

Arabidopsis Separase AESP Is Essential for Embryo Development and the Release of Cohesin during Meiosis

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To investigate how and when sister chromatid cohesion is released from chromosomes in plants, we isolated the *Arabidopsis thaliana* homolog of separase (AESP) and investigated its role in somatic and meiotic cells. AESP is similar to separase proteins identified in other organisms but contains several additional structural motifs. The characterization of two *Arabidopsis* T-DNA insertion alleles for AESP demonstrated that it is an essential gene. Seeds homozygous for T-DNA insertions in AESP exhibited embryo arrest at the globular stage. The endosperm also exhibited a weak *titan*-like phenotype. Transgenic plants expressing AESP RNA interference (RNAi) from the meiosis-specific *DMC1* promoter exhibited alterations in chromosome segregation during meiosis I and II that resulted in polyads containing from one to eight microspores. Consistent with its predicted role in the release of sister chromatid cohesion, immunolocalization studies showed that the removal of SYN1 from chromosome arms and the centromeres is inhibited in the RNAi mutants. However, the release of SYN1 during diplotene occurred normally, indicating that this process is independent of AESP. Therefore, our results demonstrate that AESP plays an essential role in embryo development and provide direct evidence that AESP is required for the removal of cohesin from meiotic chromosomes.

INTRODUCTION

The faithful transmission of chromosomes during mitosis and meiosis is essential for the survival and reproduction of eukaryotic organisms. A critical aspect of chromosome segregation is sister chromatid cohesion, which is required for the proper attachment of chromosomes to the spindle and the faithful segregation of sister chromatids to opposite poles of the cell during anaphase (reviewed in Uhlmann, 2004; Nasmyth and Haering, 2005). Sister chromatid cohesion is mediated in part by a complex of highly conserved proteins referred to as the cohesin complex. Four proteins, STRUCTURAL MAINTENANCE OF CHROMOSOME1 (SMC1), SMC3, SISTER CHROMATID COHESION1 (SCC1), and SCC3, form the core of the mitotic cohesin complex, which is used by a wide range of organisms. In yeast, the cohesin complex is found on chromosomes from S-phase to anaphase, with preferential binding in centromeric regions (Michaelis et al., 1997; Uhlmann and Nasmyth, 1998; Megee and Koshland, 1999; Blat et al., 2002). The release of chromosome cohesion at the metaphase-to-anaphase transition and the subsequent separation of sister chromatids is triggered in most organisms by separase (ESP1), which specifically cleaves the cohesin kleisin (SCC1) subunit (Ciosk et al., 1998; Uhlmann et al., 1999; Buonomo et al., 2000; Uhlmann et al., 2000; Hauf et al.,

2001). Separase belongs to the CD clan of Cys proteases (Barrett and Rawlings, 2001; Goyal, 2001). Before the metaphase-to-anaphase transition, separase protease activity is inhibited by securin. At the onset of anaphase, securin is degraded by the anaphase-promoting complex/cyclosome (APC/C), freeing separase, which cleaves SCC1, to facilitate the release of cohesion and chromosome separation (Cohen-Fix et al., 1996; Ciosk et al., 1998; Zou et al., 1999).

In budding and fission yeast, most chromosomal cohesin complexes remain on the chromosomes until they are cleaved by separase to initiate mitotic anaphase (Uhlmann et al., 1999, 2000; Tomonaga et al., 2000). In vertebrates, most cohesin complexes dissociate from chromatids during prophase in a separase-independent process (Waizenegger et al., 2000; Losada et al., 2002). The small fraction of cohesin that remains primarily in centromeric regions is released by separase at the metaphase-to-anaphase transition (Sumara et al., 2000, 2002; Ross and Cohen-Fix, 2002).

Sister chromatid cohesion is also released in several stages during meiosis. Recombination between homologous chromosomes during meiotic prophase I forms chiasmata, which hold maternal and paternal chromosomes together. The resolution of chiasmata and the separation of homologous chromosomes depend on separase cleavage of the meiotic α -kleisin subunit, REC8, in cohesin complexes along the arms at anaphase I (Buonomo et al., 2000; Kitajima et al., 2003). Centromeric cohesion is protected by the conserved SGO family of proteins until anaphase II, when separase cleavage of the REC8 protein facilitates the separation of sister chromatids (Rabitsch et al., 2003; Katis et al., 2004; McGuinness et al., 2005).

Although separase is required for meiotic chromosome segregation, other mechanisms of cohesin removal may also be operating. Studies on the distribution of cohesin proteins during

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meiosis in a number of organisms, including *Caenorhabditis elegans*, mammals, and *Arabidopsis thaliana*, have shown that similar to the situation during mitosis in animal cells, a significant amount of cohesin is either removed from or redistributed on prophase chromosomes before anaphase I in higher eukaryotes (Eijpe et al., 2000, 2003; Pasierbek et al., 2001; Cai et al., 2003; Lee et al., 2003). Furthermore, it was recently shown in budding yeast that a portion of the cohesin is also released before metaphase I in a condensin/Cdc5-dependent process (Yu and Koshland, 2005). Finally, experiments in *Xenopus* suggested that chromosome segregation at meiosis I can take place in the absence of APC activity and in the presence of high levels of separase's inhibitor securin (Peter et al., 2001; Taieb et al., 2001).

Along with its essential role in chromosome separation, separase is required for a number of additional cellular processes in different organisms. These include anaphase spindle stabilization and the coupling of anaphase to mitotic exit during mitosis in yeast (Funabiki et al., 1996; Ciosk et al., 1998; Cohen-Fix and Koshland, 1999; Uhlmann et al., 2000; Sullivan and Uhlmann, 2003; Papi et al., 2005) as well as the coordination of chromosome segregation and spindle formation during meiosis (Buonomo et al., 2003). Vertebrate separase also has a role in cell cycle progression (Papi et al., 2005; Queralt and Uhlmann, 2005). In *C. elegans*, separase is required for the proper positioning of the centrosome during the first asymmetric mitotic division and for eggshell development during embryogenesis (Siomos et al., 2001; Rappleye et al., 2002). Mutations in *Drosophila* separase result in defects in epithelial cell organization (Pandey et al., 2005). Therefore, in addition to its central role in controlling the release of sister chromatid cohesion during mitosis and meiosis, separase has been implicated in a number of different events in various organisms.

Much less is known about the cohesin machinery and how chromosome cohesion is controlled in plants. *Arabidopsis* contains four cohesin-associated α -kleisin homologs. SYN1/DIF1, which is a REC8 homolog, is essential for sister chromatid cohesion during meiosis (Peirson et al., 1997; Bai et al., 1999; Bhatt et al., 1999; Cai et al., 2003). SYN1 is expressed primarily in buds, and anti-SYN1 antibody labels chromosome axes from early leptotene to metaphase I (Cai et al., 2003). The three remaining kleisin proteins, SYN2, SYN3, and SYN4, are expressed throughout the plant (Dong et al., 2001). Antibodies to the proteins label chromosomes of somatic cells, suggesting that they may participate in mitotic cohesion (our unpublished data). The *Arabidopsis* *SMC1*, *SMC3*, and *SCC3* genes have been isolated and characterized; knockout mutants in these genes indicate that they are essential for embryo and endosperm development (Liu and Meinke, 1998; Liu et al., 2002; Chelysheva et al., 2005).

Although the basic mechanisms that control sister chromatid cohesion are conserved in a wide range of organisms, it is clear that there is still a considerable amount that we do not understand about the regulation of this process and that differences exist between organisms. Furthermore, little is known about the proteins that control chromosome structure and segregation in plants, and nothing is known about plant separase proteins. To better understand how and when cohesion is released from meiotic chromosomes in plants, and to determine the role(s) of separase in plants, we have isolated the *Arabidopsis* homolog of

separase (*AESP*) and investigated its role in somatic and meiotic cells. Analysis of two independent T-DNA insertion lines demonstrated that *AESP* is an essential gene. Plants heterozygous for the mutations exhibited embryo arrest at the globular stage of development in 25% of developing seeds, indicating that *AESP* is essential for embryo development. Reduction of *AESP* transcripts via the expression of *AESP*-RNA interference (RNAi) using the meiotic *ATDMC1* promoter resulted in reduced fertility caused by defects in meiosis. Alterations were observed in the segregation of homologous chromosomes at anaphase I and sister chromatids at anaphase II, indicating that *AESP* is required for both meiosis I and meiosis II in *Arabidopsis*. Consistent with these observations, SYN1 was found to persist on chromosomes after anaphase I. These results demonstrate that cleavage of SYN1 by *AESP* is responsible for the release of sister chromatid cohesion during meiosis.

RESULTS

Identification, Structure, and Expression of *AESP*

The *Arabidopsis* genome contains one putative separase gene (At4g22970), hereafter called *AESP*. The *AESP* cDNA was isolated and compared with the published genomic sequence of At4g22970. *AESP* consists of 27 exons and 26 introns (Figure 1A), which is different from the predicted structure (accession number NM118426). The predicted cDNA sequence (NM118426), at 5588 bp, is significantly shorter than the actual mRNA (6884 bp) as a result of the omission of exons 3, 9, 10, 16, 18, and 19. In addition, several differences between predicted and actual exon splice sites were observed.

The *AESP* cDNA (AY823256) is capable of encoding a 2180-amino acid protein. *AESP* exhibits the same level of similarity (10 to 14%) with the vertebrate, worm, fly, and yeast separase proteins as they do with each other. *AESP* is similar in size to the mammalian enzymes, which are significantly larger than those from yeast (NP011612), worm (AAK77200), and fly (AAQ72557) (Figure 1B). Although sequence conservation is observed throughout the proteins, the greatest similarity is found in the C terminus, which contains the C-50 peptidase domain. The *AESP* peptidase domain is most similar (~20% sequence identity) to those found in the mammalian enzymes (Figure 2). However, it is considerably larger (700 amino acids) than those found in most organisms (~400 to 470 amino acids). It contains a predicted 2Fe2S-ferredoxin domain that is not present in the proteins of other organisms (Figure 1B). At this time, it is not clear whether *AESP* actually binds iron, and if so, the role of the iron center. *AESP* also contains a putative EF-hand calcium binding domain. Budding yeast Esp1 contains a calcium binding domain, which is required for its association with the spindle (Jensen et al., 2001). It is possible that the EF-hand motif in *AESP* plays a similar role in plants.

AESP transcript levels were analyzed in different tissues using RT-PCR. The *ACTIN8* (*ACT8*) gene was used as an internal standard to control for the amount of cDNA used because of its relatively uniform distribution throughout the plant (An et al., 1996). Low but detectable levels of *AESP* transcript were found

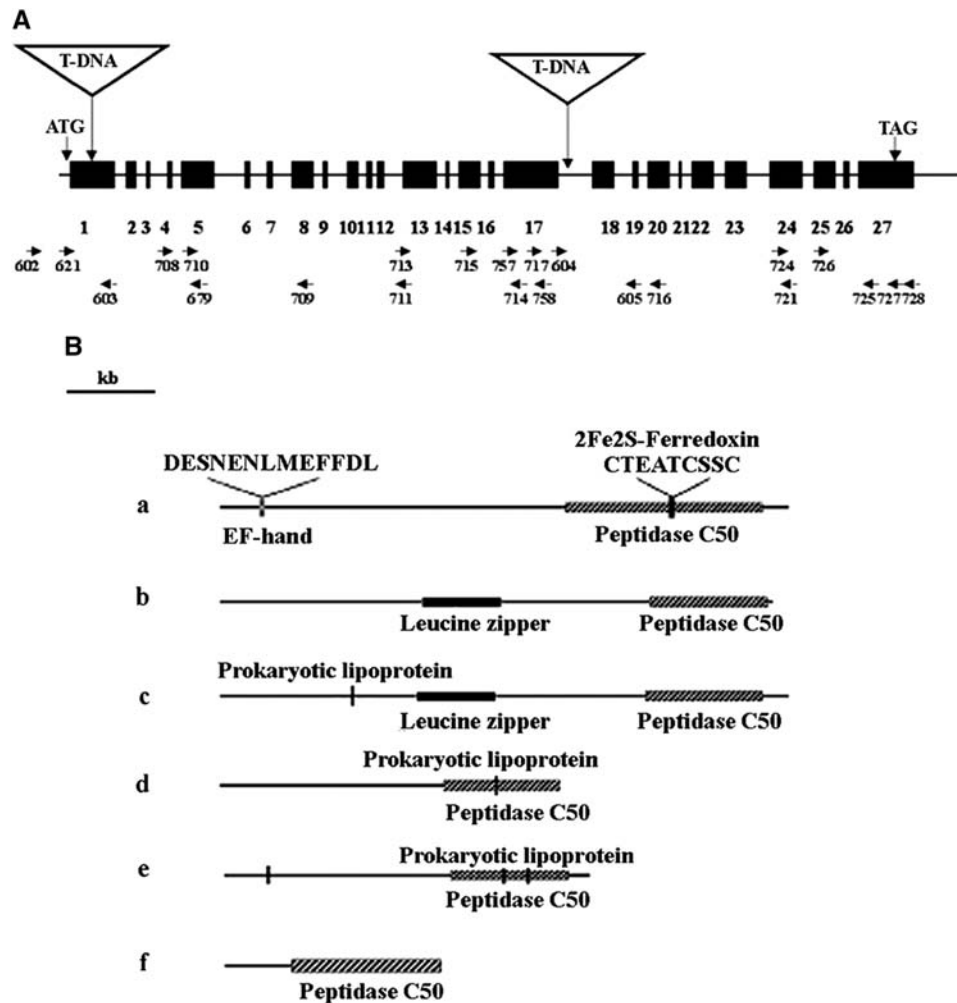


Figure 1. AESP Gene and Protein Structures.

(A) AESP gene structure. The positions of exons are shown as black boxes. The positions and directions of primers used in this study are shown as horizontal arrows. The positions of T-DNA insertions are shown as inverted triangles. The scale bar is shown at bottom.

(B) Motifs in ESP1 proteins from *Arabidopsis* (a), human (b), mouse (c), *C. elegans* (d), *Saccharomyces cerevisiae* (e), and *Drosophila* (f).

in all tissues examined (Figure 3A). Transcripts for AESP are present at approximately equal levels in roots, stems, leaves, and buds. The presence of AESP transcripts in a wide range of tissues is consistent with its predicted role in cell division and suggests that it may play a role in both mitosis and meiosis.

AESP Is Required for Seed Development

Two T-DNA insertion alleles, *Aesp-1* and *Aesp-2*, were identified in the SALK T-DNA express database and were characterized to determine whether AESP is required for mitosis. PCR amplification of the insertion sites followed by DNA sequence analysis confirmed that *Aesp-1* contains a T-DNA insert in exon 1, whereas *Aesp-2* contains a T-DNA insert in intron 17 (Figure 1A). No homozygous mutant plants were identified for either line. Therefore, seeds from heterozygous plants were collected and resown, and PCR analysis of the progeny of self-pollinated

heterozygous *Aesp-1* and *Aesp-2* plants was conducted. Thirty-five percent (41 of 116) of the progeny from *Aesp-1* plants were homozygous wild type, whereas 65% (75 of 116) of the plants were heterozygous for the *Aesp-1* insertion. Likewise, 58 (35.8%) homozygous wild-type plants and 104 (64.2%) plants heterozygous for the T-DNA insert were identified among the 162 progeny from *Aesp-2* heterozygous plants that were analyzed.

A plant homozygous for either the *Aesp-1* or *Aesp-2* T-DNA insertion was never obtained. Our inability to identify plants homozygous for the *Aesp-1* and *Aesp-2* T-DNA inserts coupled with the 1:2:0 segregation ratios that we obtained for the two independent lines suggested that the mutations may result in embryo lethality. To investigate this possibility, we examined the siliques of heterozygous plants carrying the two mutations. Wild-type plants segregating in populations of *Aesp-1* and *Aesp-2* plants produced on average 56 and 64 ($n = 50$) full-size seeds per silique, respectively. By contrast, *Aesp-1* and *Aesp-2* heterozygous

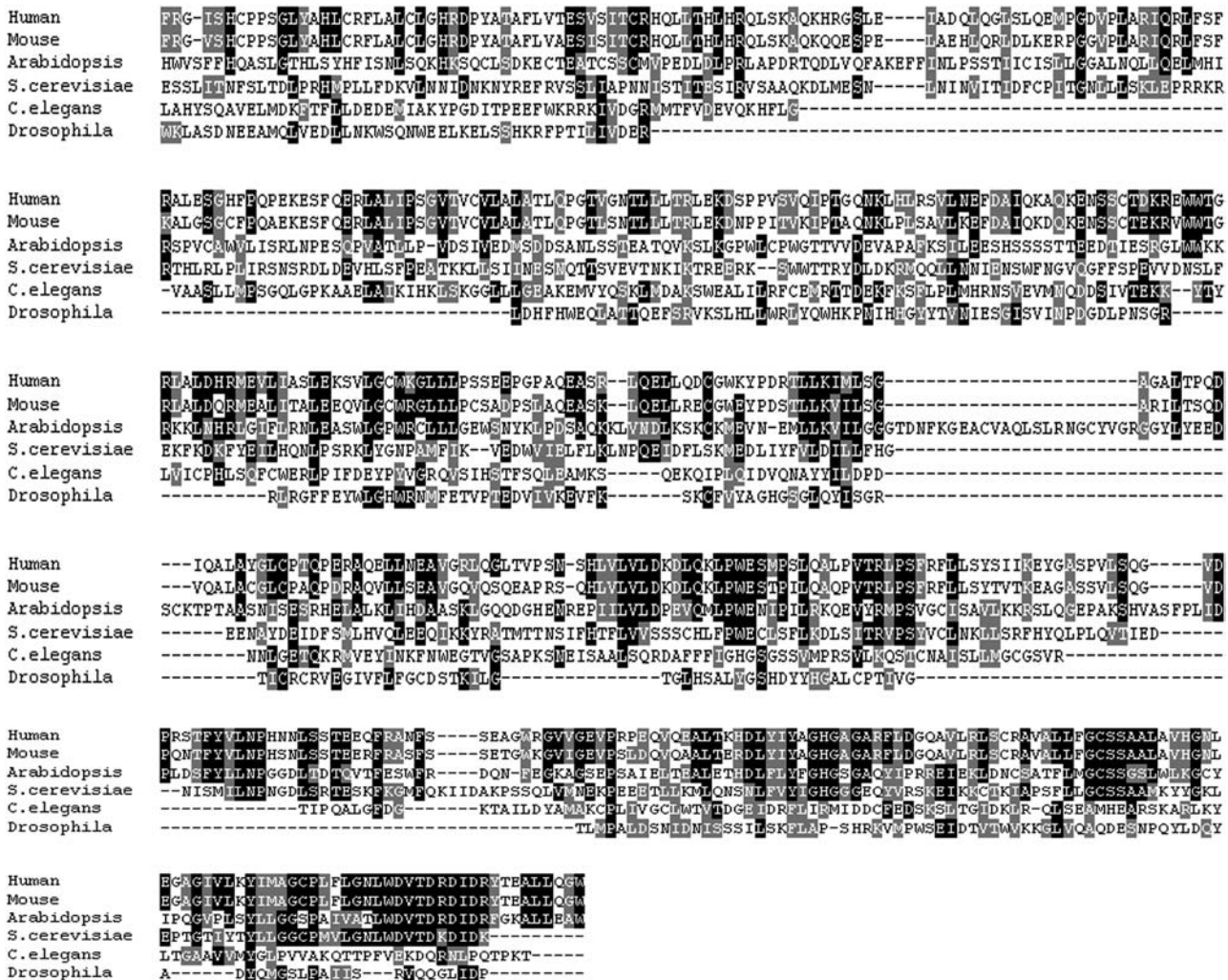


Figure 2. Amino Acid Sequence Alignment of the Peptidase C-50 Domain in ESP1 Proteins.

The peptidase domains of human (Q14674), mouse (P60330), *Arabidopsis* (AY823256), *S. cerevisiae* (NP011612), *C. elegans* (AAK77200), and *Drosophila* (AAQ72557) separase proteins are shown aligned. Identical amino acids are shaded in black, and similar amino acids are shaded in gray. Missing residues are shown as dashes. ClustalW (<http://www.ebi.ac.uk/clustalw>) and BOXSHADE 3.33 (http://www.ch.embnet.org/software/BOX_form.html) were used to produce the alignment.

plants produced on average 44 and 48 ($n = 50$) normal seeds per silique, respectively. Approximately 25% (335 of 1370) of the seeds examined in siliques of *AESP* heterozygous plants in both lines were dry and shrunken. Reciprocal crosses with heterozygous plants containing the *Aesp-1* and *Aesp-2* mutations demonstrated that they are allelic and linked to the seed defect. Backcrossing to wild-type plants indicated that the mutations are transmitted normally through the gametes. This finding is consistent with our observation that the pollen appeared normal in *Aesp-1* and *Aesp-2* plants (data not shown). These results provided strong evidence that *AESP* is an essential gene and that *Aesp* null mutations disrupt seed development.

We next compared the development of wild-type and mutant seeds in the same siliques of *Aesp-2* heterozygous plants to determine the nature of the seed defect. Early embryo develop-

ment appeared normal in all seeds. No dramatic differences were observed in seeds at the zygote, single-terminal, and two-terminal cell stages. Some variability in development was observed beginning at approximately the quadrant stage. For example, in siliques in which most seeds were at the dermatogen (16-cell) stage, others had only developed to the point at which the embryo proper had four to eight cells (data not shown), and in older siliques in which most embryos had developed to the late globular stage, others were still at early globular (Figures 4A and 4D). However, embryo development in a given silique is not completely synchronous (Bowman, 1994). Therefore, although we cannot exclude the possibility that the mutation results in a delay in development, we believe that most if not all of the early differences we observed are attributable to natural variability in seed development.

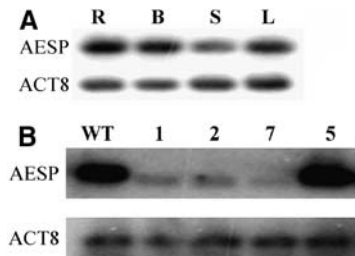


Figure 3. *AESP* Transcript Levels in Wild-Type and RNAi Plants.

(A) *AESP* transcript levels were analyzed in root (R), bud (B), stem (S), and leaf (L) using RT-PCR. The *ACT8* gene was used as an internal control.

(B) RT-PCR analysis of *AESP* mRNA levels in wild-type and transgenic plants containing an *AESP*-RNAi construct driven by the *ATDMC1* promoter. Plants 1, 2, and 7 exhibited reduced fertility and *AESP* RNA levels. Plant 5 showed normal levels of *AESP* RNA and was fertile. The *ACT8* gene was used as an internal control.

The first clear difference in development was observed at the heart stage, when ~25% of the seeds (151 of 663) arrested with the embryo at the globular stage (hereafter referred to as *Aesp-2* seeds) (Figures 4B and 4E). *Aesp-2* seeds (129 of 504) remained at the globular stage (Figure 4F) in siliques containing wild-type seeds at the torpedo and cotyledon stages (Figure 4C). No significant alterations in the appearance or morphology of the embryo proper or suspensor were observed in the vast majority (>95%) of *Aesp-2* seeds examined ($n > 300$). Embryo development in seeds of *Aesp-1* plants resembled that of *Aesp-2* plants, with ~25% (59 of 250) of the embryos arresting at the globular stage (data not shown). Therefore, *Atesp1* null mutations cause embryo arrest at the globular stage.

Alterations in endosperm development in *Aesp-2* seeds were also observed that are consistent with arrest at the globular stage. No differences in early endosperm development were observed. Two daughter nuclei were formed from the first division of the primary endosperm nucleus and found to migrate to opposite poles of the embryo sac. Later, numerous nuclei were observed, first around the periphery of the embryo sac and then dispersed throughout the embryo sac. The first major difference was observed at the early heart stage, when in wild-type embryos cellularization of the endosperm nuclei was observed (Figure 4B). By contrast, cellularization of endosperm nuclei was not observed in *Aesp-2* seeds (Figure 4E). Even in siliques containing wild-type curled cotyledon-stage embryos, the endosperm of *Aesp-2* seeds showed no signs of cellularization (Figure 4F).

A second endosperm-associated defect that was observed in *Aesp-2* seeds was an enlargement of many nuclei and nucleoli (Figures 4G and 4H). This is similar to, but not as dramatic as, the *titan* (*ttn*)-like phenotype that has been described (Liu et al., 2002). Endosperm of wild-type seeds at the late globular stage contained nuclei with an average diameter of 7.5 μm and nucleoli with an average diameter of 3.4 μm (Figure 4I). By contrast, endosperm of *Aesp-2* seeds exhibiting the *ttn*-like phenotype ($n = 75$) contained nuclei and nucleoli with average diameters of 17.2 and 7.0 μm , respectively. Enlarged endosperm nuclei with

enlarged nucleoli were found in both chalazal (Figure 4H) and peripheral (Figure 4G) positions. Similar defects were observed in the endosperm of *Aesp-1* seeds (data not shown). Therefore, *Aesp* null mutations also block the cellularization of the endosperm and result in a weak *ttn*-like phenotype in some endosperm nuclei.

***ATDMC1-AESP*-RNAi Plants Exhibit Reduced Fertility**

The embryo lethality associated with the T-DNA insertion lines limited our analysis of the role of *AESP* in chromosome segregation. Therefore, we generated transgenic plants expressing *AESP*-RNAi constructs driven by either the 35S or meiotic *ATDMC1* promoter. The *ATDMC1* promoter exhibits strong meiosis-specific expression (Klimyuk and Jones, 1997). It has been used effectively to drive the meiosis-specific expression of RNAi constructs to examine the role of DMC1, BRCA2, and CDC45 in meiotic prophase (Siaud et al., 2004; Stevens et al., 2004). Consistent with our observations with the T-DNA insertion lines, which indicated that *AESP* is an essential gene, we were unable to identify transgenic plants expressing the 35S-*AESP*-RNAi construct in >30,000 plants that were screened. By contrast, numerous transgenic plants were obtained after transformation with constructs expressing *AESP*-RNAi from the *ATDMC1* promoter.

Fifteen of the 20 independent *ATDMC1-AESP*-RNAi lines showed reduced fertility. The level of fertility varied between plants and sometimes between branches of the same plant. Of those *ATDMC1-AESP*-RNAi plants exhibiting reduced fertility, ~80% of the flowers produced reduced numbers of seeds. Flowers of *ATDMC1-AESP*-RNAi plants typically had short stamens (Figure 5B) and produced dramatically reduced levels of pollen. The pollen that was produced was typically shrunken (Figure 5D) relative to that from wild-type plants (Figure 5C). Siliques of the RNAi plants were much shorter (Figure 5E) and produced on average 6 full-size seeds per silique ($n = 50$) (Figure 5F) compared with wild-type siliques, which generated on average 55 seeds per silique ($n = 50$) (Figure 5G). When anthers of the *ATDMC1-AESP*-RNAi plants were squashed and stained with 0.1% toluidine blue, polyads containing between one and eight microspores were observed; wild-type anthers contained only tetrads of microspores. Interestingly, more than half (66%; $n = 200$) of the polyads had either five or six microspores (Figure 5H). This finding was somewhat unexpected. We had predicted that inhibition of separase activity would block nuclear division and result in a single tetraploid cell or would produce polyads with fewer than four microspores if some chromosome segregation occurred. Defects in the vegetative growth of *ATDMC1-AESP*-RNAi plants were not observed (data not shown).

AESP transcript levels were examined in the buds of *ATDMC1-AESP*-RNAi plants to determine whether the reduced fertility was correlated with a reduction in *AESP*-RNA (Figure 3B). A dramatic reduction in *AESP* RNA levels was seen in three of four plants examined containing the *ATDMC1-AESP*-RNAi construct. The three plants with reduced transcript levels exhibited obvious reduced fertility. The one *ATDMC1-AESP*-RNAi plant that did not show reduced transcript levels also did not show sterility and phenotypically resembled wild-type plants. The low levels of

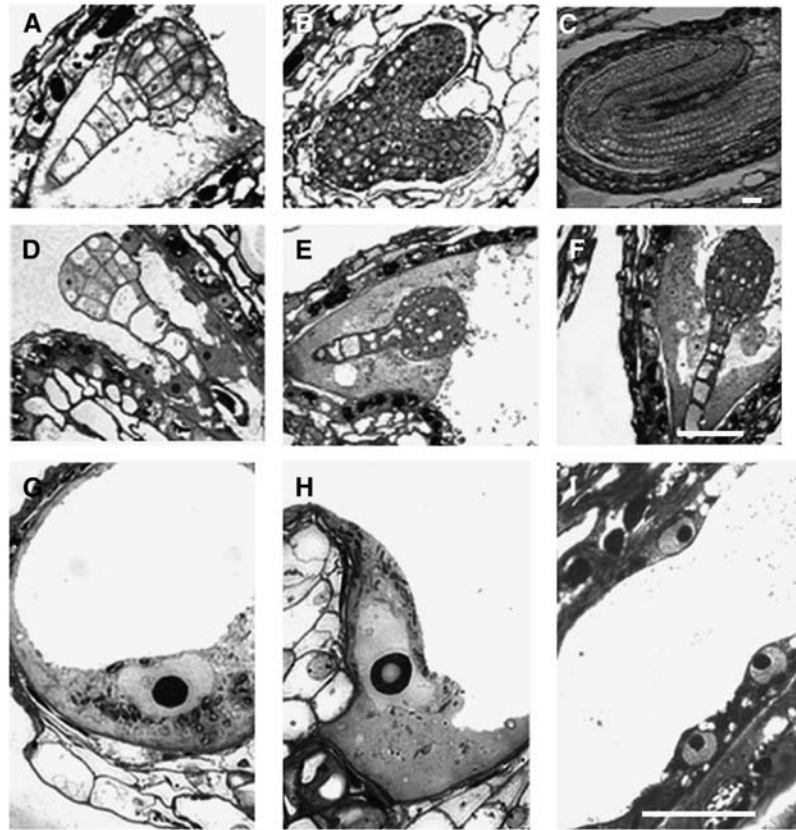


Figure 4. AESP Is Required for Seed Development.

Bright-field images of stained sections taken from embryos (**[A]** to **[F]**) and endosperm nuclei (**[G]** to **[I]**) in wild-type (**[A]** to **[C]** and **[I]**) and abnormal (**[D]** to **[H]**) seeds in the same siliques of heterozygous *Aesp-2* plants. Mutant embryos were arrested at the globular stage (**[D]** to **[F]**) relative to wild-type embryos at the globular (**[A]**), heart (**[B]**), and curled cotyledon (**[C]**) stages. Some periphery (**[G]**) and chalazal (**[H]**) endosperm nuclei and nucleoli were enlarged relative to those of wild-type embryos (**[I]**). Bars = 20 μm .

AESP transcripts found in buds of reduced-fertility plants are likely derived from somatic cells. Therefore, RT-PCR analysis of *AESP* RNA levels indicated that reduced *AESP* transcript levels are correlated with reduced fertility.

***ATDMC1-AESP-RNAi* Plants Are Defective in Meiosis I and II**

Chromosome spreads were examined in the reduced-fertility plants to investigate the effect of *AESP-RNAi* expression on meiotic chromosome segregation. No differences were observed between wild-type and *RNAi* plants during prophase I. Chromosome condensation, alignment, and synapsis appeared normal during leptotene, zygotene, and pachytene (Figures 6A and 6I). Continued chromosome condensation and the resolution of bivalents also occurred normally during diplotene and diakinesis (Figures 6B and 6J). No alterations were identified in metaphase chromosomes, which appeared to align normally (Figures 6C and 6K). The first major defect was observed at the metaphase I-to-anaphase I transition, when alterations in homologous chromosome segregation were observed. In $\sim 50\%$ (116 of 228) of the male meiocytes examined between late metaphase I and telophase I, chromosome bridges and tangled

and fragmented chromosomes were seen (Figures 6L to 6N). Cells resembling those shown in Figures 6L to 6N were never observed in wild-type plants. In $\sim 35\%$ (79 of 228) of the cells observed at anaphase I/telophase I, five intact bivalents were observed (Figure 6Q), suggesting either that the kinetochores did not attach properly to the spindle or that persistent cohesion completely blocked chromosome segregation. In $\sim 15\%$ of the cells (31 of 225), meiosis I appeared normal (data not shown).

By late anaphase II/telophase II, several types of cells were observed (Figures 6O and 6R). In most cells, a mixture of bivalents, chromosome fragments, and possibly chromosomes were observed (Figure 6O). In other cells, five intact bivalents, which were typically dispersed in different quadrants of the cell, were observed (Figure 6R). Nuclear envelopes formed around each bivalent or group of chromosomes, resulting in variable numbers of polyads (Figures 6P, 6S, and 6T) instead of the normal tetrad of microspores observed in wild-type cells (Figure 6H). Our observation that most cells contain a mixture of bivalents, chromosomes, and chromosome fragments, which arise from chromosome bridges, is consistent with, and explains, our observation of polyads with on average five to six microspores instead of the single tetraploid microspore that we initially

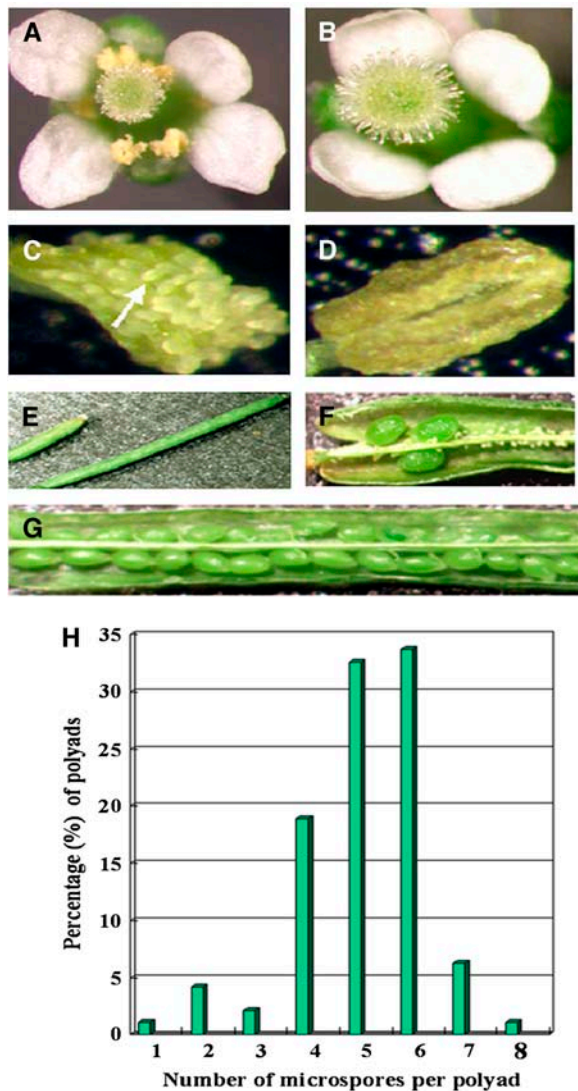


Figure 5. *ATDMC1-AESP-RNAi* Plants Exhibit Reduced Fertility.

(A) to (G) Anthers and pollen development in wild-type and *ATDMC1-AESP-RNAi* plants. Wild-type flowers had elongated stamens (A) and mature pollen grains (C, arrow), whereas *ATDMC1-AESP-RNAi* flowers had short stamens (B) and shrunken pollen grains (D). The siliques in RNAi plants were dramatically shorter (E) and generated fewer seeds (F) in contrast with wild-type siliques (G).

(H) Relative number of microspores in *ATDMC1-AESP-RNAi* mutant polyads ($n = 200$ polyads).

expected. Polyads containing one to three microspores arise from those cells exhibiting a complete failure to segregate homologous chromosomes.

In summary, reduction of *AESP* RNA levels in microsporocytes is associated with defects in chromosome segregation that are consistent with persistent sister chromatid cohesion throughout meiosis. Although the *ATDMC1-AESP-RNAi* is not 100% efficient in eliminating separase activity, most meiocytes exhibit defects in chromosome segregation. We expect that the reduc-

tion in *AESP* levels directly affects the release of cohesion in both meiosis I and meiosis II; however, we cannot exclude the possibility that the alterations observed during meiosis II are the result of abnormalities associated with meiosis I. The presence of significant numbers of cells with five intact bivalents at telophase II also raises the possibility that reduced *AESP* levels may affect the attachment of kinetochores to the meiotic spindle.

SYN1 Removal Is Defective in *ATDMC1-AESP-RNAi* Plants

The meiotic alterations suggested that *ATDMC1-AESP-RNAi* blocked the normal release of sister chromatid cohesion. Therefore, the distribution of the cohesin protein SYN1 on meiotic chromosomes of *ATDMC1-AESP-RNAi* plants was investigated to determine whether the meiotic alterations observed were in fact attributable to defects in cohesin release. SYN1 is the *Arabidopsis* homolog of REC8 and is essential for chromosome cohesion during meiosis (Bai et al., 1999; Bhatt et al., 1999). In wild-type plants, SYN1 localizes to the developing chromosome axes beginning at early leptotene and lines the chromosome axes of synapsed chromosomes at pachytene (Figure 7A). During diplotene and diakinesis, a large portion of SYN1 is released from chromosomes (Figure 7B), and at prometaphase I and metaphase I, SYN1 is found primarily at the centromeres (Figures 7C and 7D). Beginning at early anaphase I, SYN1 is no longer detected on wild-type meiotic chromosomes (Figure 7E). However, the transient observation of SYN1 in the nucleoplasm of meiotic interphase II cells suggested that low but undetectable levels of SYN1 remain on chromosomes after anaphase I (Cai et al., 2003). These localization patterns closely resemble those observed for the *Arabidopsis* SCC3 cohesin protein (Chelysheva et al., 2005) and suggest that chromatin conformation at *Arabidopsis* centromeres blocks access to cohesin antibodies.

The distribution of SYN1 on meiotic chromosomes of *ATDMC1-AESP-RNAi* plants resembled that of wild-type cells during early stages of meiosis, including pachytene (Figure 7F), diakinesis (Figure 7G), prometaphase I (Figure 7H), and metaphase I (Figure 7I). In particular, SYN1 appeared to be released from the chromosomes normally during diplotene and diakinesis. The reduction of SYN1 signal during diplotene and diakinesis in *ATDMC1-AESP-RNAi* plants suggests that cohesion is released via a two-step mechanism during meiosis I in plants, similar to the situation during mitotic prophase in animal cells (Sumara et al., 2000, 2002; Waizenegger et al., 2000; Losada et al., 2002) and meiotic prophase in yeast (Yu and Koshland, 2005). However, in contrast with wild-type plants, strong SYN1 signals were observed during anaphase I and in cells at various stages of meiosis II. Strong SYN1 labeling of the chromosomes was observed on the stretched and fragmented chromosomes during anaphase I (Figures 7J and 7K) as well as in unsegregated bivalents from telophase I through telophase II (Figures 7L to 7O). Therefore, expression of *AESP* RNAi from the *ATDMC1* promoter blocks the removal of SYN1 from the chromosomes and prevents the normal segregation of chromosomes during meiosis. These results confirm that *AESP* is the plant separase and that *AESP* function is required for the release of cohesion during meiosis.

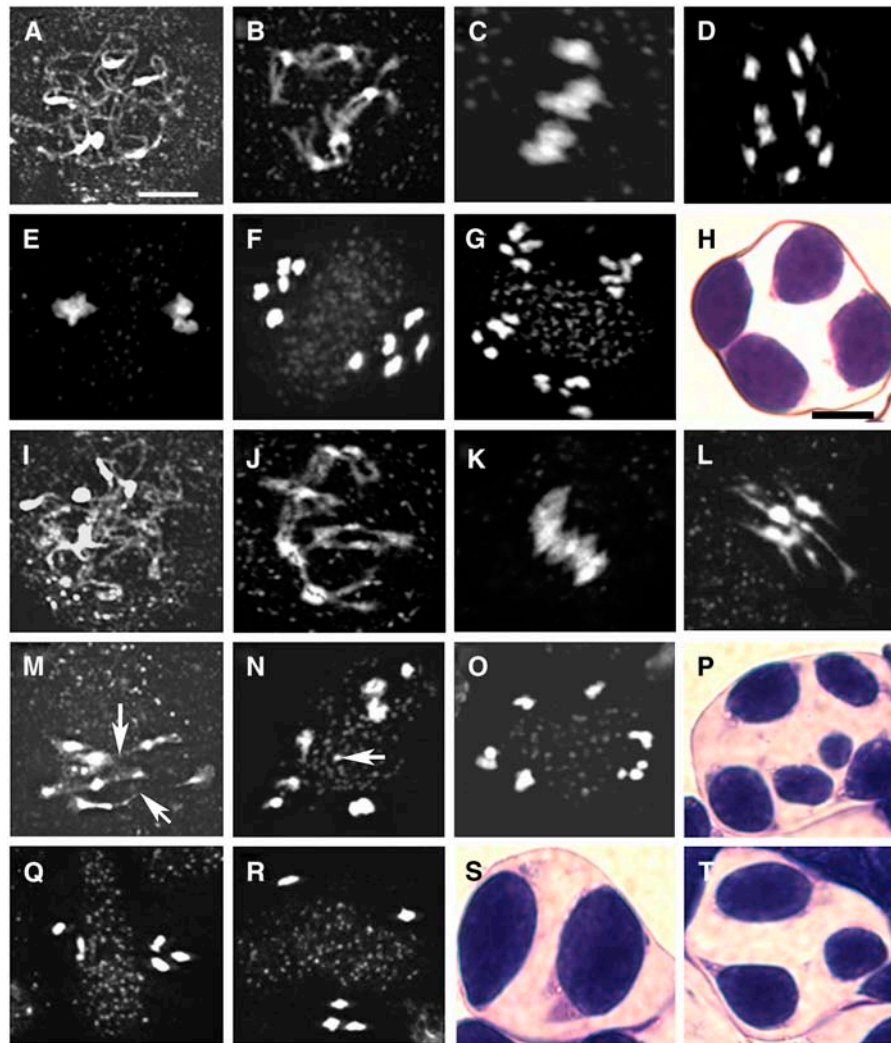


Figure 6. *ATDMC1-AESP-RNAi* Plants Are Defective in Meiosis I and Meiosis II.

Meiotic spreads of wild-type ([A] to [G]) and *ATDMC1-AESP-RNAi* ([I] to [O], [Q], and [R]) plants were prepared with Carnoy's fixative and stained with 4',6-diamidino-2-phenylindole (DAPI). Tetrads of wild-type plants ([H] and [J]) and polyads of *ATDMC1-AESP-RNAi* plants ([P], [S], and [T]) were stained with toluidine blue. No differences were detected in wild-type and mutant meiocytes during prophase I, including late pachytene ([A] and [I]) and diakinesis I ([B] and [J]), and metaphase I ([C] and [K]). Homologous chromosomes segregated evenly during anaphase I ([D] and [L]) and telophase I ([E] and [M]). Wild-type meiocytes at meiotic metaphase II ([F] and [N]), telophase II ([G] and [O]), and tetrad stage ([H] and [P]) are shown. Most meiocytes in the RNAi lines displayed more than four groups of chromosomes at telophase II ([O]) and produced polyads with more than four microspores, which contained different amounts of genetic material ([P]). Bivalents did not segregate in some meiocytes at meiosis I ([Q] and [R]) and meiosis II ([S] and [T]). Stages of the abnormal meiocytes are approximate and based on cell morphology and the stage of the surrounding tissue. Bars = 5 μ m.

DISCUSSION

Separase Is Required for the Proper Disjunction of Chromosomes during Meiosis in *Arabidopsis*

Separase has been characterized and shown to be required for proper chromosome disjunction during mitosis and meiosis in a number of organisms, including budding and fission yeast, flies,

worms, and vertebrate cells (reviewed in Losada and Hirano, 2005; Nasmyth and Haering, 2005; Queralt and Uhlmann, 2005). To investigate whether this is also the situation in plants, we generated and analyzed a number of transgenic plants expressing an *AESP-RNAi* construct expressed from the meiotic *ATDMC1* promoter. Reduced fertility was observed in 75% of the transgenic plants examined, and >80% of the meiocytes examined in reduced-fertility plants displayed meiotic defects.

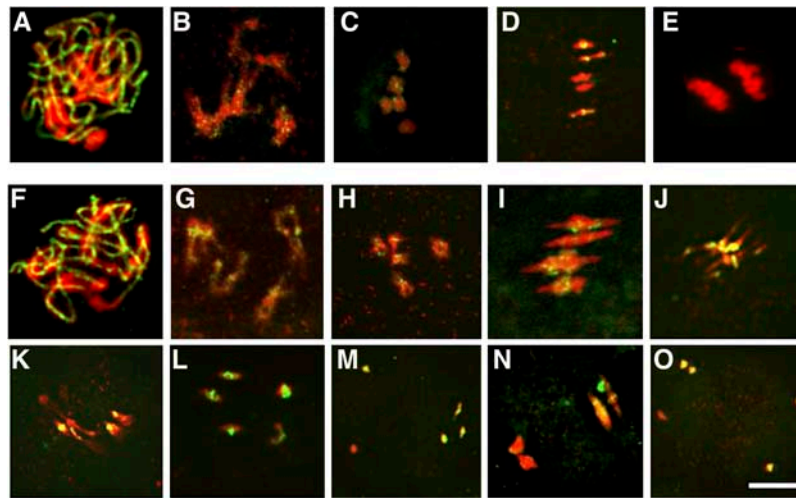


Figure 7. SYN1 Removal Is Defective in *ATDMC1-AESP*-RNAi Plants.

Meiotic spreads of wild-type (**[A]** to **[E]**) and *ATDMC1-AESP*-RNAi (**[F]** to **[O]**) plants were prepared and stained with anti-SYN1 antibody (green) and propidium iodide (red). Meioocytes in wild-type plants and the RNAi mutant exhibited similar SYN1 staining at pachytene (**[A]** and **[F]**), diakinesis (**[B]** and **[G]**), prometaphase I (**[C]** and **[H]**), and metaphase I (**[D]** and **[I]**). SYN1 labeling was not detected after anaphase I (**[E]**) in wild-type meiocytes. SYN1 was still detected on chromosome arms and at centromeric regions at anaphase I (**[J]** and **[K]**) and on intact bivalents at various stages of meiosis II in the meiocytes of RNAi plants (**[L]** to **[O]**). Bar = 5 μ m.

Therefore, similar to the situation in yeast, worms, *Drosophila*, and animal cells, separase is required for meiosis in *Arabidopsis*.

Although the requirement for separase during meiosis appears to be universal, there are differences in meiotic chromosome behavior when separase activity is inhibited or inactivated in some organisms. In budding and fission yeast, expression of a noncleavable form of REC8 or temperature-sensitive mutations in separase result in chromosome nondisjunction at meiosis I (Buonomo et al., 2000; Kitajima et al., 2003). In budding yeast, separase is required not only for the onset of anaphase but also for spindle disassembly through the FEAR (Cdc14 early anaphase release) pathway in meiosis I (Buonomo et al., 2003). By contrast, an accumulation of anaphase I spindles was not observed in a *Schizosaccharomyces pombe* separase mutant (Kitajima et al., 2003), suggesting that it is dispensable for spindle disassembly at meiosis I in fission yeast. Treatment of *C. elegans* embryos with *sep-1* RNAi blocked the normal disjunction of homologous chromosomes at meiosis I (Siomos et al., 2001). Chromosomes appeared to align properly on a normal meiosis I spindle, but their segregation was either delayed significantly or blocked completely. It was concluded that the chromosomes of *sep-1* RNAi embryos come under normal tension but fail to disjoin (Siomos et al., 2001). In mice, ~70% of oocytes overexpressing securin, the separase inhibitor, arrest at metaphase I (Terret et al., 2003).

Our analysis of meiosis in the *ATDMC1-AESP*-RNAi lines identified both defects in the release of chromosome cohesion that resulted in chromosome fragmentation and intact bivalents at both anaphase I and II. In approximately half of the meiocytes examined, sister kinetochores of homologous chromosomes appeared to attach properly to the spindles, but cohesion along the chromosome arms prevented proper homolog resolution,

resulting in stretched and broken chromosomes (Figure 6L). In ~35% of the cells examined, intact bivalents were observed. Typically, but not always, the bivalents were observed in different quadrants of the cell. After telophase II, nuclear envelopes formed around bivalents, groups of chromosomes, and chromosome fragments, resulting in variable numbers of polyads. Therefore, in contrast with the observations in most other systems, meiocytes in our RNAi lines do not arrest at metaphase I but progress through both meiosis I and II, resulting in the production of polyads. Although unique to our system, this finding is not completely unexpected, because plants appear to lack meiotic checkpoint control mechanisms found in many other organisms (Roeder, 1997; Roeder and Bailis, 2000; Armstrong and Jones, 2003).

The presence of intact bivalents in meiocytes of *ATDMC1-AESP*-RNAi plants also raises the possibility that AESP is required for the proper attachment of kinetochores to the spindle in plant cells. In most cells that failed to separate bivalents, the bivalents were observed at opposite poles, or quadrants of the cell, at anaphase I or II, respectively, suggesting that only one pair of sister kinetochores attached to the spindle. The presence of intact bivalents can also be explained if the bivalent was subjected to bipolar traction but failed to separate completely because cohesin complexes were not removed. Depolymerization of the microtubules would then allow the bivalents to disperse throughout the cell. Separase has been implicated in anaphase spindle stabilization and the coupling of anaphase to mitotic exit in budding yeast (Ciosk et al., 1998; Cohen-Fix and Koshland, 1999; Uhlmann et al., 2000; Sullivan and Uhlmann, 2003) as well as in the coordination of chromosome segregation and spindle formation during meiosis (Buonomo et al., 2003). However, a requirement for separase in the attachment of

kinetochores to the spindle has not yet been demonstrated in any organism. Further experiments are required to determine whether AESP actually plays a role in kinetochore attachment.

Separase Is Required for the Release of Cohesion during Anaphase but Not during Meiotic Prophase in *Arabidopsis*

A number of studies support the notion that proteolytic cleavage of REC8 by separase triggers homolog segregation at meiosis I (Buonomo et al., 2000; Siomos et al., 2001; Kitajima et al., 2003). However, the direct demonstration that inhibition of separase activity blocks the removal of cohesion from chromosome arms at anaphase I has been lacking. In this report, we clearly demonstrate the presence of SYN1 on unresolved bivalents in meiocytes of *ATDMC1-AESP-RNAi* lines. In contrast with wild-type plants, in which very little to no SYN1 signal is observed on chromosomes after the onset of anaphase I (Figure 7E), strong SYN1 signals were observed during anaphase I and in cells at various stages of meiosis II in meiocytes of *ATDMC1-AESP-RNAi* plants (Figures 7J to 7O). Therefore, expression of *AESP-RNAi* during meiosis clearly blocked the removal of the cohesion protein SYN1 from meiotic chromosomes during meiosis I and II. This is a direct example in which inhibition of separase activity blocked the removal of cohesin from the chromosomes.

The release of sister chromatid cohesion during meiosis appears to occur in a three-step process in *Arabidopsis*. A large portion of the arm cohesin is released from the chromosomes during prophase I (Cai et al., 2003). Residual arm cohesin is released at anaphase I to allow the separation of homologous chromosomes, whereas centromeric cohesin is released at anaphase II to allow the separation of sister chromatids. Our results indicate that the release of cohesin during diplotene and diakinesis occurs in a separase-independent process, whereas the release of cohesin at anaphase I and anaphase II is separase-dependent. It was shown recently that approximately half of the cohesin complexes are removed from the arms of meiotic chromosomes before metaphase I in budding yeast (Yu and Koshland, 2005). This process was dependent on the presence of Cdc5 and condensin. Cohesin is also released in separase-dependent and -independent processes during mitosis in vertebrate cells. During mitotic prophase, cohesin is released from chromosome arms in a separase-independent process that involves the phosphorylation of cohesin by a Polo-like kinase and of histones by Aurora kinase (Waizenegger et al., 2000; Sumara et al., 2002). Centromeric cohesin is then released at anaphase in a separase-dependent process. Our results raise the possibility that the prophase removal of cohesin during meiosis in plant cells may use a mechanism involving condensin and a Polo kinase. Studies are under way to investigate this possibility and to better understand how the formation and release of sister chromatid cohesion is regulated in plants.

AESP Is Essential for Embryo and Endosperm Development

Analysis of two different T-DNA-tagged lines demonstrated that AESP is essential for embryo and endosperm development. The overall phenotype of *Aesp* knockout plants is reminiscent of the *ttn* mutants (Liu et al., 2002). The defining features of *ttn* mutants

are arrested embryos and the presence of abnormal endosperm with giant polyploid nuclei. Mutations in the SMC proteins, which are core components of the cohesin and condensin complexes, result in a *ttn*-like phenotype (Liu et al., 2002). Other *TTN* gene products include an ARL2 class of GTP binding proteins (TTN1), a protein related to tubulin-folding cofactor D (TTN5), a protein related to the isopeptidase T class of deubiquitinating enzymes (TTN6), and two plant-specific proteins of unknown function (Tzafrir et al., 2002). Embryo development typically arrests early in most *ttn* mutants (Liu et al., 2002; Tzafrir et al., 2002). However, similar to the *Aesp* mutants, *ttn6* embryos develop to approximately the globular stage. Unlike *ttn6* embryo cells, though, which often appeared rounded and disorganized, *Aesp* embryo cells appeared relatively normal. The *ttn*-like phenotype was also less dramatic in *Aesp* mutants than in *ttn6*. Specifically, the increased size of nuclei and nucleoli was not as pronounced in *Aesp* endosperm compared with those in *ttn6* plants. Furthermore, <50% of the endosperm nuclei examined in *Aesp* plants exhibited enlarged nucleoli. Variations in the seed phenotype have also been observed in the *ttn* mutants, including *ttn6*.

Although separase, SMC1, and SMC3 all participate in the control of sister chromatid cohesion during mitosis and meiosis, the phenotype of *Aesp* null mutants differs somewhat from those of *ttn8* (SMC1) and *ttn7* (SMC3). The SMC1 and SMC3 knockouts both exhibit strong *ttn*-like phenotypes, with significant defects in early embryo development and enlarged endosperm nuclei and nucleoli (Liu et al., 2002). Our finding that *Aesp* mutant embryos develop further and that the endosperm phenotype is weaker relative to *ttn7* and *ttn8* is surprising because mutations in separase should completely block chromosome separation during mitosis. Our finding suggests that cells may contain a pool of AESP RNA or sequestered protein. Results from our analysis of *ATDMC1-AESP-RNAi* plants suggest that cells may in fact contain a pool of AESP RNA. It has been suggested that the presence of functional gametes, small embryos, and polyploid endosperm nuclei in seeds containing null mutations in genes critical for cellular division may reflect the diffusion of trace amounts of functional protein from surrounding heterozygous tissues (Liu et al., 2002). This may also be true for AESP; however, we believe that this is less likely given that the activity of separase must be tightly regulated throughout the cell cycle for proper nuclear division (Papi et al., 2005; Queralt and Uhlmann, 2005).

It was further proposed that early cellular abortion in the embryo relative to continued DNA replication and nuclear enlargement in *ttn7* and *ttn8* embryos may result from different cell cycle checkpoints operating in *Arabidopsis* embryos and endosperm (Liu et al., 2002). It is possible that different cell cycle control mechanisms operate in the embryo and endosperm and that different cell types contain different levels of the SMC and separase proteins or mRNA for the proteins. It is also possible that the observed differences are attributable to the proteins exhibiting different cellular functions. Separase has been implicated in a relatively large number of cellular events in addition to its essential role in the release of sister chromatid cohesion. It has been implicated in the positioning of the spindle body in fission yeast (Funabiki et al., 1996), the proper positioning of the centrosome and eggshell development during embryogenesis in *C. elegans* (Siomos et al., 2001; Rappleve et al., 2002), and

epithelial cell organization in *Drosophila* (Pandey et al., 2005). Separase also has been shown to play a role in controlling the mitotic cell cycle in yeast and animal cells. In budding yeast, separase acts with Cdc14 to link anaphase to mitotic exit (Stegmeier et al., 2002; Sullivan and Uhlmann, 2003; Stemmann et al., 2005). In human cells, separase appears to control multiple aspects of the G2/M program (Papi et al., 2005). These cell cycle control activities involve nonproteolytic functions of separase, including Cdk1 binding (Gorr et al., 2005; Papi et al., 2005). Therefore, it is possible that some of the embryo/endosperm alterations and the differences between the *Aesp* and *ttn7* and *ttn8* mutants are attributable to separase and possibly the SMC proteins (Lam et al., 2005) having additional roles in plant cells. Studies are currently under way to investigate additional potential roles of this important protein in plant cells.

METHODS

Plant Material

Arabidopsis thaliana ecotype Wassilewskija was used in this study. The SALK T-DNA insertion lines 071659 and 037016 were obtained from the ABRC Stock Center. Plants were grown on a commercial potting mix in a growth chamber at 20°C with a 16-h-light/8-h-dark cycle. Ten days after germination, leaves and stems were collected from rosette-stage plants. After 16 to 18 d, buds with lengths between 0.5 and 0.7 mm were collected from prebolting plants for the analysis of meiosis. Roots were collected from seeds grown on agar plates.

Molecular Analysis of AESP

A full-length *AESP* cDNA was generated using RT-PCR. The large size of *AESP* made it impossible for us to clone one full-length cDNA. Therefore, the *AESP* cDNA was initially cloned as a series of overlapping PCR fragments and subsequently confirmed by the generation of two large overlapping cDNA fragments by RT-PCR. Total root RNA was isolated (Verwoerd et al., 1989), and cDNA was produced by reverse transcription with an oligo(dT) primer (Thermoscript RT-PCR system; Invitrogen). PCR was conducted on the cDNA using a series of gene-specific primers. The products were cloned into pGEM-T Easy (Promega) and sequenced using a combination of vector- and gene-specific primers.

The distribution of *AESP* RNA in different tissues was investigated using RT-PCR. Total RNA was isolated from leaves, stems, roots, and buds. An equal amount (4 µg) of total RNA from the different tissues was used to synthesize cDNA using the Thermoscript RT-PCR system (Invitrogen). *ACT8* was used to standardize the amount of cDNA from the different tissues (An et al., 1996). *AESP* RNA levels were determined by PCR with the primers *AESP*-764 (5'-GGGCATATGCCTGGTGGT-GATCTCACCG-3') and *AESP*-765 (5'-CCCGAATTCGAGATGAAGAA-GGAAG-3'). PCR products were separated on 0.7% agarose gels, transferred to nylon membranes, and hybridized with the corresponding *ACT8* and *AESP* PCR products labeled with [α -³²P]dCTP. Radioactivity was detected with a PhosphorImager (Molecular Dynamics).

The effects of knockout mutations in *AESP* were investigated by the analysis of two independent T-DNA insertion lines. Genomic DNA was isolated from plants grown from seeds obtained for the SALK T-DNA insertion lines 071659 and 037016 and screened with a combination of plant-specific and left border primers to detect the presence of the T-DNA insertions. After the presence of the insertions was confirmed by DNA sequence analysis of the PCR fragments, the SALK T-DNA insertion lines 071659 and 037016 were renamed *Aesp-1* and *Aesp-2*, respectively. Seeds were collected from plants confirmed to be heterozygous for the

T-DNA inserts and regrown on soil to (1) rescreen for plants homozygous for the T-DNA insertions, (2) study embryo and seed development, or (3) examine seed germination by sowing the seeds on moist filter paper. An allelism test between *Aesp-1* and *Aesp-2* was conducted by performing reciprocal crosses between heterozygous plants containing the *Aesp* mutations and scoring the number of aborted seeds. Approximately 1% (4 of 632) of the seeds obtained from crosses with wild-type plants were dry and shrunken. By contrast, 125 of 506 seeds (24.7%) from crosses between *Aesp-1* and *Aesp-2* heterozygous lines were found to be defective. Backcrossing of the *Aesp-2* mutations to wild-type plants resulted in normal inheritance (48 of 105 progeny were heterozygous) of the T-DNA from both male and female parental lines.

Transgenic plants expressing *AESP*-RNAi constructs from the 35S and meiosis-specific *ATDMC1* promoters were produced and analyzed. A 435-bp fragment of the *AESP* cDNA (nucleotides 35 to 470) was amplified using the primers *AESP*-686 (5'-ACGTTAATTAAGGCGCGCCTCTCATC-GACGTCGGCG-3') and *AESP*-687 (5'-CGCAGGACCATTTAAATCCTC-CGGCAACAACCGGG-3') and cloned in both the sense and antisense directions into pFGC5941 (kindly provided by Rich Jorgenson). A modified version of the pFGC5941 *AESP* RNAi plasmid was generated in which the 35S promoter was replaced with the meiosis-specific *ATDMC1* promoter (Klimyuk and Jones, 1997). Constructs were introduced into *Agrobacterium tumefaciens* strain GV3010 and used to transform wild-type *Arabidopsis* plants (Clough and Bent, 1998). Transgenic plants were initially identified by BASTA (Farnam Companies) selection, and the presence of the transgene was then confirmed by PCR.

Microscopy

Embryo development was studied essentially as described (Liu and Meinke, 1998). Specifically, siliques were fixed in 3.5% (w/v) glutaraldehyde and 1.5% (w/v) paraformaldehyde in 0.1 M cacodylate buffer, pH 7.0, for 4 h at room temperature and rinsed with 0.1 M cacodylate buffer twice for 15 to 30 min each. Tissues were treated with 1% (w/v) OsO₄ at 0°C for 24 h followed by washing twice in distilled, deionized water for 15 to 30 min each and dehydrated with a graded ethanol series (20, 30, 50, 70, and 95% and three times with 100% ethanol) for 30 min each. The tissue was infiltrated with 25, 33.3, 50, 66.6, and 75% LR White resin in ethanol and three times with 100% resin with rotation for 16 h each. Tissues were embedded in 100% resin at 58°C for 12 h, and 0.6-µm tissue sections were made using a microtome (Reichert Ultracut) and glass knives followed by staining in 0.1% toluidine blue/0.1% borax.

Meiosis in the transgenic lines was examined and compared with that in wild-type plants using DAPI-stained meiotic chromosome spreads (Ross et al., 1996). Anthers were fixed in Carnoy's reagent (3:1 ethanol:acetic acid) for 15 min and soaked in water for 15 min twice, followed by digestion with 1.4% β-glucuronidase, 0.3% cytohelicase, 0.3% pectolyase, and 0.3% cellulase in 10 mM sodium citrate for 40 min. The samples were treated with cold 10 mM sodium citrate for 5 min twice and cleared in 60% acetic acid for 2 min, squashed, frozen on dry ice, and dried overnight. The samples were washed in 1× PBS for 15 min and stained with DAPI with 1,4-diazabicyclo-[2,2,2]-octane (DABCO). Samples were observed with an Olympus IX-81 fluorescence deconvolution microscope system. Data were analyzed with Image Pro Plus (Media Cybernetics) and organized with Photoshop. Meiotic stages were assigned based on chromosome and cellular morphology as well as the stage of the surrounding tissue.

The distribution of SYN1 antibody on the chromosomes was used to monitor the presence of the cohesin complex. Meiotic chromosome spreads were prepared as described above. The slides were then stained with anti-SYN1 antibody (Cai et al., 2003) (1:500 dilution) at 4°C overnight in a moist container. The slides were washed for 2 h with eight changes of wash buffer (1× PBS, 1 mM EDTA, and 0.1% Tween 20). The anti-SYN1 antibody was detected with Alexa-488 goat anti-rabbit antibody (1:300

dilution) at 4°C overnight. The slides were again washed for 2 h with eight changes of wash buffer and then treated with 2 µg/mL propidium iodide at 37°C for 30 min. The slides were rinsed with 1× PBS and mounted with DABCO antifade mounting medium. Samples were observed and analyzed as described above.

Accession Numbers

The Arabidopsis Genome Initiative numbers for *AESP* and *ATDMC1* are At4g22970 and At3g22880, respectively.

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