Role of non-homologous end-joining in T-DNA integration in Arabidopsis thaliana

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Chapter 1

General introduction

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Double-strand breaks (DSBs) are considered to be one of the most lethal forms of DNA damage. They can arise as a consequence of normal cellular metabolism, but occur more frequently due to external factors such as ultraviolet (UV) radiation, ionizing radiation and genotoxic chemicals (Khanna and Jackson 2001). For instance, exposure of the chest to X-rays causes 0.0008 DSBs per cell; a body CT, 0.28 DSBs per cell; a tumor PET scan, 0.4 DSBs per cell; 10 hours Airline travel, 0.002 DSBs per cell; 10 days space mission, 0.33 DSBs per cell (Ciccia and Elledge 2010). DSBs are highly toxic if unrepaired, can cause genome rearrangements and even cell death.

To prevent this, cells have evolved complex and highly conserved systems to detect these lesions, signal their presence, trigger various downstream events and finally bring about repair. There are two main pathways for DNA DSBs repair: Homologous Recombination (HR) and Non-Homologous End-Joining (NHEJ). The HR pathway precisely restores the genomic sequence of the broken DNA ends by using the sister chromatid as a template for accurate repair. In contrast, NHEJ promotes direct ligation of the DSB ends without the requirement for sequence homology and may result in small insertions and deletions at the break site. Both pathways are highly conserved throughout eukaryotic evolution but their relative importance may be different depending on the stage of the cell cycle or the cell type. Unicellular eukaryotes such as the yeast *Saccharomyces.cerevisiae* with small genomes mostly rely on HR to repair DSBs, whereas in higher eukaryotes, like mammals and plants with large genomes containing many repeat sequences, the NHEJ pathway is the predominant repair pathway.

In this chapter, we shall review first the DNA DSBs response and its repair. Two main DSBs repair pathways, HR and NHEJ, their regulation and how these pathways affect DNA integration and *Agrobacterium*-mediated T-DNA integration in particular are discussed. This will be followed by a discussion on strategies to induce DSBs artificially at specific sites in the genome and how this can be exploited to affect gene targeting. Finally we will give an outline of the thesis.

DNA damage response

The DNA damage response (DDR) is a signal transduction pathway that affects many aspects of cellular physiology (DNA repair, cell-cycle arrest, apoptosis and senescence) (Harper and Elledge 2007) (**Figure 1**). Master regulators of this pathway are members of the phosphatidylinositol 3-kinase-like protein kinases (PIKKs) family (Block *et al.* 2004). They control downstream amplification of DNA damage signals by recruitment and phosphorylation of their substrates. The current understanding of the DDR mechanism in mammalian cells is mostly based on the study of the two most important members of the PIKKs family: ATM (Ataxia Telangiectasia Mutated) and ATR (ATM and Rad-3 related) (Falck *et al.* 2005; Shiloh 2006; Matsuoka *et al.* 2007). DSBs are recognized by the MRE11-RAD50-NBS1 (MRN) complex, which then triggers the activation of ATM to activate and/or induce the levels of DNA repair proteins to bring about repair. The appearance of areas in the genome with ssDNA as a consequence of DNA damage or repair leads to the recruitment and activation of the other master regulator ATR. After their recruitment to sites of damage, ATM

and ATR then phosphorylate a number of substrates, including the downstream protein kinases CHK1 and CHK2, which generate a downstream amplification by protein activation and repression, leading to cell cycle arrest and DNA repair. Local phosphorylation of histone H2AX at damage sites leads to local accumulation of repair proteins, which is enhanced by ubiquitination and poly (ADP) ribosylation of specific damage response pathway components.

In contrast to mammalian cells, little is known about the DNA damage response in plants. So far, only few homologous genes in plants have been characterized related to the DNA damage response. The Arabidopsis orthologs of the mammalian ATM and ATR protein play an essential role in the response to DNA damage (Garcia *et al.* 2003; Culligan *et al.* 2006). WEE1 is a critical downstream target of the ATR-ATM signaling cascades (De Schutter *et al.* 2007). Despite the conserved nature of the ATM and ATR kinases, plants and animals seem to respond distinctively different to DNA stress. Many genes needed for cell cycle and DNA damage checkpoint in animals have no ortholog in plants, such as CHK1, CHK2 and p53 (Cools and De Veylder 2009). Instead plants use a downstream factor called SOG1 (suppressor of gamma response 1) as a central hub in the DNA damage response (Yoshiyama *et al.* 2013).

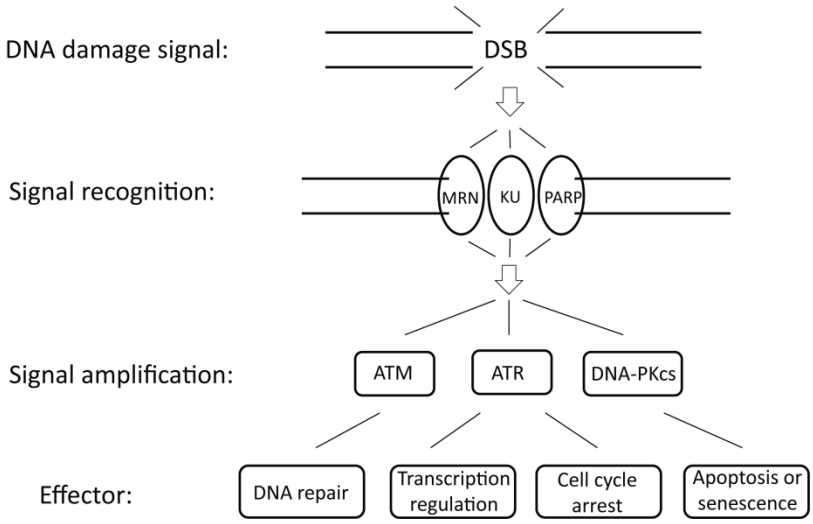

Figure 1. The DNA damage response. After signal (DSB) recognition, signal amplification occurs and effectors are producted. The presence of DSBs in the DNA is recognized by various sensor proteins, such as KU, MRN and PARP. These sensors initiate signaling pathways and activate the PIKKs: DNA-PKcs, ATM and ATR. The PIKKs amplify signals by phosphorylation of a number of downstream substrates, which ultimately leads to cell cycle arrest, DNA repair, activation of transcriptional programmes or induction of programmed cell death.

Homologous recombination

Homologous recombination (HR) promotes genome stability by facilitating error-free repair of DSBs, interstrand crosslinks (ICLs), and DNA gaps during and after DNA replication (Heyer *et al.* 2010). HR is a key repair pathway in the S and G2 phases of the cell cycle. Repair of DSBs via HR requires homologous sequences that act as a template for the reaction. In this

process, the sister chromatid is the preferred template (Kadyk and Hartwell 1992). The information from the homologous sister chromatid is copied into and replaces the damaged region resulting in precise repair. Homology search and DNA strand invasion are key steps in this process. Both are catalyzed by DNA-dependent ATPase Rad51, which is the eukaryotic RecA homolog. It can bind cooperatively to ssDNA, forming helical nucleoprotein filaments (Conway *et al.* 2004). HR can be conceptually divided into three stages and Rad51 functions in all these phases: pre-synapsis, synapsis, and post-synapsis (**Figure 2**).

In the pre-synapsis phase, the DSB is processed to generate ssDNA overhangs by 5' to 3' DNA end resection. End resection in yeast occurs by a two-step mechanism: initial resection of 50-200 nt involves the MRX (MRE11-RAD50-XRS2) complex and Sae2 (Mimitou and Symington 2008), and more extensive resection of up to several kilobases involves either Exo1 or Sgs1 in combination with Dna2 (Mimitou and Symington 2011). Functional homologs of these proteins are found in higher eukaryotes including plants as well (Mimitou and Symington 2009; Osman *et al.* 2011). After end resection, ssDNA is bound by replication protein A (RPA) to protect the ends from degradation and formation of secondary structures, which is needed for competent Rad51 filaments to assemble (Owalczykowski 1998). However, RPA bound to ssDNA also acts as a barrier against Rad51 filament assembly (Sugiyama *et al.* 1997). This inhibitory effect of RPA on Rad51 filament formation can be overcome by at least three different kinds of mediator proteins (Heyer *et al.* 2010). Rad51 paralogs and Rad52 have been identified as the key mediators of Rad51 filament formation in yeast (Sugawara *et al.* 2003). Rad52 interacts with Rad51 as well as with RPA. These interactions are required to recruit Rad51 and also accelerate displacement of RPA from ssDNA by Rad51 (Sugiyama and Kowalczykowski 2002). Besides its function as mediator, Rad52 also contributes to the later stage of recombination (Nimonkar *et al.* 2009). The third class of mediator protein is BRCA2, the human breast and ovarian cancer tumor suppressor protein, which is present in mammalian and plant cells, but not found in budding yeast (Holloman 2011; Seeliger *et al.* 2012).

The Rad51 filament facilitates fast and efficient homology search and DNA strand invasion, leading to the generation of heteroduplex DNA (D-loop). Rad54 is required for searching homology, stimulates DNA strand invasion by the Rad51 filament and also functions after synapsis (Mazin *et al.* 2003; Wolner and Peterson 2005). DNA synthesis is primed by the 3'end of the invading strand, using the donor strand as template.

After extension of the 3' invading strand, repair is finalized by one of at least three different sub-pathways of HR: classical double-strand break repair (DSBR) (Szostak *et al.* 1983), synthesis-dependent strand annealing (SDSA) (Nassif *et al.* 1994), or break-induced replication (BIR) (Kraus *et al.* 2001). In the classical DSBR pathway, Rad52 and Rad59 promote capture of the second end by the D-loop, whereafter two Holliday junctions (dHJ) are formed (Wu *et al.* 2006). These dHJs are either dissolved into non-crossover products by a RecQ helicase such as yeast Sgs1 or human BLM helicase or resolved by a structure-specific endonuclease into crossover/non-crossover products. In general, non-crossovers are much more predominant in mitotic HR (Baudat and de Massy 2007). Crossovers have the potential to generate genomic rearrangements and large-scale loss of heterozygosity (LOH) (Moynahan and Jasin 2010). To avoid crossovers events in mitotic cells, the Mph1 helicase is able to

suppress the DSBR pathway by dissociating D-loops to facilitate annealing of the extended 3' tail to complementary sequences at the other side of the DSB, leading to the SDSA sub-pathway (Prakash *et al.* 2009). It seems that the Rad51 protein has some inhibitory activity that counters capture of the second end and dHJ formation, indicating an inherent mechanistic bias toward SDSA (Wu *et al.* 2008). In the absence of a second end, the D-loop may become a replication fork, leading to the BIR sub-pathway. During BIR, the replication fork restores the integrity of a broken chromosome by copying the entire distal arm of the template chromosome, resulting in LOH (Lydeard *et al.* 2007). BIR is elevated in rad51 and mre11 mutants (Krishna *et al.* 2007).

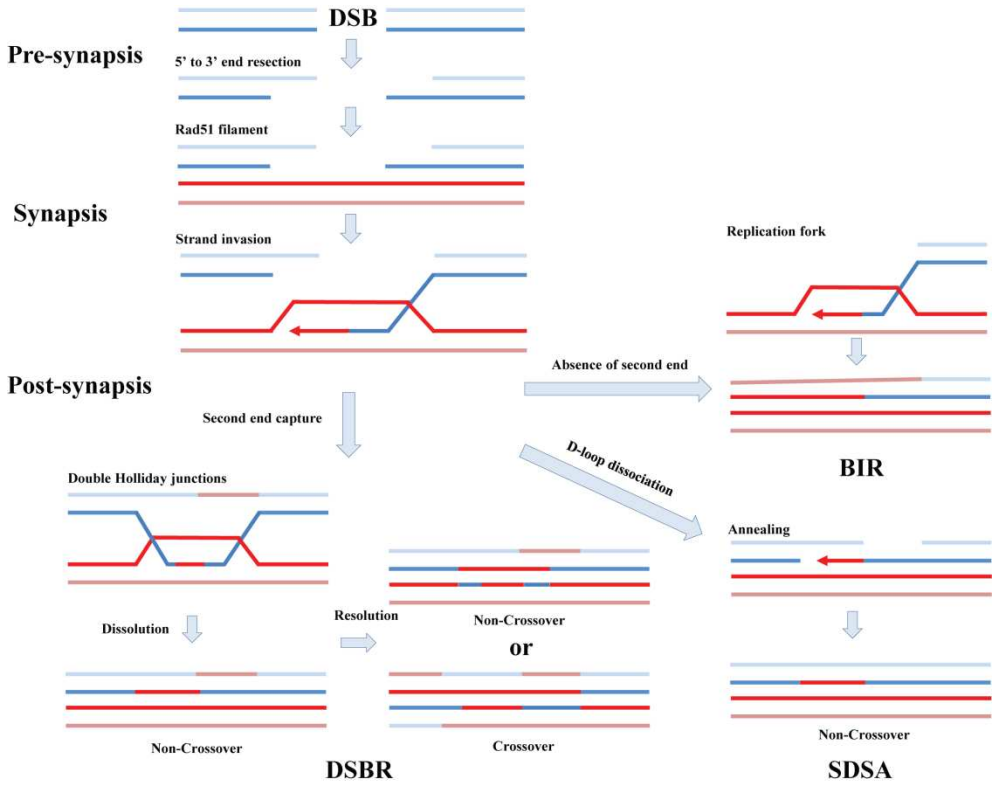

Figure 2. DSB repair by HR. DSBs can be repaired by distinctive HR pathways, including double-strand break repair (DSBR), synthesis-dependent strand annealing (SDSA) and break-induced replication (BIR). First, the DNA damage site is processed by 5' to 3' DNA end resection to generate ssDNA overhangs, which is indispensable for Rad51 filament formation. The Rad51 filament facilitates fast and efficient homology search and DNA strand invasion, leading to the generation of a D-loop structure. In the DSBR pathway, the second end is captured and a double Holliday Junction is formed. The dHJs are either dissolved into non-crossover products by BLM/Sgs1 helicase or resolved by a structure-specific endonuclease into crossover/non-crossover products. BIR happens when a second end is absent. The D-loop intermediate turns into a replication fork, leading to strand synthesis, which replicates the entire homologous template arm.

Table 1. HR proteins in human, yeast and Arabidopsis.

Homo sapiens	*Saccharomyces cerevisiae*	*Arabidopsis thaliana*	*Arabidopsis* gene number	Function
Rad51	Rad51	Rad51	At5g20850	RecA homologue, Strand invasion
MRN complex: Mre11-Rad50-Nbs1	MRX complex: Mre11-Rad50-Xrs2	MRN complex: Mre11-Rad50-Nbs1	At5g54260-At2g31970-At3g02680	DNA binding, Nuclease activities, DSB ends processing, DNA-damage checkpoints
CtIP	Sae2	Com1	At3g52115	DSB ends processing, DNA strand transition
Exo1	Exo1	Exo1A, Exo1B	At1g29630, At1g18090	DSB ends processing
BLM	Sgs1	RecQ4A	At1g10930	DSB ends processing, RecQ helicases
RPA1, RPA2, RPA3	RPA1, RPA2, RPA3	RPA1, RPA2, RPA3	At2g06510, At4g19130, At5g45400, At5g08020, At5g61000; At2g24490, At3g02920; At3g52630, At4g18590	ssDNA binding
Rad51B-Rad51C Rad51C-XRCC3 Rad51D-XRCC2	Rad55-Rad57	Rad51B-Rad51C Rad51C-XRCC3 Rad51D-XRCC2	At2g28560, At2g45280, At5g57450, At1g07745, At5g64520	ssDNA binding, Recombination mediator
Rad52	Rad52	Rad52	At1g71310, At5g47870	ssDNA binding and annealing, Recombination mediator, Interacts with Rad51 and RPA
BRCA1	-	BRCA1	At4g21070	Checkpoint mediator, Recombination mediator
BRCA2	-	BRCA2-1, BRCA2-2	At5g01630, At4g00020	Recombination mediator
Rad54	Rad54	Rad54	At3g19210	ATP-dependent dsDNA translocase, Stimulates the D-loop reaction
FancM	Mph1	FancM	At1g35530	Helicase activity, Dissociates D-loop formation and facilitates single strand annealing
PARI	Srs2	Srs2	At4g25120	Helicase activity, Dissociates D-loop formation and facilitates single strand annealing

Non-homologous end-joining

Non-homologous end-joining (NHEJ) is believed to be the major pathway for the repair of DSBs in most higher eukaryote cells. The basic mechanism of the NHEJ process looks relatively simple and straightforward: it rejoins broken ends directly, without requirement for long runs of end-resection and searching of a homologous repair template. Still many factors are required for NHEJ which demand precise cooperation and timely regulation. Although an increasing number of proteins involved in NHEJ have been identified and their interactions investigated in the past two decades, there is still much to learn about NHEJ.

In outline, the NHEJ pathway can be divided in three distinct steps (**Figure 3**). In the first step, the broken ends of DNA are bound by the Ku70/80 heterodimer to initiate the NHEJ pathway. In the second step, more NHEJ factors are recruited to perform end processing, including Artemis, polynucleotide kinase/phosphatase (PNKP), gap-filling DNA polymerases mu (Pol μ) and lambda (Pol λ), and the Mre11/Rad50/Nbs1 (MRN) complex. In the last step, the ligase4/XRCC4/XLF complex catalyzes the ligation of the processed DNA ends (Mahaney *et al.* 2009). In general, DSB repair by NHEJ can be precise, but may also cause small deletions and insertions of nucleotides at the junction, which alters the nucleotide sequence information surrounding the repair region (van Gent and van der Burg 2007). As a result, NHEJ is referred to as an error-prone DNA repair pathway. More recently, backup NHEJ pathways (b-NHEJ) for DNA repair, also called microhomology-mediated end-joining (MMEJ) or alternative-NHEJ (a-NHEJ), were identified from investigations of cells deficient for NHEJ components (**Figure 3**).

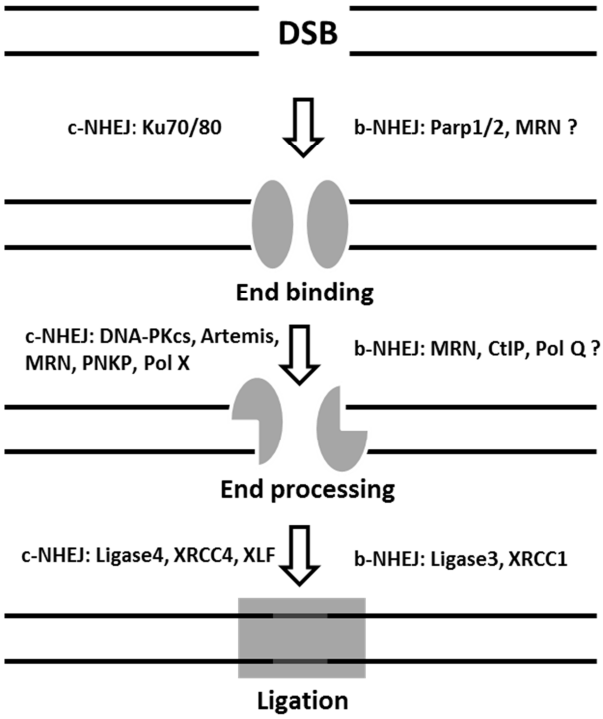

Figure 3. DSB repair by NHEJ. NHEJ comprises three distinct steps: end binding, end processing and ligation. Important factors involved in classical NHEJ and backup NHEJ, which have been identified in mammalian cells, are shown.

Classical non-homologous end-joining

Ku

The classical non-homologous end-joining (c-NHEJ) is initiated by the recognition and binding of DSBs by the Ku heterodimer (Mari *et al.* 2006; Hammel *et al.* 2010) (Figure 3). The Ku heterodimer is comprised of Ku70 and Ku80 subunits, both containing three domains: the N-terminal domain (von Willebrand domain, vWA), the Ku core domain, and the C-terminal domain. Both the N-terminus and core domains contribute to the heterodimerization of the complex. The C-terminal domain of Ku70 contains a SAF-A/B, Acinus and PIAS (SAP) domain contributing to DNA binding activity, while the Ku80 C-terminus is involved in protein-protein interactions (Zhang *et al.* 2001, 2004). The crystal structure of the human Ku heterodimer showed that the Ku heterodimer forms a ring structure on DNA broken ends in a sequence independent manner (Walker *et al.* 2001). Ku is an abundant protein that has an extraordinary affinity for dsDNA ends allowing it to quickly localize to DSBs (Mari *et al.* 2006). Once bound to a DSB, the Ku heterodimer serves as a scaffold to recruit other c-NHEJ factors to the broken end and promotes end-joining. The Ku heterodimer has been shown to interact with DNA-PKcs (Uematsu *et al.* 2007), XRCC4 (Nick McElhinny *et al.* 2000; Costantini *et al.* 2007), DNA ligase 4 (Nick McElhinny *et al.* 2000; Costantini *et al.* 2007), and XLF (Yano *et al.* 2008), directly (see below). Ku70 and Ku80 have also been identified in Arabidopsis (Bundock *et al.* 2002; Tamura *et al.* 2002; West *et al.* 2002). *Atku* mutants have been shown to display hypersensitivity to DSB-inducing treatments. The absence of the Ku protein reduced the efficiency of NHEJ in an *in vivo* plasmid-based end-joining assay in Arabidopsis (Gallego *et al.* 2003; Jia *et al.* 2012), indicating that the Ku protein plays an important role in NHEJ in Arabidopsis as well.

DNA-PKcs

Once Ku binds to a DSB, in mammalian cells DNA-PKcs is recruited to the DNA break very quickly. DNA-PKcs belongs to the phosphatidyl inositol 3-kinase-like (PIKK) family of serine/threonine protein kinases and their kinase activity is essential for c-NHEJ in mammalian system. However, fungi and plants seem to lack DNA-PKcs. DNA-PKcs phosphorylates multiple c-NHEJ proteins *in vitro,* including Ku, Artemis, XRCC4, ligase 4, and XLF. It also shows *in vitro* autophosphorylation on multiple sites (Meek *et al.* 2008; Mahaney *et al.* 2009). DNA-PKcs can also be phosphorylated by other members of the PIKK family. The phosphorylation property is important for regulating appropriate and timely access of NHEJ proteins to the DNA ends (Neal and Meek 2011).

Artemis

After the DNA break ends have been detected and captured as described above, the next step is end processing to remove non-ligatable ends. Therefore, c-NHEJ requires additional factors to produce compatible DNA termini for ligation. These factors are well characterized in mammalian systems, but poorly understood in plants. Artemis possesses 5' to 3' exonuclease activity and acquires endonuclease activity when interacting with DNA-PKcs to form phosphorylated complexes, which are responsible for DNA hairpin opening (Ma *et al.* 2002, 2005). Lack of Artemis results in accumulation of unopened DNA hairpins at DNA ends. In

plants, its homolog called sensitive to nitrogen mustard 1 (SNM1) has been reported to function in the repair of oxidative stress-induced DNA damage and positively regulates the level of NHEJ by its exonuclease activity (Molinier *et al.* 2004; Johnson *et al.* 2011).

MRN

The Mre11-Rad50-NBS1 (MRN) complex plays not only a crucial role as a sensor in the DNA damage response, the MRN complex can also bridge the DNA ends and perform end processing in collaboration with CtIP/Sae2, whereby nicking is followed by 3' to 5' exonuclease activity in NHEJ (Paull and Gellert 1998; Chen *et al.* 2001; Williams *et al.* 2008). In plants, MRN mutants display hypersensitivity to DNA damaging agents (Gallego *et al.* 2001; Bundock and Hooykaas 2002; Waterworth *et al.* 2007), but its roles in end processing remain to be determined.

PNKP

Mammalian polynucleotide kinase 3' phosphatase (PNKP) is a bifunctional end processing enzyme. PNKP possesses both 3' DNA phosphatase and 5' DNA kinase activities which are ideally suited for processing of non-ligatable DNA termini, especially 3' overhanging and blunt ended termini, and replaces them with ligatable 5'-phosphates and 3'-OH (Karimi-Busheri *et al.* 2007; Weinfeld *et al.* 2011). PNKP is able to interact with scaffolding XRCC4 as well as poly (ADP-ribose) (PAR) and those interactions facilitate the recruitment of PNKP to the DSB sites (Koch *et al.* 2004; Li *et al.* 2013). Its Arabidopsis homolog called zinc finger DNA 3′ phosphoesterase (ZDP), is a modular protein with a C-terminal 3′-phosphatase domain and an N-terminal DNA-binding domain and has been shown to bind SSBs and DSBs and to dephosphorylate 3′-phosphate ends to generate 3′-OH termini (Petrucco *et al.* 2002; Martínez-Macías *et al.* 2012).

Pol X family

The X-family of polymerases including Pol μ, Pol λ and lymphoid-specific terminal transferase (TdT), are involved in adding nucleotides or filling of gaps at DNA ends to accommodate NHEJ. These three DNA polymerases can be recruited to the DNA termini by Ku, but require the XRCC4-Lig4 complex to perform gap filling (Mahajan *et al.* 2002). As the only member of the family X DNA polymerase in plants, Pol λ was suggested to function in various repair pathways in plants and play a key role in DNA repair in the plant genome (Uchiyama *et al.* 2004; Amoroso *et al.* 2011; Roy *et al.* 2011, 2013).

Ligase4, XRCC4, and XLF

The final step in the repair of a DSB via c-NHEJ is ligation of the broken ends, which is carried out by the ligase 4-XRCC4 complex (Grawunder *et al.* 1997). DNA ligase 4 from Arabidopsis has ATP-dependent ligase activity and interacts with XRCC4 to form the Ligase 4-XRCC4 complex (West *et al.* 2000). This interaction is required for stabilization and stimulation of Ligase 4 activity. The *Atlig4* mutant is hypersensitive to the DNA-damaging agents MMS and X-rays, demonstrating that AtLIG4 is required for the repair of DNA damage (van Attikum *et al.* 2003). In animals, XLF (also called Cernunnos) has a similar structure as XRCC4, interacts with the XRCC4-Ligase4 complex and promotes NHEJ by regulating the activity of this complex (Ahnesorg *et al.* 2006).

Backup non-homologous end-joining

In the absence of the Ku complex higher eukaryotic cells still can accomplish end-joining by back up pathways. Several factors that are involved in b-NHEJ have already been identified: PARP1/2, MRN, CtIP, Ligase3, XRCC1, Pol θ (Figure 3). The molecular mechanism of b-NHEJ initiation is poorly understood, although both PARP1 and the MRN complex appear to play important roles (Deriano and Roth 2013). PARP1 has been well described as an important player of the BER/SSBR pathway responsible for the recruitment of the XRCC1-Ligase3 complex to promote repair (Caldecott 2008). Similarly, the XRCC1-Ligase3 complex also seems to contribute to the DSB ligation in the b-NHEJ pathway in mammalian cells (Audebert *et al.* 2004; Simsek *et al.* 2011). PARP1 has been reported to compete for free DNA ends with Ku and to interact with ATM which is one of the key players in the DNA damage response (Aguilar-quesada *et al.* 2007; Haince *et al.* 2008). Therefore, PARP1 may contribute to the early damage response and serve as a platform to recruit other factors to the broken ends in the b-NHEJ pathway. Recently, work in our group confirmed that the *Arabidopsis* homologs AtPARP1 and AtPARP2 are also involved in MMEJ (Jia *et al.* 2013).

Ku inhibits end resection, and in absence of Ku, the MRN complex and the MRN-interacting C-terminal-binding protein interacting protein (CtIP) probably work together to mediate DSB resection in b-NHEJ. The knockdown of Mre11 by siRNA decreased the frequency of b-NHEJ significantly, but it had no effect on the efficiency of c-NHEJ, suggesting that Mre11 is required specifically for b-NHEJ repair of DNA DSBs (Zhuang *et al.* 2009). The knockout of CtIP also results in a significant reduction in b-NHEJ (Lee-Theilen *et al.* 2011; Zhang and Jasin 2011). Human Pol λ and Pol β assist MMEJ using terminal microhomology regions (Crespan *et al.* 2012) and the interaction between PNKP and XRCC1 (Weinfeld *et al.* 2011), suggests that they may also function in end processing during b-NHEJ. Pol θ, which belongs to the DNA polymerase A-family, was reported to be involved in b-NHEJ in Drosophila and mammalian cells (Chan *et al.* 2010; Mateos-Gomez *et al.* 2015). It has been shown that the polymerase domain of Pol θ is able to independently carry out all the major stages of MMEJ in vitro (Kent *et al.* 2015).

The ligation step in the b-NHEJ pathway is catalyzed by Lig3 in mammalian cells. Since plants are lacking a Lig3 homolog, there must be other factors to take over ligation during b-NHEJ in plants. One of the candidates is SSB repair factor Ligase 1, which has been implicated in the b-NHEJ repair pathway in *Arabidopsis* (Waterworth *et al.* 2009). In mammals, Ligase 1 displays functional redundancy with Ligase 3 and might cooperate in b-NHEJ (Arakawa *et al.* 2012; Paul *et al.* 2013).

Table 2. NHEJ proteins in human, yeast and Arabidopsis.

Homo sapiens	*Saccharomyces cerevisiae*	*Arabidopsis thaliana*	*Arabidopsis* gene number	Function
Ku70	Ku70	Ku70	At1g16970	DSB end binding and protection
Ku80	Ku80	Ku80	At1g48050	DSB end binding and protection
DNA-PKcs	-	-		protein kinase
Artemis	Snm1/PSO2	Snm1	At3g26680	DNA end processing
MRN complex: Mre11-Rad50-Nbs1	MRX complex: Mre11-Rad50-Xrs2	MRN complex: Mre11-Rad50-Nbs1	At5g54260-At2g31970-At3g02680	DNA binding, Nuclease activities, DSB ends processing, DNA-damage checkpoints
PNKP	Tpp1	ZDP	At3g14890	DNA end processing
Pol λ	-	Pol λ	At1g10520	DNA polymerase, DNA end processing
53BP1	Rad9	Rad9	At3G05480	DNA end processing
DNA ligase IV	Dnl4	lig4	At5g57160	ATP-dependent DNA ligase
XRCC4	Lif1	XRCC4	At3g23100	complex with lig4
XLF/Cernunnos	Nej1	-		complex with lig4/XRCC4
Parp1	-	Parp1	At2g31320	DNA end binding, NAD+ADP-ribosyltransferase
Parp2	-	Parp2	At4g02390	DNA end binding, NAD+ADP-ribosyltransferase
Parp3	-	Parp3	At5g22470	DNA end binding, NAD+ADP-ribosyltransferase
CtIP	Sae2	Com1	At3g52115	DNA end processing
DNA ligase III	-	-		ATP-dependent DNA ligase
XRCC1	-	XRCC1	At1g80420	complex with lig3
Pol Q	-	Pol θ (Tebichi)	At4g32700	DNA polymerase, DNA end processing

Regulation of DSBs repair pathways choice

As discussed above, HR and NHEJ are two critical pathways to repair DNA DSBs and preserve genome integrity. But when DSBs arise, how is the decision made to choose between homologous recombination or NHEJ for repair? Recent studies show that the regulation of pathway choice depends on the structure of the DNA ends and the cell cycle phase.

The initiation of end resection is an important determinant for repair pathway choice (Symington and Gautier 2011). End resection is initiated by the MRN complex combined with CtIP, which counteracts NHEJ to promote HR by limited end processing. Absence of MRN-CtIP results in a complete block of homology-directed repair (Zhu *et al.* 2008; Mimitou and Symington 2008). Loss of Ku enhanced end resection suggesting that it plays an important role in regulating the balance between NHEJ and HR. In the second step of end resection, Sgs1/BLM, Dna2 and Exo1 contribute to promote HR via generating long ssDNA

ends which are poor substrates for binding by Ku (Symington and Gautier 2011). 53BP1 and BRCA1 are two factors that are enriched at DSB sites and are supposed to be regulators in the choice between NHEJ and HR, respectively (Escribano-Díaz *et al.* 2013). 53BP1 has been implicated in limiting access of nucleases to DNA ends and contributes to promote c-NHEJ. BRCA1 may mediate HR by negatively regulating 53BP1 repair activity as well as concentrating factors required for processing and the subsequent HR resection.

Another important factor affecting the choice between the HR and NHEJ pathways is the phase of the cell cycle (Chapman *et al.* 2012). HR is mainly restricted to the S and G2 phases when the sister chromatid becomes available as template, whereas NHEJ operates throughout the cell cycle but more predominantly in the G1 phase. The resection of DSBs is prevented in G1. The activity of cyclin-dependent kinases (CDKs) is thought to be the crucial factor in both yeast and human cells to promote efficient end-resection (Ferretti *et al.* 2013). It has been shown that CDKs promote the initiation of resection via phosphorylation of Sae2/CtIP and Nbs1 and also regulate long-range resection via phosphorylation of Exo1 (Buis *et al.* 2012; Falck *et al.* 2012; Wang *et al.* 2013; Tomimatsu *et al.* 2014).

Agrobacterium-mediated T-DNA integration and NHEJ pathways

Agrobacterium tumefaciens mediated genetic transformation involves the transfer of T-DNA from its tumor-inducing plasmid to the host cell nuclear genome. *Agrobacterium* is nowadays used as a vector to produce genetically modified plants. T-DNA is at random positions in the plant genome (Gelvin 2000), which may lead to mutation and position effects altering the expression of the transgenes. Therefore, there is great interest to develop methods for the controlled and targeted integration of T-DNA. In yeast this can be accomplished by providing a segment of yeast homologous DNA in the T-DNA. The HR machinery of yeast then mediates integration at the homologous site (Bundock *et al.* 1995). In plants, homologous recombination can occur between a chromosomal locus and a homologous T-DNA introduced via *Agrobacterium* (Offringa *et al.* 1990), but only with a very low efficiency. Two possible models for T-DNA integration have been proposed (Tzfira *et al.* 2004; Gelvin 2010). In the strand-invasion model, T-DNA integration depends on the microhomology between T-DNA and plant DNA sequences. It was suggested that single stranded T-DNA is preferred for integration (Rodenburg *et al.* 1989; Gheysen *et al.* 1991; Mayerhofer *et al.* 1991). In the DNA DSB repair model, the single stranded T-DNA is first converted to a double stranded T-DNA, whereafter this double strand form integrates into the genome at double strand break sites (Salomon and Puchta 1998). This was supported by the fact that DSBs are preferential targets for T-DNA integration and that T-DNA can be cut by a restriction enzyme before integration (Chilton and Que 2003; Tzfira *et al.* 2003).

Previously, it was shown in our lab that Agrobacterium T-DNA integration in yeast (*Saccharomyces cerevisiae*) depends on NHEJ proteins, such as Ku70 and DNA ligase 4 (van Attikum *et al.* 2001; van Attikum and Hooykaas 2003). Arabidopsis NHEJ mutants have subsequently been studied for T-DNA integration in plants. However, the results obtained in plant T-DNA integration by different research groups are inconsistent and reveal either no or limited negative effects (Friesner and Britt 2003; van Attikum *et al.* 2003; Gallego *et al.* 2003;

Li *et al.* 2005). Disruption of multiple DNA repair pathways at the same time did not eliminate transformation (Jia *et al.* 2012; Mestiri *et al.* 2014; Park *et al.* 2015), suggesting that there must be other unknown proteins and pathways that mediated T-DNA integration in plants.

Homologous recombination pathway and gene targeting

Gene targeting (GT) is a genetic technique based on homologous recombination to change or replace endogenous genes. It is a powerful technique and has been widely used to study gene function. Several approaches have been developed to select and detect GT events, including gene specific selection (GSS) and positive-negative selection (PNS). In GSS schemes, an endogenous target gene is replaced by a copy of the same gene with a selectable mutation. In PNS schemes, selection of homologous recombination relies on positive and negative selectable markers placed within and outside the homologous sequence, respectively. The PNS-based approach has proven to be very successful and is also used in plant species such as rice and Arabidopsis. GT can be achieved efficiently especially in yeast and a few other organisms. However, GT usually is inefficient in cells of multicellular eukaryotes, especially in those of plants and animals due to a much lower efficiency of HR than NHEJ. In order to establish a feasible tool for GT, two options have been tested to improve the frequency of GT events based on the mechanism of HR. The first option was to facilitate the HR pathway by either increasing the synthesis of proteins involved in HR (Reiss *et al.* 2000; Shaked *et al.* 2005) or by inhibiting synthesis of proteins involved in the NHEJ pathway (Jia *et al.* 2012). This was successful in fungi, where prevention of NHEJ by deletion of Ku or Lig4 resulted in very efficient GT (van Attikum and Hooykaas 2003; Kooistra *et al.* 2004; de Boer *et al.* 2010). Unfortunately, in plants hardly any improvement of GT efficiency was seen. Another approach was to enhance GT by inducing genomic DSBs at the target site, which became possible by the development of different classes of artificial nucleases (see below). In this way, the frequency of GT was increased significantly in different organisms including plants (Durai *et al.* 2005; de Pater *et al.* 2009; Urnov *et al.* 2010). GT was also achieved in *Arabidopsis thaliana* by expression of a site-specific endonuclease that not only cuts within the target but also the chromosomal transgenic donor (*in planta* GT), leading to an excised targeting vector in each plant cell (Fauser *et al.* 2012). Due to the recent developments, especially the application of genome editing tools, we expect that a method of efficient GT will be developed for plants in the near future.

Strategies for DSB induction

As DNA recombination events including transgene integration and gene targeting, is increased at break sites in the genome, it has been a strategy to induce local DNA breaks to stimulate these events. Ionizing radiation (X-ray) and genotoxic chemicals (Bleomycin, MMS, etc.) were initially used to induce such DNA breaks, but as they affect the genome in an uncontrolled manner and cause mutation this was not very successful.

Site-specific nucleases have been developed by which DSBs can be induced at a preferred site in the genome. The advent of meganucleases such as SceI for the first time

offered the possibility to induce a DSB at a specific site(s) in a large genome. Such local break led to a significant increase in DNA integration (Salomon and Puchta 1998) and in gene targeting (Smih *et al.* 1995). Since then three classes of nucleases have been used extensively: Zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and CRISPR/Cas (for ''clustered regularly interspersed short palindromic repeats/CRISPR associated'') system.

ZFNs consist of zinc finger arrays fused to the nuclease domain of the type II restriction enzyme Fok I. Each zinc finger typically recognizes three nucleotides and engineered fingers have been combined to recognize specific longer DNA sequences. ZFNs function as a dimer to create a DSB within the spacer between the binding sites of two ZFN monomers. ZFN-mediated gene modification has been reported in different eukaryotic organisms (Urnov *et al.* 2010) and also in Arabidopsis by others and our group (de Pater *et al.* 2009, 2013; Qi *et al.* 2013). Like ZFNs, TALENs are composed of DNA-binding domains linked to the Fok I nuclease domain. Each binding domain contains a variable number of amino acid repeats, each with a RVD (repeat variable di-residues) present at amino acid positions 12 and 13 of each repeat, which are able to specifically recognize a single base pair of DNA (Joung and Sander 2013). TALENs and ZFNs make DSBs with 5' overhangs. TALENs are considered to be more efficient, specific and reproducible, because TALENs are less affected by context of targeting sequences than ZFNs, as shown in yeast (Christian *et al.* 2010), human (Zhang *et al.* 2011) and *Arabidopsis* (Cermak *et al.* 2011).

More recently, a RNA-guided CRISPR/Cas nuclease system was described for inducing DNA DSBs at specific genomic loci (Jinek *et al.* 2012). CRISPR/Cas originates from a microbial adaptive immune system that uses RNA-guided nucleases to cleave foreign genetic material (Jinek *et al.* 2012). The CRISPR/Cas9 used for DSB induction in eukaryote organisms is based on bacterial type II CRISPR/Cas systems, consisting of CRISPR-associated protein Cas9 and a single guide RNA chimera (sgRNA) which was engineered from the tracrRNA (trans-activated CRISPR RNA) and crRNA (CRISPR RNA) (Jinek *et al.* 2012). Cas9 contains two endonuclease domains (HNH-and RuvC-like domains) cleaving both strands of the target DNA, directed by the sgRNA via Watson-Crick base-pairing to the target DNA sequence (Jinek *et al.* 2012). The cleavage locations are also determined by a protospacer adjacent motif (PAM) which is juxtaposed to the complementary region in the target DNA (Jinek *et al.* 2012). In contrast to ZFNs and TALENs, the CRISPR/Cas9 system is markedly easier to design. It requires only a change in the guide RNA sequence and is highly specific and efficient for a vast number of cell types and organisms. During the past three years, the CRISPR/Cas9 technique has been applied successfully in mammals, plants and yeast (review by Kim 2016).

Outline of the thesis

The non-homologous end joining (NHEJ) repair pathway has been well characterized in yeast and mammals, but there is still much to learn in plants. In plants, the main factors of c-NHEJ have been identified; our previous work showed that the b-NHEJ pathway also exist in plants, depending on Parp1 and Parp2 (Jia *et al.* 2013). As it was possible that the same DNA repair

pathways that mediate T-DNA integration in yeast might act also in plants, the first objective of this project was to analyze whether Agrobacterium-mediated transformation efficiency was affected by the absence of NHEJ factors in Arabidopsis. The second objective was to investigate the NHEJ pathways in Arabidopsis.

In **chapter 2**, AtParp3 and AtXrcc1 were functionally characterized, and double and triple mutants in NHEJ factors were obtained by crossing of single mutants. Together with the *ku70*, *ku80*, *parp1*, *parp2*, *parp1parp2*, *lig4*, *lig6* and *lig4lig6* mutants, which were characterized by our lab previously, they were all tested for T-DNA integration in a root transformation assay. In plants, *lig4lig6* double mutants are still able to perform end-joining (Jia, 2011), suggesting that there must still be another ligase to take over their function. Therefore, a putative DNA ligase gene (*LIG1a*) was studied in **chapter 3**. Mre11 has been reported to play a role both in c-NHEJ and b-NHEJ pathways in mammals. In order to analyze whether Mre11 also functions in b-NHEJ in plants, the *ku80mre11* double mutant was obtained by crossing. The function of Mre11 in plants was studied in **chapter 4**. **Chapter 5** focuses on how exactly the DNA ends join in the Arabidopsis NHEJ mutants *in vivo*, which were deficient in c-NHEJ, b-NHEJ or both. Sequence-specific nucleases were used to induced double strand breaks in Arabidopsis NHEJ mutants.

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