing at 54°C for 1 min/cycle and extension at 72°C for 2 min/cycle (10 min for last cycle).

Construction of transgenic plants

For the sense or antisense expression of the *ago1* cDNA in transgenic plants of *A.thaliana*, the insert of the 3.5 kb cDNA clone was released from the pZL plasmid vector used for the construction of the PRL2 cDNA library (Newman *et al.*, 1994). For the sense construct a *Xba*I/partial *Kpn*I digest, and for the antisense construct a *Sal*I/partial *Bam*HI digest was performed. These fragments were accordingly ligated into the binary vector pBINAR (Höfgen and Willmitzer, 1990) to obtain the proper orientation with regard to the 35S-CMV promoter. The two constructs were introduced into *Agrobacterium tumefaciens* C58C1 and used to transform *A.thaliana* Col-2 plants via vacuum infiltration (Bechtold *et al.*, 1993; Bent *et al.*, 1994). Transformed seeds (T_1 generation) were selected on MS-medium containing 1% (w/v) sucrose and 62 µg/ml kanamycin.

Northern analysis

Total RNA was isolated according to Logemann *et al.* (1987). Poly(A)$^+$ RNA was prepared with the help of a Qiagen oligotex mRNA kit (Diagen, Hilden, Germany). The RNA was separated and blotted to positively charged Nylon membranes (Hybond N$^+$; Amersham, Braunschweig, Germany) using standard protocols (Sambrook *et al.*, 1989).

Microscopy

For light microscopy, seedlings were fixed in 4% (v/v) formaldehyde/0.2% (v/v) glutaraldehyde and embedded in Historesin™ (Leica, France) according to the manufacturer's instructions. Semi-thin sections (3–5 µm) were prepared using a Jung RM microtome. Sections were stained with 0.05% (w/v) methylene blue and examined under a Nikon microphot FXA microscope. For scanning electron microscopy, seedlings were attached to the sample holder with a thin layer of clay. They were rapidly frozen in liquid nitrogen and immediately transferred to the vacuum chamber of the scanning electron microscope. Following the sublimation of ice crystals, the plantlets were coated with gold and directly examined. The leaf vascular tissue was observed with a stereomicroscope following clearing of the tissue with Herr's buffer (Herr, 1971). Immature seeds of different developmental stages were prepared from heterozygous plants and cleared over night in a buffer described by Herr (1982), prior to examination under a Nikon microphot FXA microscope.

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