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Double strand break (DSB) repair pathways in plants and their application in genome engineering

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1 Introduction

All organisms have to cope with DNA-damaging factors, with plants especially being exposed to many biological and environmental conditions causing DNA damage. Due to their sessile and phototrophic lifestyle, plants in particular have to face increased exposure to a wide variety of these factors such as UV radiation, site-specific biotic and abiotic environmental factors or reactive oxygen species. In the worst case, failure to repair the induced damage can lead to cell death. In order to ensure survival of cells or organisms, repair pathways have evolved as defense mechanisms to protect the integrity of the genetic information while still maintaining its malleability for further evolution. This means that mutations that are beneficial for survival are accumulated under selection pressure. These mutations result from error-prone repair processes and often have a direct impact on quantitative and qualitative traits of the organism.

For thousands of years, humans used this natural, dynamic variability in classical breeding and selected plants with characteristics that are interesting for agriculture. To further enhance genetic diversity, radiation such as X-rays or chemicals such as ethyl methanesulfonate (EMS) were used to increase the mutation rate. Both breeding processes of selection and mutagenesis are very complex and time-consuming, since the desired trait changes arise randomly and undirected, undesired mutations can occur elsewhere in the genome

without being noticed. Furthermore, the efficiency of classical breeding approaches depends on the amount of available functional diversity, which is limited in many elite varieties that have passed through genetic bottlenecks during domestication (Shi and Lai, 2015). Thus, the reliance on natural or randomly induced diversity is a limiting factor slowing down the breeding process (Watson et al., 2018) and contributing to an unpredictable breeding outcome (Scheben and Edwards, 2018). Consequently, in green biotechnology new breeding approaches were sought that result in precise and predictable outcomes.

Through site-specific induction of double-strand breaks (DSBs) in the genome, specific changes can be introduced. In early experiments, rare cutting endonucleases were used as tools. In plants, the homing endonuclease I-SceI from *Saccharomyces cerevisiae* was applied for the induction of DSBs (Jacquier and Dujon, 1985; Perrin et al., 1993). As a meganuclease, I-SceI recognizes an 18 nt sequence, which is often not present in the genome of the target organism. Thus, the recognition site must first be inserted into the genome, which leaves undesirable traces following DSB induction and repair, although no off-target effects are usually detected. Nevertheless, I-SceI was successfully used in plants for DSB induction (Puchta et al., 1993) and for the investigation of basic mechanisms in DNA repair (Puchta et al., 1996; Puchta, 1998; Salomon and Puchta, 1998). With the introduction of synthetic nucleases, the targeted modification of DNA was brought to the next level. The first sequence-specific nucleases developed were genetically modified meganucleases (Arnould et al., 2007; Smith et al., 2006), followed by zinc finger nucleases (ZFNs) (Kim et al., 1996) and transcription activator-like effector nucleases (TALENs) (Boch et al., 2009; Voytas, 2013). With the discovery of the clustered regularly interspaced short palindromic repeats and CRISPR-associated (CRISPR/Cas) system and its adaption as a biotechnological tool (Jinek et al., 2012), molecular biology was revolutionized. Compared to ZFNs and TALENs, the CRISPR/Cas system is characterized by its simplicity, efficiency and low costs, as well as its ability to cut multiple genes at the same time. As these synthetic sequence-specific nucleases and their use in fast and high-precision genome editing are described in detail in other chapters, we will not discuss the characteristics of these different classes of enzymes here but will refer the reader to the respective chapters in this issue and to some recent reviews (Chen et al., 2019; Schindele et al., 2020).

With the availability of artificial site-specific nucleases, it is now possible to induce breaks at almost any site in the genome at will triggering the hosts own repair mechanisms to introduce genomic changes. For effective and targeted genome editing, it is therefore essential to understand the mechanisms behind DSB-induced modifications (Puchta, 2005).

As a DSB poses a major threat to genome integrity, plants have developed a complex repair network to cope with this kind of lesion. In each eukaryotic

cell within the meristematic tissue, about 10 DSBs are generated per day (Martin et al., 1985). These DSBs can be caused by spontaneous oxidative damage, ionizing radiation or replication errors, but also two adjacent single-strand breaks (SSB) can lead to a DSB (Britt, 1996). They can occur during DNA repair and replication, but are also introduced specifically during meiosis or V(D)J recombination (Bleuyard et al., 2006). Since both DNA single strands are affected and the phosphate backbone of the DNA helix is broken, there is no intact strand as a repair template available. Therefore, the repair of DSBs is especially challenging for the organism.

DSB repair can be classified into two different main repair pathways - non-homologous end joining (NHEJ) and homologous recombination (HR) (Jasin and Haber, 2016; Puchta, 2005), which can be further divided into several sub-pathways. The DNA repair pathways are highly conserved between mammals and plants. All DSB repair pathways seem to compete with each other. The choice between these routes depends on the phase of the cell cycle and the initiation of the DNA resection (Ceccaldi et al., 2016; Grabarz et al., 2012; Kakarougkas and Jeggo, 2014). NHEJ is active throughout the cell cycle with the exception of mitosis, while HR is restricted to certain phases. In addition, the direct end joining can be completed faster than recombination and additionally NHEJ is thought to suppress HR (Tomimatsu et al., 2007). Taken together this makes NHEJ the predominant repair route in somatic cells in animals as well as plants. The second determining factor for the choice of the repair pathway is the processing of DSB ends to 3' overhangs by means of 5'-3' resection depending on which repair factors bind to the break site first (Chang et al., 2017; Ranjha et al., 2018). Unprocessed DSBs are repaired via NHEJ, whereas after resection homologies become accessible for strand exchange proteins to initiate HR. The two main repair routes of the DSB repair, NHEJ and HR, are described in more detail below.

2 DSB repair via non-homologous end joining

NHEJ is the main repair pathway of DSBs in somatic plant cells and is responsible for random integration of DNA into plant genomes. In principle, in NHEJ the two ends of a DSB are re-joined directly without using longer homologies. This can result in small insertions, deletions or a combination of both (Indels) (Salomon and Puchta, 1998). Therefore, this repair mechanism is usually described as inaccurate and error-prone (Charbonnel et al., 2010), but analyses in mammals and plants indicate that a significant part of NHEJ proceeds without loss of sequence information (Geisinger et al., 2016; Guo et al., 2018; Schmidt et al., 2019; Shou et al., 2018).

For NHEJ at least two mechanisms are known to operate in plant cells that are present in from other eukaryotes as well. These two mechanisms can be

distinguished by the repair factors that are involved as well as the resulting sequence pattern at the re-joined break ends. The classic or canonical pathway (c-NHEJ) is characterized by the direct ligation of the break ends, while the alternative pathway (a-NHEJ) uses microhomologies (MHs) near the break site for re-ligation (Fig. 1). This is why this repair pathway is also referred to as microhomology-mediated end joining (MMEJ).

2.1 Basic mechanisms of NHEJ

c-NHEJ is also described as the KU-dependent pathway as the key player in this pathway is the KU70/KU80 heterodimer. In the first step, the DSB ends are recognized and bound by the protein complex of KU70 and KU80. This fast binding protects the broken ends from nucleolytic degradation. The KU heterodimer also acts as a platform for the recruitment of other NHEJ factors (Chang et al., 2017). In vertebrates, this includes DNA-PKcs and the

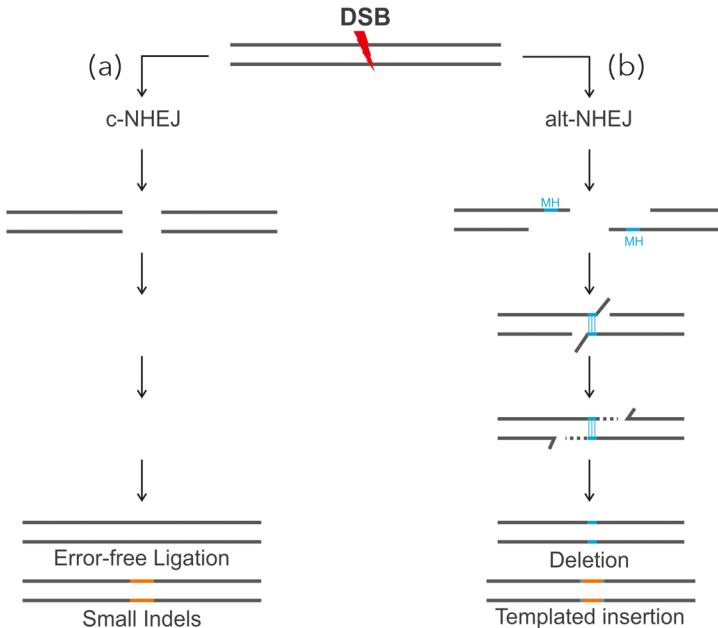


Figure 1 Non-homologous end joining (NHEJ)-mediated repair of DNA double strand breaks (DSB). In plant cells, two major NHEJ pathways are present: (a) the most abundant pathway is classical NHEJ (c-NHEJ). After binding of the KU70/KU80 heterodimer to the broken ends, they can be ligated directly leading either to error-free repair or to the induction of small insertions or deletions (Indels, orange), (b) in the absence of the KU protein, alternative pathways (a-NHEJ) take over. In contrast to c-NHEJ, more processing of the broken ends occurs and they are re-ligated via microhomologies (MH, blue) close to the break site. Therefore, a-NHEJ is error-prone and mainly leads to the deletion of the sequence between the MHs.

endonuclease Artemis as well as X-family polymerases, which act if processing of the break ends is required. KU is also involved in the final processing due to its own enzymatic activity (Roberts et al., 2010; Strande et al., 2012). For final ligation of the DSB, the KU protein recruits the ligase 4 complex comprised of LIG4, XRCC4 and XLF (Ma and Lieber, 2002). Thus, very little or no sequence information is lost. In plants, homologs of the main actors in c-NHEJ - KU70, KU80, LIG4 and XRCC4 - could be identified (Friesner and Britt, 2003; Tamura et al., 2002; West et al., 2000; West et al., 2002). However, no homologs have been detected for the other proteins, as yet. Mutant lines of the important c-NHEJ components in *Arabidopsis* show increased sensitivity to DNA-damaging agents, but do not show any phenotypic differences to the wild type (Bleuyard et al., 2006).

Due to the ubiquitous occurrence of the KU heterodimer and its rapid binding to DSBs, the repair of a DSB is usually carried out via c-NHEJ. However, if this pathway is compromised, the alternative end-joining mechanisms can take over. This KU-independent route to DSB repair is referred to in the literature with many different synonyms. These include backup-NHEJ, alternative NHEJ, microhomology-mediated end joining (MMEJ) or pol theta-mediated EJ/TMEJ. It is still not clear if those are synonyms for only one or if they describe several independent repair pathways. All alternative pathways are characterized by short MHs close to the break site.

a-NHEJ proceeds in five steps: (1) DSB recognition and end resection, (2) stabilization of the ends by annealing of MHs, (3) processing of flap structures, (4) fill-in synthesis and (5) ligation of the DSB (Wang and Xu, 2017). First, the break can be recognized via poly(ADP-ribose) polymerase 1 (PARP1) (Audebert et al., 2004; Robert et al., 2009). PARP1 is in direct competition for the binding of the break ends with KU70/KU80 (Wang et al., 2006). In addition to the detection of the DSB, PARP1 is also necessary for the subsequent recruitment of the other factors of a-NHEJ to initiate resection of the 3' ends of the DSB, which creates short single-strand overhangs. This resection takes place via the same proteins which mediate the resection of the break ends in HR, CtIP and the MRN complex (Truong et al., 2013). By processing the ends, MHs are exposed on both sides of the damage, which are used for re-ligation. In a-NHEJ, MH between 2 bp and 20 bp are sufficient to hybridize the DNA strands, while further resection of the ends of 20-100 bps is necessary for the repair via single-strand annealing (SSA) and HR (chapter 3.1). If this extended processing occurs, an inhibitory effect on a-NHEJ was found (Deng et al., 2014). The pairing of the MH serves to stabilize the two ends of the DSB. Although the underlying mechanism has not yet been clarified in detail, in mammals it is assumed that polymerase theta (Pol θ) plays an important role in this step (Black et al., 2016; Seol et al., 2018; Wyatt et al., 2016; Zahn et al., 2015). The 3'-overhangs can then be removed by nucleases such as XPF-ERCC1 and MRE11 in mammals (Bennardo et al., 2008).

The plant homologs of this nuclease RAD1/RAD10 have not yet been reported to be involved in a-NHEJ. The 3'-hydroxyl ends generated allow POL θ to bind, catalyze the elongation of the DNA and fill in the gaps (Ahmad et al., 2008; Hogg et al., 2012). In the last step, the end ligation is carried out by the Xrcc1/Ligase III complex or Ligase I (Liang et al., 2008; Masani et al., 2016).

Polymerase theta (Pol θ , encoded by the POLQ gene) was identified as the most important factor for a-NHEJ by several studies (Chan et al., 2010; Kent et al., 2015; Koole et al., 2014; Mateos-Gomez et al., 2015; Wood and Doublé, 2016; Yu and McVey, 2010). With more than 250 kDa, Pol θ is a comparatively large protein that, in addition to a polymerase domain at the C-terminus, has a helicase domain at the N-terminus (Black et al., 2016; Wood and Doublé, 2016). Pol θ can elongate both single and double-stranded DNA without a template via transferase activity. By adding individual nucleotides to the end of the break, new MHs can be created that serve a-NHEJ (Hogg et al., 2011, 2012; Kent et al., 2016). Furthermore, suppression of HR by binding of RAD51 could be demonstrated for Pol θ (Ceccaldi et al., 2015). The plant homologue to Pol θ was originally called TEBICHI (TEB). TEB is involved in processes of DNA replication, recombination and gene expression, as well as in DNA repair (Inagaki et al., 2009; Klemm et al., 2017). In addition to the increased sensitivity of *Arabidopsis teb* mutants to genotoxins, only the uptake of T-DNA into the cell could be observed, but not its integration into the genome (van Kregten et al., 2016). As a result, Pol θ is assumed to play an essential role in T-DNA integration into the plant genome.

2.2 Genomic changes induced by NHEJ

c-NHEJ leads either to perfect restoration or small indels at the break site. Indels arise because not all DSBs repaired by c-NHEJ are perfect blunt ends. Sometimes the broken ends have to be processed by adding or deleting a few nucleotides to make them available for the direct re-ligation. Moreover, it is known that the +1 bp insertion often observed in Cas9 mutagenesis experiments results from Cas9 creating a 1-nt 5' overhang that is filled in and then ligated. Nevertheless, if occurring in an ORF a deletion or insertion of only one or two nucleotides can result in a frameshift and therefore cause a complete knockout of gene function. However, a-NHEJ is a strongly error-prone pathway as the sequence information between the MHs is usually lost. Additionally, the pattern at the junction sites often reveals insertions. In the natural context, c-NHEJ is the prevalent pathway for DSB repair in somatic plant cells (Puchta, 2005; Schmidt et al., 2019) leading to no or only very small genomic changes. It is possible to shift the repair pattern to more and larger deletions by knockout of key enzymes involved in c-NHEJ. When analyzing NHEJ repair mutants of *ku70*, *lig4* or *polq* in *Arabidopsis* and also *Drosophila*, the influence by those

knockouts on repair patterns after DSB inductions could be shown (Schmidt et al., 2019; Shen et al., 2017; Yu and McVey, 2010).

The specific MH-based repair patterns of the a-NHEJ with large deletions and templated insertions can be observed in plant cells as well as for mammals (Qi et al., 2013; Shen et al., 2017). The formation of large deletions can be easily understood by the loss of the area between the MH used for the repair. However, the occurrence of the insertions, which usually shows sequence homologies to surrounding areas, is harder to comprehend.

In early experiments of DSB induction in tobacco cells, Gorbunova and Levy obtained quite large insertions originating from either genomic DNA or internal regions of transfected plasmid (Gorbunova and Levy, 1997). Shortly after, Salomon and Puchta reported insertions formed during DSB repair associated with unique as well as repetitive genomic sequences (Salomon and Puchta, 1998). Both groups found indications for annealing and priming during the formation of those insertions. Therefore, to explain the mechanism they suggested a model analogous to the SDSA model of HR (Section 3.1). Another model explaining the occurrence of direct or indirect repeats at the break site after repair was developed by Yu and McVey (2010). It is called synthesis-dependent MMEJ (SD-MMEJ) and assumed to involve POL θ for templated insertions. The mechanism is divided into two processes, loop-out and snap-back, which differ in the orientation of the MHs used (Fig. 2). With the loop-out SD-MMEJ, repair products with direct sequence repetitions are created, while with the snap-back mechanism, inverted sequence repeats can be found at the break point after the repair. These repeating sequence motifs have their origin in flanking areas in the immediate vicinity of the DSB. In addition to the simple templated insertions described, complex insertions were also obtained, which comprise several overlapping copies of the adjacent sequences. This class of complex insertion is also based on a repair model of SD-MMEJ and might result from several rounds of synthesis and dissociations from one or both sides of the DSB. DSBs can also arise if two SSBs are occurring on opposite strands at a shorter distance resulting in 3' or 5' overhangs at the DSB ends. As the CRISPR/Cas9 nuclease can be transformed by a simple mutation into a nickase such a scenario can be addressed experimentally (Ran et al., 2013; Schiml et al., 2014). In a recent study, two genomic SSBs were induced at different distances and in different regions of the *Arabidopsis* genome and the repair outcomes were analyzed (Schiml et al., 2016). SSBs on opposite DNA strands in intervals of 50–100 bp producing 5' overhangs were shown to induce mutations in up to three-quarters of the analyzed reads and to be efficient for induction of heritable mutations.

In addition to deletions, tandem sequence duplications close to the break sites were also detected. Some repair outcomes seemed to be related to c-NHEJ, others involved MHs at the break site, indicating repair mediated by MMEJ. The

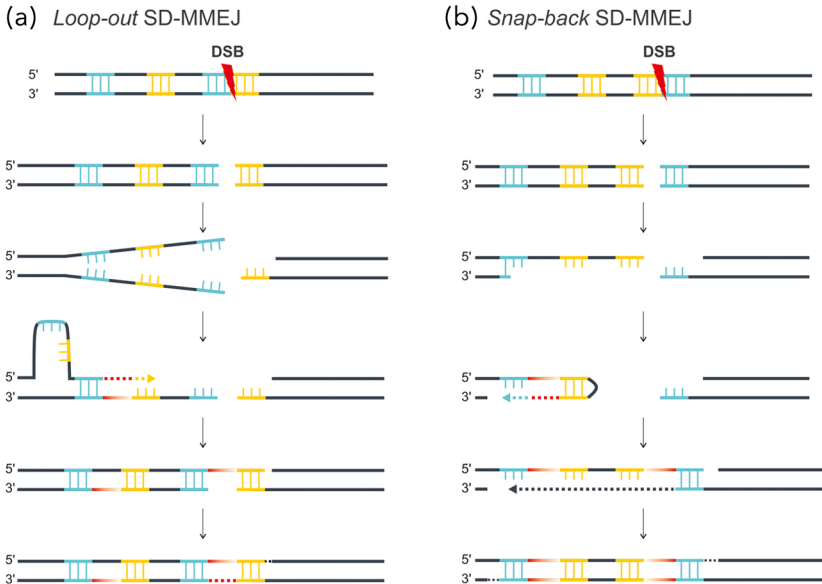


Figure 2 Mechanisms of synthesis-dependent MMEJ (SD-MMEJ). Two mechanisms are described here, which differ in the arrangement of the short homologies used: (a) in the loop-out SD-MMEJ, the sequence repeat pairs (yellow or light blue) are arranged alternately and one of the pairs spans the DSB. If the double strand of DNA is unwound, the blue sequence close to the break can pair with the homologous area, creating an outwardly curved loop (loop-out). After extending the DNA strand, the second homology (yellow) can link to the other end of the DSB, whereby the new synthesized area (red) leads to an insertion at the break point, (b) If the sequence repeats are inverted to one another, a hairpin structure can form after the ends of the break have been processed by folding over the single-stranded overhang. DNA synthesis is also started here based on the newly linked homology via a polymerase. The hairpin structure is then dissolved again and the elongated strand can pair with the opposite end of the break (snap-back). The sequence inserted in this way at the break (red) is in the opposite direction to the used template sequence.

most observed insertions originated from sequence context in close proximity. Therefore, Schiml et al. (2016) suggested two mechanisms responsible for the formation of tandem duplications after induction of paired SSBs on opposing strands (Fig. 3). In the absence of MHs at the break site, simultaneous degradation of the 5' ends and synthesis from the 3' ends followed by ligation of the blunt ends via c-NHEJ mechanisms can lead to tandem duplications (Fig. 3a). If MHs are present, a patch-mediated model for tandem duplications might explain repair (Fig. 3b) (Vaughn and Bennetzen, 2014). By hybridization of the single-stranded 5' ends via MH a more or less stable intermediate structure is formed. Repair of the internal gaps by fill-in synthesis and removal of 5' flaps at the same time give rise to staggered nicks that are finally ligated.

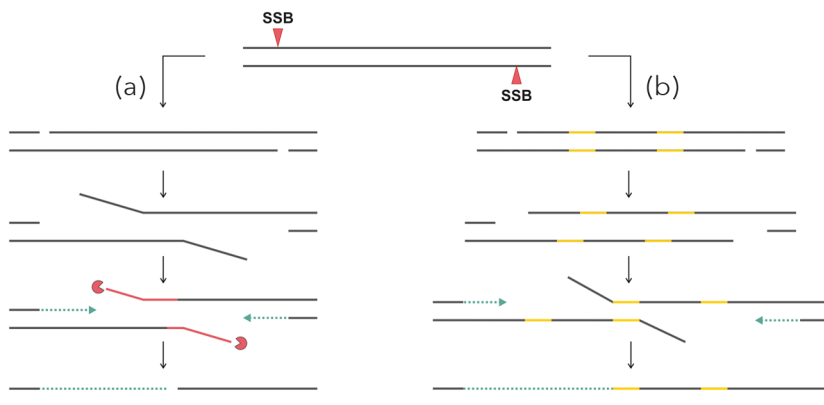


Figure 3 Repair of adjacent SSBs on different DNA strands can induce duplications. If two SSBs are induced on opposing DNA strands, a staggered DSB arises by unwinding of the complementary strands: (a) parallel resection from the 5' end (red) and elongation synthesis starting at the 3' end (green) leads to the formation of a blunt-ended break. Subsequent ligation via NHEJ results in the formation of MH-independent duplications, (b) if MHs are present, annealing of the distal MHs and subsequent DNA elongation leads to duplication of the sequence between the break sites and the homologies.

Interestingly, two SSBs on the same strand also induced similar alterations. However, the mutation frequencies for paired SSBs on the same strand were much lower than when on opposing strands with less than 1% compared to up to 77%. As high mutation frequencies correlated with the occurrence of MHs at the break site, the repair outcome and mutagenesis frequency seem to be highly dependent on the sequence context. Under natural conditions, adjacent SSBs might occur during base excision repair or through nicking errors in nucleotide excision repair. The presence of multiple short direct repeats in plant genomes (Vaughn and Bennetzen, 2014) indicates that the repair of adjacent SSBs seemed to have an important influence on the shaping of plant genomes during evolution.

SSBs occur during replication and are regularly induced during base and nucleotide excision repair (NER). As NER is a major repair pathway of UV-induced DNA damage, SSBs outnumber DSBs by orders of magnitudes in plant cells. Thus, although single SSBs themselves do not have the same potential as DSBs to induce repair-associated genomic changes these kinds of lesions have a significant influence on genome evolution, due to their sheer number.

In conjunction with the repair of adjacent SSBs, other molecular mechanisms are discussed as a source of genome variation in plants. Increased genome size is associated with polyploidization and transposable element proliferation (Adams and Wendel, 2005; Bennetzen and Wang, 2014). In contrast, transposon-mediated excision, dysploid reduction of chromosome

number, loss of entire chromosomes as well as illegitimate recombination and replication slippage might lead to genome shrinkage (Devos et al., 2002; Schubert and Vu, 2016). Additionally, in smaller genomes of cotton, DNA removal during repair could counteract genome expansion through retrotransposons (Hawkins et al., 2009). In other studies, species-specific deletion sizes in combination with insertions were observed. Analyzing the formation of deletions in *Arabidopsis thaliana* and *Nicotiana tabacum*, the size of deletions was much smaller in the >20-fold larger tobacco genome (Kirik et al., 2000). Additionally, 40% of deletions in that species coincided with insertions but nearly no insertions accompanied the larger deletions in *Arabidopsis*. The same pattern could be shown in a comparison of DSB repair outcomes between *Arabidopsis* and barley (Vu et al., 2014, 2017). Here, large deletions were detected more frequently in *Arabidopsis*, whereas barley with its larger genome showed a significantly higher amount of insertions at imprecise repaired sequences. This correlation between genome size and DSB processing via NHEJ is thought to be related to species-specific differences in exonucleolytic degradation rates of free DSB ends before downstream processing (Cavalier-Smith, 2005; Orel et al., 2003). Concluding from this data, an underestimated contribution to genome size evolution could be the inaccurate repair of DSBs.

Usually cells can repair multiple breaks simultaneously through mechanisms that keep the correct broken ends in close proximity (Williams et al., 2010). Therefore, chromosomal aberrations are unlikely to arise. Yet, these mechanisms are not always accurate or can be impaired and rearrangements can result from the illegitimate joining of broken DNA ends. This misrepair can lead to many different changes like deletions, inversions and exchange of genomic sequences as well as recombinant chromosomes or chromosome fusions. The chromosomal rearrangements can result in changes in gene regulation, new gene functions or the suppression of meiotic recombination. They are linked to evolutionary processes such as the formation of new species and adaptive divergence. By comparative analysis of the genomes of different *Arabidopsis* ecotypes and of related species, many different rearrangements were found, which occurred naturally and shaped the karyotype of these *Brassicaceae* (Lysak et al., 2006; Zapata et al., 2016).

In genome engineering, the possibility of misjoining the broken ends is used to modify chromosomes in a targeted manner and create new combinations of the fragments. The induction of large chromosomal rearrangements (CRs) is based on the systematic introduction of more than one DSB at the same time. If two DSBs are induced on one chromosome, the region between can be deleted or inverted (Fig. 4a). Interchromosomal rearrangement results from the induction of DSBs on different chromosomes, which causes fragments to be exchanged and illegitimately mated (Fig. 4b). The induction of two

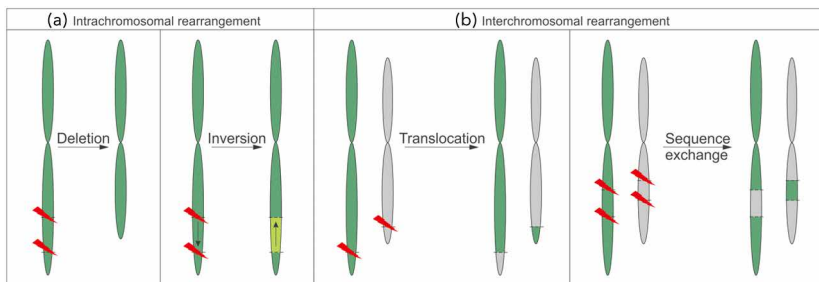


Figure 4 Induction of large chromosomal rearrangements (CRs): (a) if two DSBs are induced on one chromosome arm, intrachromosomal rearrangements can occur. The area between the fractions can either be deleted or inverted, (b) if breaks are induced on two heterologous chromosomes, the fragments can be exchanged. With one DSB on each chromosome arm, a translocation might be induced; if more breaks are located on the arms, it is possible to exchange the sequence information in between.

DSBs on heterologous chromosomes can lead to translocations. If four DSBs are induced, two on each chromosome, entire sequence regions can be exchanged.

The simplest form of a CR is the deletion. Here the sequence area between the breaks is removed and all information is lost. With this, entire genes, large gene clusters or non-coding regulatory sequences can be deleted and ultimately switched off; true loss-of-function mutant lines for functional studies can be generated without effort. Deletions of up to 1 kb are quite easy to induce both in *Arabidopsis* and in other plant species (Brooks et al., 2014; Gao et al., 2015; Kapusi et al., 2017; Upadhyay et al., 2013). In addition, deletions of up to 120 kb in *Arabidopsis* (Ordon et al., 2017; Wu et al., 2018) and up to a size of 245 kb in many other plant species such as rice, tobacco, tomato and *Medicago* can be achieved, but with a significantly lower frequency (Čermák et al., 2017; Zhou et al., 2014). Besides deletions, two breaks on one chromosome can result in the integration of the excised fragment in reverse orientations. In plants, targeted inversions up to 18 kb could be obtained using Cas9 (Gao et al., 2015; Schmidt et al., 2019; Zhang et al., 2017). In tobacco, the demonstration of the formation of DSB-induced reciprocal translocations has been achieved using the homing endonuclease I-SceI whereby NHEJ or SSA joined the previously unlinked DSB ends (Pacher et al., 2007). Very recently, it was shown that by the use of CRISPR/Cas it is indeed possible to obtain heritable chromosomal translocations in *Arabidopsis* in a controlled way, opening the avenue of chromosome engineering (Beying et al., 2020).

To achieve controlled restructuring of plant genomes, it is necessary to consider the different repair mechanisms involved. In mammals it was shown, that both c-NHEJ and a-NHEJ can potentially contribute to the creation of rearrangements (Lupski and Stankiewicz, 2005). Interestingly, a-NHEJ was

identified as the major mechanism for translocation formation in mouse embryonic stem cells and it is suppressed by c-NHEJ components such as Ku, Lig4 or Xrcc4 (Simsek and Jasin, 2010; Weinstock et al., 2007). In contrast, unlike mouse cells, translocations in human cells are generated by c-NHEJ (Ghezraoui et al., 2014). A drastic reduction in translocation frequency when both c-NHEJ and a-NHEJ components are lost could be detected in human cell lines (Brunet and Jasin, 2018), whereas murine cell lines showed an increase when cells are lacking Ku70 and Pol θ (Wyatt et al., 2016). Therefore, species-specific mechanisms could also apply to the formation of CRs in plants. In *Arabidopsis*, it was shown that under natural conditions large deletion and inversion formation was mediated by c-NHEJ. Yet, a significant increase for the frequency of inversion and translocation formation occurs when the key player of c-NHEJ, KU70, is depleted (Beying et al., 2020; Schmidt et al., 2019). Deep sequencing revealed repair patterns of the junctions with characteristics of a-NHEJ. This indicates that the a-NHEJ mechanism is significantly less selective to join the 'correct' ends during repair. Conversely, this also means that c-NHEJ is actively ensuring that the originally linked ends are re-ligated. Different factors are known in mammals that are involved in anchoring the two original broken ends during c-NHEJ and are thus able to stabilize the DSB. In this context, the KU heterodimer is essential. Two KU heterodimers initially bind to each end of the DSB. It has been possible to demonstrate biochemically, and with the aid of atomic force microscopy, the KU proteins already bound to the DNA can associate with each other (Cary et al., 1997; Ramsden and Gellert, 1998). The resulting model is based on the assumption that the two heterodimers combine to form a bridge complex, which then subsequently recruits the other repair factors. The two c-NHEJ factors XLF and PAXX, which both interact with the KU heterodimer, are certainly involved in connecting the two ends (Graham et al., 2018; Ochi et al., 2015). It was also shown that the heteromeric complex of XLF and XRCC4 is able to stably bridge two independent DNA molecules and that this bridge complex can move along the DNA. This observation suggests that in mammals the XRCC4-XLF complex forms a mobile, sleeve-like structure around the DNA that is able to hold broken ends together and that this complex is involved in the subsequent repair (Brouwer et al., 2016). So far, only homologs of the c-NHEJ factors KU70/KU80, LIG4 and XRCC4 have been identified in plants, but no homologous proteins to the two factors XLF and PAXX have been identified yet. Therefore, the XRCC4 protein alone might actually be able to support the cohesion of the two ends in plants.

In addition to the stabilization of a DSB, the location of the two broken ends in relation to one another also plays a decisive role, since the break ends should logically be in spatial proximity to be newly linked. In this context, two basic approaches as to how CRs can arise are differentiated, the

'contact-first' and the 'breakage first' theories (Sax, 1941; Serebrovski, 1929). In the 'contact-first' theory, the two chromosomal fragments, between which a structural rearrangement takes place, are already in close proximity, while the two chromosomal regions in the 'breakage-first' theory only come together after DSB induction. In the second model, the broken ends must be mobile and must be able to interact with one another. In yeast, it has been shown that chromosomal regions can move toward one another within minutes after DSB induction and that this leads to clustering of chromosome regions containing DSBs (Aten et al., 2004). A certain mobility of damaged DNA was also observed in mammalian cells, but DSBs are often described here as being more stable, which indicates that the predominant repair mechanism of c-NHEJ limits the mobility of DSBs (Lemaître et al., 2014; Robinett et al., 1996; Soutoglou et al., 2007). Increased mobility of chromosomal areas has so far been observed mainly in connection with the search for homologous areas in the context of HR (Aymard et al., 2017; Cho et al., 2014; Schrank et al., 2018). However, a recently published study also found a connection between PARP1-mediated ADP ribosylation, which leads to the formation of poly(ADP-ribose) (PAR) chains and the generation of dynamic areas in which the damaged DNA accumulates (Singatulina et al., 2019). It is assumed that movement happens at the beginning stages of DNA repair (Miné-Hattab and Rothstein, 2013).

In mammals, as well as in plants, PARP1 was shown to be involved in the repair of DSBs via a MH-based alternative end-joining repair pathway (Audebert et al., 2004; Jia et al., 2013; Wang et al., 2006). Such an accumulation of damaged DNA can have the advantage that repair factors are frequently present in these areas and thus a significantly faster and therefore more efficient repair can take place (Schrank et al., 2018; Singatulina et al., 2019). A disadvantage, however, could be that the likelihood of linking two break ends that were not originally connected to one another increases, and thus CR can also arise with a higher probability.

The same mechanisms might account for the increase of the likelihood of a chromosomal structural change if c-NHEJ is impaired in plants. On the one hand, in the absence of the KU heterodimer, no protein complex stabilizes the two break ends, which increases the chance of break ends being linked that were originally not connected. On the other hand, the clustering of damaged DNA regions that has been linked to the MMEJ in mammals could bring the two DSBs in close proximity to one another, which in turn increases the probability of a CR event occurring.

Thus, blocking c-NHEJ might be a general strategy to increase the efficiency of CRISPR/Cas-mediated plant chromosome engineering. If c-NHEJ is impaired, repair switches to the more error-prone pathway of a-NHEJ and mutations can be induced with higher frequencies. Unfortunately, this means

that repair outcomes at the junctions of CRs are less predictable in those mutants as unforeseen mutations might form depending on the sequence context. As *ku* mutants also show other genomic instabilities such as telomere dysfunction and DNA repair defects (Bundock et al., 2002; Riha et al., 2002), the use of this mutant is not advisable for practical applications in plant breeding. Using an inducible knockdown of *ku* or different c-NHEJ mutants could be another possibility to increase the efficiency of chromosome engineering in plants by simultaneously minimizing side effects.

3 DSB repair using homologous sequences

In contrast to non-homologous end joining, HR occurs rarely in somatic cells and is mainly limited to the S- and G2-phases of the cell cycle (overview in Puchta, 2005). In meiotic tissue, on the other hand, HR is essential for pairing of chromosomes, recombination and exchange of genetic information. In principle, HR is based on the involvement of a homologous sequence region, of which the intact sequence information serves as a matrix for repairing the DSB. However, HR can be divided into conservative and non-conservative mechanisms, depending on whether the repaired DNA section matches the original sequence. HR comprises several different mechanisms, which differ in the repair process and the enzymes involved. These include the two repair mechanisms prevalent in somatic tissue, single-strand annealing (SSA) and synthesis-dependent strand annealing (SDSA), as well as the double-strand break repair (DSBR) and the dissolution pathway, both of which play an important role in meiosis (Fig. 5).

3.1 Basic mechanisms of HR

Common to all HR routes is the initial 5'-3' resection, which is mediated by the MRN complex and generates single-stranded 3' overhangs, which are protected from nucleolytic degradation by the attachment of the heterotrimeric RPA (replication protein A) complex (Eschbach and Kobbe, 2014).

If complementary sequences of more than about 20 bp are present in these overhangs, the DSB can be repaired using SSA (Fig. 5b). This repair route is mechanistically similar to a-NHEJ. After the homologies have been exposed, they can pair with one another and a chimeric double-stranded DNA molecule is formed. In yeast, RAD52 seems to be involved as it is recruited to the single-stranded DNA nucleoprotein complex after end resection (van Dyck et al., 2001). Also in humans, the interaction of RAD52 with RPA was demonstrated (Ma et al., 2017). For the *Arabidopsis* homologs of RAD52 there seems to be no involvement in this process (Samach et al., 2011), but the RAD51 paralogs XRCC2, RAD51B and RAD51D were also demonstrated to play a role in SSA (Serra et al., 2013). After priming, the overhanging ends are trimmed by a dimer

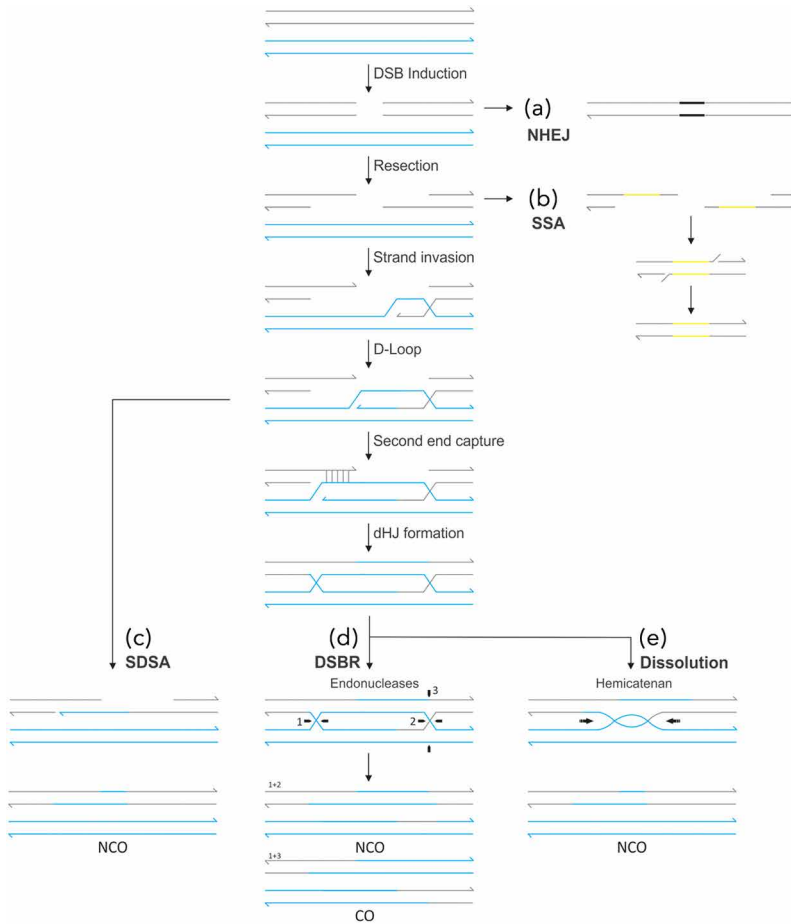


Figure 5 Overview of the repair mechanisms after a DSB: (a) If no homologous sequence is available, the break is repaired via NHEJ, (b) If homologous areas larger than 20 bp (yellow) are found on both sides of the DSB, the break can be repaired using single strand annealing (SSA). This leads to the loss of sequence information and results in mutagenic deletions. (c-e) Conservative homologous recombination (HR), which includes the three mechanisms of synthesis-dependent strand annealing (SDSA), double-strand break repair (DSBR) and dissolution, results in error-free repair of the DSB. After resection of the DSB ends and invasion of the 3' overhang into the homologous area, a displacement loop (D-Loop) is formed. The three mechanisms differ in the resolution of this intermediate. In the SDSA pathway (c), the extended invading DNA strand is detached from the donor molecule and can pair with the complementary overhang of the second break end. The gaps are then closed by DNA synthesis, resulting in a non-crossover product (NCO). Due to further synthesis of the invaded strand, the D-Loop can be expanded leading to second end capture and a double Holliday junction (dHJ) is formed. This structure can be resolved in the dissolution pathway (e) by the formation of a hemicatenane via branch migration using the RTR complex, resulting in an NCO event. In the resolution pathway (d), structure-specific endonucleases can cleave the dHJ. In addition to NCO products, in DSBR, crossovers (COs) can arise depending on the cutting pattern.

of the proteins RAD1 and RAD10 (Dubest et al., 2002; Prado and Aguilera, 1995), and the gaps are filled by DNA synthesis. Finally, the DNA backbone is ligated. As a result, the sequence information between the homologies is lost, which is why this repair route is the only one assigned to non-conservative HR (overview in Knoll et al., 2014a).

If no repetitive homologies are available at the break site, an exogenous repair matrix is required for repair via the other mechanisms of HR. The search for such a template is mediated by RAD51. RAD51 replaces the RPA encasing the single strand and mediates the search for a repair template (Renkawitz et al., 2013). The free single strand can invade into a homologous sequence section and thereby displace one strand of the double-stranded donor molecule. The resulting structure is called a displacement loop (D-loop) (Fig. 5). The strand invasion is followed by an extension of the immigrated single strand, whereby the homologous strand is serving as a matrix. From here on, the invading strand can either be released or further extended. In SDSA, the D-loop is dissolved and due to the extension the invading strand can pair with the processed, opposite end of the DSB. As information is only copied to one strand, this repair always results in a non-crossover (NCO) product.

However, if the invading strand is greatly extended, hybridization can occur between the displaced strand of the D-loop and the now complementary end of the second single strand of the break, also referred to as second end capture. This process, which occurs mainly in meiosis, leads to the formation of a repair intermediate called the double Holliday junction (dHJ, Fig. 5) by ligating the break ends. The resolution of this dHJ structure can take place via the two HR repair routes of DSBR and dissolution. The predominant mechanism, the dissolution, maintains the integrity of the genome, since only NCO products are created and the two DNA molecules are thus separated without exchanging chromosome regions. The process of dissolution is catalyzed by the RTR complex, which is highly conserved in eukaryotes (Knoll et al., 2014b). It consists of a RECQ helicase, a type 1A topoisomerase as well as the structural proteins RMI1/RMI2. The helicase pushes the two junctions of the dHJ toward each other forming an intermediate structure called a hemicatenane. The topoisomerase relaxes the resulting super spiraling of the DNA and dissolves the resulting hemicatenane structure (Chen et al., 2014). In *Arabidopsis*, all proteins like RECQ4A, TOP3 α and RMI1/RMI2 involved in the RTR complex were identified and were shown to interact in vivo (Bonnet et al., 2013; Dorn et al., 2018; Hartung et al., 2008; Röhrig et al., 2018; Schröpfer et al., 2014; Séguéla-Arnaud et al., 2015). The resolution of the dHJ is achieved in the DSBR model by structure-specific endonucleases, which are also called resolvases due to their function (Szostak et al., 1983). The induction of symmetrical cuts at the two crossing points of the dHJ leads to the separation of the two double strands. Depending on the cutting direction of the resolvases, this can result

in NCO and crossover (CO) products (Fig. 5). dHJs mainly arise in meiosis and the CO products resulting from chromosome arm exchange are required for mixing parental genomes.

By using transgenic recombination substrates with different setups, it is possible to characterize the role of different factors in these pathways *in planta*. In those pathway-specific recombination traps, a DSB can be induced by the expression of a site-specific nuclease, which leads to the restoration of a marker by either SSA or SDSA (Orel et al., 2003). In early experiments, I-SceI was used for break induction to restore a disrupted *GUS* gene. With this, the repair of one out of three DSBs induced by I-SceI was shown to be mediated by SSA in tobacco, if the break site was flanked by homology stretches (Siebert and Puchta, 1998). In contrast, under similar conditions the SDSA pathway showed a five times reduction of efficiency compared to SSA (Orel et al., 2003). Additionally, the involvement of the aforementioned factors XRCC2, RAD51B and RAD51D as well as RAD1/RAD10 in SSA could be demonstrated (Dubest et al., 2002; Serra et al., 2013). Further proteins that might have a function in this pathway are the helicase FANCM and the nuclease MUS81 (Mannuss et al., 2010). As only minor involvement could be detected, other nucleases and helicases might be able to substitute each other, obstructing the detection of key factors in SSA. MUS81 seems to be also involved in SDSA, whereas mutants of proteins that are accounted for strand exchange only show an effect in repair of the SDSA recombination trap. As a result, in *Arabidopsis* an essential role for XRCC3 and RAD54 in addition to RAD51 in SDSA was postulated (Roth et al., 2012). Other ATPases like RAD5A, which plays a role in post replicative repair (Chen et al., 2008), and FANCM, which is also involved in control of meiotic recombination (Knoll et al., 2012), are also involved in SDSA-mediated repair (Mannuss et al., 2010; Roth et al., 2012).

Next to SSA, it was demonstrated that only SDSA operates efficiently in somatic plant cells (Puchta, 1998). With an efficient repair via DSBR, somatic cells would endanger their genome stability, as this repair is prerequisite for crossover induction. The formation of crossovers between repeated ectopic sequences that are found all over plant genomes could lead to the formation of dicentric or acentric chromosomes. This is why DSBR occurs mainly during meiosis, where the exact pairing of homologous chromosomes is assured by the formation of the synaptonemal complex.

HR is dependent on the availability and accessibility of a homologous sequence. In SSA, the homologous sequence serving as a template has to be located close to the break site in direct orientation. Genomic regions harboring tandem repeats or other repetitive sequences are therefore preferentially repaired via SSA. The absolute frequency of SSA-mediated repair events was shown to be dependent on the distance between the reads. With increasing distance, longer end resection has to take place and results in a negative

correlation between deletion size and frequency (Vu *et al.*, 2017). As mentioned before, in small genomes like *Arabidopsis*, larger deletions are more common compared to the situation observed for tobacco (Kirik *et al.*, 2000), barley (Vu *et al.*, 2017) or human cells (van Overbeek *et al.*, 2016). This leads to a higher abundance of SSA between repeats over increasing distances in *Arabidopsis*. In the other cases of HR, several different repair templates might be used (Fig. 6). In S- and G2-phases of the cell cycle, the sister chromatid is available for repair. In addition, homologous sequences on the same chromosome like transposable elements or gene duplications, an allelic sequence on the homologous chromosome or ectopic sequences located on a different chromosome can serve as a template. To test the efficiencies of the different repair templates, DSB induction by I-SceI or transposons was generally used. Based on the restoration or loss of marker genes, frequencies could be quantified.

If homologies are available in close proximity to the break site, repair by HR pathways is quite efficient. Repair matrixes can be found either on the same chromosome or on the sister chromatid. If a DSB is repaired via the sister chromatid as a homologous template, no sequence change can be identified. Therefore, it is very difficult to assess frequencies of these repair events. Yet, Vu *et al.* were able to study repair involving the sister chromatid in barley by ethynyl-deoxyuridine-based staining showing frequencies of >81% (Vu *et al.*, 2014). This unprecedented result leads to the conclusion that efficiencies for repair with this kind of template in S- and G2-phases are underrated. Intrachromosomal HR

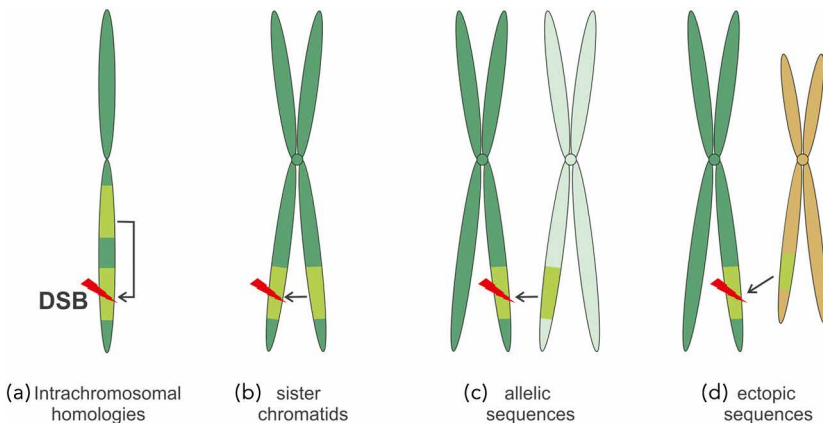


Figure 6 Possible templates for HR-mediated repair of a DSB. Depending on the cell-cycle phase, different repair templates are available for HR via the SDSA mechanism: (a) homologous sequences on the same chromosome are most frequently used for repair, as intrachromosomal regions are most accessible, (b) in G2- and S-phase, the sister chromatid is available and can be used efficiently for repair, (c) allelic sequences are primarily used during meiotic recombination, and only in very low frequencies in somatic cells, (d) ectopic sequences on another chromosome or on extrachromosomal material can also serve as templates for repair via HR.

using homology on the same chromosome as a template is the most abundant pathway of HR. In addition to direct repeats used by SSA as discussed above, other intrachromosomal homologies can serve as a template for DSB repair via SDSA. This was proven in many studies regarding intrachromosomal HR in plants (Chiurazzi et al., 1996; Mannuss et al., 2010; Orel et al., 2003; Roth et al., 2012; Siebert and Puchta, 2002).

In meiosis, mostly allelic regions function as the template for DSB repair. In somatic plant cells, allelic repair is detectable but was reported to not be significant for repair outcomes after DSB induction via I-SceI, with an estimated low frequency of 10^{-4} in an initial study (Gisler et al., 2002). The same holds true for homologous sequences within an ectopic region. Using an ectopic repair template after DSB induction, similar efficiencies for HR in somatic tissue were detected (Puchta, 1999; Shalev and Levy, 1997). Therefore, the conclusion was drawn that homologies on a different chromosome are hard to access and so in somatic cells repair using them is negligible. Yet, the experimental setup from Gisler et al. (2002) to analyze allelic repair did not provide homologies directly at the cutting site of I-SceI. In a recent study, another system was set up to test allele-dependent repair mediated by HR in tomato (Filler Hayut et al., 2017). In stark contrast to the earlier study, somatic HR was raised up to 14% per allele by DSB induction via Cas9. The higher efficiency might be because the homology was present directly at the break site in this later study. In the absence of DSB induction, somatic HR between homologous chromosomes is very low, as shown in earlier studies in tobacco (Carlson, 1974; Dullieu, 1975). However, in G2 somatic DSB can also be repaired by the sister chromatid. Indirect evidence indicates that this repair pathway is much more important than ectopic or allelic repair (Watanabe et al., 2009).

3.2 Application in gene targeting

In gene targeting (GT), HR is utilized to introduce desired changes in a precise and targeted manner. Unfortunately, low efficiencies of this method in higher plants made its application in basic research, plant biotechnology and breeding sparsely feasible. Therefore, many studies were conducted to improve frequencies of HR-based GT (for review: Huang and Puchta, 2019; Puchta and Fauser, 2013; Steinert et al., 2016; Sun et al., 2016; Wolter et al., 2018).

Following the first experiments applying GT showing these low frequencies (Beetham et al., 1999; Offringa et al., 1990; Paszkowski et al., 1988), initial improvements were made by inducing site-specific breaks at the target sequence. Using I-SceI, GT frequencies could be enhanced by up to two orders of magnitude in tobacco (Puchta et al., 1996). In many other important crops such as maize, rice and wheat, DSB induction by site-specific nucleases, for example, the CRISPR/Cas system, was used to stimulate GT (Endo et al., 2016;

Gil-Humanes et al., 2017; Li et al., 2018a; Sun et al., 2016; Svitashhev et al., 2015, 2016; Wang et al., 2017). Therefore, one strategy to improve GT is to adjust the efficiency and nature of the nuclease used for break induction. The most common used endonuclease is SpCas9 (Cas9 from *Streptococcus pyogenes*), but the application of *Staphylococcus aureus* Cas9 with its higher frequency of DSB induction (Steinert et al., 2015) was demonstrated to enhance GT frequencies over SpCas9 at least in *Arabidopsis* (Wolter et al., 2018). Both of these Cas9 orthologs induce blunt ends close to the PAM sequence. (Bothmer et al., 2017; Čermák et al., 2017). Furthermore, Cas12a which produces 5' overhangs has been reported to enhance GT (Begemann et al., 2017; Li et al., 2020; Merker et al., 2020; Wolter and Puchta, 2019). This enzyme cleaves the target DNA on the PAM distal side. In contrast to the Cas9 enzymes, the break is induced far away from the seed sequence, which serves as nucleation site for base pairing between the crRNA and the target DNA (Fonfara et al., 2016). Thus, mismatches between crRNA and target DNA arising from NHEJ repair might not hinder further cleavage. As after each cut HR is again competing with NHEJ for DSB repair, the overall chances for HR rise overtime as long as the re-cutting of the NHEJ repaired site is still possible.

In GT, a repair template for HR-induced changes has to be provided (Fig. 7). The design of this template, its activation and availability, has to be considered for efficient GT. For efficient recombination the length of the homology for donor templates has to be considered. Although no systematic study of template length was performed for plants, a homology length of at least 500 bp was

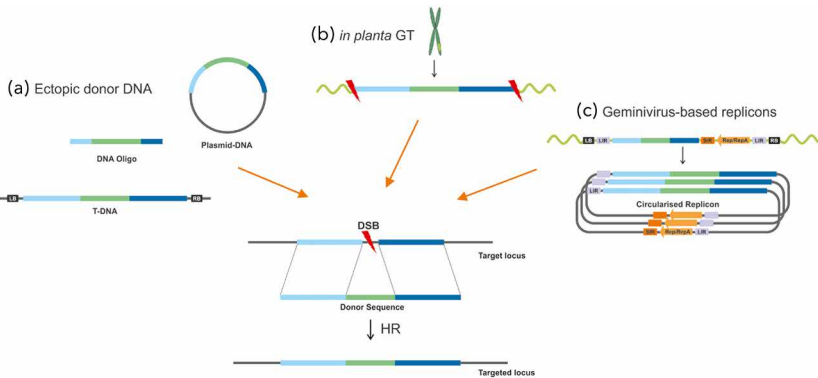


Figure 7 Gene targeting in plants and the applicable donor molecules. After DSB induction, gene targeting (GT) is mediated by HR. Therefore, the donor sequence needs to harbor homologies (blue) on both sides of the changes to be integrated (green): (a) possible templates for HR-based GT are ectopic sequences such as T-DNAs, plasmid DNA or DNA oligos that are transferred to the cell, (b) in the *in planta* GT approach, the template is already integrated into the genome and is activated through excision by nucleases, (c) the combination of *in planta* GT with geminivirus-based replicons enhances GT frequencies.

used in most studies. With sequence-specific DSBs as a very effective method to improve GT, the cutting of the repair template and the modified locus has to be prevented. In general, modification of the target sequence or for Cas9 the PAM region is the best way to abolish DNA cleavage by the nuclease. As SDSA is the main pathway responsible for GT in somatic plant cells, all changes that are supposed to be introduced in the genome should be located on one side of the DSB (Huang and Puchta, 2019). If the template is only provided transiently as an extrachromosomal repair template (Fig. 7a), degradation by the plants' intrinsic mechanisms reduces GT efficiencies. By stably integrating the donor DNA into the plant genome, the so-called '*in planta* GT' method is independent on transfected donor DNA and on transformation efficiencies as only one initial transformation event is sufficient (Fig. 7b) (Fauser et al., 2012; Schiml et al., 2014). Thus, this technique makes the application of GT in crop species that are difficult to transform more feasible. However, the template has to be activated via two cuts to release the linear sequence from the genome. These additional cleavage sites might be another reason as to why GT efficiencies are improved in this strategy. As discussed for the formation of chromosomal rearrangements, damaged DNA might accumulate in repair loci at the nuclear periphery (Caridi et al., 2018). This would bring the cut target site in close proximity to the excised repair template. Furthermore, by raising the copy number of the donor molecule, GT can be enhanced. This increase of the availability of the template was achieved using geminivirus replicons in combination with the *in planta* GT method (Fig. 7c) (Dahan-Meir et al., 2018). The successful application of geminivirus replicons for enhancing gene targeting was demonstrated before using ZFNs, TALENs and Cas9 (Baltes et al., 2014; Čermák et al., 2015).

In early experiments in plants, besides perfect events by HR, aberrant GT was also found (Puchta et al., 1996, reviewed in Puchta and Fauser, 2013). Those events showed combined NHEJ and HR repair outcomes, where one junction was repaired via HR and the other via NHEJ. The prevailing homologous repair mechanism behind GT in somatic plant cells is SDSA. Even though a homologous 3' single strand of one end of the DSB invades into the donor, the possibility persists that the repair at the other end is conducted by NHEJ. Thus, it is necessary to check GT events for correct HR-induced repair at both junction sites via sequencing of the complete modified locus. Reduction of these one-sided integration events can be achieved by selecting against random NHEJ events with negative selectable markers. This was proved to be a useful way to obtain GT in rice (Terada et al., 2002), although this technique is quite laborious. Besides SDSA, SSA can be applied to induce genomic changes as well. Instead of introducing a foreign sequence into the genome, the removal of sequences between flanking homologies can be facilitated. In a recent study, SSA was used for the removal of inserted selection cassettes

that contain flanking homologies, as a scarless alternative to the Cre-inducible recombination systems (Li et al., 2018b).

Another way of bypassing NHEJ-mediated repair and thus enhancing GT is the cell cycle phase or cell type-specific expression of the nuclease for break induction. While DSBs are repaired in G1 phase most frequently by NHEJ, in G2/M- and S-phase HR is more active compared to its near absence in G1. In yeast and other fungi, as well as in human pluripotent stem cells, higher GT efficiencies were detected in those cell-cycle phases (Tsakraklides et al., 2015; Yang et al., 2016). In mouse embryonic stem cells, GT frequencies of up to 100% could be demonstrated (Miura et al., 2018; Quadros et al., 2017). By egg-cell specific expression of the nuclease in *Arabidopsis*, promising frequencies of heritable GT events could recently be obtained (Miki et al., 2018; Wolter et al., 2018).

Additionally, suppressing NHEJ pathways or promoting the HR pathway was exploited to increase HR-mediated GT. In *Arabidopsis*, mutants of the two main factors in NHEJ, KU70 and LIG4 showed 16-times higher GT efficiencies (Qi et al., 2013). Similarly, it was reported that knockout of LIG4 via CRISPR/Cas9 in rice calli enhanced GT several folds (Endo et al., 2016). As an alternative strategy, it could be demonstrated that overexpression of some proteins involved in HR repair enhanced GT effectively, such as overexpressing the *S. cerevisiae* Rad54 protein in plants or expressing RAD54 specifically in egg cells (Even-Faitelson et al., 2011; Shaked et al., 2005). In *smc6b* mutants of *Arabidopsis*, both NHEJ-mediated mutagenesis and GT were found to be elevated at three loci (Qi et al., 2013). As in these mutants sister chromatid exchange is impaired, repair was either conducted via NHEJ or via HR-based GT with an extrachromosomal template present. Yet, DNA repair mutants and HR protein overexpression lines display genomic instabilities. Therefore, alternative ways of blocking NHEJ pathways or enhancing HR pathways utilizing the expanding toolkit of the CRISPR/Cas system, for example, are sought and might be applicable in the near future.

4 Perspectives

Harnessing the knowledge of DNA repair pathways and combining it with the increasing toolkit of different site-specific nucleases especially the CRISPR/Cas system, will open great possibilities in plant genome engineering. Editing of multiple loci simultaneously, restructuring of plant genomes, improving GT frequencies above percentage rates and many more applications are already being addressed. With this significant progress in plant breeding within sight, a new green revolution might become reality within the near future.

5 Where to look for further information

The following articles provide a good overview of the subject:

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