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REVIEW PAPER

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 To anticipate future directions in plant meiosis research, unpublished results will be included wherever possible.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 centromers 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, 2009*a*)]. 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).

Abbreviations: AE, axial element; BND, benzoyl naphthyl DEAE; CDK, cyclin-dependent kinase; ChIP, chromatin immunoprecipitation; ChIP-Seq, chromatin immunoprecipitation followed by sequencing; CO, crossover; DSB, double strand break; dsDNA, double-stranded DNA; H3K4, histone 3 Lys4; LinE, linear element; MMS, methyl methanesulphonate; MRX, protein complex consisting of Mre11/Rad50/Xrs2; NCO, non-crossover; ORF, open reading frame; RPA, replication protein A; ssDNA, single-stranded DNA; SNP, single nucleotide polymorphism; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labelling.

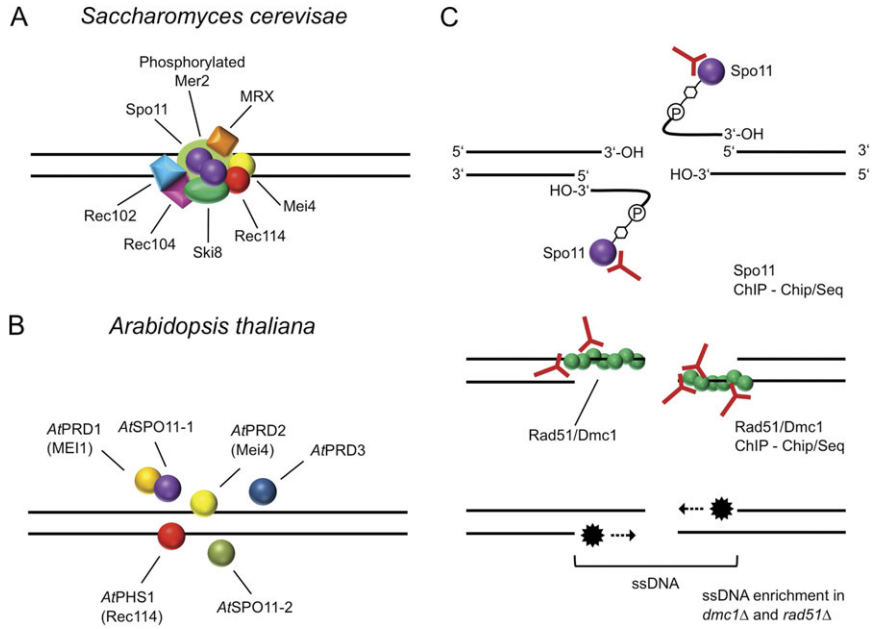

Fig. 1. Proteins involved in meiotic DNA DSB formation (related proteins are depicted in the same colour). (A) Schematic drawing of *S. cerevisiae* proteins essential for meiotic DSB formation. Please refer to the text for further explanations of spatial and temporal interactions of meiotic proteins. (B) Schematic drawing of *A. thaliana* proteins known to be essential for meiotic DSB formation. The spatial distribution of proteins is hypothetical and not supported by experimental data. So far, only the interaction of SPO11-1 and PRD1 has been demonstrated (De Muyt *et al.*, 2007). (C) Processing of SPO11-mediated DSBs. Different methods to identify meiotic DSBs are depicted next to the intermediates of meiotic recombination. Please refer to the text for further discussion.

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 ($H3K4^{me3}$) 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 $H3K4^{me3}$ 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 (Struhl, 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.

S. cerevisiae	S. pombe	A. thaliana	M. musculus	C. elegans	D. melanogaster	Function	References
Cdc28–Clb5* (CDK-S)						Kinase	Matos *et al.,* (2008); Sasanuma *et al.,* (2008); Wan *et al.,* (2008)
Cdc7–Dbf4* (DDK)						Kinase	Matos *et al.,* (2008); Sasanuma *et al.,* (2008); Wan *et al.,* (2008)
Mer2* (Rec107)						DSB formation	Cool and Malone (1992); Arora *et al.,* (2004)
Ski8* (Rec103)	Rec14*	SKI8/VIP3				DSB formation	Evans *et al.,* (1997); Tesse *et al.,* (2003); Arora *et al.,* (2004); Gardiner *et al.,* (1997); Steiner *et al.,* (2010)
Rec102*						DSB formation	Malone *et al.,* (1991); Salem *et al.,* (1999); Kee *et al.,* (2004); De Muyt *et al.,* (2009)
Rec104*						DSB formation	Malone *et al.,* (1991); Salem *et al.,* (1999); Kee *et al.,* (2004)
Rec114*	Rec7*	PHS1?				DSB formation	Malone *et al.,* (1991); Cervantes *et al.,* (2000); Davis and Smith (2001); Prieler *et al.,* (2005); Li et al., (2006); Sasanuma *et al.,* (2007); Ronceret *et al.,* (2009); Steiner *et al.,* (2010)
Mei4*	Rec24*	AtPRD2*	Mei4*			DSB formation	Menees and Roeder (1989); Martin-Castellanos *et al.,* (2005); Prieler *et al.,* (2005); Li *et al.,* (2006); Sasanuma *et al.,* (2007); De Muyt *et al.,* (2009); Kumar *et al.,* (2010); Steiner *et al.,* (2010)
Spo11*	Rec12*	SPO11-1*/ SPO11-2*	Spo11*	SPO11*	mei-W68*	DSB formation	Lin and Smith (1994); Bergerat *et al.,* (1997); Keeney *et al.,* (1997); Dernburg *et al.,* (1998); McKim and Hayashi-Hagihara (1998); Romanienko and Camerini-Otero (1999); Hartung and Puchta (2000); Steiner *et al.,* (2010)
Mre11*	Rad32	MRE11	Mre11	MRE11*	Mre11	DSB end processing	Ajimura *et al.,* (1993); Tavassoli *et al.,* (1995); Xiao and Weaver (1997); Chin and Villeneuve (2001); Ciapponi *et al.,* (2004)
Rad50*	Rad50	RAD50	Rad50	RAD50*	Rad50	DSB end processing	Kupiec and Simchen (1984); Luo et al., (1999); Gallego *et al.,* (2001); Colaiacovo *et al.,* (2002); Ciapponi *et al.,* (2004)

Table 1. *Continued*

S. cerevisiae	S. pombe	A. thaliana	M. musculus	C. elegans	D. melanogaster	Function	References
Set1			Prdm9			Methyltransferase	Nislow *et al.*, (1997); Baudat *et al.*, (2009); Mihola *et al.*, (2009); Myers *et al.*, (2009); Parvanov *et al.*, (2009)
	Rec6*					DSB formation	Lin and Smith (1994); Cervantes *et al.*, (2000); Davis and Smith (2001)
	Rec15*					DSB formation	Lin and Smith (1994, 1995a); Cervantes *et al.*, (2000); Davis and Smith (2001); Steiner *et al.*, (2010)
	Rec25?					Linear element component	Martin-Castellanos et al., (2005); Davis *et al.*, (2008)
	Rec27?					Linear element component	Martin-Castellanos *et al.*, (2005); Davis *et al.*, (2008)
	Mde2*					DSB formation	Gregan *et al.*, (2005); Steiner *et al.*, (2010)
Red1	Rec10*					Axial element, linear element protein	Rockmill and Roeder (1988); DeVeaux and Smith (1994); Lin and Smith (1995b); Lorenz *et al.*, (2004)
		AtPRD1*	Mei1*			DSB formation	Munroe *et al.*, (2000); De Muyt *et al.*, (2007)
		AtPRD3*				DSB formation	De Muyt *et al.*, (2009)
					mei-P22*	DSB formation	Sekelsky *et al.*, (1999)

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 receiving a meiotic DSB in mice and humans (Baudat *et al.*, 2009; Myers *et al.*, 2009; Parvanov *et al.*, 2009). 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 dimethlyated) (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 $H3K4^{me3}$ was correlated with high recombination activity at a given recombination hot spot (Baudat *et al.*, 2009). Analysis of $Prdm9^{-/-}$ mice showed that *Prdm9* is essential 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,

personal communication). Generally, the H3K4^{me3} 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 H3K9^{me2} 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. cerevisae* this connection is provided by the S-phase cyclin-dependent kinase Cdc28–Clb5 (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 Keeney, 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. H3K4^{me3}, 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 (Menees 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 *mer2Δ* (Li *et al.*, 2006). Rec114 is necessary for subsequent binding of Spo11 and Mre11 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 leptonema. 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

genomes, the already described *Arabidopsis AtPRD2* gene was recognized as the *Mei4* homologue and the already described *Arabidopsis AtPHS1* and maize *Zmphs1* genes as the *Rec114* homologues (Pawlowski *et al.*, 2004; De Muyt *et al.*, 2009; Ronceret *et al.*, 2009). The *PHS1* gene in maize and *Arabidopsis* is involved in pairing of homologous chromosomes. The maize mutants almost completely lack foci of the recombination protein RAD51 and, at metaphase, maize *phs1* mutant alleles show univalents (Pawlowski *et al.*, 2004). These observations are indicative of a functional conservation of *Rec114* homologues. Nevertheless, the authors of the study claim that DSB formation is not affected by mutations in the plant *PHS1* gene, but that *PHS1* is needed at the step of RAD51 nucleoprotein filament formation or RAD50 protein nuclear import, as broken DNA could be detected via TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labelling) staining (Pawlowski *et al.*, 2004; Ronceret *et al.*, 2009). *Arabidopsis* plants lacking a functional *Mei4* homologue, the *Atprd2* mutants, have no vegetative growth defects, but show short siliques, and meiotic DSB formation is blocked. Cytological analysis of the male meiocytes shows univalents and no chiasma formation (De Muyt *et al.*, 2009). Further investigations are needed to characterize both the plant Rec114 (PHS1) and Mei4 (PRD2) proteins and their impact on meiotic DSB formation.

Once Mer2, Mei4, and Rec114 are in place, they provide a platform for the binding of Spo11 (Fig. 1A). Spo11 is the catalytically active protein within the meiotic DSB complex (see below for details of the DNA cleavage reaction). Spo11, first described in *S. cerevisiae*, is related to the Top6A subunit of the archeal DNA topoisomerase type IIB (Bergerat *et al.*, 1997; Keeney *et al.*, 1997). This relationship (and other experiments discussed below) suggests that Spo11 needs to act as a dimer to catalyse DSB formation similar to type-II topoisomerases, by a transesterification reaction (Sasanuma *et al.*, 2007). The related type IIB topoisomerase consists of Top6A and Top6B subunits, but Top6B subunits have not been found in the genomes of eukaryotes, with the exception of plants (Hartung *et al.*, 2002; Yin *et al.*, 2002). In plants the Top6B homologues have no meiotic function (see below). This is consistent with the findings that the Top6B subunits provide ATPase activity, form a channel for DNA passage during the cleavage reaction, and allow subsequent re-sealing of the original DNA strand at the break site. During meiosis, this kind of re-ligation is not desired, and the processing and repair of the SPO11-mediated DSBs is performed differently (see below) (Corbett and Berger, 2003, 2004; Corbett *et al.*, 2007).

Spo11 is conserved among eukaryotes (Malik *et al.*, 2007) and has been found, for instance, in *C. elegans* (Dernburg *et al.*, 1998), in *S. pombe* (Rec12) (Lin and Smith, 1994), and in *Drosophila melanogaster* (mei-W68) (McKim and Hayashi-Hagihara, 1998). It has been found in all plants analysed so far. The *Arabidopsis* genome encodes, unlike those of mammals and yeast, where only one *Spo11* is present, three *Spo11* homologues, *AtSPO11-1*, *AtSPO11-2*, and *AtSPO11-3*, and additionally a *TOP6B* gene (Hartung and Puchta, 2000, 2001). Only *At*SPO11-1 and *At*SPO11-2 are essential for meiosis, whereas *At*SPO11-3 is needed for somatic endoreduplication and interacts with TOP6B (Grelon *et al.*, 2001; Hartung and Puchta, 2001; Stacey *et al.*, 2006). *AtSPO11-1* and *AtSPO11-2* are single-copy genes and possess all five conserved motifs previously described for Spo11 proteins (sequence identity 26–41%) (Bergerat *et al.*, 1997). *Atspo11-1*/*Atspo11-2* double mutants do not differ from the single mutants, indicating that the two proteins work together (Stacey *et al.*, 2006). It is attractive to speculate that AtSPO11-1 and AtSPO11-2 may form an obligate heterodimer for meiotic DSB formation. This idea is supported by the finding that the active tyrosine of both AtSPO11-1 and AtSPO11-2 (AtSPO11-1, Tyr103; AtSPO11-2, Tyr124) need to be functional for successful DSB formation (Fig. 1B) (Hartung *et al.*, 2007).

In yeast, the interaction of Spo11 with Mer2/Rec114 requires the Ski8 protein (Fig. 1A). Ski8 was first described as Rec103, found in a mutant screen overcoming the spore viability defects of a *rad52 spo13* haploid strain. Cloning and further characterization of Rec103 revealed that it is identical to Ski8 (Gardiner *et al.*, 1997). Ski8 seems to have a dual function, first in the mRNA decay pathway and secondly during meiosis (Arora *et al.*, 2004). Its multiple WD40 repeat structure (Evans *et al.*, 1997) allows multiple protein–protein interactions. It directly interacts with Spo11 and with Rec104, Rec114, and Mer2, as shown in yeast two-hybrid assays, apart from its non-meiosis-specific partners. Ski8 depends on Spo11 for nuclear entry and Ski8 stabilizes the association of Spo11 with meiotic chromosomes (Arora *et al.*, 2004). Interestingly, even though Ski8 is a conserved protein, functional differences have been observed. In *A. thaliana* Jolivet *et al.* identified a Ski8 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).

Rec102 and Rec104 were identified in a screen to isolate mutants defective in early steps of meiotic recombination (Malone *et al.*, 1991). They are meiosis specific, interact, and they mutually depend on each other to localize to chromatin, suggesting that they act as a functional unit (Salem *et al.*, 1999). They are needed for localizing Spo11 to the nucleus and to chromatin, and, furthermore, for Spo11 homodimer formation (Fig. 1A) (Kee *et al.*, 2004; Prieler *et al.*, 2005). Homologues of Rec102 and Rec104 have only been identified in yeasts closely related to *S. cerevisiae*.

In the budding yeast, three further proteins are essential for meiotic DSB formation (Fig. 1A). Mre11, Rad50, and Xrs2, forming the MRX complex, are conserved players in DNA repair. Mre11 was isolated in a screen for mutants with a defect in meiotic recombination (Ajimura *et al.*, 1993). Rad50 was first described by Kupiec and Simchen (1984) and found to be needed for resistance to γ-irradiation and methyl methanesulphonate (MMS). Xrs2 was first described

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 *AtRAD50* 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 nbs1-1/atm* 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* (*P*utative *R*ecombination initation *D*efect 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, synapsis, and recombination. No meiotic DSBs are formed in *swi1* mutants (Siddiqi *et al.*, 2000; Mercier *et al.*, 2001, 2003; Agashe *et al.*, 2002; Boateng *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 *rec25Δ* and *rec27Δ* 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 meiocyte 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 chromatin 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 chromatin 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 chromatin, 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 chromatin, 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 chromatin in *Atmre11-3*, *Atrad50*, and *Atcom1-1* 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 chromatin 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 Smc1, Smc3, Rec8, and Scc3. Scc1 is the kleisin of the mitotic cohesion complex and Rec8 is its meiotic paralogue (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 SYN1/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 differently 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 Sae2/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 resection 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, 2009*b*).

Following the resection of DNA strands in budding yeast, Rad52 assembles the sequence homology-dependent DSB repair machinery (Gasior *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 *AtRAD51*, *AtDMC1*, *AtXRCC3*, and *AtRAD51C* 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.*, 1995*a*, *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 (F_1 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; Jeffreys *et al.*, 2004; Tiemann-Boege *et al.*, 2006; Arnheim *et al.*, 2007; Kauppi *et al.*, 2007) (J. Drouaud 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 *rad50*$^{+}$ and *rad50S* strains. However, the signal intensity was lower in the *rad50*$^{+}$ 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 recombinases such as Rad51 and Dmc1. In the yeast *S. cerevisiae*, the turnover of these stretches of ssDNA is blocked in a *dmc1*Δ mutant strain. Therefore, ssDNA, generated at exactly the positions of a former DSB site, becomes enriched in a *dmc1*Δ 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 *dmc1*Δ meiotic cell cultures. They used benzoyl naphthoyl DEAE (BND) 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 *dmc1*Δ, and in total about five times more DSB sites were found in the *dmc1*Δ 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 *dmc1*Δ 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 througput 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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