Two *Argonaute*l genes from pea

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Introduction

The Argonaute/PIWI family of proteins was founded by the *Argonaute* genes in *Arabidopsis* and *PIWI* in *Drosophila*. These proteins have now been found in many species of plants, invertebrates and vertebrates, including humans. These proteins are known to function in RNA silencing and contain two conserved domains known as PAZ and PIWI (4). The AGO protein is a component of the RNA-induced silencing complex (RISC), which targets specific mRNAs for destruction using a microRNA (miRNA) as a guide. Recent 3-D analysis of the PAZ and PIWI domains has provided data to explain their functions. The PAZ domain binds to nucleic acids, with a preference for RNA over DNA (18). The 3-D structure of the PIWI domain is similar to that of the RNase H family of enzymes, and it is thought to function as the catalytic site for target mRNA cleavage (15).

There are 10 members of the *Argonaute* gene family in *Arabidopsis*. Some of these genes are known to play roles in development because mutants have dramatic developmental phenotypes. *AGO7/ZIPPY* controls early shoot ontogeny, and *AGO10/PINHEAD/ZWILLE* plays several roles in shoot meristem identity and maintenance (8, 11, 12, 14). Mutants of *AGO*1 show many developmental defects. Vegetative leaves often lack blades, axillary buds are lacking, cauline leaves are filamentous, abnormal inflorescences produce sterile, radial flowers, the stem is fasciated, and plants show defects in auxin responses (3, 10, 16, 19). *AGO*1 is unusual among *Arabidopsis AGO* family members in that it possesses a miRNA target site and is apparently regulated itself by miR168 (22).

Our goal was to identify *AGO*1-like genes from pea, compare their sequences to *AtAGO*1 and other *AGO*1-like genes, map them to determine if any known mutants in pea correspond to these genes, and observe expression patterns in parts of pea plants, including shoot tips of the leaf form mutants.

Materials and Methods

Pisum sativum genotypes used for this study were obtained from the Marx collection. The *tendrilled acacia* (*uni-tac*) line was W6 15272. The other lines used were from a set of near isogenic lines constructed by G.A. Marx and designated: *wildtype* (*WT*) W6 22593, *acacia/tendril-less* (*tl*) W6 22594, *afila* (*af*) W6 22597, and the double mutant, *af tl* W6 22598. Plants were grown in a standard greenhouse at 20°C in the day and 15°C at night.

To obtain gene sequences, a *WT* shoot tip cDNA library was screened as described previously (7) using the *AtAGO1* PIWI box sequence as a probe. Positive clones were then sequenced and primers were designed to do both 5' and 3' RACE to obtain the full coding sequences (5). Sequence analysis was done using the NCBI website (http://www.ncbi.nlm.gov/) and the MultAlin program (http://prodes.toulouse.inra.fr/multalin.html). The putative miRNA binding sites were identified using the Microinspector website (http://www.imbb.forth.gr/microinspector) (17).

To obtain data on gene-specific expression, plant parts, embryos and shoot tips were obtained, cDNAs prepared and PCR was done in the linear range as described previously (1, 6). Expression was calculated on the basis of control genes, which were *DEAD-box* or β-*actin* and PCR was done as described previously (6). The primers for *PsAGO1* were F: GCT CTT GGT TGC TTA GGT GA and R: TGC TGT GGA GTA AAA CAT CTC A and for *PsAGO2* were F: ATT CCC ACG CAT TAC ACA C and R: CTC GAC AAT TTT GCA GAC C. The PCR products obtained from cDNAs were 1425 bp and 800 bp, respectively.

Mapping of *AGO* sequences was performed on two recombinant inbred (RI) populations derived from the crosses JI 1794 x 'Slow' and MN313 x JI 1794. The former population consisted of 53 F_{12} lines, and segregation data for over 1000 markers is available for this population (23 and unpublished). The latter population consisted of 50 F_8 lines, and about 300 markers have been mapped on the linkage map developed for this

population. Primers used for mapping the *PsAGO1* sequence were F: GGC GGT GTG GGC CCT GGT and R: GAG CCA AGG CGC AGG AAG. These primers were selected to span introns in the genomic sequence. Primers for mapping the *PsAGO2* gene were F: TGC ATT TAT TGA GCC ACT GC and R: TGC AAG CTC ACA ACA AAA GG. These primers were expected to amplify a 1927 bp region across intron 2. Polymorphism was identified by CAPS analysis (9).

Relationships among the various *Argonaute* sequences available in the literature were determined using Phylogenic Analysis Using Parsimony (PAUP) version 4.0b10 (20). The sequences compared are listed in Table 1. Complete coding sequences were used for the comparison among the two pea sequences, the *AtZWILLE*, *AtPINHEAD* and *AtAGO*1 sequences from *Arabidopsis thaliana*, the four sequences from *Oryza sativa* and the two sequences from *Nicotiana bethamiana*. Only partial coding sequences (ESTs) were available for the *Trifolium pratense*, *Medicago truncatula*, *Phaseolus coccineus* and *Arachis hypogaea* genes, and these were compared with the two *Pisum sativum* sequences and the *AtAGO*1 and *NbAGO*1-1 sequences using only approximately 660 bp near the 3' end of the coding sequence available from each of the EST sequences. For this latter analysis the *AtAGO*1 and *NbAGO*1-1 sequences were used as the outgroup.

Table 1. List of *Argonaute* sequences used in the PAUP analysis

Sequence designation	Source	NCBI accession
PsAGO1	*Pisum sativum*	EF108450
PsAGO2	*Pisum sativum*	EF108449
T. pratense	*Trifolium pratense*	AB236789
M. truncatula	*Medicago truncatula* combined	CB895213 and BM779610
P. cocinneus	*Phaseolus coccineus*	CA905209
A. hypogaea	*Arachis hypogaea*	EG029779
AtAGO1	*Arabidopsis thaliana*	U91995
NbAGO1-1	*Nicotiana bethamiana*	DQ321488
NbAGO1-2	*Nicotiana bethamiana*	DQ321489
OsAGO1	*Oryza sativa*	NM_001054239
OsAGO4g566500	*Oryza sativa*	NM_001060116
OsAGOJO13091	*Oryza sativa*	AK111587
OsAGO2g831600	*Oryza sativa*	NM_001055156
AtPINHEAD	*Arabidopsis thaliana*	AF154272
AtZWILLE	*Arabidopsis thaliana*	NM123748.2

Results and Discussion

Complete coding sequences of two pea *AGO* genes were obtained with our methods. We call these *PsAGO*1 (EF108450) and *PsAGO*2 (EF108449). Based on the predicted amino acid sequences, these proteins were of similar lengths (1100 vs 1070 aa). They contained the two domains common to *Argonaute* genes: PAZ (aa 438 and 436 respectively) and PIWI (aa 726 and 696 respectively). BLAST comparisons of these amino acid sequences with other known AGO proteins revealed that they were most similar to AGO1 (NP_849784) in *Arabidopsis*, two AGO1-like sequences from *Nicotiana benthamiana* (ABC61502 and ABC61503) and a putative Argonaute protein from rice (BAD27856). These proteins showed strong sequence conservation in all regions except the N-terminal region, which had considerable gene specific variation (data not shown).

Comparison of the two *Pisum sativum* DNA sequences with other complete coding sequences (Fig 1A) revealed that the two clearly clustered with other *AGO1* sequences and were clearly distinct from ZWILLE

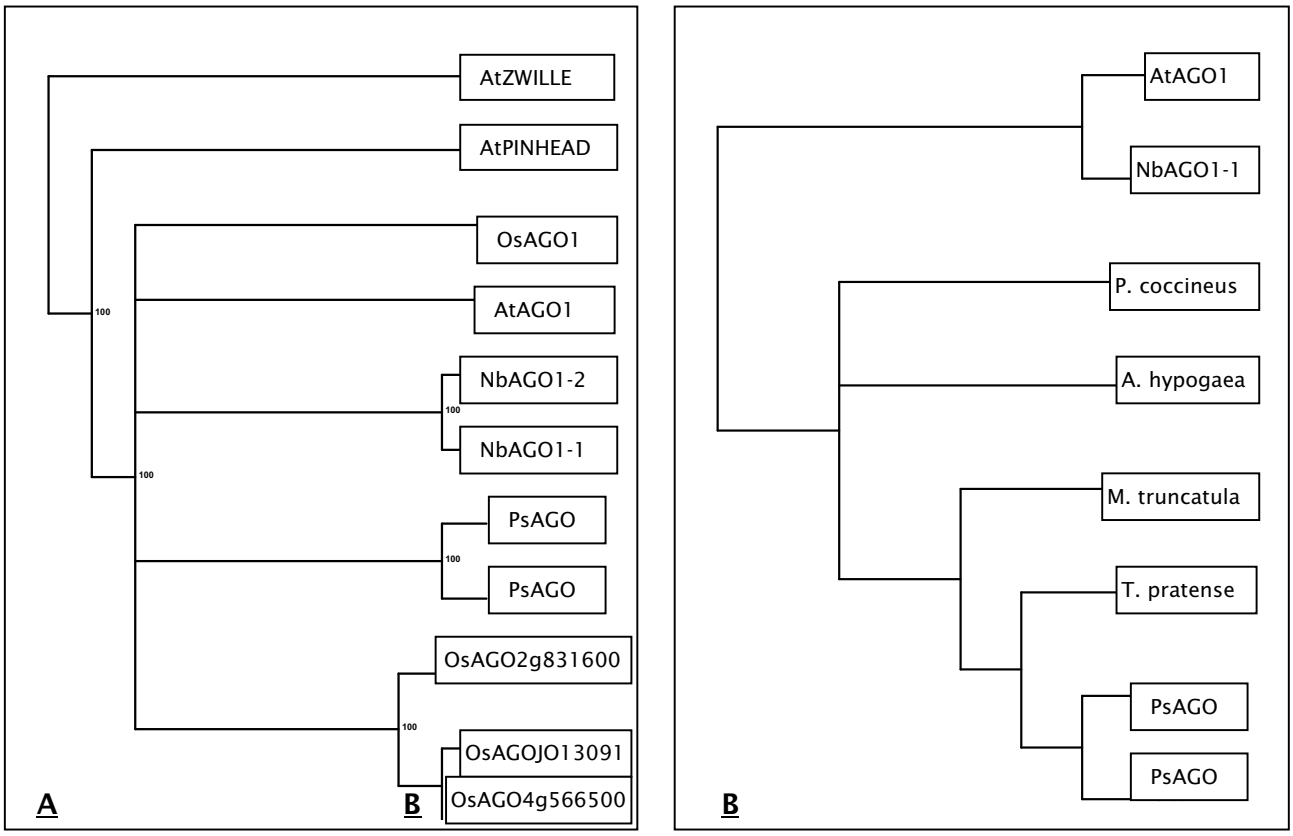

Fig. 1. Phylogenetic relationships among the Argonaute sequences based on PAUP analysis. A) The strict consensus tree for the full-length plant Argonaute genes available in the literature and B) the strict consensus tree for the comparison of approximately 600 bp of coding sequence at the 3' end of an AGO gene from each of the legume species listed. The Arabidopsis and Nicotiana sequences were used as outgroups for the latter analysis.

and PINHEAD sequences. The *Pisum* sequences were most closely related to each other, with the *AtAGO*1 sequence being the next most similar, confirming the results from the protein sequence analysis. Although two *AGO*1 genes are also found in *Nicotiana benthamiana*, the presumed duplication producing those two sequences apparently occurred independently of the duplication forming the two *Pisum* sequences. PAUP analysis of the partial coding sequences available from other legume species produced a tree that mirrored the established phylogeny of the taxa being compared (Fig. 1B). Two most parsimonious trees were detected. Only the strict consensus tree is shown in Fig. 1B. These results support the hypothesis that only *AGO*1 members of the *Argonaute* gene family are being compared in this analysis.

The *PsAGO*1 primers amplified a product of approximately 1500 bp that displayed polymorphism between JI 1794 and the domesticated lines when cut with the restriction enzyme *Hae*III . The position of the locus *Ago*1 was determined to be on linkage group (LG) VI about 4 cM from *Gty* (Table 2). In the JI 1794 x 'Slow' linkage map, the region around *Gty* appeared to be compressed, with 10 or more markers within 5 cM of *Gty*. The order of the markers was ambiguous, and it could not be determined on which side of *Gty Ago*1 was located. The data from the second RI population indicated *Ago*1 lay distal to *Gty*, towards *Rpl22* (Fig. 2).

The *PsAGO2* primers amplified a fragment of about 2000 bp in the domesticated lines but did not amplify a product in JI 1794. This presence/absence polymorphism could be easily scored without need for CAPS analysis. *Ago*2 was found to be the most distal marker on the upper arm of LGI in both RI populations (Fig 2). The combined data from both populations placed *Ago*2 approximately 3 cM from *Cop*1 (Table 2).

Neither the position of *Ago*1 nor *Ago*2 obviously matched that of known mutations in pea that modify leaf development. There is no known morphological mutation in pea that maps near *Cop*1 (23). Several mutations are located within 15 cM of *Gty*, including *rui*, *p*, and *er*1. Of these, only *rui* has a phenotype that might be expected from a mutation in an *Argonaute* gene (21, 24). Neither the roughened testa characteristic of *Gty* nor the loss of sclerenchyma in the pod walls displayed by plants homozygous for *p* are expected phenotypes for a mutation at *Ago*1. Nor would a mutation of this gene be expected to confer resistance to powdery mildew. Hence, we are investigating only the possibility of a relationship between *rui* and *Ago*1. At present the relative position of *Rui* to *Gty* is uncertain.

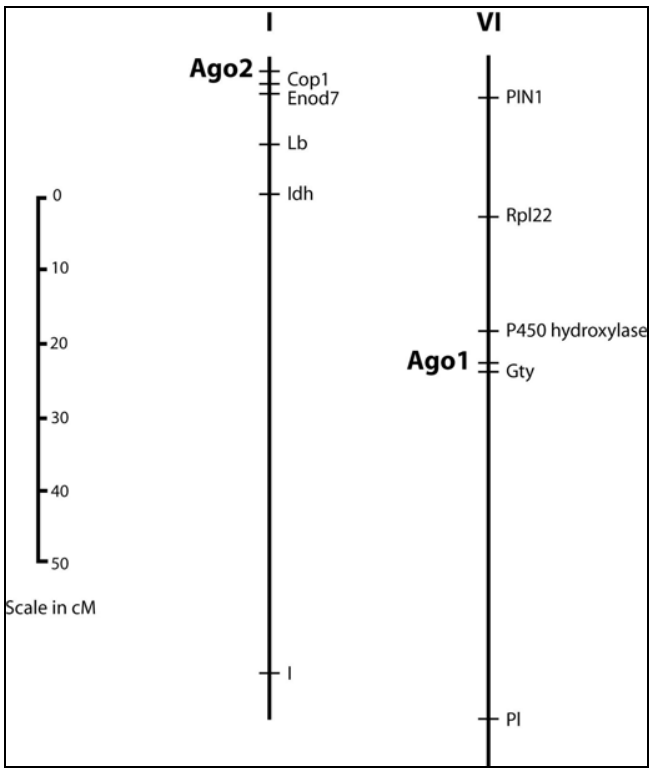

Fig. 2. Location of Ago1 and Ago2 on pea linkage groups VI and I, respectively. The scale on the left gives approximate recombination distances for both linkage groups.

Four amino acids (Y, K, Q and K) present near the 5' end of the conserved PIWI domain have been shown to be important for RNA binding affinity of the *Archeoglobus fulgidus* Piwi protein and three of these (K, Q, K) have also been shown to be important in cleavage of the target mRNA by the human AGO2 protein (13). These amino acids are also conserved in PsAGO1 and PsAGO2, as well as in the other AGO1-like proteins from the other plant species we examined (Fig.3A). Conservation of these four amino acids extends to the *Arabidopsis* AGO10/PNH/ZLL protein. Because of this conservation, it is likely that all of these proteins are active in RNAi regulation of gene expression. *AtAGO*1 mRNA is known to be regulated by a miRNA (miR168), the Microinspector program was used to evaluate the pea nucleotide sequences for potential miRNA binding sites using sequences of *Arabidopsis thaliana* miRNAs and -27 as the free energy (ΔG) limit for both *PsAGO*1 and *PsAGO*2. Both pea sequences possessed a potential miR168 binding site in approximately the same position as the one identified in *AtAGO*1 (Fig. 3B). In fact, the target sequences from pea are better matches to the *Arabidopsis* miR168 sequence than the target in *AtAGO*1 (ΔG of -34.7 vs. -21). The two *AGO*1 genes from

Table 2. Joint segregation analysis of *PsAGO* genes and closely linked standard markers

		No. of lines with designated genotype[1]				Recombination
RI population	**N**	**P1 P1**	**P1P2**	**P2P1**	**P2P2**	**Fraction + SE**
Ago*1/*Gty						
JI 1794 x 'Slow'	**43**	**22**	**1**	**1**	**29**	**3 ± 1**
MN313 x JI	**46**	**24**	**5**	**0**	**17**	**5 ± 1**
***Ago*2/*Cop*1**						
JI 1794 x 'Slow'	**49**	***14***	***4***	***2***	***29***	**6 ± 1**
MN313 x JI 1794	**48**	**22**	**0**	**1**	**25**	**5 ± 1**

[1]**Genotype designations: P1 = Maternal parent, P2 = Paternal parent. All genotypes were homozygous.**

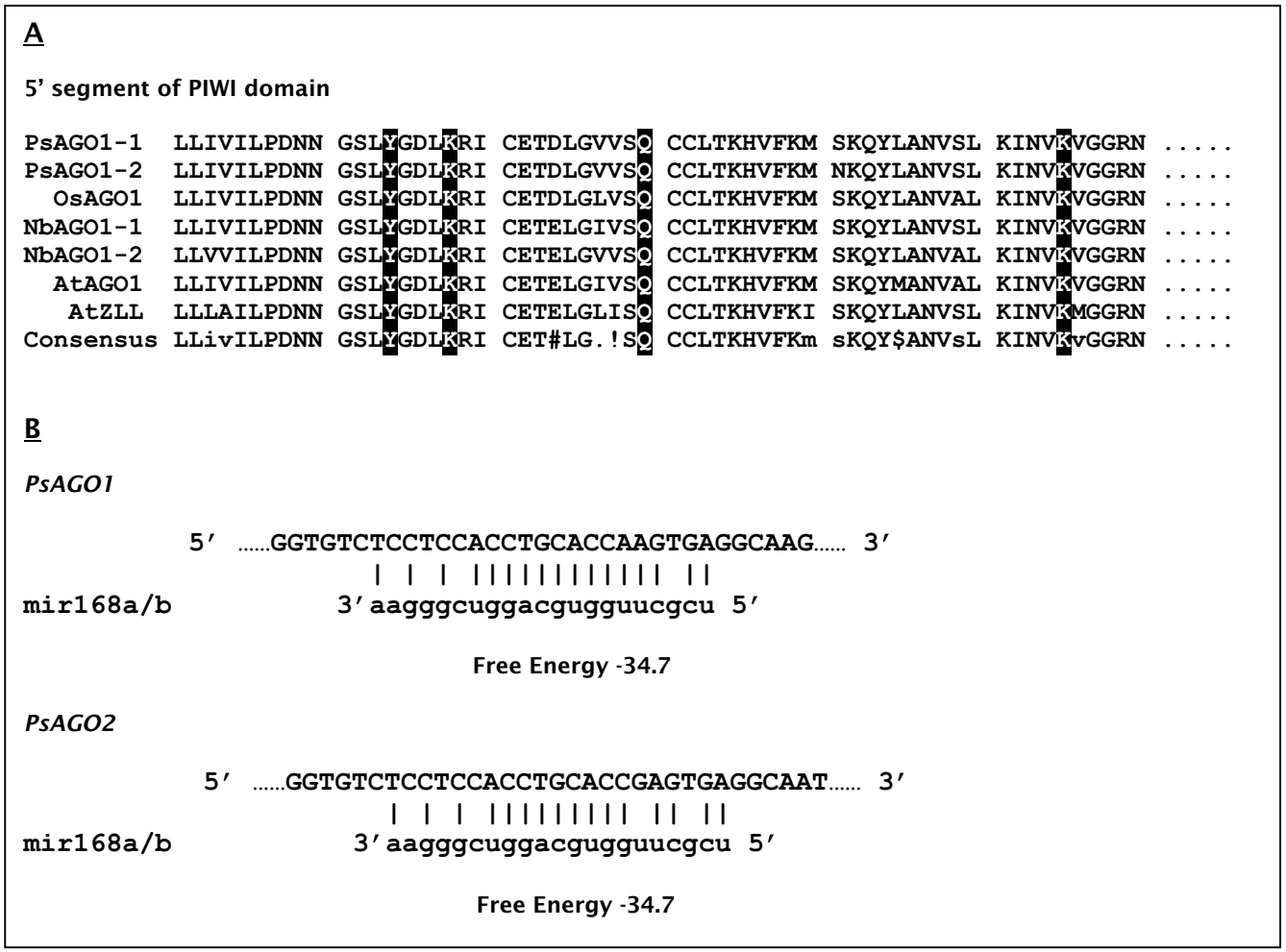

Fig. 3. Sequence comparisons. A. Alignment of predicted PsAGO1 and PsAGO2 amino acid sequences with some similar plant AGO1-like genes. B. Nucleotide sequences of PsAGO1 and PsAGO2 and match-up with that of Arabidopsis miR168.

tobacco and the putative *Argonaute* sequence from rice lack this target site (data not shown). From these data we would predict that *PsAGO*1 and *PsAGO*2 are both regulated by a single miRNA.

Both *PsAGO*1 and *PsAGO*2 were expressed in all growing plant parts and showed very similar expression patterns (Fig 4A). mRNA levels of both genes were abundant in developing and mature embryos as well (Fig. 4B). mRNA levels of *PsAGO*1 appeared to be more abundant in young embryos and the embryonic axis of mature embryos than *PsAGO*2. Bohmert et al. (3) found that *AtAGO*1 was expressed in all plant parts and at all stages of development in *Arabidopsis*. The two pea genes have overlapping and redundant expression patterns in all part of pea tested.

As *AtAGO*1 was originally described as a leaf development gene, we looked at mRNA level of *PsAGO*1 and *PsAGO*2 in the shoot tips of the leaf form mutants. mRNA levels of both genes were higher in the *af* genotypes (*af* and *af tl*) than in *WT* (Fig. 5). The mRNA levels of *PsAGO*1 were similar in *tl*, *uni-tac* and *WT*, whereas those of *PsAGO2* were lower in the *tl* and *uni-tac* genotypes than in *WT*. This pattern of differential expression was not what we had expected from the phenotype of *AGO*1 mutants in *Arabidopsis*, which have peg-shaped leaves lacking a lamina. The radial symmetry of peg-shaped leaves and of tendrils suggested that mRNA levels of these genes might be lower in *af* and higher in *tl* and *uni-tac*. Instead, the pattern of mRNA expression of these

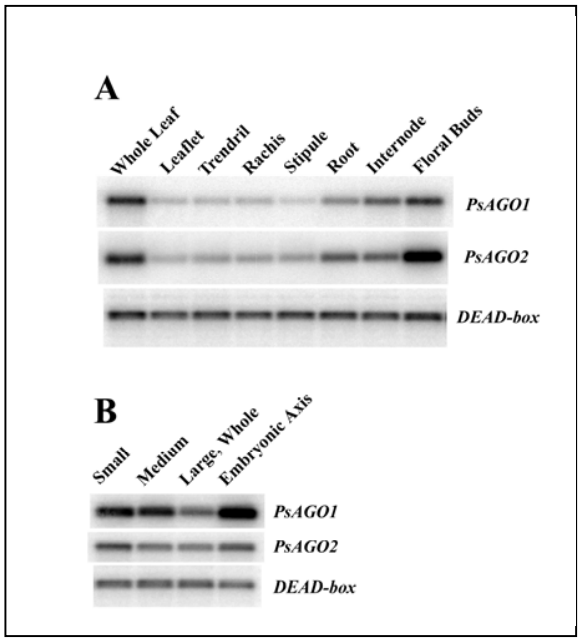

Fig. 4. PsAGO1 and PsAGO2 expression. A. Expression in various plant parts. B. Expression in different stages of developing embryos. DEAD-box was used as a control for equal cDNA used for RT-PCR and equal loading.

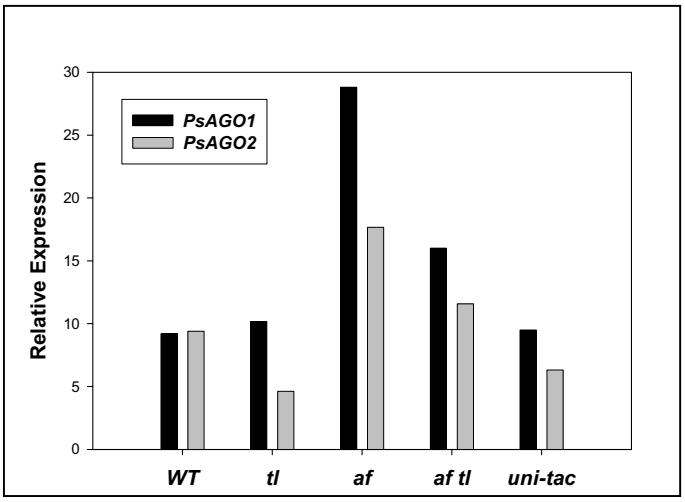

Figure 5. PsAGO1 and PsAGO2 expression shoot tips. Expression is expressed relative to mRNA of actin which was used for equal cDNA for RT-PCR and equal loading.

two genes is similar to that of *Uni*, *PsPIN*1 and *PsPK*2, which are all GA and auxin regulated (1, 2, 6), suggesting that these two genes play a role in auxin signaling. Sorin, et al. (19) have shown that in *Arabidopsis AGO*1 plays a role in two auxin responses: auxin-mediated hypocotyle elongation and adventitious root formation.

In conclusion, we have identified two pea genes similar in sequence to *AtAGO*1 which we call *PsAGO*1 and *PsAGO*2. They map to LGVI near *Gty* and on the upper arm of LGI near *Cop*1, respectively. The position of the*Ago*1 locus in pea encourages further examination of its relationship to the mutant *ruinous*. Sequence analysis suggests that both *PsAGO*1 and *PsAGO*2 function in RNAi and are themselves regulated by a miRNA, just as *AtAGO*1 is. These genes have overlapping and redundant expression patterns in developing pea and are differentially expressed in shoot tips of the classic pea leaf mutants.

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