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A novel plant gene essential for meiosis is related to the human *CtIP* and the yeast *COM1/SAE2* gene

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Obligatory homologous recombination (HR) is required for chiasma formation and chromosome segregation in meiosis I. Meiotic HR is initiated by DNA double-strand breaks (DSBs), generated by Spo11, a homologue of the archaebacterial topoisomerase subunit Top6A. In *Saccharomyces cerevisiae*, Rad50, Mre11 and Com1/Sae2 are essential to process an intermediate of the cleavage reaction consisting of Spo11 covalently linked to the 5′ termini of DNA. While Rad50 and Mre11 also confer genome stability to vegetative cells and are well conserved in evolution, Com1/Sae2 was believed to be fungal-specific. Here, we identify *COM1/SAE2* homologues in all eukaryotic kingdoms. *Arabidopsis thaliana Com1/Sae2* mutants are sterile, accumulate AtSPO11-1 during meiotic prophase and fail to form AtRAd51 foci despite the presence of unrepaired DSBs. Furthermore, DNA fragmentation in *AtCom1* is suppressed by eliminating *AtSPO11-1*. In addition, *AtCOM1* is specifically required for mitomycin C resistance. Interestingly, we identified CtIP, an essential protein interacting with the DNA repair machinery, as the mammalian homologue of Com1/Sae2, with important implications for the molecular role of CtIP.

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Introduction

The process of meiosis includes two successive nuclear divisions that follow a single round of DNA replication. During the reductional division, called meiosis I, pairs of replicated homologous chromosomes segregate, while during meiosis II, sister chromatids are separated in an equational-like division. During prophase of meiosis I, reciprocal recombination between homologous chromosomes occurs before they are segregated at meiosis I. The process of homologous recombination (HR) contributes to the genetic diversity of the meiotic products and is a prerequisite for the correct segregation of homologous chromosomes (Zickler and Kleckner, 1999; Page and Hawley, 2003).

Meiotic recombination is initiated by DNA double-strand breaks (DSBs), generated by Spo11, a homologue of the archaebacterial topoisomerase subunit Top6A (Bergerat *et al*, 1997; Keeney *et al*, 1997). *Arabidopsis thaliana* has three *SPO11*-like genes, while all other analysed species possess only one such gene (Hartung and Puchta, 2000, 2001). *Arabidopsis Atspo11-1* (Grelon *et al*, 2001) and *Atspo11-2* mutants (Stacey *et al*, 2006) exhibit defects in pairing and recombination during meiosis, while *Atspo11-3* is not involved in meiosis (Yin *et al*, 2002). In budding yeast, the help of at least nine proteins (Smith and Nicolas, 1998; Keeney, 2001; Prieler *et al*, 2005) is required for Spo11-mediated catalytic cleavage. Further processing of DSBs requires some of the same and several additional proteins, among them Com1/Sae2 (Keeney and Kleckner, 1995; McKee and Kleckner, 1997; Prinz *et al*, 1997; Cartagena-Lirola *et al*, 2006). Spo11 is found covalently linked to the 5′ termini of DNA in an intermediate of the DNA cleavage reaction. The release of Spo11 from DNA is mediated by ssDNA nick formation next to the DSB site, thereby liberating the Spo11 protein attached to a few nucleotides. In budding yeast, this release requires Rad50, Mre11 and Com1/Sae2. Mutations in these genes cause accumulation of Spo11 on DNA ends (Keeney and Kleckner, 1995; Neale *et al*, 2005; Prieler *et al*, 2005). Following DNA cleavage and Spo11 removal, a 5′-3′ exonucleolytic activity, mediated by a nuclease not yet identified, generates 3′ single-stranded DNA tails. These tails are believed to serve as probes for identifying homologous partner chromosomes, and to initiate D-loop formation and single-end invasion followed by second-end capture (Paques and Haber, 1999; Hunter and Kleckner, 2001; Neale and Keeney, 2006).

Homologues of *RAD50*, *MRE11* and their complex partner *XRS2/NBS1* have been identified in higher eukaryotes, while homologues of *Com1/Sae2* have remained elusive. Yeast two-hybrid assays have demonstrated that the *Arabidopsis* proteins AtRAD50 and AtMRE11 physically interact and that the rice OsMRE11 protein interacts with OsNBS1 (Bhatt *et al*, 2001; Daoudal-Cotterell *et al*, 2002; Akutsu *et al*, 2007). A mutation in *AtRAD50* leads to meiotic defects, sterility and sensitivity to methyl-methano-sulphonate (Gallego *et al*, 2001; Bleuyard *et al*, 2004). In vertebrates, *MRE11* is an essential gene with roles in both somatic and meiotic cells (D'Amours and Jackson, 2002), while in *A. thaliana*, *MRE11* is not essential. Depending on the *MRE11* mutant allele, plants are either sterile due to perturbed meiosis and exhibit

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severe developmental abnormalities or they only display enhanced sensitivity to genotoxic agents (Bundock and Hooykaas, 2002; Puizina *et al*, 2004). In contrast to yeast, MRE11 and RAD50 are not essential for the formation of meiotic DSBs in *A. thaliana*, but only in the ensuing processing and repair (Bleuyard *et al*, 2004; Puizina *et al*, 2004).

Yeast's *COM1/SAE2* was detected independently in three genetic screens. Two screens were designed to isolate meiotic mutants, defective after the initiation of Spo11-induced DSBs, but before resolution of recombination intermediates (McKee and Kleckner, 1997; Prinz *et al*, 1997). The phenotypes of the isolated *com1/sae2*-null mutations are similar to those conferred by the previously identified non-null mutations of *RAD50* (*rad50S*) and *MRE11* (*mre11S*) (Alani *et al*, 1990; Keeney *et al*, 1997; Nairz and Klein, 1997; Tsubouchi and Ogawa, 1998). These mutations completely block resection and turnover of meiosis-specific DSBs. The third screen aimed to identify mutants with low fidelity DSB repair during vegetative growth (Rattray *et al*, 2001). In mitotic cells, Com1/Sae2 (together with proteins of the Mre11 complex) is essential for the repair of hairpin-capped DSBs, for holding DNA ends in close proximity after DSB formation and for preventing chromosome rearrangements (Lobachev *et al*, 2002; Clerici *et al*, 2005). This explains how Com1/Sae2 could confer resistance to genotoxic treatments (Birrell *et al*, 2002; Deng *et al*, 2005). Furthermore, detailed analysis has shown that Com1/Sae2 is phosphorylated by the protein kinases Mec1 and Tel1 (yeast homologues of ATR and ATM, respectively) and that it interferes with the DNA replication and damage checkpoints during mitosis and meiosis (Baroni *et al*, 2004; Cartagena-Lirola *et al*, 2006; Clerici *et al*, 2006). Com1/Sae2 is among the first proteins to be found at somatic DSB sites, after Mre11 and Tel1, but before Rad52. Com1/Sae2 and Mre11 do not rely on each other with respect to their initial localisation to DSBs, but mutually depend on each other for their timely disappearance from DSBs (Lisby *et al*, 2004; Clerici *et al*, 2006).

The analysis of the *A. thaliana AtCOM1* gene represents the first characterisation of *COM1/SAE2* in a higher eukaryote. Our data demonstrate that a *COM1/SAE2* homologue exists in various higher eukaryotes and that it is essential for female and male meiosis in *A. thaliana*. We show that *AtCOM1* acts downstream of *AtSPO11-1* and upstream of *AtDMC1* during meiosis. We provide evidence that AtCOM1 is needed for regular turnover of AtSPO11-1 and processing of meiotic DSBs. Furthermore, we show that AtCOM1 is essential for a specific DNA repair process in somatic cells. Importantly, our findings correlate yeast's Com1/Sae2 protein with the mammalian DNA repair-related protein CtIP, whose significance in meiosis has not yet been elucidated.

Results

Identification of COM1/SAE2 homologues in higher eukaryotes

To identify homologues of the yeast *COM1/SAE2* gene, we first collected a set of fungal Com1/Sae2 homologues by applying PSI-BLAST and reciprocal proteome BLAST searches (with low-complexity filtering, E-value cut-off 0.001) (Altschul *et al*, 1997) starting with the full-length *Saccharomyces cerevisiae* Com1/Sae2 protein sequence. Alignment of 20 different fungal Com1/Sae2 homologues revealed conservation of its C-terminal half (conservation plot (Rice *et al*, 2000)), corresponding to amino acids 190–345 of the yeast Com1/Sae2 protein. Consequently, reciprocal PSI-BLAST searches (against the NCBI non-redundant database (nr), version 12/2006; low-complexity filtered, inclusion cut-off 0.001) (Altschul *et al*, 1997; Marchler-Bauer *et al*, 2002) were carried out with the conserved region of all fungal relatives (Supplementary Figure S1A). This procedure yielded a coherent set of related sequences in a wide variety of eukaryotes including the *Arabidopsis* NP_850683 (At3g52115) (Supplementary Figure S1B) and the metazoan CtIP protein (*Homo sapiens* CtIP, Rbbp8; Fusco *et al*, 1998), shown in Figure 1A aligned by their common C-terminal homology. The *Arabidopsis* and the human proteins share 54% identity and 45% similarity in their conserved C-termini and their close relation are further illustrated by cluster analysis (Figure 1B; Frickey and Lupas, 2004). *Arabidopsis* At3g52115 was picked up in a screen as being upregulated after ionising irradiation and was called *AtGR1* (*A. thaliana gamma response gene 1*) (Deveaux *et al*, 2000). Garcia *et al* (2003) demonstrated that ATM kinase is required for transcriptional induction of *AtGR1*. However, neither *AtGR1's* relation to *COM1/SAE2*, nor its importance for meiosis was noticed. We suggest calling the gene *AtGR1/COM1* and hereafter refer to it as *AtCOM1*. The CtIP protein was also not recognised as a homologue of *COM1/SAE2* despite a more detailed characterisation (see Discussion).

To underline the functional significance of the conserved *COM1/SAE2* C-terminus, yeast Δ*com1* strains (Prinz *et al*, 1997) where transformed with plasmids either carrying wild-type or mutagenised versions of the *COM1/SAE2* gene, with single, double or triple amino-acid exchanges of the most conserved residues, respectively. Whereas the cells which obtained a wild-type *COM1/SAE2* gene showed 100% spore viability (as opposed to the untransformed Δ*com1* strain which has 0.06% spore viability), the cells which obtained a mutagenised version showed strongly reduced spore viability: (1) R262,264A: 3.4%; (2) S267A: 10.4%; (3) P268A: 0.28%; (4) GF270AA: 0.03%; (5) FPS276AAA: 0.7% (Figure 1A).

Molecular characterisation of Atcom1

To study the function of the AtCOM1 protein in plants, we obtained two T-DNA insertion mutant lines from the Salk Institute Genomic Analysis Laboratory T-DNA collection (Alonso *et al*, 2003) harbouring alleles *Atcom1-1* and *Atcom1-2*, respectively. We confirmed the position of the *Atcom1-1* and *Atcom1-2* T-DNA insertions within the 2nd exon of the *AtCOM1* gene by PCR, sequenced the insertion borders and determined the structure of *Atcom1-1* by Southern analysis (Supplementary Figure S2A–C). Expression analysis by reverse transcription revealed the presence of *AtCOM1* transcripts in the all wild-type tissues examined (buds, leaves, seedlings), and a strong induction in irradiated tissues. Only a truncated mRNA, corresponding to the region upstream of the T-DNA insertion sites, was detected, in the mutant plants (Supplementary Figure S2D). As both the *Atcom1-1* and the *Atcom1-2* coding regions are interrupted by a stop codon (Supplementary Figure S2B), the mutant proteins are predicted to consist of the N-terminal 456 and 479 amino acids, respectively, both lacking the conserved C-terminus (the full-length protein has a predicted length of 588 amino acids).

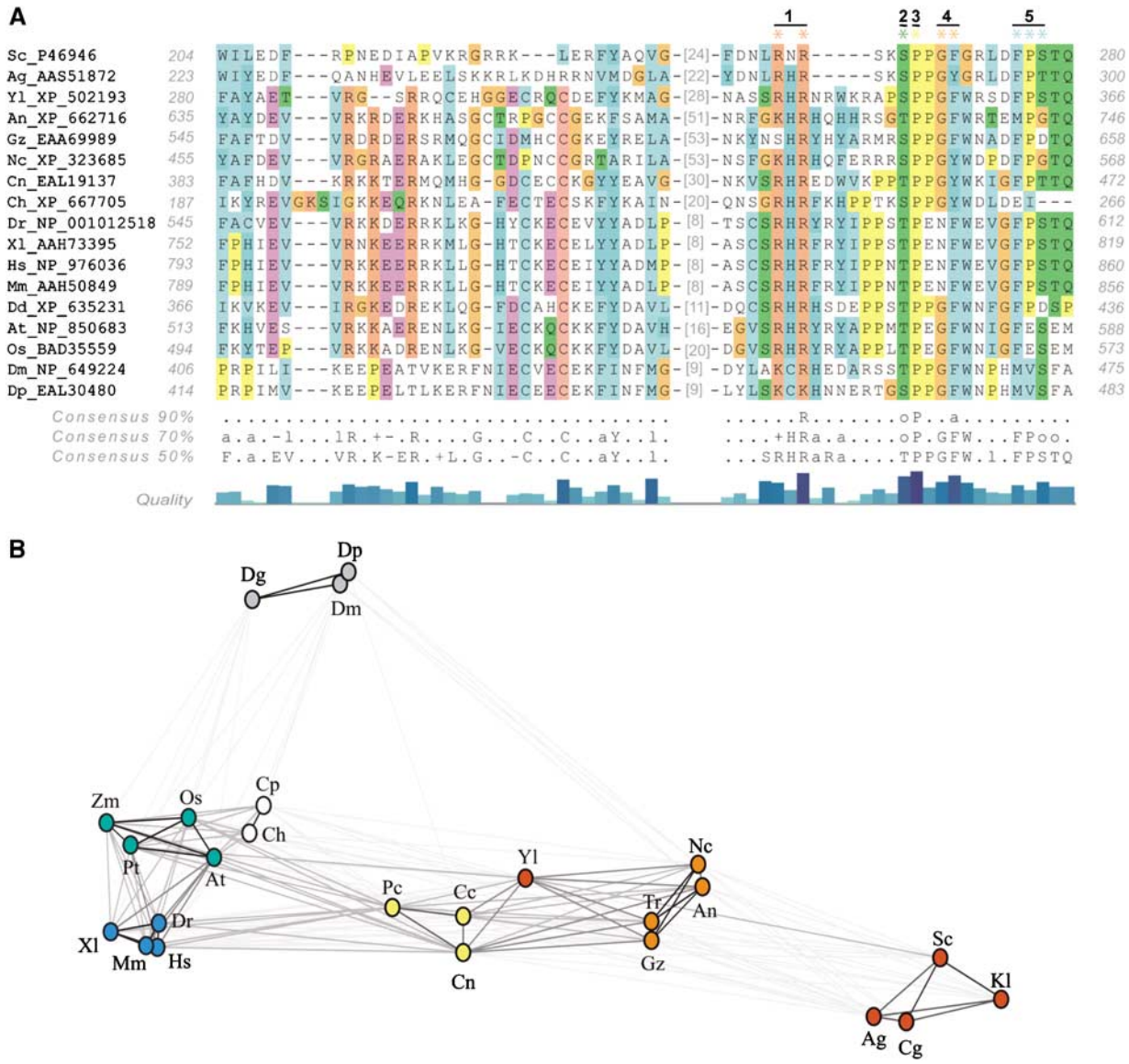

Figure 1 The Com1/Sae2 motif is conserved among eukaryotes. (**A**) Alignment of putative homologues of the *S. cerevisiae* Com1/Sae2 protein. The alignment (muscle (Edgar, 2004); clustal-like colouring) shows the C-terminal protein region of best conservation. Residue conservation above selected thresholds (50, 70, 90%) is indicated below the alignment. Relative frequency was scored for individual amino acids as well as for groups of related amino acids: alcoholic (denoted by 'o' and including the amino acids S, T), aliphatic ('l' for I, L, V), aromatic ('a' for F, H, W, Y), positive ('+' for H, K, R), negative ('−' for D, E). Numbers and asterisks above the alignment refer to amino-acid exchanges introduced into the *S. cerevisiae* protein sequence. (**B**) Cluster analysis of *COM1/SAE2* homologues. The analysis was performed with CLANS (Frickey and Lupas, 2004). Individual proteins are shown as vertices and are connected by lines reflecting pairwise sequence similarities from all-against-all BLAST searches. Attractive forces increasing with the sequence similarity between proteins lead to clustering of sequence related proteins. The strength of line shading increases with increasing sequence similarity. Abbreviations: (Ag) *Ashbya gossypii*, (An) *Aspergillus nidulans*, (At) *Arabidopsis thaliana*, (Cc) *Coprinopsis cinerea*, (Cg) *Candida glabrata*, (Ch) *Cryptosporidium hominis*, (Cn) *Cryptococcus neoformans*, (Cp) *Cryptosporidium parvum*, (Dd) *Dictyostelium discoideum*, (Dg) *Drosophila grimshawi*, (Dm) *Drosophila melanogaster*, (Dp) *Drosophila pseudoobscura*, (Dr) *Danio rerio*, (Gz) *Gibberella zeae*, (Hs) *Homo sapiens*, (Kl) *Kluyveromyces lactis*, (Mm) *Mus musculus*, (Nc) *Neurospora crassa*, (Os) *Oryza sativa*, (Pc) *Phanerochaete chrysosporium*, (Pt) *Populus trichocarpa*, (Sc) *Saccharomyces cerevisiae*, (Tc) *Trichoderma reesei*, (Xl) *Xenopus laevis*, (Yl) *Yarrowia lipolytica*, (Zm) *Zea mays*.

Atcom1 mutant plants are sterile due to chromosome fragmentation during meiosis

Plants homozygous for the *Atcom1* mutant alleles (*Atcom1-1*, *Atcom1-2*) do not show growth aberrations during vegetative development. The mutants germinate and develop at the same time and rate as wild-type plants. Rosette leaves are normal in size, shape and number, and bolting is not delayed. Inflorescences look normal, but none of the siliques of *Atcom1* mutant plants elongate, whereas shoots of wild-type plants have long siliques. The short siliques of *Atcom1-1* and *Atcom1-2* mutant plants are completely devoid of seeds (Figure 2A). This and all the other observed phenotypes were completely reversed by introduction of a genomic wild-type copy of the gene (Figure 2A). To further characterise the fertility defect, male and female gametophyte development was monitored. *Atcom1* mutant plants develop anthers that do not contain viable pollen (Figure 2A) and only aberrant embryo sacs (Supplementary Figure S3A), implying that the sterility phenotype of *Atcom1* mutant plants is based on gametogenesis defects. This conclusion is sup-

ported by the fact that *Atcom1-1* plants ($n = 8$ buds) could not be fertilised with wild-type pollen. Prior to gametogenesis, haploid cells have to be formed during the process of meiosis. A key step during meiosis is synapsis and recombination between homologous chromosomes. Cytological analysis of male meiosis fails to detect synapsed chromosomes in *Atcom1-1* plants during stages corresponding to zygotene and pachytene (Figure 3B, C, M, N). To identify homologous chromosomes, chromosome I (at an interstitial region) and chromosome II (at a subtelomeric region) were simultaneously labelled by fluorescent *in situ* hybridisation (FISH). Indeed, no close pairing of either homologous signal pair was observed in the mutant (Figure 3M, N), but close pairing was frequently seen in wild-type zygotene stages and always observed in wild-type pachytene stages (Figure 3B, C). This implies that no stable interactions between homologous chromosomes can be formed in homozygous *Atcom1-1*. While five well-defined bivalents emerge from condensation during diakinesis (Figure 3E) and metaphase I in wild type (Figure 3F), chromosomes are always connected and entangled in the *Atcom1-1* mutant during stages corresponding to diakinesis and metaphase I (Figure 3O, P). In anaphase I DNA fragments, typically telomeric fragments are left behind at the metaphase plate in *Atcom1-1* plants (Figure 3Q–S), indicating that meiotic DSBs remain unrepaired in the mutant. Figure 3R also shows a DNA bridge connecting two centromeres, which segregate to different poles. FISH analysis with a probe directed to the 180 bp repeat region of centromeres shows that some of the bridges observed in *Atcom1-1* mutants consist of centromeric DNA, indicating that bridges could either originate from separation of sister chromatids, persisting DNA catenation or from a nonhomologous repair mechanism (data not shown). DNA fragments and bridges are never observed in wild-type cells during anaphase or telophase I (Figure 3G, H). In addition to fragmentation, massive chromosome missegregation is revealed by the often asymmetric distribution of nuclear material in the first meiotic division in *Atcom1-1* cells (Figure 3). These are expected consequences of the earlier homologue pairing and DSB repair defects. Anaphase II figures were also observed, and all showed a high incidence of fragmentation and missegregation, usually culminating in the formation of more than four, poorly condensed, unequal masses of chromatin at telophase II (Figure 3U). Equivalent defects were observed during female meiosis in *Atcom1-1* mutants (Supplementary Figure S3B). We conclude that the sterility of *Atcom1-1* mutants is caused by aberrant meiosis and defective repair of meiotic DSBs.

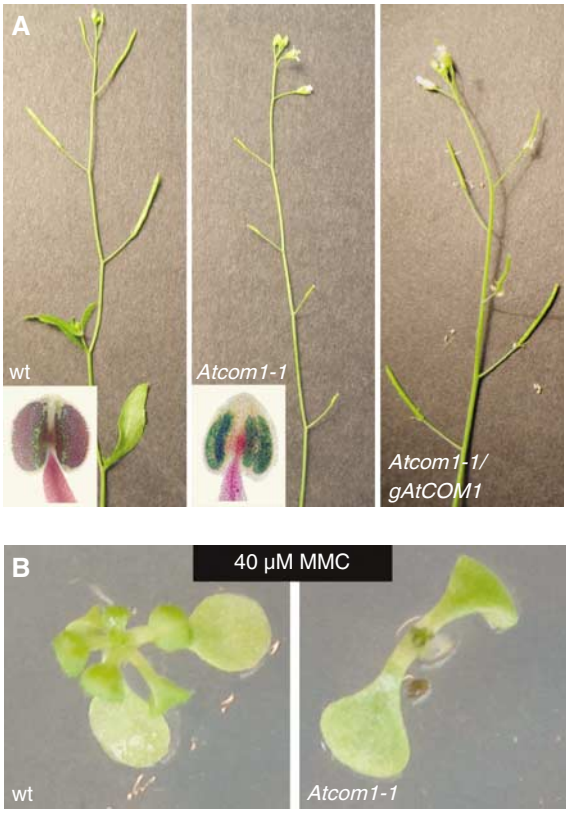

Figure 2 Phenotypes of the *Atcom1-1* mutant. (**A**) *Atcom1-1* mutants look like wild-type plants, but do not develop mature siliques. The left panel shows a stem with mature siliques of a wild-type (wt) plant. The middle panel shows the stem of an *Atcom1-1* mutant plant of the same age, which failed to develop mature siliques. The right panel shows an *Atcom1-1* mutant plant containing a fertility-restoring genomic copy of *AtCOM1*. Inlays: anthers of wild-type (wt) and *Atcom1-1* plants stained as described (Alexander, 1969). The purple-stained cytoplasm indicates viable pollen grains; green indicates empty pollen. No regular sized and viable purple pollen is present in *Atcom1* anthers. (**B**) Seedlings grown on media plates containing 40 μM MMC. Wild-type (wt) plants develop true leaves. In contrast, *Atcom1-1* mutant plants only expand their cotyledons but do not develop true leaves.

Chromosome fragmentation observed in Atcom1-1 mutants depends on SPO11-1 but not on the RecA-related DMC1 protein

If fragmentation and chromosome bridges in *Atcom1-1* meiosis were solely caused by the inability to process meiotic DSBs, reduction of DSB formation by mutating the nuclease, *AtSPO11-1* (Grelon *et al*, 2001), should strongly curb such aberrations (the *Atspo11-1-1* mutation does not completely eliminate DSBs, either due to residual activity or due to its paralog *AtSPO11-2* (Stacey *et al*, 2006)). Indeed, when we generated the homozygous *Atcom1-1 Atspo11-1-1* double mutant, DNA fragmentation was undetectable in most cells (Figure 4), showing that the inability to repair meiotic DSBs is responsible for *Atcom1-1* chromosome aberrations.

Cells repair meiotic DSBs by using the homologue rather than the sister chromatid as a template, a phenomenon called interhomologue bias (Zickler and Kleckner, 1999). We, therefore, asked whether relaxing interhomologue bias might permit repair in *Atcom1-1* meiocytes. DMC1, a meiosis-specific RecA recombinase, is specifically required for interhomologue interactions (Schwacha and Kleckner, 1997) and *Atdmc1* mutants are thought to repair all DSBs by using the sister chromatid as a template (Couteau *et al*, 1999). However, chromosome fragmentation persists in the *Atcom1 Atdmc1* double mutant meiosis (Figure 4), suggesting that *Atcom1* affects both intersister and interhomologue recombination alike. This result is expected, if AtCOM1 is not specific to the interhomologue repair pathway, but obligatory for meiotic repair as Com1/Sae2 is in yeast.

AtCOM1 is essential for regular turnover of AtSPO11-1 and normal processing of DSBs

A very early step in meiotic DSB repair after removal of Spo11 and formation of 3′ single-stranded ends is coating

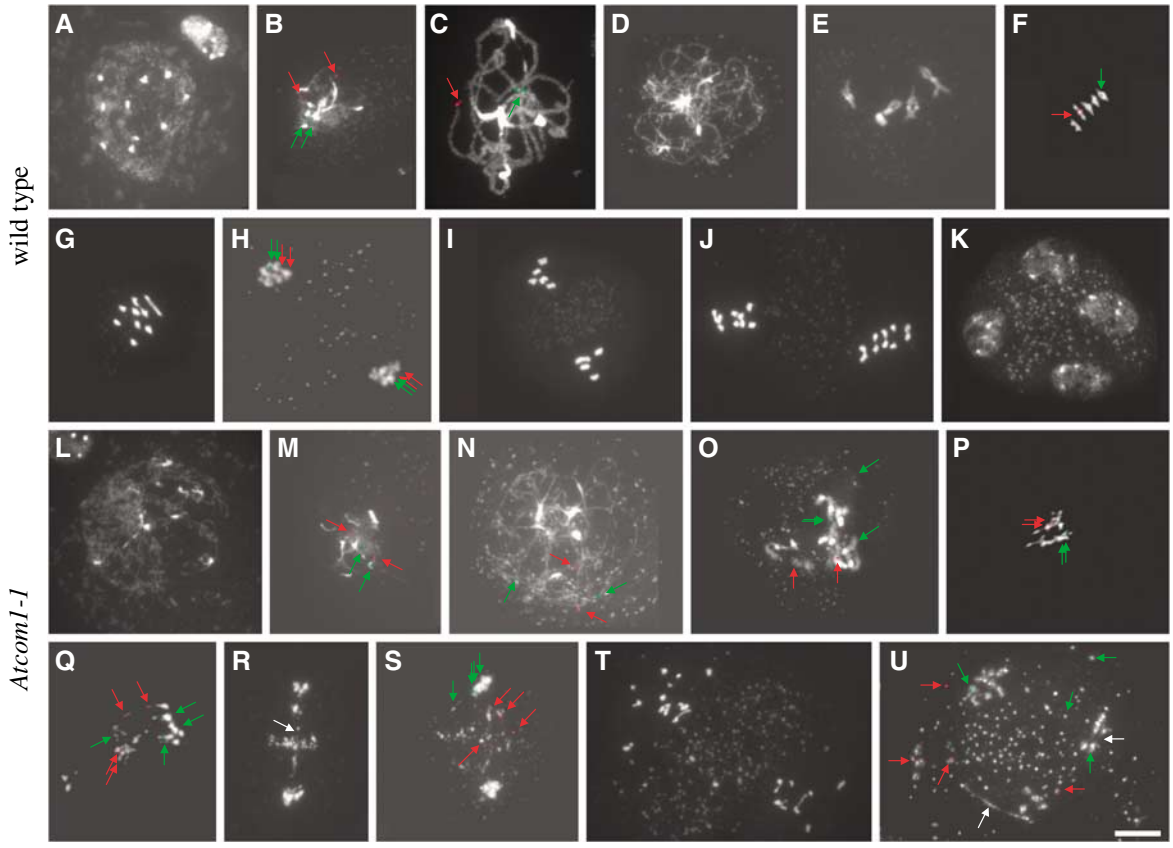

Figure 3 Meiosis in pollen mother cells of wild-type (**A–K**) and *Atcom1-1* mutant (**L–U**) plants. Wild-type: (A) leptotene; (B) zygotene; (C) pachytene; (D) diplotene; (E) diakinesis; (F) metaphase I; (G) anaphase I; (H) prophase II; (I) metaphase II; (J) anaphase II; (K) telophase II. Meiosis in the severely disrupted *Atcom1-1* mutant plants: (L) leptotene; (M) zygotene-like stage; (N) pachytene-like stage, without normal chromosome pairing; (O) diakinesis-like stage; (P) metaphase I-like stage with entangled chromosomes; (Q, R) progression of anaphase I with fragmentation of chromosomes; (S) prophase II-like stage; (T) anaphase II-like stage; (U) telophase II-like stage. Green and red arrows highlight FISH signals corresponding to an arm region of chromosome I (BAC F1N21, green) and a subtelomeric region of chromosome II (BAC F11L15, red), respectively (panels B, C, H, M–Q, S and U). White arrows highlight chromosome bridges seen during *Atcom1-1* meiosis (R, U). Meiotic progression in pollen mother cells was followed after chromosomes were stained with DAPI. Scale bar: 10 μm.

of the single strand by the recombinase Rad51 for subsequent strand invasion and D-loop formation (Li *et al*, 2004; Shinohara and Shinohara, 2004). Filament formation can be observed cytologically, as the formation of Rad51 foci upon indirect immunofluorescence (IF) staining. While large numbers of Rad51 foci appear transiently in wild-type prophase cells, only very few (comparable to those found in *Atspo11-1-1* and *Atmre11-3*) are seen in *Atcom1-1* cells (Figure 5; Supplementary Figure 4). One interpretation of this result could be that meiotic DSBs are reduced or absent. We therefore performed IF for γH2AX (Sanchez-Moran *et al*, 2007), a phospho form of a histone H2A variant representing a specific, local and fast response to DSBs (Rogakou *et al*, 1998; Fernandez-Capetillo *et al*, 2003; Friesner *et al*, 2005; Supplementary Figure 5B). This control confirmed the presence of high levels of DSBs in *Atcom1-1* cells (and in *Atmre11-3* and *AtRad50* mutant cells), similar to the staining observed in early stages in wild-type meiocytes and consistent with the observation of fragmentation in anaphase I (Figure 3Q–S). Thus, we infer that *Atcom1-1* mutants generate DSBs, but do not form Rad51 filaments efficiently.

A critical early step in repair is the removal of Spo11, which remains covalently linked after it has cleaved a step known to depend on Rad50 (; Alani *et al*, 1990), Mre11 (Nairz and Klein, 1997; Tsubouchi and Ogawa, 1998) and Com1/Sae2 (McKee and Kleckner, 1997; Prinz *et al*, 1997) in *S. cerevisiae*. In such a situation, Spo11 foci were shown to accumulate in mutant nuclei by visualising an epitope-tagged Spo11 (Prieler *et al*, 2005). We tried to address this question in *A. thaliana* using an AtSPO11-1-specific antibody (Sanchez-Moran *et al*, 2007). While AtSPO11-1 was virtually undetectable in meiocytes of wild-type cells (99.7% of prophase I cells show no AtSPO11-1 foci, $n=440$), the same antibody detected AtSPO11-1 in 98% of *Atcom1-1* meiocytes ($n=50$), some of which showed striking AtSPO11-1 hyperaccumulation (30/50 cell showed very intensive staining, 19/50 showed staining with lower intensity) (Figure 5; Supplementary Figure 5A). Interestingly, we find a similar hyperaccumulation of AtSPO11-1 in meiocytes of *Atmre11-3* and *Atrad50* mutants (Supplementary Figure 5A). We summarise that available evidence places the defect of *Atcom1-1* before AtRAD51 filament formation and suggests a problem with AtSPO11-1 removal. Thus, our study suggests that yeast Com1/Sae2 and *Arabidopsis* AtCOM1 are required for equivalent mechanisms during meiotic DSB repair.

AtCOM1 is required for resistance to mitomycin C

Aside from meiosis, the *S. cerevisiae COM1/SAE2* gene is required for genomic stability and for resistance to DNA

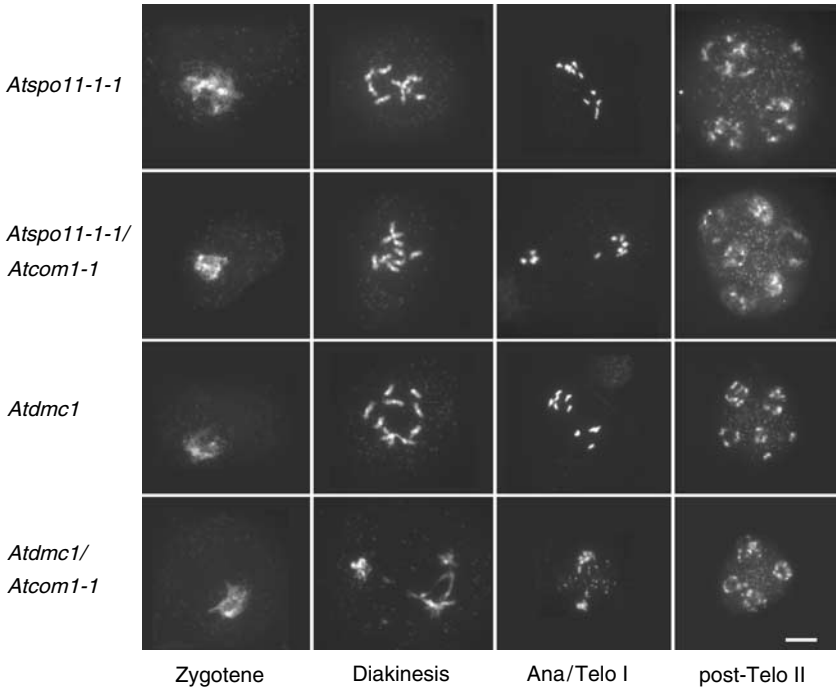

Figure 4 AtCOM1 acts downstream of AtSPO11-1 and upstream of AtDMC1. Comparison of meiotic progression in the *Atspo11-1-1* mutant (upper panel) and in the *Atcom1-1Atspo11-1-1* double mutant (second panel from top). In both, zygotene-like stages are followed by progressive condensation and formation of univalents, which subsequently segregate at random, forming polyads at the end of meiosis II. Comparison of meiotic progression in the *Atdmc1* mutant (third panel from top) and in the *Atcom1-1Atdmc1* double mutant (lowest panel). Whereas *Atdmc1* mutants form univalents, which segregate at random to give rise to polyads at the end of meiosis II, the *Atcom1-1Atdmc1* double mutant resembles *Atcom1-1*, with fragmented chromosomes. Chromosomes are stained with DAPI. Scale bar: 10 μm.

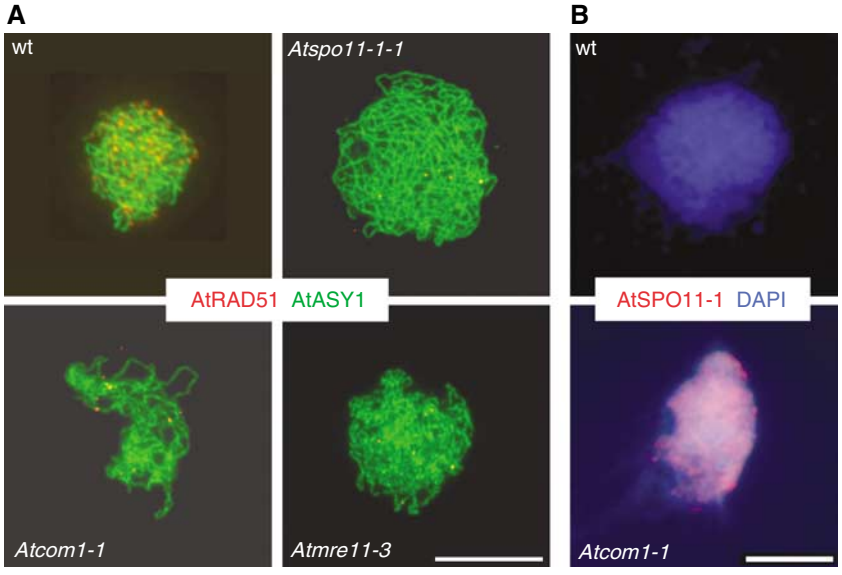

Figure 5 Atcom1-1 cells fail to form AtRAD51 foci, but accumulate AtSPO11-1. (**A**) *Atcom1-1* cells fail to form AtRAD51 foci. Immunofluorescent staining of spreads of meiotic cells with antibodies directed against the axial element protein Asy1 (green) and against AtRad51 (red). Whereas in wild type (wt) numerous AtRAD51 foci are observed in zygotene, representing loci of meiotic DNA repair and recombination, only very few foci are seen in *Atspo11-1-1*, *Atcom1-1* and *Atmre11-3* mutants. Scale bar: 10 μm. (**B**) AtSPO11-1 is enriched in prophase I of *Atcom1-1* mutant meiosis. Immunofluorescent staining of spreads of cells in prophase I stages of wild type (wt) and *Atcom1-1* meiocytes with an antibody directed against AtSPO11-1 (red). DNA was counterstained with DAPI (blue). Scale bar: 10 μm.

cross-linking agents, including mitomycin C (MMC) in vegetative cells (Lobachev *et al*, 2002; Wu *et al*, 2004). Figure 2B shows wild-type and *Atcom1-1* seedlings, which were germinated on media containing 40 μM MMC. In contrast to wild type, true leaves do not develop in the mutant, while expansion of cotyledons, which does not require cell division, is unaffected. The strong MMC sensitivity was 100% penetrant for homozygous *Atcom1-1* mutants, whereas heterozygous or

wild-type plants were resistant. However, no difference in sensitivity to a number of other genotoxic agents was found in a series of experiments described in the Supplementary data. In addition, in yeast, diploid *com1/sae2*Δ mutants were only very weakly sensitive to X-ray or a radiomimetic alkylating agent in our hands (McKee and Kleckner, 1997; Prinz *et al*, 1997). Work in recent years has implicated Com1/Sae2 in the processing of very selective lesions such as hairpin capped DNA ends (data not shown; Rattray *et al*, 2001; Lobachev *et al*, 2002) or interstrand cross-links (Wu *et al*, 2004). The selective sensitivity of vegetative cells of both *Atcom1-1* and yeast *com1/sae2*Δ mutants to MMC is further evidence for the conservation of their roles in evolution and, furthermore, may reflect a specificity of AtCOM1 for the repair of only a subset of DNA lesions.

Discussion

This study describes the first identification and characterisation of a *Com1/Sae2* homologue in a higher eukaryote, the *AtCOM1* gene of *A. thaliana*. This study provides evidence that both female and male meiosis are affected by the *Atcom1* mutation, which thus leads to sterility. *Atcom1* mutants do not form any viable microspores or macrospores, as judged from our analysis of gametogenesis. Furthermore, *Atcom1* mutants do not form seeds, even when pollinated with wild-type pollen. Analysis of DAPI-stained chromosomes reveals that during progression of both male and female meiosis, severe chromosome fragmentation occurs. Chromosome pairing, visualised by DAPI and FISH analysis, never occurred in *Atcom1* mutants. Chromosome fragmentation and failure of pairing indicate that meiotic DSBs are produced, but downstream events such as DNA end processing or heteroduplex formation are impaired in *Atcom1* mutants. Epistasis analysis clearly shows that the DNA fragmentation observed in *Atcom1* mutant plants depends on the activity of the AtSPO11-1 protein. This result places Atcom1 downstream of Atspo11-1. In contrast, *Atcom1-1 Atdmc1* double mutants show severe chromosome fragmentation and, therefore, resemble the *Atcom1-1* mutant rather than the *Atdmc1* mutant. This result implies that meiotic DSBs are formed in *Atdmc1* mutants, and that their repair depends on AtCOM1. This result places AtCOM1 upstream of the RecA-related DMC1 protein.

The mechanistic role of Com1/Sae2 is still unknown. Nevertheless, it has been established that Com1/Sae2 is needed for the removal of Spo11 from DNA during meiosis in yeast. As an intermediate of the DNA cleavage reaction, Spo11 is covalently bound to the 5′ end phosphate of the DNA at the DSB site. Removal of the Spo11 protein occurs by nicking the DNA next to the cleavage site, thereby releasing the catalytic protein attached to a few nucleotides (Neale *et al*, 2005). If Com1/Sae2 is not present, this reaction does not occur and subsequent steps in meiotic progression are blocked. The present study reveals that the plant protein AtCOM1 is involved at the same stage during meiosis. Moreover, evidence is presented that *Atcom1-1* mutant plants accumulate the AtSpo11-1 protein during prophase I of meiosis. Furthermore, the strand invasion mediator AtRAD51, which forms numerous foci on meiotic chromosomes and is visible from leptotene to pachytene in wild-type meiocytes (Li *et al*, 2004), cannot be detected in *Atcom-1-1* mutants. These results imply that processing of DSBs is impaired in mutant plants because of AtSPO11-1 not being removed from DNA ends at DSB sites, thereby blocking processing of DNA ends. Future experiments are needed to resolve the question as to whether the second, meiotically active *Arabidopsis* SPO11 protein, AtSPO11-2, behaves similarly to AtSPO11-1 in an *Atcom1* mutant background.

Somatic function of the AtCOM1 protein

Expression data for the *AtCOM1* gene, presented by Deveaux *et al* (2000) and confirmed by this study, showed strong accumulation of the *AtCOM1* transcript upon exposure to IR in somatic tissues. Furthermore, data presented by Garcia *et al* (2003) show that transcription of *AtCOM1* is strictly dependent on ATM, a conserved protein kinase known to mediate signalling of DNA lesions (Shiloh, 1998). Thus, to unravel a potential somatic function of the AtCOM1 protein, the *Atcom1-1* mutant was tested for resistance to various genotoxic treatments. Under the conditions tested, only the presence of MMC led to strong inhibition of mutant seedling growth. In contrast, all other treatments including IR, did not affect the growth of homozygous *Atcom1-1* mutants compared to heterozygous or wild-type plants. This may reflect a specificity of AtCOM1's involvement in the repair of a subset of DNA lesions. It is conceivable that, upon DNA damage, ATM-mediated signalling leads to broad transcriptional induction of DNA repair-related genes, and that it depends on the actual type of DNA damage, which of the corresponding gene products is effectively needed for repair. In somatic tissues, AtCOM1 seems to be needed for the repair of interstrand DNA cross-links, the dominant effect of MMC, but not for other types of DNA damage, which are predominantly formed by IR, HU, cisplatin, bleomycin or aphidicolin. The selective sensitivity of vegetative cells of both *Atcom1-1* and yeast *com1/sae2*Δ mutants to DNA cross-linking agents provides further evidence for the conservation of their roles in evolution.

COM1/SAE2 homologues are present in higher eukaryotes

As demonstrated in Figure 1B, *AtCOM1* is much more closely related to the vertebrate *COM1/SAE2* homologues than to the fungal branch. The mammalian relative of AtCOM1 and, thus, also of yeast Com1/Sae2, is CtIP (Fusco *et al*, 1998; Schaeper *et al*, 1998), which has been strongly implicated in DNA repair. Initially, CtIP was identified as a protein that binds to Rb, BRCA1 and CtBP1 (Fusco *et al*, 1998; Schaeper *et al*, 1998; Meloni *et al*, 1999; Yu and Baer, 2000). CtIP's interaction with the transcriptional regulators CtBP, Rb and LMO4 involves it in the regulaton of cell-cycle and DNA repair-related proteins (Yu *et al*, 1998; Sum *et al*, 2002; Liu and Lee, 2006). CtIP is most highly expressed in thymus and testis (Wong *et al*, 1998), where DNA DSBs are naturally generated. Yu *et al* (2006) show that CtIP is ubiquitinated in a BRCA1/BARD1-dependent manner following DNA damage and that this modification leads to its association with chromatin, not to its degradation. Upon DNA damage, CtIP associates with BRCA1/BARD1, Rad50, Mre11 and NBS1 (Greenberg *et al*, 2006). Furthermore, CtIP's interaction with BRCA1 seems to be essential for the activation of a DNA damage checkpoint in G2 (Yu and Chen, 2004; Greenberg *et al*, 2006), possibly mediated by CtIP's phosphorylation by ATM, a process that depends on BRCA1 (Foray *et al*, 2003). Homozygous *Ctip*$^{-/-}$-knockout mice die during

embryonic stage 4 and heterozygous *Ctip*$^{+/-}$ mice are prone to developing tumours (Chen *et al*, 2005). While all previous data are consistent with a role of CtIP in DSB repair similar to Com1/Sae2, these observations have not provided a clue regarding the molecular role of CtIP. Both CtIP and Com1/Sae2 are phosphorylated by ATM/Tel1 kinases in response to DNA damage (Foray *et al*, 2003; Baroni *et al*, 2004), cooperate with Mre11 and Rad50 (Lobachev *et al*, 2002; Lisby *et al*, 2004; Greenberg *et al*, 2006) and play a role in DNA damage checkpoint signalling (Yu and Chen, 2004; Clerici *et al*, 2006). CtIP, however, also interacts physically with BRCA1, Rb and CtBP1, which are not part of the yeast DNA repair repertoire. Thus, CtIP and possibly AtCOM1 may have acquired accessory functions in addition to those conserved with yeast Com1/Sae2. So far, a detailed analysis of CtIP function has been complicated by the embryonic lethality caused by homozygous CtIP deficiency in mice. However, heterozygous CtIP leads to increased incidence of cancer (Chen *et al*, 2005). Our findings lead to the prediction that CtIP is required for meiotic DSB repair and for resistance to certain genotoxic agents (e.g. MMC). CtIP may function by modulating the nuclease activity of Mre11 and may be an important factor in processing hairpin capped DNA ends and DNA ends blocked by covalently bound proteins (such as in topoisomerase II in the presence of topo II poisons). Interestingly, deletion of *RAD50* is embryonic-lethal (Luo *et al*, 1999) and a hypomorphic mutation of *MRE11* severely compromises embryo viability (Theunissen *et al*, 2003) in mammals, just as deletion of *CtIP* (Chen *et al*, 2005), but all three genes are not essential in yeast and in plants. This is consistent with evolutionary conservation of a potential regulatory role of Com1/Sae2 for the Mre11 complex. The function of yeast Com1/Sae2 may overlap with that of flap endonuclease Fen1, because they display synthetic lethality, a relation that might also be conserved between mammalian Fen1 and CtIP. Mammalian Fen1 suppresses tumour progression (Kucherlapati *et al*, 2002). The phenotype of *Atcom1-1* provides information which links yeast Com1/Sae2 and mammalian CtIP. Based on the detailed knowledge on Com1/Sae2, we suggest CtIP to be required for genome stability as a tumour suppressor, for fertility through meiotic DNA repair and possibly for V(D)J recombination, because in yeast Com1/Sae2 is instrumental together with Mre11 to process hairpins (Rattray *et al*, 2001; Lobachev *et al*, 2002 our own observations, manuscript in preparation). Com1/Sae2 and CtIP have been studied separately, but the knowledge of their relationship should strongly stimulate both hitherto separated fields.

Materials and methods

Plant growth conditions and plant transformation

All plants were germinated on soil after 2 days of stratification in water at 4°C. Plants were grown at 22°C with a 16:8 h light: dark photoperiod. Seeds of the SALK_061706 (*Atcom1-1*) and SALK_122386 (*Atcom1-2*) lines were obtained from the Nottingham Arabidopsis Stock Center (Nottingham, UK). Mutants and double mutants were identified by PCR (see Supplementary data). Transformation of plants heterozygous for the *Atcom1-1* mutation with pBIB-Hyg/gAtCOM1, utilising *Agrobacterium tumefaciens* strain GV3101, was performed as described previously (Bechtold *et al*, 1993).

Vector construction

For construction of the complementing pBIB-Hyg/gAtCOM1, a 4811 bp genomic DNA fragment derived from BAC clone ATF4F15 (ABRC, Columbus, Ohio) by digestion with *Bsm*I and subsequent T4 polymerase treatment, was cloned into the *Sma*I restrictions site of the plant binary vector pBIB-Hyg (Becker, 1990) and subsequently used for transformation.

Analysis of meiotic chromosomes

DAPI staining of male meiotic chromosomes was performed as described previously (Ross *et al*, 1996). Female meiotic chromosomes were observed as described previously (Motamayor *et al*, 2000), except that pictures were taken with a Zeiss Axioplan 2 microscope, including an LSM 510 Laser module. Data processing was performed with Zeiss LSM 510 Meta and Helicon Focus software. FISH and fluorescent immunolocalisation analysis of ASY1 and AtRAD51 was performed as described previously (Kerzendorfer *et al*, 2006), except that both the anti-RAD51 antibody (rat) and the anti-ASY1 antibody (rabbit) were used in 1:500 dilutions. The polyclonal rabbit antibody against AtSPO11-1 (a gift from F.C.H Franklin) was diluted 1:100 and a polyclonal rabbit anti-phospho-H2AX (Ser 139) (Upstate) antibody was diluted 1:100. Immunolocalisation analysis of AtSPO11-1 and γH2AX antibodies was carried out on squashed prophase I meiocytes from *Atcom1-1* mutant plants as described previously (Page *et al*, 1998; Sanchez-Moran *et al*, 2007).

Yeast spore viability assay

Yeast strain SK1 (with the following relevant genotype: *MATa/alpha*, *ura3/ura3*, *com1::KanMX4/com1::KanMX4*), was transformed with plasmid pRS316, including the *COM1/SAE2* coding sequence (with additional 682 bp upstream and 251 bp downstream regions of the COM1/SAE2 coding sequence) between the *Sac*II and *Hpa*I restriction sites. Indicated point mutations were introduced by standard techniques and verified by sequencing. Yeast spore viability was determined as described by Prinz and co-workers (Prinz *et al*, 1997).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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