Accurate Chromosome Segregation at First Meiotic Division Requires AGO4, a Protein Involved in RNA-Dependent DNA Methylation in *Arabidopsis thaliana*

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ABSTRACT The RNA-directed DNA methylation (RdDM) pathway is important for the transcriptional repression of transposable elements and for heterochromatin formation. Small RNAs are key players in this process by regulating both DNA and histone methylation. Taking into account that methylation underlies gene silencing and that there are genes with meiosis-specific expression profiles, we have wondered whether genes involved in RdDM could play a role during this specialized cell division. To address this issue, we have characterized meiosis progression in pollen mother cells from *Arabidopsis thaliana* mutant plants defective for several proteins related to RdDM. The most relevant results were obtained for *ago4-1*. In this mutant, meiocytes display a slight reduction in chiasma frequency, alterations in chromatin conformation around centromeric regions, lagging chromosomes at anaphase I, and defects in spindle organization. These abnormalities lead to the formation of polyads instead of tetrads at the end of meiosis, and might be responsible for the fertility defects observed in this mutant. Findings reported here highlight an involvement of AGO4 during meiosis by ensuring accurate chromosome segregation at anaphase I.

KEYWORDS AGO4; *Arabidopsis thaliana*; centromere; meiosis; RdDM

RNA-DIRECTED DNA methylation (RdDM) confers transcriptional repression in all sequence contexts (Matzke *et al.* 2009; Law and Jacobsen 2010). In this specialized RNA-interference pathway, the base pairing between 24-nt, small-interfering RNAs (siRNAs) and nascent scaffold transcripts directs the DNA methylation complex to target loci (Law and Jacobsen 2010; Matzke and Mosher 2014). In *Arabidopsis thaliana*, the two catalytic subunits of RNA polymerase IV (Pol IV), NRPD1A and NRPD1B, generate single-strand RNAs which serve as templates for RNA-dependent RNA polymerase 2 to produce double-strand RNAs (dsRNAs). These dsRNAs are subsequently cleaved by DICER-LIKE 3 (DCL3)

into 24-nt siRNAs, exported to the cytoplasm, and loaded onto AGO4, AGO6, or AGO9 (Matzke *et al.* 2009; Law and Jacobsen 2010; Zhang and Zhu 2011; Pikaard *et al.* 2012; Ye *et al.* 2012). Afterward, these complexes are imported back to the nucleus to target transcripts generated by Pol V at the same loci, before they are released from the chromatin (Wierzbicki *et al.* 2008, 2009; Liu *et al.* 2014). In addition to the sequence complementarity between the 24-nt siRNAs and the nascent Pol V transcripts, Pol V subunit NRPE1 possesses an AGO4-binding motif (known as the AGO hook), located in the carboxy-terminal region (El-Shami *et al.* 2007). AGO4 is then able to recruit the DNA methyltransferase DOMAINS REARRANGED METHYLTRANSFERASE 2 (DRM2) to establish *de novo* DNA methylation (for a detailed characterization of this pathway see Bologna and Voinnet 2014; Borges and Martienssen 2015).

RdDM affects transcription of transposons and repeated DNA elements through *de novo* methylation of cytosines in all sequence contexts (CG, CHG, and CHH contexts; where H denotes A, T, or C). In this DNA methylation, the methyltransferases DRM1 and DRM2 play a key role (Cao and Jacobsen 2002a; Henderson *et al.* 2010; Law and Jacobsen 2010). However, other methyltransferases, such as CHROMOMETHYLTRANSFERASE

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Figure 1 Meiotic stages in PMCs from *Ler*, *ago4-1*, and *ago4-2*. (A, B, F, J, N, R, V) Representative images from *Ler* PMCs. (C, D, G, H, K, L, O, P, S, T, W, X) Representative images from *ago4-1* PMCs. (E, I, M, Q, U, Y) Representative images from *ago4-2* PMCs. (A, C, E) Pachytene. (B) Enlarged centromeric and pericentromeric regions from A. (D) Enlarged centromeric and pericentromeric regions from B. (F–I) Diplotene. (J–M) Metaphase I. (N–Q) Anaphase I. Red arrows indicate (O) a chromosome bridge, (P) delayed segregation of homologous chromosomes, and (Q) lagging chromosomes. (R–U) Metaphase II. Red arrows indicate laggards in S, T, and U. (V–Y) Tetrads. Red arrows indicate chromatin accumulation in W and some micronuclei in X and Y. Bar, 5 μ m. (Z) Analysis of chromosome condensation at metaphase I and polyad formation in *ago4-1* and *ago4-2*. Differences were observed in both mutants compared to the respective WT backgrounds. ** $P < 10^{-2}$, *** $P < 10^{-3}$.

3 (CMT3) and DNA METHYLTRANSFERASE 1 (MET1) are involved in CHG and CG methylation maintenance, respectively (Jones *et al.* 2001; Cao and Jacobsen 2002b; Cao *et al.* 2003). Furthermore, CHG methylation, previously established by DRM2, is recognized by the H3K9 histone methyltransferase

KRYPTONITE (KYP), reinforcing the repressed chromatin state of methylated DNA (Cao *et al.* 2003; Sasaki *et al.* 2012).

On these grounds, we have wondered whether genes involved in siRNA biogenesis and RdDM could be important during meiotic division in *A. thaliana*. In fission yeast and

animals, the regulation of histone methylation is necessary for meiotic recombination (Wahls *et al.* 2008; Acquaviva *et al.* 2013; Crichton *et al.* 2014). In maize, the absence of proteins involved in DNA methylation leads to alterations in sporogenesis and megagametogenesis (García-Aguilar *et al.* 2010). In this species, defective mutants for *AGO104* (ortholog of *AGO9*) display an apomixis-like phenotype, revealing that this gene is essential during meiosis (Singh *et al.* 2011). In *A. thaliana*, it has been reported that methylation status might influence meiotic homologous recombination (HR). The hypomethylated *decrease in DNA methylation 1* (*ddm1*) and *met1* mutant plants display a total number of reciprocal genetic exchanges or crossovers (COs) similar to wild-type (WT) plants. However, they show differences in the recombination frequency along chromosomes with respect to WT plants (Melamed-Bessudo and Levy 2012; Mirouze *et al.* 2012; Yelina *et al.* 2012). Additionally, plants defective for *AGO9*, a protein from the same phylogenetic clade as *AGO4* which is expressed in the germline, show a slight increase in the mean chiasma frequency per cell with respect to WT plants (Oliver *et al.* 2014). Here we present results that reveal the influence of a protein required for RdDM, *AGO4*, on chromatin organization at both centromeric and pericentromeric regions; highlighting its importance in ensuring an accurate chromosome segregation.

Materials and Methods

Plant materials

All the mutants evaluated are in Col background except *ago4-1* which is in *Ler* (Zilberman *et al.* 2003). *ago4-2* is a dominant mutation resulting from the substitution of a Glu at position 641 (located inside the PIWI domain) by a Lys (Agorio and Vera 2007). Mutant seeds were kindly donated by Pablo Vera (Universidad de València, Spain). The remaining mutants correspond to transfer-DNA (T-DNA) insertion lines and they were obtained from Salk Institute Genomic Analysis Laboratory and provided by the Nottingham *Arabidopsis* Stock Centre (Alonso *et al.* 2003). In this work, we have analyzed the following single mutants: *ago4-1*, *ago4-2*, *ago4-1t*, *ago6-2*, *dcl3-1*, *kyp-4*, *npr1a-8*, and *nrpe1-11*; and the triple mutants: *cmt3-11 drm1-2 drm2-2*, *met1-3 drm1-2 drm2-2*, and *kyp-6 drm1-2 drm2-2*. Plants were cultivated on a soil mixture of vermiculite and commercial soil (3:1) and grown in a greenhouse under a 16 or 8 hr light/dark photoperiod, at 18–20° with 70% humidity. Primers listed in Supplemental Material, Table S1 and primers from the T-DNA left border sequence, LBB1.3 (5'ATTTTGCCGATTCGGAAC3', for SALK lines) or LB2 (5'GCTTCCTATTATATCTTCCCAAATTACCAATACA3' for SAIL lines), were used for genotyping.

Seeds from *Ler* and *ago4-1* plants ($n = 215$ and $n = 218$, respectively) were put on plates containing germination medium (GM) to assess the germination percentage. The number of germinated seeds in each background was evaluated at 9, 11, 13, and 18 days after sowing.

Table 1 Percentage of metaphases I with decondensed chromosomes

Mutant	Metaphases I with decondensed chromosomes (%)	<i>n</i>
<i>kyp-4</i>	4	137
<i>ago6-2</i>	6	109
<i>kyp6-1 drm1-2 drm2-2</i>	9	66
<i>dcl3-1</i>	22	105
<i>ago4-2</i>	27	177
<i>nrpe1-11</i>	44	71
<i>ago4-1</i>	47	121

n, number of cells analyzed.

Cytological analysis

Pollen viability was quantified by Alexander (1969) staining with some modifications (Peterson *et al.* 2010). Fixation of flower buds, slide preparations, and fluorescence *in situ* hybridization (FISH) were performed according to Sánchez-Morán *et al.* (2001). The following DNA probes were used: pTa71 [45S ribosomal DNA (rDNA); Gerlach and Bedbrook 1979], pCT4.2 (5S rDNA; Campbell *et al.* 1992), pAL1 (centromeric DNA repeat; Martínez-Zapater *et al.* 1986), and pLT11 (telomeric DNA repeat; Richards and Ausubel 1988). Chromosome preparations for subsequent immunolocalizations of histone H3 modifications, centromeric histone H3 variant (CENH3), and α -tubulin were obtained by a squash technique as described by Manzanero *et al.* (2000), with minor modifications (Oliver *et al.* 2013). ASY1, ZYP1, RAD51, and DMC1 proteins were immunodetected according to the spreading protocol described by Armstrong *et al.* (2002) (Table S2). Secondary antibodies were FITC conjugated (1:50; Sigma Chemical, St. Louis, MO) and Cy3 conjugated (1:300, Sigma). Immunolocalization of 5-methyl cytosine (5mC) was previously described in Oliver *et al.* (2014).

Sensitivity to γ -rays

Seeds from *Ler* and *ago4-1* were surface sterilized in 2.5% sodium hypochlorite solution for 5 minutes. After three washes in sterile water and overnighting at 4°, the seeds were exposed to 150, 300, and 500 Gy (2.94 Gy/min) from a ¹³⁷Cs source (IBL-437C, CIS Bio International) and sown on GM plates. The number of true leaves and the fresh weight of the seedlings were recorded 14 days after treatment.

Quantitative PCR

Expression analyses were performed as previously described by Pradillo *et al.* (2012). Details of the primers and probes used are shown in Table S3. Fold variation was considered over a calibrator using the $\Delta\Delta C_t$ method (Livak and Schmittgen 2001).

Statistical analyses

Statistical analyses used in this work were performed using the software SPSS Statistics 17.0.

Data availability

Table S1 contains the sequences of the primers used for genotyping. Information about the antibodies used is provided in

Table 2 Mean chiasma frequencies per cell, per bivalent, and per bivalent arm (short vs. long) in *Ler*, *Col*, and mutants analyzed in this study

	Bivalents										C	n
	1 ^a		2		3		4		5			
	s	l	s	l	s	l	s	l	s	l		
Ler	—	—	0.56	1.02	0.97	1.00	0.52	0.95	0.86	1.16	9.38	63
	2.35	(0.25)	1.57	(0.17)	1.97	(0.21)	1.48	(0.16)	2.02	(0.21)		
ago4-1	—	—	0.62	0.90*	0.74***	1.05	0.60	0.88	0.84	1.07	8.84**	90
	2.13*	(0.24)	1.52	(0.17)	1.80*	(0.20)	1.48	(0.17)	1.91	(0.22)		
Col	—	—	0.61	1.14	0.90	1.26	0.48	1.01	0.97	1.30	10.20	69
	2.52	(0.25)	1.75	(0.17)	2.16	(0.21)	1.49	(0.15)	2.28	(0.22)		
nprp1a-8	—	—	0.78*	1.10	0.97	1.17	0.67*	1.05	1.00	1.32	10.68*	60
	2.63	(0.25)	1.88	(0.18)	2.13	(0.20)	1.72*	(0.16)	2.32	(0.22)		
kyp-4	—	—	0.80*	1.11	0.97	1.13	0.63	1.08	1.00	1.31	10.53	75
	2.51	(0.24)	1.91	(0.18)	2.11	(0.20)	1.71*	(0.16)	2.31	(0.22)		
ago6-2	—	—	0.80*	1.12	0.86	1.10*	0.74**	1.16	1.00	1.22	10.34	50
	2.34	(0.23)	1.92	(0.19)	1.96*	(0.19)	1.90**	(0.18)	2.22	(0.21)		
kyp-6 drm1-2 drm2-2	—	—	0.76	1.13	0.97	1.13	0.52	1.13	0.96	1.28	10.34	67
	2.37	(0.23)	1.90	(0.18)	2.18	(0.21)	1.66	(0.16)	2.24	(0.22)		
met1-3 drm1-2 drm2-2	—	—	0.79*	1.09	0.97	1.21	0.56	1.15	1.00	1.12*	10.29	34
	2.35	(0.23)	1.88	(0.18)	2.18	(0.21)	1.71	(0.17)	2.12	(0.21)		
dcl3-1	—	—	0.80*	1.07	0.92	1.12	0.69**	1.03	0.96	1.25	10.27	75
	2.43	(0.24)	1.87	(0.18)	2.04	(0.20)	1.72*	(0.17)	2.21	(0.22)		
nrpe1-11	—	—	0.75	1.15	0.91	1.11*	0.61	1.11	0.98	1.12**	10.14	66
	2.39	(0.24)	1.91	(0.19)	2.02	(0.20)	1.71*	(0.17)	2.11*	(0.21)		
cmt3-11 drm1-2 drm2-2	—	—	0.62	1.04	0.94	1.10*	0.60	1.02	0.96	1.19	9.88	52
	2.42	(0.24)	1.65	(0.17)	2.04	(0.21)	1.62	(0.16)	2.15	(0.22)		
ago4-2	—	—	0.78*	0.97**	0.86	1.04**	0.67*	1.03	0.99	1.10**	9.61**	69
	2.17***	(0.23)	1.75	(0.18)	1.90**	(0.20)	1.70*	(0.18)	2.09*	(0.22)		

The values in parentheses are the bivalent chiasma frequencies as proportions of the total cells. s, short arm; l, long arm; C, mean cell chiasma frequency; n, number of cells.

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

^a Chromosome 1 is considered as a whole because it is not possible to distinguish the chromosome arms.

Table S2. Table S3 contains the sequences of the primers used in the expression analyses and numbers corresponding to the TaqMan probes (Roche).

Results

Plant fertility and seed germination in mutants of genes related to RdDM

We have analyzed the following single mutants of genes related to RdDM: *ago4-1* (knockout, *Ler* background), *ago4-1t*, *ago4-2* (knockdown, *Col* background), *ago6-2*, *dcl3-1*, *kyp-4*, *nprp1a-8* (defective for one of the catalytic subunits of Pol IV), and *nrpe1-11* (defective for the largest subunit in Pol V). We have also examined triple mutants defective for different methyltransferases: *cmt3-11 drm1-2 drm2-2*, *met1-3 drm1-2 drm2-2*, and *kyp-6 drm1-2 drm2-2*. Only the single mutant *ago4-1* and the triple mutant *met1-3 drm1-2 drm2-2* showed reduced fertility. The semisterility of the triple mutant may be explained by defects in gametogenesis and embryo viability (Saze *et al.* 2003; Xiao *et al.* 2006). However, in *ago4-1* we detected interplant variation in the number of nonviable pollen grains (2–21%), suggesting meiotic defects (five flowers analyzed; Figure S1), and also a reduction in the percentage of germinated seeds with respect to WT plants: 46.38 vs. 98.13% (18 days after sowing, $t = 14.77$,

$P < 1 \times 10^{-3}$; Figure S2). Furthermore, a proportion of *ago4-1* seeds (20.4%) showed smaller size and dehydrated appearance, 2.3% presented two root apical meristems (RAMs), and 0.9% did not show any RAM ($n = 216$; Figure S2).

Cytological analysis of meiosis

Pollen mother cells (PMCs) from *ago4-1* plants displayed the highest number of meiotic alterations among all mutants analyzed, as follows: (i) A different chromatin compaction at centromeric and pericentromeric regions at pachytene (Figure 1, A and C). In WT PMCs there are two strongly DAPI-stained regions per bivalent, corresponding to pericentromeric regions that flank the centromere, which is faintly DAPI stained (Figure 1B). However, in *ago4-1*, centromeric and pericentromeric regions were not clearly distinguished because they showed a similar DAPI staining intensity (Figure 1D). (ii) Chromosome decondensation from diplotene to metaphase I (Figure 1, G, H, and K); 47% of meiocytes at metaphase I ($n = 121$) showed this feature (Table 1). In addition, some bivalents displayed centromeric regions with forked appearance (Figure 1L). (iii) Presence of interchromosomal bridges (Figure 1O), lagging chromosomes (Figure 1P), and occasional fragments at anaphase I (Figure 1, S and T). The percentage of these cells was ~30% ($n = 26$). However, this percentage could be

overestimated, since defects on chromosome segregation might increase the duration of this stage. (iv) Presence of chromatin accumulations and isolated chromosomes at late telophase II. These defects are responsible for the formation of polyads in 47.7% of cells analyzed ($n = 300$; Figure 1, W and X).

The mutant *ago4-2* showed meiotic alterations similar to those described in *ago4-1*, although to a minor extent: slight differences in the conformation of centromeric and pericentromeric regions at pachytene with respect to WT; chromosome decondensation from diplotene to metaphase I, exhibiting 27% of decondensed metaphases I ($n = 177$; Table 1; Figure 1, I and M); interchromosomal bridges and lagging chromosomes at anaphase I that originate the abnormalities observed at metaphase II (Figure 1, Q and U); and ~3% of polyads ($n = 300$; Figure 1Y). Female meiosis was apparently normal in both *ago4* mutants (Figure S3), but the results are not conclusive due to the low number of cells analyzed.

The mutants *dcl3-1*, *ago6-2*, *nprp1-11*, *kyp-4*, and *kyp-6 drm1-2 drm2-2* also displayed chromatin decondensation in a percentage of diplotene-metaphase I PMCs (Table 1; Figure S4). Interchromosomal anaphase I bridges were observed in *ago6-2*, *kyp-6 drm1-2 drm2-2*, and *kyp-4* (Figure S4, F–H), but, in contrast to *ago4-1* and *ago4-2*, chromosome fragments and isolated chromosomes were absent. Finally, meiosis seemed to be cytologically normal in *nprp1a-8*, *ago4-1t* (mutant with the T-DNA insertion into the promoter), *cmt3-11 drm1-2 drm2-2*, and *met1-3 drm1-2 drm2-2*.

Chiasmata were scored according to previously established criteria (Sánchez-Morán *et al.* 2001, 2002). Between one and three plants per mutant were analyzed to estimate mean cell chiasma frequencies at metaphase I. Only three mutants showed significant differences in this parameter with respect to WT plants: *ago4-1* and *ago4-2* exhibited a significant decrease ($t = 2.92$, $P = 4 \times 10^{-3}$ and $t = 3.11$, $P = 2 \times 10^{-3}$, respectively), whereas *nprp1a-8* presented a significant increase ($t = 2.44$, $P = 16 \times 10^{-3}$). The remaining mutants analyzed displayed a general tendency toward a slight, although nonsignificant, increase of this parameter (Table 2). At the chromosome level, there was a significant increase of chiasma frequency in the short arms of chromosomes 2 (*nprp1a-8*, *kyp-4*, *ago6-2*, *met1-3 drm1-2 drm2-2*, *dcl3-1*, and *ago4-2*) and 4 (*nprp1a-8*, *ago6-2*, *dcl3-1*, and *ago4-2*). By contrast, *ago4-1* and *ago4-2* showed a decrease in chiasma frequency in both chromosome 1 and the long arm of chromosome 2. Regarding chromosome 3, there was a significant reduction in the short arm (*ago4-1*) and in the long arm (*ago6-2*, *nprp1-11*, *cmt3-11 drm1-2 drm2-2*, and *ago4-2*). Finally, *met1-3 drm1-2 drm2-2*, *ago4-2*, and *nprp1-11* displayed a decrease in the long arm of chromosome 5.

Since the most relevant results obtained, from a meiotic point of view, are those that referred to *ago4* mutants, particularly *ago4-1*, we decided to perform a more accurate study based on the following issues: (i) characterization of centromeric and pericentromeric regions, (ii) histone modifications

Figure 2 FISH to detect centromeres (pAL1) and telomeres (PLT11) at pachytene. (A, D) WT. (B, E) *ago4-1*. (C, F) *ago4-2*. Centromeres are showed in green and telomeres in red. White arrows indicate pAL1 signals. Bar, 5 μ m.

during meiosis, (iii) synapsis and HR, and (iv) chromosome segregation. Additionally, we have analyzed possible defects in mitosis and DNA repair.

Characterization of centromeric and pericentromeric regions

As mentioned before, centromeric and pericentromeric regions of *ago4-1* and *ago4-2* displayed a different DAPI staining with respect to WT, especially at pachytene. To gain insight into this phenomenon, we performed a FISH using the centromeric sequence of 180 bp (pAL1), and a telomeric probe (PLT11) as a positive control. The overall size of the centromeric signals was conspicuously smaller in the *ago4* mutants than in WT plants, while no apparent differences in the size of the telomeric signals were observed (Figure 2). We also detected differences among the chromosomes.

We performed an immunodetection of 5mC, because heterochromatic regions in plant chromosomes are usually enriched in this DNA modification. In Col, Ler, and *ago4-2*, 5mC was restricted to pericentromeric regions (Figure 3, A–D); but in *ago4-1* it was located at both pericentromeric and centromeric regions (Figure 3, E–H). Additionally, both mutants displayed a normal distribution of CENH3, even in meiocytes with abnormal chromosome segregations (Figure S5, Figure S6, and Figure S7).

Analysis of histone modifications during meiosis

Dimethylated H3K9 (H3K9me2) is the main histone H3 modification regulated by RdDM and it is located at heterochromatic, pericentromeric regions. We did not detect any variation in the distribution of this histone modification between *ago4* and WT plants (Figure S8). The distribution was also similar for the following euchromatic H3 modifications (Oliver *et al.* 2013): H3K4me2, H3 acetylated, and H3K27me3 (Figure S9, Figure S10, and Figure S11). We have also analyzed the chromosome distribution of H3S10ph, a modification associated with condensation in mammals

Figure 3 Immunolocalization of 5mC in PMCs from WT and *ago4-1* plants. (A, B) *Ler*. (C, D) Details of A and B. (E, F) *ago4-1*. (G, H) Details of E and F. Regions of the further enlarged pictures are indicated. White arrows point out centromeres. Bar, 5 μ m.

(Cobb *et al.* 1999) and with the process of sister-chromatid cohesion in plants (Kaszás and Cande 2000; Manzanero *et al.* 2000). In *A. thaliana*, this post-translational modification appears at diplotene-diakinesis and remains until the beginning of anaphase I. It reappears again at metaphase II to finally be absent at anaphase II (Oliver *et al.* 2013). This pattern was broadly similar in *ago4-1*, *ago4-2*, and WT pachytene PMCs (Figure 4; Figure S12), although a slight delay in H3S10ph disappearance was observed at anaphase I in *ago4-1* (Figure 4). This delay was probably associated to chromosome regions involved in interchromosomal bridges (Figure 4, A–L). The pattern of appearance/disappearance at the second meiotic division was similar to that observed in WT plants (Figure 4, M–T).

Synapsis and HR

Since *ago4-1* and *ago4-2* were the only mutants that showed a significant reduction in the mean cell chiasma frequency as compared to WT plants, we decided to analyze HR and synapsis by means of the immunolocalization of different proteins. The recombinases RAD51 and DMC1 were loaded normally onto meiotic axes and synapsis progressed correctly according to the detection of ASY1 and ZYP1, proteins associated to the lateral and central elements of the synaptonemal complex, respectively (Figure S13).

In *ago4-1* we have also analyzed the expression of some representative genes involved in HR: *SPO11-1*, *ATM*, *ATR*, *BRCA1*, *BRCA2B*, *RAD50*, *RAD51*, *RAD51C*, *DMC1*, *MSH4*, *MLH3*, *MUS81*, *SMC6A*, and *SMC6B*. In bud samples (enriched in meiocytes) only *SPO11-1* (2.52-fold), and *SMC6B* (2.36-fold) were overexpressed; while *DCM1* (4.43-fold), and *MUS81* (2.11-fold) only were in leaf samples (Figure S14). *ATR* (0.40-fold), *RAD51C* (0.35-fold), and *SMC6B* (0.33-fold) were underexpressed in leaf samples.

Meiotic chromosome segregation

To assess whether abnormalities in chromosome segregation at anaphase I observed in *ago4-1* were related to alterations in the structure and/or function of the spindle, we examined this structure by α -tubulin immunolocalization. The most intriguing finding was the presence of microtubule bundles

with an altered disposition; located in transversal orientation with respect to the division axes at anaphases I and II (Figure 5). The immunodetection of α -tubulin in the polyads revealed that some of the four pollen grains that originated were multinucleate (Figure S15). However, we did not detect abnormal mature pollen grains with more than three nuclei. Hence, it is feasible to think that any irregular pollen grains observed might degenerate before the occurrence of pollen mitoses (Figure S16).

Mitosis and sensitivity to γ -rays

Simultaneously to meiotic characterization, a cytological analysis of mitosis in *ago4-1* and *ago4-2* was conducted. Prophase was apparently normal, but we observed associations between decondensed chromosomes at metaphase (36%; $n = 50$), anaphases with delayed chromatid segregations (36% in *ago4-1*, 32% in *ago4-2*; $n = 50$) and interchromatid bridges (5% in both mutants; $n = 50$). However, telophases were apparently normal (Figure 6).

Due to the presence of these mitotic defects we decided to evaluate possible deficiencies in DNA repair by irradiating seeds of *ago4-1* and *Ler* with γ -rays. This DNA-damaging agent has a high mutagenic potential in plants and produces a large number of lesions which mainly generate double-strand breaks (DSBs). Among the different doses of irradiation, only at 450 Gy the mutant showed a significant decrease with respect to WT plants in both the number of leaves and the fresh weight per plant ($t = 2.53$, $P = 13 \times 10^{-3}$; Figure S17).

Discussion

The semisterility displayed by *ago4-1* had only been associated to developmental floral organ defects (Zilberman *et al.* 2004). However, our results suggest that it can also be related to the formation of unviable pollen grains (Figure S1). Furthermore, *ago4-1* seeds showed a germination delay (Figure S2) and a considerable variability at morphological level; similar to that observed in *superman* (*sup*) mutants, defective for a zinc-finger protein (Gaiser *et al.* 1995). Indeed, AGO4 is involved in silencing the *SUP* gene (Zilberman *et al.* 2003).

Figure 4 Immunolocalization of H3S10ph in *ago4-1*. (A–L) Anaphase I. Arrows indicate bridges or chromosome fragments. (M–P) Metaphase II. (Q–T) Polyad. Bar, 5 μ m.

Centromeric and pericentromeric regions

Among all mutants analyzed, the most conspicuous meiotic alterations were observed in *ago4* mutants (Figure 1), especially in *ago4-1* PMCs. Observations reported here suggest that differences in chromatin organization at both centromeric and pericentromeric regions with respect to WT could play a role in this scenario (Figure 2 and Figure 3). The main components of the *Arabidopsis* centromere are a sequence of 180 bp in thousands of tandemly repeated copies and transposons like *Athila*, *Tat*, *Tim*, or *Copia*. Only 15% of 180-bp sequences are connected to CENH3 (Nagaki *et al.* 2003; Schubert *et al.* 2012). In contrast to the centromere, pericentromeric regions can show different condensation levels (Schubert *et al.* 2012). These regions have a low gene density and are rich in transposons and repeated DNA sequences (May *et al.* 2005). In *ago4-1*, the signal size corresponding to the 180-bp sequence observed by FISH was considerably smaller than in the WT (Figure 2). This reduction in DNA-FISH signals could reflect a partial centromeric deletion, although changes in chromatin conformation could also hinder the accessibility of the probe to the complementary DNA sequence. However, apparent changes in the location pattern of CENH3, required for kinetochore assembly, were not detected (Figure S5, Figure S6, and Figure S7). Zilberman *et al.* (2003) reported that *ago4-1* does not affect methylation levels at the 180-bp sequence, but we have observed a decrease in 5mC at pericentromeric regions, associated with a punctate pattern at centromeres (Figure 3). This anomalous 5mC distribution could be related to the alterations detected

during anaphase I (Figure 1, Figure 3, and Figure 5). Mutations in fission yeast of genes coding for proteins involved in the siRNA pathway produce abnormalities in chromosome segregation, and also a loss of pericentromeric heterochromatin silencing detected by a decrease in the signal corresponding to H3K9me2 (Volpe *et al.* 2003; Fukagawa *et al.* 2004; Kanellopoulou *et al.* 2005). However, we have not detected dissimilarities in the distribution pattern of this and other H3 modifications in *ago4* mutants (Figure S8, Figure S9, Figure S10, and Figure S11).

Chromatin architecture

The final effect of RdDM is methylation of DNA that is determinant in chromatin compaction (Liu *et al.* 2016). siRNAs are involved in the recruitment of chromatin modification complexes that lead to the formation of heterochromatin (Wassenegger 2005; Matzke and Mosher 2014). The general chromatin decondensation observed from diplotene to metaphase I was a common feature in the mutants of genes involved in RdDM, although the percentage of decondensed nuclei was variable among them. The highest percentages corresponded to *ago4-1* and *nrpe1-11* (Table 1; Figure 1 and Figure S4). However, we did not observe differences between *ago4-1* and WT PMCs in the distribution pattern of H3ac, despite histone deacetylase 6 (HDA6) being necessary for the propagation of the *de novo* methylation directed by RNA (Aufsatz *et al.* 2002). Likewise, we did not detect differences for other H3 post-translational modifications (Figure S8, Figure S9, and Figure S11). These results concur with those reported in other mutants that

Figure 5 Immunolocalization of α -tubulin and CENH3 in PMCs from Ler and *ago4-1*. (A–H) WT. (I–T) *ago4-1*. (A–D, M–P) Anaphase I. The white arrow indicates a microtubule bundle in opposite orientation from the spindle. (E–H, Q–T) Anaphase II. (I–L) Metaphase I. The white arrow indicates a microtubule bundle in opposite orientation from the spindle, depicted by a double-headed arrow. Bar, 5 μ m.

show alterations in the siRNA machinery (Naumann *et al.* 2005; Pontes *et al.* 2009).

HR

Drosophila mutants defective in PIWI-interacting RNA/repeat associated siRNA components do not show important changes in CO frequency (Cross and Simmons 2008). In *A. thaliana*, we have found only three mutants for genes involved in RdDM with significant variations in the mean cell chiasma frequency with respect to WT plants: *ago4-1* and *ago4-2* showed a decrease, while *npr1-8* displayed an increase (Table 2). The results obtained here also reveal that the same bivalent, and their chromosome arms, may behave in a different way in different mutants (Table 2). This indicates the existence of factors controlling meiotic recombination at the arm/chromosome level that are differentially regulated in the mutants. In this sense, *met1* and *ddm1* mutants present a reduction in DNA methylation at heterochromatic pericentromeric regions and changes in CO frequency along chromosomes, although their overall chiasma frequency is similar to that observed in WT plants (Melamed-Bessudo and Levy 2012; Mirouze *et al.* 2012; Yelina *et al.* 2012).

Maintenance of chromosome architecture is important for the correct function of meiotic proteins. Nevertheless, and despite the reduction in CO frequency, *ago4-1* and *ago4-2* showed full synapsis and the recombinases RAD51 and DMC1 were normally loaded onto chromatin (Figure S13). Furthermore, expression analysis of genes involved in meiotic HR did not reveal any clear tendency (Figure S14). Additionally, *ATR*,

RAD51C, and *SMC6B* are underexpressed in the somatic line of *ago4-1* (Figure S14), despite the mutant not being hypersensitive to γ -rays (Figure S17). Similar results have been found in other mutants affected in siRNA biogenesis, in which there is a low spontaneous HR frequency and DNA repair enzymes are not overexpressed (Wei *et al.* 2012; Yao *et al.* 2016).

Chromosome segregation

ago4 mutants exhibited some abnormalities at mitotic anaphases, although telophases were normal (Figure 6). They also displayed defects during meiosis, especially at anaphase I, involving the presence of interchromosomal bridges and a delay in chromosome segregation (Figure 1). These abnormalities were also observed, although to a minor degree, in *nrpe1-11*, *kyp-4*, and *kyp-6 drm1-2 drm2-2* (Figure S4). Chromosome bridges at anaphase I have been described in mutants defective in the repair of programmed DSBs. In these mutants, the bridges are mostly accompanied by chromosome fragments that are detected at late prophase I (Schommer *et al.* 2003; Puizina *et al.* 2004; Wang *et al.* 2012). However, in *ago4-1* chromosome fragmentation was almost nominal and restricted to anaphase I (Figure 1 and Figure 4).

In *A. thaliana*, H3 phosphorylation occurs all along the chromosomes from diplotene to telophase I, and from late prophase II to metaphase II, disappearing at telophase II (Caperta *et al.* 2008; Oliver *et al.* 2013; Figure S12). Manzanero *et al.* (2000) and Kászas and Cande (2000) have suggested that in plants, H3 phosphorylation is related to

Figure 6 Cytological analyses of mitosis in somatic cells from *ago4-1*. (A–D) WT. (E–H) *ago4-1*. (I–L) *ago4-2*. (A, E, I) Prophase. (B, F, J) Metaphase. (C, G, K) Anaphase. Arrows indicate (G) a lagging chromatid and (K) an interchromatid bridge. (D, H, L) Telophase. Bar, 5 μ m. (M) Cells were scored to analyze the decondensation at metaphase, delays in chromatid segregation, and presence of interchromatid bridges at anaphase. There were statistical differences with respect to WT in chromosome condensation at metaphase (*ago4-1*) and anaphase alterations (*ago4-1* and *ago4-2*). *** $P < 10^{-3}$.

sister chromatid cohesion. Thus, it is tempting to speculate that the association of H3S10ph to bridges and chromosome fragments at anaphase I could reflect the difficulties in the separation of sister chromatids (Figure 4). Other factors in this scenario could be members of the Aurora family (Demidov *et al.* 2005) that are responsible for the cell-cycle-dependent phosphorylation of H3 at serine 10 (Demidov *et al.* 2009) and for ensuring correct chromosome segregation (Demidov *et al.* 2014). In addition, depletion of Aurora B in *Drosophila* and *Caenorhabditis elegans* results in reduced H3S10ph and is related to defects in chromosome segregation (Wei *et al.* 1999; Adams *et al.* 2001; Giet and Glover 2001). Also, a mutation of H3S10 in *Tetrahymena* produces segregation defects (Wei *et al.* 1999). In *Arabidopsis* meiosis, the alterations in the dynamics of this modification observed in *ago4-1* could be responsible for the improper spindle formation (Figure 5). Alterations in chromatin condensation, failures in spindle morphogenesis, and polyad formation were also observed in the *ago104* maize mutant; and in the *Arabidopsis* mutants *radially swollen 4* (*rsw4*) and *tardy asynchronous meiosis 1* (*tam1*), defective for a separase and a cyclin, respectively.

These mutants also show delayed chromosome segregation (d'Erfurth *et al.* 2010; Singh *et al.* 2011; Yang *et al.* 2011). This phenomenon, although less extreme, is also displayed by defective mutants for CENH3 (Ravi *et al.* 2010; Lermontova *et al.* 2011). In mammals, defects in spindle formation and chromosome alignment have also been detected in the female meiosis of mutants defective for endogenous siRNAs (Stein *et al.* 2015).

In conclusion, the changes in the organization of centromeric and pericentromeric chromatin observed in *ago4* are probably the origin of the failures observed in spindle organization. Alterations in kinetochore-microtubule interactions could be responsible for chromosome segregation defects, manifested cytologically as chromosome bridges and fragments. Cytokinesis at the end of the meiotic process would lead to the formation of four meiotic aberrant products. Altogether these results suggest that AGO4 may possess a meiosis-associated cellular function that seems to be independent of other proteins also involved in RdDM. In this sense, recent publications have highlighted the existence of siRNAs generated via an alternative route independent of DCLs [siRNAs independent of DCLs (sidRNAs)]. These sidRNAs are associated with AGO4 and capable of directing DNA methylation, playing key roles in the initiation of RdDM (Ye *et al.* 2015). Additionally, although in general AGO6 is redundant with AGO4 in RdDM, they can play nonredundant roles in regulating the same RdDM target and may act sequentially to mediate siRNA-guided DNA methylation (Duan *et al.* 2015). It could explain the differences in the mutant phenotypes that we have analyzed. Although further studies should be needed to decipher the detailed mechanism how AGO4 influences *Arabidopsis* meiosis, this study marks the way.

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Figure S1 Alexander staining of *ago4-1* pollen grains. (A) Ler anther. (B) *ago4-1* anther. (C) *ago4-1* pollen grains with different sizes. Black arrows indicate unviable small pollen grains. Blue arrow indicates a viable pollen grain bigger than normal. Bars = 25 μ m.

Figure S2 Germination analysis and seed morphology in *ago4-1*. (A) Percentage of germinated seeds in *Ler* (green) and *ago4-1* (red). (B) Representative images of seeds from *Ler* (above) and *ago4-1* (below). Green arrows indicate the longitude of two seeds with different size. White arrows indicate the two root apical meristems from one seed. Red arrows indicate two small dehydrated seeds. Bar = 300 μ m. (C) Analysis of seed morphology. ***, $p < 10^{-3}$.

Figure S3 Representative images of female meiosis in *ago4-1*. (A) Zygotene. (B) Pachytene. (C) Diplotene. (D) Metaphase I. (E) Prophase II. (F) Metaphase II. Bars = 5 μ m.

Figure S4 Representative images of metaphase I and anaphase I PMCs from *dcl3-1*, *ago6-2*, *kyp-4*, *nrpe1-11*, and the triple mutant *kyp-6 drm1-2 drm2-2*. (A-E) Representative images of decondensed metaphases I. (F-H) Representative images of anaphases I with interchromosomal bridges. (A) *dcl3-1*. (B, F) *ago6-2*. (C, G) *kyp-4*. (D, H) *kyp-6 drm1-2 drm2-2*. (E) *nrpe1-11*. Bars = 5 μ m. (I) Nuclei at metaphase I were classified in two groups according to the degree of chromosome condensation (*cdd*: *cmt3-11 drm1-2 drm2-2*; *mdd*: *met1-3 drm1-2 drm2-2*; *kdd*: *kyp-6 drm1-2 drm2-2*). Statistical differences respect to the WT were observed in *dcl3-1*, *ago6-2*, *kyp-4*, *nrpe1-11*, and *kyp-6 drm1-2 drm2-2*. * $p < 5 \times 10^{-2}$; **, $p < 10^{-2}$; ***, $p < 10^{-3}$.

Figure S5 Immunolocalization of CENH3 in Col PMCs. (A-C) Pachytene. (D-F) Metaphase I. (G-I) Metaphase II. (J-L) Tetrad. Bars = 5 μ m.

Figure S6 Immunolocalization of CENH3 in *ago4-1* PMCs. (A-C) Pachytene. (D-F) Metaphase I. (G-I) Metaphase II. (J-L) Polyad with multiple micronuclei, some of them without CENH3 signals. Bars = 5 μ m.

Figure S7 Immunolocalization of CENH3 in *ago4-2* PMCs. (A-C) Pachytene. (D-F) Metaphase I. (G-I) Metaphase II. (J-L) Tetrad. Bars = 5 μ m.

Figure S8 Immunolocalization of H3K9me2 at pachytene. (A-C) *Ler.* (D-F) *ago4-1*. (G-I) *ago4-2*. Bars = 5 μ m.

Figure S9 Immunolocalization of H3K4me2 at pachytene. (A-C) *Ler.* (D-F) *ago4-1*. (G-I) *ago4-2*. Bars = 5 μ m.

Figure S10: Immunolocalization of H3Ac at pachytene. (A-C) *Ler*. (D-F) *ago4-1*. (G-I) *ago4-2*. Bars = 5 μ m.

Figure S11 Immunolocalization of H3K27me3 at pachytene. (A-C) *Ler.* (D-F) *ago4-1*. (G-I) *ago4-2*. Bars = 5 μ m.

Figure S12 Immunolocalization of H3S10Ph in *ago4-1* PMCs. (A-C) Pachytene. (D-F) Metaphase I. (G-I) Prophase II. (J-L) Metaphase II. Bars = 5 μm.

Figure S13 Immunolocalization of different proteins involved in synapsis and recombination at prophase I stages: ASY1, ZYP1, RAD51 and DMC1. (A-C, G-I, M-O) *Ler*. (D-F, J-L, P-R) *ago4-1*. Bars = 5 μ m.

Figure S14 Relative transcript levels of representative HR genes in bud (blue) and leaf (green) samples in *ago4-1*. The results of a quantitative RT-PCR analysis after quantification and normalization to WT expression levels are shown. The data are means of three experiments, and error bars show SEM.

Figure S15 Immunolocalization of α -tubulin in *ago4-1* polyads. (A) DAPI. (B) α -tubulin. (C) DAPI (gray). The four meiotic products generated after finishing cytokinesis are distinguished. Red arrowheads indicate some micronuclei. Bar = 5 μ m.

Figure S16 Mature pollen grains from *Ler* and *ago4-1*. (A) *Ler*. (B) *ago4-1*. SN, sperm nuclei; VN, vegetative nucleus. Bars = 5 µm.

Figure S17 Analysis of *ago4-1* γ -ray sensitivity. (A) Seedlings of Ler (left of plates) and *ago4-1* (right of plates) 14 days after irradiation. (B) Percentages of leaf number per plant. (C) Percentages of fresh weight per plant.

Table S1 Primers used for genotyping mutants

MUTANT	LINE	LP 5'-3'	RP 5'-3'
<i>nrpd1a-8</i>	SALK_057637	AACACAAACTTCACAAATAAATT	GGAGTTCAAAGACAACGATCG
<i>dcl3-1</i>	SALK_005512	ATACATTGGTGGAGGGGTT	CATCACCATCCTGTAGTTTGG
<i>ago4-1t</i>	SALK_007523	GTAGATACCCTCATTCTCCAGC	AAGCACACTCACAAGACCCTA
<i>ago6-2</i>	SALK_031553	TGACGGAGAAGAGAAATGAGCT	CCAGATAAGAGGGACATACCGT
<i>nrpe1-11</i>	SALK_029919	ATTTCTTCTTTGATGGGGGAG	TGCGTGGATATGACCATTG
<i>kyp-4</i>	SALK_044606	TTTATTCGAGCCAACATTTGC	AGTTCGGTTGACACATTTTGG
<i>met1-3</i>	SAIL_809_E0	GCGTGTACCAGTTTCAAGGAG*	TAAAGAGCCCAGTTGTGAAGC ^a
<i>drm1-2</i>	SALK_021316		
<i>drm2-2</i>	SALK_150863		

^a Primers used to check the T-DNA insertion in *MET1*. This line is double homozygous for *drm1-2* and *drm2-2* alleles and heterozygous for *met1-3* allele.

Table S2 List of antibodies used

ANTIBODY	WORKING DILUTION	OBTAINED IN	SOURCE
anti-ASY1	1:1000	Rat	Dr. Chris Franklin
anti-ZYP1	1:250	Rabbit	Dr. Chris Franklin
anti-DMC1	1:300	Rabbit	Dr. Chris Franklin
anti-RAD51	1:300	Rabbit	Dr. Chris Franklin
anti-H3K9me2	1:200	Rabbit	Millipore
anti-H3K4me2	1:200	Rabbit	Abcam
anti-H3K4me3	1:200	Rabbit	Abcam
anti-H3K27me3	1:200	Rabbit	Millipore
anti-H3Ac	1:200	Rabbit	Millipore
anti-H3S10Ph	1:200	Rabbit	Upstate
anti-CENH3	1:300	Rabbit	Millipore (Dr. Andreas Houben)
α-tubulin	1:100	Mouse	Sigma
5-methyl cytosine	1:50	Mouse	Eurogenetec (Dr. Tomás Naranjo)

Table S3 Primers and probes used for qPCR analyses

GENE	AGI CODE	PRIMERS 5'-3'	UPL (TAQMAN PROBE)
ATM	AT3G48190	AGGGTGGTGAGATGAGAAGC TCTGTGTCAATTGCGTCTTGT	98
ATR	AT5G40820	TTCAGCGCCCAAAGAAGA GGCTTGCAGAGGAATGGATA	3
BRCA1	AT4G21070	CCAAGAAATTGGTCTTATCTTGC AGTTCCGCAAATTCTGCAAT	100
BRCA2B	AT5G01630	CACCTTAAAACCCGCAGTG AGGTGATTTACAAGCACCGATT	140
DMC1	AT3G22880	TCAACGTTGCTGTCTACATGACT GACCACCTGCTGGCTTTTT	31
MLH3	AT4G35520	GACTGAAGCAGACCTCACTTTG GCCTTCAAATCGACAAGAGG	47
MSH4	AT4G17380	CAAGAATGGGGACAATGGAT TGCATTATGAAAGCGGTCTCT	151
MUS81	AT4G30870	GATATGTACCCAACGCTTTTGTC CTTCTTGCGCCGAGACAT	29
RAD50	AT2G31970	GCAGTGCAGGTCAAAAGGTT GGCCCATCCAGGTTTGTAG	136
RAD51	AT5G20850	CATGCCACCACAACAAGG ACATGGCGAGCTTATCACTTTAC	91
RAD51C	AT2G45280	TCAACTAGCGCTTGCTTTAGG AATACAGAATGACTCGGTTGGTG	54
SMC6A	AT5G07660	TGCCTCAAGATGCAACAAAC AAAGTCGAGAAAGACCGTTCC	150
SMC6B	AT5G61460	TCGCACGAGAGGATAAAGAAA TGA CTCAAAGCCGAGGATG	68
SPO11-1	AT3G13170	TTCCCAAACAGTGTCTTTTGC TTCAAGTTCCAACCTCCATTG	143