The *Arabidopsis* SYN1 cohesin protein is required for sister chromatid arm cohesion and homologous chromosome pairing

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Summary

The faithful transmission of chromosomes during mitosis and meiosis requires the establishment and subsequent release of cohesion between replicated chromosomes. Sister chromatid cohesion is mediated, in large part, by the cohesin complex, which consists of four highly conserved proteins: SMC1, SMC3, SCC1/REC8 and SCC3. Mitotic cohesin complexes contain SSC1, whereas meiotic cohesin complexes contain the related REC8 protein. As part of studies to identify and characterize proteins required for meiosis in plants, we previously identified a putative *Arabidopsis* REC8 homolog, referred to as *syn1*. Preliminary cytological studies indicated that *syn1* plants exhibit defects in meiotic chromosome cohesion and condensation that result in fragmentation of the chromosomes and the formation of polyads. In the experiments presented here we show that SYN1 encodes a protein that localizes to arms of meiotic chromosomes from approximately meiotic interphase to anaphase I. The protein is not detected at the centromeres or after metaphase I. Furthermore, fluorescence in situ hybridization experiments on microsporocytes from *syn1* plants demonstrate that the mutation eliminates arm cohesion as early as interphase, whereas centromere cohesion is maintained until approximately anaphase I. These results indicate that although the main role of SYN1 is in chromosome arm cohesion, it is also important for maintaining cohesion at the centromeres during late stages of meiosis I.

Key words: Meiosis, *Arabidopsis*, Cohesins, Synapsis

Introduction

The faithful transmission of chromosomes during mitosis and meiosis is essential for the survival of eukaryotic organisms. A critical aspect of chromosome segregation is sister chromatid cohesion, which is required for proper attachment of chromosomes to the spindle and the faithful segregation of sister chromatids to opposite poles of the cell during anaphase (reviewed by Orr-Weaver, 1999; van Heemst and Heyting, 2000). Sister chromatid cohesion is mediated, in part, by a group of highly conserved proteins, referred to as the cohesin complex. Four proteins (SMC1, SMC3, SCC1 and SCC3) form the core of the mitotic cohesin complex, which is utilized by a wide range of organisms. In *S. cerevisiae*, the cohesion complex is found on chromosomes from S phase to anaphase, with preferential binding in centromeric regions (Michaelis et al., 1997; Toth et al., 1999; Uhlmann and Nasmyth, 1998). 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, a cysteine protease, which specifically cleaves SCC1 (Ciosk et al., 1998; Hauf et al., 2001; Uhlmann et al., 1999; Uhlmann et al., 2000).

Sister chromatid cohesion also serves a critical role in meiosis; however, there are several important differences between its roles in meiosis and mitosis. In the first meiotic division, which is a reductional division, homologous chromosomes segregate. Attachment of sister kinetochores to spindles occurs with the same polarity, termed monopolar attachment, ensuring that homologs and not sister chromatids segregate in this first division. Therefore, whereas sister chromatids are attached to microtubules emanating from opposite poles during mitosis, they attach to microtubules from the same pole during meiosis I. Also, with few exceptions, recombination between homologous chromosomes occurs during meiotic prophase to form chiasmata, which hold maternal and paternal chromosomes together. In order for homologs to separate during anaphase I, chiasmata between homologous chromosomes must be resolved and sister chromatid cohesion released along the arms. Therefore, meiotic divisions require sister chromatid cohesion to be released in two steps. In the first step cohesion is released along the arms to facilitate resolution of chiasmata while it is maintained at the centromeres. Destruction of centromeric cohesion at anaphase II then allows the separation of sister chromatids in an equational division.

Sister chromatid cohesion during meiosis is mediated by cohesin complexes that are similar to their mitotic counterparts and share many of the same subunits (reviewed in Lee and Orr-Weaver, 2001; Orr-Weaver, 1999). However, meiotic cohesin contains at least one, and in some instances more, meiosis-specific forms of the core cohesin proteins, including REC8 for

SCC1 and in animal cells SMC1β for SMC1 and STAG3 for SCC3 (Prieto et al., 2001; Revenkova et al., 2001). All meiotic cohesin complexes studied to date contain the meiosis-specific REC8 cohesin. Mutations in REC8 have been identified and characterized in a number of organisms (Bai et al., 1999; Bhatt et al., 1999; Klein et al., 1999; Lin et al., 1992; Parisi et al., 1999; Pasierbek et al., 2001; StoopMyer and Amon, 1999; Watanabe and Nurse, 1999). In *S. cerevisiae* and *S. pombe*, *rec8* mutations result in reduced recombination, alterations in synaptonemal complex formation and premature separation of sister chromatids (Molnar et al., 1995; Klein et al., 1999; Watanabe and Nurse, 1999). In *C. elegans*, depletion of REC8 using RNAi resulted in the formation of univalents and chromosome fragmentation at diakinesis (Pasierbek et al., 2001). Finally, *Arabidopsis* plants containing mutations in the REC8/SCC1 ortholog, referred to as SYN1/DIF1, exhibit alterations in chromosome condensation and cohesion that lead to chromosome fragmentation at metaphase I (Bai et al., 1999; Bhatt et al., 1999).

REC8 localization studies on meiotic chromosomes have been conducted in several organisms. *S. pombe* Rec8 is present from the time of premeiotic DNA synthesis until after meiosis I (Parisi et al., 1999). It is localized as foci throughout chromosomes, with the highest concentration at the centromeres (Watanabe and Nurse, 1999). Likewise, *S. cerevisiae* Rec8 is found as punctate foci along chromosomes in early prophase I. It subsequently localizes to centromeric regions where it persists until approximately anaphase II (Klein et al., 1999). REC8 localization patterns in *C. elegans* were similar to those observed in yeast. Specifically, REC8 was partially lost along chiasmata-distal portions of the arms at anaphase I and at the centromeres at metaphase II (Pasierbek et al., 2001).

Differences have also been reported concerning the release of sister chromatid cohesion during meiosis. In yeast and *C. elegans* the anaphase promoting complex (APC)-activated separase pathway is required for the release of cohesin at the onset of anaphase I (Buonomo et al., 2000; Siomos et al., 2001). In contrast, experiments in *Xenopus* have suggested that chromosome segregation at meiosis I takes place in the absence of APC activity and in the presence of high levels of securin, the separase inhibitor (Peter et al., 2001; Taieb et al., 2001). This suggests that removal of cohesin from the arms of *Xenopus* chromosomes during meiosis I may occur by a mechanism similar to that observed for the removal of cohesin from other vertebrate arms during mitotic prophase (Darwiche et al., 1999; Losada et al., 1998; Sumara et al., 2000; Waizenegger et al., 2000; Warren et al., 2000). Finally, phenotypic differences have also been observed in cells containing mutations in cohesin subunits. For example, in *rec8* mutants of *S. cerevisiae*, chromosomes segregate randomly at meiosis I (Klein et al., 1999), whereas *in S. pombe rec8* mutants, sister chromatids segregate equationally at anaphase I (Watanabe and Nurse, 1999). Therefore, a number of differences in the distribution and release of REC8 as well as the effect of *rec8* mutations have been identified in the relatively few organisms studied to date. This suggests that, while the general nature and properties of meiotic cohesin complexes are similar, differences probably exist in the way cohesion is controlled in different organisms.

As part of studies to better understand sister chromatid cohesion in plants, we have further characterized the role of a putative *Arabidopsis* REC8 ortholog, SYN1, which we previously identified in a T-DNA-tagged, meiotic mutant (*syn1*) of *Arabidopsis* (Bai et al., 1999; Peirson et al., 1997; Peirson et al., 1996). Preliminary cytological studies indicated that *syn1* plants exhibit defects in chromosome cohesion and condensation that result in fragmentation of the chromosomes and the formation of polyads (Bai et al., 1999; Peirson et al., 1997). In the experiments described below, we show that SYN1 encodes a protein that localizes to arms of meiotic chromosomes from approximately S phase to anaphase I. The protein is not detected at the centromeres or after metaphase I. Furthermore, fluorescence in situ hybridization (FISH) experiments on microsporocytes from *syn1* plants demonstrate that the mutation eliminates arm cohesion as early as leptotene whereas centromere cohesion is maintained until approximately anaphase I.

Materials and Methods

Plant material

Seeds of wild-type and *syn1 Arabidopsis thaliana*, ecotype Wassilewskija (WS) were grown on a commercial potting mix in a growth chamber at 22°C with a 16:8 light:dark cycle. The *syn1* mutation is a T-DNA-tagged mutation that has been previously described (Bai et al., 1999). Approximately 15-18 days after germination, buds with lengths between 0.3 and 0.7 mm were collected from prebolting plants, fixed and analyzed as described below.

SYN1 antibody production

A portion of the SYN1 cDNA containing amino acids 178 to 353 was cloned into pET24b (Novagen), transformed into BL21(DE3)pLysS cells and overexpressed as a histidine-tagged protein. Upon induction the overexpressed protein accumulated in the insoluble fraction. Inclusion bodies were collected from overexpressing cells, washed, solubilized in PBS containing 8 M urea and purified using nickel chromatography. The isolated protein was further purified by SDS polyacrylamide gel electrophoresis and used to inject New Zealand White rabbits using standard procedures (Harlow and Lane, 1988). The antibody was affinity purified using the *E. coli* expressed protein and found to be specific for SYN1; it did not cross-react with the three other SYN1-like *Arabidopsis* proteins expressed in *E. coli*.

Immunolocalization

Inflorescences of 15-18 day old plants were fixed for 2 hours in Buffer A (Dernburg et al., 1996) containing 4% paraformaldehyde, washed twice and stored at 4°C in Buffer A until needed. Buds were staged by squashing and staining an individual anther in acetic orcein. The remaining anthers were squashed between two perpendicular poly-L-lysine slides. Male meiocytes were covered with a thin layer of agarose and treated with β-glucuronidase (Peirson et al., 1997). After washing in 1× PBS, the slides were blocked in 1× PBS containing 5% BSA for 60 minutes and then incubated overnight at 4°C in a moist chamber with anti-SYN1 antibody, diluted 1:250 in blocking buffer. After washing, the slides were treated with Alexa-488-labeled goat anti-rabbit secondary antibody for 2 hours at room temperature. After washing, the DNA was stained with 2 μg/ml propidium iodide (PI) and the slides mounted in DABCO antifade mounting media. Samples were viewed with a Nikon PMC-2000 Confocal Microscope System. Individual optical z-sections were captured, the three-dimensional data were stacked (maximum intensity) using Image Pro Plus and were represented as two-dimensional images.

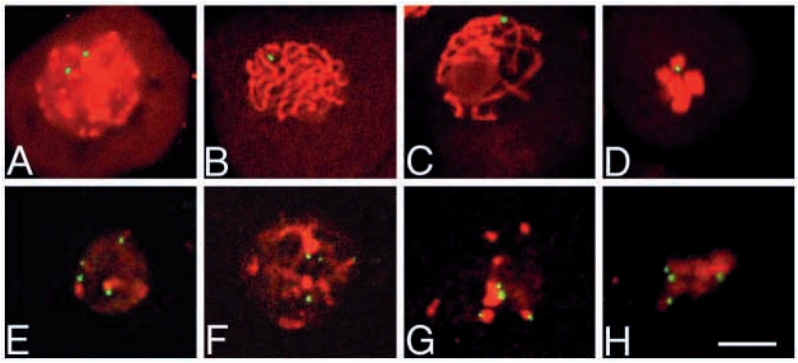

Fig. 1. Fluorescence in situ hybridization of BAC probes to chromosome arms in wild-type (A-D) and *syn1* (E-H) meiocytes. Hybridization of BAC probes was detected with fluorescein-labeled streptavidin (green). Chromosomes were stained with PI (red). (A,E) Early leptotene; (B,F) pachytene; (C,G) early diplotene; (D,H) prometaphase. Normal-appearing chromosomes at zygotene, pachytene, diplotene or diakinesis were never observed in *syn1* plants. Therefore, assignment of the stage of the cell cycle is approximate and based on chromosome morphology and the stage of surrounding cells in the anther. Bar, 5 μm.

Fluorescence in situ hybridization (FISH)

Inflorescences were fixed in acetic alcohol (ethanol:glacial acetic acid, 3:1) for 2 hours at room temperature and stored at –20°C after replenishing the fixative. Staged buds were subjected to FISH using previously published procedures (Caryl et al., 2000; Fransz et al., 1996). The following probes were used in this study: (1) pAL1 containing a pericentromeric 180 bp repeat (Martinez-Zapater et al., 1986); and (2) BAC probes F15E21 and MFG13 corresponding to the lower arms of chromosomes one and five, respectively. Southern blotting demonstrated that the BAC clones hybridized to a single copy region of the genome. The pAL1 probe was generated by primary PCR amplification using the M13 forward and reverse primers followed by random primer labeling in the presence of biotin-labeled dUTP (Roche). The BAC probes were digested with *Eco*R1 to fragment the DNA followed by random primer labeling in the presence of biotin-labeled dUTP. Biotin-labeled probes were used in hybridization solution at 10 μg/ml and detected with 10 μg/ml fluorescein-labeled streptavidin. Slides were counterstained with PI, mounted and viewed as above.

Dual FISH and immunolocalization

Buds were fixed and spread as described above for immunolocalization experiments. In situ hybridization using DNA probes was conducted essentially as described previously (Dernburg et al., 1996). Specifically, agarose-covered, poly-L-lysine slides containing spread PMCs were washed twice in Buffer A, twice in 20% deionized formamide/2× SSC, twice in 40% deionized formamide/2× SSC/0.1% Tween-20 and twice in 50% deionized formamide/2× SSC/0.1% Tween-20. Hybridization solution (50% deionized formamide/2× SSC/0.1% Tween-20/10% Dextran Sulfate) containing a PCR fragment (15 μg/ml) corresponding to the pericentromeric 180 bp repeat labeled with digoxygenin-labeled dUTP (Roche) was added and the specimen covered with a coverslip and sealed with rubber cement. The slides were incubated at 40°C for 30 minutes, denatured at 96°C for 6 minutes and then hybridized overnight at 37°C. After hybridization the slides were washed in 50% deionized formamide/2× SSC at 37°C and 20% deionized formamide/2× SSC/0.1% Tween-20, 2× SSC/0.1% Tween-20 and 2× SSC/5% BSA, all at room temperature. They were then incubated in binding solution (2× SSC/0.1% Tween-20/5% BSA) containing 20 μg/ml mouse anti-digoxygenin (Roche) for 1 hour at 37°C. After washing, the slides were incubated in binding solution containing 20 μg/ml Texas Red goat anti-mouse IgG (Jackson Labs) for 1 hour at room temperature, washed in 2× SSC/0.1% Tween-20 and incubated overnight at 4°C in binding solution containing anti-SYN1 antibody (1:250 dilution). After washing the slides were incubated in 20 μg/ml Alexa-488-labeled goat anti-rabbit IgG (Molecular Probes) in binding solution at 37°C for 1 hour, washed, mounted and viewed as above.

Results

Previously we identified alterations in microsporocytes of *syn1* plants that begin just prior to leptonema (Bai et al., 1999). In contrast to wild-type microsporocytes, in which chromosomes appear as long thin threads that loop out of a dense synizetic knot, chromosomes in *syn1* plants typically appeared tangled, and the synizetic knot was darkly stained. In wild-type plants, paired homologous chromosomes were readily apparent during zygonema, whereas at approximately zygonema in the mutant, a series of intertwined knots was observed; chromosomes rarely appeared as thread-like structures, and paired chromosomes were not observed. From approximately anaphase I, approximately 15 to 20 chromosomes and chromosome fragments were observed in *syn1* plants. These results suggested that the *syn1* mutation caused defects in chromosome cohesion, condensation and possibly homologous chromosome pairing, which ultimately result in chromosome fragmentation. In order to evaluate this possibility further, FISH experiments were conducted using chromosome arm and centromere-specific probes. Furthermore, antibodies to SYN1 were generated and used in immunolocalization experiments to determine the distribution and timing of the association of the protein with chromosomes.

Syn1 plants are defective in arm cohesion but maintain centromere attachment until anaphase I

Sister chromatid cohesion and homologous chromosome pairing was investigated using Alexa-488-labeled BAC clones, which correspond to the arms of chromosomes one and five. Similar results were obtained with both probes. In wild-type plants two FISH signals were typically observed in cells during meiotic interphase and early leptonema when probes corresponding to chromosome arms were used (Fig. 1A). During zygonema in wild-type meiocytes, the number of arm signals was reduced from two to one as homologous chromosomes paired (Fig. 1B). One signal was typically observed through diakinesis (Fig. 1C-D). In contrast, when probes corresponding to chromosome arms were used in FISH against *syn1* microsporocytes, four distinct signals were normally observed from meiotic interphase to metaphase (Fig. 1E-H). The presence of four arm signals was consistent with a lack of sister chromatid cohesion. Occasionally cells with two or three signals were observed; however, most cells contained four (average=3.9, Table 1). This indicated that in addition to a lack of sister chromatid arm cohesion, the arms of homologous chromosomes failed to pair in *syn1* plants.

Cohesion and pairing of centromeric regions was also examined by FISH using PAL1, a 180 bp tandemly repeated

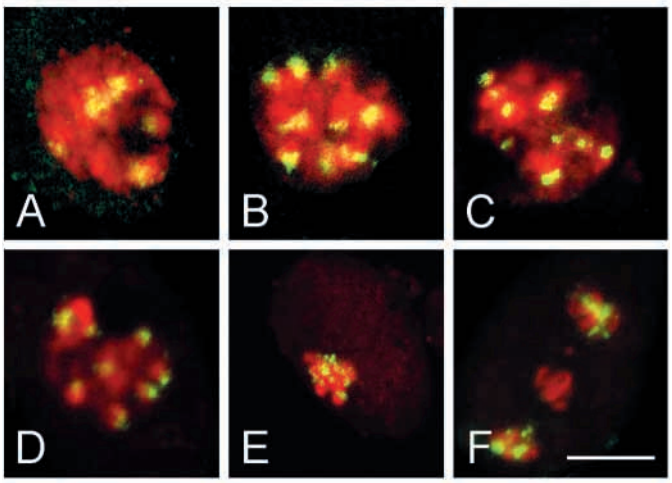

Fig. 2. Fluorescence in situ hybridization of centromere probes to chromosomes in *syn1* meiocytes. Hybridization of a centromere probe (pAL1) was detected with fluorescein-labeled streptavidin (green). Chromosomes were stained with PI (red). (A) An example of an early leptotene meiocyte showing six centromere signals; (B) zygotene; (C) diplotene; (D) diakinesis; (E) metaphase; (F) telophase I. Assignment of the stage of the cell cycle is approximate and based both on chromosome morphology and the stage of surrounding cells in the anther. Bar, 5 μm.

Table 1. Centromere and arm FISH at different meiotic stages of wild type and *syn1* male meiocytes

	Interphase	Leptotene	Zygotene	Pachytene	Diplotene/ diakinesis
Wildtype					
Arm	2.0 (*n*=78)	2.0 (*n*=46)	1.4 (*n*=41)	1.0 (*n*=55)	1.0 (*n*=61)
Centromere	9.8 (*n*=56)	9.6 (*n*=61)	4.2 (*n*=48)	4.9 (*n*=57)	5.2 (*n*=60)
syn1					
Arm	3.7 (*n*=63)	3.9 (*n*=56)	3.9 (*n**=89)		3.9 (*n*=41)
Centromere	9.9 (*n*=70)	9.7 (*n*=62)	9.5 (n*=84)		9.7 (*n*=56)

The average number of signals observed at each stage of meiosis is shown. The total number of signals was determined by adding signals from all optical planes of the cells obtained by confocal microscopy. The number of cells examined at each stage is shown within brackets. *Meiocytes exhibiting a typical pachytene arrangement of chromosomes are never observed in *syn1* plants, therefore cells post-leptotene, but prior to diplotene/diakinesis, are included in the zygotene numbers.

sequence that localizes to the central domain of the pericentromeric heterochromatin of all 10 *Arabidopsis* chromosomes (Fransz et al., 1998). In wild-type plants approximately 10 centromere signals (eight to 10) were observed during meiotic interphase and leptonema (Table 1). As expected, during zygotene and pachytene between two and five centromere signals were observed, with averages of 4.2 and 4.9 respectively. During diplotene/diakinesis in wild-type meiocytes, five to six signals were typically observed. These results are consistent with those previously observed in wild-type *Arabidopsis* (Armstrong et al., 2001). *Syn1* meiocytes resembled wild-type plants during meiotic interphase and leptotene, exhibiting approximately 10 (six to 10) centromere signals (Fig. 2A-B). This result contrasts with results obtained with arm-specific probes and suggests that sister chromatid cohesion at the centromeres of *syn1* meiocytes was maintained during prophase. Consistent with a general lack of pairing, *syn1* meiocytes continued to exhibit approximately 10 centromere signals throughout prophase (Fig. 2C-D; Table 1). In a number of cells, fewer than 10 centromere signals (six to nine) were observed (Table 1), suggesting that some pairing of centromeric regions may have occurred. However, we believe that it is more likely that the reduced number of signals in these cells is not due to chromosome pairing, but rather because of the generally intertwined and sticky nature of chromosomes in *syn1* meiocytes, and possibly general centromere clustering.

Approximately eight (five to 10) centromere signals were detected in optical sections of *syn1* meiocytes at metaphase I (Fig. 2E). In contrast, 12 to 18 signals were present in the 43 cells observed at telophase I (average=14.8; Fig. 2F). Because of the highly condensed nature of the chromosomes at telophase I, we believe that this number may actually be an under-representation of the true number of centromere signals. Nonetheless, the presence of more than 10 centromere signals indicates that although sister chromatids remain associated at their centromeres up to metaphase I, centromere cohesion is lost by telophase I. During metaphase I, centromere signals were always found in the condensed chromosome mass at the center of the cell (Fig. 2F), suggesting that the presence of a centromere was sufficient for movement of the chromosome to the spindle assembly. Consistent with this is the observation that centromere signals were always found at the spindle poles during anaphase I. In contrast, acentric chromosome fragments, present in *syn1* meiocytes, failed to attach to the spindles, although they too were often found in the center of the cell (Fig. 2F). The absence of centromere sequences confirmed that they represented chromosome fragments rather than univalents. Taken together these results confirm that *syn1* meiocytes are defective in sister chromatid cohesion and homologous chromosome pairing; however, sister chromatids remain attached at their centromeres up to metaphase I.

SYN1 localizes to the arms of meiotic chromosomes from approximately interphase to metaphase I

To examine the distribution of SYN1 on chromosomes during meiosis, antibodies were raised to the central portion (amino acids 176-353) of SYN1. This region was chosen because it is the least conserved portion of the protein. *Arabidopsis* contains four SCC1/REC8 paralogues. Like all SCC1/REC8 proteins, the greatest similarity is found at the N- and C-terminal regions of the proteins. In contrast the central portions of the proteins show very little (less than 15% identity) sequence conservation (Dong et al., 2001). Consistent with this observation, the SYN1 antibodies did not crossreact with *E. coli* expressed protein for the three other *Arabidopsis* cohesin proteins, SYN2, 3 and 4 (data not shown).

Immunolocalization experiments on wild-type micro-sporocytes with SYN1 antibody revealed a strong signal in the nucleus beginning at approximately meiotic interphase. Meiocytes at interphase displayed diffuse chromatin and SYN1 labeling (Fig. 3A). Although some labeling was observed in the centrally located nucleolus, the SYN1 signal was clearly

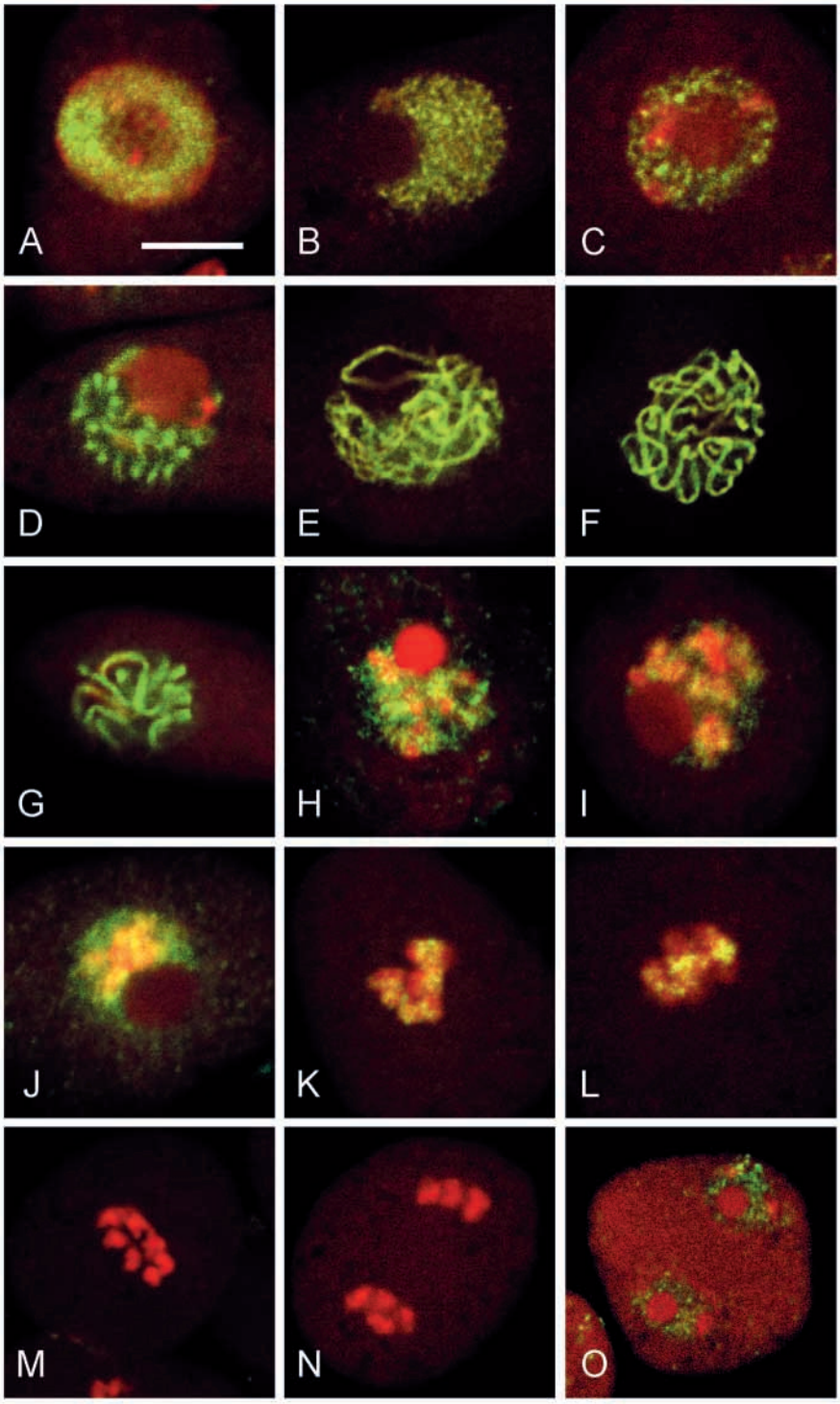

Fig. 3. Fluorescence immunolocalization of SYN1 antibody (green) on meiotic spreads of wild type *Arabidopsis* chromosomes counterstained with PI (red). (A) Interphase; (B) early leptotene; (C) leptotene; (D) early zygotene; (E) zygotene; (F) pachytene; (G) late pachytene; (H) early diplotene; (I) diplotene; (J) diakinesis; (K) prometaphase; (L) metaphase; (M) anaphase I; (N) telophase I; (O) meiotic interphase II. Bar, 5 μm.

stronger in the surrounding nucleoplasm. During early leptotene, the SYN1 signal associated with the condensing chromatin (Fig. 3B). As meiocytes proceeded through leptotene, SYN1 labeling went from a relatively diffuse pattern (Fig. 3C) at early stages to approximately 100 large foci at the leptotene/zygotene transition (Fig. 3D). During zygotene and pachytene, the SYN1 signal was distributed over most of the chromosomes (Fig. 3E-G). As the chromosomes began to condense during diplotene, SYN1 labeling was reduced and began to shift from the chromosomes into the nucleoplasm (Fig. 3H). As the cells proceeded through diplotene and diakinesis, labeling in the nucleoplasm became progressively stronger until it completely filled the nucleus (Fig. 3I,J). No labeling was detected in the nucleolus. By the time the nuclear envelope had broken down SYN1 was only detected on prometaphase chromosomes, suggesting that after release the protein is degraded (Fig. 2K). As cells proceeded through metaphase I the SYN1 signal became progressively weaker until it was no longer detectable by the beginning of anaphase I (Fig. 3L-N). SYN1 was never observed on chromosomes after the onset of anaphase I. However, we were able to detect very weak SYN1 staining in the nucleus of interphase II cells (Fig. 3O). Staining of cells at this stage was always weak and short-lived.

SYN1 labeling was never observed in somatic cells of the anther or in *Arabidopsis* cell cultures (data not shown). This was consistent with results from in situ hybridization experiments, in which SYN1 transcripts were only detectable in the locules of stage 8 and 9 anthers (data not shown). Likewise, SYN1 labeling was not detected in meiocytes of *syn1* plants (data not shown), which confirms that the antibody is specific for SYN1. Taken together these results support phylogenetic evidence (Bai et al., 1999), indicating that SYN1 is the *Arabidopsis* REC8 ortholog.

Results shown in Fig. 3H-J indicated that most SYN1 was lost from the chromosomes during diplotene/diakinesis. Fig. 4A-D clearly shows that during diplotene SYN1 labeling associated with the condensing chromosomes is dramatically reduced. By diakinesis most of the SYN1 labeling is not associated with the chromosomes, rather it appears to be free in the nucleoplasm. At these stages signal was never associated with the nucleolus. During metaphase I, SYN1 was detectable on the chromosomes, but not in the nucleoplasm (Fig. 4E,F). In contrast to its localization during zygotene and pachytene, SYN1 labeling was more narrowly focused on metaphase chromosomes. By early anaphase I SYN1 was clearly no longer detectable (Fig. 4H). SYN1 signal was never observed in the approximately 50 anaphase I cells that were examined. However, during meiotic interphase II, SYN1 was briefly

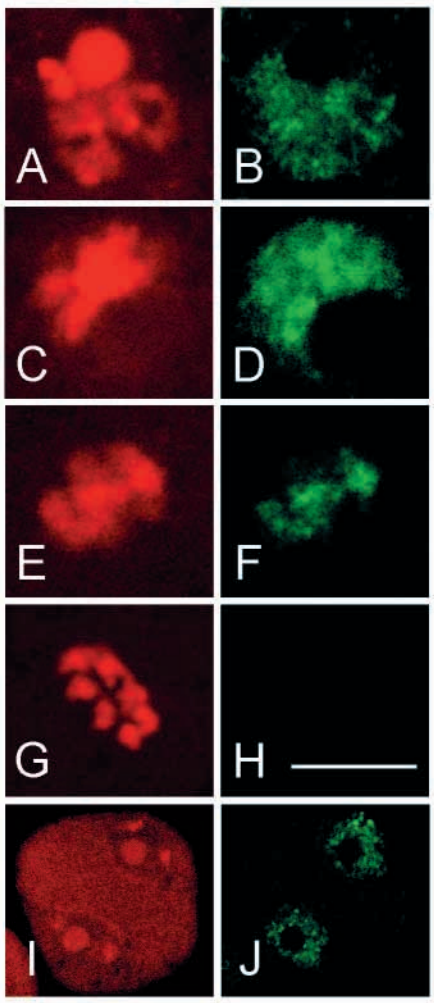

Fig. 4. Fluorescence immunolocalization of SYN1 antibody (B,D,F,H,K; green) on meiotic spreads of wild type *Arabidopsis* chromosomes (A,C,E,G,J) counterstained with PI (red). (A,B) Diplotene; (C,D) diakinesis; (E,F) metaphase I; (G,H) anaphase I; (J,K) meiotic interphase II. Bar, 5 μm.

detected in the nucleus of meiocytes. This signal was very transient and disappeared before metaphase II (data not shown). Therefore, SYN1 was detectable in meiocytes from approximately interphase I to interphase II. Most of the protein appeared to disassociate from the chromosomes at diplotene/diakinesis, and labeling of the chromosomes was not detected after metaphase I.

Results from our immunolocalization studies suggested that SYN1 was localized preferentially along the arms of meiotic chromosomes and not at the centromeres. In order to investigate this possibility further we conducted dual SYN1 immunolocalization/centromere FISH experiments. Centromere labeling patterns and the distribution of SYN1 in the dual immunolocalization/centromere FISH experiment resembled the results obtained for the individual experiments. During leptotene, zygotene and early pachytene (Fig. 5A-C), SYN1 labeling was clearly evident on chromosome arms. However, the dispersed nature of the chromosomes made it difficult to determine if the centromeres were also labeled with the SYN1 antibody. Beginning at late pachytene (Fig. 5D), as the centromeres started to become distinguishable from the chromosome arms, SYN1 labeling was not detected at centromeric regions (verified by 3D analysis; however only 2D projections are shown). Likewise, as overall SYN1 levels decreased during diplotene, diakinesis and metaphase, labeling was found primarily along the chromosome arms and not at the centromeres (Fig. 5E-H). In the approximately 350 centromeres examined in >70 cells observed at these stages, overlap between the SYN1 and centromere signals was observed 38 times (11%). In cells that were oriented such that the centromeres were clearly distinguishable from the arms, no overlap in labeling was detected between SYN1 and the centromere repeat clone. Therefore, SYN1 is generally not detectable at the centromeres.

Discussion

SYN1 is the *Arabidopsis* meiotic cohesin

Components of the cohesin complex and general features associated with the establishment of cohesion and its subsequent removal are generally conserved amongst different organisms and between mitosis and meiosis. However, differences in the distribution of cohesin proteins, the mechanism of their removal and the phenotype of cells containing mutations in cohesin subunits have also been observed. This suggests that subtle differences exist in the ways that sister chromatid cohesion is controlled. In order to investigate meiotic cohesins in plants we have characterized the effect of the *syn1* mutation on homologous chromosome pairing and sister chromatid cohesion and investigated the distribution of the protein on meiotic chromosomes of *Arabidopsis*, which contains four *SCC1/REC8*-like genes. Results presented here confirm previous studies, which suggested that SYN1 is a meiotic cohesin (Bai et al., 1999). SYN1 transcripts are detectable only in locules of stage 8 and 9 anthers (data not shown), which contain meiocytes at premeiotic interphase and meiosis respectively (Armstrong and Jones, 2003). Furthermore, SYN1 is detected in meiocytes from approximately meiotic interphase to metaphase I (Fig. 2); it is not detected in somatic cells. Finally, inactivation of SYN1 disrupts sister chromatid arm cohesion beginning at approximately interphase (Fig. 1). Therefore, SYN1 is an *Arabidopsis* meiotic cohesin.

Defects in sister chromatid cohesion interfere with homologous chromosome pairing in *Arabidopsis*

Varying phenotypes have been observed for *rec8* mutants in other organisms. For example, when REC8 is depleted using RNAi in *C. elegans*, meiotic chromosomes undergo presynaptic alignment, but not synapsis (Pasierbek et al., 2001). Separation of sister chromatids is observed as early as leptotene but is typically not wide spread until diakinesis. In contrast, approximately 70% of *S. cerevisiae* cells carrying a *rec8* mutation exhibit FISH labeling patterns consistent with defects in both cohesion and chromosome pairing/alignment (Klein et al., 1999). Interestingly, in *S. pombe* cells containing the *rec8-110* mutation, pairing of interstitial and centromeric chromosome regions was strongly impaired, whereas pairing at chromosome ends was less impaired (Molnar et al., 1995).

Our results indicate that the phenotype of *syn1* meiocytes is

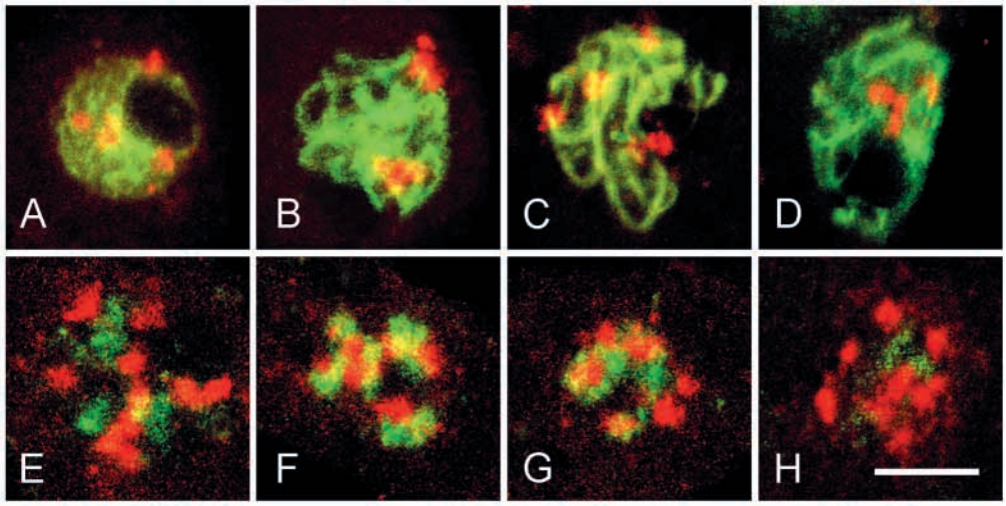

Fig. 5. Simultaneous immunolocalization of SYN1 antibody (green) and fluorescence in situ hybridization of centromere probes (red) to meiotic chromosomes. (A) Zygotene; (B) early pachytene; (C) late pachytene; (D) early diplotene; (E) diakinesis; (F) prometaphase; (G) metaphase I; (H) early anaphase I. Bar, 5 μm.

similar to that observed in *S. cerevisiae*; specifically, SYN1 is required for sister chromatid arm cohesion and homologous chromosome pairing. Results from FISH experiments on *syn1* plants revealed, in general, four arm-specific signals throughout prophase consistent with both a lack of sister chromatid arm cohesion and homologous chromosome pairing. Approximately 10 (six to 10) centromere signals were observed from meiotic interphase to anaphase I, when on average 15 (12 to 18) signals were observed (Table 1). These results are also consistent with a lack of homologous chromosome pairing but suggest that sister chromatids remain attached at their centromeres until anaphase I. At this point it is not clear why we see differences in cohesion between chromosome arms and the centromeres. One possibility is that the centromeres remain topologically intertwined during prophase. It is also possible that another protein helps link the centromeres prior to anaphase I in *Arabidopsis*.

SYN1 is not detectable at the centromeres of prophase chromosomes or in association with chromosomes after anaphase I

SYN1 is clearly detectable in the nuclei of meiocytes at approximately interphase. It is likely that SYN1 is present as early as S phase, as appears to be the case in *S. pombe* (Parisi et al., 1999). However, because it is difficult to accurately assess the stage of cells within this period, we can not conclude with certainty that SYN1 is present during S phase. As meiocytes proceed through leptotene, SYN1 labeling progresses from a relatively diffuse pattern to approximately 100 large foci at the leptotene/zygotene transition (Fig. 2D). During zygotene and pachytene, chromosome arms are completely covered with SYN1 (Fig. 2E-G). During diplotene and diakinesis, much of the SYN1 signal is released and moves into the nucleoplasm as the chromosomes condense (Fig. 2H). By prometaphase I, unbound SYN1 is no longer detected in the cell. It is, however, still localized to discreet regions of the chromosomes where it persists until the metaphase/anaphase transition. By the beginning of anaphase I, SYN1 was no longer detectable (Fig. 2M). These results for SYN1 were generally consistent with those observed in other organisms.

S. cerevisiae Rec8 was initially found as punctate foci during leptotene and zygotene (Klein et al., 1999). By pachytene it formed a continuous line along the longitudinal axes of the chromosomes. By anaphase I, Rec8 labeling was dramatically reduced but still detectable as a number of small foci that remained visible in the vicinity of the centromeres until shortly after the onset of anaphase II. In *S. pombe*, Rec8 was highly enriched in centromeric regions from pre-meiotic S phase through metaphase II (Parisi et al., 1999). Finally, *C. elegans* REC8 was found as dots along unsynapsed chromosome axes during leptotene/zygotene (Pasierbek et al., 2001). During pachytene, REC8 antibodies associated with the SC. From anaphase I through metaphase II, REC8 signals were less intense and restricted to the region between the centromere and the chiasmata. Therefore, in both *S. cerevisiae* and *C. elegans* REC8 appeared to localize to the SC during pachytene, and REC8 was localized to the centromeres of all three organisms from anaphase I to metaphase II. Although the resolution of our experiments does not allow us to say with certainty that SYN1 is associated with the SC, on the basis of its distribution and the results from other systems, this is highly likely.

The greatest difference between our results and those obtained in *S. cerevisiae, S. pombe* and *C. elegans* is our observation that SYN1 antibody does not localize to centromeric regions or to meiotic chromosomes after metaphase I. This raises the interesting possibility that SYN1 is not involved in maintaining centromeric cohesion and that one of the other three *Arabidopsis* cohesin proteins (SYN2, SYN3 or SYN4) may be responsible for centromeric cohesion. If true, then this would suggest that meiotic chromosome cohesion is controlled differently in plants than in other organisms. However, several observations suggest that this is not the case. First, SYN1 staining was detected in the nucleus of interphase II cells (Fig. 3O). Although this staining was always weak and short-lived, it was reproducible. This suggests that low levels of the protein may be present on the chromosomes of anaphase I cells but that it is inaccessible to the antibody. This is similar to results obtained with antibodies to vertebrate SCC1, which failed to detect the protein on metaphase chromosomes (Darwiche et al., 1999; Losada et al., 1998; Sumara et al., 2000). Through the use of myc-tagged SCC1 Waizenegger et al. were, however, able to demonstrate

that SCC1 is present on metaphase I chromosomes and that it localizes to the centromeres (Waizenegger et al., 2000). Our observation that the SYN1 signal is relatively weak during diplotene is consistent with the theory that chromosome conformation and possibly the location of the protein on the chromosomes has a major effect on the observed signal. During pachytene the SYN1 signal is very strong (Fig. 3F), whereas during diplotene the total level of SYN1 signal is dramatically reduced as the chromosomes condense (Fig. 3H). SYN1 signal is again relatively high at late diakinesis when most of the signal is no longer associated with the chromosomes (Fig. 3J). Finally, similar to other *rec8* mutants, meiocytes in *syn1* plants exhibit defects in centromere cohesion beginning at approximately anaphase I. This indicates that SYN1 plays an important role in centromere cohesion. Therefore, we believe that SYN1 is present at the centromeres of meiotic chromosomes but that it is not detectable with our antibody. We are currently investigating this question further through the use of GFP- and epitope-tagged versions of SYN1 and detailed localization studies for SYN2, SYN3 and SYN4.

Most SYN1 crossreactive material is released from *Arabidopsis* chromosomes beginning at approximately diplotene. From diplotene to prometaphase I SYN1 labeling is very strong in the nucleoplasm. This is in contrast to results obtained in *C. elegans* where REC8 was observed along the axes of diplotene/diakinesis chromosomes. It was not until the cells entered metaphase I that REC8 labeling appeared to become progressively less intense. The release of SYN1 from the chromosomes coincident with chromosome condensation during diplotene/diakniesis resembles the situation in mitotic cells of animals where the bulk of cohesin is removed from chromosomes during prophase (Losada et al., 1998). This early release of cohesin from chromosome arms is referred to as the prophase pathway and is separase independent (Waizenegger et al., 2000). The segregation of chromosomes at meiosis I in *Xenopus* also appears to take place in the absence of APC activity and in the presence of high levels of securin, the separase inhibitor (Peter et al., 2001; Taieb et al., 2001). In contrast, in *S. cerevisae* and *C. elegans*, the resolution of chiasmata along meiotic chromosome arms depends on the cleavage of REC8 by separase at the onset of anaphase I (Buonomo et al., 2000; Siomos et al., 2001). At this time it is not known if the removal of SYN1 from meiotic chromosome arms is separase dependent. The *Arabidopsis* genome does, however, contain a putative separase homologue, suggesting that the APC-mediated pathway is utilized for the release of centromeric and/or possibly arm cohesion. Experiments are underway to investigate this question.

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References

Armstrong, S., Franklin, F. and Jones, G. (2001). Nucleolus-associated telomere clustering and pairing preceed meiotic chromosome synapsis in *Arabidopsis thaliana*. *J. Cell Sci.* **114**, 4207-4217.

Armstrong, S. and Jones, G. (2003). Meiotic cytology and chromosome behavior in wild-type *Arabidopsis thaliana*. *J. Exp. Botany* **54**, 1-10.

Bai, X., Peirsion, B., Dong, F., Cai, X. and Makaroff, C. A. (1999). Isolation and characterization of SYN1, a RAD21-like gene essential for meiosis in *Arabidopsis*. *Plant Cell* **11**, 417-430.

Bhatt, A. M., Lister, C., Page, T., Fransz, P., Findlay, K., Jones, G. H., Dickinson, H. G. and Dean, C. (1999). The DIF1 gene of Arabidopsis is required for meiotic chromosome segregation and belongs to the REC8/RAD21 cohesin gene family. *Plant J.* **19**, 463-472.

Buonomo, S. B. C., Clyne, R. K., Fuchs, J., Loidl, J., Uhlmann, F. and Nasmyth, K. (2000). Disjunction of homologous chromosomes in meiosis I depends on proteolytic cleavage of the meiotic cohesin Rec8 by separin. *Cell* **103**, 387-398.

Caryl, A. P., Armstrong, S. J., Jones, G. H. and Franklin, F. C. H. (2000). A homologue of the yeast HOP1 gene is inactivated in the Arabidopsis meiotic mutant asy1. *Chromosoma* **109**, 62-71.

Ciosk, R., Zachariae, W., Michaelis, C., Shevchenko, A., Mann, M. and Nasmyth, K. (1998). An ESP1/PDS1 complex regulates loss of sister chromatid cohesion at the metaphase to anaphase transition in yeast. *Cell* **93**, 1067-1076.

Darwiche, N., Freeman, L. A. and Strunnikov, A. (1999). Characterization of the components of the putative mammalian sister chromatid cohesion complex. *Gene* **233**, 39-47.

Dernburg, A. F., Sedat, J. W. and Hawley, R. S. (1996). Direct evidence of a role for heterochromatin in meiotic chromosome segregation. *Cell* **86**, 135-146.

Dong, F., Cai, X. and Makaroff, C. (2001). Cloning and characterization of two Arabidopsis genes that belong to the RAD21/REC8 family of chromosome cohesin proteins. *Gene* **271**, 99-108.

Fransz, P., Alonso-Blanco, C., Liharska, T. B., Peeters, A. J. M., Zabel, P. and Hans de Jung, J. (1996). High resolution physcial mapping in *Arabidopsis thaliana* and tomato by fluorescence in situ hybridization to extended DNA fibers. *Plant J.* **9**, 421-430.

Fransz, P., Armstrong, S., AlonsoBlanco, C., Fischer, T. C., TorresRuiz, R. A. and Jones, G. (1998). Cytogenetics for the model system *Arabidopsis thaliana*. *Plant J.* **13**, 867-876.

Harlow, E. and Lane, D. (1988). Antibodies: A laboratory manual. Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory.

Hauf, S., Waizenegger, I. C. and Peters, J. M. (2001). Cohesin cleavage by separase required for anaphase and cytokinesis in human cells. *Science* **293**, 1320-1323.

Klein, F., Mahr, P., Galova, M., Buonomo, S. B. C., Michaelis, C., Nairz, K. and Nasmyth, K. (1999). A central role for cohesions in sister chromatid cohesion, formation of axial elements and recombination during yeast meiosis. *Cell* **98**, 91-103.

Lee, J. and Orr-Weaver, T. (2001). The molecular basis of sister-chromatid cohesion. *Annu. Rev. Cell Dev. Biol.* **17**, 753-777.

Lin, Y., Larson, K., Doer, R. and Smith, G. (1992). Meiotically induced rec7 and rec8 genes of *Schizosaccharomyces pombe*. *Genetics* **132**, 75-85.

Losada, A., Hirano, M. and Hirano, T. (1998). Identification of *Xenopus* SMC protein complexes rerquired for sister chromatid cohesion. *Genes Dev.* **12**, 1986-1997.

Martinez-Zapater, J. M., Estelle, M. A. and Somerville, C. R. (1986). A highly repeated DNA sequence in *Arabidopsis thaliana*. *Mol. Gen. Genet.* **204**, 417-423.

Michaelis, C., Ciosk, R. and Nasmyth, K. (1997). Cohhesins: Chromosomal proteins that prevent premature separation of sister chromatids. *Cell* **91**, 35-45.

Molnar, M., Bahler, J., Sipiczki, M. and Kohli, J. (1995). The rec8 gene of *Schizosaccharomyces pombe* is involved in linear element formation, chromosome pairing and sister-chromatid cohesion during meiosis. *Genetics* **141**, 61-73.

Orr-Weaver, T. L. (1999). The ties that bind: Localization of the sister-chromatid cohesin complex on yeast chromosomes. *Cell* **99**, 1-4.

Parisi, S., McKay, M. J., Molnar, M., Thompson, M. A., van der Spek, P. J., van Drunen-Schoenmaker, E., Kanaar, R., Lehmann, E., Hoeijmakers, J. H. J. and Kohli, J. (1999). Rec8p, a meiotic recombination and sister chromatid cohesion phosphoprotein of the Rad21p family conserved from fission yeast to humans. *Mol. Cell. Biol.* **19**, 3515-3528.

Pasierbek, P., Jantsch, M., Melcher, M., Schleiffer, A., Schweizer, D. and Loidl, J. (2001). A *Caenorhabditis elegans* cohesion protein with functions in meiotic chromosome pairing and disjunction. *Genes Dev.* **15**, 1349-1360.

Peirson, B. N., Owen, H. A., Feldmann, K. A. and Makaroff, C. A. (1996).

Characterization of three male-sterile mutants of *Arabidopsis thaliana* exhibiting alterations in meiosis. *Sex. Plant Repro.* **9**, 1-16.

Peirson, B. N., Bowling, S. E. and Makaroff, C. A. (1997). A defect in synapsis causes male sterility in a T-DNA-tagged *Arabidopsis thaliana* mutant. *Plant J.* **11**, 659-669.

Peter, M., Castro, A., Lorca, T., le Peuch, C., Magnaghi-Jaulin, L., Doree, M. and Labbe, J. (2001). The APC is dispensable for first meiotic anaphase in *Xenopus* oocytes. *Nat. Cell Biol.* **3**, 83-87.

Prieto, I., Suja, J. A., Pezzi, N., Kremer, L., Martinez, C., Rufas, J. S. and Barbero, J. L. (2001). Mammalian STAG3 is a cohesin specific to sister chromatid arms in meiosis I. *Nat. Cell Biol.* **3**, 761-766.

Revenkova, E., Eijpe, M., Heyting, C., Gross, B. and Jessberger, R. (2001). Novel meiosis-specific isoform of mammalian SMC1. *Mol. Cell. Biol.* **21**, 6984-6998.

Siomos, M. F., Badrinath, A., Pasierbek, P., Livingstone, D., White, J., Glotzer, M. and Nasmyth, K. (2001). Separase is required for chromosome segregation during meiosis I in *Caenorhabditis elegans*. *Curr. Biol.* **23**, 1825-1835.

StoopMyer, C. and Amon, N. (1999). Meiosis: Rec8 is the reason for cohesion. *Nat. Cell Biol.* **1**, E125-E127.

Sumara, I., Vorlaufer, E., Gieffers, C., Peters, B. H. and Peters, J. M. (2000). Characterization of vertebrate cohesin complexes and their regulation in prophase. *J. Cell Biol.* **151**, 749-761.

Taieb, F., Gross, S., Lewellyn, A. and Maller, J. (2001). Activation of the anaphase promoting complex and degradation of cyclin B is not required for progression from meiosis I to II in *Xenopus* oocytes. *Curr. Biol.* **11**, 508-511.

Toth, A., Ciosk, R., Uhlmann, F., Galova, M., Schleiffer, A. and Nasmyth, K. (1999). Yeast Cohesin complex requires a conserved protein, Eco1p(Ctf7), to establish cohesion between sister chromatids during DNA replication. *Genes Dev.* **13**, 320-333.

Uhlmann, F. and Nasmyth, K. (1998). Cohesion between sister chromatids must be established during DNA replication. *Curr. Biol.* **8**, 1095-1101.

Uhlmann, F., Lottspeich, F. and Nasmyth, K. (1999). Sister-chromatid separation at anaphase onset is promoted by cleavage of the cohesin subunit Scc1. *Nature* **400**, 37-42.

Uhlmann, F., Wernic, D., Poupart, M. A., Koonin, E. V. and Nasmyth, K. (2000). Cleavage of cohesin by the CD clan protease separin triggers anaphase in yeast. *Cell* **103**, 375-386.

van Heemst, D. and Heyting, C. (2000). Sister chromatid cohesion and recombination in meiosis. *Chromosoma* **109**, 10-26.

Waizenegger, I. C., Hauf, S., Meinke, A. and Peters, J. M. (2000). Two distinct pathways remove mammalian cohesin from chromosome arms in prophase and from centromeres in anaphase. *Cell* **103**, 399-410.

Warren, W. D., Steffensen, S., Lin, E., Coelho, P., Loupart, M. L., Cobbe, N., Lee, J. Y., McKay, M. J., OrrWeaver, T., Heck, M. M. S. et al. (2000). The *Drosophila* RAD21 cohesin persists at the centromere region in mitosis. *Curr. Biol.* **10**, 1463-1466.

Watanabe, Y. and Nurse, P. (1999). Cohesin Rec8 is required for reductional chromosome segregation at meiosis. *Nature* **400**, 461-464.