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Redundant and Specific Roles of the ARGONAUTE Proteins AGO1 and ZLL in Development and Small RNA-Directed Gene Silencing

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Abstract

The Arabidopsis ARGONAUTE1 (AGO1) and ZWILLE/PINHEAD/AGO10 (ZLL) proteins act in the miRNA and siRNA pathways and are essential for multiple processes in development. Here, we analyze what determines common and specific function of both proteins. Analysis of ago1 mutants with partially compromised AGO1 activity revealed that loss of ZLL function re-establishes both siRNA and miRNA pathways for a subset of AGO1 target genes. Loss of ZLL function in ago1 mutants led to increased AGO1 protein levels, whereas AGO1 mRNA levels were unchanged, implicating ZLL as a negative regulator of AGO1 at the protein level. Since ZLL, unlike AGO1, is not subjected to small RNA-mediated repression itself, this cross regulation has the potential to adjust RNA silencing activity independent of feedback dynamics. Although AGO1 is expressed in a broader pattern than ZLL, expression of AGO1 from the ZLL promoter restored transgene PTGS and most developmental defects of ago1, whereas ZLL rescued only a few AGO1 functions when expressed from the AGO1 promoter, suggesting that the specific functions of AGO1 and ZLL are mainly determined by their protein sequence. Protein domain swapping experiments revealed that the PAZ domain, which in AGO1 is involved in binding small RNAs, is interchangeable between both proteins, suggesting that this common small RNA-binding domain contributes to redundant functions. By contrast, the conserved MID and PIWI domains, which are involved in 5'-end small RNA selectivity and miRNA cleavage, and the non-conserved N-terminal domain, to which no function has been assigned, provide specificity to AGO1 and ZLL protein function.

Introduction

Small RNA-directed gene regulation is a major process in plant development and viral defense [1,2]. A central component in these pathways is the activity of ARGONAUTE (AGO) proteins, which bind small RNAs and mediate repression of the complementary RNA targets [3,4]. In Arabidopsis, 10 AGO genes have been identified [5]. AGO1 [6] associates with numerous microRNAs (miRNAs) and short interfering RNAs (siRNAs) to mediate target repression via mRNA cleavage and inhibition of translation [3,4,7]. Binding of AGO1 to miR168, which targets AGO1 mRNA, establishes a homeostatic AGO1 regulatory loop [8,9]. AGO4 and AGO6 function in small RNA mediated chromatin regulation whereas AGO7 associates specifically with miR390 and directs cleavage of the non-protein coding TASI3 precursor RNA to generate trans-acting short interfering RNAs (tasiRNAs) [5]. Recently, ZLL was implicated in miRNA-directed translational inhibition [7] and repression of miR165/166 levels [10].

AGO1 and ZLL protein sequences are highly similar, including the PAZ and MID domains, which bind small RNAs in AGO1 [11], and the PIWI domain, which is required for target mRNA cleavage in AGO1 [3,4]. By contrast, their N-terminal domains do not display sequence similarities. Both genes differ in their expression patterns and developmental functions. AGO1 is expressed broadly during plant development, and ago1 loss-of-function mutants display pleiotropic defects in development and in virus defense [6,12]. Seedlings of the null allele ago1-1 form only a few finger-like leaves and about 10% of seedlings lack a shoot meristem. ago1 mutants are deficient in transgene posttranscriptional gene silencing (PTGS) of L1 35GSU5, a standard reference
for systemic sense transgene PTGS in Arabidopsis [13], the ta siRNA pathway, and cell autonomous miRNA-directed repression [5]. In contrast to ago1-1, the hypomorphic allele ago1-27, which expresses an AGO1 protein with reduced mRNA cleavage activity, displays more subtle developmental defects [12].

Expression of ZLL is limited to the provasculature and, weaker, to the adaxial (upper) sides of leaves, and ceases as tissue differentiation takes place [14,15]. In the Landsberg erecta (Ler) accession, zll mutant seedlings display differentiated cells or complete organs in place of the shoot meristem stem cells with allele specific penetrance [14–16]. Recent studies indicate that ZLL function in the provascular is necessary and sufficient to maintain shoot meristem stem cells during embryogenesis [17]. Furthermore, ZLL acts in a sequential manner with AGO1 during embryogenesis to potentiate WUSCHEL (WUS) dependent signaling from the stem cell organizer to the stem cells in the developing shoot meristem primordium [17]. ago1 zll double mutants of strong alleles result in early embryony arrest, suggesting that both proteins also have redundant activities during early embryo development [14]. Recent findings demonstrated that both proteins function in miRNA-directed repression of Ca/Zn SUPEROXIDE DISMUTASE 2 (CSOD2) and SCARECROW-LIKE 6 (SCL6-IV) mRNAs and proteins [7]. In contrast to ago1 mutants, however, L1 transgene PTGS is not compromised in zll mutants [12].

Here, we address specific and overlapping functions of ZLL and AGO1 in development and RNA silencing pathways. Our results indicate that in ago1 hypomorphic mutants, loss of ZLL function restores leaf development and siRNA and miRNA pathways and leads to increased AGO1 protein levels, implicating ZLL as a negative regulator of AGO1. Analyses of chimeric gene constructs indicate that the PAZ domain, which is thought to mediate small RNA binding, is exchangeable between both proteins, whereas the MID-PIWI and N-terminal domains appear to contribute to their specific functions.

Results

zll suppresses leaf defects of ago1 hypomorphs

To study genetic interactions between ZLL and AGO1, we analyzed different mutant combinations. Since double mutants of strong zll and ago1 alleles in the Ler ecotype are embryo lethal [14], we analyzed mutant alleles in the Col ecotype, where ZLL loss of function alone does not greatly affect development (Figure 1A and Figure S1), unlike in the Ler accession, where shoot meristem stem cells are defective [14,15]. Despite the reduced effect of zll mutations in Col compared with Ler, ago1-1 zpp-1 double mutant embryos also arrested at the late globular stage with defects in cell division, cell elongation, and expression of both WOX5 and WUS genes, which mark root and shoot stem cell niches, respectively (Figure S2). None of these effects were observed in any single mutant, indicating redundant functions of ZLL and AGO1. To avoid embryo lethality obtained in double mutants with the null allele ago1-1 [6] and to enable the analysis of genetic interactions during postembryonic development, we used the hypomorphic ago1-27 mutant in combinations with zll and ago1-27 alleles. ago1-27 mutants are defective in small RNA-directed regulation [9,12] and, in contrast to the severe growth and developmental defects of ago1-1, display increased leaf margin serration, reduced leaf width, abnormal flower phyllotaxis, and reduced fertility compared to wildtype [12]. By contrast, seedlings of zll and single mutants did not display any noticeable leaf defects (Figure 1 and Figure S1A) [18] and only infrequently a defective shoot meristem (0.2%, n>1000) [14,15]. Surprisingly, ago1-27 zpp-1 and ago1-27 zlp-1 double mutants revealed that both zll mutations partially suppressed the increased leaf margin serration of ago1-27 (Figure 1B and Figure S1A), rather than enhancing it as we expected for two related AGO proteins involved in RNA silencing. By contrast, neither the phyllotaxis nor the fertility defects of ago1-27 were restored by the zll mutations (data not shown).

zll mutations restore transgene PTGS and miRNA-mediated gene silencing in hypomorphic ago1 mutants

To study ZLL and AGO1 interactions at the level of RNA silencing, we first analyzed PTGS of the L1 35S:GUS transgene. Our previous studies indicated that PTGS of the L1 35S:GUS transgene was compromised in ago3, rdr6, hen1, and ago1 mutants but not in zll single mutants [12,19,20]. The newly identified ago1-40 EMS mutation causes an A to V amino acid change at position 863 of the protein, resulting in increased mRNA levels and protein activity and decreased siRNA levels for the L1 35S:GUS transgene (Figure 2 and Table S1). Unlike previously identified ago1 mutations that impair L1 PTGS with 100% efficiency, about 50% of ago1-40 adult plants at each generation had triggered PTGS, allowing us to test whether zll mutations affected L1 PTGS in ago1-40. To avoid any potential interference between the 35S promoters embedded in the T-DNA of the available insertional zll mutants in Col and the L1 35S:GUS transgene [21], we backcrossed five times to L1 the EMS-induced zll-3 mutant, which was isolated in the Ler accession [15]. L1/zll-3 Col had similar GUS mRNA levels, protein activity and siRNA levels as silenced L1 controls (Figure 2) [12].

GUS mRNA levels in L1 ago1-40 zpp-1 Col had similar GUS mRNA levels, protein activity and siRNA levels as silenced L1 controls (Figure 2) [12].

GUS mRNA levels in L1 ago1-40 zll-3 Col double mutants were reduced in comparison to L1 ago1-40 mutants to nearly the level of silenced L1 controls (Figure 2). This increase in L1 silencing in the double mutant correlated with increased levels of GUS siRNAs. Seven days after germination (DAG), GUS siRNA levels were more than 10-fold higher than in L1 ago1-40 mutants, reaching levels comparable to silenced L1 controls by DAG, and by 15 DAG even exceeding L1 control levels (Figure 2). Thus, loss of ZLL function restored L1 gene silencing compromised in ago1-40.

To address whether zll mutations also were able to restore the miRNA pathway in ago1 hypomorphs, we analyzed miRNA levels and miRNA-regulated target genes in the ago1-27 zpp-1 double
mutants. miR398 levels were reduced and CSD2 mRNA and protein levels were substantially elevated in ago1-27 compared to zllago10-3 and zllago10-1 single mutants and wildtype (Figure 3A and Figure S3). By contrast, in both ago1-27 zllago10-3 and ago1-27 zllago10-1 double mutants, CSD2 mRNA and protein levels were reduced and miR398 levels were elevated, compared to ago1-27 alone (Figure 3A and Figure S3). The zllago10-3 mutation also restored miR164 accumulation and miR164-directed CUC2 silencing to wildtype levels in the ago1-27 background (Figure 3B). To extend our investigation to the whole-genome level, a transcriptome analysis was performed using Col wildtype, ago1, zll and ago1zll double mutants. AGO1 protein levels were increased in both ago1-27 zllago10-3 and ago1-40 zll-3-Col double mutants compared to the corresponding ago1 single mutants (Figure 4 and Figure S4). AGO1 mRNA and miR163 levels, however, were not significantly different (Figure 4). This indicates that ZLL is a negative regulator of AGO1 at the protein level, consistent with the role of ZLL in translational inhibition [7].

| Genotype          | n  | Depth of serration in mm± SD |
|-------------------|----|-----------------------------|
| Col               | 12 | 0.0±0a                      |
| zll ago10-1       | 20 | 0.0±0a                      |
| ago1-27           | 20 | 0.38±0.13b                  |
| ago1-27 zll ago10-1 | 20 | 0.1±0.12a                   |

The depth of the first two serrations of the oldest leaf was measured in 18-days-old plants. Differences are significant between a and b p<0.01 (ANOVA, Tukey). n = number of leaves analyzed.

Figure 1. Loss of ZLL function partially restores leaf development in ago1-27. (A) Null mutations in the ZLL gene result in seedlings lacking primary shoot apical meristems in Ler, whereas no seedling defects are discernible in the Col ecotype. All seedlings are 12 days after germination. Genotypes are indicated. Images are at the same magnification. (B) 18-day-old seedlings of Col, zllago10-1, ago1-27, and ago1-27 zllago10-1. Serration of leaves is reduced markedly in ago1-27 zllago10-1 compared to ago1-27, resulting in a rounded leaf shape similar to Col. Images are at the same magnification. Quantifications of the results with standard deviations are indicated.

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Roles of Arabidopsis ZLL and AGO1 Proteins

Protein sequence and specific expression patterns determine the functional differences between AGO1 and ZLL

To determine whether the specific effects of ago1 and zll mutations could be explained by the expression patterns of AGO1 and ZLL, we first compared the expression patterns of pZLL:YFP-ZLL and pAGO1:CFP-AGO1 reporter genes. Both reporter constructs rescued the corresponding mutants, indicating that the fusion proteins are functional (Table S3) [17]. YFP-ZLL and CFP-AGO1 proteins were detected in a largely overlapping punctuate pattern outside the nucleus of expressing cells (Figure 5E-5J). As previously reported, pZLL:YFP-ZLL is initially expressed throughout the embryo, but becomes limited to provascular strands and the adaxial side of the cotyledons at the globular stage (Figure 5A). By contrast, pAGO1:CFP-AGO1 is expressed in the whole embryo with the strongest signal in the provascular cells from globular stage to early torpedo stage (Figure 5C). Thus, ZLL and AGO1 expression patterns overlap partially, with the AGO1 expression pattern being broader than the one of ZLL, in agreement with miRNA localization results [14].

To evaluate the significance of the broad AGO1 expression pattern, we expressed AGO1 from the ZLL promoter and found that AGO1 could replace ZLL and vice versa. AGO1 expression from the ZLL promoter (pZLL:AGO1) rescued shoot meristem formation in the zll-1 mutant in the majority of cases (Table 2). By contrast, expression of ZLL from the AGO1 promoter (pAGO1-ZLL) in the strong ago1-1 allele resulted only in a slight reduction of leaf radialization compared to untransformed ago1-1 (Figure S6), but did not rescue any other developmental defect. Furthermore, in the ago1-27 hypomorph, pAGO1:ZLL was unable to rescue altered flowering time, reduced rosette size (Figure S7), L1 PTGS (Figure 6A and 6B) or CSD2 regulation (Figure 6C). Thus, whereas AGO1 can largely replace ZLL function in stem cell development, ZLL appears unable to efficiently replace the developmental, miRNA and PTGS functions of AGO1. Intriguingly, although pAGO1-ZLL did not restore CSD2 silencing in ago1-27, it fully restored miR398 accumulation to wildtype levels (Figure 6C). These results suggest that the intrinsic differences of AGO1 and ZLL proteins determine their specific contribution to small RNA and development pathways.

Next, we addressed whether differences within ZLL and AGO1 protein sequences are responsible for differences in their functions by analyzing whether AGO1 could replace ZLL and vice versa. AGO1 expression from the ZLL promoter (pZLL:AGO1) rescued shoot meristem formation in the zll-1 mutant in the majority of cases (Table 2). By contrast, expression of ZLL from the AGO1 promoter (pAGO1-ZLL) in the strong ago1-1 allele resulted only in a slight reduction of leaf radialization compared to untransformed ago1-1 (Figure S6), but did not rescue any other developmental defect. Furthermore, in the ago1-27 hypomorph, pAGO1:ZLL was unable to rescue altered flowering time, reduced rosette size (Figure S7), L1 PTGS (Figure 6A and 6B) or CSD2 regulation (Figure 6C). Thus, whereas AGO1 can largely replace ZLL function in stem cell development, ZLL appears unable to efficiently replace the developmental, miRNA and PTGS functions of AGO1. Intriguingly, although pAGO1-ZLL did not restore CSD2 silencing in ago1-27, it fully restored miR398 accumulation to wildtype levels (Figure 6C). These results suggest that the intrinsic differences of AGO1 and ZLL proteins determine their specific contribution to small RNA and development pathways.

The PAZ domain, but not the ZLL MID-PIWI- or N-terminal domains, is exchangeable between ZLL and AGO1 proteins

To address whether and if any ZLL and AGO1 protein domains have similar functions, we analyzed the ability of chimeric proteins composed of AGO1 and ZLL domains to rescue the respective mutant defects. As expected from the pZLL:AGO1 result, most chimeric ZLL:AGO1 proteins (where one AGO1 protein domain was embedded in a ZLL protein backbone) driven from the ZLL promoter rescued shoot meristem formation of the zll-1 mutant (Table 2). The marked exception was the AGO1 N-terminal domain (pZLL:AGO1N) that could not efficiently replace the corresponding ZLL N-terminal domain (Table 2). This finding was unexpected since the complete AGO1 protein largely replaced ZLL, and might indicate that the function of the N-terminal domain is sensitive to the correct protein context.

On the converse, only the ZLL PAZ domain within the AGO1 backbone (pAGO1:AGO1ZLLP) efficiently rescued developmental defects not only of the ago1-27 hypomorph (Figure 7L and Figure S7) but also of the null ago1-1 allele (Figure 7E, Table 1, and Figure S5). The ZLL:PAZ domain also largely restored L1 PTGS and GUS siRNA accumulation, and CSD2 silencing and miR398 accumulation in ago1-27 (Figure 6). PTGS restoration, however, was delayed compared to the developmental rescue (Figures 6A and 7), consistent with previous findings that PTGS is more sensitive than development to compromised AGO1 activity [12]. By contrast, replacing the N-terminal or MID-PIWI domains of AGO1 with the corresponding ZLL regions (pAGO1:AGO1ZLLN and pAGO1:AGO1ZLL MID-P) only restored bilateral leaf development but not sterility of ago1-1 mutants (Figure 7C, 7F, and 7G, Table 1, and Figure S5), or any developmental defects of ago1-27 mutants (Figure 7M and 7O and Figure S7). In addition, neither the N-terminal domain nor the MID-PIWI domains of ZLL were able to restore L1 PTGS and GUS siRNA accumulation or CSD2 silencing in ago1-27 (Figure 6). Since previous studies have indicated that PAZ, MID and PIWI domains function together in small RNA binding [11,22,23], we constructed a pAGO1:AGO1ZLL PAZ-PWI chimera where the AGO1 genomic region containing PAZ, MID and PIWI domains was replaced by the corresponding ZLL genomic sequence (Figure S8). pAGO1:AGO1ZLL PAZ-PWI resulted in similar effects as pAGO1:AGO1ZLL MID-PWI (Table 1, Figures 6 and 7, and Figure S7). This suggested that the failure of the ZLL MID-PIWI domains to restore the majority of ago1 defects was not due to an
incompatibility with the AGO1 PAZ domain or the disruption of the region connecting the PAZ and PIWI domains. Notably, although the pAGO1:AGO1ZLL PAZ-PIWI did not rescue CSD2 silencing, it restored miR398 accumulation in ago1-27 (Figure 6C).

In summary, these results indicate that the ZLL PAZ domain has the capacity to fulfill AGO1 functions in development, the miRNA pathway, and PTGS whereas the ZLL N-terminal and MID-PIWI domains are largely incompatible with AGO1 activity.

Discussion

As part of the small RNA-directed RNA silencing machinery, the closely related ZLL and AGO1 proteins fulfill important roles during Arabidopsis development. Previous studies of mutant phenotypes indicate the presence of both, redundant, specific, and even opposite functions of ZLL and AGO1. Here, we investigate the diversity of ZLL and AGO1 functions and show that ZLL acts as a negative regulator of AGO1, and that the activities of the two proteins are determined by both functionally equivalent and distinct domains.

Redundant functions of ZLL and AGO1

We find that double mutant combinations of strong zll and ago1 alleles are embryo lethal with strong patterning defects, revealed by abnormal expression of marker genes for the shoot and root meristem stem cell niche. This indicates that ZLL and AGO1 have a significant set of redundant functions required during early embryo development, in line with previous reports [14]. Although we have been unable to directly determine the small RNAs bound to ZLL due to the instability of the ZLL protein, we present several lines of indirect evidence suggesting that ZLL and AGO1 have partially redundant functions in small RNA-mediated silencing, and that ZLL domains are capable of binding a subset of small RNAs bound by AGO1: [1] Our protein domain swapping experiments indicate that the PAZ domain, which has been shown to bind small RNAs in several AGO proteins [11], is interchange-
Figure 4. Loss of ZLL function leads to increased AGO1 protein levels in *ago1* hypomorphic mutants. Immuno blot of AGO1 protein in seedlings of the indicated mutant lines and their Col control. Coomassie blue–stained RUBISCO small subunit (RbcS) serves as a loading control. Normalized values of AGO1 protein to RbcS controls (with Col controls set at 1.0) are indicated. Quantitative RT-PCR of *AGO1* mRNA in seedlings of the indicated mutant lines. Average values of three technical replicates were normalized to EF1α control values, and standard deviations are shown. RNA gel blot analysis of miR168 in seedlings of the indicated mutant and control lines. Normalized values of miR168 to U6 RNA (with Col controls set at 1.0) are indicated.
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Figure 5. Expression of AGO1 and ZLL reporter proteins in heart stage *Arabidopsis* embryos. (A–D) Epifluorescence images of Col seedlings showing expression of YFP-ZLL and CFP-AGO1 from the respective endogenous promoter and with swapped promoters as indicated. Images (A–D) are at the same magnification. (E–G) Confocal images of heart stage embryo cells expressing pZLL:YFP-ZLL outside the nuclei, which are marked by blue DAPI staining. (H–J) Confocal images showing overlapping expression of pZLL:YFP-ZLL (yellow) and pAGO1:CFP-AGO1 (colored red for clarity).
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Table 1. Restoration of leaf formation in ago1-1 seedlings expressing AGO1/ZLL chimeras.

| Plant Genotype | n  | Leaf Numbers ± SD |
|----------------|----|-------------------|
| Col            | 8  | 7.6 ± 0.9         |
| ago1-1         | 54 | 5.0 ± 0.2         |
| ago1-1/pAGO1:AGO1 | 15 | 7.1 ± 0.9         |
| ago1-1/pAGO1:AGO1 ZLL PAZ | 27 | 7.1 ± 1.0         |
| ago1-1/pZLL:AGO1 | 22 | 7.3 ± 0.7         |
| ago1-1/pAGO1:AGO1 ZLL PAZ-PIWI | 15 | 3.0 ± 1.0         |
| ago1-1/pAGO1:AGO1 ZLL-PIWI | 22 | 3.2 ± 1.3         |
| ago1-1/pAGO1:AGO1 ZLL N | 27 | 3.3 ± 0.9         |
| ago1-1/pAGO1:ZLL | 59 | 0.1 ± 0.4         |

Leaf numbers were counted 20 days after germination. Plants were germinated on 0.5xMS medium and then transferred to soil.

| n, number of analyzed plants from 2–3 independent primary transformants. SD, standard deviation. |

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able between ZLL and AGO1, providing fully active proteins, (2) miR5398 accumulation is restored to wildtype levels in an ago1 hypomorph by expression of pAGO1:ZLL, pAGO1:AGO1ZLL PAZ, pAGO1:AGO1ZLL MID-PIWI and pAGO1:AGO1ZLL PAZ-PIWI chimeras, and (3) both AGO1 and ZLL negatively regulate AGO1.

Opposing effects of ago1 and zll mutations

In addition to redundant functions of AGO1 and ZLL, our results using hypomorphic ago1 alleles to circumvent embryo lethality demonstrate opposing effects of ago1 and zll mutations. First, loss of ZLL function re-establishes both PTGS of the L1 transgene and miRNA-directed repression of a subset of target mRNAs deregulated in ago1-27, including miR398- and miR164 directed repression of their CSD2 and CUC2 targets, respectively. Furthermore, we observe partial suppression of hypomorphic ago1 leaf serration defects by zll mutations, which could be due to the partial re-establishment of miR164-directed CUC2 regulation in ago1 zll double mutants (Figures 1 and 3 and Figure S1) [24]. These opposite effects of ago1 and zll mutations are consistent with recent findings showing that mRNAs of leaf polarity-related HD-ZIP transcription factors and the corresponding mIR165/166 are affected oppositely in ago1 and in zll in single mutants (S. Bosca and T.L. unpublished) [9,10,25]. A plausible explanation for the restoration of developmental and RNA silencing defects caused by reduced AGO1 activity is provided by our finding that loss of ZLL activity results in upregulation of AGO1 protein levels in ago1-27. This negative regulation of AGO1 by ZLL suggests that homeostasis of AGO activity involves cross-regulation between different AGO proteins, which in the case of ZLL affects AGO1 protein but not mRNA levels, consistent with the recent implication of ZLL in translational repression [7]. Importantly, since ZLL expression itself is not a target of small RNA-mediated repression whereas AGO1 is [9,26], ZLL has the potential to provide an input into RNA silencing activity that is independent of negative feedback dynamics and thus might serve to mediate, for example, developmental tuning of RNA silencing.

However, silencing of all miRNA targets deregulated in ago1-27 is not restored by the absence of ZLL function. One possible explanation is that upregulation of AGO1 protein levels in ago1 zll double mutants does not restore AGO1 activity completely to wildtype levels, which might be required for efficient silencing of a subset of target genes. Alternatively, since the miRNA pathway is cell autonomous [27,28], the re-establishment of silencing of miRNA targets is expected to be limited to tissues where AGO1 and ZLL are co-expressed but will not take place in tissues where only AGO1 is expressed. This explanation is consistent with the ZLL:AGO1 analysis, where limiting AGO1 expression to the ZLL domain in ago1 mutants restored systemic L1 PTGS but did not fully restore miR398 accumulation and CSD2 repression. Future experiments comparing AGO1, ZLL and miRNA tissue-specific expression will help to discriminate between these two possibilities.

Determinants of specific AGO1 and ZLL activities

Even though the sequences of ZLL and AGO1 proteins are closely related, the corresponding single mutants display different developmental defects. The pleiotropic ago1 mutants are defective in leaf morphology, general growth, and fertility, whereas zll mutants in the Ler accession display specific developmental defects in shoot apical meristem, flower, and silique development with allele specific penetrance. In contrast to the interchangeable PAZ domain, the non-conserved N-terminal domains, for which a function has yet to be assigned, cannot be exchanged between AGO1 and ZLL without loss of activity. Similarly, exchange of the MID and PIWI domains, which in AGO1 have been shown to provide selectivity for small RNAs possessing a 5’ U [22] and to function as a slicer domain that cleaves mRNA, respectively [3,4], also cannot provide fully active proteins. This indicates that these domains contribute to functional differences. It is possible that the inability of the ZLL MID-PIWI fragment to replace the AGO1 domains reflects different preferences for 5’ nucleotide selectivity. Since the consensus amino acid residues essential for mRNA cleavage in several AGO1 proteins [29] are present in the ZLL PIWI domain, it is conceivable that both AGO1 and ZLL have the capacity to silence via mRNA cleavage and translational inhibition, but that each protein has a different preference for one of the two mechanisms, in line with recent findings [7].

Future dissection of AGO1 and ZLL properties will help to reveal how the interplay between AGO1 and ZLL proteins influences silencing specificity and efficiency in development.

Materials and Methods

Plant material

The following mutants in the Col ecotype have been described previously: ago1-1 [6], ago1-27 [12], and zll-1 [18], ago1-27 zll-15 mutants were generated using the zll-15 mutant (SALK_519798), which expresses a mis-spliced transcript that lacks part of exons 13 and 14 creating a frame-shift mutant with much milder than ago1-27 [12] zll-1, zll-3, and zll-15 mutants were isolated in the Ler accession as described [15]. Plants on soil were grown as described previously [30]. Plants on agar plates were grown on 1/2xMS supplemented with Gamborg vitamins (Sigma) and 10 g/l sucrose if indicated.

RNA analysis and GUS activity quantification

For RNA gel blot analyses, frozen tissue was homogenized in a buffer containing 0.1 M NaCl, 2% SDS, 50 mM Tris-HCl (pH 9), 10 mM EDTA (pH 8) and 20 mM beta mercaptoethanol and RNAs were extracted two times with phenol. RNA gel blot analyses and quantification of GUS activity were performed as described [31]. Hybridization signals were quantified using a Fuji phosphor imager and normalized to a U6 oligonucleotide probe.
Figure 6. Restoration of L1 PTGS in ago1-27 by AGO1<sup>ZLL</sup> chimeras. (A) Average GUS protein activity (nmol MU/min/μg protein) in 7-day-old (white bars), 15-day-old (black bars), and 21-day-old (hashed bars) seedlings of L1 and L1/ago1-27 and L1/ago1-27 harboring the different chimeric AGO1<sup>ZLL</sup> genes as indicated. For all constructs, 2–3 independent homozygote T3 lines and a minimum of 12 plants per line were analyzed. Standard deviations are shown. nd, not determined in 21-day-old seedlings. Dev. rescue, Developmental rescue; Y, yes; N, no. (B) Northern blot analyses of GUS mRNA and siRNA accumulation in 7- and 15-day-old seedlings of L1, L1/ago1-27, and L1/ago1-27 lines harboring the different chimeric AGO1<sup>ZLL</sup> genes as indicated. At least two biological replicates are shown for each chimera at each time point. 25S rRNA and U6 RNA were used as a loading control for mRNAs and siRNAs, respectively. (C) Northern blot analyses of miR398 and immuno blot analysis of CSD2 protein accumulation in 15-day-old seedlings of L1, L1/ago1-27 and L1/ago1-27 lines harboring the different chimeric AGO1<sup>ZLL</sup> genes, as indicated. Normalized values of miR398 and CSD2 protein to U6 and RbcS controls, respectively, (with L1 controls set at 1.0), are indicated. At least two biological replicates are shown for each chimera. U6 RNA and coomassie blue–stained RUBISCO small subunit (RbcS) serve as loading controls.

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GUS mRNA and GUS activity analyses were performed on the aerial parts of 7-day-, 15-day- and 21-day-old seedlings grown on Bouturage media (Duchefa) in 16 hours light, 8 hours dark at 22°C. For the CSD2 and miR398 analyses, seeds were germinated on media [32] without sucrose in both the presence and absence of 0.5 μM CuSO4, and plants were grown in 16 hours light, 8 hours dark at 22°C for 12 days at which time the aerial portion of the seedlings were harvested and homogenized in liquid nitrogen. For the CUC2, miR164, AGO1 and miR168 analyses, plants were grown for 10 days on media [32] in the presence of 0.5 μM CuSO4. For cDNA synthesis, RNAs were extracted with the Plant RNeasy kit (Qiagen), treated with DNAseI (Invitrogen) and 1 μg of DNA-free RNA was reverse transcribed with oligo-dT (Invitrogen). Quantitative real time (QRT)-PCR, was performed on a MasterCycler ep realplex (Eppendorf) with the RealMAster SYBR ROX mix (5PRIME) according to the manufacturer’s protocol. Each reaction was performed on 5 μl of 1:60 dilution of the cDNA and synthesized in a 20 μl total reaction. Specific oligonucleotide pairs were: EF1a: 5’- CTGGAGGTTTTGAGGCTGGTAT -3’;

Table 2. Frequency of shoot meristem defects in zll-1 seedlings expressing ZLL/AGO1 chimeras.

| Plant Genotype                  | n  | Seedlings Lacking a Shoot Meristem (%) ± SD |
|--------------------------------|----|------------------------------------------|
| zll-1/empty vector             | 7  | 81 ± 8.9a                                |
| zll-1/pZLL:ZLL                 | 7  | 0.0 ± 0.0b                               |
| zll-1/pZLL:AGO1                 | 14 | 22 ± 18.6c                               |
| zll-1/pZLL:AGO1 PAZ            | 8  | 2.1 ± 4.2b                               |
| zll-1/pZLL:AGO1 MID-PIWI        | 8  | 2.5 ± 7.1b                               |
| zll-1/pZLL:AGO1 N              | 8  | 64 ± 6.5c                                |

The fraction of 12-day-old seedlings lacking a shoot meristem is indicated based on >50 seedlings for each transgenic line.

*Seedlings displaying a phenotype suggestive of AGO1 cosuppression are not included.

a,b,c,dDifferences are significant between a, b, and c, respectively (ANOVA, Tukey).

n, number of independent transgenic lines analyzed.

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Figure 7. Rescue of ago1 seedling defects by AGO1ZLL chimeras. (A–C,J) Wildtype Col and ago1 mutant controls. (D–H) ago1-1 plants harboring chimeric constructs as indicated. (K–P) ago1-27 plants harboring chimeric constructs as indicated. Images are at the same magnification. 12-days-old plants are shown.

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Protein extraction and immuno blotting

Protein was extracted in buffer containing 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 10% glycerol, Sigma Protease Inhibitor (CSD2) or 20 mM Tris-HCl pH 7.5, 300 mM NaCl, 5 mM MgCl2, 0.1% IGEPAL CA-630, 5 mM DTT, Sigma Protease Inhibitor (AGO1). Protein concentrations were determined using the Quick-Amp One-Color Labelling Kit (Agilent Technologies) and hybridized to Agilent custom microarrays. Three replicates were performed for each genotype.

Microscopy and image analysis

For fluorescence studies, embryos where dissected from ovules using fine tip syringes in 10% glycerol, mounted on slides and analyzed using an AxioImager microscope (Zeiss) with YFP or CFP filter sets. Images were taken using Axiovision 4.4 software (Zeiss) and figures were generated using Photoshop 7.0 (Adobe). For confocal pictures, a Leica TCS SP2 AOSB spectral confocal microscope was used. Embryos were stained with DAPI (1 mg/ml) for 5 minutes and mounted in 50% glycerol in 1×PBS.

Construction of fluorescent protein genes and chimeric genes

All AGO1 and ZLL sequences for both the fluorescent protein fusion and chimeric constructs are derived from the Col accession. AGO1 and ZLL chimeric constructs were made by exchanging five genomic domains; the 5’ sequence upstream of the ATG, the N-terminal, PAZ and the MID-PIWI domains and the 3’ region downstream of the stop codon. For cloning, restriction sites were introduced within introns at the appropriate positions (Table S4 and Figure S8). During the course of this work, we re-sequenced the ZLL Ler gene and several new ZLL cDNA clones and found that the original report of six amino acid differences between the ZLL Col and ZLL Ler proteins [15] was in error. The ZLL Ler amino acid sequence is identical to that of ZLL in Col, as previously published [14].

Supporting Information

Figure S1 Loss of ZLL function partially restores leaf development in ago1-27. (A) 20-day-old seedlings of Col and zllago10-3 and 24-day-old seedlings of ago1-27, ago1-27 zllago10-3 and ago1-27 zllago10-3. Serration of leaves is reduced markedly in both ago1-27 zllago10-3 and ago1-27 zllago10-3 compared with ago1-27. (B) RT-PCR was performed on RNA from Col and zllago10-3 inflorescences. Tubulin was used as control. Quantitative RT-PCR was performed on RNA from Col and zllago10-3 12-day-old seedlings. Values were normalized to EF1a control.

Found at: doi:10.1371/journal.pgen.1000646.s001 (1.91 MB TIF)

Figure S2 ZLL and AGO1 act redundantly in embryogenesis. (A–D) Late globular stage. The formation of the lens-shaped cell [scar marked in blue] is disturbed in segregating embryos of ago1-1/ago1-1 zllago10-3 plants where the hypophysis divides longitudinally instead of transversely, compared to wildtype and single mutants. Provascular cells stay isodiametric and do not elongate in putative double embryos (marked in red) compared to wildtype and single mutants. (E–H) Late torpedo stage. Putative double mutant embryos arrest development without initiating organs as spherical structures. Segregating ago1-1 embryos display a broader apex and a wider angle between the cotyledons than wildtype or zll/mutants. (I–L) Expression of WOX5-NLS-GFP in the QC cells and the upper suspensor cells is undetectable in putative double mutant embryos in contrast to wildtype and single mutants. (M–P) Expression of gWUS::GFP53 in the organizing center of the shoot meristem is expanded in putative double mutant embryos in comparison to wildtype and single mutants. Genotype of the mother plants and the percentage of embryos displaying the given phenotype are indicated. N: number of analyzed embryos; scale bar: 10 µm.

Found at: doi:10.1371/journal.pgen.1000646.s002 (2.07 MB TIF)

Figure S3 zll mutations enhance miR398-directed silencing of CSD2 when AGO1 function is partially compromised. (A) RNA gel blot analysis of miR398 in the indicated mutant and control lines in the presence (0.5 µM) or absence (0 µM) of CuSO4. U6 hybridization was used as a loading control. Normalized values of miR398 to U6 RNA (with Col controls set at 1.0) are indicated. Immuno blot of CSD2 protein in the indicated mutant lines and their Col control in the presence (0.5 µM) or absence (0 µM) of CuSO4. Coomassie blue-stained RUBISCO small subunit (RbcS) serves as a loading control. (B) Quantitative RT-PCR of CSD2 mRNA in the indicated mutant lines in the presence (0.5 µM, black bars) of CuSO4. Average values of three technical replicates were normalized to EF1a control values and standard deviations are shown.

Found at: doi:10.1371/journal.pgen.1000646.s003 (0.22 MB TIF)

Figure S4 Loss of ZLL function in ago1 hypomorphic mutants increases AGO1 protein levels. Immuno blot of AGO1 protein in inflorescences of the indicated mutant lines and their Col control. Biological replicates are shown for ago1-40 zllago10-3 and ago1-40.
Coomassie blue-stained RUBISCO small subunit (RbsS) serves as a loading control. 

Found at: doi:10.1371/journal.pgen.1000646.s004 (0.12 MB TIF)

**Figure S5** Rescue of ago1 defects by AGO1ZLL chimeras. 40-day-old wildtype Col, ago1-27 and ago1-1 mutant plants harboring chimeric constructs as indicated. 

Found at: doi:10.1371/journal.pgen.1000646.s005 (4.55 MB TIF)

**Figure S6** ago1-1 seedlings expressing pAGO1-ZLL. (A) 20-day-old seedlings of ago1-1 and ago1-1/pAGO1-ZLL. Radialization of the leaves is reduced in ago1-1/pAGO1-ZLL compared to ago1-1. 

(B) Quantification of leaf length per width in ago1-1 and ago1-1/pAGO1-ZLL seedlings. n, number of analyzed plants. Plants were grown on 0.5×MS medium supplemented with 1% sucrose and Gamborg vitamins and then transferred to soil, which allows for limited leaf development in the ago1-1 mutant. 

Found at: doi:10.1371/journal.pgen.1000646.s006 (0.92 MB TIF)

**Figure S7** Rescue of ago1 defects by AGO1ZLL chimeras. (A) The flowering times of Col, ago1-27, and ago1-27 harboring the indicated constructs. Two to three independent lines per construct and eight plants per line were analyzed. (B) The rosette diameter of 24-day-old plant. Har Col, ago1-27, and ago1-27 harboring the indicated constructs. Two to three independent T2 lines for each construct were analyzed. Standard deviations are given. The blue and orange dashed lines indicate the rosette diameter of Col and ago1-27, respectively. The dark grey and light grey boxes indicate rescued and non-rescued development, respectively. 

Found at: doi:10.1371/journal.pgen.1000646.s007 (0.30 MB TIF)

**Figure S8** Schematic of chimeric AGO1 and ZLL constructs. (A) The fragments used and the introduced restriction sites are shown. Functional domains are indicated. (B) Genomic organization of the ZLL gene. (C) Genomic organization of the AGO1 gene.

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