Have a break: determinants of meiotic DNA double strand break (DSB) formation and processing in plants

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Abstract

Meiosis is an essential process for sexually reproducing organisms, leading to the formation of specialized generative cells. This review intends to highlight current knowledge of early events during meiosis derived from various model organisms, including plants. It will particularly focus on cis- and trans-requirements of meiotic DNA double strand break (DSB) formation, a hallmark event during meiosis and a prerequisite for recombination of genetic traits. Proteins involved in DSB formation in different organisms, emphasizing the known factors from plants, will be introduced and their functions outlined. Recent technical advances in DSB detection and meiotic recombination analysis will be reviewed, as these new tools now allow analysis of early meiotic recombination in plants with incredible accuracy. To anticipate future directions in plant meiosis research, unpublished results will be included wherever possible.

Key words: DNA double strand break, DNA repair, meiosis, recombination.

Introduction

In eukaryotes a specialized cell division, called meiosis, ensures the formation of generative cells. Meiosis is a two-step division, with homologous centromeres being segregated during the first, and sister centromeres during the second division. As there is no intervening DNA replication between the two meiotic divisions, each of the final division products contains only half of the initial DNA content. For a given diploid organism, the developing generative cells are then haploid. It is important to note that during meiosis, genetic information between maternal and paternal chromosomes is mutually exchanged, leading to novel combinations of genetic traits in the following generation. Two genetically diverse generative cells fuse during the process of fertilization, re-establish the organism’s original genome content, and constitute an individual with a unique genetic set-up (Zickler and Kleckner, 1999; Page and Hawley, 2003).

Recombination between homologous chromosomes depends on the formation of DSBs. DSBs are formed by a protein complex, with Spo11 proteins representing the catalytically active subunits (see below). As an intermediate of the DNA cleavage process, Spo11 proteins remain covalently linked to the 5’ termini of single-stranded DNA (ssDNA) at the incision sites and have to be removed (Fig. 1C) (Bergerat et al., 1997; Keeney et al., 1997; Neale et al., 2005). To release Spo11 from the DNA ends, DNA is nicked at a distance from the incision site by the MRX complex [Mre11–Rad50–Nbs1/Xrs2 in conjunction with Com1/Sae2 (Alani et al., 1990; Cao et al., 1990; Ivanov et al., 1992; McKee and Kleckner, 1997; Nairz and Klein, 1997; Prinz et al., 1997; Longhese et al., 2009; Mimitou and Symington, 2009a)]. It is most probably the endonuclease activity of Mre11 that mediates the ssDNA nick formation and the 3’–5’ exonuclease activity of Mre11 that resects the ssDNA towards the Spo11 protein. Spo11 is then released from the nascent cleavage site with a short DNA oligonucleotide remaining attached to the Spo11 protein (Fig. 1C).
To yield long stretches of ssDNA that can probe for matching strands on homologous chromosomes (or in some cases on sister chromatids) three proteins have gained attention. It seems that the exonucleases Exo1 and Dna2 together with the helicase Sgs1 are instrumental in 5′–3′ strand resection, starting at the Mre11-mediated ssDNA lesions to yield long single-stranded overhangs (Cromie et al., 2008; Mimitou and Symington, 2008; Zhu et al., 2008; Longhese et al., 2010; Manfrini et al., 2010). The ssDNA is bound with high affinity by replication protein A (RPA) (reviewed in Fanning et al., 2006; Broderick et al., 2010), a prerequisite for the loading of the strand exchange proteins Rad51 and Dmc1 (Fig. 1C). In yeast, this loading is mediated by Rad52 and accessory proteins, among them Rad54, Rad55, Rad57, Rad59, and Rdh54/Tid1 (reviewed in Krogh and Symington, 2004). In higher eukaryotes, the BRCA2 protein has been found to be essential for this step (a detailed description of these post-DSB steps is beyond the scope of this review and they have been reviewed in, for example, Longhese et al., 2010). Specialized meiotic DNA repair proteins, together with other DNA repair factors, mediate strand invasion, strand elongation by DNA synthesis, capture of the second DNA end, and subsequent repair and ligation of two different DNA strands to yield novel allelic combinations (reviewed in Hunter, 2006).

Setting the stage for meiotic recombination

Once a cell has been committed to undergo meiosis and DNA replication has been licensed and initiated (Borde et al., 2000; Sanchez-Moran et al., 2004; Boselli et al., 2009; Chi et al., 2009; Ronceret et al., 2009; Bergner et al., 2010; Merritt and Seydoux, 2010), certain regions in the genome become disposed to being cleaved by the meiotic DSB machinery. Until recently, the nature of cis-requirements for meiotic DSB formation remained elusive. The general understanding about what makes a certain genomic region more prone to receiving a break (generally referred to as ‘hot spot’ of meiotic recombination) than another (‘cold spots’) was the presence of an open chromatin status (Ohta et al., 1994; Fan and Petes, 1996; Keeney and Kleckner, 1996). This status may be acquired by transcription factor binding (without a need for transcription) (White et al., 1993), intrinsically open chromatin due to sequence constraints, or open chromatin due to histone modification(s) (α-, β-, and γ-hotspots; reviewed in Petes, 2001). Recently the Nicolas lab demonstrated that trimethylation of Lys4 on histone H3 (H3K4me3) is enriched at meiotic DSB hot spots in promoter regions in Saccharomyces cerevisiae (see below for technical details of how the DSB sites have been analysed) (Borde et al., 2009). Deletion of the SET1 methyltransferase led to a genome-wide decrease of hot spot activity, with hot spots located in promoter regions (the majority of S. cerevisiae hot spots) being mostly affected. Interestingly, the H3K4me3 marks were present even before pre-meiotic DNA replication. Furthermore, it is worth mentioning that other histone modifications such as histone acetylation or ubiquitination influence hot spot activity as well, and that some hot spots were not affected by the set1 deletion (Sturh, 1998; Sollier et al., 2004; Yamashita et al., 2004; Borde et al., 2009).
Table 1. Compilation of proteins needed for meiotic DSB formation in various organisms and selected references for further reading
Proteins depicted with an asterisk are essential for meiotic DSB formation. Proteins depicted with a question mark are suspected to be essential, but experimental proof is lacking.

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<th>S. cerevisiae</th>
<th>S. pombe</th>
<th>A. thaliana</th>
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<td>DSB formation</td>
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The yeast genome is small, and genes and their regulatory sequences are tightly packed, leaving hardly any space for heterochromatin (Zickler and Kleckner, 1999). The genomes of higher eukaryotes, in contrast, are often extremely large, and the coordination of DSB formation throughout the genome may rely even more on epigenetic marks. Work performed by the groups of de Massy, Myers and Petkov/Paigen shed new light on the question of how certain chromosomal regions become more prone to recombination.

In parallel, using different approaches, these groups found that the allelic status of a gene termed PRDM9 was responsible for the efficient initiation of recombination at a certain locus in the mouse and human genomes (Baudat et al., 2009; Myers et al., 2009; Parvanov et al., 2009). In a remarkable approach, Myers and co-workers analysed >30,000 human hot spots (deduced from linkage disequilibrium patterns) of certain well-documented human populations for the occurrence of a consensus motif in close proximity to highly recombinogenic regions and found a degenerate 13-mer motif to be associated with ~40% of all human recombination hot spots (Myers et al., 2005, 2008, 2009). In a second step, demonstrating the power of bioinformatics, a candidate list of zinc finger DNA-binding proteins that could specifically recognize the identified motif was established for the human genome, with PRDM9 as the prime candidate. The de Massy group identified a genetic locus responsible for meiotic recombination activity in a certain region of the mouse genome. The activity-determining locus on chromosome XVII was mapped between 12.2 Mb and 16.8 Mb. This region contains the Prdm9 gene, which encodes a protein with a SET-methyltransferase domain and a tandem array of 12 C2H2 zinc fingers (the human version contains 13 zinc fingers, with a tandem repeat structure similar to that observed in mice). Prdm9 trimethylates H3K4 (that has already been mono- or dimethylated) (Hayashi et al., 2005) and is expressed specifically in germ cells during meiotic prophase. The zinc finger domain has been found to be variable in different mouse strains (and variable in different human populations). It is this variability that allows binding to certain genomic loci with higher or lower affinity, leading to more or less H3K4 trimethylation. A high level of H3K4me3 was correlated with high recombination activity for progression through meiotic prophase, but some DSBs were detected in Prdm9−/− spermatocytes, suggesting that Prdm9 is not absolutely required for DSB formation (Baudat et al., 2009). This is in line with the presence of a Prdm9 signature in only 40% of human recombination hot spots (see above) (Myers et al., 2005, 2008, 2009).

In plants, no PRDM9 homologue has been identified so far. There are 176 genes encoding C2H2 zinc finger proteins (Englbrecht et al., 2004; Ciftci-Yilmaz and Mittler, 2008) and ~47 genes encoding a SET methyltransferase domain (Ng et al., 2007), but there is no clear homologue combining both features within one single reading frame (C. Uanschou, 2009).
personal communication). Generally, the H3K4me3 modification seems to mark transcriptionally active chromatin, and plants interpret this signal in the same way (Zhang et al., 2006, 2009; Borde et al., 2009). It will be interesting to see if plants use this mark in the meiotic context as well. Interestingly, a study published recently highlighted the impact of histone H3 acetylation on meiotic crossover (CO) formation in Arabidopsis thaliana (Perrella et al., 2010). Hyperacetylation did not lead to a general increase in CO formation, but differentially to more COs on one chromosome, and to less on two others. This observation is in line with data from different organisms indicating that histone acetylation has an effect on meiotic recombination (Yamada et al., 2004; Mieczkowski et al., 2007; Merker et al., 2008) but that the modification has different consequences depending on the genomic context. In Caenorhabditis elegans, recent work suggests that chromatin modifications play an important role during meiosis. The him-17 mutant with reduced H3K9me2 is defective for meiotic recombination and chromosome segregation due to a defect in DSB formation (Reddy and Villeneuve, 2004) and the xnd-1 mutant has elevated levels of H2AK5 acetylation and has an altered DSB and recombination landscape (Wagner et al., 2010).

Deployment of meiotic DSB proteins

Meiotic DNA DSBs may only be introduced after DNA replication, and therefore the DSB-forming machinery has to be connected to the cell cycle and replication control. In the yeast S. cerevisiae this connection is provided by the S-phase cyclin-dependent kinase Cdc28-Cbi5 (CDK-S) and Dbf4-dependent kinase Cdc7-Dbf4 (DDK), both needed for the initiation of (pre-meiotic) DNA replication. The protein Mer2, an essential factor for meiotic DSB formation, is phosphorylated by CDK-S (Henderson et al., 2006). This phosphorylation primes Mer2 for an additional phosphorylation by DDK (Wan et al., 2008). It was speculated that replication fork-associated CDK and DDK may coordinate replication and hot spot maturation (Murakami and Keeny, 2008). The negative patch, generated on Mer2 by addition of the phosphate residues, allows the interaction with two further essential DSB complex members, Rec114 and Mei4 (Fig. 1A) (Matos et al., 2008; Sasanuma et al., 2008; Wan et al., 2008).

Mer2/Rec107 has been independently described by Engebrecht et al. (1991) and Malone et al. (1991) in S. cerevisiae. It is required for chromosome synapsis and initiation of meiotic recombination. A null mutation of MER2 leads to meiotic lethality (Rockmill et al., 1995). In yeast two-hybrid interaction assays it has been shown that Mer2 interacts with itself, Mei4, Xrs2, and Rec114 (Li et al., 2006). A plant or mammalian counterpart for Mer2 has not been identified yet. The actual molecular link between Mer2 positioning (or the positioning of other DSB factors) and chromatin modifications (e.g. H3K4me3, as outlined above) remains unknown. The challenging task for the future will be to find the factors that can recognize histone modifications and attract (or repel) the meiotic DSB machinery. Furthermore, it seems reasonable to assume that similar mechanisms to those described above are in place in plants. It is still an open question as to whether targeted histone modifications govern DSB formation in plants with higher probability at certain loci. It seems undisputed that DNA replication and DSB formation are linked in plants. This is supported by reports on SPO11 deposition and DSB formation following DNA replication, visualized by bromodeoxyuridine (BrdU) incorporation, in A. thaliana (Sanchez-Moran et al., 2008). In A. thaliana ~61 core cell cycle genes have been described (Vandepoele et al., 2002; Inze and De Veylder, 2006). The distinct and shared roles of the 12 CDKs and of the at least 30 cyclins, and their impact on plant meiosis is still under investigation. A CDC7 homologue has been found, but its role during meiosis has not been characterized yet. Identifying the molecular mechanisms and factors that actually couple the cell cycle, DNA replication, and meiotic DSB formation in plants will be an important task for future research.

Phosphorylated Mer2 attracts Mei4 and Rec114 (Fig. 1A). Rec114 and Mei4 were first identified in two genetic suppressor screens, and epistatic analysis suggested that they are needed together with Spo11 (Meneses and Roeder, 1989; Malone et al., 1991). Later, it was shown that Rec114, Mei4, and Mer2 associate with chromatin in the absence of other proteins essential for DSB formation, although Mei4 binding is reduced in mer2d (Li et al., 2006). Rec114 is necessary for subsequent binding of Spo11 and Mer11 to future DSB regions in the genome and for Spo11 homodimer formation (Borde et al., 2004; Prieler et al., 2005; Sasanuma et al., 2007). Interestingly, Rec114 overexpression suppresses DSB formation, suggesting a dual role for Rec114 (Bishop et al., 1999), first as a scaffold protein of the DSB complex and secondly as a negative regulator of DSB formation. Rec114 shows sequence homology to the Schizosaccharomyces pombe Rec7 protein (Molnar et al., 2001). Rec7 localizes to nuclei, associates with linear elements (LinEs; the rudimentary axial elements of S. pombe) of meiotic chromosomes, and is required for DSB formation (Cervantes et al., 2000; Davis and Smith, 2001; Lorenz et al., 2006). Steiner et al. found that Rec7 interacts with Rec24, a S. pombe meiotic DSB protein, which is related to the S. cerevisiae Mei4 (Kumar et al., 2010; Steiner et al., 2010). Recently, the de Massy lab published the identification and characterization of the mouse orthologues of Mei4 and Rec114 (Kumar et al., 2010). Murine Mei4 and Rec114 are expressed in testis and embryonic ovary, and they interact with each other when expressed in HeLa cells. Cytological analysis showed that MEI4 is localized to the lateral elements of the synaptonemal complex, with the highest number of foci in leptotene. MEI4 does not co-localize with DMC1 and RPA and does not require SPO11 for localization. Greatly reduced γH2AX staining in Mei4−/− mice meiocytes indicates a severe defect in DSB formation. Additionally, Mei4 knock-out mice are defective in homologous synapsis (Kumar et al., 2010). Aligning the mouse Mei4 and Rec114 sequences with plant
and in *Drosophila melanogaster* et al. (1997), in *Arabidopsis*, and in *C. elegans* is involved in pairing of homologous chromosomes. The meiotic non-somatic functions of Top6 homologues have been named Rec14, or in *Sordaria macrospora* (Jolivet et al., 1999), these proteins are essential for meiosis, whereas *Mei4* homologues, also known as Vip3. They characterized two different allelic mutations (*vip3-2* and *vip3-3*). As in corresponding mutants in *S. pombe*, where the gene has been named *Rec14*, or in *Sordaria macrospora*, these mutants grew poorly, but they displayed no meiotic defect (Jolivet et al., 2006).

Rec102 and Rec104 were identified in a screen to isolate equivalent homologue, also known as Vip3. They characterized two different allelic mutations (*vip3-2* and *vip3-3*). As in corresponding mutants in *S. pombe*, where the gene has been named *Rec14*, or in *Sordaria macrospora*, these mutants grew poorly, but they displayed no meiotic defect (Jolivet et al., 2006).

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as a DNA repair gene, and the study of Ivanov et al. (1992) showed that Xrs2 has also a meiotic function. Homologues of Rad50, Mre11, and Xrs2/Nbs1 have been identified in all eukaryotes. In humans, plants, S. pombe, Mus musculus, and D. melanogaster the interaction partner of MRE11 and RAD50 is NBS1, which displays only limited homology to Xrs2 (Carney et al., 1998; Vissinga et al., 1999; Ueno et al., 2003; Ciapponi et al., 2006; Akutsu et al., 2007). In A. thaliana it was demonstrated that these proteins physically interact (Gallego et al., 2001; Daoudal-Cotterell et al., 2002; Waterworth et al., 2007). A mutation in ATRAD5 leads to meiotic defects, sterility, and sensitivity against MMS (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 non-essential. Depending on the MRE11 mutant allele, plants are sterile, due to perturbed meiosis, and severely affected in development or display only enhanced sensitivity to genotoxic agents (Bundock and Hooykaas, 2002; Puizina et al., 2004). Arabidopsis NBS1 mutant lines exhibit hypersensitivity to a DNA cross-linking reagent but no meiotic defects. Analysis of an Arabidopsis nbs1-1atm double-mutant revealed a role for AtNBS1 in meiotic recombination but no involvement in DSB formation. Interestingly, the requirement of the MRX complex for DSB formation is not universal. In organisms other than S. cerevisiae and C. elegans, such as A. thaliana, Tetrahymena, and S. pombe, the MRX complex is dispensable for meiotic DSB formation and only instrumental for meiotic DSB processing (Chin and Villeneuve, 2001; Puizina et al., 2004; Young et al., 2004; Lukaszewicz et al., 2010).

Exhaustive genetic screens in A. thaliana identified novel genes that are essential for meiotic DSB formation but seem unrelated to known meiotic genes from other organisms. Primary screens for reduced fertility and secondary screens for the absence of meiotic DNA breaks identified the genes AtPRD1, AtPRD2, and AtPRD3 to be essential for SPO11-mediated meiotic DSB formation (Fig. 1B) (De Muyt et al., 2007, 2009). AtPRD1 (Putative Recombination initiation Defect 1) has low similarity to mammalian Mei1. The mouse Mei1 was characterized by Libby et al. (2002) and was isolated in a mutant screen for infertility. The N-terminus of AtPRD1 interacts with AtSPO11-1 in a yeast two-hybrid assay, thereby identifying for the first time an interaction partner of a SPO11 protein in plants. The functional relevance of this interaction is unknown (De Muyt et al., 2007). AtPRD2 was later recognized as a homologue of Mei4 (see above) (Kumar et al., 2010). AtPRD3 is a protein of unknown function, similar to the previously identified rice PAIR1 gene, but with no homologues outside the plant kingdom (Nonomura et al., 2004; De Muyt et al., 2009).

In different independent screens, the plant SWI1 gene has been found to affect plant fertility. The data suggest that SWI1 is required for meiotic chromatin remodelling, sister chromatid cohesion, chromosome pairing, synopsis, and recombination. No meiotic DSBs are formed in swil mutants (Siddiqi et al., 2000; Mercier et al., 2001, 2003; Agashe et al., 2002; Boeteng et al., 2008).

In the yeast S. pombe a couple of proteins needed for DSBs have been identified that do not share sequence homology with proteins from S. cerevisiae, mammals, or plants. Among them are Rec6, Rec15, Rec25, Rec27, and Mde2. All of these five proteins are very small and harbour no motifs to suggest any biological function. Rec6 and Rec15 are required for DSB formation (Cervantes et al., 2000; Davis and Smith, 2001), and Rec15 interacts with Mde2 (Steiner et al., 2010). Furthermore, Rec25 and Rec27 are important, but not absolutely essential for meiotic recombination in S. pombe. Both deletions exhibit a similar phenotype, namely aberrant asci with abnormal spore number and morphology, resulting from reduced meiotic recombination. Although no DSB formation has so far been observed in rec25A and rec27A mutants, recombination still occurs at low levels. Nevertheless, both Rec25 and Rec27 are absolutely necessary for Rec10 localization to LinEs and both co-localize with Rec10 during LinE formation (Davis et al., 2008). Rec10, a homologue of the S. cerevisiae axial element protein Red1, is meiosis specific and localizes to LinEs (Lorenz et al., 2004). In contrast to what has been observed in S. cerevisiae for Red1, a rec10 deletion shows no DSB formation, whereas in Red1 mutants DSB formation still occurs, but is reduced to 20–60% of wild-type levels (Schwacha and Kleckner, 1997; Woltering et al., 2000; Hunter and Kleckner, 2001). A rec10 deletion has the same recombination defect as a rec12 mutant and the gene is indispensable for DSB formation. No plant homologue of Red1 has been found yet. A dimerization partner of yeast Red1 is the HORMA domain protein Hop1 (de los Santos and Hollingsworth, 1999). The C. elegans htp3 gene displays homology to yeast’s HOP1, and is needed for DSB formation (Goodyer et al., 2008). Deletion of the fission yeast Hop1 homologue reduces DSB frequency (Latypov et al., 2010). In plants a gene displaying limited sequence similarity to Hop1 has been identified and named ASY1. ASY1 is one of the axial element proteins (like Hop1 in yeast), but has not been found to affect levels of meiotic DSB formation (Caryl et al., 2000; Armstrong et al., 2002; Sanchez-Moran et al., 2008).

The Drosophila gene mei-P22 was isolated in a large scale P-element mutagenesis screen for mutants with a high frequency of X-chromosome non-disjunction in the female germline (McKim et al., 1998). It is a 35.7 kDa protein, which cytologically localizes to meiotic chromosomes and it is necessary for the induction of DSB formation in the Drosophila female. Crossing over in such mutants can be restored to a level of 50% of that of the wild type by γ-irradiation (Liu et al., 2002).

Meiotic DNA DSB formation and processing

As outlined above, meiotic DSB formation is essential for subsequent recombination. Interestingly, an excess of breaks, compared with the number of reciprocal recombination products, is observed in most organisms analysed. In
the yeast *S. cerevisiae* it has been estimated that a single meiotic cell forms ~140–170 (Buhler et al., 2007) to 180–270 (Weiner and Kleckner, 1994) breaks but only ~90–95 COs (Chen et al., 2008; Mancera et al., 2008). Studies in *A. thaliana*

established between 150 (Sanchez-Moran et al., 2007) and 250 (Chelysheva et al., 2007; Vignard et al., 2007) breaks per meiocyte and ~10 COs (Higgins et al., 2004; Mercier et al., 2005; Wijeratne et al., 2006). Interestingly, *C. elegans* seems to be an exception, with an estimation of ~12 breaks and six COs per meiocyte (Mets and Meyer, 2009).

As mentioned above, in *S. cerevisiae* Spo11 needs at least nine other proteins for catalysis of DSB formation (Fig. 1A; Mre11, Rad50, Xrs2, Rec102, Rec104, Rec114, Mei4, Mer2, and Ski8) (Keeney, 2001). The topology of the active cleavage complex has not been clarified yet. The meiotic chromat is organized in loops and axes (Moens and Pearlman, 1988; Zickler and Kleckner, 1999), with cohesin molecules (Klein et al., 1999) and meiosis-specific axial element proteins (Smith and Roeder, 1997) forming the axis and chromat loops emanating from there. The DNA sequences associated with cohesin were mapped with a resolution of ~1 kb (Blat and Kleckner, 1999; Glynn et al., 2004). While the DSB proteins form foci on meiotic prophase chromat, their localization has only recently been analysed by chromatin immunoprecipitation (ChIP). Mre11 was found to localize to DSB hot spots in one study (Borde et al., 2004) and equally strongly to hot spots and cohesin sites in another (Mendoza et al., 2009). Spo11 was reported to bind to DSB hot spots and cohesin sites (Kugou et al., 2009), while Mer2, Rec114, and Mei4 in fact avoid binding to most strong DSB hot spots, while localizing to sites flanking the hot spots, usually coinciding with cohesin sites (S. Panizza and F. Klein, unpublished results). The latter observation supports a model in which Spo11-mediated cleavage occurs after loop sequences are transiently recruited to the DSB machine located at the chromosome axis (S. Panizza and F. Klein, unpublished results). This model is related to a series of models put forward first by Zickler and Kleckner (1999), who proposed that DSBs are made at the chromosome axis, and then assuming that hot spot sequences are close to axis protein-binding sites, which was later found not to be the case.

In plants, the distribution of DSB proteins on meiotic chromat, and their interdependencies, have not been analysed yet. An AtSpo11-1-specific antibody has been generated (Sanchez-Moran et al., 2008), but so far it was only used to determine the massive enrichment of AtSpo11-1 on meiotic chromat in *Atmre11-3, Atrad50*, and *Atcom1* mutants (Uanschou et al., 2007), but not for precise localization of SPO11-1 and not for determining the dependencies of the meiotic cleavage complex in the context of chromat and other protein factors.

A major component of meiotic chromosome axes are cohesins, which are better known for their role in sister chromatid cohesion (Orr-Weaver, 1999; van Heemst and Heyting, 2000) and which consist of four highly conserved proteins, namely Snc1, Snc3, Rec8, and Sec3. Scc1 is the kleisin of the mitotic cohesion complex and Rec8 is its meiotic parologue (Guacci et al., 1997; Michaelis et al., 1997; Klein et al., 1999). In yeast, Hop1 and Red1 are two meiosis-specific axial element (AE) proteins, which are required for full levels of DSB formation and interhomologue bias. The two proteins co-localize to AEs and interact as well in co-immunoprecipitation and in yeast two-hybrid experiments (Smith and Roeder, 1997; de los Santos and Hollingsworth, 1999). In the model plant *Arabidopsis*, six Scc1/Rec8 homologues have been identified, with SYNL1/DIF1 representing the orthologue of Rec8 (Bai et al., 1999; Bhatt et al., 1999). *syn1/dif1/rec8* mutants display defects in cohesion, chromosome condensation, and DNA repair, but DSBs are still formed. ASY1, as described above, has been identified as a homologue of yeast’s Hop1. To date, it is not clear whether plant cohesin and axial element proteins positively support meiotic DSB formation. It is unknown if fewer DSBs are formed in *Atrec8* and *asy1* mutants, respectively (Chelysheva et al., 2005).

Once the DSB complex has formed, Spo11 is anticipated to form a homodimer, in analogy to the defined structure of Top6A (Nichols et al., 1999; Corbett and Berger, 2004). Furthermore, it is believed, that Spo11-mediated DNA cleavage occurs via a transesterification mechanism, as described for type-IIB topoisomerases (Corbett and Berger, 2004). In yeast, it is the side chain of Tyr135 of Spo11 that carries out a nucleophilic attack on the DNA phosphodiester backbone. In the course of this reaction, the 5′ phosphorus of the DNA becomes covalently linked to the tyrosine via a phosphodiester link, thereby generating a protein–DNA intermediate and a nick in the DNA strand. It is anticipated that the nucleophilic attacks occur simultaneously on both DNA strands, thereby generating a DNA DSB. As for Top6A, the Spo11 DSB complex has been shown to generate breaks with a two-nucleotide 5′ overhang (Liu et al., 1995). Whereas in the case of topoisomerases the broken DNA ends are held together via the Top6B subunits and are resealed in a reversion of the transesterification described above, the meiotic Spo11-containing DSB complex lacks analogues of Top6B subunits and the DSB is therefore processed differentially and subsequently yields recombinogenic ssDNA strands (Corbett and Berger, 2004). An intermediate of the reaction represents Spo11 covalently attached to the 5′ end of the DNA strand at the break site. Release of Spo11, attached to a short oligonucleotide derived from the DNA adjacent to the DSB site, determines the irreversibility of the cleavage reaction (Fig. 1C) (Neale et al., 2005). The MRX complex together with Sac2/Com1 mediates the needed strand incision at a distance from the site of Spo11 activity (Neale et al., 2005). To date it is not clear if the incision takes place close to the initial site of Spo11 activity, thereby generating the final size of the ssDNA oligonucleotide attached to Spo11 when released, or if the incision takes place further away from the break site with a subsequent need for 3′–5′ exonuclease activity. Unpublished results from our lab indicate that the latter possibility is more likely to occur in yeast (B. Edlinger et al., unpublished results). In such a scenario, the endonucleolytic activity of MRX/Com1 would be exerted first at a distance.
Starting from this nicked DNA site, the exonucleolytic 3’–5’ activity of the MRX complex would work towards the Spo11 protein, and the 5’–3’ exonucleolytic activity of Exo1 and other factors would be directed outwards away from the Spo11-mediated DSB. In *S. cerevisiae* Spo11 has been found with two different classes of oligonucleotides attached, approximately half of 7–12 nucleotides in length and the other half between 21 and 37 nucleotides in length (Neale et al., 2005), whereas in *S. pombe* only one class of oligonucleotide could be detected (between 17 and 27 nucleotides in length, with an average of 23) (Milman et al., 2009; Rothenberg et al., 2009). This indicates that the meiotic DSB complex may be structurally different in various organisms, which is in line with the fact that the conservation of proteins involved in DSB formation is often low or absent (Bleuyard et al., 2004; Young et al., 2004; Jolivet et al., 2006).

Following Spo11-mediated DSB formation and release of Spo11, with the help of MRX/Com1, 3’ ssDNA becomes exposed. The length of the 3’ ssDNA is determined by the activity of the ressection machinery that exhibits exonucleolytic activity in the 5’–3’ orientation and is mediated during meiosis most probably by Sgs1–Dna2 and Exo1 (Mimitou and Symington, 2008; Zhu et al., 2008; Farah et al., 2009). The resulting 3’-ended ssDNA tails are believed to serve as probes to identify homologous partner chromosomes and to initiate D-loop formation and single end invasion followed by second end capture (Paques and Haber, 1999; Hunter et al., 2001; Krogh and Symington, 2004; San Filippo et al., 2008; Mimitou and Symington, 2009b).

Following the resection of DNA strands in budding yeast, Rad52 assembles the sequence homology-dependent DSB repair machinery (Gasiör et al., 1998). Rad52 orthologues are known in vertebrates (Van Dyck et al., 1999) but not in *A. thaliana* (Ray and Langer, 2002). Either DSB repair is independent of Rad52 in *A. thaliana* or this protein has, thus far, not been detected in database searches. However, other protein factors involved in DNA strand exchange, such as proteins of the RecA-like recombinase protein family, have been identified in plants (Osakabe et al., 2002). The completion of the Arabidopsis genome sequence revealed 12 genes with a conserved RecA domain. Five of them are closely related to the bacterial RecA protein (three of the five possess target sequences for mitochondria or chloroplasts). The other seven proteins have known homologues in man and have been analysed in *A. thaliana*. Mutations in *AtRADS1*, *AtDMC1*, *AtXRC3*, and *AtRADS1C* lead to severe meiotic defects (Couteau et al., 1999; Bleuyard and White, 2004; Li et al., 2004; Abe et al., 2005; Osakabe et al., 2005). After DNA DSB formation and resection, Rad51 is loaded onto ssDNA. Rad51 plays a role in both somatic and meiotic recombination, whereas the closely related strand exchange factor Dmc1 is exclusively loaded onto ssDNA during meiosis (Fig. 1C) (Sato et al., 1995a, b, c; Dresser et al., 1997; Paques and Haber, 1999) and is required, together with other factors, for interhomologue recombination (but not intersister recombination) (Schwacha and Kleckner, 1997; reviewed in San Filippo et al., 2008).

Methods to analyse sites of meiotic DSB formation

As outlined, meiotic DSB formation is essential for subsequent recombination to occur. Knowing the sites of meiotic DSB formation allows analysis of determining factors and, in the future, may lead to novel plant breeding strategies by targeting meiotic recombination to desired loci in the genome of crop plants. Approximate locations of (a limited set of) DSB sites in the genome of a given organism can be deduced from the recombination products. This has been done extensively in various organisms including plants (Baudat and Nicolas, 1997; Gerton et al., 2000; Drouaud et al., 2006). With an increasing density of genetic markers, recombination maps in Arabidopsis now have the power to identify genetic exchange points in a window of about only 2 kb (Drouaud et al., 2006). Recombination events can be monitored in the offspring (F1 generation) of two genetically distinct ecotypes by determining the exchange rate of known genetic markers. Historically, these markers have been phenotypic traits, but advances in molecular analysis and genome sequencing projects led to the discovery of a vast number of single nucleotide polymorphisms (SNPs) or small insertions or deletions (INDELs), of which many can be monitored simultaneously (Drouaud et al., 2006).

The physical distances between the analysed markers define the resolution of the recombination map. In 2007 the Weigl and Nordborg labs published a set of ~1 million non-redundant SNPs for different accessions of Arabidopsis. To examine sequence variation in this model plant, they performed high density array re-sequencing of ~20 different accessions (ecotypes). They observed that ~4% of the genome is highly dissimilar or deleted relative to the reference genome (Clark et al., 2007). In a subsequent publication, using a similar, but larger, data set, historic recombination events have been deduced from regions with linkage disequilibrium. Furthermore, it has been demonstrated, that the historic hot regions correlate well with recent recombination events (Kim et al., 2007). In another study, an in-depth analysis of recombination utilized ~100 recombinant inbred lines. Genomic DNA from these lines was hybridized to microarrays representing open reading frames (ORFs) of *A. thaliana* ecotype Columbia. The initial cross of the mapping population has been performed with the ecotypes Columbia and Landsberg, with the latter genome hybridizing to many probes of the microarray sequences with lower affinity. This allowed the genome-wide differentiation of the two initial genomes and interrogation of initial recombination events (Singer et al., 2006).

Another approach utilized a microarray-based readout for recombination in *S. cerevisiae*. The innovative aspect was that not diploid offspring but haploid cells, the products of meiosis, were analysed. Furthermore, since the four cells from one individual meiosis (tetrads) were analysed, CO events, non-crossover (NCO) events, gene
conversion and CO interference could be studied (Chen et al., 2008; Mancera et al., 2008).

Analysing recombination directly in the haploid products of meiosis is also possible in higher organisms, via sperm or pollen typing techniques (Li et al., 1988; Cui and Li, 1998; Jeffrey et al. 2004; Tiemann-Boege et al., 2006; Arnheim et al., 2007; Kauppi et al., 2007) (J. Droitau and C. Mezard, personal communication). Because of the high number of post-meiotic cells that can be studied, this technique allows a more efficient determination of meiotic recombination, compared with pedigree analysis.

The drawbacks of the approaches outlined above are (i) that polymorphic markers have to be present and known in the organism of interest; (ii) that recombination does not necessarily reflect DSB initiation sites; and (iii) that the polymorphisms needed for such studies may influence DSB formation and recombination distribution and frequency. These methods have been instrumental for recombination analysis, but they have not provided high-resolution maps to identify the actual underlying DSB sites and omitted all those DSB sites not leading to exchange of genetic information. However, detailed information on DSB sites is required to identify underlying cis- and trans-determinants of meiotic DSB formation. Below, a range of methods is outlined that are dedicated to analyse directly and identify meiotic DSB sites throughout the genome.

Work performed in S. cerevisiae in the labs of Simchen and Nicolas (Zenvirth et al., 1992; Baudat and Nicolas, 1997) revealed meiotic DSB sites on yeast chromosomes. Chromosomes and chromosome fragments from synchronized yeast cultures, containing a mutation that enriches for meiotic DSBs, rad50S, were separated by pulsed-field gel electrophoresis and detected via Southern blotting. The rad50S hypermorphic mutation allows DSB formation to occur, but subsequent processing steps are blocked. With this direct approach for DSB detection, 76 DSB regions have been identified. Furthermore, these experiments revealed the existence of cold and hot domains with respect to DSB formation and the quantitative differences of various hot spots. Most DSBs in S. cerevisiae were found in intergenic promoter-containing intervals and some of the hot DSB sites were known also to be hot recombination sites.

A much more refined technique was published in 2000 by the Petes lab (Gerton et al., 2000). In this study, a tagged Spo11 protein was immunoprecipitated from synchronized rad50S yeast cultures. The intermediate of meiotic DSB formation, Spo11 covalently attached to the 5′ ends of DNA, was thereby enriched and allowed analysis of the bound DNA (Fig. 1C). The Spo11-associated DNA was fragmented, amplified by PCR, and labelled. The DNA samples were then applied to microarrays, comprised of 6400 DNA sequences representing yeast ORFs (DeRisi et al., 1997). A total of 177 hot spots of DSB formation and 40 cold spots were identified. In more detail, each chromosome has at least one hot spot of DSB formation and there is a significant correlation between chromosome size and number of hot spots. Large chromosomes have relatively few hot spots per kb as compared with small chromosomes. The average distance between hot spots was determined to be 54 kb, and for intervals including the centromere, ~117 kb (Lichten and Goldman, 1995; Gerton et al., 2000). As already found (Sharp and Lloyd, 1993) for chromosome III, hot regions show a positive correlation with high GC content (Gerton et al., 2000). Caveats concerning this technique to map meiotic DSBs are as follows. First, microarrays may contain a biased set of probes, as in the case described above (e.g. ORFs only). Only an unbiased microarray, using genomic probes, with equal spacing and, preferentially, overlap of the probes, will yield a high resolution map. For organisms with larger genomes and with high sequence redundancy, the quality of microarray-based assays will always depend on the available microarray platform. Although, custom-made arrays are available, the standard genomic arrays for Arabidopsis provide on average a probe of 25 nucleotides in length every 35 nucleotides. As discussed below, deep sequencing of immunoprecipitated DNA will most probably substitute microarray-based techniques for many applications. Secondly, DSB mapping in the rad50S background needs fragmentation of DNA prior to immunoprecipitation of DNA, and the resolution of DSB maps therefore crucially depends on thorough fragmentation of genomic DNA. The average fragment size will define the broadness of the hybridization signal. Thirdly, and most importantly, the DSB mapping approach outlined above depends on the rad50S mutant allele. Later studies demonstrated that in S. cerevisiae DSB formation is reduced in rad50S mutants (Blitzblau et al., 2007; Buhler et al., 2007). Similar studies have subsequently been performed in S. pombe (Cervantes et al., 2000; Cromie et al., 2007; Ludin et al., 2008), and DSB maps based on electrophoretic separation of DSB-generated fragments and on immunoprecipitation of tagged Rec12 (Spo11) were found to correlate. DSB sites are separated by at least 65 kb, mostly situated within large intergenic regions and underrepresented in coding DNA regions. The intervening regions undergo almost no breakage (Cromie et al., 2007). This is in contrast to the situation in S. cerevisiae, with most of the DSBs in promoter regions and a much higher density of DSBs over the genome. Later, the Smith lab mapped meiotic DSB sites in S. pombe wild-type cultures (Hyppa et al., 2008). Intriguingly, the locations of DSBs were found to be indistinguishable in rad50S and rad50S strains. However, the signal intensity was lower in the rad50S strains, most probably due to ongoing DNA repair. It should be noted that Rad50 is not needed for DSB formation in S. pombe; however, the rad50S allele has the same defect of inhibiting post-DSB processing events. It may therefore be assumed that in organisms with no need for the MRX complex for DSB formation, rad50S and mre11S mutant alleles may be potent tools to enrich for SPO11–DNA intermediates at meiotic DSB sites and to represent the DSB landscape of these organisms faithfully.

In principle, the outlined approaches could also be performed in plants. It should be emphasized that in higher plants RAD50 (and also MRE11) is not needed for meiotic...
DSB formation, only for the ensuing processing, together with MRE11 and COM1/SAE2 (Gallego et al., 2001; Bundock and Hooykaas, 2002; Puizina et al., 2004). Mutations in either of the three corresponding genes lead to accumulation of AtSPO11-1 on meiotic chromatin (Uanschou et al., 2007), suggesting conservation of DSB processing. The Atcom1-1 mutation seems best suited to be used, as plants do not display any somatic aberrations during unchallenged life. In contrast, mre11-3 and rad50 mutants display pleiotropic somatic defects under normal growth conditions (Gallego et al., 2001; Bundock and Hooykaas, 2002; Puizina et al., 2004). Tagged and functional AtSPO11 proteins have been generated (BE and PS, unpublished results) and efforts are currently underway to set up the experimental framework for genome-wide DSB identification in a higher plant.

A different approach took advantage of another intermediate of meiotic DNA repair. After DSB formation, break processing generates a long stretch of ssDNA, which serves as a probe for finding a DNA template for repair. As briefly outlined, meiotic DNA repair depends on recombinational activity, such as Rad51 and Dmc1. In the yeast S. cerevisiae, the turnover of these stretches of ssDNA is blocked in a dmc1Δ mutant strain. Therefore, ssDNA, generated at the exact positions of a former DSB site, becomes enriched in a dmc1Δ mutant and is amenable to biochemical analysis (Fig. 1C). Buhler et al. (2007) and, in a similar approach, Blitzblau et al. (2007) developed a technique to isolate ssDNA from synchronized dmc1Δ meiotic cell cultures. They used benzoyl naphthyl DEAE (BND) cellulose to enrich ssDNA tracts, and amplified, labelled, and hybridized them to the Agilent 44k, a yeast whole-cellulose to enrich ssDNA tracts, and amplified, labelled, and hybridized them to the Agilent 44k, a yeast whole-genome oligonucleotide array. It turns out that in S. cerevisiae the DSB landscape is more subtle than anticipated from the rad50S maps. For instance, regions close to the centromeres and telomeres, previously thought to be devoid of DSBs, were found to contain DSB sites, even though these regions have a very low recombination rate (Baudat and Nicolas, 1997; Gerton et al., 2000; Borde et al., 2004; Robine et al., 2007). Importantly, all of the hot regions previously identified in the rad50S background were also found in dmc1Δ, and in total about five times more DSB sites were found in the dmc1Δ background. Caveats of this technique are that different ssDNA-containing intermediates may not be equally stable. Furthermore, 5′ to 3′ end resection continues over time in dmc1Δ mutants (Bishop et al., 1992; Shinohara et al., 1992), and early-forming DSBs might therefore be associated with more ssDNA than late-forming DSBs (Buhler et al., 2007).

In principle, meiotic ssDNA could be isolated from any organism that provides sufficient amounts of staged meiotic cells. It should be emphasized, however, that in plants, loss of DMC1 does not lead to accumulation of ssDNA or to a block in meiotic progression, but to RAD51-dependent DSB repair via the sister chromatid (Couteau et al., 1999; Siaud et al., 2004). So-called accessory proteins, such as MND1 and HOP2 (AHP2 in Arabidopsis), support DMC1 and to a lesser extent RAD51 during meiotic DNA repair (Schommer et al., 2003; Kerzendorfer et al., 2006; Vignard et al., 2007). Mutations in MND1, for instance, lead to unrepaired DSBs and might be used to enrich for ssDNA.

Another intermediate of meiotic recombination are the meiotic nucleoprotein filaments, ssDNA associated with the recombinases RAD51 and/or DMC1 (reviewed in Sehorn and Sung, 2004). Antibodies against RAD51/DMC1 are commercially available for many organisms and can be used to immunoprecipitate these nucleoprotein filaments and analyse the associated DNA (Fig. 1C). This has been done in the Dernburg lab for the model organism C. elegans (A. Dernburg, personal communication), in the Petukhova lab for mouse (G. Petukhova, personal communication), and in the Pawlowski lab for maize (W. Pawlowski, personal communication). The detailed and genome-wide analysis in mouse provides the first genome-wide map of meiotic DSB formation and, furthermore, confirmed the existence of a target motif for Prdm9 (see above). In C. elegans, the Dernburg lab generated the first genome-wide DSB map and, furthermore, identified a DNA sequence motif in the centre of hot spot regions, indicative of a conservation of the Prdm9-like mechanism, first described in the mouse. The analysis for maize is still ongoing, but it is anticipated that only these high-resolution techniques will generate DSB maps with a resolution and density that will allow analysis of putative DNA motifs in the centre of DSB hot spots.

The most recent advances in DSB detection methods turned back to the core enzyme of meiotic DSB formation. As outlined, two Spo11 proteins become covalently linked to the 5′ ends of DNA at either side of the duplex DNA at a given DSB site. DSB processing releases these two Spo11 proteins with a short DNA oligonucleotide attached to the active tyrosine residue (Bergerat et al., 1997; Keeney et al., 1997; Corbett and Berger, 2004; Neale et al., 2005; Corbett et al., 2007). The short DNA oligonucleotides exactly represent the regions of meiotic DSB activity and, moreover, the 5′ ends of these oligonucleotides represent the nucleotide of Spo11 activity. High throughput sequencing of these Spo11-associated oligonucleotides would allow the establishment of a high-resolution map of meiotic DSB sites (Fig. 1C). The Keeney lab (S. Keeney, personal communication) and our lab (B. Edlinger et al., unpublished results) established protocols to ligate, with high efficiency, amplification and sequencing adaptors to Spo11-associated oligonucleotides after immunoprecipitating Spo11 from synchronized S. cerevisiae or S. pombe cultures. The amplification products are sequenced using deep-sequencing platforms and the mapped reads represent the meiotic DSB landscape with nucleotide resolution. ChIP followed by deep sequencing (ChIP-Seq) was one of the first applications for next-generation sequencing, and the first results were published in 2007 (Barski et al., 2007; Johnson et al., 2007; Mikkelsen et al., 2007; Robertson et al., 2007). Compared with ChIP-on-chip (ChIP with subsequent hybridization to a microarray), ChIP-Seq has a higher resolution, generates fewer artefacts, and has a better coverage. Additionally, for ChIP-Seq, only a very low amount of input DNA is needed, it has a better dynamic range, and multiplexing is possible. Importantly,
the technique does not require prior knowledge of DSB sites and, moreover, reduces the danger of biased results (Park, 2009).

Efforts are currently under way to implement the method described above in the model plant A. thaliana (BE and PS, unpublished results). The technically challenging experiments seem justified, judging from the wealth of information that has been and still is gained from detailed analysis of DSBs, performed in other organisms. In the plant field it is, for instance, still unknown which kind of trans- and cis-acting factors determine the ‘hotness’ of a certain genomic region. So far, the research has been driven by genuine interest, but plant breeders are becoming more and more interested in efficiently exploiting naturally occurring beneficial traits of crop plants. Understanding meiotic recombination may provide the basis to develop the tools to modify recombination rates at desired loci in crop genomes.

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