The *Arabidopsis SKP1* homolog *ASK1* controls meiotic chromosome remodeling and release of chromatin from the nuclear membrane and nucleolus

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Summary

During early stages of meiotic prophase I the nucleus undergoes considerable reorganization, including the clustering of telomeres, the release of contacts between chromosomes and the nuclear membrane, the reorganization of the nucleolus, and chromatin remodeling. Using a light squashing technique for the analysis of meiotic chromosomes along with fluorescent in situ hybridization, transmission electron microscopy and immunolocalization studies with antibodies to modified histones, we demonstrate that ASK1 is essential for early nuclear reorganization events. A relatively large number of meiotic alterations have been identified in *ask1-1* plants. We show that many of these defects, including alterations in homolog pairing, nucleolus migration and the mis-segregation of chromosomes, may arise from alterations in chromatin structure and the inability of chromosomes to resolve and release properly from the nuclear membrane and nucleolus during leptotene. These results raise the interesting possibility that ASK1 controls chromatin structure by targeting of either an early regulator of meiotic progression or possibly matrix attachment proteins for destruction.

Key words: *ASK1*, Nucleolus, Meiosis, Ribosomal DNA, Histone modification

Introduction

Meiosis is a central feature in the sexual reproduction of all eukaryotic organisms. Accurate chromosome segregation during meiosis is essential for the viability of progeny and the ultimate long-term survival of a species. Cytological and molecular studies in a number of organisms have defined numerous events that are crucial for the correct partitioning of genetic material (Dawe et al., 1994; Zickler and Kleckner, 1998; Zickler and Kleckner, 1999). In most organisms, the chromosomes undergo a profound reorganization in early meiotic prophase I. Some of the earliest, and least understood, meiotic events involve the reorganization of the nucleus after DNA replication to allow the synapsis and recombination of homologous chromosomes during zygotene and pachytene of prophase I.

During early prophase I in budding yeast the centromeres lose their intimate association with the spindle pole body (SPB) and become dispersed throughout the nucleus (Hayashi et al., 1998; Jin and Loidl, 1998). The telomeres, which are typically grouped into several perinuclear clusters in mitotically dividing cells (Gotta et al., 1996), attach to the nuclear envelope and then congregate in the vicinity of the SPB to form a 'chromosomal bouquet' at the leptotene/zygotene transition (Trelles-Sticken and Scherthan, 1999). During pachytene the paired telomeres become redistributed randomly in the nucleus. In budding yeast, bouquet formation depends on the presence of the meiosis-specific telomere protein Ndj1/Tam1 (Chua and Roeder, 1997; Conrad et al., 1997; Trelles-Sticken et al., 2000) and actin polymerization (Trelles-Sticken et al., 2005a). In plants, the maize *PAM1* gene is required for meiotic bouquet formation (Golubovskaya et al., 2002).

Telomere attachment to the inner nuclear membrane and their subsequent clustering in a narrow region of the nuclear envelope to form a chromosomal bouquet is observed in many organisms (Scherthan, 2001; Zickler and Kleckner, 1998; Zickler and Kleckner, 1999). Bouquet formation is believed to facilitate telomere pairing, which is followed by the movement and juxtapositioning of homologous centromere regions at early zygotene and the subsequent synapsis of homologous chromosomes during zygotene and pachytene (Goldman and Lichten, 2000; Loidl, 1990; Rockmill and Roeder, 1998; Trelles-Sticken et al., 2000).

A number of analyses have shown a close relationship between premeiotic S phase and the subsequent pairing and initiation of recombination between homologous chromosomes (Borde et al., 2000). For example, the absence of the B-type cyclins Clb5 and Clb6, which are required for premeiotic S phase in budding yeast, leads to defects in double-strand break (DSB) induction, recombination and synaptonemal complex (SC) formation (Smith et al., 2001; Stuart and Wittenberg, 1998). Deletion of the histone methyltransferase Set1 leads to a delay in premeiotic S-phase and subsequent alterations in centromere and telomere distribution (Trelles-Sticken et al., 2005b). Likewise, defects in

the establishment of sister chromatid cohesion, which occurs during premeiotic S-phase also have major effects on chromosome juxtapositioning and synapsis during later stages of prophase. Therefore, the configuration of the chromatin established during premeiotic S phase and the subsequent nuclear reorganization events that occur in early prophase I are critical for chromosome synapsis and recombination later in meiosis.

While chromosome dynamics and reorganization of the nuclear architecture have been observed during meiosis in a number of eukaryotes (reviewed by Loidl, 1990; Scherthan, 2001; Zickler and Kleckner, 1998) much less is known about factors that control these processes. In particular, cytological studies have been used to study nuclear organization during meiosis in plants for decades; however, essentially nothing is known about the genes that regulate the movement of chromosomes and chromatin reorganization during early meiosis in plants. In this report we present the results of studies that were designed to provide insight into factors that control meiotic nuclear organization in plants.

The *Arabidopsis ASK1* (*Arabidopsis SKP1-like1*) gene encodes a homolog of the human and yeast Skp1 proteins (Yang et al., 1999) and is involved in a number of aspects of plant growth and development, possibly through the ubiquitin-mediated proteolysis of proteins by the proteasome (del Pozo and Estelle, 2000; Hershko and Ciechanover, 1998; Sullivan et al., 2003; Zheng et al., 2002). Plants homozygous for the *ask1-1* mutation are defective in vegetative growth and flower development (Ni et al., 2004; Zhao et al., 1999; Zhao et al., 2001). More related to our current studies, *ASK1* is also essential for male fertility in *Arabidopsis* (Wang et al., 2004; Yang et al., 1999; Zhao et al., 2003b). The *ask1-1* mutant was initially found to be defective in chromosome separation during male meiosis (Yang et al., 1999). Subsequently, defects in homolog juxtaposition, synapsis and SC formation along with alterations in the distribution and removal of cohesin complexes were identified in the *ask1-1* mutant, suggesting that ASK1 acts relatively early in meiotic prophase (Wang et al., 2004; Zhao et al., 2006). Consistent with this hypothesis, ASK1 protein was recently found to localize in early prophase I cells (Wang and Yang, 2006).

However, the relatively wide range of defects in *ask1-1* mutant plants has made it difficult to assign a specific function to the protein. Several of the meiotic alterations observed in *ask1-1* plants, including defects in meiotic chromosome condensation, homolog synapsis and segregation are similar to defects observed in yeast cells with defects in meiotic condensin complexes as a result of the *ycs4-2* mutation (Yu and Koshland, 2003). This raised the possibility that the *ask1-1* mutation may also affect premeiotic nuclear structure and/or reorganization of the nucleus during meiosis. In this report we present the results of studies designed to test this hypothesis. Analysis of DAPI stained meiotic chromosomes, transmission electron microscopy (TEM), fluorescence in situ hybridization (FISH), and immunolocalization using several anti-histone antibodies demonstrate that *ASK1* is essential for the proper release of chromatin from the nuclear membrane and nucleolus, and the remodeling of meiotic chromosomes. Given that *ASK1* is thought to participate in the ubiquitin-mediated proteolysis of proteins, our results suggest that it may be responsible for the removal of proteins that link chromatin with the nuclear membrane and nucleolus. These results, along with those from previous studies, indicate that *ASK1* plays an important role in the reorganization of the nucleus that begins during leptotene.

Results

Meiotic chromosomes in *ask1-1* plants exhibit prolonged attachment to the nuclear membrane and the nucleolus

Meiotic prophase I is often observed using chromosome spreads of cells fixed in Carnoy's solution. During this procedure, the cell wall is removed by enzymatic digestion and the cytoplasm is cleared with 50-60% glacial acetic acid to allow better spreading of the chromosomes (Ross et al., 1996). Comparison of wild-type and *ask1-1* meiotic chromosomes using this technique did not reveal noticeable alterations in chromosome morphology from interphase to late leptotene in mutant plants (Fig. 1aA,E; B,F) (Wang et al., 2004; Zhao et al., 2003a). With this method the first noticeable *ask1-1* defect was observed at mid-zygotene, when chromosomes appeared thinner than in wild-type cells (Fig. 1aC,G) (Wang et al., 2004; Zhao et al., 2003a), suggesting that chromosome condensation might be abnormal. Subsequent defects were readily observed at pachytene, where in contrast to the juxtaposed homologs observed in the wild-type meiocytes (Fig. 1aD), unpaired chromosomes were observed in *ask1-1* meiocytes (Fig. 1aH).

Given the nature of the meiotic defects observed in *ask1-1* plants, we reasoned that defects may also be present prior to zygotene but not observed because the structural integrity of the nucleus and its chromosomes are not well preserved in chromosome spreads. To test this idea, we used additional procedures to fix and analyze meiocytes. When wild-type meiocytes were fixed in 4% paraformaldehyde in Buffer A, squashed and dried directly on slides we obtained good resolution of the DAPI stained chromosomes, while still maintaining the structural integrity of the cell. The nucleus of wild-type meiocytes has a granular appearance at meiotic interphase, with several brightly stained chromocenters near the nuclear envelope and a lightly stained, centrally positioned nucleolus (Fig. 1bA). During leptotene the chromosomes became more brightly stained with clear evidence of condensing chromatin (Fig. 1bB). The nucleolus became unstained and gradually moved towards one side of the nucleus as leptotene progressed (Fig. 1bC). By zygotene the chromosomes became partially resolved from the nuclear envelope and moved opposite to the nucleolus (Fig. 1bD,I). Typically only 1-2 fibers of the condensing chromosomes were observed extending into the nucleolus. As expected, chromosomes paired at pachytene (Fig. 1bJ), then desynapsed and continued to condense at diakinesis (Fig. 1bK,L). During late zygotene, pachytene, diplotene, and diakinesis the nucleolus remained unstained and at the periphery of the nucleus (Fig. 1bI-L). Therefore, fixation using 4% paraformaldehyde in Buffer A can preserve the structure and integrity of the nucleus, nucleolus, and chromosomes and works well for the analysis of meiotic nuclear organization.

While *ask1-1* meiocytes prepared using paraformaldehyde fixation appeared relatively normal at premeiotic interphase (Fig. 1bE), alterations were readily apparent by early leptotene in mutant cells. Specifically, *ask1-1* meiocytes had a distinct donut-shaped (or double-ring) appearance with strong DAPI staining of the nuclear membrane and the nucleoplasm

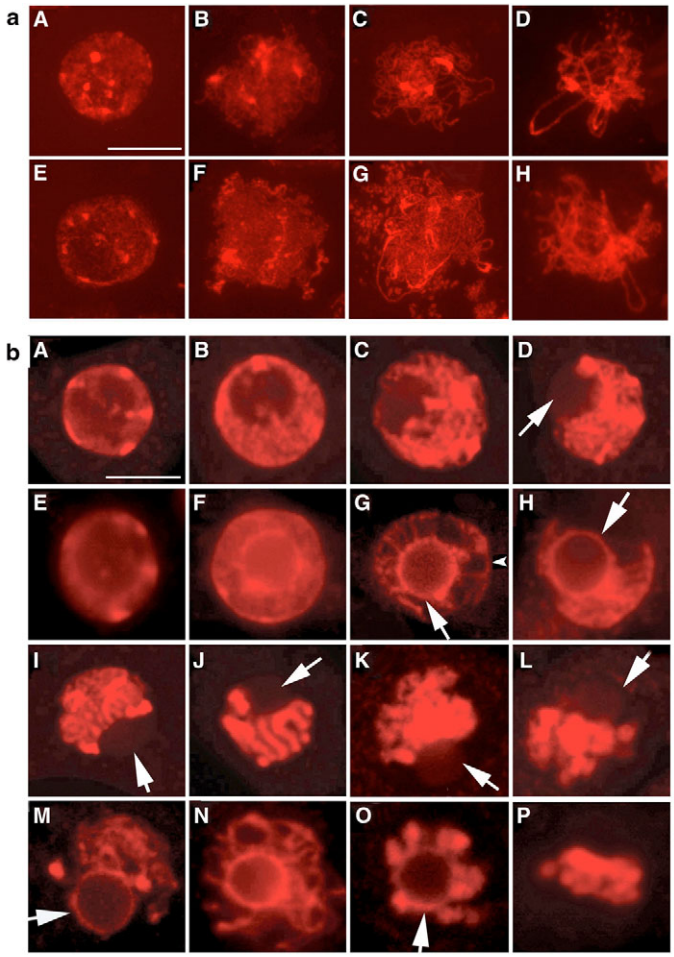

Fig. 1. Alterations in nucleolar structure of *ask1-1* plants are observed in intact male meiocytes. (a) Prophase I spreads of meiotic chromosomes in wild-type (A-D) and *ask1-1* (E-H) cells prepared using Carnoy fixation with enzymatic digestion and acid clearing. (A,E) interphase; (B,F) leptotene; (C,G) zygotene; (D,H) pachytene. Alterations in the association of chromatin with the nucleolus and the nuclear membrane are not observed. (b) Meiotic chromosome analysis in wild-type (A-D and I-L) and *ask1-1* (E-H and M-P) cells fixed in parafomaldehyde and lightly squashed without additional treatment. (A,E) interphase; (B,C,F,G) leptotene; (D,H,I,M) zygotene; (J,N) pachytene; (K,L,O,P) diakinesis. Large arrows indicate the position of the nucleolus and nucleolus-associated chromatin in *ask1-1*. Small arrow points to the attachment of chromatin to the nuclear envelope in *ask1-1*. Meiotic stage assignment for *ask1-1* meiocytes is based on chromatin structure and the stage of the surrounding tapetal cells (Wang et al., 2004). Bars, 10 μm.

surrounding the nucleolus (Fig. 1bF). The nucleoli also stained more strongly than those of wild-type cells. These results suggest a stronger attachment of chromosomes to the nuclear membrane and nucleolus in the *ask1-1* mutant meiocytes. During zygotene *ask1-1* meiocytes retained their donut-shaped appearance with progressively stronger DAPI staining around the outside of the nucleolus, which typically remained in the center of the nucleus (Fig. 1bG). Chromosome fibers during prophase I were typically observed extending between the nuclear membrane and the nucleolus in a spoke-like fashion, suggesting an extended and enhanced association of the chromosomes with both the nuclear envelope and the nucleolus. In some *ask1-1* meiocytes the chromosomes appeared to release from a portion of the nuclear membrane and condense to one side of the nucleus during late prophase I (Fig. 1bH). However, the chromatin remained attached to the nucleolus, which retained its central position in the nucleus throughout prophase I. As the chromosomes in *ask1-1* meiocytes continued to condense, they were eventually released from the nuclear membrane, but remained attached to the outside of the nucleolus (Fig. 1bM,N) forming a single-ring structure. The nucleus of *ask1-1* meiocytes at approximately diakinesis contained a central nucleolus that was surrounded by highly condensed chromatin (Fig. 1bO). This tight association between condensed chromosomes and the nucleolus remained until approximately metaphase I, when the nucleolus appeared to disassemble (Fig. 1bP). These defects are not observed using the standard chromosome spreading technique. Therefore, using paraformaldehyde fixation and lightly squashed cells we were able to identify alterations in nuclear conformation as early as leptotene in *ask1-1* meiocytes that included the extended and enriched attachment of chromosomes to both the nuclear membrane and nucleolus. This indicates that chromatin is not able to resolve properly from nuclear membrane and nucleolus in the absence of ASK1.

To obtain further evidence for defects in nuclear organization in the *ask1-1* mutant, we performed TEM. Wild-type and *ask1-1* meiocytes were analyzed starting at meiotic interphase, which coincides with stage 7 of floral development (Sanders et al., 1999; Smyth et al., 1990). No apparent differences were observed during interphase between wild-type and *ask1-1* meiocytes. At meiotic interphase in both wild-type and *ask1-1* plants, the meiocyte nucleus is characterized by a centrally positioned, prominent nucleolus (Fig. 2A,E). The chromatin is evenly distributed throughout the nucleus. Chromosomes became visible as a result of chromatin condensation and the formation of proteinacious cores (axial elements) between sister chromatids during leptotene in wild-type and *ask1-1* plants (Fig. 2B,F). No noticeable defects in axial element formation were observed in *ask1-1* plants at this stage. However, unlike wild-type meiocytes where the axial elements were dispersed in the nucleus opposite the peripheral nucleolus during mid to late leptotene (Fig. 2B-D), the axial elements/chromatin in *ask1-1* meiocytes were typically attached to the nuclear membrane and nucleolus (Fig. 2F-H). Axial elements/chromatin were usually observed wrapped around the outside of the nucleolus (Fig. 2F, thin arrow), but could also be seen entering/leaving the nucleolus (Fig. 2F,G).

During the leptotene/zygotene transition the nucleolus migrated to a peripheral position in the nucleus in wild-type meiocytes (Fig. 2B), but maintained its central location in the *ask1-1* mutant (Fig. 2F-H). As *ask1-1* meiocytes progressed through pachytene and early diplotene, chromosome connections with the electron dense nucleolus persisted, became more pronounced, and often resembled the spokes in a wheel configuration observed in DAPI-stained cells. Similar to the results from our light microscope studies, the nuclei of *ask1-1* meiocytes contained a centrally positioned nucleolus surrounded by highly condensed chromosomes at diplotene (Fig. 2H). By contrast, condensed bivalents were always observed spread throughout the nucleus of wild-type meiocytes

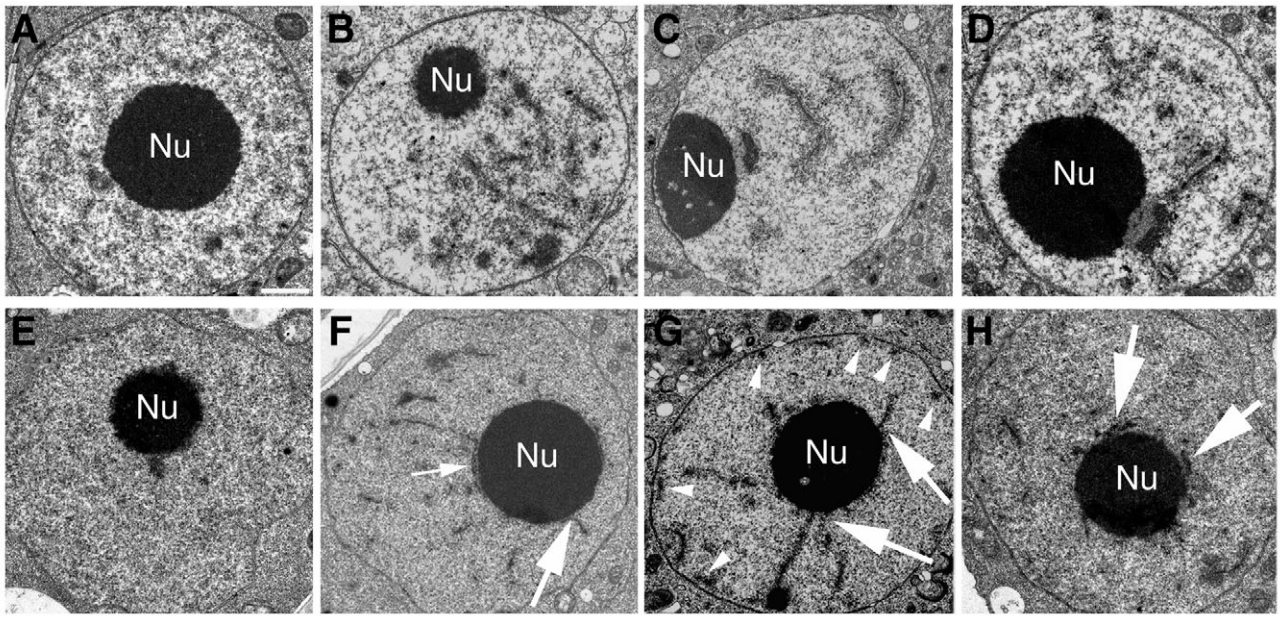

Fig. 2. Electron micrographs of *Arabidopsis* meiocytes showing chromosomes at different stages of prophase I. (A-D) Wild type. (A) Interphase; (B) laptotene/zygotene, note most chromosomes are resolved away from and to one side of the nucleolus; (C) pachytene; (D) diplotene/diakinesis. (E-H) *ask1-1*. (E) Interphase; (F) zygotene, showing the close association of unsynapsed chromosome ends or rDNA with the nucleolus; (G) pachytene; (H) diplotene. Note the multiple attachment sites of chromatin to the nuclear envelope (small arrows) and the nucleolus (large arrows). Nu, nucleolus. Bar, 1 μm.

(Fig. 2D). The persistent attachment of axial elements/chromatin to the nuclear membrane and nucleolus in TEM images of *ask1-1* meiocytes corresponding to wild-type prophase I cells (Fig. 2G), confirms observations from the DAPI analysis of paraformaldehyde-fixed *ask1-1* meiocytes, which identified spoke-like chromosome structures.

The *ask1-1* mutation affects rDNA structure and causes alterations in the nucleolar organizer regions

The alterations in nuclear structure identified above raised the possibility that *ask1-1* meiocytes may exhibit alterations in rDNA structure. Therefore, FISH experiments using a probe for the *Arabidopsis* rDNA repeat were conducted to test this hypothesis. With the exception of the 5S RNA genes, the *Arabidopsis* rRNA genes are arranged in long tandem arrays comprising the two nucleolar organizer regions (NORs) on chromosomes II and IV (Maluszynska and Heslop Harrison, 1991). The rDNA probe typically identified two to four relatively compact signals that were associated with the nucleolus in wild-type meiocytes prior to late leptotene (Fig. 3A). By late leptotene the NOR's had condensed into a single, relatively large spot that remained associated with nucleolus throughout zygotene and pachytene in wild-type meiocytes (Fig. 3B,C). Two spots, corresponding to the NORs on paired chromosomes II and IV, respectively, were observed as the chromosomes desynapsed during diplotene/diakinesis (Fig. 3D,I). Four groups of rDNA signals were observed at anaphase I as the homologous chromosomes separated (Fig. 3J,K), while eight signals were observed during meiosis II (Fig. 3L). Each microspore nucleus contained one rDNA signal at the end of meiosis (data not shown).

The rDNA labeling patterns of interphase cells were relatively normal in *ask1-1* meiocytes and typically contained two to four signals in the vicinity of the nucleolus (data not shown). Alterations in rDNA structure (NORs) were first observed during early leptotene in *ask1-1* meiocytes. The rDNA signals were typically diffuse and often observed as a partial ring near the nucleolus (Fig. 3E). The rDNA/NORs became more diffuse and spread out around the nucleolus as meiocytes progressed through prophase I (Fig. 3F-H), until approximately late diakinesis when they formed a single compact spot that remained throughout the rest of meiosis (Fig. 3L-O). No separation of rDNA/NORs was observed during anaphase I indicating that they could not be resolved and ultimately fragmented from one or more of their parental chromosomes (Fig. 3N,O). Usually only one *ask1-1* polyad nucleus contained an rDNA signal at the end of meiosis (data not shown). Therefore *ask1-1* meiocytes exhibit major alterations in rDNA structure during meiosis.

ask1-1 meiocytes display continued telomere association with the nucleolus during prophase I

Unlike many plants, classical telomere clustering at the bouquet stage of leptotene/zygotene appears to be absent in *Arabidopsis*. Instead, telomeres first associate with the nucleolus prior to mid-leptotene. As meiosis progresses they lose this association and become randomly dispersed in the nucleus (Armstrong et al., 2001). Our finding that *ask1-1* meiocytes exhibit alterations in nucleolar structure, including the persistent association of chromatin with the outside of the nucleolus raised the possibility that telomeres may not be released normally from the nucleolus in *ask1-1* plants. A telomere repeat probe was therefore used in FISH experiments to test this possibility. In addition to telomere signals at the

chromosome ends the probe also produced two large signals corresponding to internal telomere-like sequences adjacent to the centromere of chromosome I (Armstrong et al., 2001).

FISH was performed both on chromosome spreads prepared after Carnoy fixation/enzymatic digestion (Fig. 4a) and in cells prepared by paraformaldehyde fixation (Fig. 4b). The distribution of telomere signals was similar in wild-type and *ask1-1* chromosome spreads prepared by Carnoy's fixation. During meiotic interphase and early leptotene telomere signals (up to 20) were typically clustered around the nucleolus in both wild-type and *ask1-1* plants (Fig. 4aA,E). Telomere signals were reduced to 10 or less and found dispersed throughout the nucleus beginning in mid-leptotene and continuing throughout prophase in wild-type meiocytes (Fig. 4aB-D). Although some *ask1-1* meiocytes (20-25%) exhibited continued clustering of the telomeres around the nucleolus during zygotene (Fig. 4aF), most *ask1-1* cells resembled wild-type cells with telomere signals found throughout the nucleus (Fig. 4aG,H).

The clustering of telomere signals around the nucleolus was more pronounced before early leptotene in both wild-type and *ask1-1* meiocytes fixed with paraformaldehyde (Fig. 4bA,E). The average total number of telomere signals in the nucleoplasm during interphase and early leptotene in paraformaldehyde-fixed meiocytes was very similar (1.90 versus 1.94) for wild-type and *ask1-1* meiocytes, respectively (n = 20). The remaining telomere signals were associated with the nucleolus. During subsequent stages of prophase I (late leptotene to diplotene) telomeres in wild-type meiocytes were typically scattered in the nucleus opposite the peripheral nucleolus (Fig. 4bB-D). The average total number of the telomere signals in the nucleoplasm increased from approximately two to nine during leptotene (n = 19), indicating that the telomeres were released from the nucleolus during this stage in wild-type meiocytes. By contrast, meiocytes in *ask1-1* plants displayed a consistent association of the telomeres with the nucleolus throughout prophase I (Fig. 4bE-H). In most cells the telomere signals were either positioned around the surface of the nucleolus (Fig. 4bE,H) or were found in the region of the nucleolus (Fig. 4bF). On average, 2.90 telomere signals were observed in the nucleoplasm of *ask1-1* meiocytes (n = 62). In a small number of *ask1-1* cells (<5%), the telomeres were found in the vicinity of the nucleolus, but did not appear tightly associated (Fig. 5bG). Therefore, telomeres do not release normally from the nucleolus in *ask1-1* meiocytes. Furthermore, the normal interaction of telomeres with the nucleolus appears to be generally weak and is best observed after cross-linking with paraformaldehyde.

ask1-1 meiocytes exhibit alterations in histone modification patterns

Our finding that meiocytes in *ask1-1* plants exhibit alterations in nuclear structure, organization of the rDNA loci and telomere association with the nucleolus suggested that the mutation may have direct or indirect effects on chromatin structure. To test more directly for defects in chromatin structure, we conducted immunolocalization studies with antibodies to several modified histone proteins, including: histone H3 dimethyl-lysine 9 (H3-Dm-K9), histone H3 dimethyl-lysine 4 (H3-Dm-K4), acetyl-histone 3 (AH3), and acetyl-histone 4 (AH4), which are known to associate with different chromatin structures (Probst et al., 2004; Soppe et al., 2002).

Identical labeling patterns were observed between wild-type and *ask1-1* plants for AH4 and H3-Dm-K4. Antibody for AH4 yielded weak nuclear labeling of the euchromatin in both plants; it was not associated with chromocenters (data not shown). This is consistent with labeling patterns for tetra-acetylated histone 4 in *Arabidopsis* mesophyll protoplasts (Probst et al., 2004). No signal was observed during later stages of meiosis in either wild-type or *ask1-1* plants (data not shown). Differences were also not observed in the distribution of H3-Dm-K4 between wild-type and the *ask1-1* mutant. Labeling was typically strong on chromosomes during meiotic interphase I and leptotene (Fig. 5aA,B). The signal became progressively weaker as the chromosomes condensed during zygotene, and was weakest on synapsed pachytene chromosomes (Fig. 5aC). However, the H3-Dm-K4 signal increased

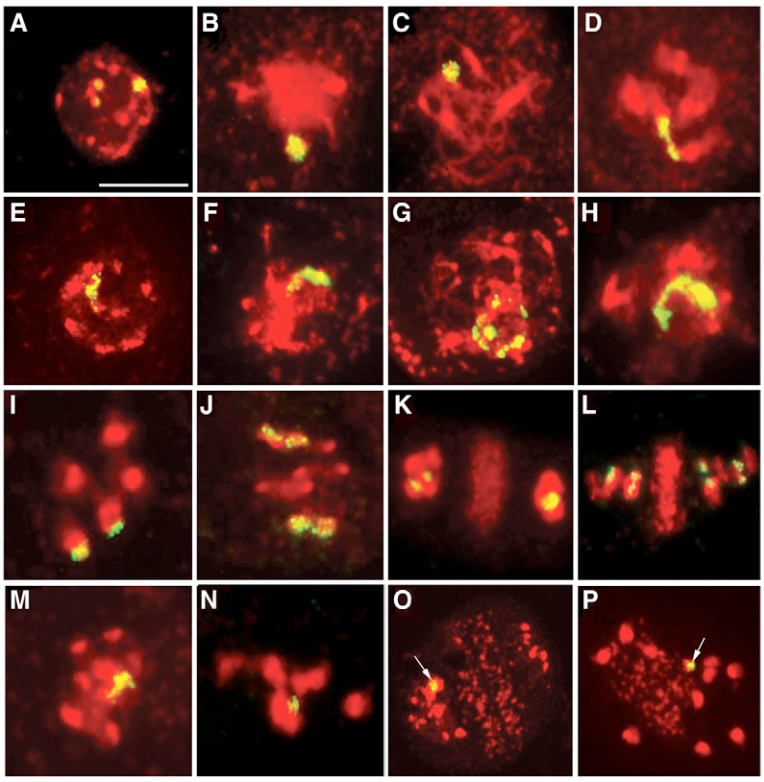

Fig. 3. Alterations in rDNA structure in meiocytes of *ask1-1* plants. FISH using an rDNA probe on meiotic chromosomes in wild-type (A-D,I-L) and *ask1-1* (E-H,M-P) plants. Merged photos of biotin-labeled rDNA signals (green) on chromosomes counterstained with DAPI (red) are shown. (A,E) Early leptotene; (B,F) zygotene; (C,G) pachytene; (D,H) diakinesis; (I,M) premetaphase; (J,N) metaphase I/early anaphase I; (K,O) telophase I; (L,P) metaphase II. Arrow shows the single rDNA region. Eleven chromosomes are observed suggesting the breakage of rDNA from chromosomes II and IV. Assignment of meiotic stages in *ask1-1* is based on chromosome morphology and the stage of the surrounding tapetal cells (Wang et al., 2004). Bar, 10 μm.

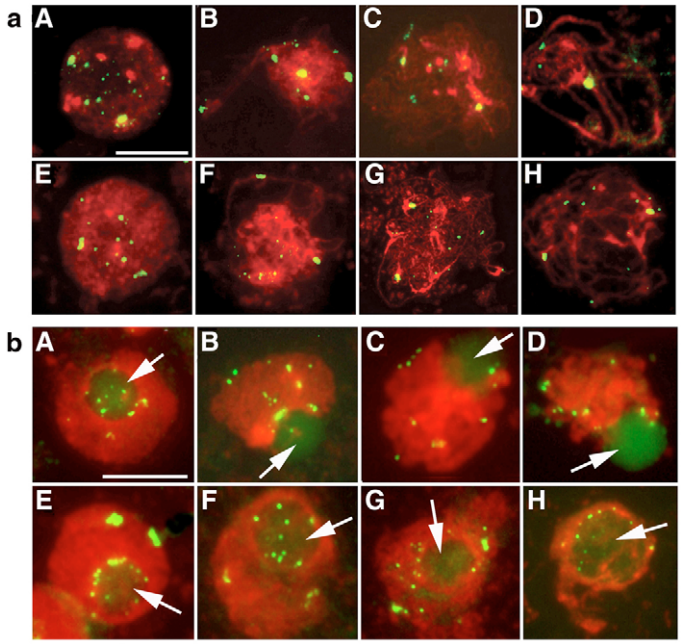

Fig. 4. Telomeres exhibit a prolonged attachment to the nucleolus in the meiocytes of *ask1-1* plants. (a) Telomere FISH on meiotic chromosomes in wild-type (A-D) and *ask1-1* (E-H) cells prepared using standard fixation techniques and chromosome spreading. (A,E) Interphase; (B,F) leptotene; (C,G) zygotene; (D,H) pachytene. No significant differences are observed in the distribution of telomere signals. (b) Telomere FISH on meiotic chromosomes in paraformaldehyde-fixed wild-type (A-D) and *ask1-1* (E-H) cells. Arrows indicate the position of the nucleolus. (A,E) Interphase; (B,F) leptotene; (C,G) zygotene; (D,H) pachytene. Note the persistent association of telomere signals with the nucleolus in *ask1-1* meiocytes but not in wild-type meiocytes. Assignment of meiotic stages in *ask1-1* is based on chromosome morphology and the stage of the surrounding tapetal cells. Bars, 10 μm.

at diakinesis and metaphase I (Fig. 5aD). Similar to the situation with antibody for AH4, no labeling of the chromocenters was observed.

Differences were, however, identified in AH3 and H3-Dm-K9 labeling patterns. In wild-type cells the anti-AH3 antibody labeled chromosomes in both somatic and meiotic cells. The labeling pattern was generally uniform over the chromosomes with the exception that the nucleolus and chromocenters were not labeled. In somatic cells strong labeling was observed during all stages of the cell cycle (data not shown). In wild-type meiotic cells AH3 was detected during interphase (Fig. 5bA), but little to no signal was observed during leptotene, zygotene, pachytene and diplotene (Fig. 5bB,C,G). Signal corresponding to AH3 was again detected on highly condensed chromosomes at metaphase I and telophase I (Fig. 5bH,I). In *ask1-1* meiocytes strong labeling was also observed during meiotic interphase I (Fig. 5bD). However, in contrast to wild-type plants, AH3 signal was also consistently observed from leptotene through telophase I (Fig. 5bE,F; J,L). AH3 signals were also abnormal in appearance at later stages of meiosis, typically appearing as several spots on the highly condensed chromosomes at metaphase I and telophase I (Fig. 5bK,L).

Consistent with previous reports, H3-Dm-K9 was found

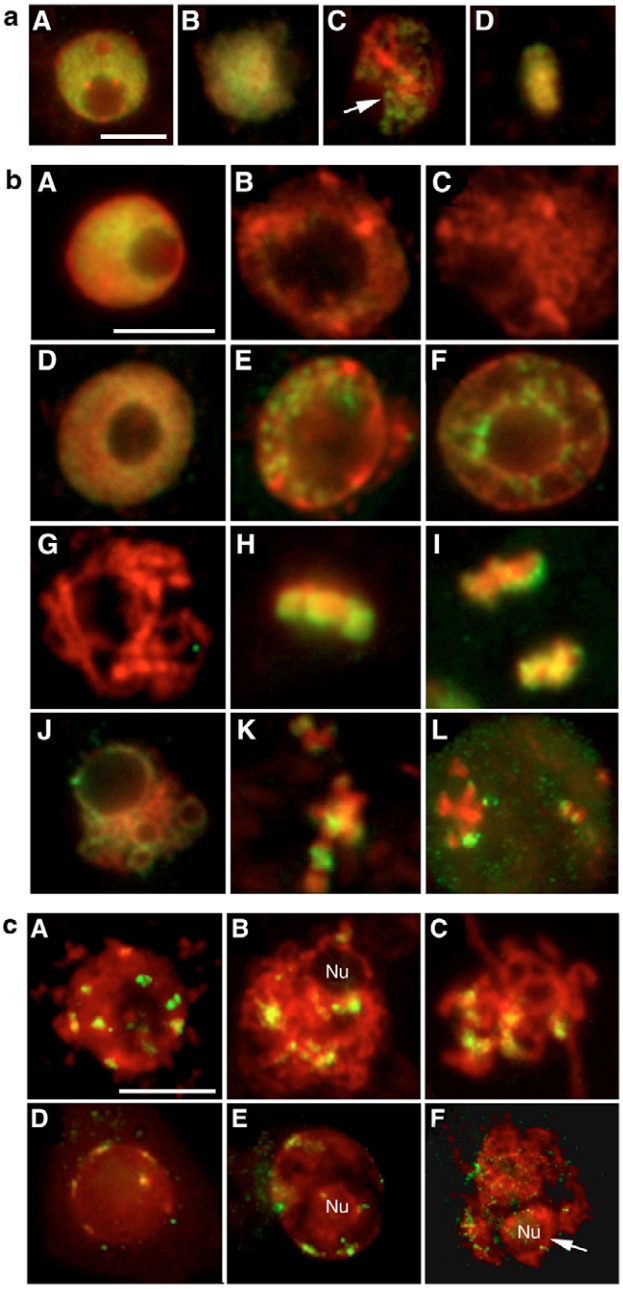

Fig. 5. Immunolocalization of modified histones during meiosis. Merged photos of anti-histone antibody (green) and meiotic chromosomes (red) are shown. Assignment of meiotic stages in *ask1-1* meiocytes is based on chromosome morphology and the stage of the surrounding tapetal cells. (a) Immunolocalization of dimethyl-histone H3 lysine 4 in wild-type meiocytes. (A) interphase; (B) leptotene/zygotene transition; (C) pachytene. (D) metaphase I. Arrow indicates weak signal at pachytene. *ask*1-1 meiocytes exhibited similar labeling patterns (data not shown). (b) Immunolocalization of acetylated histone 3 (positions 9 and 14) in wild-type (A-C,G-I) and *ask1-1* (D-F,J-L) meiocytes. (A,D) Interphase; (B,E) leptotene; (C,F) zygotene; (G,J) pachytene; (H,K) metaphase I; (I,L) telophase I. (c) Immunolocalization of dimethyl-histone 3 lysine 9 in wild-type (A-C) and *ask1-1* (D-F) meiocytes. (A,D) Interphase/early leptotene; (B,E) zygotene; (C,F) pachytene. Note the nucleolus in *ask1-1* is also stained with DAPI (red) at later stages, suggesting the persistent association of chromatin with nucleolus in the mutant. Nu, nucleolus. Bars, 10 μm.

primarily at the chromocenters in wild-type cells (Soppe et al., 2002). In wild-type meiocytes during interphase, there were approximately 10 groups of H3-Dm-K9 signals, corresponding to heterochromatic regions (centromeric and pericentromeric) (Fig. 5cA). The signals decreased in number and increased in intensity as the chromosomes paired and condensed. Four to eight relatively strong large signals, along with a number of weak foci scattered near the chromosome centers, were observed during zygotene (Fig. 5cB). By pachytene, five large diffuse signals corresponding to the centromeres were observed (Fig. 5cC). By contrast, a punctate pattern of small, dispersed H3-Dm-K9 signals was observed in *ask1-1* meiocytes. These signals were generally greater in number and weaker in intensity than those in wild type. At interphase the dispersed signals were mostly observed along the nuclear membrane (Fig. 5cD). During leptotene and zygotene small, weak and stretched H3-Dm-K9 signals were also observed on the nuclear membrane and at the edge of the nucleolus (Fig. 5cD), suggesting that heterochromatin/chromocenters may be preferentially associated with nuclear membrane and the nucleolus. However, by zygotene the signals were weak and widely dispersed (Fig. 5cF). Therefore, alterations in the distribution of modified histone 3, in particular those associated with heterochromatin, were also observed in *ask1-1* meiocytes.

Discussion

Arabidopsis plants homozygous for the *ask1-1* mutation exhibit a wide range of defects including alterations in flower development, hormonal regulation and male meiosis (Gray et al., 1999; Ni et al., 2004; Yang et al., 1999; Zhao et al., 1999; Zhao et al., 2001). The pleiotropic nature of the *ask1-1* mutation has complicated studies to understand its molecular function. In particular, *ask1-1* plants exhibit several meiotic alterations, including defects in chromosome condensation, homolog juxtaposition, synapsis and segregation and the migration of the nucleolus (Wang and Yang, 2006; Wang et al., 2004; Yang et al., 1999; Zhao et al., 2006). On the basis of these phenotypes, several potential roles have been proposed for ASK1 during male meiosis, including a regulator of homolog separation (Yang et al., 1999) and a repressor of recombination (Wang and Yang, 2006). In our current study we show that ASK1 is required for overall chromosome conformation and the remodeling of meiotic chromosomes by controlling the release of chromatin from the nucleolus and nuclear membrane beginning at leptotene. The prolonged and enriched attachment of chromatin to both the nuclear membrane and nucleolus in *ask1-1* meiocytes is the earliest meiotic defect reported for this mutation. These results explain many of the observed meiotic alterations in *ask1-1* plants and suggest that a primary role of *ASK1* is to control meiotic chromatin structure.

Alterations in chromatin structure are likely a primary effect of the *ask1-1* mutation

Most, if not all of the meiotic alterations described for the *ask1-1* mutation, including failure of the nucleolus to migrate to the periphery of the nucleus, and defects in homolog juxtapositioning and synapsis can be explained by the defects in chromatin structure that we observed. In particular, we believe that many of the defects are a consequence of the failure of chromosomes to release from the nucleolus and/or nuclear membrane during leptotene. Results from our rDNA FISH experiments showed that the rDNA loci were abnormally dispersed around the periphery of the nucleolus during early to mid-prophase (Fig. 3E-G) and then collapsed into single condensed spot at approximately diakinesis (Fig. 3M-P) in *ask1-1* meiocytes. Likewise, the telomeres typically remained associated with the periphery of the nucleolus throughout prophase and did not disperse normally into the nucleus in *ask1-1* meiocytes (Fig. 4bE-H). Finally, results of DAPI-stained, unspread cells demonstrated an abnormal association between the chromatin and the nuclear membrane in *ask1-1* plants (Fig. 1bG). This restricted chromosome movement likely impedes the homologous chromosome search process, which in turn would result in defects in homolog juxtapositioning and synapsis. This restricted chromosome movement is also expected to block physically the migration of the nucleolus to the nuclear periphery at the leptotene to zygotene transition. Therefore, many of the defects observed in *ask1-1* meiocytes can be explained by physical constraints on the chromosomes brought about by their interaction with the nucleolus and possibly the nuclear membrane.

Alterations associated with the nucleolus, the prolonged attachment of chromatin to the nuclear membrane and the failure of the telomeres to migrate in the nucleus are not readily observed in chromosome spreads (Fig. 1a) (Wang et al., 2004; Yang et al., 1999; Zhao et al., 2003a). They are, however, observed when the cells are fixed in paraformaldehyde and observed with both light microscopy and TEM. This suggests that the chromosomal association with the nucleolus and nuclear membrane involves relatively weak protein interactions that are not preserved when samples are fixed in Carnoy's solution and are lost during the chromosome spreading procedure. Crosslinking with paraformaldehyde appears to maintain these interactions and allows them to be visualized. These results highlight the importance of using a number of different techniques to analyze meiotic chromosomes. They also suggest that alterations based on weak interactions may often be missed using standard chromosome spreading techniques.

What is the role of ASK1 in meiosis?

The *ASK1* gene encodes a homolog of the human and yeast Skp1 proteins (Yang et al., 1999). It is the most conserved and highly expressed of the 21 *Arabidopsis ASK* genes (Kong et al., 2004; Zhao et al., 2003b). Skp1 proteins together with Cullin1, an F-box protein, and Rbx1 are components of SCF complexes, which are putative E3 ubiquitin-protein ligases that target proteins for degradation by the 26S proteasome. Skp1 proteins have been shown to participate in the control of a number of regulatory events, including the cell cycle, signal transduction and transcription (Bai et al., 1996; Hershko and Ciechanover, 1998; Schulman et al., 2000; Shen et al., 2002; Zheng et al., 2002). Our results indicate that *ASK1* is important for controlling meiotic chromatin structure and the general reorganization of chromosomes during leptotene, including deacetylation of H3 during prophase I, migration of the telomeres from the nucleolus, rDNA structure and organization, and the release of chromatin from the nucleolus and nuclear membrane.

ASK1 could control meiotic chromosome structure in a

number of ways. First, ASK1 could regulate the degradation of a protein that controls the leptotene to zygotene transition. In budding yeast, the single *SKP1* gene promotes mitotic cell cycle progression by facilitating the degradation of an inhibitor of S-phase cyclins, thereby allowing DNA replication (Bai et al., 1996; Feldman et al., 1997; Skowyra et al., 1999; Tsvetkov et al., 1999). Pachytene progression in *C. elegans* has also been shown to require a Skp1 homolog, although its exact role in meiosis has not been determined (Nayak et al., 2002). Therefore, it is possible that an *ASK1*-containing meiotic SCF complex mediates the degradation of a protein(s) that inhibits the leptotene to zygotene transition. The observed alterations in chromatin structure/reorganization would then be a consequence of the block in this transition.

Alternatively, *ASK1* could regulate the interaction of meiotic chromosomes with the nuclear matrix/membrane. The attachment of chromosomes to the nuclear envelope is important for controlling chromatin arrangement during mitosis and meiosis. In yeast, animals and many plants, homologous chromosome pairing during meiosis is immediately preceded by chromosomal reorganization and the clustering of telomeres on the nuclear envelope (Bass et al., 1997; Bass et al., 2000; Cowan et al., 2001; Martinez-Perez et al., 1999; Scherthan et al., 1996; Trelles-Sticken and Scherthan, 1999; Zickler and Kleckner, 1998). This bouquet arrangement of chromosomes is believed to facilitate homologous chromosome synapsis by bringing the ends of the chromosomes into close proximity (Scherthan, 2001). Telomere clustering on the nuclear envelope has not been observed during meiosis in *Arabidopsis* (Armstrong et al., 2001); however our results indicate that the release of telomeric regions from the nucleolus and chromatin reorganization at the nuclear envelope is an important aspect of meiosis in *Arabidopsis*. Our results using light and electron microscopy and FISH experiments demonstrated a persistent association of rDNA sequences and the telomeres with the nucleolus and the general association of chromatin with the nuclear envelope in *ask1-1* meiocytes.

In animal cells, chromatin is attached to the nuclear matrix via lamins, type V intermediate filament proteins (reviewed in Mattout-Drubezki and Gruenbaum, 2003). While the plant nuclear matrix is morphologically similar to those of vertebrates (reviewed in Moreno Diaz de la Espina, 1995) and contains proteins antigenically related to animal nuclear matrix components (Minguez and Moreno Diaz de la Espina, 1993; Minguez and Moreno Diaz de la Espina, 1996), plant genomes lack nuclear lamin homologs. However, several coiled-coil proteins that may act as lamins have been identified in plants (Blumenthal et al., 2004; Masuda et al., 1997; Mattout-Drubezki and Gruenbaum, 2003). Therefore, *ASK1* may facilitate the degradation of one or more proteins that link chromatin to the nuclear matrix/membrane, thereby allowing normal meiotic nuclear reorganization to take place.

It is also possible that *ASK1* affects meiotic chromatin structure by regulating one or more chromatin remodeling proteins. Most of our understanding of proteins involved in modifying chromatin structure has come from studies on somatic cells (Fischle et al., 2003; Hsieh and Fischer, 2005). Typically heterochromatin is enriched in hypoacetylated histones, methylated DNA and histone H3 methylated at lysine 9. By contrast, euchromatin is characterized by highly acetylated histone H4 and histone H3 methylated at lysine 4 (Nishioka et al., 2002; Peters et al., 2002). Very little is known about how changes in chromatin structure affect meiosis or how these changes are controlled in any organism. Studies in budding yeast have shown that mutations in *Set1*, which is responsible for methylation of H3 lysine 4, block bouquet formation (Trelles-Sticken et al., 2005b). Likewise, telomere clustering at the spindle pole body during meiosis requires the methylation of H3 on lysine 9 in *S. pombe* (Tuzon et al., 2004). Therefore, telomere heterochromatin structure appears to be important for meiotic telomere clustering in yeast cells.

Mutations in chromatin remodeling proteins have been found to cause alterations similar to some of those observed in *ask1-1* plants. For example, mutations in HDA6 result in hyperacetylation of histone H4 and an increase in histone H3 methylation at lysine 4 at the nucleolar organizer regions, resulting in decondensation of the NORs (Probst et al., 2004). While dramatic alterations in rDNA structure are observed in *ask1-1* plants, specific changes in histone H4 acetylation, or histone H3 lysine 4 methylation patterns were not observed at the rDNA loci. Rather increased histone H3 acetylation levels were observed in *ask1-1* meiocytes as well as an apparent decrease in heterochromatin, as evidenced by DAPI staining and H3 lysine 9 methylation patterns, at the centromeric and pericentromeric regions in the mutant.

The *Arabidopsis* hypomethylation mutants, *ddm1* and *met1* also share some properties with *ask1-1* plants, including a reduction in lysine 9 methylated H3-labeled heterochromatin and the dispersion of pericentromeric sequences away from heterochromatic chromocenters (Soppe et al., 2002). *DDM1* encodes a *SWI/SNF*-like chromatin-remodeling factor (Jeddeloh et al., 1999), while *MET1* encodes a maintenance DNA methyltransferase (Finnegan and Kovac, 2000). While increased levels of histone H3 acetylation were not reported for either mutant, increased levels of acetylated histone H4 were observed at the chromocenters (Soppe et al., 2002). Like *ask1-1*, the *ddm1* and *met1* mutants also exhibit alterations in flower morphology, leaf shape and sterility (Finnegan et al., 1996; Kakutani et al., 1996; Ronemus et al., 1996).

The failure of *ask1-1* meiotic chromosomes to form heterochromatin and condense properly could result from the continued acetylation of histone 3. Chromosome condensation is typically associated with a substantial reduction in H3 and H4 acetylation in mitotic cells (Kruhlak et al., 2001). Although the role of histone acetylation has not been studied during meiosis in plant cells, it has been demonstrated that histone hyperacetylation impairs chromosome condensation and sister chromatid separation, resulting in mitotic arrest, in tobacco cells (Li, Y. et al., 2005). Furthermore, inhibition of histone deacetylation resulted in defects in chromosome condensation and persistent cohesion along sister chromatid arms after centromere separation in human fibroblasts (Cimini et al., 2003). Therefore, it is possible that *ASK1* controls meiotic chromatin structure by regulating chromatin-remodeling complexes.

In summary, we have shown that many of the meiotic defects associated with the *ask1-1* mutation arise from alterations in chromatin structure and the inability of chromosomes to resolve and release properly from the nuclear membrane and nucleolus during leptotene. These results raise the interesting possibility that ASK1 controls chromatin structure by

regulating the interaction of chromosomes with the nuclear matrix/membrane. ASK1 could affect chromatin structure in a number of ways, including the targeting of either a regulator of meiotic progression or possibly matrix attachment proteins for destruction. While additional molecular and biochemical studies are necessary to further define the specific function of *ASK1* in meiosis our results show that *ASK1* functions earlier than was first believed and provide significant new insights into the role of this important protein.

Materials and Methods

Plant material and growth conditions

The wild-type and *ask1-1* mutant plants used in this study are of *Arabidopsis thaliana* ecotype Landsberg *erecta*. Plants were grown in Metro-Mix 200 soil (Scotts-Sierra Horticultural Products Co., Maryville, OH) at 22°C with 16 hours of light and 8 hours of darkness.

Chromosome analysis and immunolocalization

Meiotic chromosome spreading was performed on floral buds fixed in Carnoy's fixative (ethanol:chloroform:acetic acid: 6:3:1) and prepared as previously described (Ross et al., 1996). An alternative way of visualizing meiotic chromosomes was performed on cells that were fixed in 4% paraformaldehyde in Buffer A (Dernburg et al., 1996) and then squashed without any additional treatment. Chromosomes and cell squashes were stained with DAPI [(1.5 μg/ml) 4,6-diamino-2-phenylindole dihydrochloride, Vector Laboratories, Inc. Burlingame, CA] and were observed with an Olympus BX51 epifluorescent microscope system. Images were captured with a Spot camera system (Diagnostic Instruments, Inc.) and processed with Adobe Photoshop software (Adobe System, San Jose, CA). Meiocytes were staged as previously described (Ross et al., 1996). The assignment of meiotic stages in *ask1-1* meiocytes was based on the stage of the surrounding tapetal cells (Wang et al., 2004) as well as meiotic chromosome morphology.

Immunolocalization of modified histone proteins was performed on methanol:acetone fixed cells as previously described (Lam et al., 2005). The rabbit antibodies were raised against dimethyl-histone H3-Lys9 (H3-Dm-K9), dimethyl-histone H3-Lys 4 (H3-Dm-K4), acetyl-histone 3 (AH3, positions 9 and 14) and acetyl-histone 4 (AH4, positions 5, 8, 12 and 16) were obtained from Upstate Group LLC (www.upstate.com) and used at 1:500 dilutions. The slides were incubated at 4°C overnight and washed eight times (20 minutes each) with 1× PBS buffer. The primary antibody was detected with Alexa-488-labeled goat anti-rabbit secondary antibody (1:500) overnight at 4°C. After washing, images were captured as above.

Fluorescence in situ hybridization (FISH)

Chromosome spreads were prepared from anthers isolated from inflorescences that were fixed in Carnoy's fixative 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; Li, W. X. et al., 2005). FISH experiments were also performed on anthers that were fixed in 4% paraformaldehyde in Buffer A and lightly squashed onto poly-L-Lysine coated slides. Wild-type and *ask1*-meiocytes were staged as described above. The paraformaldehyde-fixed cells were treated with proteinase K (20 μg/ml) for 30 minutes at 37°C and washed three times with 1× PBS and distilled H_2O. After drying, the cells were subjected to hybridization. The BAC clone F15B18, which contains rDNA repeats, was used as the template for PCR amplification of rDNA sequences. The primers CCTCCGGCGCTGTTACTTTG and GTTTCAGCCT-TGCGACCATACTC were used to generate a primary PCR product that was subsequently subjected to random primer labeling in the presence of Biotin-High Prime-labeled dUTP (Roche, Indianapolis, IN 46250). The biotin-labeled probe was used in hybridization solution at 5 μg/ml and detected with 10 μg/ml fluorescein-labeled streptavidin. Telomere sequences were detected by hybridization with the FITC-labeled oligonucleotide probe, FITC-$(CCCTAAA)_6$ at 5 μg/ml, which was previously described (Armstrong et al., 2001). Slides were counterstained with PI, mounted and viewed as above.

Transmission electron microscopy

Fixation and infiltration procedures used for TEM were according to Owen and Makaroff (Owen and Makaroff, 1995) with minor modifications. Whole inflorescences were submerged in fixative (2.8% glutaraldehyde in 0.1 M HEPES buffer, pH 7.2 and 0.02% (v/v) Triton X-100) for 2 hours at room temperature and then transferred to fresh fixative for a day at 4°C. Samples were rinsed three times for 15 minutes in the same buffer and post-fixed in 1% OsO_4 in HEPES buffer for a day at 4°C. The samples were rinsed, dehydrated through a graded acetone series with 10% increments for 15 minutes each and infiltrated in Spurr's resin. All buds were dissected from individual inflorescences and numbered according to their size before embedding. Meiotic stages were assigned based on the developmental stage of the surrounding anther cells. Samples were initially analyzed with a light microscope by examining thick sections of buds that were stained with 0.1% toluidine blue in 1% sodium tetraborate. Ultrathin sections were then generated with a diamond knife on a Reichert Jung Ultracut microtome, placed on copper-mesh grids, stained with 2% uranyl acetate in 50% ethanol for 16 minutes and with Reynold's lead citrate for 12 minutes, and examined with a JEOL 1200 EXII transmission electron microscope (JEOL Ltd, Tokyo, Japan) at 80 kV. Images were captured by a CCD-camera (Tietz Video and Image Processing Systems, Gauting, Germany).

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