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Analysis of the molecular function of ATM, ATR and FANCD2
during meiosis in *Arabidopsis thaliana*

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1. Table of Contents

1. TABLE OF CONTENTS.....	3
2. SUMMARY	7
3. ZUSAMMENFASSUNG	9
4. INTRODUCTION	11
4.1. DNA repair.....	11
4.1.1. DNA DSB repair in yeast.....	12
Non-homologous end joining in <i>S. cerevisiae</i>	12
Homologous recombination in <i>S. cerevisiae</i>	12
Regulation of NHEJ and HR in <i>Saccharomyces cerevisiae</i>	14
Mec1/Tel1 checkpoint signaling.....	14
4.1.2. DNA repair in mammals.....	16
Non-homologous end joining in mammals.....	17
Checkpoint signaling by ATM and ATR	17
Homologous recombination in mammals	19
Fanconi anemia	21
4.1.3. DNA repair in <i>Arabidopsis</i>	22
Non-homologous end joining and the MRN complex in <i>Arabidopsis</i>	23
Homologous recombination in <i>Arabidopsis</i>	24
AtATM and AtATR are involved in DNA repair.	26
4.2. Meiosis is the reductional cell division.....	27
4.2.1. Initiation of meiosis	27
4.2.2. Homologous recombination in meiosis.....	28
4.2.3. Synaptonemal complex formation	30
4.2.4. ATM and ATR are involved in meiotic recombination.....	31

4.2.5. Generation of four haploid spores in meiosis	33
4.3. The initial characterization of <i>AtFANCD2</i>	34
5. RESULTS	37
5.1. Fanconi anemia in <i>Arabidopsis</i>	37
5.1.1. Isolation and characterization of new mutant alleles of <i>AtFANCD2</i> ...	37
5.1.2. Seed per silique ratio of <i>AtFANCD2</i> mutant alleles is reduced in the <i>Atatm</i> mutant background.	38
5.1.3. Meiotic defects observed in <i>Atatm</i> and <i>Atatm Atfancd2-1</i> plants depend on AtSPO11-2	39
5.1.4. Markers of synapsis and initiation of recombination can be detected in <i>Atatm-2 Atfancd2-1</i> mutants.	42
5.1.5. New strategies to determine the molecular function of AtFANCD2 ..	45
5.1.6. Identification of other Fanconi anemia protein homologs in <i>Arabidopsis</i> 47	
5.1.7. Yeast two-hybrid search for interaction partners	49
5.2. ATM and ATR in <i>Arabidopsis</i>	51
5.2.1. Meiotic chromosome fragmentation in <i>Atatm Atatr</i> is suppressed in an <i>Atspo11-2</i> background	51
5.2.2. <i>Atatm-2 Atatr-2</i> double mutants fail to polymerize ZYP1 and do not form a Synaptonemal complex	54
5.2.3. Recombination is initiated in <i>Atatm-2 Atatr-2</i> double, but not in <i>Atatm-2 Atatr-2 Atspo11-2-3</i> triple mutants.	55
5.2.4. Phosphorylation of SQ/TQ sites is abrogated in <i>Atspo11-2</i> , <i>Atatm Atatr</i> and <i>Atatm Atatr Atspo11-2</i>	57
6. DISCUSSION	60
6.1.1. AtATM promotes DNA repair in <i>Arabidopsis</i> meiosis	60
6.1.2. AtFANCD2 plays a role in meiosis that acts in parallel to AtATM ...	61
6.1.3. AtFANCD2 interaction with a phosphatase PP2A subunit opens a connection to chromatin modification	62

6.1.4. New <i>Arabidopsis</i> homologs of Fanconi anemia pathway proteins are found by bioinformatic tools	62
6.1.5. AtATR acts in parallel with AtATM to promote meiotic synapsis and DNA repair	63
6.1.6. An <i>Atspo11-2-3</i> mutant rescues meiotic chromosome fragmentation in <i>Atatm-2</i> , <i>Atatm-2 Atfancd2-1</i> and <i>Atatm-2 Atatr-2</i>	64
6.1.7. Evidence of a Rad51-dependent and ATM/ATR-independent DNA repair pathway in <i>Arabidopsis</i> meiosis.....	64
6.2. Concluding words	65
 7. MATERIALS AND METHODS	 66
7.1. Media	66
Plant media	66
Yeast media	66
Bacterial media	67
7.2. Plant work.....	67
7.2.1. Growth conditions	67
7.2.2. Seed sterilization.....	67
7.2.3. <i>In planta</i> transformation – floral dip method.....	67
7.3. Yeast work	68
7.3.1. Yeast transformation.....	68
7.3.2. Yeast glycerol stock.....	68
7.3.3. Yeast-2-hybrid mating test.....	69
7.4. Bacterial work	69
7.4.1. Agrobacteria	69
Preparation of competent cells	69
Transformation of competent <i>Agrobacteria</i>	69
7.5. Protein work	70
7.5.1. Protein extraction.....	70

Ferguson extraction	70
7.5.2. SDS polyacrylamide gel electrophoresis (SDS-PAGE)	70
7.5.3. Western blot	70
7.6. Cytological procedures	71
7.6.1. Squashing preparation of pollen mother cells and DNA staining	71
7.6.2. Spreading preparation of pollen mother cells and immunostaining	72
7.7. PCR verification of plant genotypes	72
7.7.1. FANCD2	72
7.7.2. Fanconi Anemia genes.....	73
7.7.3. ATM, ATR, RAD51, SPO11-2	73
7.8. Supplementary data	74
7.8.1. List of abbreviations.....	74
7.8.2. Antibodies	75
7.8.3. Primers.....	75
7.8.4. List of alleles	77
7.8.5. Plasmid maps	78
7.8.6. Supplementary figures.....	84
8. LITERATURE	86

2. Summary

Arabidopsis has proven to be a powerful organism to study DNA repair and recombination since most genes related to these processes are not essential (e.g. do not lead to apoptosis and cell cycle arrest). Furthermore, most of the DNA repair proteins present in humans are found in the genome of *Arabidopsis*, including those that are not found in yeast.

One of these genes is *AtFANCD2*, the *Arabidopsis* homologue of human *FANCD2* (Fanconi anemia D2). Fanconi anemia is a genetic disease characterized by chromosome instability and severe pathological conditions. Upon DNA damage the FANCD2 protein is known to be mono-ubiquitinated and phosphorylated by checkpoint kinases ATM (Ataxia telangiectasia mutated) and ATR (ATM and Rad3 related). It was shown to co-localize together with DNA repair proteins and form nuclear foci, but the molecular function of the protein is still unclear.

In *Arabidopsis*, mutations in *AtFANCD2*, *AtATM* or *AtATR* do not lead to obvious growth defects, but mutant *Atatm* plants show only 20% fertility when compared to wild type. This fertility defect is further exacerbated in *Atatm Atfancd2* double mutants and complete sterility is observed in *Atatm Atatr* double mutants. A role of FANCD2 in meiosis has so far not been recognized.

Consistent with these observations, cytological analysis of pollen mother cells (PMCs) shows wild type-like meiotic progression for *Atfancd2* and *Atatr*. *Atatm* and the *Atatm Atfancd2* double mutant appear normal until pachytene, showing regular synapsis (ZYP1-polymerization) and recombination (RAD51-foci formation), but in later stages chromosome fragmentation and anaphase bridges become visible, being more pronounced in the double mutant.

In contrast, meiotic chromosomes in *Atatm Atatr* PMCs do not pair and synapse, but the number of AtRAD51 recombination foci are not reduced. In later meiotic stages of *Atatm Atatr*, massive DNA fragmentation accounts for the complete sterility of these plants.

There are two possible explanations for the observed chromosome fragmentation in *Atatm* single and double mutants, namely persisting DNA damage from pre-

meiotic stages or unrepaired DNA double-strand breaks generated by the meiosis-specific SPO11 nuclease. Introducing an *Atspo11-2* mutation into the *Atatm*, *Atatm Atfancd2* and *Atatm Atatr* plants reveals that DNA fragmentation is suppressed in all three plant lines.

Interestingly, the recombinase AtRAD51 appears in numerous foci in an *Atatm Atatr Atspo11-2* triple mutant, indicative for DNA lesions from pre-meiotic stages. Obviously, these breaks are reliably repaired during meiosis (most probably from the sister chromatid), whereas the breaks generated by AtSPO11 depend on AtATM and AtATR for repair during meiosis. Introducing an *Atrad51* mutation into the afore-mentioned backgrounds will help us understand the requirement for repair of SPO11 independent DNA breaks during meiosis.

3. Zusammenfassung

In der Modellpflanze *Arabidopsis thaliana* hat sich die Erforschung von DNA-Reparatur und Rekombination bewährt, da genetische Veränderungen in diesen biochemischen Prozessen in der Pflanze selten zu Apoptose oder Blockierung des Zellzyklus führen. Zusätzlich werden die meisten humanen DNA-Reparaturgene auch im *Arabidopsis*-Genom kodiert, darunter auch solche, die z.B. in der Hefe fehlen.

Eines dieser Gene ist *AtFANCD2*, das homologe *Arabidopsis*-Gen des humanen *FANCD2* (Fanconi-Anämie D2). Fanconi-Anämie ist eine genetische Krankheit, die durch Chromosomen-Instabilität und körperliche Fehlbildungen gekennzeichnet wird. Als Antwort auf DNA-Schäden wird das Protein FANCD2 ubiquitinyliert und von den Checkpointkinasen ATM (*Ataxia telangiectasia*-mutiert) und ATR (ATM und Rad3-bezogen) phosphoryliert. Es konnte bereits gezeigt werden, dass sich das Protein zusammen mit anderen DNA-Reparaturproteinen in Foci im Zellkern befindet, allerdings ist die konkrete Aufgabe von FANCD2 in diesen Foci noch rätselhaft.

Mutationen in den *Arabidopsis*-Genen *AtFANCD2*, *AtATM* oder *AtATR* beeinträchtigen die normale Entwicklung der Pflanzen nicht. Die Fertilität in mutierten *Atatm*-Pflanzen ist jedoch im Vergleich zu Wildtyp-Pflanzen auf ein Fünftel reduziert. Dieser Fertilitätsdefekt verschlimmert sich in *Atatm Atfancd2* Doppelmutanten, und *Atatm Atatr* Doppelmutanten sind völlig steril. Interessanterweise wurde eine Beteiligung von FANCD2 in meiotischen Prozessen bisher noch nicht beobachtet.

Im Einklang mit diesen Ergebnissen zeigte die zytologische Analyse von Pollenmutterzellen keine Veränderungen im Fortschreiten der Meiose bei *Atfancd2* und *Atatr* Mutanten. *Atatm* und *Atatm Atfancd2* Doppelmutanten erscheinen ebenfalls Wildtyp-ähnlich bis zum Pachytän, mit normaler Synapsis (ZYP1-Polymerisation) und Rekombination (RAD51-Foci-Bildung). In der weiteren meiotischen Entwicklung wird jedoch Chromosomen-Fragmentation sichtbar, wiederum verstärkt in der *Atatm Atfancd2* Doppelmutante im Vergleich zu *Atatm*.

Die homologen Chromosomen von *Atatm Atatr* Pollenmutterzellen paaren sich hingegen nicht, obwohl die Zahl der RAD51-Foci nicht reduziert scheint. Massive

DNA-Fragmentierung in späteren meiotischen Stadien bedingt die Infertilität dieser Pflanzen.

Zwei mögliche Ursachen für die beobachtete Chromosomen-Fragmentation scheinen wahrscheinlich, einerseits unreparierte DNA-Schäden aus premeiotischen Zellstadien, zweitens fehlerhafte Reparatur von Doppelstrangbrüchen (DSBs) der Meiose-spezifischen Nuklease SPO11. Das Einkreuzen einer *Atspo11-2* Mutation in Pflanzen des Genotyps *Atatm*, *Atatm Atfancd2* und *Atatm Atatr* unterdrückt diese Fragmentierung; folglich ist mangelhafte Reparatur von meiotischen DSBs der Grund für die Fertilitätsdefekte.

Erstaunlicherweise konnten zahlreiche RAD51-Foci in *Atatm Atatr Atspo11-2* Trippelmutanten detektiert werden, was möglicherweise auf premeiotische DNA-Schäden in diesem Genotyp hinweist. Anscheinend werden diese Brüche verlässlich in der Meiose repariert (wahrscheinlich über das Schwesterchromatid), wohingegen die Reparatur AtSPO11-induzierter Brüche von AtATM und AtATR abhängt. Das Einkreuzen einer *Atrad51* Mutation in diesen genetischen Kontext soll ermöglichen, die Rolle von RAD51 in der Reparatur von SPO11-unabhängigen meiotischen DSBs zu beleuchten.

4. Introduction

4.1. DNA repair

In all living organisms (except some viruses), DNA is the basis of all genetic information. It is of vital importance for a cell to maintain the integrity of this information and to protect it from damage that may arise from intra- or extracellular influences. If damage has occurred, organisms employ their DNA repair machinery, consisting of different sets of proteins that can restore an intact DNA molecule. Following detection of a DNA lesion, this repair machinery can excise, recombine and ligate DNA. Depending on the nature of the damage it can also stop cell proliferation or induce apoptosis.

A malfunction of just one of the repair proteins can result in DNA mutation and thereby alter gene expression of a cell. Consequently, inherited mutations of DNA repair genes often confer a high incidence of cancer.

While DNA single-strand breaks have been shown to be the more frequent DNA damage, they are very efficiently repaired and do not pose a lethal risk to the cell. This section will outline DNA repair pathways involved in correcting DNA double-strand breaks (DSBs). DSBs can be introduced intentionally in the cell as in V(D)J recombination, meiotic recombination (see Section 4.2), or topoisomerase action. They can also be the result of exogenous stress conditions, either directly after exposure to radiation or certain chemicals, or during the repair of stalled replication forks (when the cell encounters obstacles during S-phase). (Cromie *et al.*, 2001)

The repair of DNA double-strand breaks involves specific signaling cascades and the activation of either of two distinct repair, namely non-homologous end joining (NHEJ) and homologous recombination (HR) (Jeggo, 1998). They are tightly regulated on cell cycle, genetic and developmental levels (Haber, 2000).

4.1.1. DNA DSB repair in yeast

Saccharomyces cerevisiae has shown to be a valuable model to study DNA repair, as it is a simple eukaryotic system, with many genes and basic processes conserved between human and yeast.

As in humans, also in yeast DNA double-strand breaks are formed programmatically (meiotic recombination, mating type switching), or as damage from extracellular influences. Likewise, the repair of these lesions can involve proteins of homologous recombination or non-homologous end joining pathways, mostly depending on the cell cycle context. In contrast to humans, homologous recombination is generally considered the preferred pathway in *S. cerevisiae* if a related DNA sequence is present (e.g., on the homologous chromosome in diploid yeast, or on the (identical) sister chromatid during S-phase and G₂-phase). This makes yeast a prime model to study HR.

Most interestingly, DSB repair pathways in yeast share several proteins, and the interplay of these proteins is not yet fully understood.

Non-homologous end joining in *S. cerevisiae*

The purpose of the NHEJ pathway is the religation of DNA double-strand breaks. Yeast Ku complex occupies the DNA ends. The MRX protein complex (Mre11p, Rad50p, Xrs2p) that is indispensable during homologous recombination fulfills a bridging function, tethering the two molecules together. Endonucleases (Rad27p) and filling enzymes (Pol4) produce microhomology or end-compatibility and ligase Dnl4 finally ligates the break in this error-prone pathway. (Hefferin and Tomkinson, 2005)

Homologous recombination in *S. cerevisiae*

The purpose of the HR pathway is the repair of potentially lethal DNA double-strand breaks, using a homologous template for repair.

After formation of DSBs, DNA ends are processed to form 3' single-stranded tails. The MRX complex and Sae2 initiate 5' degradation, with Mre11 and/or Sae2 acting as a structure-specific nuclease (Haber, 1998; Mimitou and

Symington, 2008), Rad50 tethering the ends (Chen *et al.*, 2001), and Xrs2 adapting the complex to signals of the cell cycle (see below) (D'amours and Jackson, 2001). Helicase Sgs1 and nucleases Dna2 and Exo1 further process the 5' ends to generate 3' single-stranded tails (see Figure 4-1) (Zhu *et al.*, 2008).

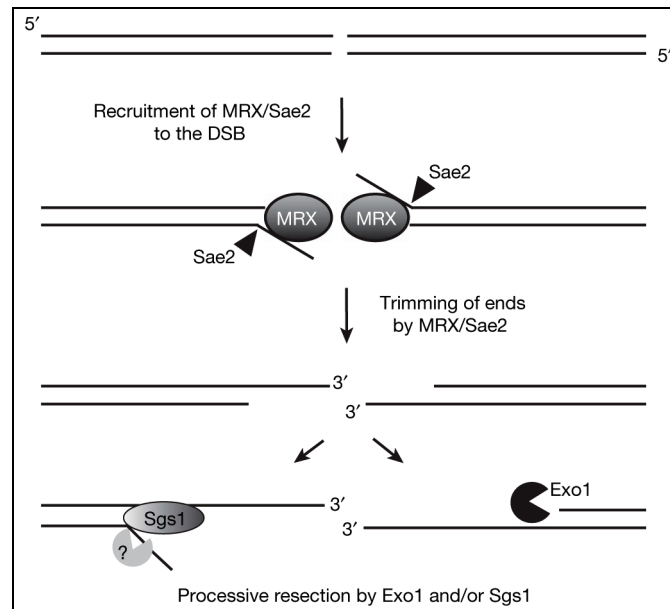


Figure 4-1 Double-strand break resection by MRX, Sae2/Com1, Sgs1, Dna2 and Exo1. This two-step reaction produces 3' ssDNA overhangs. (Taken from Mimitou and Symington, 2008)

RPA coats the so-formed single-strand DNA, thereby removing secondary ssDNA structures. Rad52 is thought to bind and displace RPA, at the same time recruiting the recombinase Rad51 (Sugiyama and Kowalczykowski, 2002). Rad51, a homolog of bacterial RecA, polymerizes on ssDNA depending on ATP and the Rad55/57 heterodimer (Sung, 1997). This nucleoprotein filament then interacts with another DNA molecule to initiate synapsis. Rad54 and Rdh54 facilitate this search for homologous sequences, promote formation of a displacement loop (D-loop) (Petukhova *et al.*, 1998) and translocate nucleosomes (Jaskelioff *et al.*, 2003).

The emerging joint DNA duplex is termed Holliday junction (HJ) (Holliday, 1974). Following DNA repair synthesis using the homologous template, the strand can be displaced and anneal back to the second resected end, ligate and form an intact molecule. This process is called synthesis-dependent strand

annealing (SDSA) (Allers and Lichten, 2001). Alternatively, the newly synthesized strand captures the second, non-invading end, forming a stable double Holliday junction (dHJ) (Szostak *et al.*, 1983). Branch migration elongates the conversion track before the junction sites are finally resolved, restoring an intact chromosome.

Regulation of NHEJ and HR in *Saccharomyces cerevisiae*

Homologous recombination and non-homologous end joining have long time been considered two competing processes. Concurrently, it seems obvious that the choice between the two depends on the cell cycle stage, as HR needs a homologous template for repair. *S. cerevisiae* has provided important insight into these mechanics. Regulation has been observed e.g. in G1 for haploid yeast, where NHEJ is the pathway of choice (Karathanasis and Wilson, 2002). Consequently, cell cycle proteins (Cdc28) and ploidy indicator proteins (mating-type regulated Nej1) are key determinants of the nature of DNA damage response (Daley *et al.*, 2005).

Another level of regulation is the DSB recognition and initial processing. Central to this aspect are the MRX complex proteins and the checkpoint kinases Mec1 and Tel1 (see below).

Mec1/Tel1 checkpoint signaling

DNA damage checkpoints protect genomic integrity by pausing the cell cycle and by activating and modulating repair pathways. Consistent with other eukaryotes, chromatin-associated Mec1 and Tel1 are the central proteins governing and signaling these events. (Mcgowan and Russell, 2004)

Mutant Mec1 (*esr1-1*) was first characterized in a screen for sensitivity to alkylating agents and UV light and associated defects in meiotic recombination (Kato and Ogawa, 1994). Being a PI3K-like kinase (PIKK) related to human ATR (see below), it phosphorylates and thereby activates a range of DNA repair proteins upon DNA damage sensing. An important target of Mec1 phosphorylation is Rad53, a kinase that mediates Mec1 checkpoint signaling by direct interaction with the cell cycle machinery. Mec1 is an essential gene; the

haploid null mutant is lethal (Kato and Ogawa, 1994), and a functional protein is required also in the absence of obvious DNA damage, possibly to ensure proper replication fork progression (Cha and Kleckner, 2002).

Tel1 was first shown to be defective in telomere length maintenance, and to be homologous to yeast Mec1 and human ATM (Greenwell *et al.*, 1995). Like Mec1, Tel1 is a very large protein with a PI3K homology domain. Tel1 has a checkpoint function that depends on DSBs and the Mre11 nuclease (Usui *et al.*, 2001). While a specific requirement for Tel1 has been shown for DNA damage response during S-phase (Nakada *et al.*, 2003), the *tel1Δ* allele shows significant checkpoint defects during other cell cycle stages only in the presence of hypomorphic *mec1* mutations (Nakada *et al.*, 2003). This led to the conclusion that Mec1 is the main DNA repair kinase and that Tel1 plays a limited role in this context (Carballo and Cha, 2007).

In a single-cell analysis, Lisby *et al.* (2004) show appealing data that the MRX complex localizes first to the DSB, activating Tel1. Tel1, on the other hand, channels the repair machinery into HR. As Ira *et al.* describe, this decision might also be a function of CDK availability (summarized in Garber *et al.*, 2005). Dissociation of Tel1 and MRX, and localization of Mec1 and other effectors precedes recruitment of Rad52 epistasis group proteins and synapsis.

Despite the differences in their timely activation, Mec1 and Tel1 share a variety of phosphorylation targets. Many research groups have tried to dissect the biological and biochemical relevance of Mec1 and Tel1 dependent regulation of MRX complex proteins, Sae2, replication protein A (RPA) and others (Baroni *et al.*, 2004; Bartrand *et al.*, 2006; Cartagena-Lirola *et al.*, 2006; Usui *et al.*, 2001). DNA damage-dependent phosphorylation of histone H2A variants (Tsukuda *et al.*, 2005) is of special interest, because it puts DSB repair in the context of epigenetic mechanisms.

Phosphorylated γ H2AX is one of the earliest marks of a double-strand break (Fillingham *et al.*, 2006). This chromatin modification elongates several kilobases around a DSB in yeast. It ensures the recruitment of DSB recognition and repair proteins (Fernandez-Capetillo *et al.*, 2004), and it is implicated in retaining repair

and signaling proteins until repair is complete (Nussenzweig and Paull, 2006) and γ H2AX is dephosphorylated (Keogh *et al.*, 2006).

4.1.2. DNA repair in mammals

While yeast is considered a lower eukaryotic model system, its DNA repair pathways are well conserved also in higher eukaryotes. In yeast, as in humans, double-strand breaks are repaired either by non-homologous end joining or homologous recombination. Most of the proteins central to these pathways are conserved between yeast and humans (e.g. Harrison and Haber, 2006; Su, 2006). Yet, evolution, multicellularity and genome size have adapted various aspects of DNA damage response in time. While conserved proteins can sometimes exert non-conserved functions, new effector molecules add new layers of complexity to the DNA repair machinery.

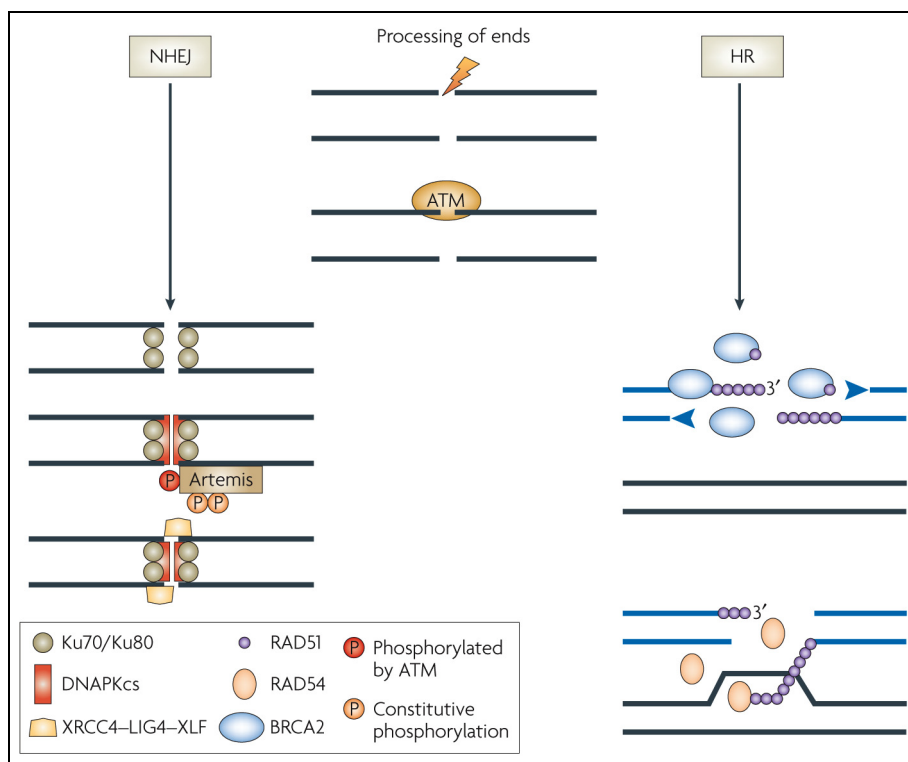


Figure 4-2 A double-strand break can be repaired via non-homologous end joining (NHEJ) or via homologous recombination (HR) in mammals. Both pathways can be activated in diploid mammalian cells, but HR requires an intact related

DNA sequence as a repair template. (Modified from Löbrich and Jeggo, 2007, figure 2)

Non-homologous end joining in mammals

When a DSB occurs, mammals can efficiently repair it via NHEJ. Ku proteins bind to dsDNA ends and recruit the DNA-dependent protein kinase (DNA-PKcs), a damage-sensing PIKK with no specific homolog in yeast. It is required to activate nucleolytic processing by the Artemis nuclease. This prepares DNA ends for ligation by Ligase IV and associated XRCC4-XLF (see Figure 4-2). (Sekiguchi and Ferguson, 2006)

The process of non-homologous end joining is not only activated in mammalian DNA damage repair, but also in V(D)J-recombination of antigen receptors during early development (Rooney *et al.*, 2005). Proteins of the NHEJ pathway are required to repair programmatically introduced DSBs, thus generating the incredible diversity of Ig genes that form the basis of adaptive immunity. Consequently, mutations in the genes coding for NHEJ proteins confer both sensitivity to DSB inducing agents as well as inherited immunological disorders. (O'Driscoll *et al.*, 2004)

Checkpoint signaling by ATM and ATR

As in yeast, also in mammals PI3K-like kinases are the crucial proteins activating the DNA repair network upon DSBs or replication fork stalling. Apart from DNA-PKcs, the signaling kinase involved in NHEJ (see above), these are the serine/threonine kinases ATM and ATR. The genetic disorders associated with biallelic hypomorphic mutations of *ATM* and *ATR* are called *Ataxia telangiectasia* and ATR-Seckel syndrome.

ATM and ATR were long time thought to be part of different repair pathways responding to distinctive stimuli. It was shown that ATM is primarily activated at the site of DSBs and triggers their repair (e.g. Cortez *et al.*, 1999), and that ATR governs the cell's response to stalled replication forks (Kumagai and Dunphy, 2006). This view, however, must integrate recent findings that ATR is also

recruited to DSB repair sites (Jazayeri *et al.*, 2006), and that ATM is phosphorylated by ATR following stalled replication forks (Stiff *et al.*, 2006). New models of ATM and ATR regulation are therefore emerging that incorporate this interplay (Hurley and Bunz, 2007).

Considering that *ATM* knockout mice are viable (Xu *et al.*, 1996) whereas homozygous disruptions of *ATR* are lethal (Brown and Baltimore, 2000) led to the notion that ATR has an important function apart from its role in DNA repair. Indeed, ATR was shown to trigger a signaling cascade that indirectly, via Chk1 kinase and Cdc25A phosphatase, regulates the level of cyclin-dependent kinases in S-phase (Syljuasen *et al.*, 2005), thereby regulating the progression of replication (Hurley and Bunz, 2007).

In response to a DNA DSB, activation of ATM occurs in different ways. Chromatin decondensation surrounding the DSB is thought to induce autophosphorylation and monomerization of ATM (Bakkenist and Kastan, 2003). Also, DSB recognition by MRN (see below) was shown to activate ATM and target it to the site of the break (Lee and Paull, 2005), possibly via binding to Nbs1 (Dupré *et al.*, 2006). BRCA1 could be an additional factor that recruits ATM to the DSB site (Kitagawa *et al.*, 2004).

ssDNA is bound by the RPA complex and leads to activation of ATR via ATRIP. This is observed also upon replication fork stalling, independently of ATM; though the downstream cascade differs slightly from DSB repair pathway (see below).

Checkpoint signaling of ATR and ATM is mediated via Chk1 (ATR), Chk2 and p53 (ATM) phosphorylation. By this means, cells can arrest the progression of the cell cycle to allow efficient repair of a lesion, or they can also induce cell death if the damage is too critical. (Reviewed in Su, 2006)

A hallmark of DSBs, and phosphorylated by the DNA damage-associated PIKKs (especially by ATM) is the histone variant H2AX. Thereby generated γ H2AX localizes to DSB-surrounding chromatin over Megabase long stretches, shortly after DSB formation (Stiff *et al.*, 2004). γ H2AX is thought to act as a specialized chromatin component that can efficiently constrain DNA repair factors to the site of the lesion (Kinner *et al.*, 2008). An important repair factor attracted by γ H2AX

is the breast cancer susceptibility protein 1 (BRCA1), an E3 ubiquitin ligase and DNA binding protein (Boulton, 2006). BRCA1's involvement in both NHEJ and HR (Shrivastav *et al.*, 2008) makes it a candidate for the regulation of pathway choice.

Homologous recombination in mammals

HR utilizes a homologous DNA template for repair and is therefore employed preferentially during S-phase, G2/M-phase and meiotic prophase.

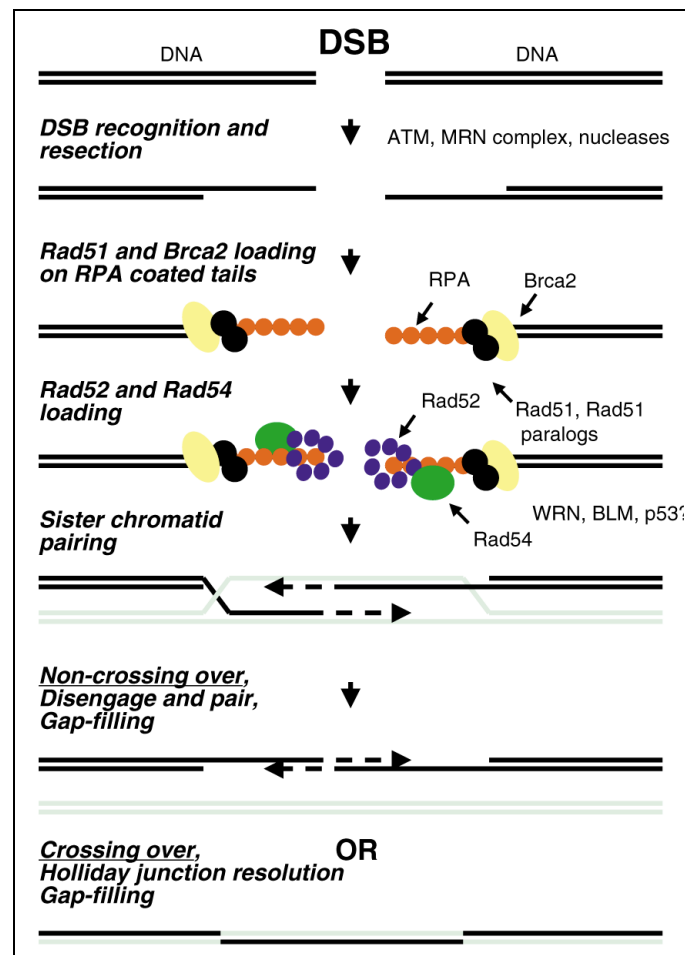


Figure 4-3 Model of homologous recombination in mammals. ATM, γ H2AX and BRCA1 are the first known factors at a DSB. ATM senses and activates the HR pathway, and γ H2AX and/or BRCA1 attract repair proteins. The MRN complex, CtIP and other nucleases generate 3' ssDNA overhangs that are coated with RPA. BRCA2 and Rad51 paralogs help loading of RAD51 onto the DNA.

RAD51 initiates recombination and synapsis with an intact DNA template (e.g., the sister chromatid in G₂ phase). RAD54 and RAD54B contribute to D-loop formation. Repair is completed either by crossing over (CO) or by synthesis-dependent strand annealing (NCO). (Taken from Valerie and Povirk, 2003, figure 4)

A complex of MRE11, RAD50 and NBS1 (MRN) proteins is required for recognition, tethering and signaling of a DSB (Moreno-Herrero *et al.*, 2005; Paull and Lee, 2005). Generation of ssDNA by MRN, CtIP and other nucleases (Valerie and Povirk, 2003) (probably analogous to yeast 5' resection, see above) brings about ATR/ATRIP, 9-1-1, and Rad17 complexes recruitment (Su, 2006). The function of the vertebrate RAD52 homolog is still not clear (Rijkers *et al.*, 1998), and so is the function of the vertebrate RAD51 paralogues (RAD51B/C/D, XRCC2/3). Deletion studies point to a RAD51 presynaptic filament establishing function (reviewed in Sung and Klein, 2006). Mutations in any of these genes can confer a higher risk of cancer as well as developmental pathologies. A similar function is fulfilled by the breast cancer susceptibility protein 2 (BRCA2/FANCD1) that acts as a mediator of recombination with intriguing structural features. BRCA2 contains a motif that mimics a RAD51 binding site, thus probably promoting RAD51 oligomerization (Pellegrini *et al.*, 2002). Other BRCA2 motifs can bind to a dsDNA/ssDNA junction *in vitro* and presumably target RAD51 to these sites during DNA repair (San Filippo *et al.*, 2008; Yang *et al.*, 2002).

Similar to yeast Rad54, human RAD54 and its paralog RAD54B are important in the context of chromatin remodeling and D-loop formation by RAD51 (Wesoly *et al.*, 2006; Zhang *et al.*, 2007).

Pathologies that are associated with defects in homologous recombination are Werner and Bloom syndromes. The corresponding genes, *WRN* and *BLM*, encode repair helicases of the RecQ family that are required for resolving HR intermediates (see Figure 4-3) and repair of stalled replication forks, respectively (Reliene *et al.*, 2007).

Fanconi anemia

A medical condition associated with a failure to repair stalled replication forks is Fanconi anemia (FA). FA is a severe human disease characterized by chromosomal instability, bone marrow failure, congenital skeletal abnormalities and a predisposition for cancer (Fanconi, 1967). At least 13 complementation groups or genes responsible for these clinical manifestations have been identified up-to-date (FANCA, -B, -C, -D1 (BRCA2), -D2, -E, -F, -G, -I, -J (BACH1/BRIP1), -L, -M, -N) (San Filippo *et al.*, 2008). Cell lines with mutated forms of either of these genes show a characteristic hypersensitivity to interstrand-crosslinking agents (ICLs) and thus imply a functional involvement in DNA repair processes.

Although cloning of the first Fanconi anemia gene dates back to 1992 (Strathdee *et al.*, 1992), with many more to follow in the recent years, the biochemical functions of the gene products have remained mostly unknown. On one hand, this is an indication of the complexity of the genetic disorder. On the other, the analysis is constricted by limited homology of most Fanconi anemia homologs in lower eukaryotes (Lee *et al.*, 2007) and the failure to establish an *in vitro* reconstitution of the pathway.

The FA/BRCA network, whose major signaling cascade comprises the FA proteins, is predominantly active in the S-phase of the cell cycle (Taniguchi *et al.*, 2002). It was shown to be responsive to DSBs as well as to replication fork stalling due to ICLs (Ishiai *et al.*, 2008). While FA cells are not necessarily HR defective (Niedzwiedz *et al.*, 2004), they show ICL hypersensitivity and a characteristic radial chromosome structure (breakage-induced chromatid fusions) (Niedernhofer *et al.*, 2005).

Recent data showed that the FA pathway is activated by ATR-dependent phosphorylation of FANCI (Ishiai *et al.*, 2008) and ATR- (or ATM-) dependent phosphorylation of FANCD2 (Pichierri and Rosselli, 2004). FANCI and FANCD2 were shown to co-IP as the so-called 'ID' complex (Smogorzewska *et al.*, 2007). Phosphorylation triggers mono-ubiquitination of FANCD2 by the FA core complex, an E3 ubiquitin ligase complex containing

FANCA/B/C/E/F/G/M and FANCL (the ligase). This, in turn, results in translocation of the ID complex to chromatin, where it can be detected immunologically in repair-associated foci together with BRCA1 and RAD51 (Taniguchi *et al.*, 2002).

As in the model of Niedernhofer *et al.* (Niedernhofer *et al.*, 2005), repair of ICL-induced replication stalling requires unwinding of DNA around the lesion (possibly mediated by FANCM helicase, in addition to its ability to sense and signal DNA damage (Collis *et al.*, 2008)); endonucleolytic cleavage to generate a free double-strand DNA end; error-prone translesion synthesis to create an intact template strand; and repair of the DSB via homologous recombination, probably involving BRCA1 and FANCI.

Important insights into the biology of Fanconi anemia come from *C.elegans*, a simple eukaryote that encodes homologs of human FANCD1/BRCA2 (*brc-2*), FANCD2 (*fcd-2*), FANCI (putative, *W02D3.10*), FANCI (*dog-1*) and FANCM (putative, *drh-3*) (Youds *et al.*, 2008). The *C.elegans* FA pathway responds to DSBs only during embryogenesis when DNA replication is increased (Lee *et al.*, 2007). *fcd-2* deletion mutants show, however, a sensitivity to ICL agents in somatic development (Collis *et al.*, 2006) and in meiosis (Lee *et al.*, 2007). Collis *et al.* (2006) could also show that the FCD-2 protein is mono-ubiquitylated in response to ICLs and forms foci that colocalize with other repair proteins. Checkpoint activation in *fcd-2* mutant S-phase is unaffected, pointing to a direct repair function of FCD-2 instead of a signaling function.

Unpublished data from the lab of Adriana La Volpe (2008) shows that the NHEJ pathway is upregulated in *fcd-2* mutants, and that FCD-2 is involved in inter-sister repair downstream of BRC-1 (*brc-1* is the homolog of mammalian BRCA1, see above). It is possible that FCD-2 is involved in regulating the choice between HR and NHEJ repair.

4.1.3. DNA repair in *Arabidopsis*

Similar to *C.elegans*, *Arabidopsis thaliana* has been extensively used as a model system to study DNA repair. Forward and reverse genetics have allowed

assessing the functions of various DNA repair proteins in plants, and researchers benefit from the publicly available mutant collections. Their results have an impact on both the understanding of DNA repair as well as on applied plant science (gene targeting) (Schuermann *et al.*, 2005).

Experimentally, analyses of DNA repair in plants include sensitivity assays or transcriptional activation assays (microarrays, e.g. Culligan *et al.*, 2006) following treatment with DNA damaging agents at high or low doses, respectively. Mutants that are implicated in meiotic HR can be tested for sterility.

In general, DNA repair pathways and proteins are conserved between plants and animals, notwithstanding that research on this topic is still fragmentary. NHEJ and HR have both been observed in plants (Bleuyard *et al.*, 2006). The preference for somatic DSB repair via NHEJ seems to be conserved between vertebrates and plants (Schuermann *et al.*, 2005).

Non-homologous end joining and the MRN complex in *Arabidopsis*

The NHEJ pathway in *Arabidopsis* is thought to function similarly as in yeast and mammals. Homologs of Ku70 and Ku80, AtKU70 and AtKU80, were shown to form a heterodimer (Tamura *et al.*, 2002) and to be required for NHEJ (Gallego *et al.*, 2003). Consistent with the major role of NHEJ in higher eukaryote DSB repair, AtKU70/80 show transcriptional activation after DSB formation with bleomycin or MMS (Tamura *et al.*, 2002). Similarly, *Arabidopsis* DNA ligase IV and AtXRCC4 are upregulated upon γ -irradiation and interact in yeast two-hybrid and affinity-column chromatography (West *et al.*, 2000). A homolog of the mammalian DNA-PKcs kinase has not yet been found in *Arabidopsis*.

As described above, yeast MRX complex is important for both NHEJ and HR. Homologs of Mre11 and Rad50 have been identified in *Arabidopsis* (Bundock and Hooykaas, 2002; Gallego *et al.*, 2001), whereas the third component of the complex shows similarities only to mammalian NBS1 (Akutsu *et al.*, 2007). Mutant *Atmre11* and *Atrad50* plants were shown to be MMS hypersensitive, thus implying a conserved function in DSB repair. Experiments on dysfunctional telomeres have shown that this role of AtMRE11 requires DNA microhomology

for efficient end-joining (Heacock *et al.*, 2004).

Mutant *Atnbs1* plants are only mildly MMS sensitive, but show an elevated sensitivity to interstrand-crosslinking agents (MMC) (Waterworth *et al.*, 2007).

Homologous recombination in *Arabidopsis*

Many proteins involved in HR are conserved from yeast to human (see above), and most of them have (putative) homologs in *Arabidopsis*. One exception is Rad52: the sequenced *Arabidopsis* genome does not encode a homologous protein.

The process of homologous recombination is also conserved between mammals and *Arabidopsis*, and knock-outs of *Arabidopsis* DNA repair genes often confer a comparable sensitivity to genotoxic agents as mutants of their mammalian counterparts do.

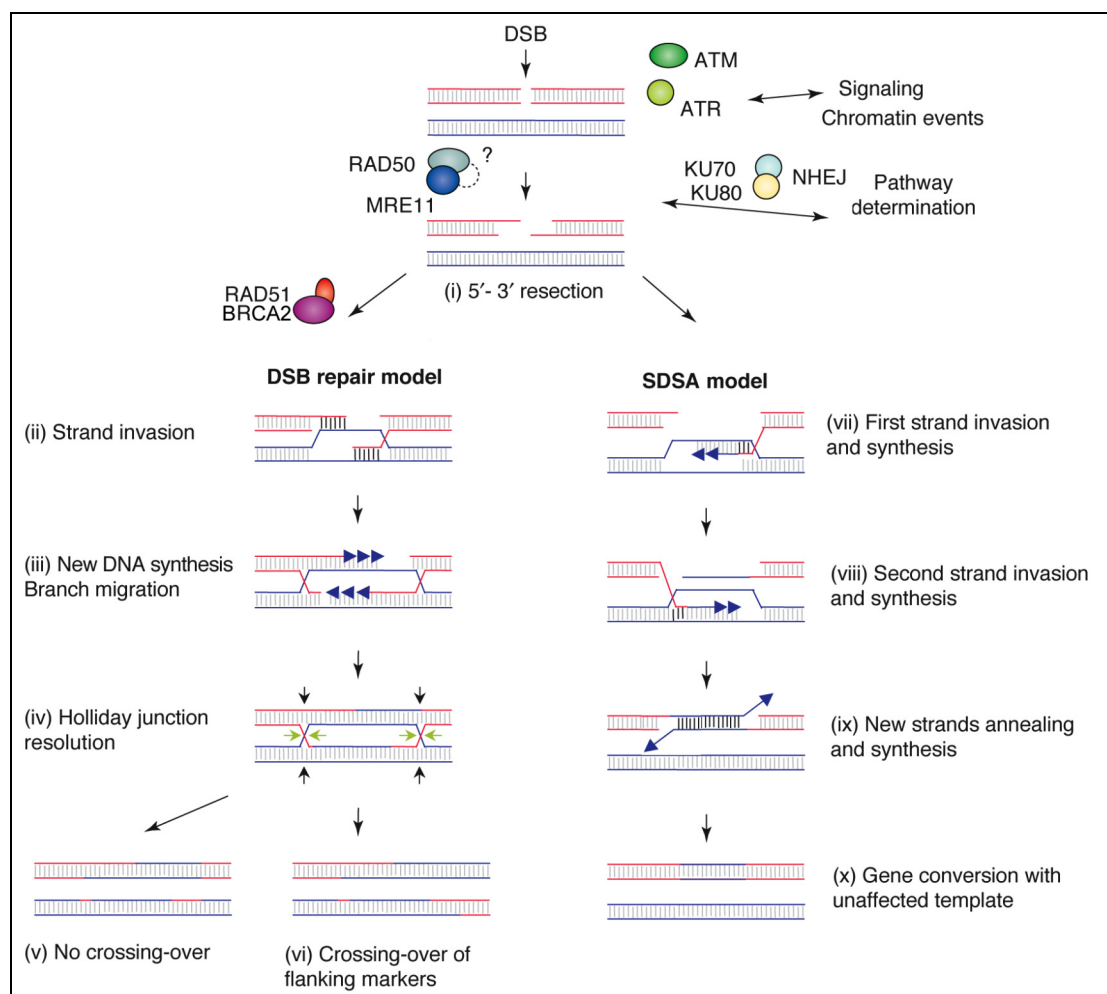


Figure 4-4 Model of homologous recombination in *Arabidopsis*. ATM acts as a DSB sensor, activating downstream events. MRN complex proteins initiate end

processing. End conformation, current cell cycle stage and other determinants channel repair to NHEJ (Ku-complex etc.) or HR pathways. BRCA2, RAD51 and RAD51 paralogs initiate synapsis. Synapsis is completed to form double HJs and HJ resolution. Alternatively, strands fall off the repair template after sufficient DNA synthesis and anneal, forming an intact dsDNA molecule. (Modified from Schuermann *et al.*, 2005, figure 2)

Besides the MRN complex proteins, whose function in homologous recombination has been shown in meiosis and, for AtMRE11, also in somatic tissue (Bleuyard *et al.*, 2004; Puizina *et al.*, 2004) (see below), homologs of the human RAD51 paralogs are also found in *Arabidopsis*. Bleuyard *et al.* (2005) could show that *Atrad51B/C* and *Atxrcc2/3* are hypersensitive to interstrand-crosslinking agents, but not to γ -irradiation. Interestingly, only mutations in the *AtRAD51C* and *AtXRCC3* genes – that supposedly form the ‘CX3’ complex – fail to complete meiosis, while mutant *Atrad51B/D* and *Atxrcc2* show no impairment in meiotic progression.

Yeast two-hybrid analysis showed that AtRAD51 and AtBRCA2 interact (Dray *et al.*, 2006), indicating that the mechanism of BRCA2 as a mediator of recombination (see above) is conserved between *Arabidopsis* and humans. A function for *Arabidopsis* AtRAD51 in somatic development has been detected only in the repair of ICLs (Markmann-Mulisch *et al.*, 2007). However, a *Physcomitrella patens* RAD51 mutant (*Pprad51AB*) from the same study (2007) shows hypersensitivity to DSB induction and impaired vegetative development. The authors argue that *Physcomitrella* plants are evolving slower than *Arabidopsis* plants and therefore preferentially employ an error-free repair mechanism. Therefore, the mutation of *RAD51* shuts off the main DSB repair pathway in *Physcomitrella*, but not in *Arabidopsis* plants.

A recent paper showed that AtRAD54 interacts with AtRAD51 in yeast two-hybrid analysis. Interestingly, the *Arabidopsis* protein can also complement MMS sensitivity in a Rad54-deficient yeast cell line, indicating a conserved function (Klutstein *et al.*, 2008).

AtATM and AtATR are involved in DNA repair.

In analogy to humans and yeast, PIKKs govern the plant cell's response to DNA damage. The *Arabidopsis* counterparts of human ATM and ATR are essential for the repair of DSBs (Garcia *et al.*, 2003) and DNA replication fork stalling (Culligan *et al.*, 2004), respectively.

Two recent papers (Culligan *et al.*, 2006; Ricaud *et al.*, 2007) illuminated the transcriptional activation mediated by AtATM and AtATR following DNA damage (i.e. ionizing radiation). Both experimental setups led to the conclusion that AtATM is the essential transcriptome effector, while AtATR only mildly contributes to the expression of damage response proteins. Ricaud *et al.* (2007) propose that the impact on transcription reflects phosphorylation by AtATM and subsequent auto-regulation of the corresponding protein. Interestingly, Garcia *et al.* (2003) could not detect any influence of an *Atatm-1* mutation on transcripts of recombination genes (*AtSPO11-1*, *AtRAD51*, *AtDMC1*; see below) in young inflorescences.

H2AX phosphorylation and foci formation is a rapid mark of DNA DSBs (see above). In accordance with AtATR playing only a secondary role in DSB response, γ H2AX foci formation after γ -irradiation is dependent mainly on phosphorylation by AtATM; still, averaged 10% of wild-type foci are reliant on AtATR (Friesner *et al.*, 2005).

Unlike human ATM and ATR, knockout mutants of plant PIKKs show no impairment during unperturbed vegetative life. Mutant *Atatm* plants are hypersensitive to DSBs (γ -irradiation, MMS) but not to UV-B (Garcia *et al.*, 2003). In contrast, *Atatr* mutant plants are only mildly sensitive to γ -irradiation and hypersensitive to replication blocking (aphidicolin, hydroxyurea, UV-B) (Culligan *et al.*, 2004). Moreover, AtATR regulates a DNA damage checkpoint in G2: wild type, but not *Atatr* mutant root cells arrest in G2 phase after aphidicolin treatment (Culligan *et al.*, 2004).

While the analysis of human *ATM ATR* double mutants is impeded by the fact that *ATR* conditional knockout cells ultimately exit the cell cycle (Brown and Baltimore, 2003), *Atatm Atatr* double mutants show an increased level of bridged chromosomes during mitosis (Vespa *et al.*, 2005).

4.2. Meiosis is the reductional cell division.

Meiosis is a specialized cell division in sexually reproducing eukaryotes. A diploid cell undergoes a meiotic DNA replication phase that is followed by two consecutive divisions: meiosis I is the segregation of homologous chromosomes, meiosis II separates sister chromatids. This yields four haploid cells or spores that can further divide mitotically to form the gametes.

The reduction to a haploid chromosomal state poses a fundamental problem for a diploid cell. While sister chromatids are bound together after S-phase by cohesin proteins and can thus be identified and separated in mitosis and meiosis II, proper segregation of homologous chromosomes in meiosis I is far more complicated. Two processes are crucial, namely homologous recombination and Synaptonemal complex (SC) formation. They coordinate homology-based pairing of non-sister chromatids and thus allow the formation of a physical connection (crossover) between paternal and maternal chromosomes (Zickler and Kleckner, 1999).

4.2.1. Initiation of meiosis

The meiotic cell cycle starts with an elongated premeiotic S-phase that includes loading of the meiosis-specific cohesin subunit Rec8. The *Arabidopsis Atrec8 (dif1)* mutant is characterized by univalent segregation during meiosis I and chromosome fragmentation (Bhatt *et al.*, 1999).

Cytologically, initiation of meiosis can be discerned at the beginning of meiotic prophase in leptotene, where chromosomes begin to condense and chromosome axes begin to form.

To start the meiotic HR program, numerous DSBs are formed, mediated by Spo11 in yeast (Keeney, 2001). Three Spo11 homologs are encoded by the *Arabidopsis* genome, but only AtSPO11-1 and AtSPO11-2 are involved in meiotic recombination (Grelon *et al.*, 2001; Hartung and Puchta, 2000; Stacey *et al.*, 2006). Unlike yeast and mammals, the activity of both AtSPO11 homologs is required for proper chromosome segregation: complementation of either *Atspo11-*

1-3 and *Atspo11-2-3* single mutants with the respective genomic DNA construct requires an intact catalytically active Tyr site (Hartung and Puchta, 2000; Hartung *et al.*, 2007).

In yeast, several other proteins are required for the initiation of meiotic DSB formation and localize in a cleavage complex, among these the MRX complex proteins (Keeney, 2001). Mutations of components of the Synaptonemal complex (Hop1, Red1) have a reduced number of programmed DSBs (Mao-Draayer *et al.*, 1996). In *Arabidopsis*, AtMRE11 is not required for Spo11-dependent DSB generation (Puizina *et al.*, 2004), yet it localizes to meiotic chromosomes independent of the presence of Spo11 and DSBs (Lohmiller *et al.*, 2008). Similarly, localization of AtASY1, the homolog of yeast Hop1, and formation of DSBs occur independently of DSBs (Sanchez-Moran *et al.*, 2007).

4.2.2. Homologous recombination in meiosis

Meiotic HR resembles homologous recombination after DNA damage-induced DSBs (see above).

An important difference is the need for the removal of Spo11 that remains covalently linked to DNA after DSB formation. This reaction requires the action of MRX complex and Com1/Sae2 in yeast (Hunter, 2006). Mutant alleles of *Arabidopsis AtRAD50* (Bleuyard *et al.*, 2004), *AtMRE11* (Puizina *et al.*, 2004) and *AtCOM1* are deficient in Spo11-induced DSB repair, and *Atcom1* shows accumulation of AtSPO11-1 foci (Uanschou *et al.*, 2007), thus providing evidence of a conserved mechanism of meiotic DSB processing. Together with Spo11 removal, these proteins are involved in resection of 5'-ends of DSBs to generate 3'ssDNA.

These single-stranded tails are immediately occupied by replication-protein A (RPA), thereby removing secondary structures of the ssDNA. RPA is displaced from DNA with the help of recombination mediators (e.g. BRCA2, San Filippo *et al.*, 2008) and Rad51 and its meiosis-specific homolog Dmc1 nucleate the 3' overhangs. Immuno-cytochemistry shows a large number of Rad51 and Dmc1 foci in zygotene stage (see below) (Franklin *et al.*, 1999; Vignard *et al.*, 2007).

Unlike Rad51-dependent mitotic homologous recombination that uses mainly genetic information of the sister chromatid as a template, meiosis requires bias towards a homologous chromatid (Schwacha and Kleckner, 1994); Dmc1 is important for this interhomolog (IH) bias (Schwacha and Kleckner, 1997). However, a recent study about Rad51's and Dmc1's recombinase activity *in vitro* shows no obvious differences in their filament formation, suggesting that this bias is likely mediated by accessory factors (see Figure 4-5) (Sheridan *et al.*, 2008). The Mnd1-Hop2 complex is such an accessory factor that binds to DNA and stimulates Dmc1 recombinase activity (Pezza *et al.*, 2006), and the absence of Hop2 leads to non-homologous pairing *in vivo* (Tsubouchi and Roeder, 2003).

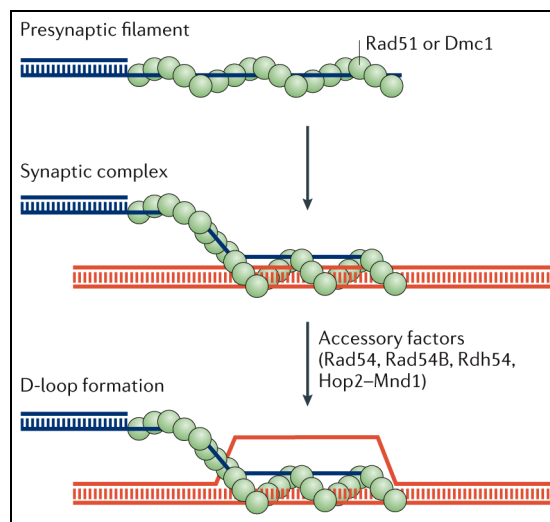


Figure 4-5 Initiation of recombination and D-loop formation. (Taken from Sung and Klein, 2006)

Homologs of Rad51 and Dmc1 have been identified in *Arabidopsis*. While *Atrad51* mutant plants show chromosome fragmentation during meiotic prophase, indicating a complete failure to repair Spo11-induced DSBs (Li *et al.*, 2004), *Atdmc1* mutants segregate intact univalent chromosomes due to repair of DSBs via inter-sister recombination (Couteau *et al.*, 1999; Vignard *et al.*, 2007). AtMND1 and AHP2 (homologs of Mnd1 and Hop2, respectively) interact in *Arabidopsis* (Kerzendorfer *et al.*, 2006), and AtDMC1 foci formation requires AtMND1 (Vignard *et al.*, 2007).

Other factors that contribute directly or indirectly to filament loading and strand exchange in *Arabidopsis* are Rad51 paralogs AtRAD51C and AtXRCC3 (Bleuyard *et al.*, 2005) and the regulator of recombination, AtBRCA2 (Siaud *et al.*, 2004). AtXRCC3 stimulates AtDMC1 foci formation in the presence of AtMND1, but its precise function is not yet clear (Vignard *et al.*, 2007). AtBRCA2 was shown to interact with both AtRAD51 and AtDMC1 in yeast two-hybrid experiments (Dray *et al.*, 2006), reinforcing its notion as a mediator of recombination.

4.2.3. Synaptonemal complex formation

While HR is required for proper meiotic chromosome segregation in yeast, plants and mammals, there is evidence of an early recognition of homologous chromosomes, independent of DSB formation, in all these organisms (Zickler, 2006). This rough alignment involves the telomeres that attach usually to the nuclear envelope (or the nucleolus in *Arabidopsis*), visible as ‘telomere bouquet’, at species-specific phases in meiosis. It is proposed that this step facilitates homolog recognition and chromosome pairing (Ma, 2006).

During leptotene stage of meiotic prophase, DSBs are formed and initiate homology search using the recombination machinery. These numerous associations along the chromosomal length are thought to promote pairing and synapsis. A proteinaceous structure called ‘Synaptonemal complex’ (SC) nucleates and extends along the homologous chromosomes, strengthening their interaction and contributing to the interhomolog bias (Zickler and Kleckner, 1999).

During the leptotene to zygotene transition, proteins constituting the ‘axial elements’ (AE) of the SC are installed as stretches along the chromatids. In yeast, these are comprised of Hop1 and Red1 proteins (Smith and Roeder, 1997). Phosphorylation of Hop1 by Mec1/Tel1 is required for Dmc1-dependent interhomolog repair of DSBs, the latter being mediated probably by the effector kinase Mek1 (Carballo *et al.*, 2008). This emphasizes the intricate regulatory network that controls homolog pairing and crossover formation.

SC component proteins share similar secondary structures among eukaryotes, but they do not necessarily have homologous sequences. In *Arabidopsis*, a homolog of yeast Hop1 has been identified and named ASY1. The *asy1* mutant is asynaptic, i.e. it does not form an SC and chromosomes segregate as univalents in meiosis I (Caryl *et al.*, 2000). A function in regulating the activity of AtDMC1 (and possibly AtRAD51) has recently been reported (Sanchez-Moran *et al.*, 2007).

Zygotene can be defined as the meiotic stage where AE proteins are polymerized continuously along the aligning homologous chromosomes. These filaments constitute the 'lateral elements' (LE) of the SC, and are gradually connected by transverse filament proteins, forming the 'central element' of the Synaptonemal complex. In yeast, Zip1 homodimers form this joint that connects the lateral elements of homologous chromosomes (Sym and Roeder, 1994). The *Arabidopsis* genome encodes two Zip1 structure homologs, *ZYP1A* and *ZYP1B*, which are functionally redundant. ZYP1 localizes to synapsed chromosomes, and *ZYP1^{RNAi}* plants shows defects in recombination (Higgins *et al.*, 2005).

During single-end invasion and dHJ formation, the central element elongates fully between the bivalents. This stage of full synapsis is termed pachytene.

4.2.4. ATM and ATR are involved in meiotic recombination.

An essential role for ATM and/or ATR in meiosis has been reported from a variety of organisms (Carballo and Cha, 2007).

ATM^{-/-} mice are infertile, as synapsis is abnormal, chromosomes fragment, and meiocytes arrest at the zygotene/pachytene stage (Xu *et al.*, 1996). Interestingly, immunolocalisation studies showed that in the absence of ATM, RAD51, DMC1 and ATR do not localize to the axial elements, whereas they do in wild type (Barlow *et al.*, 1998). It is proposed that in meiosis, PIKK proteins perform mainly a checkpoint role; however, a direct function in DSB repair cannot be ruled out.

Yeast Mec1 and Tel1 phosphorylate a variety of proteins involved in meiotic recombination (e.g. Mre11, Xrs2, Sae2, RPA, Hop1, Mek1). Since only *mec1Δ*

mutants show meiotic defects, while *tel1Δ* mutants divide normally in meiosis, primarily Mec1 seems to contribute to meiotic progress (Carballo and Cha, 2007). Carballo & Cha recently suggested that Mec1 is a promoter of meiotic recombination and synapsis, rather than negatively regulating meiotic progression as a checkpoint protein. Remarkably, they observed that a *mec1Δ tel1Δ* double knockout (in an *sml1Δ* background that abolishes *mec1Δ*-dependent lethality) is deficient in progression through meiotic S-phase (Carballo and Cha, 2007). Even though these cells progress normally through mitosis, certain meiosis-specific events during S-phase are not activated, and the cell cycle arrests when the cell is committed to meiosis.

Arabidopsis mutant alleles of *AtATM* show frequent chromosomal fragmentation during meiosis I, but a fraction of cells analyzed still produce fertile gametes (Garcia *et al.*, 2003). *Atatr* knockout plants, on the other hand, are fully fertile. Yet this does not exclude a role for AtATR in meiosis, as *Atatm Atatr* double knockout plants produce no viable seeds and progress through meiosis showing no synapsis and extensive fragmentation (Culligan *et al.*, 2004; Culligan and Britt, 2008) (see Figure 4-6). Introducing a mutant *Atspo11-1-1* allele that substantially reduces DSB formation in meiosis, the observed meiotic phenotype of *Atatm* and *Atatm Atatr* mutants can be corrected (Figure 4-6 F-H). This indicates that the *Arabidopsis* PIKKs have overlapping functions in suppressing chromosome fragmentation in meiotic DSB repair. (Culligan and Britt, 2008)

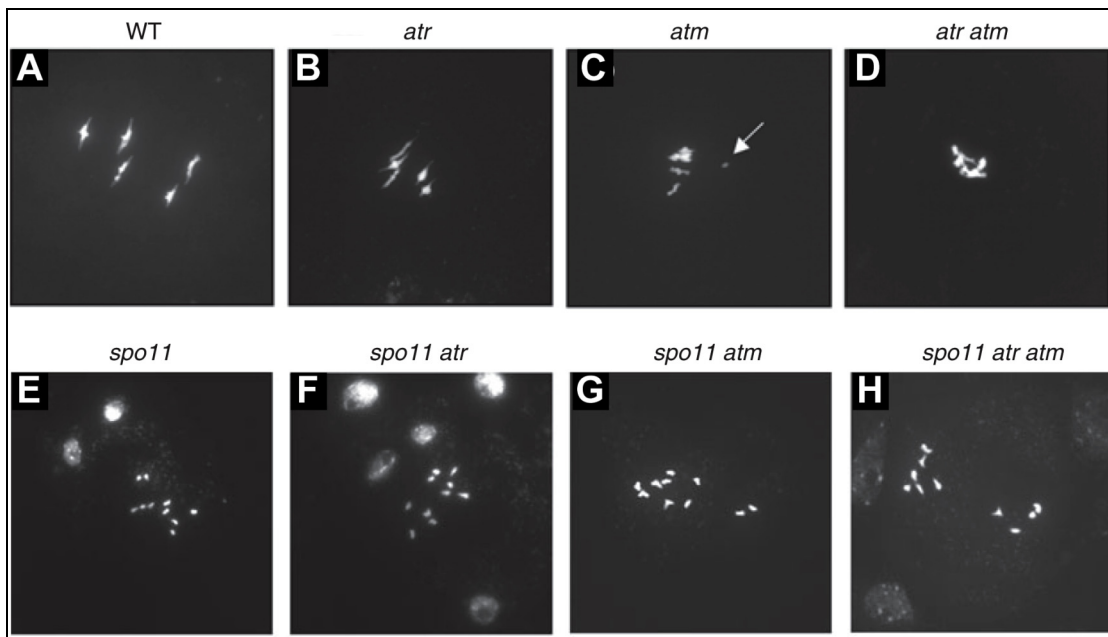


Figure 4-6 Chromosomal aberrations in *Atatm* and *Atatr Atatm* mutants is suppressed in an *Atspo11-1-1* background. Metaphase I stages from pollen mother cells, forming five bivalent chromosomes in wild type and *Atatr* mutants (A,B); forming chromatin fragments (arrow) and aggregates in *Atatm* and *Atatr Atatm* mutants (C,D); and segregating ten univalents in *Atspo11-1-1*, *Atspo11-1 Atatr*, *Atspo11-1 Atatm* and *Atspo11-1 Atatr Atatm* mutant plants (E-H). (Modified from Culligan and Britt, 2008)

4.2.5. Generation of four haploid spores in meiosis

Following pachytene stage (see above), the Synaptonemal complex is dissolved, and homologous chromosomes remain attached at sites of crossover (CO), the cytologically defined chiasmata.

In meiosis as in mitosis, repair of DSBs can result in formation of double Holliday junctions or in synthesis-dependent strand annealing (see above). At least in meiosis, this decision is highly regulated, because only the formation of stable dHJs ensures proper segregation of homologous chromosomes in meiosis I. Therefore, part of the Spo11-induced lesions and at least one DSB per bivalent chromosomes have to be repaired through this pathway. The major part, however, is thought to be repaired by synthesis-dependent strand annealing (McMahill *et al.*, 2007).

Chromosomes decondense in diplotene, only to be packed as x-shaped bivalents during diakinesis. At metaphase I, chromosomes align on the equatorial plane and the homologous chromosomes are pulled to the spindle poles in anaphase I. A mitosis-like division in meiosis II allows the formation of haploid tetrads that will develop into gametes. (Ma, 2006)

4.3. The initial characterization of *AtFANCD2*

Kerzendorfer *et al.* (Kerzendorfer and Schweizer, 2007) identified a gene with 25% protein identity to human FAND2 and named it *AtFANCD2* (At4g14970). Expression profiling could detect a transcript in all plant organs tested, but especially in proliferating tissue.

Analysis of the mutant allele *Atfancd2-1* (SALK_113293) revealed no impairment in growth and development of the plants, or in the analysis of DNA damage sensitivity and meiotic repair. As human FANCD2 function is known to be controlled by ATM and ATR (see above), the epistatic relationship between *AtFANCD2*, ATM and ATR was assessed genetically by producing double mutants.

Atatm mutant plants only generate ~15% seeds compared to wild type (Garcia *et al.*, 2003). In an *Atatm-2 Atfancd2-1* double mutant, fertility was further reduced approximately 7 times (2.5%). Cytological analysis of mutant pollen mother cells revealed that pachytene nuclei were normal, whereas diakinesis chromosomes showed interconnections, resulting in chromosome fragmentation during anaphase I (see Figure 4-7). These phenotypes were aggravated in the double mutant compared to the *Atatm* single mutant, in all cells tested. Therefore, *AtFANCD2* and *AtATM* might be involved in parallel pathways in response to meiotic DSBs. (Kerzendorfer and Schweizer, 2007)

Atatr mutants are sensitive to a variety of exogenous DNA damaging agents (Culligan *et al.*, 2004). The sensitivity to hydroxyurea or ICLs was not altered in *Atatr-2 Atfancd2-1* double mutants, suggesting that FANCD2 might act in the ATR pathway of replication fork repair. (Kerzendorfer and Schweizer, 2007)

By contrast, the Ku complex is a central component of the NHEJ pathway, also in plants (Gallego *et al.*, 2003). Hence, a knockout of Ku and of FANCD2 could confer a higher DNA damage sensitivity. Indeed, the double mutant *Atku80-5 Atfancd2-1* shows an elevated sensitivity to interstrand-crosslinking (Kerzendorfer and Schweizer, 2007).

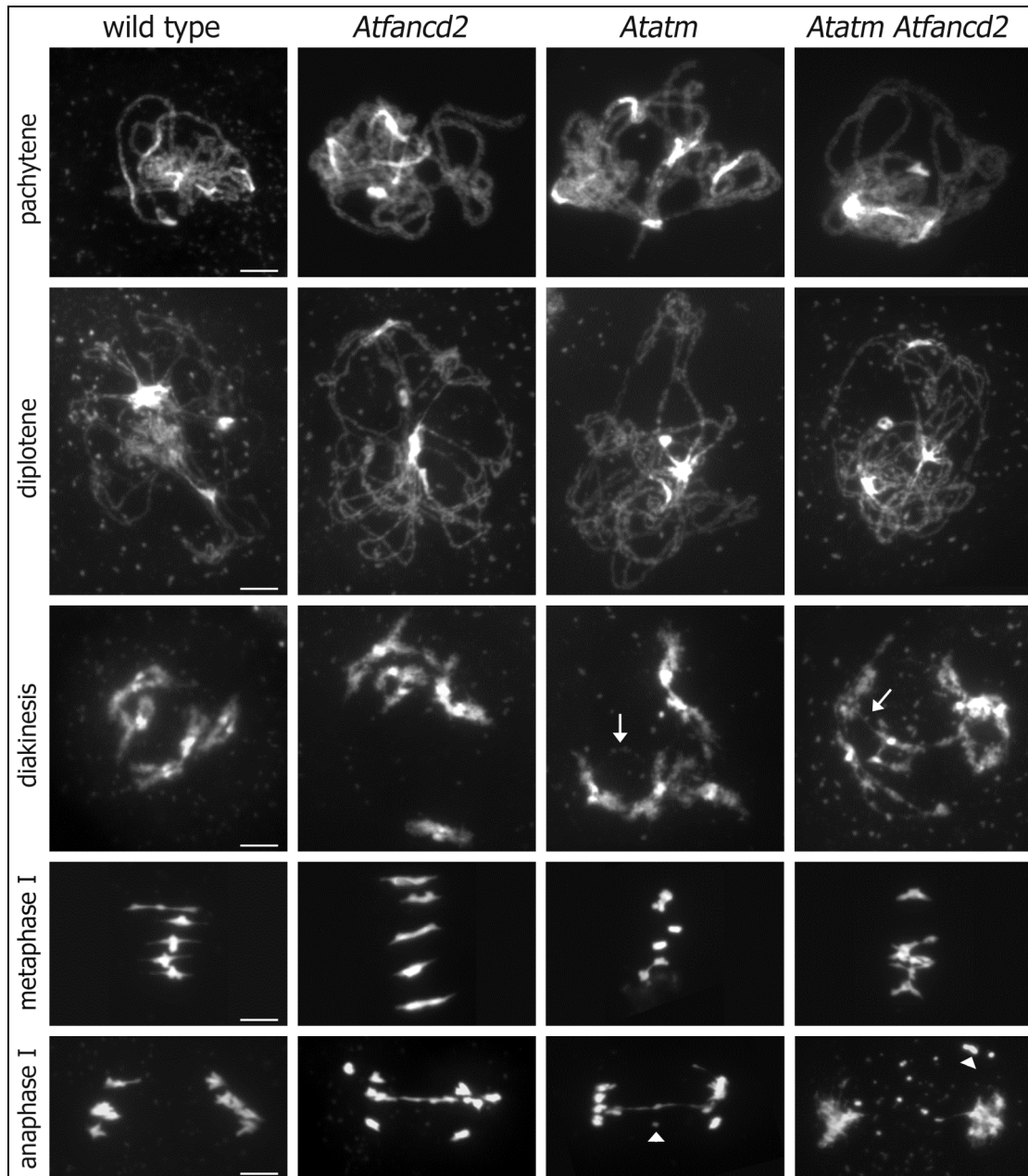


Figure 4-7 *Atfancd2* affects meiosis in the *Atatm* mutant background. Meiosis in wild type, *Atfancd2-1*, *Atatm-2* and *Atatm Atfancd2* pollen mother cells (selected stages shown). Pachytene synapsis is visible in all genotypes. *Atatm* and *Atatm Atfancd2* mutants show interconnected chromosomes in diakinesis (arrows). *Atatm* and

Atatm Atfancd2 show chromatin bridges and fragmentation (arrowheads), particularly in the double mutant. Size bar 5 μ m. (Modified from Kerzendorfer and Schweizer, 2007)

5. Results

5.1. Fanconi anemia in *Arabidopsis*

5.1.1. Isolation and characterization of new mutant alleles of *AtFANCD2*

In order to extend our knowledge of the *Arabidopsis* FANCD2 homolog and to confirm previous results, we analyzed a new *AtFANCD2* mutant allele, *Atfancd2-2*, and generated a transgenic plant encoding an RNAi construct against *FANCD2*, *AtFANCD2^{RNAi}* (see Figure 5-1).

Atfancd2-2 is a T-DNA insertion line from the [GABI-Kat](#) collection. The ~6kb T-DNA insertion was mapped to exon 19, possibly giving rise to a truncated AtFANCD2 protein. The homozygous mutant does not show any impairments in growth or fertility (Kerzendorfer and Schweizer, 2007), corroborating previous results found with *Atfancd2-1*.

The *AtFANCD2^{RNAi}* transgenic plant consists of a T-DNA insertion (introduced non-specifically in the genome) that encodes an artificial microRNA precursor against the *AtFANCD2* cDNA and a resistance gene. This small RNA uses the miRNA processing machinery to mature and target a complementary mRNA, thereby mediating its cleavage and essentially a knock-down of the gene's expression (Schwab *et al.*, 2006). The miRNA sequence was designed using the online available [Web MicroRNA Designer](#) and cloned by PCR according to the procedure described in (Schwab *et al.*, 2006). The PCR product was integrated in vector pCR2.1 (Invitrogen) and transferred to the binary vector pGreen0229::35S, combining the precursor miRNA with the consecutive 35S promoter and a resistance gene (phosphinotricin herbicide-resistance). The plasmid was shuttled into the plant genome using *Agrobacterium tumefaciens* and the floral dip procedure. Importantly, the resistant offspring grew and reproduced normally.

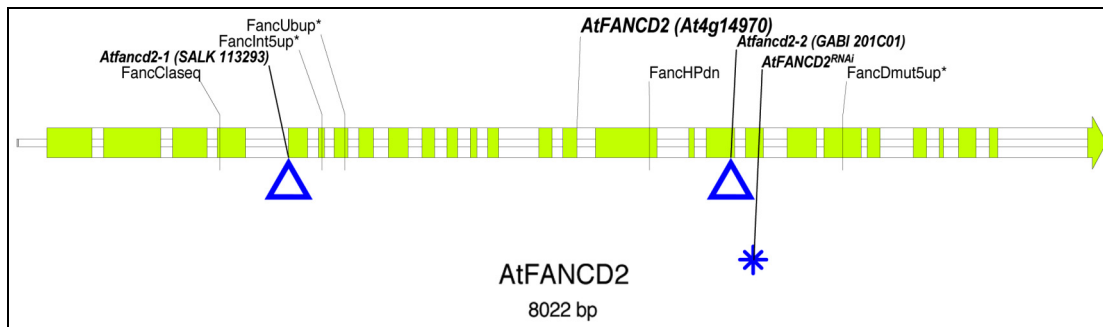


Figure 5-1 *AtFANCD2* mutant allele localization and primers. Drawing of genomic DNA (exons in green). Asterisks indicate insertion sites or miRNA-directed sequence, respectively. Genotypes are determined with the depicted primers and a T-DNA specific primer (see **Materials and Methods**).

5.1.2. Seed per silique ratio of *AtFANCD2* mutant alleles is reduced in the *Atatm* mutant background.

Earlier observations suggest that mutant *Atfancd2-1* compromises fertility in an *Atatm-2* background (Kerzendorfer and Schweizer, 2007). Seed per silique ratio resembles wild type in the *Atfancd2-1* single mutant, but drops to one seed per silique in *Atfancd2-1 Atatm-2* double mutants. This contrasts the *Atatm-2* single mutant that produces between seven and nine seeds per silique (Garcia *et al.*, 2003; Kerzendorfer and Schweizer, 2007).

For the purpose of confirming these results, *Atatm-2* heterozygous plants were crossed to *Atfancd2-2* as well as *AtFANCD2^{RNAi}* mutants and propagated to select for double homozygous plants. Phenotypic analysis indicates that the fertility defect is consistent in these double mutants. Plants develop normally but generate shorter siliques (see Figure 5-2), indicative of a reduced amount of viable offspring per silique.

Thorough analysis of seed ratios in *Atatm-2 Atfancd2-2* mutant plants and comparison with *Atatm Atfancd2-1* siliques reveals a significant and constant reduction of seed levels between the alleles and compared to the *Atatm-2* single mutant (see Figure 5-2). With seed per silique ratio normalized towards wild type, *Atfancd2-1* (96.3%) and *Atfancd2-2* (105.6%) mutant plants are fully fertile. *Atatm-2* mutant plants (15.2% to 21.5%; independent experiments) show a five- to

six-fold reduction of fertility compared to wild type, consistent with results from (Garcia *et al.*, 2003; Kerzendorfer and Schweizer, 2007). This reduction of fertility is aggravated in *Atatm-2 Atfancd2-1* (3.9%) and *Atatm-2 Atfancd2-2* (4.6%) double mutants, corresponding to a 25- to 20-fold reduction compared to wild type, or to about a five-fold reduction compared to the *Atatm-2* single mutant.

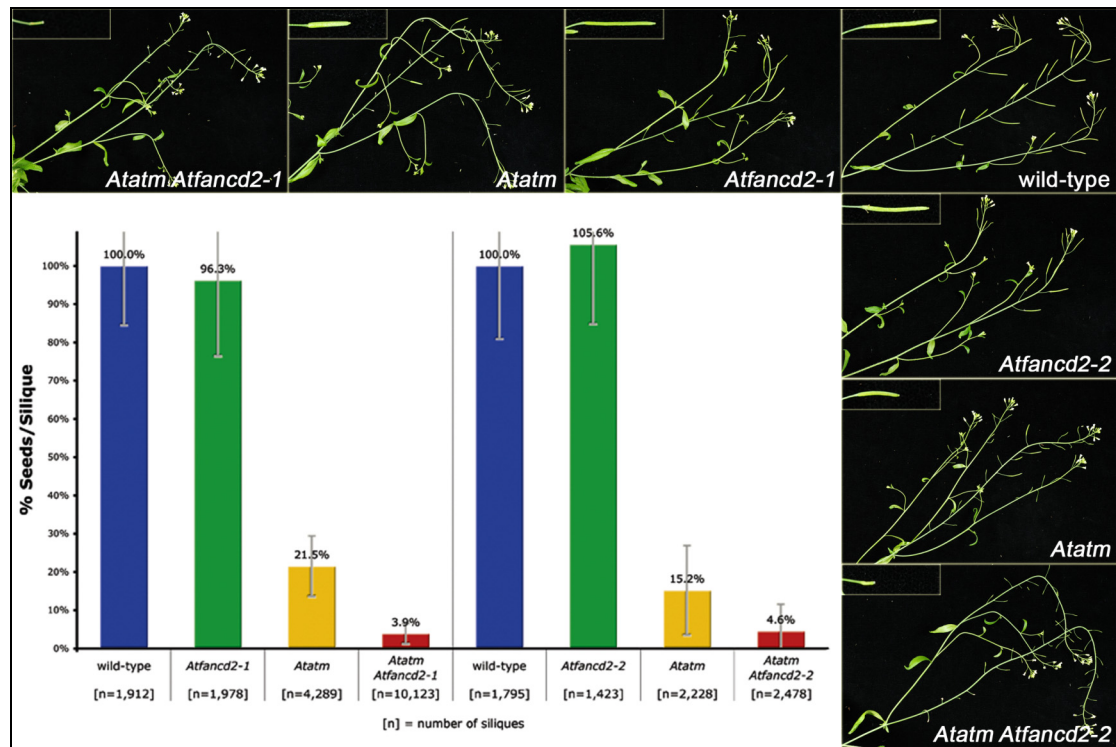


Figure 5-2 Seed per silique ratios and phenotypic analysis of *Atfancd2-1*, *Atfancd2-2* and *Atatm-2* single mutants and of *Atatm-2 Atfancd2-1* and *Atatm-2 Atfancd2-2* double mutants. Mean seed per silique ratio of wild type plants is set as 100%. Ratios of wild type and *Atfancd2* mutant lines are similar. As expected, seed levels decline in *Atatm* mutants, but this is greatly exacerbated in the *Atfancd2 Atatm* double mutants.

5.1.3. Meiotic defects observed in *Atatm* and *Atatm Atfancd2-1* plants depend on AtSPO11-2

The fertility defect of *Atatm* (Garcia *et al.*, 2003) and *Atatm Atfancd2-1* (Kerzendorfer and Schweizer, 2007) plants was shown to be the result of anaphase bridges and fragmented chromosome bodies, visible in meiotic

prophase of mutant pollen mother cells (see Figure 4-7). The DNA lesions causing this fragmentation could either stem from a failure to repair AtSPO11-induced meiotic DSBs or from DNA damage that arises before meiosis, possibly during pre-meiotic S-phase.

By eliminating the programmed meiotic double-strand breaks we unraveled the cause of the fragmentation. We generated crosses between *Atatm-2 Atfancd2-1* and *Atspo11-2-3* (GABI_749C12, Hartung *et al.*). Pollen mother cells of *Atspo11-2*, double and triple homozygous plants were analyzed cytologically for defects in chromosome segregation during meiosis. In *Atspo11-2-3* mutants, chromosomes do not synapse and paired sister chromatids segregate randomly during anaphase I (Hartung *et al.*, 2007) (see Figure 5-3 A,J). *Atatm-2* and *Atatm-2 Atfancd2-1* mutants show pairing of homologous chromosomes in pachytene (Figure 4-7), but these structures are absent in *Atatm-2 Atspo11-2-3* double (Figure 5-3 B) and *Atatm-2 Atfancd2-1 Atspo11-2-3* triple (C) mutant pollen mother cells. While *Atatm-2* and *Atatm-2 Atfancd2-1* mutants show fragmenting, but partly pairing homologous chromosomes in diakinesis (Figure 4-7), characteristic ten univalents are visible in diakinesis of *Atspo11-2-3*, *Atatm-2 Atspo11-2-3* and *Atatm-2 Atfancd2-1 Atspo11-2-3* mutants (Figure 5-3 G–I). In an *Atspo11-2-3* mutant background, *Atatm-2* and *Atatm-2 Atfancd2-1* plants segregate meiotic chromosomes analogous to the *Atspo11-2-3* single mutant (J–L). Rarely, interconnected chromosomes become apparent (arrowhead in L), but these structures are also reported for the *Atspo11-2-1* single mutant (Stacey *et al.*, 2006).

These observations led to the conclusion that the chromosome fragmentation in *Atatm-2* and *Atatm-2 Atfancd2-1* mutants is due to a deficiency to repair AtSPO11-induced DNA double-strand breaks.

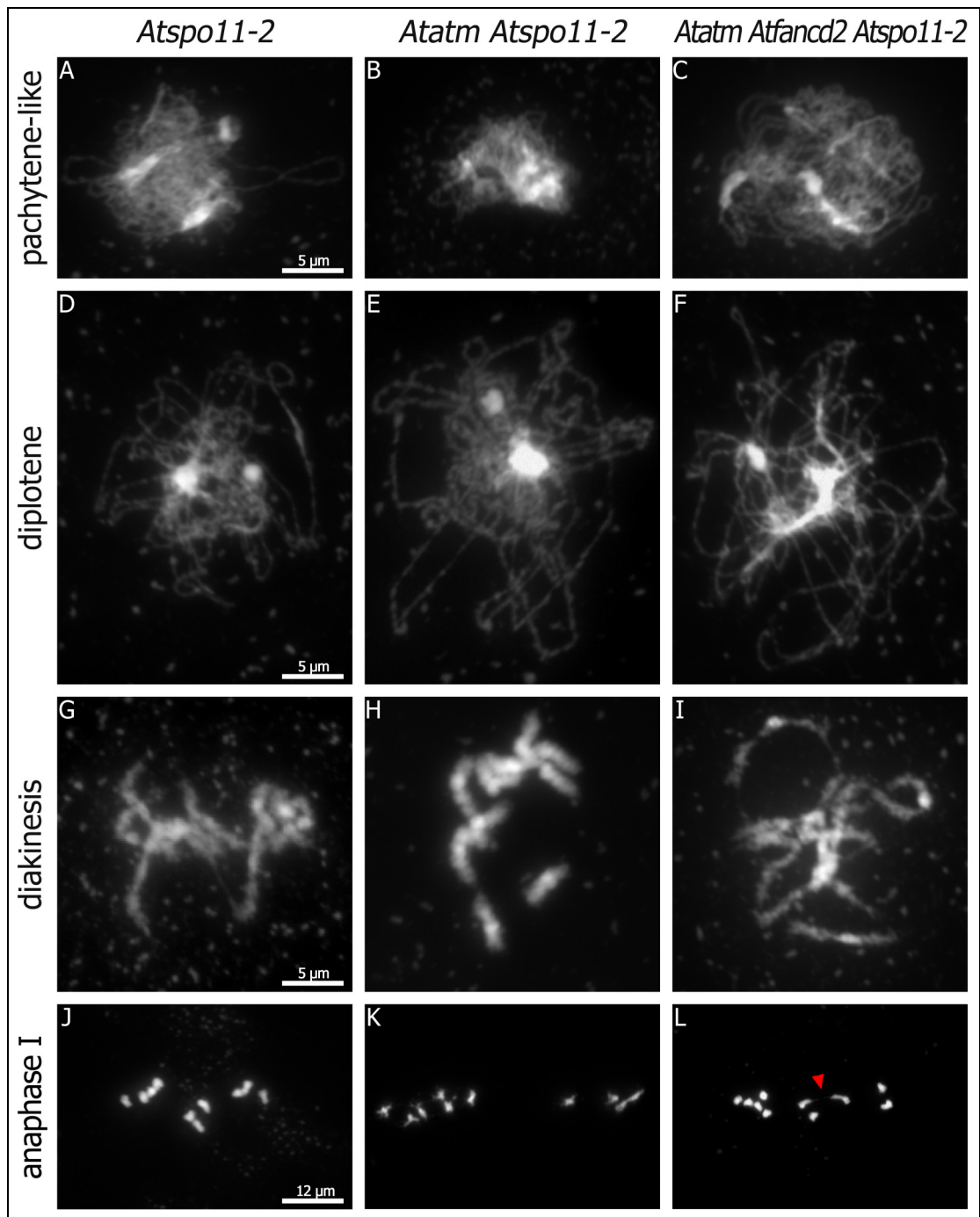


Figure 5-3 Meiotic chromosome fragmentation of *Atatm* and *Atatm Atfancd2* plants depends on *AtSPO11-2*. Squash preparations of pollen mother cells were stained with 4',6 Diamidino-2-phenylindol (DAPI). Genotypes are all homozygous; *Atspo11-2-3* for (A,D,G,J), *Atatm-2 Atspo11-2-3* for (B,E,H,K) and *Atatm-2 Atfancd2-1 Atspo11-2-3* for (C,F,I,L). During meiotic prophase, no pairing of the homologs can be detected (A–C). While chromatin morphology is similar to wild type during diplotene (D–F), ten univalent chromosomes become visible during diakinesis (G–I). Subsequently, these are randomly pulled to the spindle poles,

often resulting in aneuploidy (J–L). (L) Interconnected chromosomes can sometimes be detected (arrowhead), and this has been observed also by others (Stacey *et al.*, 2006).

5.1.4. Markers of synapsis and initiation of recombination can be detected in *Atatm-2 Atfancd2-1* mutants.

The observed meiotic defects in *Atatm-2* and *Atatm-2 Atfancd2-1* pollen mother cells depend on AtSPO11-2. This indicates that AtATM and AtFANCD2 are directly involved in the repair or signaling of SPO11-induced DSBs.

Mutants of genes involved in the repair of SPO11-induced DSBs typically fail to pair homologous chromosomes (e.g., *Atrad51c-1*, Bleuyard *et al.*, 2005; *Atmnd1*, Kerzendorfer *et al.*, 2006; *Atcom1-1*, Uanschou *et al.*, 2007; *Atnbs1-1 Atatm-3* double mutant, Waterworth *et al.*, 2007). By contrast, *Atatm-2* (Garcia *et al.*, 2003), *Atatm-2 Atfancd2-1* (Figure 4-7) (Kerzendorfer and Schweizer, 2007) and *Atxrcc3* (Bleuyard and White, 2004) mutant plants show pairing of homologous chromosomes when staining pollen mother cells for DNA. Synapsis and recombination are central to correct homolog pairing in meiosis. We therefore analyzed two crucial players in these processes, namely ZYP1 and AtRAD51, via immuno-cytochemistry (see below). ZYP1 forms the central element of the tripartite Synaptonemal complex. Hence it is a marker for synapsis and its complete elongation is used as an indicator of completed pachytene stage (Osman and Subramani, 1998). AtRAD51 and AtDMC1 are the essential recombinase proteins involved in meiotic HR, and RAD51 localizes to *Arabidopsis* (Mercier *et al.*, 2003) and maize (Franklin *et al.*, 1999) pachytene chromosomes as few foci. This can also be observed in mouse meiosis (Barlow *et al.*, 1997) and probably indicates sites of future crossing over.

Synaptonemal complex formation seems unaffected in *Atatm* and *Atatm Atfancd2-1* mutants as ZYP1 protein can be detected all along the synapsed chromosomes in many of the analyzed cells (see Figure 5-4).

AtRAD51 protein can be detected normally throughout zygotene as numerous chromatin-associated foci (see Supplementary Figure 7-13) (Kerzendorfer *et al.*,

2006). Advanced homologous recombination depletes AtRAD51 protein as is visible in our fully synapsed wild type nuclei (Figure 5-4). A similar progression of DSB repair occurs probably in the mutant cells, as AtRAD51 foci number in *Atfancd2-1*, *Atatm-2* and *Atatm-2 Atfancd2-1* is comparable to wild type.

The observation that synapsis and initiation of recombination are unaffected in *Atfancd2-1*, *Atatm-2* and *Atatm-2 Atfancd2-1* mutants does not ultimately exclude a role of ATM and FANCD2 as promoters of synapsis and homologous recombination, as proposed for yeast Mec1 (Carballo and Cha, 2007) or for *C.elegans fcd-2* (see above). AtATR or another regulator could perform a similar function that compensates for the loss of AtATM and AtFANCD2.

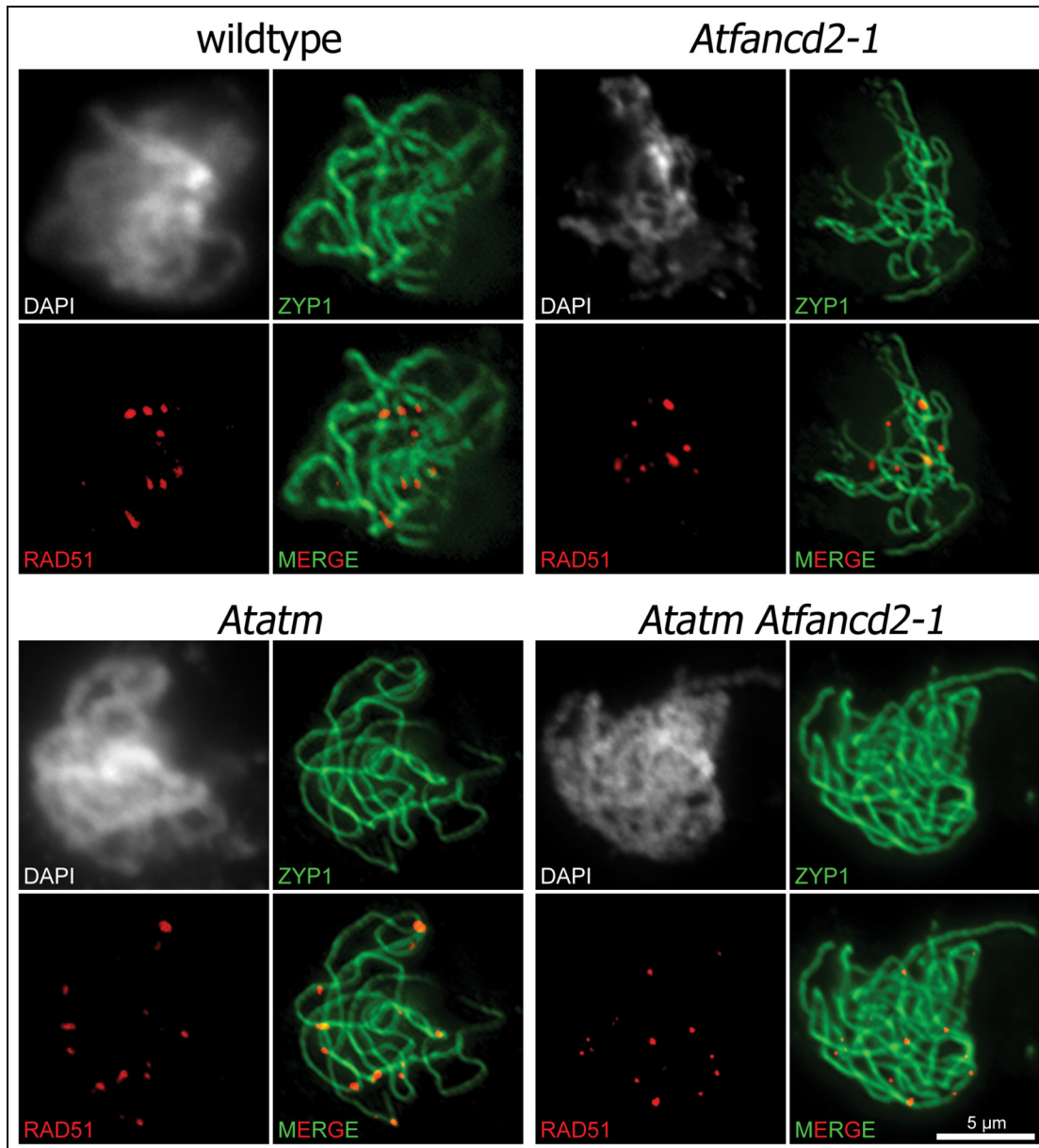


Figure 5-4 AtRAD51 foci generation and ZYP1 filament formation is unaffected in *Atatm* and *Atatm Atfancd2-1* mutants. Pollen mother cells of wild type, *Atfancd2-1*, *Atatm* and *Atatm Atfancd2-1* double mutants were probed with antibodies against AtRAD51 and ZYP1. Chromatin was counter-stained with DAPI. Fully elongated ZYP1 staining was used to identify pachytene chromosomes. AtRAD51 foci number is reduced during *Arabidopsis* pachytene stage, and the number of foci is comparable between wild type and mutant nuclei.

5.1.5. New strategies to determine the molecular function of AtFANCD2

Human FANCD2 can be detected biochemically either in the native form or with posttranslational modifications (phosphorylation or mono-ubiquitination) upon DNA damage. An antibody against *Arabidopsis* FANCD2 would allow the detection of the protein in different genetic or environmental contexts and could be helpful to determine its localization and molecular function in meiotic cells.

Western blot detection of AtFANCD2 is limited by the unavailability of a positive control and possibly by confined protein expression in particular plant cell types or tissues.

A polyclonal antibody against an N-terminal region of AtFANCD2 has been raised before, but it did not allow the detection of the endogenous protein in plant protein extracts (Kerzendorfer and Schweizer, 2007). We analyzed the presumable AtFANCD2 protein sequence with bioinformatic tools to identify regions that are likely to be on the surface of the folded protein. [Eurogentec](#) synthesized these three peptides (see Figure 5-5, Supplementary table Antibodies), immunized two rabbits and purified the corresponding antibodies on columns.

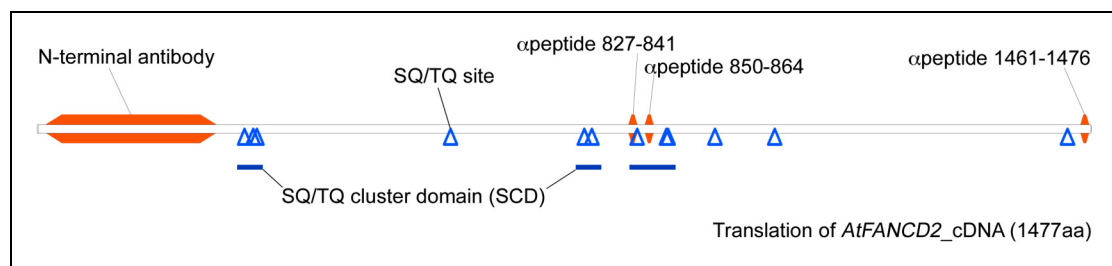


Figure 5-5 Translation of *AtFANCD2* cDNA and localization of antigens and SQ/TQ motifs. Localization of (poly)peptides used for antibody generation is shown in orange. Possible sites for SQ/TQ phosphorylation by ATM/ATR are indicated in blue (see **Discussion**).

These antibodies were used to detect the endogenous protein on Western blots of whole plant extracts (see Protein work). Unfortunately, no signal was found by chemiluminescent detection. It is possible that the expression level of

AtFANCD2 is below the detection limit or that the protein is mainly expressed in particular plant tissues. However, semi-quantitative RT-PCR results of Kerzendorfer and Schweizer (2007) and publicly available Genevestigator microarray data (www.genevestigator.ethz.ch) confirm that the *AtFANCD2* gene is ubiquitously transcribed within the plant body.

In order to possess a positive control to test the sensitivity and specificity of the peptide antibodies, we used protein extracts of yeast strains transfected with a yeast two-hybrid plasmid containing the cDNA of FANCD2. We also utilized protein extracts of IPTG-inducible *E.coli* expression systems (Rosetta™, Novagen) expressing a C-terminal fragment of FANCD2 (see Figure 7-12, Figure 7-14). Both protein extracts did not produce a signal when tested with the FANCD2 α peptide antibodies in Western blotting experiments.

***Atatm Atfancd2-1* mutant plants cannot be complemented by an *AtFANCD2*–HA₃ T-DNA construct.**

The *AtFANCD2* cDNA has been cloned before via reverse transcription. The coding sequence was tagged with a triple hemagglutinin-tag (HA-tag) and transformed into *Atfancd2-1* mutant plants. (Kerzendorfer and Schweizer, 2007)

We analyzed protein extracts of these plants in a Western blot experiment with an HA antibody (see Protein work, Antibodies). Unfortunately, a distinctive signal that was missing in *Atfancd2-1* and *Atfancd2-2* mutant plants without *AtFANCD2*–HA₃ T-DNA construct could not be detected.

As the *Atfancd2* mutation alone provides no apparent phenotype, complementation analysis relies on the meiotic defect of *Atfancd2* in the *Atatm-2* mutant background. Therefore, we crossed plants harboring a tagged cDNA construct with *Atfancd2-1 Atatm-2* double mutant plants. We observed no rescue of fertility in plants homozygous for *Atfancd2-1* and *Atatm-2* and carrying at least one cDNA construct. Thus, the *AtFANCD2*–HA₃ T-DNA construct cannot complement the observed fertility defects of *Atfancd2-1 Atatm-2* mutant plants.

FANCD2 α peptide antibodies do not produce a specific signal in immuno-cytochemistry experiments.

We employed the FANCD2 α peptide antibodies also in immuno-cytochemistry experiments (see below). A weak chromatin-associated staining could be detected in certain experimental settings. Unfortunately, these signals were also detectable in pollen mother cells of *Atfancd2-1* mutant plants. It is possible that a truncated form of AtFANCD2 is expressed in these plants and detected by the peptide antibodies.

5.1.6. Identification of other Fanconi anemia protein homologs in *Arabidopsis*

BLAST search against genbank allowed us to find possible homologs of several members of the Fanconi anemia pathway (see above). Table 5-1 shows the results of Ncbi BLAST search. In Figure 5-6 the annotated genomic organization of *AtFANCE*, *AtFANCI*, *AtFANCIJ*, *AtFANCL* and *AtFANCM* is indicated.

<i>Arabidopsis thaliana</i> gene	TAIR accession #	human accession # and protein length	E-value
<i>AtFANCE</i>	At4g29560	Q9HB96, 536aa	1.3E-02
<i>AtFANCI</i>	At5g49110	Q9NVI1, 1328aa	2.0E-48
<i>AtFANCIJ</i>	At1g20720	Q9BX63, 1249aa	3.0E-133
<i>AtFANCL</i>	At5g65740	Q9NW38, 375aa	7.0E-44
<i>AtFANCM</i>	At1g35530	Q8IYD8, 2048aa	1.0E-128

Table 5-1 Five new Fanconi anemia proteins are found in the *Arabidopsis* genome.

BLAST results of human Fanconi anemia proteins. E-values of homology are indicated.

We isolated a collection of mutants in these genes using publicly available databases (www.arabidopsis.org) and seed banks (see Figure 5-6 and List of alleles). An additional knock down allele of *AtFANCL*, *AtFANCL^{RNAi}* was generated in parallel to *AtFANCD2^{RNAi}* (see above) using artificial microRNA technology (see below and [Web MicroRNA Designer](#)).

Phenotypical analysis showed no impairment of vegetative growth and development in homozygous mutants. Similar to the *Atfancd2* mutant alleles, these newly identified FA mutants are fertile and produce wild type-like siliques.

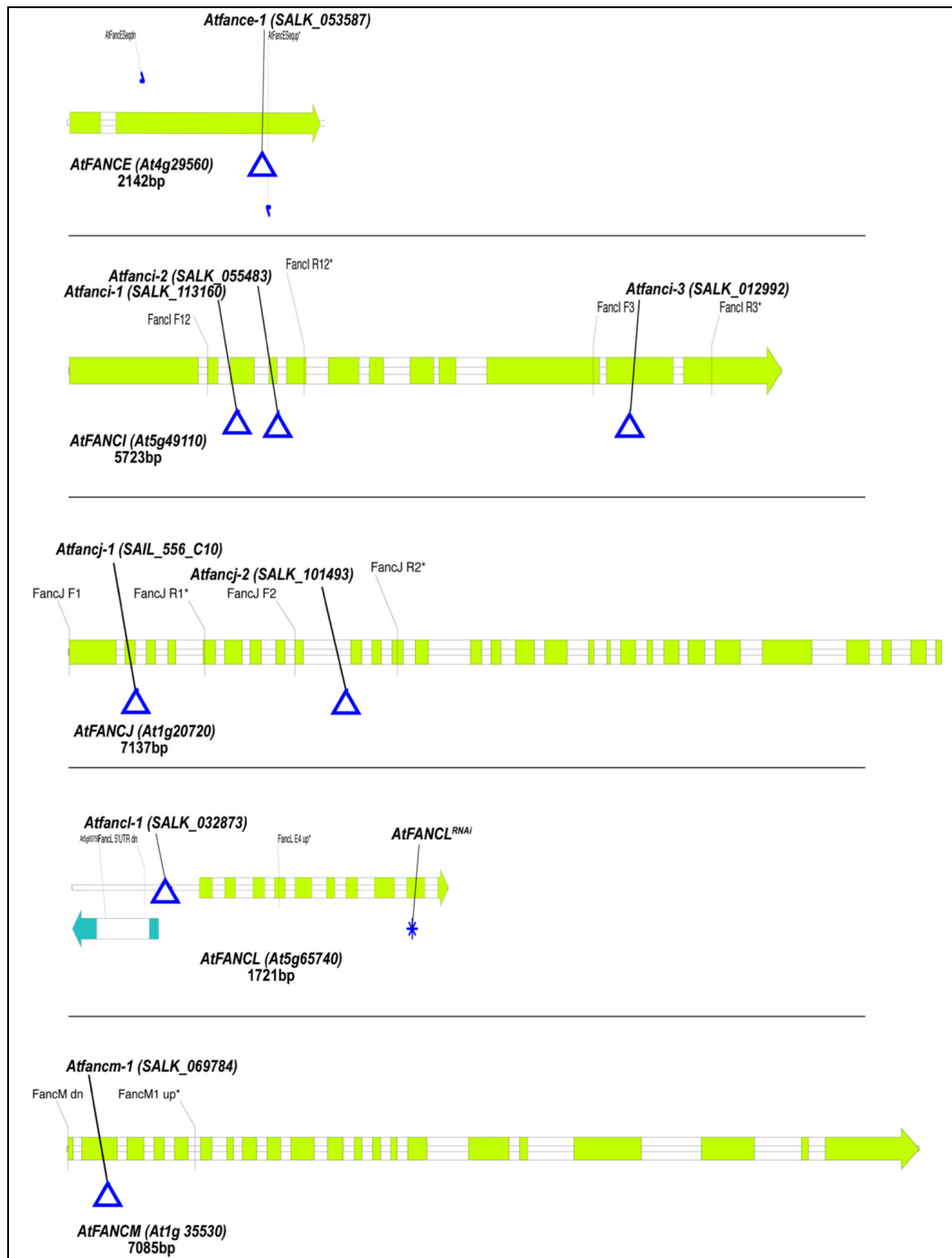


Figure 5-6 Schematic drawings of Fanconi anemia genes in Arabidopsis. Green and white bars indicate exon/intron structure as annotated. Asterisks mark the site of T-DNA insertion (or amiRNA sequence) of the isolated mutants. Primers for genotyping are specified.

We crossed several of these mutants (*Atfanci-1/2/3*, *Atfancj-2*, *Atfancl-1*, *AtFANCL^{RNAi}*, *Atfancm-1*) to *Atatm-2* to investigate a possible conserved role of the entire Fanconi anemia pathway in meiosis (no homozygous double mutants yet). Functions of helicase FANCM or E3 Ubiquitin ligase FANCL in governing meiotic processes would be interesting results. Future work on these proteins will enlighten their involvement in these processes.

5.1.7. Yeast two-hybrid search for interaction partners

In the course of defining the molecular function of AtFANCD2, a yeast two-hybrid co-transformation screen was performed by O. Krsicka in our lab. Using the *AtFANCD2* cDNA cloned by C. Kerzendorfer (Kerzendorfer and Schweizer, 2007) he screened a cDNA expression library (H. Sommer, MPIZ Cologne; see Plasmid maps). Several positive interactions were observed and publications discussing the corresponding genes were analyzed for indications of an involvement in the Fanconi anemia pathway (see above).

A candidate of the AtFANCD2 yeast two-hybrid screen is phosphatase PP2A subunit B'γ (At4g15415). PP2A, a heterotrimeric phosphatase, was identified before as a regulator complex of γH2AX concentration in human cells (Chowdhury *et al.*, 2005), and a similar protein complex was found to directly dephosphorylate γH2AX in yeast (Keogh *et al.*, 2006). This study also positions loss of γH2AX as an essential event between synapsis and completion of DNA repair. Considering that *Atatm Atfancd2* meiotic cells show normal synapsis but deteriorate during subsequent steps, this draws a parallel between AtFANCD2 and γH2AX. It is tempting to draft a model of action that connects these two pathways. Analysis of γH2AX kinetics shows that it localizes to long stretches around DSBs, but is not found in very close proximity (Shroff *et al.*, 2004). FANCD2, on the other hand, is recruited later to repair foci and co-localizes with proteins actively involved in DNA repair. A FANCD2-dependent regulation of chromatin modification and DNA repair dynamics via interaction with PP2A is worth considering. Cloning of AtPP2A subunit B'γ (cDNA provided by [NASC](#)) into a Y2H vector (see Plasmid maps) and mating tests (see below) with

AtFANCD2 cDNA could not yet confirm the result of our screen. Future work including mutant *Arabidopsis* alleles of the PP2A phosphatase, possibly in an *Atatm* background, might help solve this issue.

cDNA from *AtFANCL* (equally provided by [NASC](#)) was also included in our mating tests (see Plasmid maps). Noteworthy, a heretofore-unreported self-interaction of FANCL could be observed.

5.2. ATM and ATR in *Arabidopsis*

FANCD2 mono-ubiquitination upon DNA damage is known to depend on ATR and RPA (Andreassen *et al.*, 2004). Similar to *Atfancd2* mutants, *Atatr* does not show any meiotic defects (Culligan *et al.*, 2004; Culligan and Britt, 2008). To compare the meiotic phenotype of *Atfancd2* and *Atatr* mutants, we generated the double mutant *Atatm-2 Atatr-2*. A cooperating role in promoting DNA repair has been reported before, and synthetic sterility was observed in the double mutant (Culligan *et al.*, 2004) (see above). Cytological and statistical analyses of the fertility defects in *Atatm Atatr* have been published (Culligan and Britt, 2008) (see above) during the work on the present study, and our findings greatly confirm these results. We also present interesting immuno-cytochemistry data about molecular aspects of AtATM and AtATR action during meiosis. Additionally, we gathered promising information about an AtATM and AtATR independent mode of repair.

5.2.1. Meiotic chromosome fragmentation in *Atatm Atatr* is suppressed in an *Atspo11-2* background

Homozygous *Atatm Atatr* double mutants are sterile, producing no viable offspring (Culligan *et al.*, 2004). We analyzed pollen mother cells of *Atatm-2 Atatr-2* double mutant plants, making squash preparations stained with DAPI (see below). No obvious impairment can be seen up to zygotene, judging from chromatin condensation (Figure 5-7 A) and compared to wild type meiotic progression (see Figure 4-7). Structures corresponding to paired homologous chromosomes cannot be observed in the double mutant, the cells rather seem to skip synapsis and progress to diplotene (Figure 5-7 C). This is in contrast to the *Atatm-2* (see Figure 4-6 and Figure 4-7) and *Atatr* (Culligan and Britt, 2008) single mutants, where synapsis is complete. In subsequent meiotic stages we detect massive chromosome fragmentation and missegregation (see Figure 5-7 E,G), while *Atatm-2* single mutants also frequently show some fragmentation (see Figure 4-7), and no fragmentation is seen in *Atatr* single mutants (Culligan and Britt, 2008).

To investigate whether these deficiencies are due to a failure to repair SPO11-induced double-strand breaks, we introduced an *Atspo11-2-3* mutation into *Atatm-2 Atatr-2* double mutants and analyzed preparations of triple mutant pollen mother cells. In analogy to *Atatm-2 Atfancd2-1* (see Figure 5-3) chromosome fragmentation was prevented in this mutant and univalents segregated randomly during meiosis I as in *Atspo11-2-3* single mutant. We can therefore conclude that the chromosome fragmentation seen in *Atatm-2 Atatr-2* meiosis stems from unrepaired meiotic double-strand breaks.

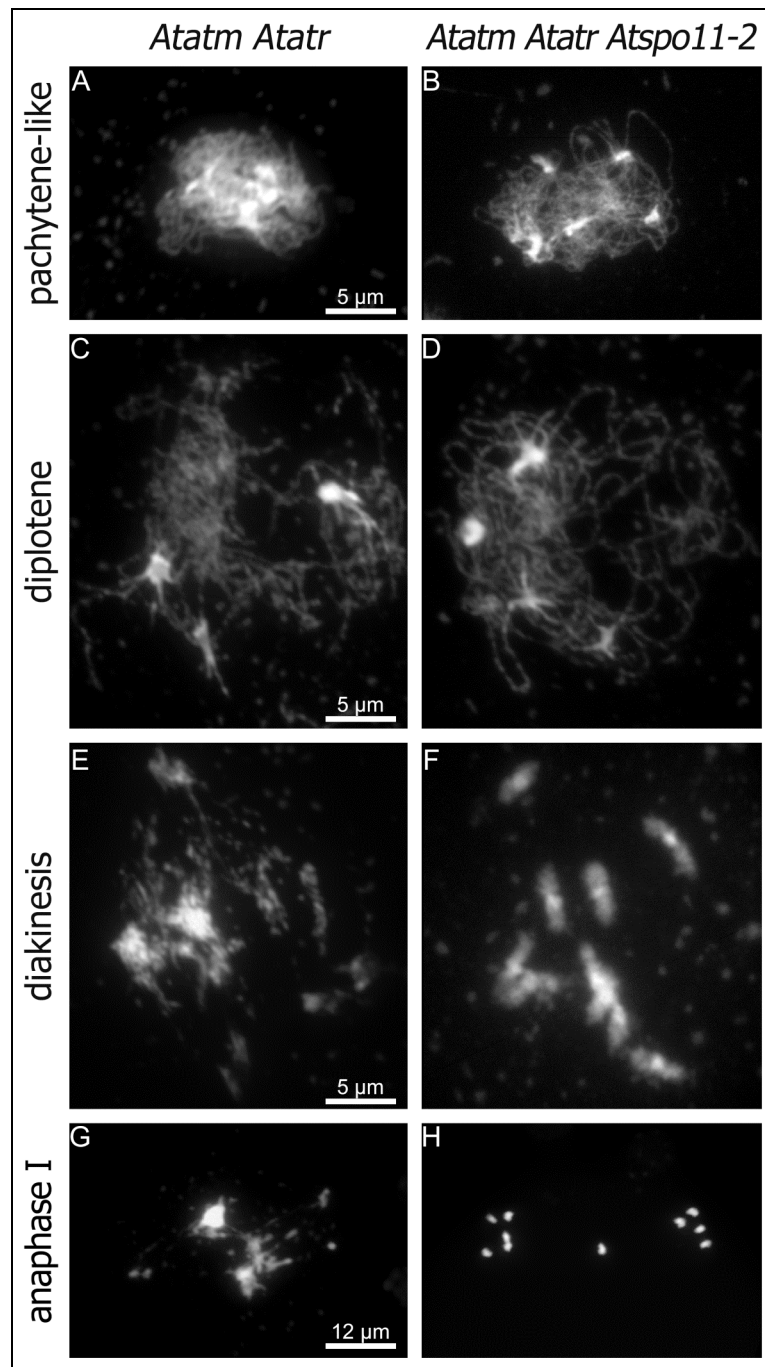


Figure 5-7 Meiosis in *Atatm Atatr* and *Atatm Atatr Atspo11-2* pollen mother cells. Both mutant plants show normal zygotene nuclei, but no chromosome synapsis can be detected (A,B). In *Atatm Atatr*, genomic integrity is lost in subsequent stages (E) and a big chromosomal mass is not captured by the spindle apparatus (G). In contrast, *Atatm Atatr Atspo11-2* triple mutants show hardly any fragmentation and segregate univalent chromosomes in anaphase I (H). Size bars are 5μm.

5.2.2. *Atatm-2 Atatr-2* double mutants fail to polymerize ZYP1 and do not form a Synaptonemal complex

While pachytene chromosome structures can clearly be detected in the *Atatm* mutant, no pairing was visible in the *Atatm-2 Atatr-2* double mutant. We investigated whether this reflects a failure to induce Synaptonemal complex formation. Two homologs of yeast Synaptonemal complex proteins have been found in *Arabidopsis*. ASY1 localizes to chromatin starting from leptotene and is therefore considered an axial element protein (see above). AtZYP1 constitutes the central element of the SC. It polymerizes as transverse filaments during homolog pairing, becoming fully elongated at pachytene (see Figure 5-8).

Axis formation and synapsis resemble the wild type situation in *Atatm-2* (Figure 5-8) and *Atatm-2 Atfancd2-1* (see Figure 7-15). Thus, chromosomal fragmentation seems to be independent of Synaptonemal complex formation in these mutants.

Atatm-2 Atatr-2 mutants also show mature axis formation as judged by the polymerization of ASY1 protein. However, no or scarce AtZYP1 protein can be detected (red foci in Figure 5-8 *Atatm Atatr*), confirming that synapsis depends on functional AtATM and AtATR in *Arabidopsis*.

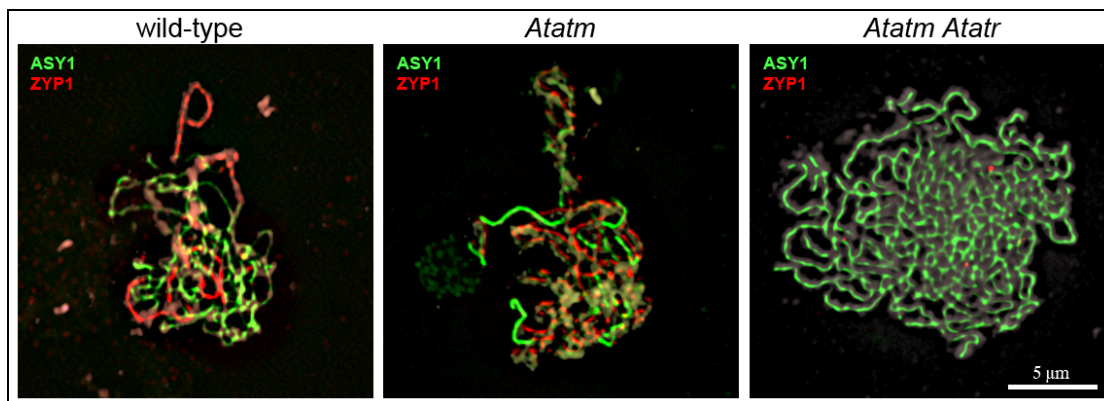


Figure 5-8 Axis formation and Synaptonemal complex generation in wild type, *Atatm* and *Atatm Atatr* meiocytes. Merged images of ASY1 (green) and AtZYP1 (red) localization and DNA (white). ASY1 protein can be detected in wild type, *Atatm* and *Atatm Atatr*, indicative of intact chromosome axis formation. In contrast, only wild type and *Atatm-2* mutant pollen mother cells stain for AtZYP1 filaments, whereas AtZYP1 immunolocalisation is seen as red foci in *Atatm-2 Atatr-2*.

5.2.3. Recombination is initiated in *Atatm-2 Atatr-2* double, but not in *Atatm-2 Atatr-2 Atspo11-2-3* triple mutants.

ATM and ATR regulate homologous recombination in both mitosis and meiosis in mammals (see above). The central recombinase during both somatic and meiotic HR is RAD51, and while many regulatory targets of ATM and ATR are involved in processes that precede RAD51-mediated initiation of recombination (e.g. MRE11, RPA), it is not known whether RAD51 itself is misregulated in mutants of ATM and/or ATR.

We analyzed AtRAD51 foci formation using immuno-cytochemistry (see below). Surprisingly, AtRAD51 foci seem to be as numerous in *Atatm-2 Atatr-2* as they are in wild type pollen mother cells (nuclear spreads, see Figure 5-9). It thus appears that the loading of RAD51 to sites of Spo11-induced DSBs is unaffected in this mutant.

Atspo11-1 or *Atspo11-2* single mutants are thought to completely lack meiotic DSB formation (see above and Figure 5-3 J). This is confirmed by the observation that *Atspo11 Atrad51* double mutants show no fragmentation (Hartung *et al.*, 2007). It is therefore expected that no AtRAD51 staining is visible in an *Atspo11-2-3* mutant. While some AtRAD51 foci are visible when looking at the RAD51 image layer of *Atspo11-2-3* (Figure 5-9), we argue that this is a background noise of the antibody.

When looking at the *Atatm Atatr Atspo11-2* triple mutant, the number of AtRAD51 foci is elevated compared to the *Atspo11-2-3* single mutant pollen mother cell. Certainly, an explanation for this observation could be deduced from the fact that *Atatm Atatr* double mutants show a small fraction of cells with failed mitotic DNA repair (mitotic anaphase bridges in (Vespa *et al.*, 2005)) – independently of Spo11, as it is visible also in our triple mutant (Supplementary Figure 7-16). Consequently, DNA damage that could arise during premeiotic S-phase would persist in *Atatm Atatr* double mutants up to the initiation of meiosis. However, this observation does not match with the notion that *Atatm-2 Atatr-2 Atspo11-2-3* triple mutants proceed through meiosis like the *Atspo11-2* single mutant, unless DNA repair takes place that works independently of

AtATM/AtATR but depends on AtRAD51. As no pairing of homologous chromosomes is visible, the sister chromatid could serve as a template for repair. To test this hypothesis, we are currently generating a quadruple mutant, combining *Atatm-2 Atatr-2 Atspo11-2-3* with an *Atrad51* mutant allele.

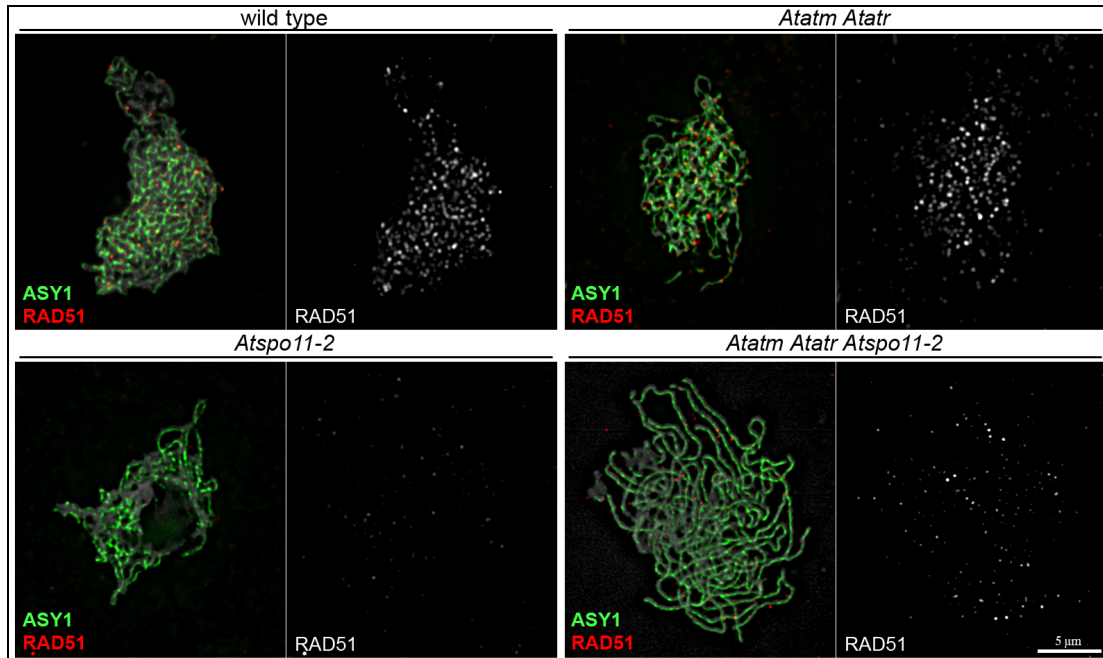


Figure 5-9 Qualitative analysis of AtRAD51 foci formation in wild type, *Atatm Atr*, *Atspo11-2* and *Atatm Atatr Atspo11-2* mutants. Merged images: AtRAD51 foci (red) are visualized on pollen mother cells, counter-stained with ASY1 antibody (green) and DAPI (white). AtRAD51 staining is also shown as separate images. Wild type picture from T. Siwiec. See text for explanation.

DMC1 is the second central recombinase in meiotic homologous recombination. The protein is needed for the inter-homolog bias during meiosis, but this effect is thought to be mediated by accessory proteins, not DMC1 itself (see above). We wanted to test if AtDMC1 protein is equally present in elevated number in *Atatm-2 Atatr-2 Atspo11-2-3* triple mutants as in AtRAD51. The analysis is still in progress and not all mutants have been analyzed yet, but preliminary results indicate that AtDMC1 is not present in *Atatm-2 Atatr-2 Atspo11-2-3* pollen mother cells (see Figure 5-10) and therefore might not be involved in ATM/ATR-independent repair.

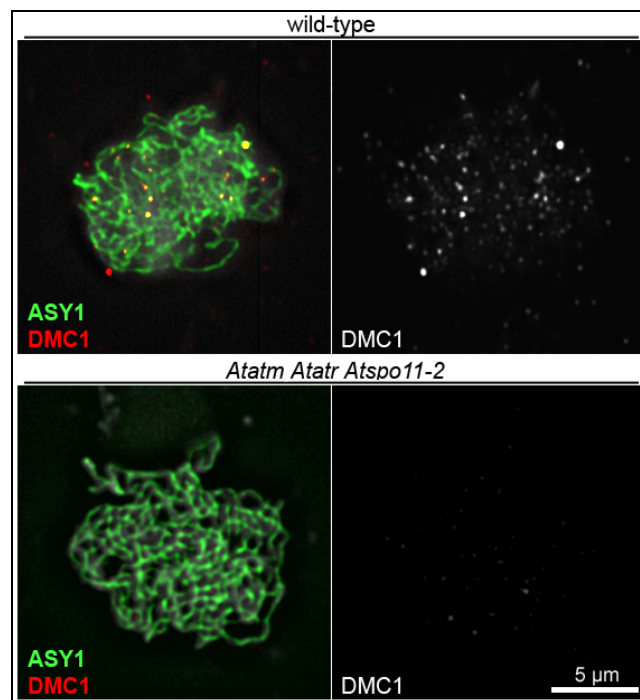


Figure 5-10 AtDMC1 localization in wild type and *Atatm Atatr Atspo11-2* triple mutant. AtDMC1 antibody is stained in red, and cells are counterstained with α ASY1 (green) and DAPI (white). See text for details.

5.2.4. Phosphorylation of SQ/TQ sites is abrogated in *Atspo11-2*, *Atatm Atatr* and *Atatm Atatr Atspo11-2*

ATM and ATR are serine/threonine kinases, and they show a preference for SQ or TQ sites on their targets. A recent study in yeast showed that site-specific mutagenesis of only one of these motifs in axial element protein Hop1 is sufficient to confer spore lethality (Carballo *et al.*, 2008).

We investigated whether SQ/TQ-motif phosphorylation is also occurring in plants and if this depends on AtATM and/or AtATR. For this purpose, we employed the pSQ/TQ antibody used in the aforementioned study (see Antibodies). As can be seen in Figure 5-11, serine/threonine phosphorylation can be detected along chromosome axes in wild type. In analogy to yeast Hop1, we anticipate that this signal corresponds to *Arabidopsis* axial element protein ASY1. In addition, ASY1 has numerous SQ/TQ sites that could serve as regulation motifs for AtATM/AtATR (see Figure 5-12). Western blot experiments with an

ASY1 antibody and the analysis of *asy1* mutant pollen mother cells will allow us to confirm this assumption.

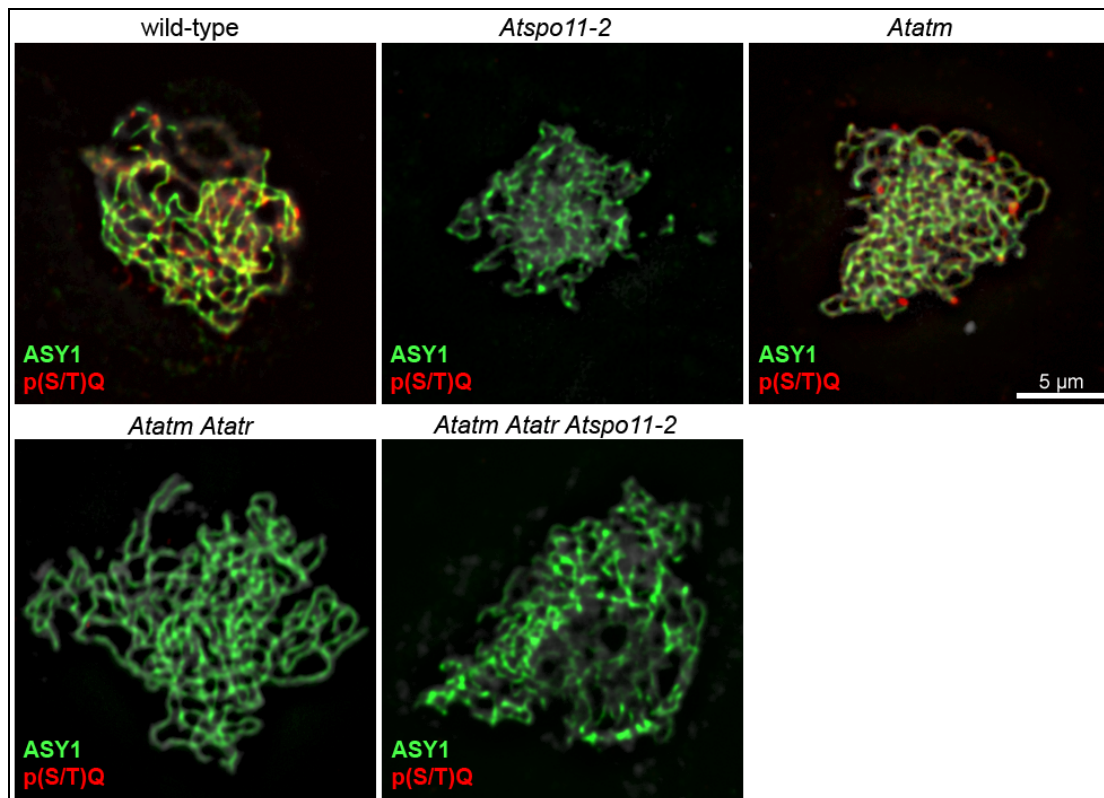


Figure 5-11 anti-pSQ/TQ antibody distribution in pollen mother cells of wild type, *Atspo11-2-3*, *Atatm-2*, *Atatm-2 Atatr-2* and *Atatm-2 Atatr-2 Atspo11-2-3*. Immunocytochemistry images of pSQ/TQ (red), counterstained with ASY1-antibody (green) and DAPI (white). See text for details.

When looking at *Atspo11-2-3* mutant meiocytes, we do not detect phosphorylated SQ/TQ sites. This suggests once more that ATM/ATR kinase activity depends on SPO11 in meiosis. By introducing interstrand-crosslinks via cisplatin uptake in pre-meiotic S-phase, we will test whether this activity is also specific to extrinsically induced double-strand breaks.

Interestingly, also *Atatm-2* mutant pollen mother cells stain for the pSQ/TQ antibody. It is probable that AtATR compensates for the lack of AtATM in these cells, therefore we will test the *Atatr-2* single mutant for reduced pSQ/TQ antibody detection.

SQ/TQ phosphorylation is certainly abrogated in the *Atatm Atatr* double mutant (Figure 5-11), thereby confirming that the antibody detects ATM/ATR phosphorylation sites in *Arabidopsis*.

Finally, we analyzed the phosphorylation status of SQ/TQ motifs also in the *Atatm Atatr Atspo11-2* triple mutant where no signal was detected.

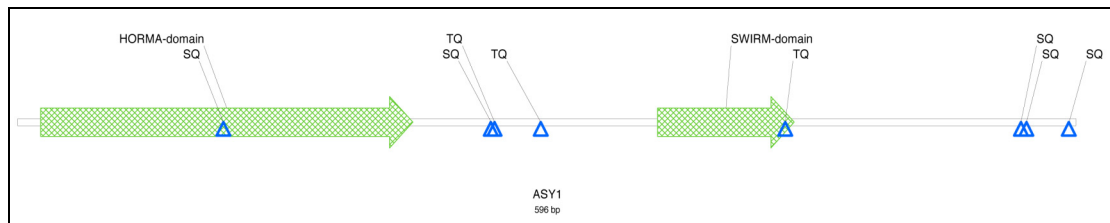


Figure 5-12 Annotated ASY1 protein sequence illustration with domains and SQ/TQ sites. As noted in (Carballo *et al.*), yeast Hop1 protein sequence contains 8 SQ/TQ motifs, and so does ASY1.

While our results in cytology (Figure 5-11) and the bioinformatic analysis (Figure 5-12) point to ASY1 as the central recipient of ATM/ATR-phosphorylation in meiotic prophase, other proteins involved in meiosis also share such sites, among them AtFANCD2 (see Figure 5-5). It will be interesting to compare the ATM/ATR-phosphorylation target antibody to AtFANCD2 antibodies (see above) and to connect ATM/ATR regulation to the action of the Fanconi anemia pathway in *Arabidopsis thaliana*.

6. Discussion

A hallmark of efficient meiotic progression is the requirement for DNA repair proteins. This is evident in plants, where mutant alleles of proteins involved in homologous recombination often confer meiotic, not mitotic deficiencies during unchallenged development, eventually leading to fertility defects. Cytological and biochemical analysis of these mutants allowed dissecting their molecular function. Studying their involvement in these competing and collaborating pathways is interesting and challenging.

Mutations underlying the human diseases *Ataxia telangiectasia*, Seckel syndrome and Fanconi anemia led to the discovery of the essential genes *ATM*, *ATR* and *FANCD2*. While phenotypic analysis of these genes relies on the study of hypomorphic mutations in humans, knockout mutants are available in *Arabidopsis thaliana* and other model organisms. Experimental approaches in these organisms allow a detailed analysis of the pleiotropic effects of these mutants.

6.1.1. AtATM promotes DNA repair in *Arabidopsis* meiosis

The involvement of murine and *Arabidopsis* ATM and of the yeast homolog Mec1 in meiosis was reported some time ago (Carballo and Cha, 2007; Garcia *et al.*, 2003; Xu *et al.*, 1996). While *Atm*^{-/-} mice and *mec1Δ* yeast strains show aberrant synapsis (Grushcow *et al.*, 1999; Xu *et al.*, 1996), this is not the case for *Atatm-2* mutant plants. We showed by immuno-staining correct association of components of the Synaptonemal complex with chromosomes.

Generally, ATM is believed to fulfill a dual role during meiosis; it must regulate cell cycle progression and promote DNA repair by coordinating the activities of DNA repair proteins. A cell cycle checkpoint function has not yet been reported for AtATM, likely because homologs of central checkpoint response proteins (p53, p21) have not been found in *Arabidopsis*. Barlow *et al.* (1997) recreated the *Arabidopsis* situation in mouse, generating *Atm*^{-/-} *p53*^{-/-} and *Atm*^{-/-} *p21*^{-/-} double knockouts. While *Atm*^{-/-} single knockout mice show no pachytene synapsis and

frequently become apoptotic, *Atm*^{-/-} *p53*^{-/-} and *Atm*^{-/-} *p21*^{-/-} double knockouts show synapsis marker immunolocalisation and less apoptosis (Barlow *et al.*, 1997).

This resembles the phenotype of *Arabidopsis Atatm-2* pollen mother cells that appear wild type-like at pachytene, but often show chromosome fragmentation during later stages.

6.1.2. AtFANCD2 plays a role in meiosis that acts in parallel to AtATM

Human FANCD2 is an enigmatic protein, and its structural domains do not suggest a specific molecular function. It localizes to sites of DNA damage and it is tightly regulated by the kinases ATM/ATR as well as the Fanconi ubiquitin ligase complex. While FANCD2 is clearly involved in ICL repair, a function in meiosis has remained elusive, aside from the observation that FANCD2 knockout mice have a reduction in testes weight (Houghtaling *et al.*, 2003).

We failed to detect a meiotic phenotype in the single *Atfancd2* mutant in *Arabidopsis*. However, we observed a synthetic effect of reduced fertility in an *Atatm-2* mutant background (Figure 5-2). This is in contrast to *Atatr-2 Atfancd2-1* double mutants (Kerzendorfer and Schweizer, 2007) that show no fertility defect. We assume therefore that AtFANCD2 acts in the same DNA repair pathway as AtATR, and that this pathway acts in parallel of the AtATM kinase pathway.

Chromosome fragmentation in meiotic stages subsequent to pachytene explains the observation of reduced fertility in *Atatm-2 Atfancd2-1*. This fragmentation is exacerbated compared to the meiotic phenotype of the *Atatm* single mutant (see Figure 4-7). This corroborates the hypothesis that AtATM and AtFANCD2 act in partly overlapping, but parallel pathways in meiosis. Interestingly, mutations in both genes still allow the generation of a small amount of offspring, and these seeds can be efficiently propagated at least to the 6th generation. Apparently, residual repair activity is active in these cases. Indeed, we see regular synapsis and wild type AtRAD51 recombination in our mutants (see Figure 5-4).

6.1.3. AtFANCD2 interaction with a phosphatase PP2A subunit opens a connection to chromatin modification

While numerous reports discuss the regulation of FANCD2 and its localization to DNA repair foci (Ishiai *et al.*, 2008), the mechanism of action of the Fanconi anemia pathway has been difficult to unravel. A recent article compares regulation and localization of FANCD2 and γ H2AX, finding parallels throughout their activation cycle (Cohn and D'Andrea, 2008).

γ H2AX is known to be inactivated through the action of PP2A phosphatase (Fillingham *et al.*, 2006). It is interesting in this context that a yeast two-hybrid screen in our lab using AtFANCD2 as bait pulled out a PP2A subunit (see above). It is possible that FANCD2 and γ H2AX share the same mode of dephosphorylation. It is, however, equally possible that AtFANCD2 is a regulator of γ H2AX, e.g. indirectly modifying its phosphorylation status and thereby directing repair proteins. The Y2H result still needs to be verified with full-length proteins. One caveat for this verification, and for the entire screening procedure, is that we do not know if FANCD2 post-translational modifications are a pre-requisite for protein-protein interactions. Screening for genetic interactions *in planta* is therefore worth considering.

6.1.4. New *Arabidopsis* homologs of Fanconi anemia pathway proteins are found by bioinformatic tools

While it has been known for quite some time that FANCD2 homologs are present in worm and plant genomes, we identified homologs of FANCE, FANCI, FANCIJ, FANCL and FANCM in *Arabidopsis* (see Table 5-1). Using BLAST search and T-DNA mutant collection databases, we obtained mutant alleles for all of the above-mentioned genes. Judging from their development and fertility, no phenotype was apparent, similar to *Atfancd2* mutants. Whether or not this resemblance extends also to the meiotic phenotype of *Atfancd2* is currently being examined in *Atatm* mutant backgrounds. If this is the case, it will be the first evidence of a meiotic function for these genes, except for FANCIJ/BACH1,

which is partially homologous to Synaptonemal complex protein 1 (Cantor *et al.*, 2001).

6.1.5. AtATR acts in parallel with AtATM to promote meiotic synapsis and DNA repair

Both ATM and ATR are central DNA repair kinases conserved from yeast to human. They are variably activated and mutant alleles show differing phenotypes, but they share a variety of phosphorylation targets and can compensate for each other's loss, and hence it is difficult to discern between their molecular functions.

Whereas ATR is an essential gene in humans during unchallenged life, no *Arabidopsis Atatr* mutants have been isolated that show deficiencies during unperturbed mitosis or during meiosis. However, *Atatr* mutants are hypersensitive to replication blocking by aphidicolin, hydroxyurea or UV-B (Culligan *et al.*, 2004). However, *atm atr* double mutants were first analyzed in *Arabidopsis*, and were found to be sterile (Culligan *et al.*, 2004). Results from Culligan and Britt (Culligan and Britt, 2008) as well as our independent findings (Figure 5-7) show that this is due to massive chromosome aberrations in the course of meiotic prophase I. We could not detect synapsis by immunological staining (Figure 5-8), but observed a high number of AtRAD51 foci (Figure 5-9), indicative of active initiation of recombination in *Atatm Atatr*.

We conclude from these results that AtATM and AtATR act in partly redundant pathways in meiosis, and that AtATM can fully compensate for the loss of ATR, whereas AtATR can promote synapsis but not efficient DNA repair in the absence of AtATM.

6.1.6. An *Atspo11-2-3* mutant rescues meiotic chromosome fragmentation in *Atatm-2*, *Atatm-2 Atfancd2-1* and *Atatm-2 Atatr-2*.

To investigate the nature of the observed chromatin fragmentation, we eliminated SPO11-induced double-strand break formation by introducing an *Atspo11-2-3* mutation (Hartung *et al.*, 2007) into *Atatm-2*, *Atatm-2 Atfancd2-1* and *Atatm-2 Atatr-2* mutants. This suppressed fragmentation and channeled meiosis into univalent segregation (Figure 5-3 and Figure 5-7). Thus, AtATM, AtATR and AtFANCD2 are required for efficient repair of double-strand breaks induced by SPO11.

Culligan and Britt report in their study (2008) that chromosomal fragmentation of *Atatr-3 Atatm-1* is suppressed only partially when introducing an *Atspo11-1-1* mutation. The analysis of anaphase I pollen mother cells in *Atatm-1 Atspo11-1-1* and *Atatr-3 Atatm-1 Atspo11-1-1* mutants shows that some cells still segregate fragmented chromosomes (Culligan and Britt, 2008). We did not see a difference in chromosome fragmentation between *Atspo11-2-3* single mutant and *Atatm-2 Atatr-2 Atspo11-2-3* triple mutant PMCs (Figure 5-7) and suggest that the contradictory observations are due to an incomplete knockout of AtSPO11-1 in the *Atspo11-1-1* allele (Grelon *et al.*, 2001).

6.1.7. Evidence of a Rad51-dependent and ATM/ATR-independent DNA repair pathway in *Arabidopsis* meiosis

We found an elevated amount of AtRAD51 foci between zygotene chromosomes from *Atspo11-2-3* and *Atatm-2 Atatr-2 Atspo11-2-3* mutants (Figure 5-9). Even though meiotic chromosome segregation is highly similar in the respective pollen mother cells (Figure 5-7), more AtRAD51 protein is detected in *Atatm-2 Atatr-2 Atspo11-2-3* triple meiocytes when compared to *Atspo11-2-3*. We infer that premeiotic DNA lesions occur in the absence of AtATM and AtATR that are efficiently repaired via RAD51-dependent inter-sister recombination. Presumably, the end structure of these lesions resembles MRN-processed DSB ends and therefore activation of end processing (e.g., ATM-dependent phosphorylation of

MRE11) is not needed in these cases. An *Atatm-2 Atatr-2 Atspo11-2-3 Atmre11* quadruple mutant will help to confirm this assumption.

From the lack of AtDMC1- (Figure 5-10) and phosphorylated SQ/TQ-motif staining (Figure 5-11) in *Atatm-2 Atatr-2 Atspo11-2-3*, we conclude that DMC1 recombination or extrinsic SQ/TQ phosphorylation are not involved in this pathway.

6.2. Concluding words

Diverse effects on DNA DSB repair are attributed to the proteins analyzed in this study, ATM, ATR and FANCD2. We characterized their molecular function and genetic interactions in plants, and showed that they are required for correct meiotic segregation and double-strand break repair. Being non-essential genes in *Arabidopsis thaliana*, we could create mutant combinations that are not possible in other organisms. This allowed us to determine the requirement of ATM, ATR and FANCD2 for SPO11-induced DSB repair, and also allowed us to examine a meiotic repair pathway that effectively repairs DNA lesions mediated by Rad51-dependent inter-sister repair.

7. Materials and Methods

7.1. Media

Plant media

ARA medium: 4.3g/l MS basal salts (Duchefa Biochemie), 0.5g/l MES-monohydrate (w/o vitamins), 1% sucrose, 1ml/l vitamin B5 (1000x stock, Gamborg); pH 5.6-5.8 set with 1M KOH; 6g/l plant agar; autoclave.

T-DNA transformants with Hygromycin resistance are selected on ARA medium containing 25mg/l Hygromycin (Duchefa Biochemie) and 250mg/l Amoxycillin as an *A.tumefaciens* antibiotic.

Sensitivity assays against interstrand-crosslinking agent susceptibility are conducted on ARA medium with a final concentration of 40μM MMC (13.37mg/l, Sigma).

Yeast media

YPAD medium: 1% yeast extract, 2% peptone, 2% glucose, 40mg/l Adenine sulfate, 20g/l agar; autoclave.

YSD-dropout media: 1.7g/l yeast nitrogen base w/o ammonium sulfate, 5g/l ammonium sulfate, 20g/l glucose, pH 5.8 set with 2N NaOH; autoclave; add 100ml/l 10x drop-out mix; add 0.1ml of 3M 3-AT (3-amino-1,2,4-triazol) for -Leu -Trp -His medium.

10x Drop-out mix: Excluding respective compounds for auxotrophy selection, amino acids and adenine are mixed and autoclaved: 300mg/l L-Isoleucine, 1.5g/l L-Valine, 600mg/l L-Adenine hemisulfate salt, 200mg/l L-Arginine HCL, 200mg/l L-Histidine HCL monohydrate, 1g/l L-Leucine, 300mg/l L-Lysine, 200mg/l L-Methionine, 500mg/l L-Phenylalanine, 2g/l L-Threonine, 200mg/l L-Tryptophane, 300mg/l L-Tyrosine, 200mg/l L-Uracil.

Freeze medium: 25% glycerol in YPAD.

Bacterial media

LB medium: 1% peptone, 0.5% yeast extract, 1% NaCl, 1.5% agar; autoclave; add adequate amount of antibiotics (final conc.: Amp 100mg/l; Kan 50mg/l).

YEB medium: 0.5% beef extract, 0.1% yeast extract, 0.5% peptone, 0.5% sucrose, 1.5% agar; autoclave; add 2ml/l 1M MgSO₄; add adequate amount of antibiotics (final conc.: Kan 50mg/l; Rif 50mg/l; Tet 12mg/l)

7.2. Plant work

7.2.1. Growth conditions

Arabidopsis thaliana (Columbia ecotype, except denoted otherwise) is grown under long-day conditions – 16h light, 8h dark; 21°C; 60-80% humidity; 5800lux with 4x Phillips TLD 36W and 2x Sylvana GroLUX 36W lamps. Seeds are pre-germinated in H₂O for 2 days at 4°C and sown on Frux ED 63 Einheitserde soil.

7.2.2. Seed sterilization

A small amount of seeds (up to 50µl) are treated with 500µl calcium hypochloride solution (3% of a saturated Ca(OCl)₂ solution, 1‰ of 20% Triton-X) and shaken for 15 min. After 15s of centrifugation, the solution is discarded. The seeds are washed three times with 500µl dH₂O and dried in the laminar at least overnight.

7.2.3. *In planta* transformation – floral dip method

An *Agrobacterium* colony is pre-cultured in LB medium containing selective antibiotics, diluted in 500ml of this medium and incubated at 28°C for 1-2 days. After centrifuging for 25 min at 3krpm, the cell pellet is washed with a 5% sucrose

solution, collected by centrifugation for 10 min and slowly resuspended in 200ml of the same solution with 0.02% Silwet L-77.

Flower buds of about 4–6-week-old plants are dipped into the bacterial suspension for 1 min and the plants are covered with plastic bags. After 1 day, the bags are cut to allow circulation and after another 2 days the bags are removed. The plants are kept under normal growth conditions until the offspring is harvested.

7.3. Yeast work

7.3.1. Yeast transformation

Yeast strains are inoculated in 6ml YPAD and grown at 30°C overnight. The culture is diluted in 50ml YPAD and incubated until it reaches an OD_{660nm} of 0.5–0.6. After centrifugation for 2 min 2000rpm RT the cells are resuspended in 20ml sterile dH₂O, centrifuged, resuspended in 1ml 100mM LiAc/TE, centrifuged and resuspended again in LiAc/TE on ice.

3–10µl plasmid are mixed with 5µl carrier ssDNA (salmon sperm, pre-cooked), 100µl competent yeast cells and 700µl 40% PEG4000 in 100mM LiAc/TE by pipetting. After up to 1h at 30°C, cells are incubated for 15 min at 42°C (heatshock), centrifuged shortly (20s) and the resuspended pellet is plated on selective medium. Incubation time is 2–3 days at 30°C.

7.3.2. Yeast glycerol stock

A well-grown yeast ON culture is scraped off the plate and resuspended in freeze medium.

7.3.3. Yeast-2-hybrid mating test

Haploid yeast strains containing a plasmid that encodes a fusion protein of cDNA of interest and GAL4 binding domain (BD) or activation domain (AD) are grown on selective media. A strain coding for the BD and a strain coding for the AD are plated together on YPAD medium. After 24h at 30°C, the mixed strains are transferred to a YSD –Leu –Trp plate where only mated diploid cells will grow. After another 2-3 days at 30°C, the colonies can be tested for interaction of bait- and prey-tagged proteins on YSD – Leu –Trp – Ade and YSD –Leu –Trp –His +3-AT media, respectively.

7.4. Bacterial work

7.4.1. Agrobacteria

Preparation of competent cells

Agrobacterium tumefaciens strain GV3101 containing helper plasmid C59C1 is inoculated in 10ml YEB-Rif/Tet and 2mM MgSO₄ for 2 days at 28°C. 4ml culture is diluted in 100ml YEB-Rif/Tet and 2mM MgSO₄ and grown for another 3.5h. The cells are collected by centrifugation at 5krpm for 10 min at 4°C and resuspended in 1ml YEB-Rif/Tet and 2mM MgSO₄ on ice. 200µl aliquots are frozen in N₂liq and stored at -80°C.

Transformation of competent *Agrobacteria*

A vial of competent cells is thawed at RT and gently mixed with 2-5µg DNA. The cells are frozen for 1 min in N₂liq and heat-shocked for 5 min at 37°C. 800µl YEB are added and the cells are incubated for 2h at 28°C and plated on selective medium for min. 2 days.

7.5. Protein work

7.5.1. Protein extraction

Ferguson extraction

150mg of plant material ground in N_2 liq are mixed with 200 μ l Ferguson solution (50mM Tris-Cl pH 6.8, 4% SDS, 10% Mercaptoethanol). The mixture is cooked for 10 min at 95°C while shaking and centrifuged for 10 min at maximum speed. The supernatant is mixed with an equal amount of 2x Laemmli sample buffer (50% glycerol, 20mM DTT, 2% SDS, 125mM Tris-Cl pH 6.8) and used for SDS-PAGE.

7.5.2. SDS polyacrylamide gel electrophoresis (SDS-PAGE)

For an 8% resolving gel, 2.3ml dH_2O are mixed with 1.3ml 30% acrylamide mix, 1.3ml Tris-Cl pH8.8 1.5M and 50 μ l 10% SDS. 50 μ l 10% APS (ammonium persulfate) and 3 μ l TEMED are added, the resolving gel is poured into a gel preparation apparatus (Biorad) and overlaid with dH_2O . The surface of the polymerized gel is washed well with dH_2O and dried with filter paper. 2ml stacking gel are prepared from 1.4ml dH_2O , 330 μ l 30% acrylamide mix, 250 μ l Tris-Cl pH6.8 1M, 20 μ l 10% SDS, 20 μ l 10% APS and 2 μ l TEMED and poured on the resolving gel, using a plastic comb for the slots.

Preheated samples are run on gel with 1x electrophoresis buffer (5x stock solution from 7.55g Tris, 36g glycine, 2.5g SDS ad 500ml with dH_2O) at 15mA for 1.5h.

7.5.3. Western blot

A Western blot cassette (Biorad) is assembled with a layer of foam, a piece of filter paper, the resolving gel, a PVDF membrane (pre-soaked in transfer buffer for 10min), another piece of filter paper and another layer of foam. The sandwich is transferred to a transfer chamber (Biorad), filled up with transfer buffer (3.08g

Tris, 14.41g glycine and 850ml with dH₂O, add 150ml MeOH before use) and run at 380mA for 1.5h.

The membrane is blocked with blocking solution (3% milk powder in PBS-T) for 1h on a shaking stand. The first antibody is thawed on ice and diluted in blocking solution. The membrane is incubated with the antibody solution at 4°C ON. The next day, the membrane is washed three times 5min with remaining blocking solution. The second antibody is diluted in blocking solution and applied on the membrane for 1.5h at room temperature. The membrane is then washed twice with PBS-T for 10min and protein-antibody interaction is visualized using ECL Western Blotting Substrate (Pierce) and the antibody-conjugated horseradish peroxidase (HRP).

7.6. Cytological procedures

7.6.1. Squashing preparation of pollen mother cells and DNA staining

Inflorescences of 5-6 week old plants are harvested in the morning and fixed overnight in fresh fixative (ethanol : acetic acid 3 : 1). Buds of up to 0.5mm size are dissected in fresh fixative and washed twice in citrate buffer (10mM sodium citrate buffer, pH 4.8) in a watchglass. Buds are digested with enzyme mix (0.3% cellulase, 0.3% pectolyase in citrate buffer) for 90 min at 37°C in a moisture chamber. The buds are washed with ice-cold citrate buffer and around 5 buds are transferred to a slide (pre-cleaned with ethanol) and tapped with a mounted needle to release pollen mother cells. 7µl 60% acetic acid are added and the slide is placed on a 45°C hot plate for 5 seconds. 100µl ice-cold fixative are pipetted around the cells and the slide is dried. DNA is stained with 7µl DAPI-solution (2µg/ml in Vectashield mounting medium), a coverslip is added and the slide is squashed in a drying block.

7.6.2. Spreading preparation of pollen mother cells and immunostaining

Inflorescences of 5-week-old plants are harvested on a moist filter paper. The inmost buds are dissected and mixed on a slide with 7µl digestion mix (0.4% cytohelicase, 1% polyvinylpyrrolidone, 1,5% sucrose). Buds are mashed with a metal rod and incubated for 3 min after addition of another 7µl digestion mix. Meiocytes are spread with 10µl Lipsol solution (1% Lipsol in borate buffer pH 9). The spreading is stopped and cells are fixed with 4% paraformaldehyde pH 8, and the slide is dried. An hour later it is washed carefully in phosphate buffer saline for 7 min before a mixture of primary antibodies diluted in blocking solution (1% BSA in PBS) is added and incubated at 4°C overnight. Slides are washed again in PBS for 7 min and Cy3- and FITC-coupled secondary antibodies are added sequentially and incubated at 37°C in a moist chamber, with a washing step between and afterwards. 7µl DAPI-solution is added and slides are sealed with coverslip and nail polish.

7.7. PCR verification of plant genotypes

7.7.1. FANCD2

For *Atfancd2-1* plants, the wild type allele is determined with primers FancUbup and FancClaseq, giving a 945bp product, or intron primer FancInt5up and FancClaseq for complementation lines, giving a 778bp product, and the mutant allele is confirmed using primers FancUbup and SALK LBa1, giving a product of about 750bp.

For *Atfancd2-2* plants, the wild type allele is determined with primers FancHPdn and FancDmut5up, producing 1445bp, and the mutant allele is verified with primers FancHPdn and T-DNA specific GABIttest1, giving ~1kb.

7.7.2. Fanconi Anemia genes

For *Atfance* plants, the wild type allele is determined with primers AtFancESeqdn and AtFancESequp, giving a 816bp product, and the mutant allele is confirmed using primers AtFancESequp and SALK LBa1, giving a product of about 400bp.

For *Atfanci-1* and *Atfanci-2* plants, the wild type allele is determined with primers FancI_F12 and FancI_R12, producing 798bp, and the mutant allele is verified with primers FancI_R12 and SALK LBc1 for *Atfanci-1*, giving ~650bp, and with primers FancI_F12 and SALK LBc1 for *Atfanci-2*, giving ~750bp.

For *Atfancj-1* plants, the wild type allele is determined with primers FancJ_F1 and FancJ_R1, giving a 1130bp product, and the mutant allele is confirmed using primers FancJ_R1 and SAIL LB3, giving a product of about 750bp.

For *Atfancj-2* plants, the wild type allele is determined with primers FancJ_F2 and FancJ_R2, producing 865bp, and the mutant allele is verified with primers FancJ_R2 and SALK LBa1, giving ~750bp.

For *AtfancL-1* plants, the wild type allele is determined with primers FancL_5'UTR_dn and FancL_E4_up, giving a 950bp product, and the mutant allele is confirmed using primers FancL_5'UTR_dn and SALK LBa1, giving a product of about 600bp.

For *Atfancm-1* plants, the wild type allele is determined with primers FancM_dn and FancM1_up, producing 1080bp, and the mutant allele is verified with primers FancM_dn and SALK LBa1, giving ~700bp.

7.7.3. ATM, ATR, RAD51, SPO11-2

For *Atatm-2* plants, the wild type and the mutant allele are determined in a three-primer PCR with ATM-F1, ATM-R1 and SALK LBc1, giving a 795bp product for the wild type allele and a product of about 600bp for the mutant.

For *Atatr-2* plants, the wild type allele is determined with primers atr2_1 and atr2_3, producing 765bp, and the mutant allele is verified with primers atr2_1 and SALK LBa1, giving ~700bp.

For *Atrd51* plants, the wild type allele is determined with primers pcr_atrad51_1 and pcr_atrad51_2, giving an 850bp product, and the mutant allele is confirmed using primers rad51TDNA and RAD51gen, giving a product of about 800bp.

For *Atspo11-2-3* plants, the wild type and the mutant allele are determined in a three-primer PCR with Spo11-2_GABI_dn, Spo11-2_GABI_up and GABItest1, producing 1072bp for the wild type allele and ~500bp for the mutant.

7.8. Supplementary data

7.8.1. List of abbreviations

- ICL inter-strand crosslinking
- ICLs inter-strand crosslinking agents
- DSB double-strand break
- ssDNA single-strand DNA
- dsDNA double-strand DNA
- FA Fanconi anemia
- NHEJ non-homologous end joining
- HR homologous recombination
- D-loop displacement loop
- HJ Holliday junction
- SDSA synthesis-dependent strand annealing
- PIKK phosphoinositide-3-kinase like kinase
- SC Synaptonemal complex
- IH interhomolog
- AE axial elements
- LE lateral elements
- CO crossover
- NCO non-crossover

7.8.2. Antibodies

antibody	organism	company	dilution	publication
<u>used for immuno-cytochemistry</u>				
α ASY1	rabbit		1:500	(Armstrong <i>et al.</i> , 2002)
α ZYP1	rat		1:500	(Higgins <i>et al.</i> , 2005)
α RAD51	rat		1:500	
α rat AlexaFluor566	goat	Invitrogen	1:500	
α rabbit AlexaFluor488	goat	Invitrogen	1:500	
α DMC1	rabbit		1:20	(Chelysheva <i>et al.</i> , 2007)
α ASY1	rat		1:500	(Higgins <i>et al.</i> , 2004)
α rat AlexaFluor488		Invitrogen	1:400	
α rabbit AlexaCy3		Invitrogen	1:500	
α Phospho-(Ser/Thr) ATM/ATR Substrate Antibody #2851S		Cell Signaling	1:100	(Carballo <i>et al.</i> , 2008)
α RAD51	rabbit		1:200	(Mercier <i>et al.</i> , 2003)
<u>used for Western blot</u>				
α FANCD2-peptide 1 #EP064074	rabbit	Eurogentec	1:5'000	
α FANCD2-peptide 2 #EP064075	rabbit	Eurogentec	1:5'000	
α FANCD2-peptide 3 #ep064073	rabbit	Eurogentec	1:5'000	
α FANCD2-Nterm	rabbit		1:3'000	(Kerzendorfer and Schweizer, 2007)
α rabbit HRP			1:100'000	
α nti-HA (12CA5)	mouse		1:300	
α mouse HRP			1:30'000	

7.8.3. Primers

primer name	sequence	alleles analyzed
<u>plant genotype verification</u>		
ATM-F1	5' - GCT TCA AGG TTG GGC AGT TCC - 3'	<i>Atatm-2</i>
ATM-R1	5' - GCA AAC AGC ATA ACA AAA CAC TTC C - 3'	<i>Atatm-2</i>
Lbc1	5' - TGG ACC GCT TGC TGC AAC TCT -3'	<i>Atatm-2</i> , <i>Atfanci-1</i> , <i>Atfanci-2</i>
atr2_1	5' - GGA TCA AGT ACT ACT GAC TCA G -3'	<i>Atatr-2</i>
atr2_3	5' - CAA CTC ATT TTG AAT ATG AGA G -3'	<i>Atatr-2</i>
LBa1	5' - TGG TTC ACG TAG TGG GCC ATC G - 3'	<i>Atatr-2</i> , <i>Atfancd2-1</i> , <i>Atfance</i> , <i>Atfancj-2</i> , <i>Atfancl-1</i> , <i>Atfancm-1</i>

FancUbp	5' - AAC CTG CCT CCT TAC TAT CAT C - 3'	<i>Atfancd2-1</i>
FancClaseq	5' - AGG AGC TGA ATA GTC TGG AG - 3'	<i>Atfancd2-1</i>
FancInt5up	5' - GCC CAC CTT ATG CAA ATT ATC AAT - 3'	<i>Atfancd2-1</i>
FancHPdn	5' - ATT TAA GTT CAG GCC TCT TCT TCC - 3'	<i>Atfancd2-2</i>
FancDmut5up	5' - CCC AGT TTG GCA TGT AGA TTG - 3'	<i>Atfancd2-2</i>
GABItest1	5' - CCC ATT TGG ACG TGA ATG TAG ACA C - 3'	<i>Atfancd2-2, Atspo11-2-3</i>
AtFancESeqdn	5' - AGA GAG ATG CAC ATA ACC TGC - 3'	<i>Atfance</i>
AtFancESequp	5' - TCC GCT CTT GTG TTC TTC CAC - 3'	<i>Atfance</i>
FancI_F12	5' - GAG ATT GCA AGT GGC TAC CTG - 3'	<i>Atfanci-1, Atfanci-2</i>
FancI_R12	5' - TTT ACC TGA AGA TCA CGG CTG - 3'	<i>Atfanci-1, Atfanci-2</i>
FancI_F3	5' - ATG AAG CCC ATG ATC ACA GAC - 3'	<i>Atfanci-3</i>
FancI_R3	5' - TTC ACG CTT TTG GAT ACG AAG - 3'	<i>Atfanci-3</i>
FancJ_F1	5' - ATG GTT TCC TCA ACC AGC AAA TC - 3'	<i>Atfancj-1</i>
FancJ_R1	5' - TGA AGT GAT GGA TGA GAT GTG ATT - 3'	<i>Atfancj-1</i>
SAIL-LB3	5' - TAG CAT CTG AAT TTC ATA ACC AAT CTC GAT ACA C - 3'	<i>Atfancj-1</i>
FancJ_F2	5' - GGA CTG ATA AGC TGG ATT GGA - 3'	<i>Atfancj-2</i>
FancJ_R2	5' - CCC AGT TGG TAA TCT AAG ATG TGA C - 3'	<i>Atfancj-2</i>
FancL_5'UTR_dn	5' - AAA TTA AGC AGG AAG ATT CAC G - 3'	<i>AtfancL-1</i>
FancL_E4_up	5' - CCT TCA ATC TTG AAC TTG TTG A - 3'	<i>AtfancL-1</i>
FancM_dn	5' - ATG GGA TCT AGG GTT CCA ATA G - 3'	<i>Atfancm-1</i>
FancM1_up	5' - CAA GTG GTA ATT TGA GAA CAA AC - 3'	<i>Atfancm-1</i>
pcr_atrad51_1	5' - GGT TCC ATC ACG GAG TTA TAT GG - 3'	<i>Atrad51</i>
pcr_atrad51_2	5' - AGC CAT GAT ATT CCC ACC AAT C - 3'	<i>Atrad51</i>
rad51TDNA	5' - CCC ATT TGG ACG TGA ATG TAG ACA C - 3'	<i>Atrad51</i>
RAD51gen	5' - GCA GGT ATT GCT TCT GTT GAT GTA - 3'	<i>Atrad51</i>
Spo11-2_GABI_down	5' - GCT CGT GGA AGA TCG TGT GTT C - 3'	<i>Atspo11-2-3</i>
Spo11-2_GABI_up	5' - CCT GCA TAG GAA AGT GGA GAT TAG GAC - 3'	<i>Atspo11-2-3</i>

generation of artificial microRNA

FD2-1_miR-s	5' - gaT AAT ACT GGA TGT GTT CGG ACt ctc tct ttt gta ttc c - 3'
FD2-2_miR-a	5' - gaG TCC GAA CAC ATC CAG TAT TA t caa aga gaa tca atg a - 3'
FD2-3_miR*s	5' - gaG TAC GAA CAC ATC GAG TAT TTt cac agg tcg tga tat g - 3'
FD2-4_miR*a	5' - gaA AAT ACT CGA TGT GTT CGT ACt cta cat ata tat tcc t - 3'
FL1-miR-s	5' - gaT ACA TGT GTA ATC TGT TCG TCt ctc tct ttt gta ttc c - 3'

7.8.5. Plasmid maps

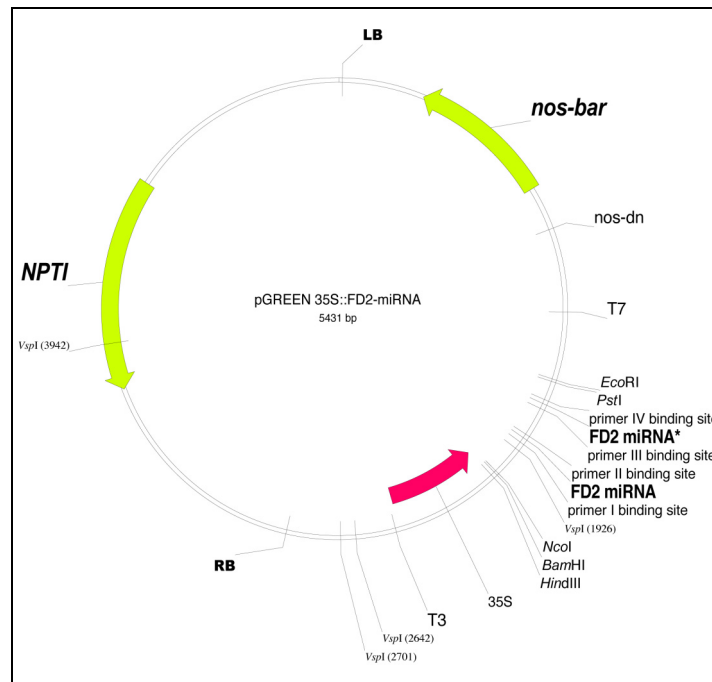


Figure 7-1 pGREEN-35S::*FANCD2*^{RNAi} encoding sense and antisense artificial microRNA against *FANCD2* mRNA under a constitutive CaMV 35S promoter, Basta® resistance and kanamycin resistance.

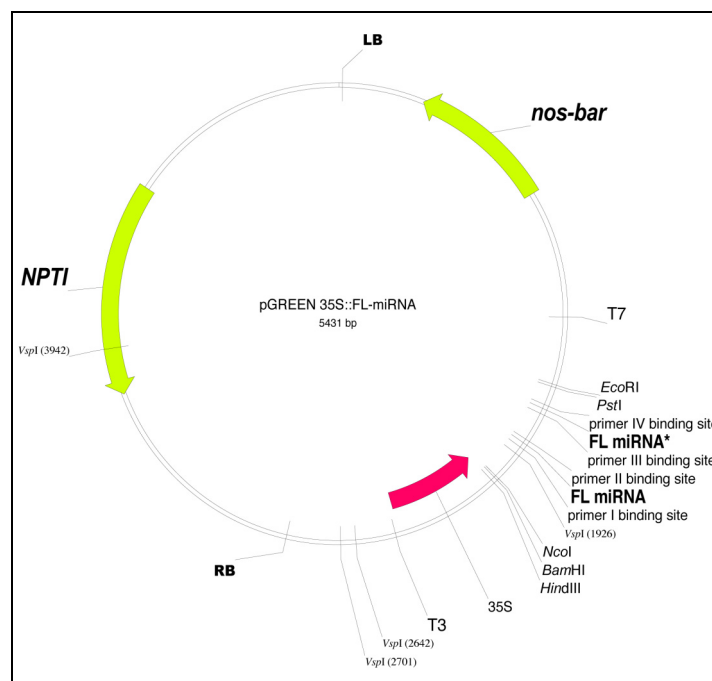


Figure 7-2 pGREEN-35S::*FANCL*^{RNAi} encoding sense and antisense artificial microRNA against *FANCL* mRNA under a constitutive CaMV 35S promoter, Basta® resistance and kanamycin resistance

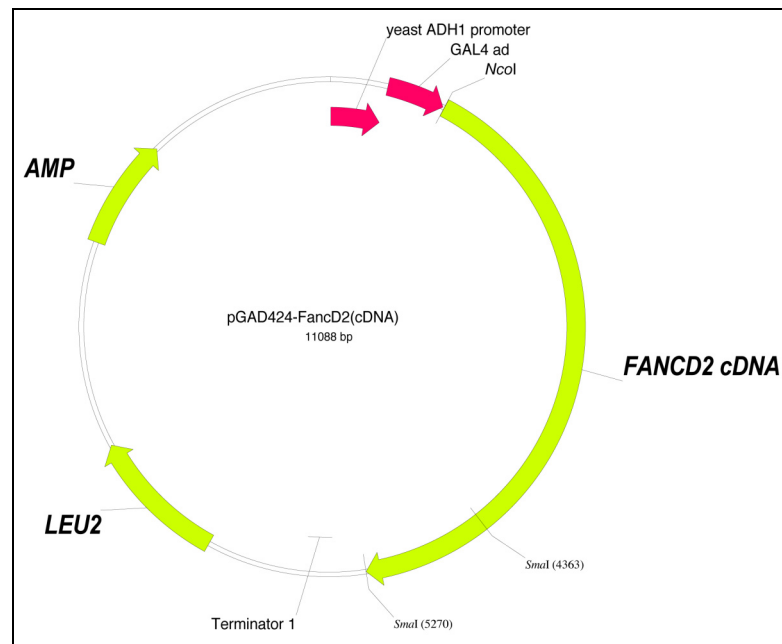


Figure 7-3 pGAD424-*FANCD2*(cDNA) encoding a fusion protein of yeast GAL4 activation domain and FANCD2 under a yeast ADH1 promoter, LEU2 for Leucine-auxotroph yeast strains and ampicillin resistance. Cloning sites are shown. (Courtesy of Kerzendorfer and Schweizer, 2007).

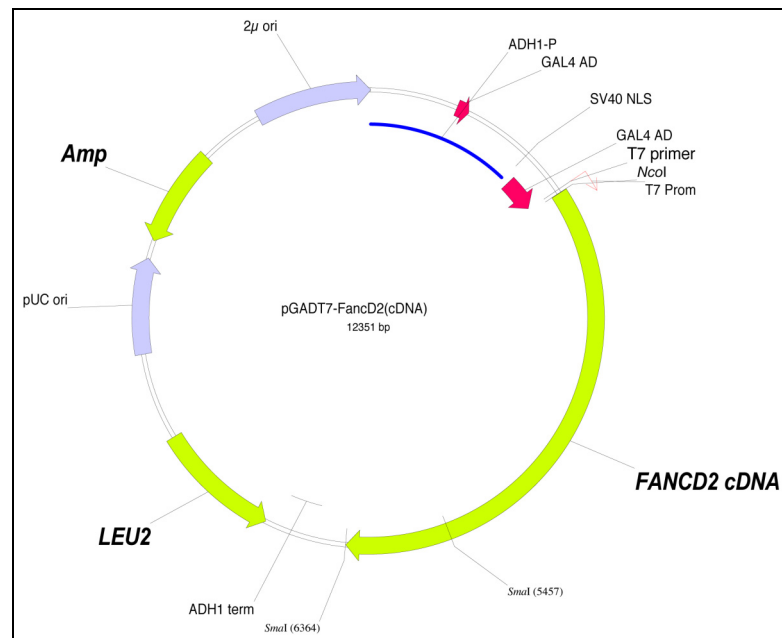


Figure 7-4 pGADT7-*FANCD2*(cDNA) encoding a fusion protein of yeast GAL4 activation domain and FANCD2 under a yeast ADH1 promoter, LEU2 for selection in Leucine auxotroph yeast strains and ampicillin resistance. Cloning sites are shown. (Courtesy of Kerzendorfer and Schweizer, 2007).

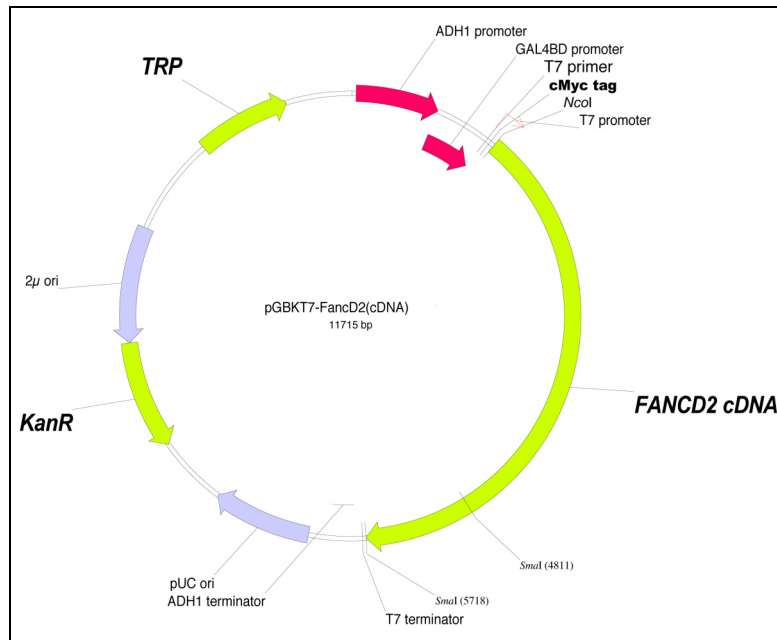


Figure 7-5 pGBKT7–*FANCD2*(cDNA) encoding a fusion protein of yeast GAL4 binding domain and *FANCD2* under a yeast ADH1 promoter, TRP1 for selection in Tryptophane-auxotroph yeast strains and kanamycin resistance. Cloning sites are shown. (Courtesy of Kerzendorfer and Schweizer, 2007).

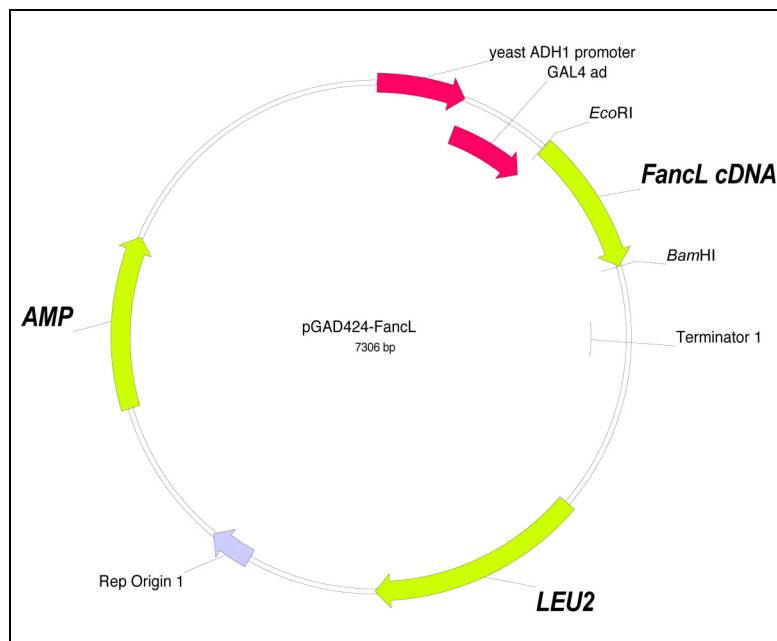


Figure 7-6 pGAD424–*FANCL* encoding a fusion protein of yeast GAL4 activation domain and *FANCL* under a yeast ADH1 promoter, LEU2 for selection in Leucine auxotroph yeast strains and ampicillin resistance. Cloning sites are shown.

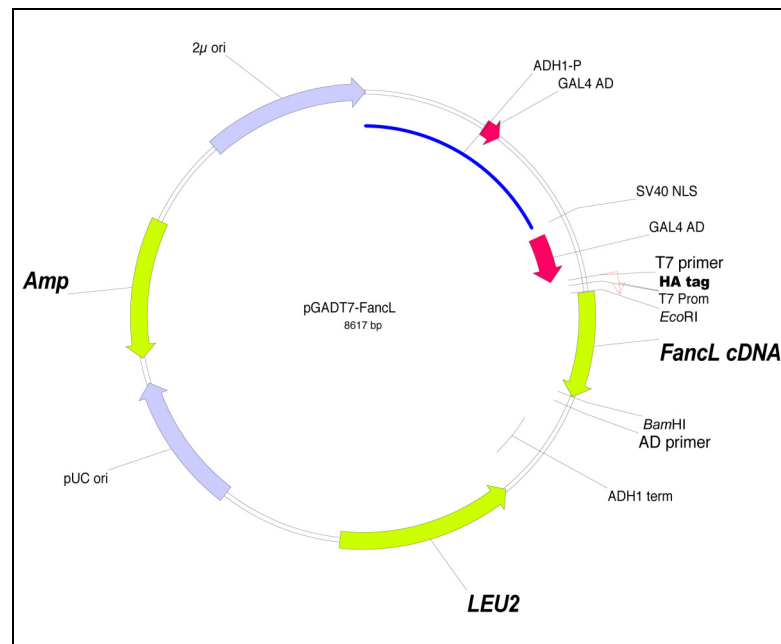


Figure 7-7 pGADT7-*FANCL* encoding a fusion protein of yeast GAL4 activation domain, HA-Tag and FANCL under a yeast ADH1 promoter, LEU2 for selection in Leucine auxotroph yeast strains and ampicillin resistance. Cloning sites are shown.

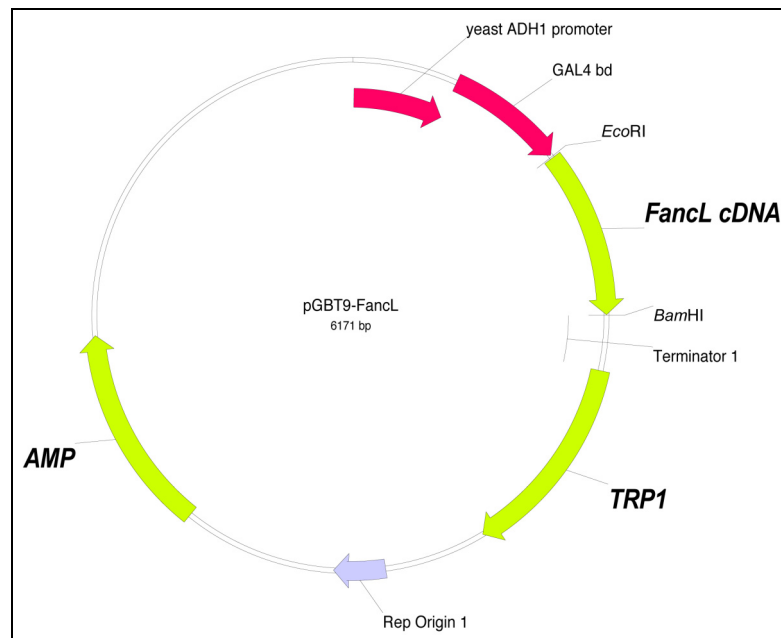


Figure 7-8 pGBT9-*FANCL* encoding a fusion protein of yeast GAL4 binding domain and FANCL under a yeast ADH1 promoter, TRP1 for selection in Tryptophane-auxotroph yeast strains and ampicillin resistance. Cloning sites are shown.

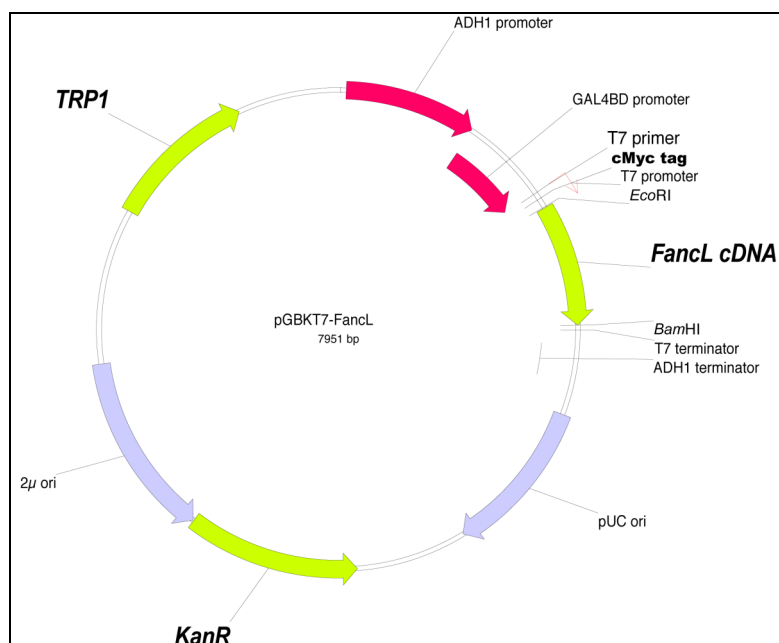


Figure 7-9 pGBKT7–FANCL encoding a fusion protein of yeast GAL4 binding domain, cMyc-Tag and FANCL under a yeast ADH1 promoter, TRP1 for selection in Tryptophane-auxotroph yeast strains and kanamycin resistance. Cloning sites are shown.

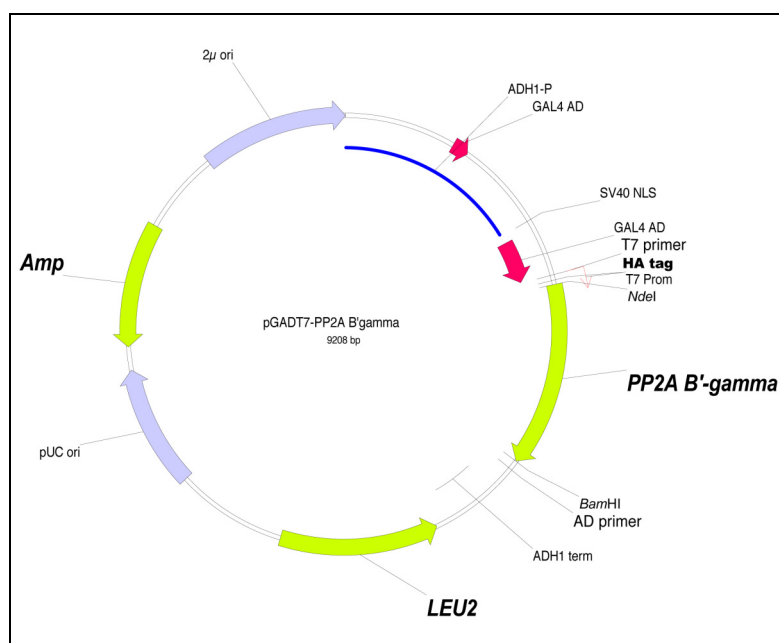


Figure 7-10 pGADT7–PP2A-B'γ encoding a fusion protein of yeast GAL4 activation domain, HA-Tag and PP2A subunit B'γ under a yeast ADH1 promoter, LEU2 for selection in Leucine-auxotroph yeast strains and ampicillin resistance. Cloning sites are shown.

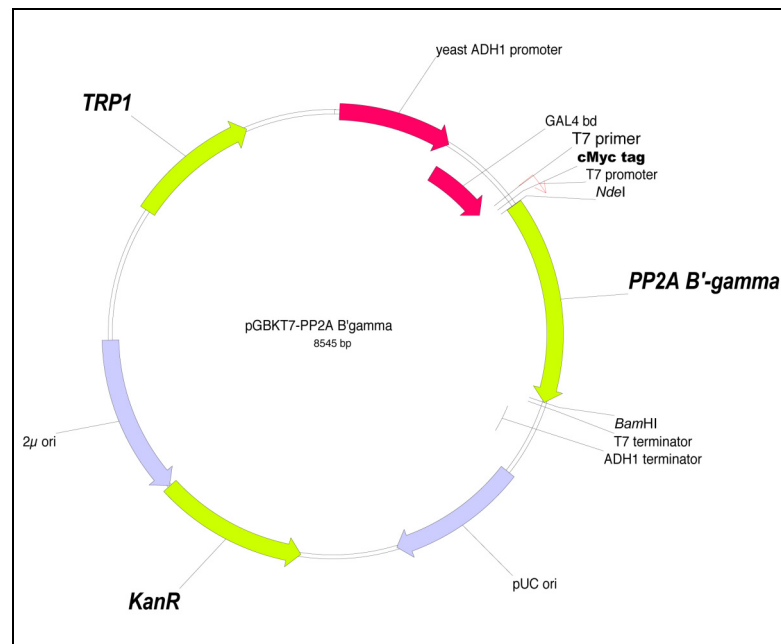


Figure 7-11 pGBKT7–PP2A-B' B'γ encoding a fusion protein of yeast GAL4 binding domain, cMyc-Tag and PP2A subunit B'γ under a yeast ADH1 promoter, TRP1 for selection in Tryptophane-auxotroph yeast strains and kanamycin resistance. Cloning sites are shown.

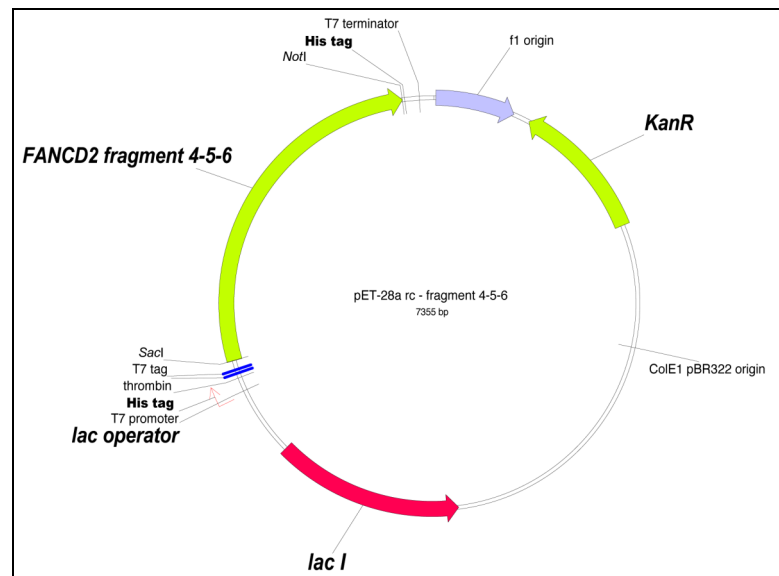


Figure 7-12 pET28a–FANCD2 fragment 4-5-6 encoding a His-tagged FANCD2 fragment (fragment 4-5-6, see Kerzendorfer and Schweizer, 2007) under an IPTG-inducible Lac-promoter and conferring kanamycin resistance. Cloning sites are indicated.

7.8.6. Supplementary figures

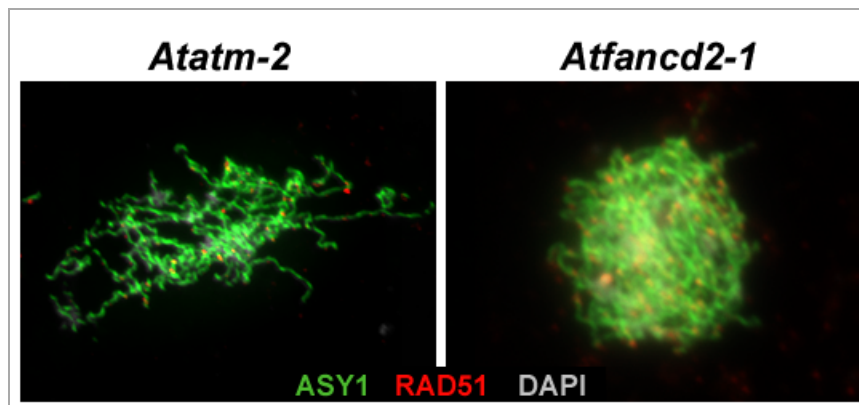


Figure 7-13 ASY1 polymerization and AtRAD51 foci formation is unaffected in *Atatm-2* and *Atatm-2 Atfancd2-1* mutants.

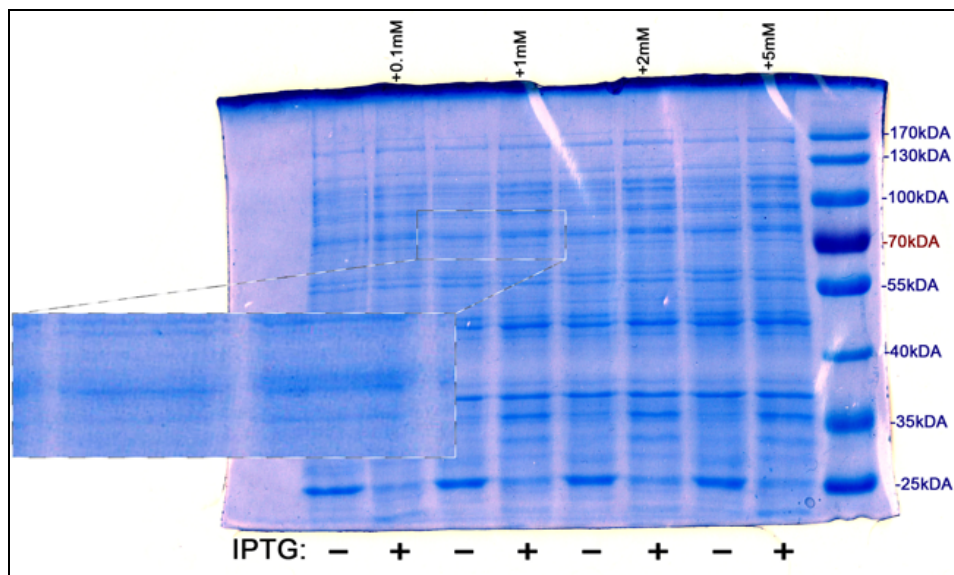


Figure 7-14 Coomassie-stained SDS-PAGE gel of protein extracts of *E. coli* RosettaTM (Novagen) with pET28a-FANCD2 fragment 4-5-6 (see Figure 7-12) and IPTG induction, concentrations indicated.

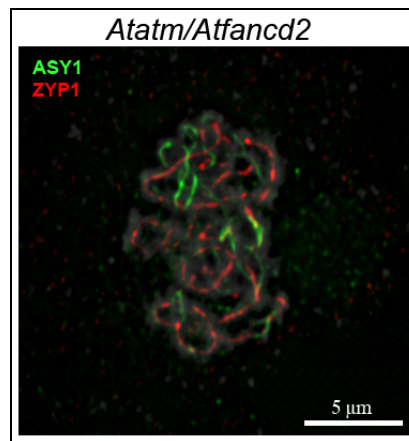


Figure 7-15 Axis formation and Synaptonemal complex generation in *Atatm-2* *Atfancd2-1*. Immunolocalization of ASY1 and ZYP1 on pollen mother cells.

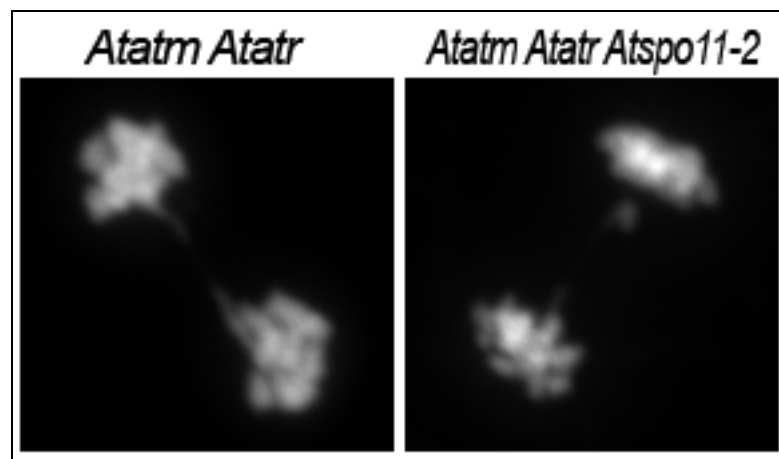


Figure 7-16 Mitotic anaphase bridges in DAPI spreads of *Atatm-2 Atatr-2* and *Atatm-2 Atatr-2 Atspo11-2-3* PMCs.

8. Literature

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