Chapter 1
Double-Strand Break Repair and Its Application to Genome Engineering in Plants

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Abstract The induction of double-strand breaks (DSBs) is the basis for the targeted modification of plant genomes. At the same time, the efficient repair of DSBs is important for the survival of all organisms. To efficiently employ DSB repair for genome manipulation using synthetic nucleases, detailed knowledge of the repair process is required. Many aspects of the mechanisms and factors involved in DSB repair have been elucidated in plants over the last two decades. Here, we seek to summarize our current knowledge about the process of DSB repair via non-homologous end joining (NHEJ) as well as homologous recombination (HR) and place this knowledge in the context of strategies applied for genome engineering in plants. While the induction of a unique DSB is generally sufficient for editing single genes, the induction of multiple DSBs can be applied for the engineering of genomes. There is no question that the controlled induction of DSBs exhibits great potential for restructuring plant genomes.

Over the last several years, genome manipulation has been revolutionized by the development of different types of site-specific nucleases for the controlled induction of double-strand breaks (DSBs). This development has especially significant consequences for organisms in which gene targeting (GT) has not been established as a feasible technique. While in many species, such as bacteria or yeast, homologous sequences must be included in a DNA sequence to achieve integration into the genome, random integration of any type of DNA takes place in most multicellular eukaryotes. The establishment of GT in mouse embryonic stem cells represented a major breakthrough (Doetschman et al. 1987; Thomas and Capecchi 1987), allowing not only the knockout of any gene of interest but also various types of controlled genome rearrangements by means of site-specific recombinases (for a recent review, see Turan and Bode 2011). Unfortunately, in many other eukaryotes, GT has not been established at a feasible integration frequency despite decades of attempts (for a review of the situation in plants, see Puchta and Fauser 2013).

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1 DSB Induction as a Tool for Genome Manipulation in Plants

It has long been established in yeast that homologous recombination (HR) can be enhanced by the induction of DSBs at specific positions in the genome, which occurs in a controlled manner during mating type switching (for a review, see Paques and Haber 1999). The first indications that DSB induction might also lead to enhanced HR frequencies in plants came from studies involving DNA transposons in maize (Athma and Peterson 1991). An important step in the analysis of DSB repair in plants was the use of site-specific endonucleases, such as the homing endonuclease I-SceI (Perrin et al. 1993). I-SceI contains an 18-mer recognition site and was originally isolated from yeast mitochondria. The I-SceI ORF integrates itself into the mitochondrial 21S rRNA gene by inducing a DSB. This DSB is repaired via HR using a copy of the 21S rRNA gene as a matrix that already includes the I-SceI ORF as an intron. Thus, I-SceI is able to disseminate in the mitochondrial DNA pool (Jacquier and Dujon 1985). The demonstration that I-SceI can induce DSBs in plant cells in vivo (Puchta et al. 1993) showed that a tool was available to the plant community that not only enables various analyses of the basic mechanism of DSB repair but can also be used for genome manipulations. Although other site-specific nucleases have been applied for this purpose (Chilton and Que 2003; Chiurazzi et al. 1996), I-SceI remained the gold standard for studies on DSB repair in plants for quite some time. Transgenic plant lines were produced for homing endonuclease-induced repair experiments that contained an artificial I-SceI site coupled to marker gene sequences within a transgene. Experiments were designed in such a way that the marker was restored due to DSB repair after DSB induction (in some cases, a negative marker gene was also destroyed). As marker restoration was coupled mostly to only one specific type of repair reaction, the respective repair efficiencies could be determined. Moreover, when selection markers were used, the recombinant cells could be isolated and propagated, and the genetic changes could be analyzed in detail at the genome level. Using I-SceI, a proof of principle experiment could be performed showing that by inducing DSBs at a specific site in the genome, GT can be enhanced by orders of magnitude (Puchta et al. 1996). Interestingly, it was also demonstrated that even without the use of homologous sequences, a T-DNA can integrate into an endonuclease-induced DSB (Salomon and Puchta 1998; Chilton and Que 2003; Tzfira et al. 2003).

As discussed in detail in other chapters of this volume, different types of artificial nucleases have been developed over the years and applied more recently for genome engineering in plants. We will not discuss the characteristics of these different classes of enzymes in any detail here but will refer the reader to the respective chapters on meganucleases (MNs, Chap. 2), zinc finger nucleases (ZFNs, Chap. 3), transcription activator-like effector nucleases (TALENs, Chap. 4), and the CRISPR/Cas system (Chap. 5) as well as to a series of recent reviews in the literature (Gaj et al. 2013; Tzfira et al. 2012; Voytas 2013; Puchta and Fauser 2014). In 2005, the group led by Dan Voytas was able to demonstrate that an artificial locus in the tobacco

genome could be targeted using a ZFN with high efficiency (Wright et al. 2005). Four years later, the first reports related to targeting natural genes were published by Dan Voytas for tobacco (Townsend et al. 2009) and by scientists at Dow AgroScience for maize (Shukla et al. 2009). Additionally, using ZFNs, it could be demonstrated by inducing DSBs—first in a transgenic sequence (Lloyd et al. 2005) and later in natural genes (Osakabe et al. 2010; Zhang et al. 2010)—that error-prone repair often results in a loss of gene function. Subsequently, it was also demonstrated that TALENs (Shan et al. 2013a; Mahfouz et al. 2011; Zhang et al. 2013) and engineered homing endonucleases (D'Halluin et al. 2013) as well as the CRISPR/Cas system (Li et al. 2013; Nekrasov et al. 2013; Shan et al. 2013b; Fauser et al. 2014; Feng et al. 2014; Zhang et al. 2014) can be used in a similar manner for these types of genome manipulations in plants.

To better understand the basis of DSB-induced genome engineering in plants, it is important to understand the mechanism of DSB repair in detail. There are two principle ways in which such repair occurs: via HR or via nonhomologous end joining (NHEJ). While in the former pathway, the reaction takes place between sequences that are totally or at least nearly identical to each other, in the latter, the sequence information does not play a major role in the rejoining of the two double strands. Interestingly, there are several mechanisms of HR and NHEJ that can be differentiated. Below, we seek to describe these mechanisms in detail and to summarize current knowledge regarding factors that are involved in these pathways (for reviews, see also Waterworth et al. 2011; Lieberman-Lazarovich and Levy 2011; Puchta 2005).

2 Mechanisms of DSB Repair Involving Homologous Sequences

In principle, there are three main mechanisms of DSB repair involving the use of a homologous template: single-strand annealing (SSA), synthesis-dependent strand annealing (SDSA), and the so-called double-strand break repair (DSBR) model. DSBR is a prominent mechanism for meiotic recombination (Osman et al. 2011). In yeast, there are indications that DSBR also operates in somatic cells, although at a low frequency (Bzymek et al. 2010). While in SDSA and DSBR, the homologous repair template can be supplied in *cis* or *trans*, in the case of the SSA mechanism, recombination only occurs between two directly repeated homologous sequences. These homologies are generally in close proximity on the same chromosome. All three pathways are depicted in Fig. 1.1.

Following DSB induction in all pathways, single-stranded overhangs are produced via exonuclease-catalyzed resection. In SSA, both ends of the break must carry complementary sequences. These molecules can then directly anneal to one another, and a chimeric DNA molecule is formed. If this molecule contains 3′-overhangs, the respective parts of the sequence will be trimmed; otherwise, single-stranded

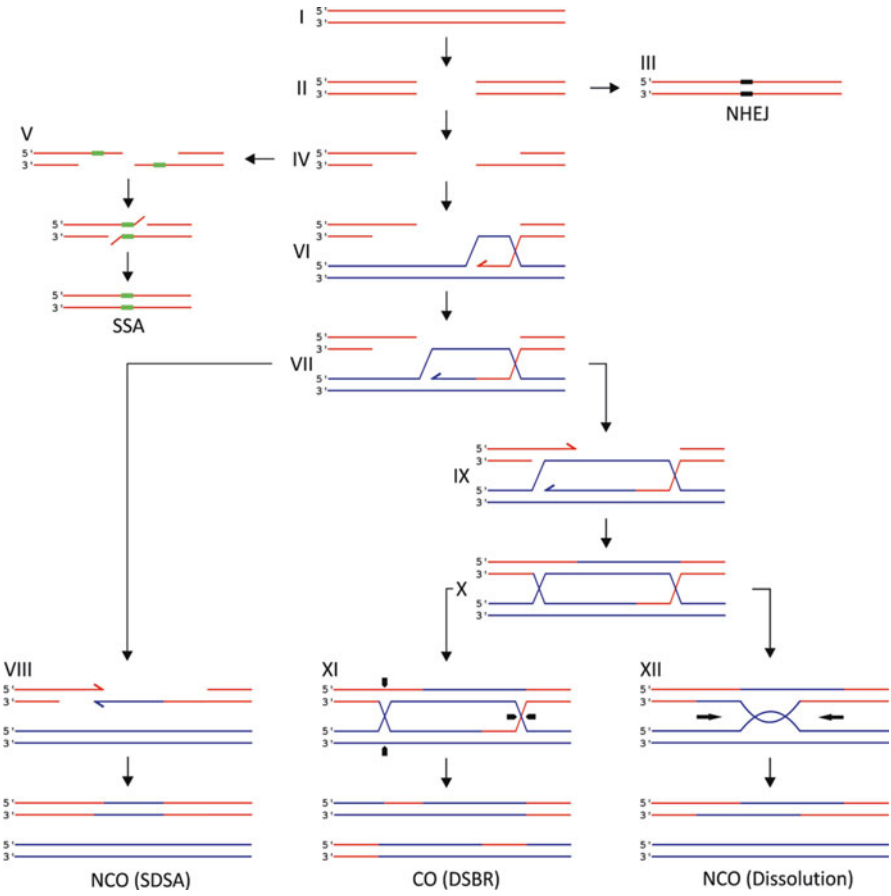

Fig. 1.1 Double-strand break (DSB) repair mechanisms. DSBs can be repaired either via homologous recombination (HR) or nonhomologous end joining (NHEJ). In principle, DSB repair via HR occurs via one of three main mechanisms: the single-strand annealing (SSA), synthesis-dependent strand annealing (SDSA), and double-strand break repair (DSBR) model. A model to explain the dissolution of a double Holliday junction (dHJ) was also added. Following the induction of a DSB (*II*), the cell must choose between NHEJ (*III*) and HR (*IV–XII*). Figure 1.4 depicts the NHEJ pathway in detail. In the case of HR-mediated DSB repair, single-stranded overhangs are produced via exonuclease-catalyzed resection (*IV*). In line with the SSA pathway (*V*), direct annealing of two single-stranded molecules can occur when complementary sequences are present at both ends of the break. After annealing, 3′-overhangs are trimmed, and single-stranded gaps are filled through DNA synthesis. Sequence information that is flanked by the homologies is lost. Therefore, SSA is also classified as a nonconservative HR-mediated DSB repair mechanism. In the case of the conservative HR pathways, a 3′-end invasion into a homologous double strand takes place, resulting in a D-loop (*VI*). Repair synthesis is initiated using the homologous strand as a matrix (*VII*). In contrast to DSBR and dHJ dissolution, SDSA only copies genetic information from the homologous matrix to the invaded single strand, generally leading to noncrossover (NCO) events following re-hybridization with the other end of the break (*VIII*). Once DNA synthesis occurs at the other broken end (*IX*, *X*), a dHJ is formed. This dHJ can either be resolved (in the case of DSBR) or dissolved (in the case of dHJ dissolution). In the case of DSBR, HJ-processing endonucleases are required to make symmetrical cuts. Depending on the orientation of the cuts, DSBR leads to CO or NCO events (*XI*). Dissolution occurs via the action of a RECQ DNA helicase that forms a hemicatenate and a class 1 topoisomerase to carry out strand separation, resulting in an NCO event

regions will be filled in via DNA synthesis. As a consequence, all information shared between the formerly repeated sequences will be lost (Fig. 1.1 V). SSA is a quite efficient mechanism: a study performed in tobacco using I-SceI indicated that up to one out of three DSBs is repaired via SSA under these circumstances (Siebert and Puchta 2002). SSA can, in principle, also occur between two DNA molecules that are not linked. These molecules could be transfected plasmid DNAs (Puchta and Hohn 1991) or T-DNAs (Tinland et al. 1994) as well as broken chromosomes (Pacher et al. 2007).

In the case of DSBR and SDSA, 3′-end invasion of a single strand into a homologous double strand occurs, resulting in a D-loop (Fig. 1.1 VII). Reparative synthesis is initiated using the newly paired strand as a template. From this point onward, the two pathways deviate: whereas in the case of SDSA, the genetic information of the matrix is only copied to one strand (Fig. 1.1 VIII), in DSBR, DNA synthesis also occurs at the other broken end, so that information is copied from both strands of the matrix (Fig. 1.1 XI). This second-end synthesis has major consequences for the further processing of the recombination intermediates. While in SDSA, the extended strand hybridizes with a single strand resulting from the resection of the other end of the break, in the case of DSBR, a double Holliday junction (dHJ) is formed. This structure can be either resolved or dissolved. For resolution, HJ-processing endonucleases are required (Fig. 1.1 XI). Dissolution occurs via the action of a RECQ DNA helicase that forms a hemicatenate and a class 1 topoisomerase that carries out strand separation, resulting in gene conversion (Fig. 1.1 XII). While in SDSA, the reaction always results in gene conversion, in the case of DSBR, the resolution pathway can end in a crossover event. Hence, larger parts of the recombining chromosomes are exchanged, which is of the utmost biological significance, as in meiosis, it is required for mixing parental genomes (Osman et al. 2011).

An interesting question is, of course, whether the SDSA or the DSBR mechanism is mainly used for the repair of DSBs in somatic cells. To discriminate between the two pathways, one must create an experimental situation in which the two mechanisms predict different outcomes. While in DSBR, homologous interactions between the two ends of the DSB are required, in the case of SDSA, the copying process is restricted to one end. Thus, *Agrobacterium*-mediated experiments were performed in tobacco using a T-DNA with homology to only one end of the break. The frequency of the repair of an I-SceI-induced DSB was compared with the results of experiments in which a T-DNA harboring homologous sequences to both ends of the break in the target locus was used. The recombination frequencies obtained with the T-DNA showing homology to both ends of the DSB were only one-third higher than those obtained using the one-ended construct (Puchta 1998). This small difference could be easily explained by one-sided invasion of the unique second homologous end of the respective T-DNA. Thus, homology to only one end of the DSB is sufficient for an efficient HR reaction to occur in plant cells. Somatic homologous DSB repair is initiated by a one-sided initiation event and does not require the other end of the break. These results are only in accordance with the SDSA model, as it is based on one-sided initiation and is therefore able to describe recombination events due to HR as well as to a combination of HR and NHEJ events.

Additionally, general considerations argue against an efficient DSBR mechanism in somatic plant cells. For example, if DSBR operated efficiently in somatic plant cells, crossovers would occur regularly. Additionally, repeated sequences are found at multiple ectopic positions in plant chromosomes, and crossovers between such repeated ectopic sequences would result in di- and acentric chromosomes. Thus, an efficient DSBR pathway would endanger genome stability in somatic cells.

Using the SDSA model, the results of GT experiments in somatic plant cells can be explained. Here, not only were perfect HR-mediated integration events found at the target locus, but recombinants in which only one end of the targeting vector was integrated via homology were often characterized. Alternatively, after copying sequences from the transgenic locus, the vector was observed to be integrated elsewhere in the genome (ectopic targeting). Depending on the experimental system employed, one-sided events could represent the major class of GT events (for review, see Puchta and Fauser 2013). An efficient strategy for reducing one-sided events in addition to random integration events is the use of negative selectable markers for GT (for details, see Chap. 6 of this volume). Additionally, in several studies involving DSB-induced targeting in tobacco, one-sided events were found in a fraction or even in the majority of cases (Puchta et al. 1996; Reiss et al. 2000; Wright et al. 2005).

3 The Chromosomal Site of the Template Makes a Difference

For SSA, the homologous template for the repair reaction of course has to be located in close proximity in direct orientation. This restriction does not apply for the SDSA and DSBR models. During meiosis, controlled recombination between homologs is the main repair mechanism. There are also indications that a portion of the DSBs are repaired during meiosis using the sister chromatid as template—at least in yeast (Goldfarb and Lichten 2010). A prerequisite for efficient allelic recombination in meiosis is the alignment of homologs and the formation of the synaptonemal complex (for a review on meiotic recombination in plants, see Osman et al. 2011).

DSBs must be induced in somatic plant cells for gene and genome engineering. In the case of HR, several different types of homologous sequences might be employed if an SDSA-like repair mechanism operates (Fig. 1.2). In G2 and S phase of the cell cycle, homology to the sister chromatid could be exploited (Fig. 1.2a). However, as this mechanism does not lead to any sequence changes, the frequencies of such events could not be determined for plants. Nevertheless, it must be assumed that this type of repair is likely the most efficient means of repair. Apart from a sequence on the same chromosome (Fig. 1.2b), an allelic sequence on the homologous chromosome (Fig. 1.2c) or an ectopic sequence on a different chromosome (Fig. 1.2d) could be employed. Using I-SceI, the efficiency of different types of templates could be determined based on the restoration of marker genes.

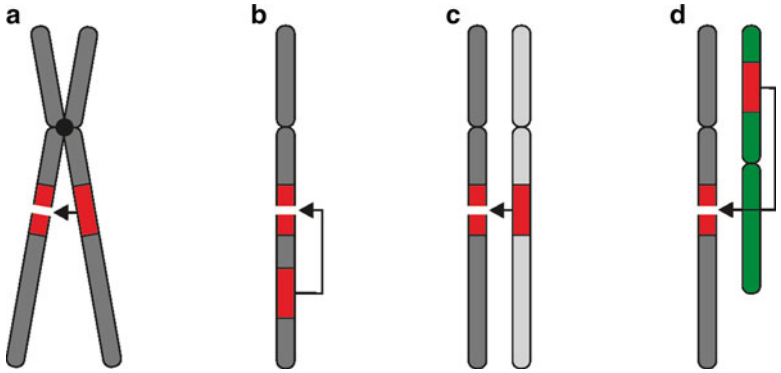

Fig. 1.2 Templates for homologous recombination. Several different types of homologous sequences can be used during the operation of an SDSA-like repair mechanism. (**a**) Homologous sequences on the sister chromatid appear to be used efficiently in G2 and S phases of the cell cycle. (**b**) Intrachromosomal homologous sequences are other potential templates for DSB repair. They are used at frequencies up to the percent range (**c**). Allelic sequences are available in diploid cells, but in contrast to meiotic recombination, they are used in very few cases in somatic cells. (**d**) Ectopic sequences on a different chromosome are also employed as a template, but at least as rarely as allelic sites

To test the efficiency of ectopic DSB-induced HR, a donor and an acceptor locus containing an I-SceI site and carrying partly homologous sequences of a nonfunctional kanamycin resistance gene were transformed independently into tobacco and combined by crossing. DSBs were induced via *Agrobacterium*-mediated transient expression of I-SceI in cell culture, and recombinants were selected for by kanamycin. The study revealed that approximately 1 out of 10,000 DSBs is repaired by the use of an ectopic sequence. Detailed molecular analysis of the recombinants indicated that HR did not occur in all cases at both ends of the DSB, and a combination of HR and NHEJ also took place (Puchta 1999). This finding is a clear indication of the operation of an SDSA-like mechanism (see above). In an independent study conducted in *Arabidopsis* based on DSB induction by a transposon, a similar efficiency of DSB-induced ectopic HR was reported (Shalev and Levy 1997).

As during meiosis, DSBs are mainly repaired using allelic sequences from the homolog, how efficiently such a template can be exploited in somatic plant cells is an interesting question. To answer this question, transgenic tobacco cell culture was used. The experimental setup was such that two transgenes in allelic positions were combined. One transgene carried the negative selectable marker cytosine deaminase (codA), together with an I-SceI site that was originally produced for the analysis of NHEJ (Salomon and Puchta 1998). The other transgene was derived from the former transgenic line. Here, the I-SceI site was destroyed following DSB induction, and the selection marker became nonfunctional due to a deletion associated with the repair event. After combining the two alleles via crossing, DSBs were induced through the transient expression of I-SceI. Selection was performed based on the loss of the marker. Using this approach, it was indeed possible to isolate

DSB-induced allelic recombination events. However, the vast majority of events resulted in a loss of function of the marker gene due to NHEJ. The frequency of allelic recombination was estimated to be approximately 10^{-4}, similar to that of ectopic recombination following DSB repair in the same species (Gisler et al. 2002). Thus, in contrast to meiosis, allelic HR is not a significant DSB repair pathway in somatic cells. It appears that there is not a great difference when homology is present in an allelic versus an ectopic position: as long as it is on a different chromosome it is hardly accessible for DSB-induced HR. With respect to genome engineering, these results indicate that ectopic and allelic HR pathways are not sufficiently efficient to successfully induce controlled genomic changes. Nevertheless, there is also no risk that these pathways will interfere with other applications of DSB-induced manipulation and lead to unwanted genome rearrangements.

The situation is clearly different when homologies are supplied in close proximity to the break: such sequences, either on the same chromosome or on the sister chromatid, are more efficient matrixes for repair. Due to experimental limitations, it has not been possible to address sister chromatid recombination in plant cells in connection with DSB induction for a long time. Very recently, by applying I-SceI it could be demonstrated for barley that a DSB is predominantly repaired using the sister chromatid as a template during S- and G_2-phase (Vu et al. 2014). Thereby, reciprocal chromatid exchanges occur but its molecular mechanisms have not been elucidated yet. However, a series of studies has been published on endonuclease-induced intrachromosomal HR in plants (Chiurazzi et al. 1996; Orel et al. 2003; Roth et al. 2012; Siebert and Puchta 2002). SDSA appears to be approximately five to ten times less efficient than SSA under comparable conditions (Orel et al. 2003).

4 Extrachromosomal Templates

An efficient way to supply the cell with a matrix for HR-mediated DSB repair is to use an incoming T-DNA from *Agrobacterium tumefaciens* or transfected plasmid DNA. A T-DNA that is directly transformed into cells was found to be a better template for homologous DSB repair by several orders of magnitude than an ectopic chromosomal site carrying the same DNA template (Puchta 1999; Puchta et al. 1996). This situation most likely arises because the incoming T-DNA is more readily accessible for the copying processes than a chromosomal site, which can potentially be attributed to steric hindrances related to the use of an ectopic site as template. It might also occur because the ectopic site is chromatin packed or because the incoming DNA is recognized in some way by the cell as damages and, thus, actively recruits DNA repair factors that make recombination reactions more efficient.

Of great interest in this respect is the recently developed *in planta* GT technique (Fauser et al. 2012). Here, a linear DNA molecule is excised from the plant genome using a site-specific endonuclease at the same time as a break is induced in the target locus. Hence, the GT reaction can occur in vivo during plant development. If the induced targeting event is transferred to the progeny, genetically modified seeds can

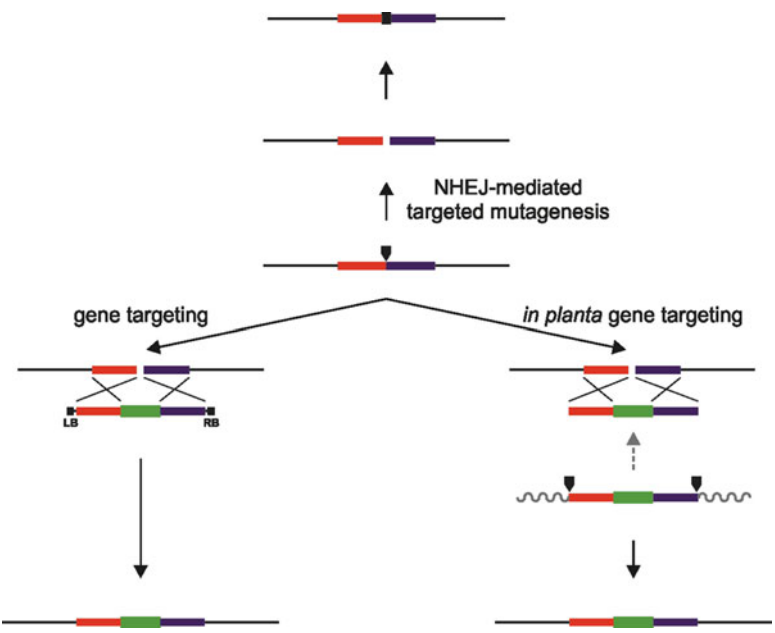

Fig. 1.3 Strategies of gene engineering. Endonuclease-mediated DSB induction can be employed for either targeted mutagenesis or GT. In the case of targeted mutagenesis, NHEJ leads to mutagenic religation of the broken ends. Typically, deletions occur, but insertions as well as nucleotide exchanges are also observed. Two different NHEJ pathways are depicted in Fig. 1.4. However, DSBs can also be used to activate a target locus for GT. DNA molecules transferred into cells carrying homologous sequences to the target locus integrate into the activated target site. On the other hand, the *in planta* GT system allows the simultaneous release of a linear GT vector and activation of a target site. Under both GT strategies, the GT vector can be designed for the site-specific integration of transgenes or to modify the target locus in a predefined manner (e.g., via AS exchange)

be harvested directly. This system was developed in *Arabidopsis* using the scorable marker ß-glucuronidase (GUS, uidA) and I-SceI, and this method should be applicable to any endogenous locus and custom-made nuclease. Its principle is depicted in Fig. 1.3. It is based on a transgene carrying sequences homologous to the target locus that is flanked by two recognition sites for a custom-made endonuclease that also cuts the locus of interest. Expression of the enzyme, which can be modulated by inducible, organ-specific, or constitutive promoters, should lead to the simultaneous release of a linear GT vector and the induction of a DSB at the target locus. Using a constitutive promoter for different target/donor combinations, up to one GT event per 100 seeds could be recovered. Hundreds of seeds exhibiting GT events could therefore be obtained. Molecular analysis of recombinant lines indicated that HR occurred at both ends of the DSB in almost all of these lines (19 out of 20 tested). Additionally, no extra copies of the vector were integrated elsewhere in the genome because in the case of a hemizygous, single-copy transgene, only one copy of the target vector can be set free per genome. Thus, the number of unwanted random integration events is minimized, thus differing from classical GT approaches,

in which multiple copies of a vector are often transferred into a single cell. Moreover, in contrast to classical GT approaches that rely on the generation of a greater number of transformation events, *in planta* GT requires only a single transformation event in principle. Hence, the GT vector as well as the ORF of a synthetic nuclease can be provided simultaneously. As *in planta* GT is more efficient than DSB-induced ectopic recombination, it appears that neither the number of copies nor the chromatin package itself are factors that hinder the DNA from acting as an efficient template. It seems more likely that the steric accessibility of the template is enhanced by the excision and that the excision might recruit repair factors to the template that could enhance the recombination reaction.

5 Factors Involved in Homologous Recombination

Intensive research has been taking place in recent decades, especially in yeast and mammals to identify the key factors involved in HR and define their roles in detail. Here, we wish to exclusively discuss the role of the most prominent factors in relation to what is known about their function in DSB repair in plant cells. The most direct way to characterize the role of individual factors in the different repair pathways is to use recombination traps in which a DSB can be induced by the expression of a site-specific endonuclease, and a marker is restored either via the SSA or SDSA pathway (Orel et al. 2003).

Interestingly, knowledge regarding the factors involved in SSA is quite limited. Early work by Charles White's group indicated that the RAD1/RAD10 heterodimer is as structure-specific flap-like endonuclease that trims the complementary strand prior to ligation (Dubest et al. 2002). Very recently the same group was also able to demonstrate that *Arabidopsis* RAD51 paralogues XRCC2, RAD51B, and RAD51D are involved independently of RAD51 in SSA (Serra et al. 2013). Other results indicate that the nuclease MUS81 and the FANCM helicase play some minor role in SSA (Mannuss et al. 2010). It might well be that different helicases and nucleases can substitute for each other under this quite robust and simple mechanism. SSA and SDSA share the feature of 3′-resection of the double-stranded ends, so that single strands become available for the recombination reaction to proceed. Work in mammals and yeast indicates the existence of two sub-pathways, one involving the DNA exonuclease exo1 and the other involving DNA replication helicase/nuclease 2 (DNA2) and a RECQ helicase (BLM in mammals and SGS1 in yeast) (for a recent review, see Blackwood et al. 2013).

Although it has been shown that the two EXO1 *Arabidopsis* homologs, Exo1A and Exo1B, are involved in the resection of telomeres (Kazda et al. 2012), direct evidence of their involvement in HR is still missing. The fact that overexpression of the rice protein OsRecQl4 (BLM counterpart) and/or OsExo1 (Exo1 homolog) can enhance intrachromosomal HR was taken as an indication that these proteins might, in fact, be involved in end resection in plants (Kwon et al. 2012). Indeed, *Arabidopsis* plants with a deficit of RECQ4A show some deficiency in both the SSA and SDSA

pathways. However, at this time, we cannot exclude the possibility that one of the other six RECQ-like helicases present in the *Arabidopsis* genome (Knoll and Puchta 2011) might also show some function in resection.

Invasion of the resected single strand in the double-stranded matrix is only required for SDSA. In all eukaryotes, RAD51, the homolog of the bacterial recombinase RECA, is involved in this key reaction. In the strand exchange reaction, a set of other factors is also required, which has been characterized in great detail in eukaryotes (for a recent review, see Suwaki et al. 2011). Indeed, recent experiments using pathway-specific recombination traps have demonstrated that the eukaryotic strand exchange protein RAD51 is extremely important for SDSA in *Arabidopsis*, but not for SSA. The same holds true for the SWI2/SNF2 chromatin remodeler AtRAD54 (Roth et al. 2012). BRCA2 is involved in the formation of RAD51 filaments. This function appears to be conserved in plants (e.g., Seeliger et al. 2011). Similar to AtRAD51 itself, its paralogs RAD51C and XXRC3 are essential for SDSA, but not for SSA. It is tempting to speculate that the same holds true for the other paralogs, RAD51B, RAD51D and XRCC2, as general deficiencies of HR have been reported (Da Ines et al. 2013; Durrant et al. 2007). Apart from RAD54, other ATPases appear to play a role in SDSA, including AtRAD5A, which is involved in "post-replicative" strand switching (Mannuss et al. 2010), and AtFANCM (Roth et al. 2012), which is involved in the control of the recombination reaction (Knoll et al. 2012). The nuclease MUS81 is also required for the processing of recombination intermediates within the SDSA pathway (Mannuss et al. 2010).

With respect to genome manipulation, knowledge of the roles of the factors involved in HR is a prerequisite for regulating DSB-induced DNA repair via modulation of the repair machinery of the cell. A classic example of the improvement of GT is provided by the expression of the yeast RAD54 ATPase in *Arabidopsis*, whereby Avi Levy's group was able to demonstrate that GT can be enhanced by the expression of this heterologous chromatin remodeler (Even-Faitelson et al. 2011; Shalev and Levy 1997). An early attempt to enhance DSB-induced GT involved expression of the bacterial strand exchange protein RecA. Unfortunately, this approach did not result in higher targeting frequencies. However, RECA overexpression shifted the obtained product classes towards more recombination events where both ends of the break were repaired via HR. Thus, although the efficiency of the reaction was not altered, its quality was enhanced (Reiss et al. 2000).

As NHEJ, in contrast to HR, often results in genomic change at the break site (see below), when performing NHEJ-mediated targeted mutagenesis, it is attractive to reduce the efficiency of HR pathways. The group led by Dan Voytas was able to achieve this result using a mutant deficient in the "structural maintenance of chromosomes" gene SMC6B. SMC6A and SMC6B are closely related and partly redundant factors required for DNA repair and HR in *Arabidopsis* that appear to be involved in sister chromatid interactions. While it has been reported that SMC6B mutants show a dramatic defect under conditions where sister chromatid recombination can be used for the restoration of a marker (Watanabe et al. 2009), the mutant shows only mild impacts on intrachromatid recombination (Roth et al. 2012). Interestingly, NHEJ-mediated targeted mutagenesis and GT was found to be enhanced at three

different loci of the *Arabidopsis* genome in an smc6b mutant (Qi et al. 2013). The easiest explanation for this phenomenon is that if the sister chromatid is not available to carry out the repair mechanism, an extrachromosomal copy can be used more efficiently as an alternative template. Alternatively, in the case of a lack of template, the DSB is repaired not via HR, but via NHEJ. This study provides a nice demonstration of the importance of template availability in the choice of pathways.

6 DSB Repair via Nonhomologous End Joining

NHEJ is the main mechanism of DSB repair in somatic plant cells and is also required for the random integration of DNA into plant genomes. DSB repair via NHEJ exhibits the characteristic that no extensive stretches of homology are required during the repair reaction. Ends are rejoined more or less directly, which can result in small deletions and, in some cases, also insertions (see below). Such a mechanism ensures that breaks can be efficiently repaired in an absence of homology with little genetic information being lost. However, it also poses the risk that genomic rearrangements might occur if several DSBs are repaired at the same time. This mechanism further provides new options for the control of genome engineering (see below).

Interestingly, at least two different pathways of NHEJ operate in plant cells that are known from other eukaryotes as well. These two pathways can be distinguished by the pattern of the resulting repair junctions as well as by the factors that are involved (Fig. 1.4). The classical NHEJ (cNHEJ) pathway involves minimal processing of broken ends before ligation occurs. This pathway is characterized by the involvement of the Ku70/Ku80 heterodimer. The function of this protein complex is to protect dsDNA from degradation by binding at the broken ends. If necessary, the ends are subsequently processed to become ligateable. For rejoining to occur, a specific ligase (Ligase 4) is necessary (Fig. 1.4). Thus, very little genetic information is lost, and small numbers of identical base pairs ("microhomologies") are found at the junctions only on rare occasion. Nevertheless, if a DSB occurs within an ORF, deletions of one or two nucleotides result in a frameshift and, thus, depending on the position of the break in the ORF, can often lead to complete knockout of gene function.

The main characteristic of the alternative NHEJ pathway (aNHEJ) (Mladenov and Iliakis 2011) is the regular occurrence of microhomologies, combined with the deletion of some nucleotides. In the case of the less well-characterized aNHEJ pathway, a certain amount of 3′-resection of the broken ends occurs, and a junction is formed by annealing of the two single strands involving a few complementary nucleotides. Following end trimming, religation occurs, and microhomologies can be observed at the junction site (Fig. 1.4). This process leads to the occurrence of deletions. Thus, more genetic information is lost more frequently when aNHEJ is used for DSB repair, rather than cNHEJ. Therefore, aNHEJ can be regarded as an extremely mutagenic means of DSB repair. Both NHEJ pathways appear to be con-

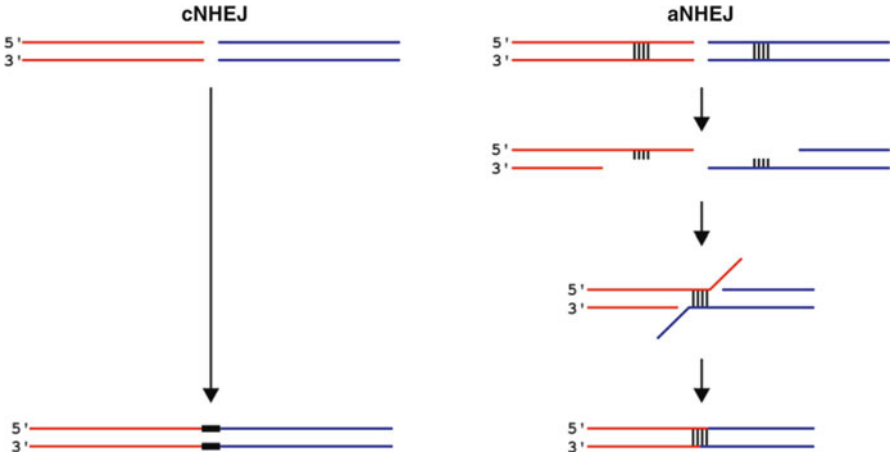

Fig. 1.4 DSB repair via nonhomologous end joining (NHEJ). At least two different NHEJ pathways operate in plant cells. Both are depicted here: the canonical, or classical NHEJ (cNHEJ) pathway and the alternative NHEJ (aNHEJ) pathway. The Ku70/Ku80 heterodimer and Ligase 4 are characteristic of cNHEJ leading to minimal processing of the broken ends prior to ligation. Typically, very little information is lost, and microhomologies are not involved. In contrast, broken ends undergo more processing in aNHEJ, and microhomology-mediated religation occurs. Therefore, larger deletions are often observed following aNHEJ-mediated DSB repair

served among eukaryotes. Recent experiments demonstrated that the poly ADP ribose polymerase I (PARPI) and XRCC1 proteins are actors in this pathway in plants, as previously shown for mammals (Jia et al. 2013; Charbonnel et al. 2011). There is increasing evidence that the two NHEJ pathways compete with each other: in a Ku80 *Arabidopsis* mutant, a 2.6-fold increase in the error-prone rejoining frequency, associated with end-degradation, was documented (Osakabe et al. 2010). Additionally, HR and NHEJ compete for DSBs. The group led by Dan Voytas achieved a fivefold to 16-fold enhancement of DSB-induced GT in a ku70 mutant and a threefold to fourfold enhancement of GT in the lig4 mutant (Qi et al. 2013). It is an important open question whether the different DSB pathways function at the same efficiency in different plant species or cell types during development (Kirik et al. 2000; Lloyd et al. 2005). Moreover, evidence was recently provided that there might be a third NHEJ pathway that is responsible for the joining of at least some DNA ends when cNHEJ and aNHEJ are knocked out (Charbonnel et al. 2011).

The fact that DSB repair via NHEJ can also be associated with insertions is important both for understanding genome evolution as well as for the application of this mechanism. This statement holds true for genomic sequences that are located elsewhere in the genome as well as for incoming T-DNAs (Salomon and Puchta 1998). Interestingly, in most of these cases, microhomologies were found at the junctions between break sites and inserts, which can be taken as a hint that either an aNHEJ mechanism or a mode of copying similar to the SDSA model described

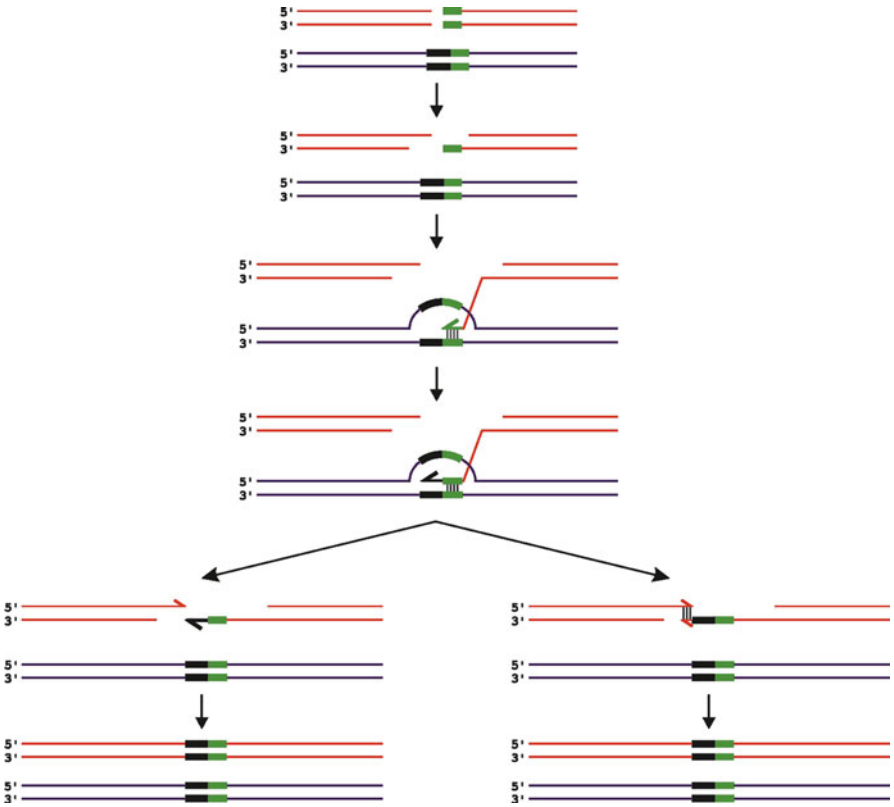

Fig. 1.5 SDSA-like insertions. Single-stranded overhangs are produced after DSB induction and the invasion of the 3′-end via microhomologies initiates a SDSA-like repair mechanism leading to insertions within the original DSB site. Any genomic sequence as well as extrachromosomal DNA can be used as template for the repair reaction. Microhomologies may also mediate second end capture

above for homologous DSB repair might be responsible for this phenomenon. As unique genomic sequences can still be found next to those inserted in the break site at their original location in the genome following DSB repair, a copying mechanism appears to be the most prevalent mode of repair (Salomon and Puchta 1998) as it is shown in Fig. 1.5. Thus, NHEJ can be applied similarly to HR for DSB-induced gene stacking (Chilton and Que 2003; Tzfira et al. 2003; Weinthal et al. 2013).

The potential of NHEJ for genome engineering is especially promising, as more than one DSB can be induced at a time in a plant genome. The principle underlying such applications is simple: in a certain number of cases, not the original linked DSB ends are rejoined, but instead, joining occurs between ends that were not linked previously. Thus, new combinations of genetic information are obtained. This phenomenon can be applied to achieve various types of planned genome rearrangements.

7 From Gene Engineering to Genome Engineering: Inducing More Than One DSB at a Time

The introduction of more than one DSB is a prerequisite for moving from the engineering of single genes to that of genomes. This step can be achieved by targeting either multiple identical sites within the genome using a unique synthetic enzyme or multiple different sites via the application of more than one nuclease. In particular, using the CRISPR/Cas system, it is possible to induce multiple DSBs at different sites simultaneously (Mali et al. 2013; Cong et al. 2013; Wang et al. 2013; Li et al. 2013).

Indeed, the repair of more than one DSB at a time might represent a challenge that cells have to face regularly. Multiple types of DNA damage might arise concurrently, particularly if a cell has to cope with genotoxic stress, but also during DNA replication. Cells are adapted to simultaneously repair several breaks through mechanisms that hold the correct broken ends together (Williams et al. 2010). Thus, the likelihood of complex genome rearrangements due to misrepair or misjoining of the broken ends is reduced. Nevertheless, as these mechanisms are not especially efficient, creating such a situation artificially provides a unique opportunity for genome engineering. It therefore becomes possible to achieve a tremendous number of different types of changes, including deletions, inversions, and exchanges of genomic sequences, and even of chromosome arms (Fig. 1.6).

A series of reports have been published that can be regarded as proof of concept experiments for genome manipulations based on the induction of a number of site-specific DSBs. The most prominent, and simplest, example is the programmed deletion

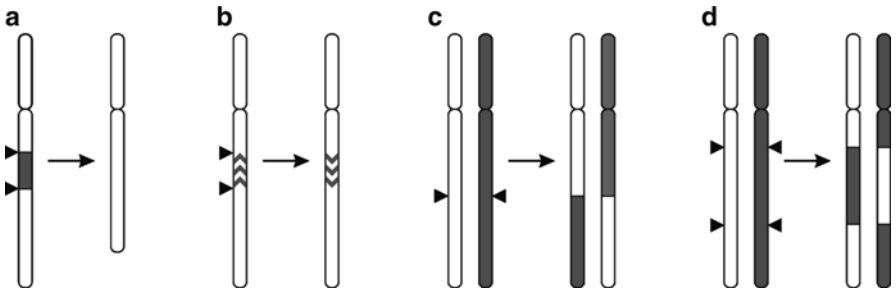

Fig. 1.6 Strategies of genome engineering. Future applications of genome engineering in plants will focus on larger deletions, the inversion of specific genomic regions, the exchange of chromosome arms or the exchange of sequence information between homologs of different accessions or cultivars as well as different chromosomes. Deletions can be induced by inducing two DSBs at a defined distance, whose repair can lead to the elimination of all sequence information between the respective recognition sites (**a**). If this sequence is not eliminated but is reintegrated the "wrong way round" an inversion takes place (**b**). If two DSBs are induced on two different chromosomes, chromosome arms may be exchanged (**c**). By inducing four DSBs (two per chromatid), sequences can also be exchanged between chromosomes (**d**)

of sequences such as marker genes following transformation. Through the induction of two DSBs in relatively close proximity, the sequences between the respective sites can be deleted from the plant genome (Petolino et al. 2010; Siebert and Puchta 2002). In principle, to achieve the controlled deletion of sequences, two differing types of repair reactions can be employed. The most direct way is to join broken ends following elimination of the internal sequence via NHEJ. However, in the presence of direct repeats, the direct annealing of repeated sequences via the SSA mechanism is also possible to obtain a deletion with a junction that can be predicted. Thus, if genomic sequences are organized in tandem repeats, as observed in certain gene clusters, the number of repeats is reduced. The deletion of single genes from the genome should become routine using artificial nucleases. An interesting option for genome engineering is the deletion of larger genomic regions. The size of these deletions might be limited in that genes that are essential for obtaining viable progeny cannot be eliminated. Through the induction of two DSBs, an inversion of the intervening sequence can also be achieved, as demonstrated in mammalian cells (Lee et al. 2012).

In addition to excising or inverting sequences, the controlled induction of more than one DSB can be applied to exchange sequences within a plant genome. This has been demonstrated in a proof of concept experiment involving tobacco chromosome arms, in which two unlinked transgenes, each carrying a restriction site for an endonuclease and parts of an intron containing kanamycin resistance genes were combined via crossing. The transgenes were constructed in such a way that the kanamycin resistance gene could be restored by joining two previously unlinked broken ends, either via SSA or via NHEJ (Pacher et al. 2007). The frequencies obtained using this approach indicate that DSB-induced translocation is up to two orders of magnitude more frequent in somatic cells than DSB-induced ectopic gene conversion (Puchta 1999). Indeed, both SSA and NHEJ events were recovered. Despite the fact that no selection was applied for the joining of the two other ends, the respective linkages could be detected in most of the tested cases, demonstrating that the respective exchanges were indeed reciprocal, as expected.

There are also applications in genome engineering that require the induction of more than two DSBs. One example discussed above is the *in planta* GT technique, which requires the induction of at least three DSBs. By inducing four chromosomal DSBs, it should be possible to exchange chromosomal segments. Here, DSBs should be induced at both ends of each sequence to be exchanged. Such an experiment has not been previously reported. However, the group led by Tzvi Tzfira described an exchange reaction between a chromosomal marker flanked by two ZFN recognition sites that could be exchanged with a DNA sequence in a T-DNA flanked by the same recognition sites (Weinthal et al. 2013).

The ways in which synthetic nucleases can be applied to achieve genome modifications are increasing. It is tempting to speculate that in the long run, DSB-induced genome engineering will result in synthetic plant genomes constructed from the most attractive alleles of a gene pool within, or even beyond, species boundaries.

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