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Cloning and characterization of two Arabidopsis genes that belong to the RAD21/REC8 family of chromosome cohesin proteins

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Abstract

Sister chromatid cohesion is required for proper chromosome segregation during cell division. One group of proteins that is essential for sister chromatid cohesion during mitosis and meiosis is the RAD21/REC8 family of cohesin proteins. Two cohesin proteins are found in yeast; one that functions mainly in mitosis while the other participates in meiosis. In contrast, only one cohesin gene appears to be present in Drosophila. In previous studies we identified an Arabidopsis cohesin protein that is required for meiosis. In this report we describe the isolation and characterization of two additional Arabidopsis cohesin genes. The structure of the genes suggests that they arose via a gene duplication event followed by extensive sequence evolution. Transcripts for the two genes are present throughout the plant and are highest in regions of active cell division, suggesting that the proteins may participate in chromosome cohesion during mitosis.

Keywords: Mitosis; Meiosis; Sister chromatid; Chromosome cohesion

1. Introduction

The establishment of sister chromatid cohesion between replicated copies of chromosomes is essential for their proper segregation during mitosis and meiosis. It is required for the proper alignment of chromosomes on the spindle and its subsequent dissolution after attachment of the sister kinetochores to the spindles is crucial for the faithful segregation of sister chromatids to opposite poles of the cell during anaphase (reviewed in Orr-Weaver, 1999; van Heemst and Heyting, 2000). Studies in yeast indicate that sister chromatid cohesion arises concomitant with DNA replication and is lost during anaphase I of mitosis and anaphase II of meiosis (Guacci et al., 1997; Michaelis et al., 1997).

Studies on mitotic cells have provided most of the available information on factors involved in sister chromosome cohesion (reviewed in Hirano, 1999; Nasmyth, 1999). In yeast six genes (*SMC1, SMC3, MCD1/SCC1, SCC2, SCC3* and *ECO1/CTF7*) have been identified that are required for sister chromatid cohesion during mitosis (Guacci et al., 1997; Michaelis et al., 1997; Skibbens et al., 1999; Strunnikov et al., 1993; Toth et al., 1999). The products of four of these genes (*SMC1, SMC3, MCD1/SCC1* and *SCC3*) bind as a cohesion complex to multiple sites along the chromosomes from S phase to anaphase (Michaelis et al., 1997; Toth et al., 1999). Recently it has been shown that the cohesion complex associates preferentially with centromeric regions of chromosomes and that cohesion is critically important at the centromeres (Blat and Kleckner, 1999; Megee et al., 1999; Tanaka et al., 1999). A complex of five proteins is required for mitotic sister chromatid cohesion in *Xenopus* egg extracts; three of the proteins are homologs of SMC1, SMC3 and MCD1/SCC1 (Losada et al., 1998). In contrast to the observations in yeast, the *Xenopus* cohesin complex associates with chromatin during S phase, but most of the complex dissociates from chromosomes during prophase even though cohesion persists until anaphase (Losada et al., 1998).

Recently, information has become available on proteins involved in meiotic cohesion, with a number of similarities having been identified between the mitotic and meiotic complexes (Orr-Weaver, 1999; van Heemst and Heyting, 2000). For example, SMC3 is required for sister chromatid cohesion during mitosis and meiosis in yeast (Klein et al., 1999). In addition, we along with several other groups have

Abbreviations: BAC, bacterial artificial chromosome; C-, carboxy-; DIG, digoxigenin; IPCR, inverse polymerase chain reaction; kbp, kilobase pair; MS, Murashige Skoog; ml, milliliter; N-, amino; NLS, nuclear localization signal; PCR, polymerase chain reaction; RT-PCR, reverse transcription polymerase chain reaction; μg, microgram; WS, Wassilewskija

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shown that a meiotic version of SCC1 is essential for early events during meiosis (Bai et al., 1999; Bhatt et al., 1999; Klein et al., 1999; Parisi et al., 1999; StoopMyer and Amon, 1999).

MCD1/SCC1 belongs to a group of proteins generally referred to as cohesins. The first gene of this class to be characterized, *S. pombe RAD21,* was originally identified as an essential gene involved in DNA double-strand break (DSB) repair (Birkenbihl and Subramani, 1992). Later it was shown to be required for chromosome cohesion during mitosis (Tatebayashi et al., 1998). In addition to RAD21, *S. pombe* contains a second cohesin gene (*REC8*) that is required for meiosis (Parisi et al., 1999; Watanabe and Nurse, 1999). *Rec8* mutations result in impaired meiotic chromosome pairing, reduced recombination, alterations in linear element structures in centromere-adjacent regions and premature separation of sister chromatids (Molnar et al., 1995; Watanabe and Nurse, 1999). REC8 is present from the time of premeiotic DNA synthesis until after meiosis I (Parisi et al., 1999). Interestingly, it appears that both RAD21 and REC8 function during meiosis in *S. pombe*. REC8 is found associated mainly with the centromeres while RAD21 is found toward the teleomeres (Watanabe and Nurse, 1999).

S. cerevisiae also contains two different cohesins. In addition to *MCD1/SCC1*, which is required for both chromosome condensation and sister chromatid cohesion during mitosis (Guacci et al., 1997; Heo et al., 1998; Michaelis et al., 1997), *S. cerevisiae* contains a *REC8* homolog required for meiosis. *Rec8* cells are defective in sister chromatid cohesion and the formation of synaptonemal complexes and lateral elements (Klein et al., 1999). REC8 is found as punctuate foci along the lengths of the chromosomes in early prophase I. It subsequently localizes to the centromeric regions where it persists until approximately anaphase II (Klein et al., 1999). SCC1/MCD1 also appears to play some role in meiosis as spore viability is only 50% in *scc1* mutants (Klein et al., 1999).

Less is known about the number and function of cohesins in higher eukaryotes. As described above, a RAD21-like protein (XRAD21) is part of the mitotic cohesion complex in *Xenopus* (Losada et al., 1998). Mouse (mHR21) and human (HR21) counterparts have been isolated that may be involved in V(D)J and meiotic recombination (McKay et al., 1996). A second human cohesin, *hREC8*, has also been identified, which is expressed in meiotic and post-meiotic spermatids and the thymus, (Parisi et al., 1999). Finally, we have demonstrated that a cohesin (SYN1) is essential for meiosis in Arabidopsis (Bai et al., 1999). Plants homozygous for the *syn1* mutation are male and female sterile and show defects in chromosome cohesion, condensation and pairing beginning at leptonema of meiosis I (Bai et al., 1999). Fragmentation of the chromosomes is observed at metaphase I, resulting in the production of up to eight microspores containing variable amounts of DNA (Peirson et al., 1997). Two *SYN1* transcripts were identified that differ in their 5′-most exons. One transcript is expressed at low levels in most tissues, while the other is expressed only in prebloting buds, suggesting that while the major role of *SYN1* is in meiosis, it may also have a minor role in mitosis.

Given that two cohesin proteins, one mitotic and one meiotic have been identified in yeast and only one cohesin gene appears to be present in the Drosophila genome, we were interested in determining the number and function of the cohesin genes in plants. Specifically we are interested in determining how the cohesins function in plants and identifying potential differences in these functions during mitosis and meiosis. As a first step in this process we have begun the characterization of the Arabidopsis *SYN* gene family. In this report we describe the isolation and characterization of two additional members of this family, *SYN2* and *SYN3*. The three Arabidopsis cohesins show extensive similarity at their N- and C- termini and very little similarity in the middle portion of the proteins. Transcripts for *SYN2* and *SYN3* are present throughout the plant and are highest in regions of active cell division, suggesting that *SYN2* and *SYN3* may participate in chromosome cohesion during mitosis. However, while *SYN1* is able to complement *S. cerevisiae mcd1* mutant cells, *SYN2* and *SYN3* can not. This raises the possibility that SYN2 and/or SYN3 may not play a direct role in chromosome cohesin during mitosis.

2. Materials and methods

2.1. Plant material

Arabidopsis thaliana L. Heynh. ecotype Wassilewskija (WS) was the source of all plant materials. Plants were grown on a commercial potting mix in a growth chamber at 20°C with a 16:8 light:dark cycle. Approximately 15–18 days after germination, buds were collected from bolting plants. Leaves were harvested from rosette-stage plants, while roots and seedlings were harvested from seeds sown on sterile agar plates. All samples were harvested, frozen in liquid N_2 and stored at −80°C until needed.

2.2. Isolation of SYN2 and SYN3 genomic and cDNA sequences

The *SYN2* genomic DNA sequence was identified on chromosome 5 (P1 clone MHK7, accession # AB011477) during a BLAST search of Arabidopsis sequences. Sequences corresponding to the 3′end of *SYN3* were initially identified in a BAC end sequence through BLAST searching using the conserved C-terminal sequence of SYN1. The entire DNA sequence for this region of the genome was not available at the time. Therefore, BAC clone T31H5TR was obtained from the Arabidopsis Stock Center and *SYN3* subcloned and sequenced. Subclones containing *SYN3* were identified through Southern blotting with probes corresponding to the 3′end of the gene generated via PCR.

DNA sequencing was conducted using an ABI310 Genetic Analyzer on deletion clones. All regions of a 4.3 kbp region were sequenced on both strands at least once. Contigs and restriction maps were generated using DNA STAR. Potential exons in the genomic DNA were identified by NetPlantGene v.1.0b. The Neighbor Joining tree was produced using average pair wise distances with the software program PAUP* 4.0 (Swofford, 1999) and bootstrap support was evaluated for 100 replicates.

SYN2 and *SYN3* cDNAs were isolated in a series of RT-PCR experiments. Primers corresponding to predicted exon sequences were used for reverse transcription and subsequent PCR amplification experiments. The *SYN2* and *SYN3* cDNAs were each generated in two steps. *SYN2* cDNA sequences were isolated by reverse transcription with primers 2-3 and 2-4, followed by PCR using primers 2-1/2-3 and 2-2/2-4, respectively (Fig. 1). Likewise, *SYN3* cDNAs were generated by reverse transcription with primers 3-12 and 3-13 followed by PCR with primers 3-NdeI/3-12 and 3-14/3-13, respectively (Fig. 1). All reverse transcription experiments were conducted on total RNA that was isolated from buds. Full-length cDNA sequences for *SYN2* and *SYN3* were generated by recombinant PCR using the overlapping RT-PCR fragments generated above and primer sets 2-1/2-4 and 3-NdeI/3-13, respectively. Amplification products were cloned in pBluescript plasmids and sequenced in their entirety. The sequences reported here have been deposited under the accession numbers AF281154 (*SYN2*) and AF281155 (*SYN3*).

The 5′ ends of the *SYN2* and *SYN3* cDNAs were mapped using inverse PCR (IPCR). The 5′ terminus of the *SYN2* transcript was isolated by IPCR on cDNA that was generated by reverse transcription with primer 2-12. After second strand synthesis and self-ligation, PCR was conducted with primer set 2-10/2-11 (Fig. 1). The 5′ terminus of the *SYN3* transcript was isolated by IPCR on cDNA that was generated by reverse transcription with primer 3-11 followed by PCR with primer set 3-9/3-10 (Fig. 1). Following PCR amplification the products were cloned into pGEM-T and analyzed by DNA sequencing.

2.3. Expression studies

Total RNA isolated from buds, leaves, shoots, etiolated shoots and roots was treated with RNA'se free DNA'se and quantitated. RNA preparations (2.5 μg) were standardized by northern blotting using the rRNA's to ensure equal loadings. Equal amounts of total RNA (4 μg) was then used to analyze *SYN2* and *SYN3* transcript levels using RT-PCR. *SYN2* transcripts were analyzed by reverse transcription with primer 2-7 followed by PCR with primer pair 2-5/2-7. Likewise *SYN3* was analyzed by reverse transcription with primer 3-12 followed by PCR with primer pair 3-10/3-12. Amplification products were analyzed by Southern blotting using α ^{32}P-dATP-labeled cDNA probes. After hybridization and washing radioactivity was detected using a Molecular Dynamics Phosphorimager.

2.4. In situ hybridization

Roots and shoots were collected from three day old seedlings grown on MS plates. Whole inforescences were collected from soil grown plants 15 days post germination. Tissue was fixed in paraformaldehyde, dehydrated in a graded ethanol series, cleared in xylene/chloroform and infiltrated with Paraplast Plus. *In situ* hybridization experiments were carried out on 10 μm dewaxed and rehydrated sections using DIG-labeled RNA probes essentially as described (McFadden, 1995). *SYN2* and *SYN3* sense and antisense probes were generated by in vitro transcription of linearized template DNAs in the presence of DIG-labeled dUTP according to the Boehringer Mannheim application manual. The templates used in the hybridization experiments were cDNA fragments of 865 bp (nucleotides 1657–2522) and 2099 bp (nucleotides 170–2269) for *SYN2* and *SYN3*, respectively. After overnight hybridization and washing at 55°C, the slides were incubated for 1 h. at 37°C with 10 μg/ml mouse anti-DIG antibody, washed and incubated for 1 h. at 37°C with 10 μg/ml Alexa 488-labeled, goat anti-mouse IgG. After washing the slides were mounted and viewed with a Nikon PMC-200 confocal microscope system. The images shown are the sum of the signals captured with the red (background) and green (Alexa 488) channels.

2.5. Complementation studies

Yeast expression constructs for *SYN1*, *SYN2*, *SYN3* and *MCD1* were prepared by transferring fragments that contained the complete protein-coding regions of the genes from pBluescript vectors into the yeast expression vector pYES2. Positive clones were identified by colony hybridization and the cloning junctions were confirmed by DNA sequencing. The pYES2 constructs were then transferred to the temperature-sensitive *mcd1* line, 985-7C (Guacci et al., 1997) and control cells (INVSC1) using Li acetate transformation. The ability of the constructs to complement the *mcd1* mutation was tested by growing 985-7C cells containing the expression constructs in dropout media, supplemented with glucose and galactose at either 30 or 37°C.

3. Results

3.1. Cloning and sequence analysis of the Arabidopsis SYN2 and SYN3 genes

In order to better understand the role of plant cohesins in chromosome condensation and sister chromatid cohesion we have isolated and characterized two additional members of the Arabidopsis *SYN* gene family. The entire genomic

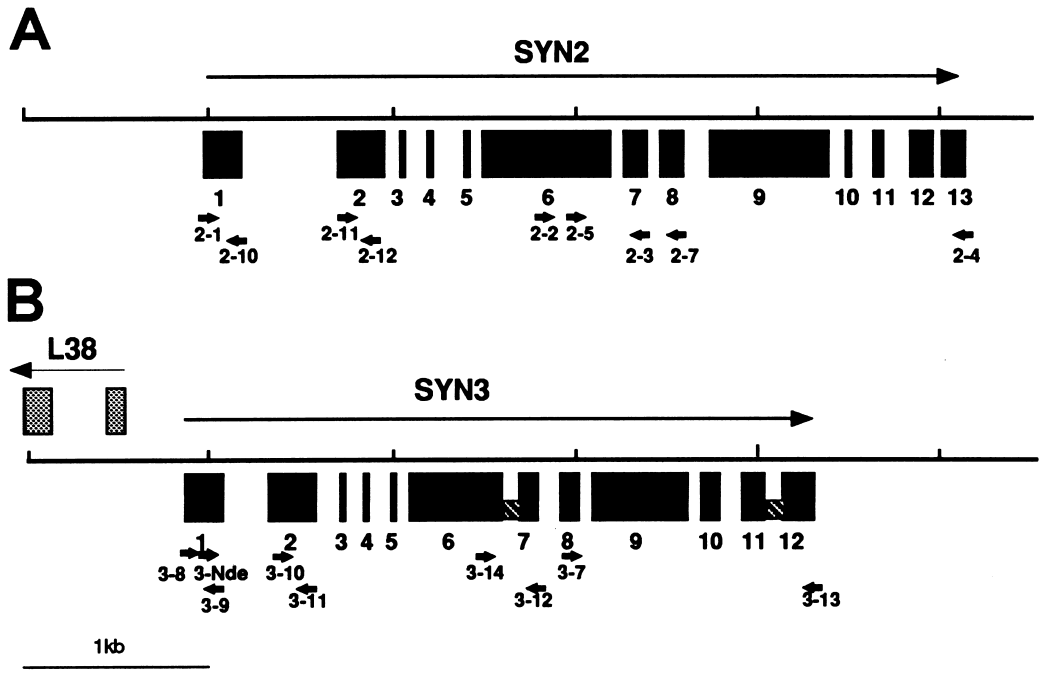

Fig. 1. Maps of the SYN2 and SYN3 loci and exon patterns. (A) Map of a 5.5 kbp region of chromosome 5 showing the *SYN2* locus. (B) Map of a 5.8 kbp region of chromosome 3 corresponding to *SYN3*. The direction of transcription is shown with an arrow above the maps. The position of exons in *SYN 2* and *SYN3* are shown as black boxes below the maps. Introns 6 and 11 of *SYN3*, which were found to be partially spliced, are shown as half boxes shaded in diagonal stripes. Two exons for the ribosomal protein L38 are shown as shaded boxes above the *SYN3* map. The position and direction of primers used in this study are shown as horizontal arrows below the maps. The map is in 1 kbp intervals.

sequence for *SYN2* was identified on the P1 clone MHK7 (accession # AB011477) through a BLAST search using sequences corresponding to the conserved amino terminus of RAD21/REC8 proteins. Based on exon sequences predicted by NetPlantGene, primers were designed and used in RT-PCR experiments to isolate *SYN2* cDNAs. *SYN2* is approximately 4300 bp long and consists of 13 exons and 12 introns (Fig. 1). It encodes a transcript of approximately 2600 nt that has the potential to produce a 809 amino acid protein. The 5′ end of the *SYN2* transcript was mapped by IPCR to a site 92 bp 5′ to the start codon.

SYN3 was identified in a BAC end sequence (T31H5TR) through BLAST searching using the conserved C-terminal sequence of SYN*1*. Because the entire DNA sequence for this region of the genome was not available at the time, a 4.3 kbp fragment of the BAC clone containing *SYN3* was subcloned and sequenced. Primers corresponding to predicted exon sequences were then used in RT-PCR experiments to isolate *SYN3* cDNAs. *SYN3* is approximately 3.5 kbp long and consists of 12 exons and 11 introns (Fig. 1). It encodes a transcript of approximately 2300 nt that has the potential to produce a 692 amino acid protein. The 5′ end of the *SYN3* transcript was mapped by IPCR to a site 179 bp 5′ to the start codon. Located approximately 300 bp 5′ to *SYN3* is a gene encoding the ribosomal protein L38.

Three different forms of the *SYN3* transcript were identified during cDNA isolation. Approximately half of the cDNA's isolated corresponded to fully processed message. Four of the eight cDNAs generated for the 5′half of the transcript using primers 3-NdeI and 3-12 contained intron 6. The presence of this intron introduces a stop codon that would result in the production of a 339 amino acid protein. In addition, two of the eleven cDNA's generated for the 3′ half of the transcript using primers 3-7 and 3-13 contained intron 11. The presence of this intron maintains the reading frame, but results in the insertion of 27 amino acids in the C-terminus of the protein. Two cDNAs were isolated using primers 3-14 and 3-13 that contained both intron 6 and intron 11. At this time we do not know the significance of this apparent differential splicing. It is unclear if the unspliced transcripts represent slow processing of introns 6 and 11 or if they represent functional message. Only fully processed transcripts were identified for *SYN1* (Bai et al., 1999) and *SYN2*, indicating that if the unspliced transcripts are the result of slow splicing, then this is specific to *SYN3*. The splice sites for introns 6 and 11 contain consensus splice site sequences. Therefore, slow splicing does not appear to result from alterations in the splice sites. Given that half of the *SYN3* transcripts characterized encode an altered form of the protein, it raises the interesting question of whether multiple forms of SYN3 are produced and if so what role they play in the cell.

The size and distribution of introns and exons in *SYN2* and *SYN3* is quite similar and very different from that seen for *SYN1* (Bai et al., 1999). It appears that six introns are at identical positions in the two genes (Fig. 2). The two clear-

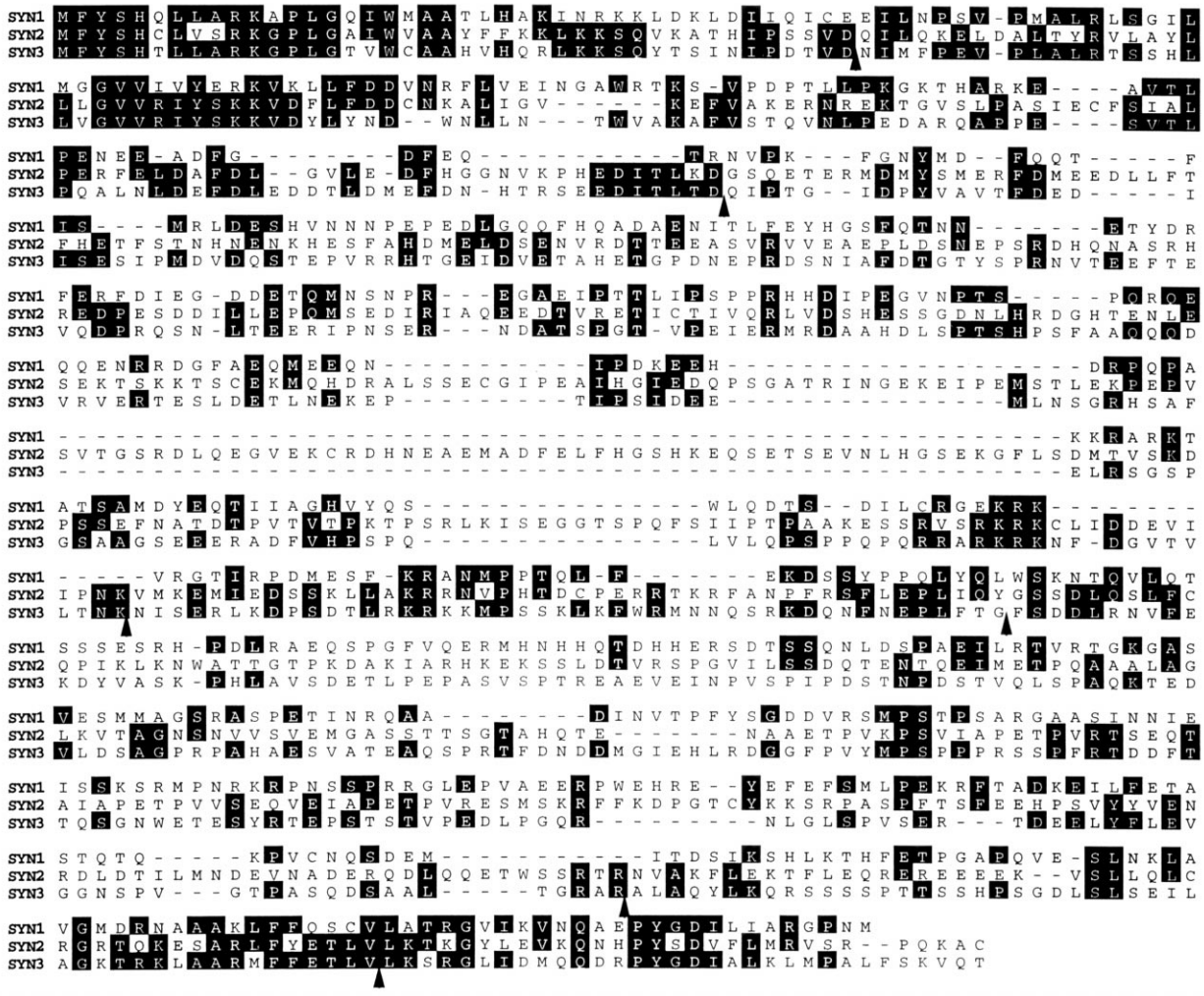

Fig. 2. Alignment of Arabidopsis SYN1, SYN2 and SYN3 proteins. The deduced amino acid sequences of SYN1, SYN2 and SYN3 were aligned using Clustal. Residues present at the same position in two or more sequences are boxed and shaded black. Gaps are shown as a '−'. Intron positions that are identical in both SYN2 and SYN3 are marked with an arrow head. The sequences reported here have been deposited under the accession numbers AF281154 (*SYN2*) and AF281155 (*SYN3*).

est examples of this are the first and last introns. The first intron occurs between amino acid residue 41 and 42 in each gene. The last intron, which is 47% identical at the DNA sequence level between the two genes, occurs between the first and second nucleotides of a conserved valine. In addition, four other introns are found at similar places in the aligned sequences. This suggests that *SYN2* and *SYN3* arose from a gene duplication event.

3.2. Analysis of the SYN2 and SYN3 proteins

The polypeptide encoded by *SYN2* has a predicted mass of 91,283 Da, while the protein encoded by *SYN3* has a predicted mass of 77,118 Da. Overall the proteins are relatively hydrophilic, containing large numbers of polar and charged amino acids. Comparison of the predicted SYN2 and SYN3 proteins with sequences in the databases using BLAST searching confirmed that they do in fact belong to the RAD21/REC8 family of proteins. An alignment of SYN1, SYN2 and SYN3 is shown in Fig. 2. As observed for all cohesin proteins the greatest similarity is present at their N- and C-termini. The central portions of the proteins exhibit the least sequence conservation and all of the length variability between the proteins is due to insertions/deletions in the middle of the proteins. The central portions of the proteins do however share several common features. They are all hydrophilic and relatively acidic in nature. Signals for nuclear localization are also found in the central portions of the proteins. Potential nuclear localization signals (NLS) are present at positions 353 and 382 in SYN2 and 459 in SYN3, respectively. In addition, a bipartite NLS is present between amino acids 490 and 507 in SYN2.

The similarities in gene structure suggest that *SYN2* and *SYN3* may have arisen through a gene duplication event. Therefore we expected that the predicted proteins would

be more closely related to each other than to *SYN1*. However when Arabidopsis SYN1, SYN2 and SYN3 were compared with each other we found that the amino acid similarity between SYN2 and SYN3 is no greater than that observed between the two proteins and SYN1. SYN2 and SYN3 are 18% identical while the two proteins share 16 and 19% identity with SYN1, respectively. As expected, there is very little DNA sequence similarity between the two genes.

3.3. Expression of SYN2 and SYN3

In order to gain insight into the role(s) of *SYN2* and *SYN3,* their expression patterns were examined using RT-PCR and *in situ* hybridization. Similar to results obtained for *SYN1* (Bai et al., 1999), northern blot analysis of *SYN2* and *SYN3* mRNA levels indicated that the transcripts are present at very low levels (data not shown). When RT-PCR was used to analyze mRNA levels, *SYN2* and *SYN3* transcripts were found throughout the plant, including buds, leaves, roots, shoots and sliques (Fig. 3). *SYN2* transcripts were present at relatively similar levels in all tissues examined with mature leaves exhibiting the lowest signal. *SYN3* transcript levels were approximately the same in buds, roots, seedlings and sliques, with buds and sliques exhibiting the highest levels. However, *SYN3* transcript levels are at least 10-fold lower in mature leaves than in other tissues (Fig. 3).

The presence of *SYN2* and *SYN3* transcripts throughout the plant and their similarity to cohesins suggested that the two proteins may function in sister chromatid cohesion during mitosis. In order to assess this possibility more directly, *in situ* hybridization experiments were conducted. DIG-labeled *SYN2* and *SYN3* antisense and sense RNA probes were used against longitudinal sections of various plant tissues, including roots, seedlings and buds. The antisense probes for both genes produced relatively strong signals in all tissues, with the greatest signal present in the meristematic regions (Fig. 4). In contrast, the sense probes produced signal comparable to the autofluorescence seen with no probe. In roots, the greatest signal is observed in actively dividing cells, in the procambium, and in the epidermal (protoderm) and endodermal (ground meristem) regions. Less signal is observed in the vascular tissue and essentially no signal is observed in the root cap. In shoots the greatest signal was observed in the shoot apical meristem with lower, but detectable, levels found in mature, vacuolized cells. Relatively strong signals were also found throughout developing buds. The highest signal was observed in the floral meristem with correspondingly less signal in more mature cells. These expression patterns are consistent with SYN2 and SYN3 playing a role in mitosis.

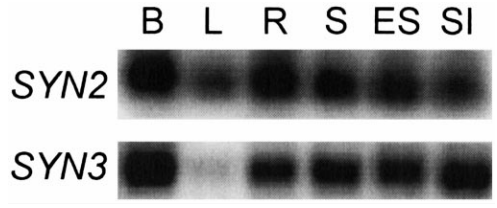

Fig. 3. PCR analysis of *SYN2* and *SYN3* expression patterns. Reverse transcription was conducted on total RNA (4 μg) from light grown (S) and etiolated (ES) seedlings, buds (B), sliques (Sl) mature leaves (L) and roots (R). PCR was then conducted using gene-specific primers as described in Section 2. The PCR products were separated on a 1.0% agarose gel, transferred to nylon membranes and probed with labeled cDNA fragments corresponding to the amplification products.

Differences in the size of the amplified PCR fragments and the relative specific activities of probes used in these experiments makes it difficult to accurately compare *SYN2* and *SYN3* transcript levels. However, based on the relative signals obtained in RT-PCR and *in situ* hybridization experiments, it appears that *SYN2* and *SYN3* are expressed at comparable levels in actively dividing cells.

3.4. Complementation studies

In order to further investigate the role of *SYN2* and *SYN3* we determined whether they are able to complement a temperature-sensitive, *S. cerevisiae mcd1* mutation. When expressed from the GAL1 promoter in the pYES2 vector, *SYN2* and *SYN3* were unable to restore growth to the temperature-sensitive *mcd1* cell line 985-7C at restrictive temperatures (data not shown). However, when Arabidopsis *SYN1* was expressed under the same conditions, slow growth was restored to *mcd1* cells at 37°C. While growth of the SYN1-expressing cells was dramatically slower at 37°C than cells expressing MCD1, SYN1 was clearly able to complement the *mcd1* mutation. In contrast, no growth was observed from SYN2 and SYN3-expressing cells at restrictive temperatures. All cell lines grew at permissive temperatures. This suggests that while SYN1, which is required for chromosome condensation and cohesion during meiosis (Bai et al., 1999) can replace MCD1 during mitosis in *S. cerevisiae*, SYN2 and SYN3 can not. Results of western blots of *S. cerevisiae* cells expressing SYN1 and SYN2 probed with antibody to the proteins indicated that they are expressed at approximately the same levels in the yeast cells (data not shown). Therefore, the inability of *SYN2* to complement the *mcd1* mutation does not result from a lack of expression. Because the SYN1 and SYN2 antibodies do not cross-react with SYN3, it is not however clear if SYN3 is expressed at levels similar to the other two. While further experiments are required, these results raise the possibility that SYN2 and possibly SYN3 may not be directly responsible for mitotic chromosome cohesion or that differences may exist in the mitotic cohesion complexes between yeast and plants. However, we can not rule out the possibility that SYN2 and SYN3 may not be folded and/or modified properly in yeast.

4. Discussion

As part of studies to better understand chromosome condensation and sister chromatid cohesion in plants we

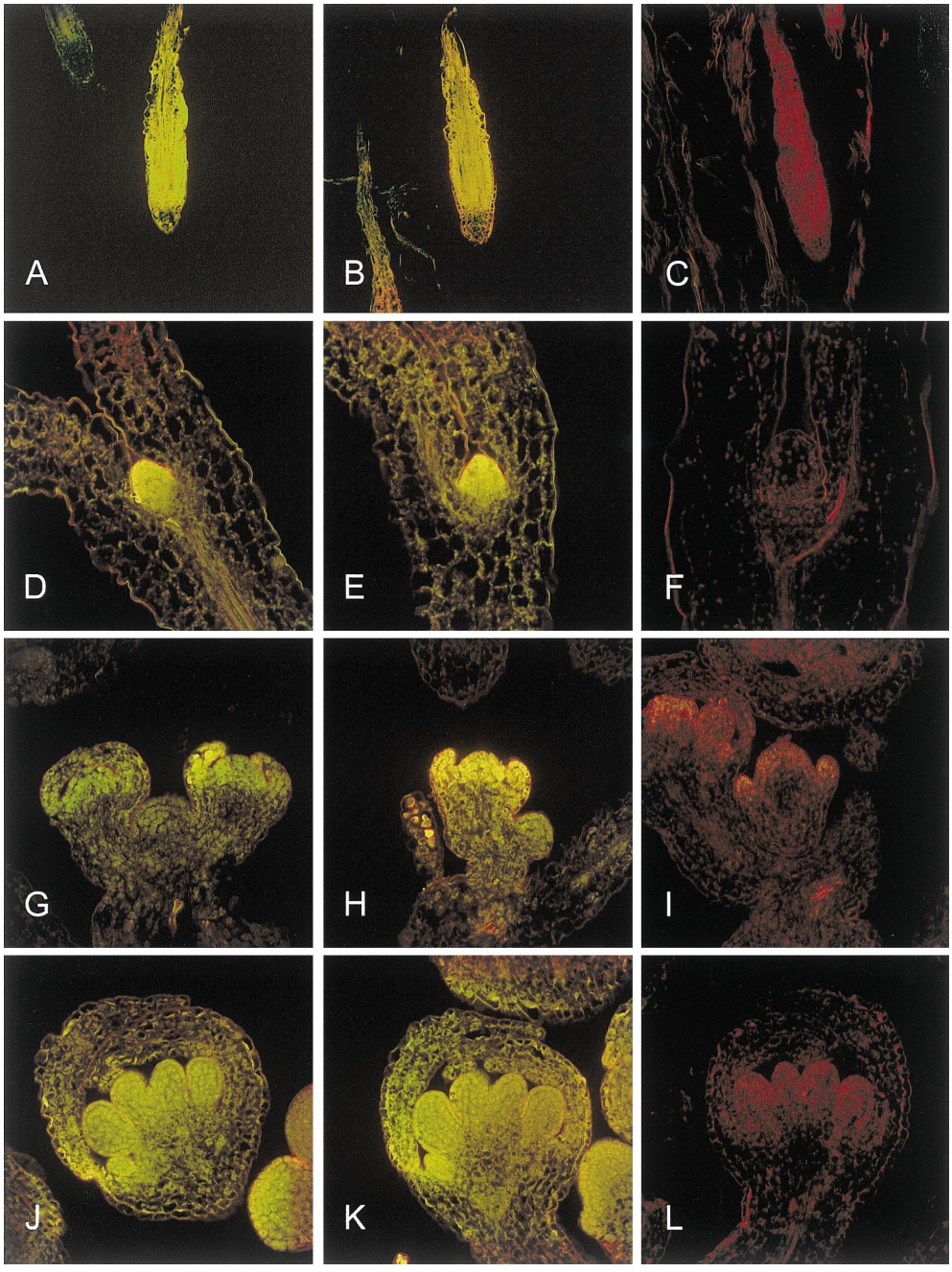

Fig. 4. *In situ* hybridization of *SYN2* and *SYN3* mRNA. Longitudinal sections of roots (A–C), shoots (D–E), floral meristems (G–I) and immature buds (J–L) were hybridized with DIG-labeled antisense RNA corresponding to *SYN2* (A, D, G, J) or *SYN3* (B, E, H, K) or *SYN2* sense RNA (C, F, I, L). DIG-labeled probes were detected with Alexa 488-labeled goat anti-DIG antibody. Images were captured on a PMC2000 confocal system and are the sum of signals captured with the red (autofluorescence) and green (FITC channels). Signals obtained with *SYN3* sense probes were identical to those of *SYN2* and are therefore not shown.

have isolated and characterized two additional members of the cohesin family of proteins in Arabidopsis. The cohesins represent a group of proteins that are required for sister chromosome cohesion during mitosis and meiosis. RAD21 homologues have been identified in yeast and a number of eukaryotes, including mammals (McKay et al., 1996), *Xenopus* (Losada et al., 1998), *Drosophila* (Warren et al., 2000) and *C. elegans*. More recently a second class of cohesins, sometimes referred to as REC8 proteins, have been identified in several organisms. Studies in yeast (Parisi et al., 1999; Watanabe and Nurse, 1999) and our results from Arabidopsis (Bai et al., 1999) have demonstrated that they are required for sister chromatid cohesion during meiosis.

To date two different cohesin proteins have been identified in *S. cerevisiae* (Guacci et al., 1997; Klein et al., 1999; Michaelis et al., 1997), *S. pombe* (Birkenbihl and Subramani, 1992; Watanabe and Nurse, 1999) and mammals (McKay et al., 1996; Parisi et al., 1999). Based on genome

sequence information for *S. cerevisiae*, it is quite clear that only two cohesin genes exist in yeast, one that participates mainly in mitosis and one that functions during meiosis. The number and role(s) of cohesin proteins in higher eukaryotes is much less clear. To date genes for two different cohesin proteins have been identified in mammals, while analysis of the recently completed Drosophila genome sequence indicates that only one RAD21/REC8-like gene may be present in the genome of this organism.

We previously reported on the isolation and characterization of *SYN1*, a cohesin that is essential for chromosome condensation and cohesion during meiosis in Arabidopsis (Bai et al., 1999). Results on *SYN2* and *SYN3* presented here demonstrate that there are at least three members of the cohesin family in Arabidopsis. Based on their expression patterns it appears that *SYN2* and *SYN3* may function during mitosis. Transcripts for the two genes are present throughout the plant with the highest levels found in meristematic regions (Fig. 4). Specifically, the greatest signals were observed behind the root cap and in the epidermal and endodermal cells of roots and in the shoot apical and floral meristems. Signals above background were also observed in mature cells of roots, leaves and buds suggesting that expression of *SYN2* and *SYN3* is not limited to actively dividing cells. In addition, *SYN2* and *SYN3* were unable to complement the *S. cerevisiae mcd1* mutation. These results raise the possibility that SYN2 and SYN3 may not participate in sister chromatid cohesion during mitosis, or if they do then they may also have a more general role in chromosome structure. Transcripts for the human cohesin, *HR21*, were also found throughout the cell cycle with the highest levels in cells at G2 (McKay et al., 1996). Consistent with the theory that some cohesins may function in ways other than, or in addition to sister chromatid cohesion, is the observation that HR21 has recently been reported to be a nuclear matrix protein (Sadano et al., 2000). Experiments are currently underway to more directly address the role(s) of SYN2 and SYN3 in Arabidopsis.

Phylogenetic analyses have been used to classify some cohesins as REC8- or RAD21-like and support putative roles in meiosis or mitosis, respectively (Parisi et al., 1999). A neighbor joining tree of known cohesins shows that in general the RAD21 proteins tend to group together, as do several REC8 proteins. For example, the mammalian, *Xenopus*, *Drosophila* and *C. elegans* RAD21 proteins all group together and human REC8 is more closely related to *S. pombe* REC8 than to human RAD21. However, because the overall similarity between the cohesins is low, it is difficult to use this type of comparison to draw firm conclusions for some of the proteins. In particular, the Arabidopsis proteins do not fit well with this prediction model. The similarity analysis shown in Fig. 5 indicates that Arabidopsis SYN2 is most closely related to *S. pombe* RAD21 and that it groups with the RAD21-like proteins. SYN1 and SYN3 are the most dissimilar of the proteins and can not be grouped with any of the other cohesins with any confidence.

Based on functional analyses it is clear that SYN1 (Bai et al., 1999) and the yeast REC8 proteins (Molnar et al., 1995; Watanabe and Nurse, 1999) are essential for meiosis and that the yeast RAD21 proteins (Guacci et al., 1997; Michaelis et al., 1997) are required for mitosis. *SYN2* and *SYN3* expression patterns suggest that they may participate in mitosis. In addition, intron/exon patterns provide strong evidence that the two genes are the result of a gene duplication event. The lack of similarity between SYN2 and SYN3 suggests that if the two genes did arise through a gene duplication event, then extensive sequence evolution has occurred. Perhaps more interesting is the fact that SYN1 exhibits so little similarity with the REC8 proteins, although in theory they perform common functions during meiosis. This raises the possibility that differences may exist in the meiotic cohesion complex between plants and yeast/animals. Furthermore while the neighbor joining analysis suggests that SYN2 is most similar to *S. pombe* RAD21 and therefore may participate in mitosis, the generally low levels of similarity observed between some of the proteins limits our ability to use similarity analyses to accurately assign function to some cohesins.

Based on our results and those on cohesin proteins in yeast it is clear that similarities exist in the mechanisms that operate in the control of sister chromatid cohesion during mitosis and meiosis. However, differences in the distribution of cohesin proteins and the phenotype of cells defective in cohesion have also been identified (Bai et al., 1999; Guacci et al., 1997; Klein et al., 1999; Michaelis et al.,

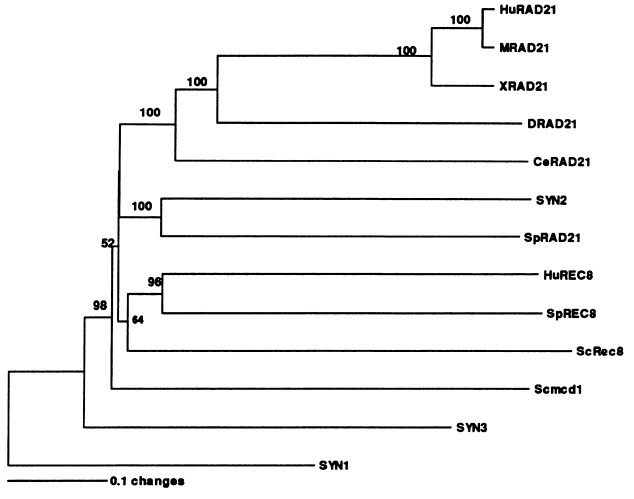

Fig. 5. Cohesin Neighbor Joining Tree. RAD21- and REC8-like proteins from human (HuRAD21 (McKay et al., 1996), HuREC8 (Parisi et al., 1999)), Mouse (MRAD21 (McKay et al., 1996)), Xenopus (XRAD21 (Losada et al., 1998)), Drosophila (DRAD21 (Warren et al., 2000), *C. elegans* (CeRAD21(GeneBank U40029)), *S. Pombe* (SpRec8 (Watanabe and Nurse, 1999), SpRAD21 (Birkenbihl and Subramani, 1992)), *S. cerevisiae* (ScREC8 (GeneBank YRP007C), ScMCD1 (Guacci et al., 1997)) and Arabidopsis SYN1 (Bai et al., 1999), SYN2 and SYN3 were aligned with Clustal and an average distance neighbor joining tree was generated using PAUP 4.0. Bootstrap values, evaluated for 100 replicates are shown at branch points.

1997; Watanabe and Nurse, 1999). In *Xenopus*, the 14S cohesin complex, which contains XRAD21, associates with chromatin during S phase, but most of the complex dissociates before the onset of mitosis (Losada et al., 1998). In contrast, MCD1/SCC1 remains bound to the chromosomes until anaphase in yeast (Guacci et al., 1997; Michaelis et al., 1997). Likewise, in contrast to the cell-cycle dependent expression observed for MCD1/SCC1 (Guacci et al., 1997; Michaelis et al., 1997), no significant changes were observed throughout the cell cycle for the mouse cohesin protein, PW29 (Darwiche et al., 1999). MCD1/SCC1 (Guacci et al., 1997) and SYN1 (Bai et al., 1999) are required for both chromosome condensation and cohesion, while XRAD21 appears to only function in chromosome cohesion (Losada et al., 1998). In addition, a mouse cohesin has been isolated that contains an EF-hand Ca^{2+} binding domain (Yu et al., 1995), which is not found in any of the other cohesins studied to date. Finally, several genes have been identified in Drosophila that are required for the establishment and/or maintenance of sister chromatid cohesion during meiosis that are not required for mitosis. Taken together these results indicate that while a number of similarities exist in the machinery that controls chromosome cohesion during meiosis and mitosis, differences in these two processes also exist. Likewise, these observations predict that species-specific differences exist in the way chromosome cohesion is controlled during meiosis and mitosis. As a first step in better understanding these differences we have isolated and characterized two plant cohesin proteins (SYN2 and SYN3) that appear to function during mitosis. Experiments are currently underway to better understand the role(s) of SYN2 and SYN3 in chromosome structure and function.

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References

Bai, X., Peirsion, B.N., Dong, F., Cai, X., 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., 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.

Birkenbihl, R.P., Subramani, S., 1992. Cloning and characterization of *rad21* an essential gene of *Schizosaccharomyces pombe* involved in double-strand-break repair. Nucleic Acids Res. 20, 6605–6611.

Blat, Y., Kleckner, N., 1999. Cohesins bind to preferential sites along yeast chromosome III, with differential regulation along arms versus the centric region. Cell 98, 249–259.

Darwiche, N., Freeman, L.A., Strunnikov, A., 1999. Characterization of the components of the putative mammalian sister chromatid cohesion complex. Gene 233, 39–47.

Guacci, V., Koshland, D., Strunnikov, A., 1997. A direct link between sister chromatid cohesion and chromosome condensation revealed through the analysis of *MCD1* in *S. cerevisiae*. Cell 91, 47–57.

Heo, S.-J., Tatebayashi, K., Kato, J., Ikeda, H., 1998. The *RHC21* gene of budding yeast, a homologue of the fission yeast *rad21* gene, is essential for chromosome segregation. Mol. Gen. Genet. 257, 149–156.

Hirano, T., 1999. SMC-mediated chromosome mechanics: a conserved scheme from bacteria to vertebrates? Genes Dev. 13, 11–19.

Klein, F., Mahr, P., Galova, M., Buonomo, S.B.C., Michaelis, C., Nairz, K., 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.

Losada, A., Hirano, M., Hirano, T., 1998. Identification of *Xenopus* SMC protein complexes required for sister chromatid cohesion. Genes Dev. 12, 1986–1997.

McFadden, G., 1995. In situ hybridization. In Methods in Cell Biology. Academic Press, New York, pp. 165–183.

McKay, M.J., Troelstra, C., van der Spek, P., Kanaar, R., Smit, B., Hagemeijer, A., Bootsma, D., Hoeijmakers, J., 1996. Sequence conservation of the *rad21 Schizosaccharomyces pombe* DNA double-strand break repair gene in human and mouse. Genomics 36, 305–315.

Megee, P.C., Mistrot, C., Guacci, V., Koshland, D., 1999. The centromeric sister chromatid cohesion site directs Mcd1p binding to adjacent sequences. Mol. Cell. 4, 445–450.

Michaelis, C., Ciosk, R., Nasmyth, K., 1997. Cohesins: chromosomal proteins that prevent premature separation of sister chromatids. Cell 91, 35–45.

Molnar, M., Bahler, J., Sipiczki, M., 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.

Nasmyth, K., 1999. Separating sister chromatids. Trends Biochem. 24, 98–104.

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., vanderSpek, P.J., van Drunen-Schoenmaker, E., Kanaar, R., Lehmann, E., Hoeijmakers, J.H.J., 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.

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

Sadano, H., Sugimoto, H., Sakai, F., Nomura, N., Osumi, T., 2000. NXP-1, a human protein related to Rad21/Scc1/Mcd1, is a component of the nuclear matrix. Biochem. Biophys. Res. Com. 267, 418–422.

Skibbens, R., Corson, L., Koshland, D., Hieter, P., 1999. Ctfp is essential for sister chromatid cohesion and links mitotic chromosome structure to the DNA replication machinery. Genes Dev. 13, 307–319.

StoopMyer, C., Amon, N., 1999. Meiosis: Rec8 is the reason for cohesion. Nat. Cell Biol. 1, 125–127.

Strunnikov, A.V., Larionov, V.L., Koshland, D., 1993. *Smc1* – an essential yeast gene encoding a putative head-rod-tail protein is required for nuclear division and defines a new ubiquitous protein family. J. Cell Biol. 123, 1635–1648.

Swofford, D.L., 1999. PAUP*, Phylogenetic Analysis Using Parsimony (* and Other Methods) Version 4, Sinauer Associates, Sunderland, MA.

Tanaka, T., Cosma, M.P., Wirth, K., Nasmyth, K., 1999. Identification of cohesin association sites at centromeres and along chromosome arms. Cell 98, 847–858.

Tatebayashi, K., Kato, J., Ikeda, H., 1998. Isolation of a *Schizosaccharomyces pombe rad21* ts mutant that is aberrant in chromosome segregation, microtubule function, DNA repair and sensitive to hydroxyurea: possible involvement of RAD21 in ubiquitin-mediated proteolysis. Genetics 148, 49–57.

Toth, A., Ciosk, R., Uhlmann, F., Galova, M., Schleiffer, A., 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.

van Heemst, D., Heyting, C., 2000. Sister chromatid cohesion and recombination in meiosis. Chromosoma 109, 10–26.

Warren, W.D., Lin, E., Nheu, T.V., Hime, G.R., McKay, M.J., 2000. *Drad21*, a Drosophila *rad21* homologue expressed in S-phase cells. Gene 250, 77–84.

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

Yu, S., Ozawa, M., Naved, A.F., Miyauchi, T., Muramatsu, H., Muramatsu, T., 1995. cDNA cloning and sequence analysis of a novel calcium binding protein with oligoproline motif. Cell Struct. Fun. 20, 263–268.