http://doi.org/10.1093/plphys/kiab572 PLANT PHYSIOLOGY 2022: 188: 1769–1779

Plant Physiology®

Update

Nonhomologous end joining as key to CRISPR/Cas-mediated plant chromosome engineering

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Abstract

Although clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein (Cas)-mediated gene editing has revolutionized biology and plant breeding, large-scale, heritable restructuring of plant chromosomes is still in its infancy. Duplications and inversions within a chromosome, and also translocations between chromosomes, can now be achieved. Subsequently, genetic linkages can be broken or can be newly created. Also, the order of genes on a chromosome can be changed. While natural chromosomal recombination occurs by homologous recombination during meiosis, CRISPR/Cas-mediated chromosomal rearrangements can be obtained best by harnessing nonhomologous end joining (NHEJ) pathways in somatic cells. NHEJ can be subdivided into the classical (cNHEJ) and alternative NHEJ (aNHEJ) pathways, which partially operate antagonistically. The cNHEJ pathway not only protects broken DNA ends from degradation but also suppresses the joining of previously unlinked broken ends. Hence, in the absence of cNHEJ, more inversions or translocations can be obtained which can be ascribed to the unrestricted use of the aNHEJ pathway for double-strand break (DSB) repair. In contrast to inversions or translocations, short tandem duplications can be produced by paired single-strand breaks via a Cas9 nickase. Interestingly, the cNHEJ pathway is essential for these kinds of duplications, whereas aNHEJ is required for patch insertions that can also be formed during DSB repair. As chromosome engineering has not only been accomplished in the model plant Arabidopsis (*Arabidopsis thaliana*) but also in the crop maize (*Zea mays*), we expect that this technology will soon transform the breeding process.

Introduction

Genome editing might become a fundamental pillar in plant breeding to face the future challenges in food supply concerning the alarming growth rate of the world population and globally changing climate conditions (Hickey et al., 2019; Zaidi et al., 2020). To address the global increase in food demand and to compensate the expected global temperature rise of 2°C by 2050 (Bastin et al., 2019), breeders and scientists are trying to improve the yield and quality as well as pathogen resistance and abiotic stress tolerance of major food crops (Pingali, 2012; Newbery et al., 2016; Zaidi et al., 2020).

For a long time, high-yielding traits had been selected by classical breeding methods. However, this field has been revolutionized by the application of site-specific nucleases for the induction of targeted genetic change (Zhang et al., 2019; Atkins and Voytas, 2020; Schindele et al., 2020; Gao, 2021). The targeted induction of double-strand breaks (DSBs) enables the recruitment of the cell's own repair machinery. In eukaryotes, two main repair pathways exist for DSB repair:

Received September 29, 2021. Accepted November 9, 2021. Advance access publication December 6, 2021

Advances

- Two DSBs induced on the same chromosome facilitate the deletion or inversion of the intermediate region.
- Two DSBs induced on different chromosomes facilitate reciprocal translocations.
- The induction of staggered single-strand breaks on the same chromosome allows the formation of tandem duplications via cNHEJ.
- Blocking cNHEJ enhances the linkage of previously unlinked sequences.
- CRISPR/Cas-mediated chromosome engineering allows breaking or forming genetic linkages for breeding.

nonhomologous end-joining (NHEJ) and homologous recombination (HR). Which of the two mechanisms occurs is determined by the cell cycle phase and the cell type (Trenner and Sartori, 2019). In somatic plant cells, DSBs are mainly repaired via NHEJ, whereas DSBs induced during meiosis are repaired by HR.

The induction of targeted genetic changes depends on the efficiency and specificity of the utilized site-specific nuclease. The latter presented a major obstacle prior to the discovery of the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein (Cas) system in 2012, comprising a RNA-guided Cas endonuclease to induce a targeted DSB (Jinek et al., 2012). The first biotechnological applications were tested with the type II single nuclease Cas9 from *Streptococcus pyogenes*. The sequence specificity of the nuclease is ensured by the complementary binding of the chimeric single-guide RNA (sgRNA). Guided by the sgRNA to the target sequence, the Cas9 enzyme catalyzes the DSB induction if a protospacer adjacent motif is present next to the complementary region (Jinek et al., 2012).

CRISPR/Cas-based genome engineering was not only rapidly applied in the model organism Arabidopsis (*Arabidopsis thaliana*; Li et al., 2013; Fauser et al., 2014), but also in crops, such as rice (*Oryza sativa*), maize (*Zea mays*), tomato (*Solanum lycopersicum* L.) and even cotton (*Gossypium hirsutum* L.), and banana (*Musa acuminate*; Jaganathan et al., 2018), to induce mutations based on erroneous NHEJ. Pioneering results have been achieved by simultaneous editing of multiple loci, e.g. in the de novo domestication of the wild tomato *Solanum pimpinellifolium* (Zsögön et al., 2018) and various salt-tolerant or disease-resistant accessions (Li et al., 2018). A Cas9-based multiplexing approach was used to target different genes whose knockout is responsible for improved traits and yield of crops. Altered morphology, increased fruit number and size as well as an optimized nutritional content could be achieved in a remarkably short time.

The mechanisms of NHEJ and their application in modifying individual genes

Since most genome engineering methods rely on targeted DSB induction and subsequent cellular repair, it is of enormous importance to understand these repair pathways in order to assess the outcome of the intended modification. DSB repair is highly conserved between plants and mammals, with different pathways competing for successful repair (Ceccaldi et al., 2016; Zhao et al., 2020). Thus, a DSB can either be repaired via HR, which mainly acts in the late S and G2 phase of the cell cycle as sister chromatids can be used as a repair template, or by the error-prone NHEJ, which dominates in somatic plant cells (Puchta, 2005; Beying et al., 2021). In plants, as in mammals, two NHEJ-based DSB repair sub-pathways are known (Figure 1; Zhao et al., 2020). In classical NHEJ (cNHEJ; Figure 1A), the break ends are rapidly bound by the abundant, ring-shaped heterodimer KU70/KU80 (Walker et al., 2001), which recruits a wide variety of other repair factors and subsequently facilitates the break to be re-ligated by DNA LIGASE 4 (LIG4; Grawunder et al., 1997). A cNHEJ repair may result in small deletions or insertions next to perfect ligations.

In contrast, repair via the alternative NHEJ (aNHEJ) pathway results in larger deletions since microhomologies, present at the break sites, are used for annealing (Figure 1B). This leads to the loss of the intermediate sequence. Here, the break ends can be bound by poly(ADP-ribose)-polymerase 1 (PARP1; Audebert et al., 2004; Robert et al., 2009), a polymerase competing with the KU heterodimer (Wang et al., 2006). Recruited by PARP1, the 5′- *to* 3′-resection of the DSB can occur, creating short single-strand overhangs (Truong et al., 2013). The exposed microhomologies can anneal, with the resulting repair intermediate being stabilized by polymerase Q (PolQ; Zahn et al., 2015; Black et al., 2016; Wyatt et al., 2016; Seol et al., 2018). After the protruding 3′-ends have been degraded by nucleases (Bennardo et al., 2008), PolQ-initiated fill-in synthesis can begin (Ahmad et al., 2008; Hogg et al., 2012). Finally, the break can be ligated by a Xrcc1/Ligase III complex or Ligase I (Liang et al., 2008; Masani et al., 2016). Moreover, POLQ is essential for the integration of T-DNA, following *Agrobacterium tumefaciens*-mediated floral dip transformation of Arabidopsis (van Kregten et al., 2016; Nishizawa-Yokoi et al., 2021).

Since most DSBs in somatic plant cells are repaired by NHEJ, this pathway serves as the basis for a wide variety of chromosomal modifications. Apart from mutagenesis approaches that exploit the error susceptibility of NHEJ-mediated repair to disrupt the protein open reading frame for functional characterization, the induction of DSBs and their repair can be used to facilitate precise insertions, deletions and replacements.

The integration of a target sequence at a defined site can be achieved by inducing a single DSB in the target sequence (Salomon and Puchta, 1998). Insertion efficiencies of 2.2% were achieved by an intron targeting-based method in rice (Li et al., 2016). Another approach enabled efficient

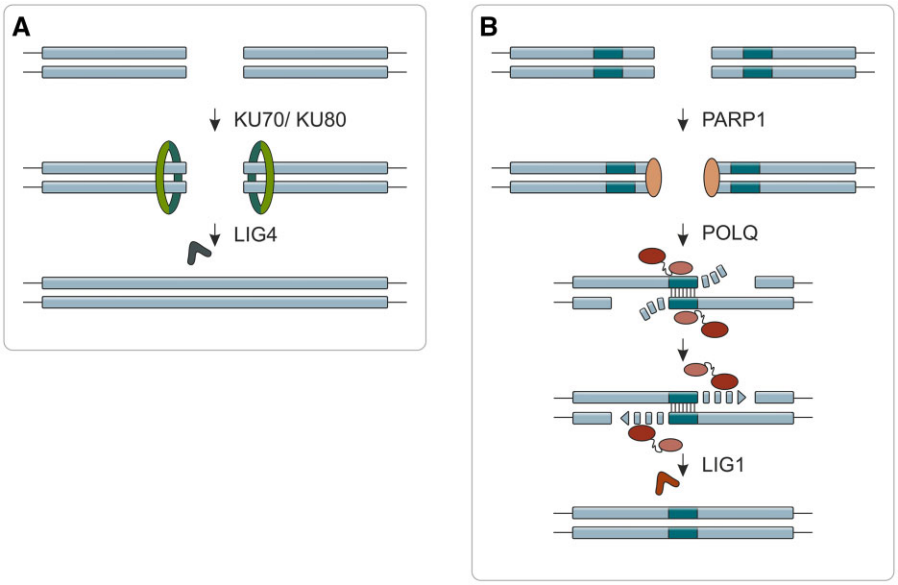

Figure 1 DSB repair via NHEJ. A DSB can be repaired via cNHEJ (A) or aNHEJ (B). In cNHEJ-mediated repair, the broken ends are bound by the KU70/KU80 heterodimer (green) and re-ligated by LIG4 (gray). Depending on whether the ends need to be processed prior to re-ligation, small insertions and/or deletions may occur in addition to error-free repair. In contrast, microhomologies (dark blue) at the break site are used in aNHEJ-mediated repair. Here, the break ends are bound by the polymerase PARP1 (orange), initiating the 5′- *to* 3′-resection of the ends. The annealing of the exposed microhomologies takes place, stabilized by the polymerase POLQ (red), whereby intervening regions can get lost. After filling the gaps via a POLQ-mediated fill-in synthesis, the break can be ligated. As areas between the microhomologies are resected, aNHEJ-mediated repair results in large deletions or more complex insertions.

integration in up to 25% of the analyzed samples by modifying DNA ends of the donor with a phosphorothioate linkage and 5′-*p*hosphorylation. Thus, it is now feasible to integrate regulatory elements upstream of agronomically important genes to manipulate the expression pattern in crops (Lu et al., 2020). Next to NHEJ-based strategies, HR-based approaches can be pursued for error-free and predictable modification of target sequences. In recent years, some promising approaches lead to the optimization of gene targeting efficiencies, creating another attractive tool for plant breeding (Huang and Puchta, 2019; Dong and Ronald, 2021).

The induction of two DSBs can lead to the deletion of the intervening sequence (Figure 2; Siebert and Puchta, 2002). Targeted formation of deletions can be used in basic research for functional analyses (Durr et al., 2018), the induction of smaller deletions is also an attractive approach for practical applications in molecular breeding. For example, by using CRISPR/Cas9 in a multiplex approach in tomato, deletions within regulatory elements of promoters could be induced, altering tomato yield and fruit quality (Rodríguez-Leal et al., 2017). Also, large deletions have been successfully induced in crops, ranging from 245 kb in rice (Zhou et al., 2014) to 1 Mb in soybean (*Glycine max* (L.) Merr.; Duan et al., 2021). Moreover, deletions can be combined with the integration of a defined sequence at the break sites. To do so, a suitable donor is introduced into the cell that can be integrated in place of the deleted region. To ensure that the NHEJ-based sequence mutations do not affect coding regions, CRISPR/Cas-based intron targeting was used in rice to exchange exon sequences (Li et al., 2016).

In addition to these modifications, induction of two breaks can lead to different chromosomal rearrangements (Figure 2; Rönspies et al., 2021). Thus, the induction of staggered single-strand breaks (SSBs) enables the formation of duplications (Schiml et al., 2016). Simultaneous induction of two DSBs on the same chromosome can result in the integration of the intervening sequence in the reverse orientation, leading to an inversion (Schmidt et al., 2019a), whereas the induction of two DSBs on different chromosomes can lead to reciprocal translocations (Beying et al., 2020). In the subsequent sections, we will take a closer look at these kinds of induced changes.

NHEJ-mediated duplications

Effective adaptation to changing environmental conditions over many generations can be achieved by the evolution of plant genomes through chromosomal restructuring and gene copy variation. A particularly rapid change in genome structure was observed in CHROMATIN ASSEMBLY FACTOR 1 Arabidopsis mutants, which resulted in large tandem duplications in addition to a significant reduction of ribosomal genes up to 20% compared to the wild-type. The duplication of more than one hundred genes resulted in an increased transcript number which lead to, among other things, an increased resistance to pathogens (Picart-Picolo et al., 2020). Most likely, these duplications are due to induction of random DSBs in the mutant, resulting from its defect

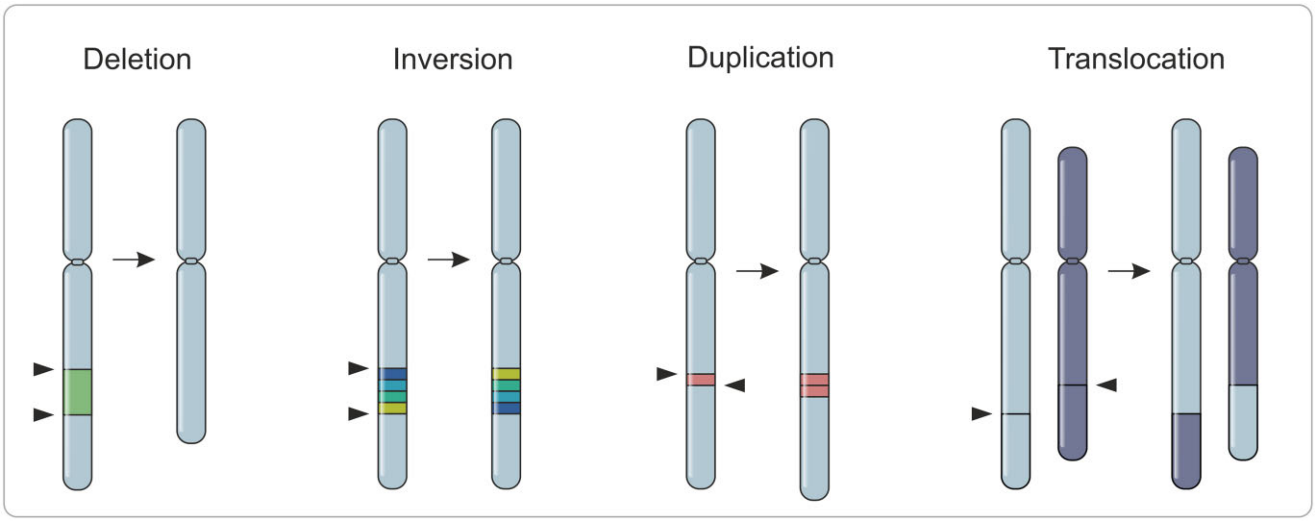

Figure 2 Possible chromosomal rearrangements after targeted break induction. If two DSBs (black triangle) are induced on the same chromosome, the intervening sequence can be deleted or inverted. Induction of two SSBs on opposite DNA strands of the same chromosome can result in duplication of the intervening sequence, whereas induction of two DSBs on nonhomologous chromosomes can result in a translocation by exchanging the ends of the chromosomes.

in chromatin organization. It is tempting to speculate that duplication of these segments originates from translocations between sister chromatids or homologs. The duplicated region might be excised from one sister chromatid and reintegrated in the other one via NHEJ-based repair. Thus, the formation of duplications could be achieved by inducing DSBs at both ends of the target region. Evidence for the feasibility of this approach was recently demonstrated in a study in Arabidopsis. Here, DSBs were induced flanking a segment of 2.3 kb or 8.5 kb (Lynagh et al., 2018). Both approaches indicated a successful duplication of the segment in somatic tissue. Furthermore, the smaller fragment of 2.3 kb was successfully transmitted to the next generation in one line.

Bioinformatic analysis of natural DNA insertions revealed that short tandem duplications are overrepresented in rice (Vaughn and Bennetzen, 2014). Sometimes, DSB repair is associated with insertions, which can arise by synthesis-dependent strand annealing (SDSA)-like mechanism. During this process, sequences from distant parts of the genome can be copied into the break site (Gorbunova and Levy, 1997; Salomon and Puchta, 1998). If copying of these regions occurs discontinuously, patch insertion patterns can be formed at the repaired site (Figure 3A). In contrast, the formation of tandem duplication could not be explained by such a mechanism. Therefore, an alternative model was proposed in which the formation of tandem duplications results from defective repair of adjacent SSBs in opposite strands (Vaughn and Bennetzen, 2014). Indeed, a paired Cas9 nickase approach, which was used to induce neighboring genomic SSBs on opposite strands, showed that the majority of observed insertions were simple tandem duplications between nicks (Schiml et al., 2014, 2016). Figure 3B shows the mechanism explaining these duplications. Starting from the SSB, DNA is synthesized from both ends, resulting in a duplication of the sequence between the staggered nicks. It can differ in length, depending on the extent of the 5′-end resection.

In a recent study, Wolter et al. (2021) defined the role of different DSB repair pathways in insertion formation. The paired nickase approach was applied to a variety of mutants in different repair proteins to induce 5′-staggered ends with a nick distance of 50 bp. In the wild-type, tandem duplications and, to a lesser extent, patch insertions were mainly detected, in addition to deletions. Mutation patters of representative members of the cNHEJ pathway (KU70 and LIG4), the aNHEJ pathway (X-Ray Repair Cross Complementing 1 (XRCC1) and PARP1) and the HR pathway (Radiation Sensitive 51 (RAD51) and RAD54) were analyzed. In the case of both cNHEJ mutants, *ku70* and *lig4*, next generation sequencing (NGS) analysis revealed a drastically different mutation pattern, with a distinct reduction in insertions and an increased number of deletions compared to the wild-type and all other tested mutants. Detailed analysis of these insertions showed that, in contrast to the wild-type, in which around 90% of all insertions were tandem duplications, in both cNHEJ mutants the occurrence of tandem duplications was dramatically reduced in comparison to patched insertions. In contrast, analysis of mutants devoid of the aNHEJ factors XRCC1 and POLQ, showed almost exclusively tandem duplications with a complete lack of patch insertions. Thus, the presence of the cNHEJ pathway is a prerequisite for tandem duplication formation, whereas aNHEJ plays no role in this process. The authors suggest that the KU70/KU80 heterodimer either directly protects longer single-stranded overlaps from nucleolytic degradation and/or promotes the fill-in reaction. On the other hand, aNHEJ, and here POLQ as a central factor, are required for

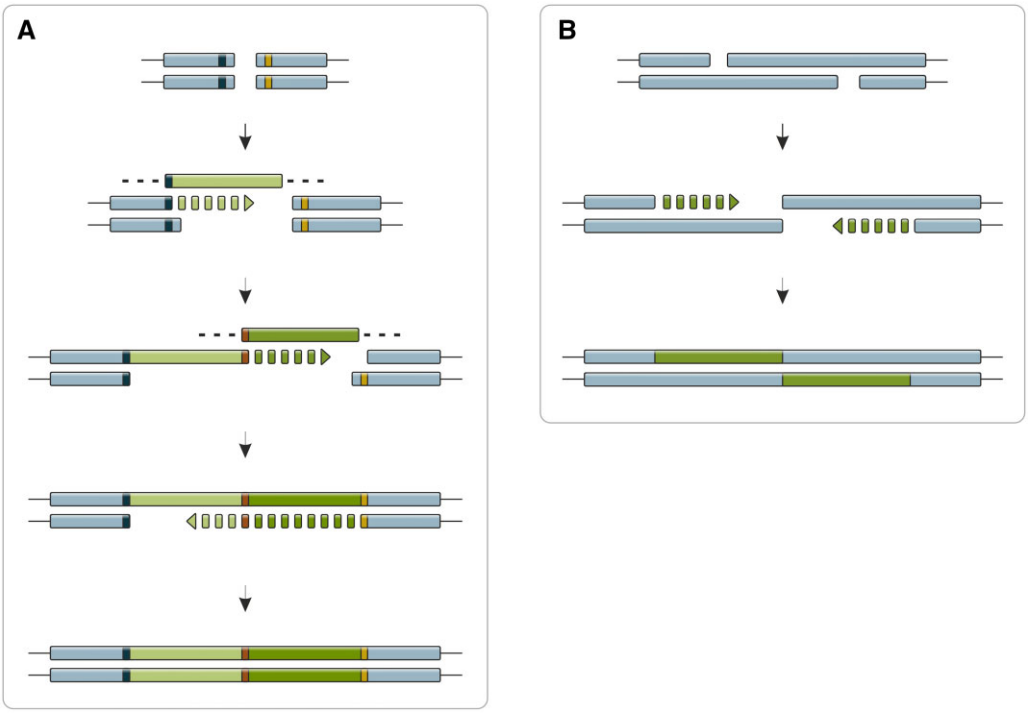

Figure 3 Possible mechanisms for the formation of insertions. A, The repair of a DSB via an SDSA-like mechanism can result in the formation of patch insertions. In this process, microhomologies at the break site (blue and yellow) may allow hybridization with distant sequences (green) in the genome. Depending on the microhomologies used, different ectopic sequences can be copied as templates and integrated into the break site. B, Tandem duplications can arise when DSBs with staggered 5′-overhanging ends are repaired in a microhomology-independent manner. Once the complementary regions are separated, the 5′-overhangs can be degraded, while fill-in synthesis starts at the 3′-end. After synthesis, the ends can be directly re-ligated resulting in the formation of duplications, depending on the length of the 5′-overhang.

the formation of patch insertions, whereas the binding of the KU heterodimer might even hinder the formation of this class of insertions. As patch insertions are a regular outcome of DSB repair, in contrast to tandem duplications, which were only detected after the induction of paired nicks, it is likely that POLQ is generally required for their formation in plants.

The fact that the presence of cNHEJ is essential for the formation of tandem duplications is consistent with recently published data on mammalian cells (Schimmel et al., 2021). Tandem duplications preferentially arise at DSBs with 3′-protruding ends in a Ku80-dependent manner (Schimmel et al., 2017). Subsequently, it was shown that DNA polymerase α (Pol α)-primase can be activated near DSBs with 3′-overhangs and initiate the fill-in synthesis, generating blunt ends that can be repaired via the cNHEJ (Schimmel et al., 2021). Unfortunately, it has not yet been elucidated which polymerases are required for tandem duplication formation at 5′-overhangs, but there are some indications that the DNA repair polymerase λ and μ are involved in mammals (Schimmel et al., 2017).

In terms of practical applications, an interesting question is how far apart the paired nicks can be induced on opposite DNA strands, so that duplications arise at a reasonable frequency. Whereas distances of 50 and 100 bps turned out to be efficient, there was a steep reduction in their occurrence in the case of 250 and 600 bps (Schiml et al., 2016; Wolter et al., 2021). Despite this limitation to about 100 nucleotides, the controlled induction of tandem duplications by a paired-nick approach is a promising tool for applications in genome engineering. Also, this methods appears to be particularly suitable for the manipulation of promoter regions (Rodríguez-Leal et al., 2017; Wolter et al., 2019). Duplication of transcription factor binding sites could help to enhance gene expression for crop improvement.

NHEJ-mediated inversions

Large genomic changes play a substantial role in plant biodiversity. Especially, inversions are associated with environmental adaptation and niche specification (Schubert and Vu, 2016). A consequence of large-scale inversions in different plant species is hybrid sterility, centromere shifting as well as the formation of new open reading frames, but also disruption of already existing genes, resulting in alteration of expression profiles, and, in some cases, the formation, or breakage of genetic linkages. The most prominent inversion of *A. thaliana* is the heterochromatic knob hk4S inversion on the short arm of chromosome IV (Fransz et al., 2016). This inversion with a size of 1.17 Mb is found in the Columbia accession, but not in the Landsberg accession and is associated with a pericentromeric shift. To demonstrate the feasibility of inversions for chromosome engineering

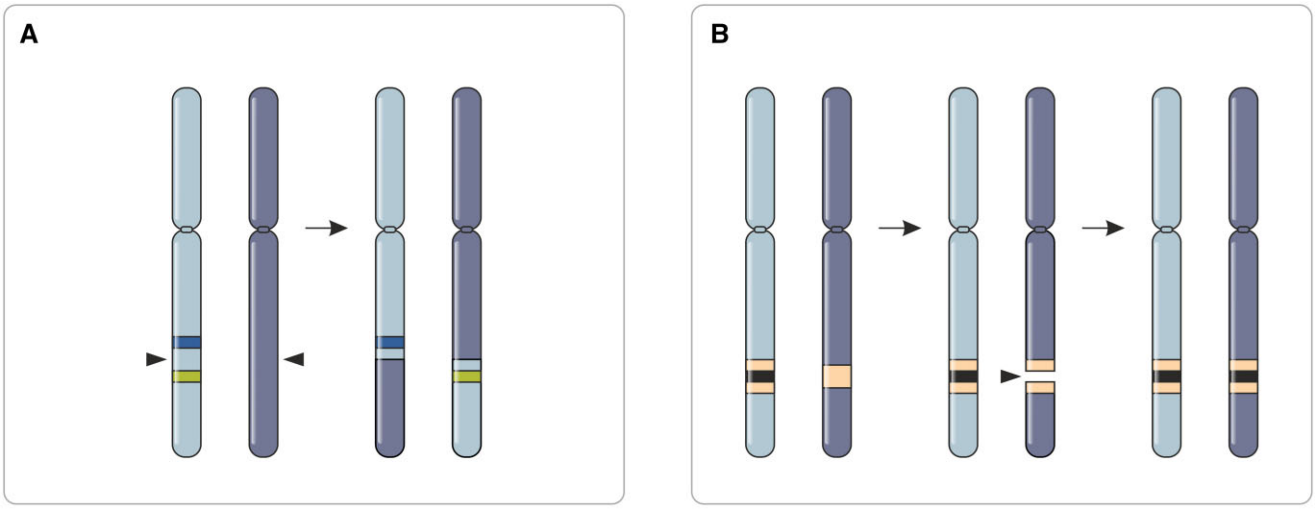

Figure 4 HR-based applications for plant chromosome engineering. A, To modify genetic linkage, DSBs (black triangles) can be induced on both homologous chromosomes. Repairing the break via meiotic HR, the homologous chromosome can be used as a repair template and targeted CO can be formed. Thus, breaking or creating genetic linkage of attractive traits is possible. B, Gene drive enables the introduction of a genetic modification into a natural population. Thereby, a gene-drive cassette (black) codes for a targeted Cas nuclease and is initially located on only one of the homologous chromosomes. Once the Cas nuclease is expressed, a DSB can be induced in the second chromosome at the same homologous site. Using the first chromosome as a template, a HR-based repair of the break copies the gene-drive construct into the second chromosome. The gene drive cassette is now present on both chromosomes and is thus inherited by all offspring.

purposes, Schmidt et al. (2019a) established a CRISPR/Cas9-based system to generate targeted heritable inversions. In a proof-of-concept experiment, the induction of two DSBs, 3 kb apart, within a single chromosome was tested, resulting in up to 7% deletions and up to 2% inversions. Molecular analysis of the newly formed junctions of the inverted sequences revealed that most of the inversions were devoid of deletions or other mutations, indicating that cNHEJ plays a key role. To verify this finding, the same approach was performed in the DNA repair mutant *ku70*. Surprisingly, digital droplet (dd)PCR showed a two-fold increase in the formation of inversions at the two tested loci, indicating that the KU70/KU80 heterodimer is also required for tethering the broken ends during the repair process. Consistently, in animals, a single XRCC4-like factor (XLF) dimer recruited by the KU70/KU80 heterodimer has been shown to promote tight alignment of DNA ends. A mutation of the KU binding site for XLF affected end-joining efficiency and accuracy (Graham et al., 2018; Nemoz et al., 2018). In the absence of KU70, the chance of a ligation of the unlinked broken ends is increased, resulting in more inversions. However, this improvement comes with a price: due to the lack of protection of the broken DNA ends by the KU70/KU80 heterodimer, the majority of inversions contained deletions within the newly formed junctions.

To obtain heritable inversions, a strategy was used that was first developed to obtain rare gene targeting events in Arabidopsis. Here, the use of the egg cell-specific EGG CELL1.1 (EC1.1) promoter, fused to the EC1.2 enhancer for tissue-specific expression of the Cas9 nuclease, resulted in a three-fold increase in gene targeting frequency and, thus, heritable gene targeting events (Wolter et al., 2018). Indeed, replacement of the constitutive promotor with the egg cell-specific promotor restricting the expression of the Cas9-nuclease to the early stage of plant development, allowed more efficient and heritable induction of inversions in wild-type plants. In the experimental setup, inversions of defined sequences comprising up to 18 kb were induced at two different loci. Thus, inversion events were detected in up to 10% of the tested progeny of individual T1 plants. A total of 25 plants with a fully inverted sequence were identified. Sequencing of six junctions indicated error-free repair of the break sites. Hence, inversions can now be induced precisely and more efficiently in wild-type plants (Schmidt et al., 2019a). Apart from the replacement of the Cas-driving promotor, prior testing of the nuclease cutting efficiency on the target sequence turned out to be of great importance for the success of the approach.

Later, this system was used to revert the large 1.7-kb heterochromatic knob hk4S inversion in the accession Col-0 on the short arm of chromosome IV (Schmidt et al., 2020). In total, seven different heritable inversion events were obtained, equating to a 0.5% inversion frequency. Analysis of the newly formed junctions showed that 10 of 14 junctions were formed by precise ligation, whereas the remaining four junctions contained minor deletions or insertions. Fluorescence in situ hybridization (FISH) analysis revealed the successful reversion of the hk4S knob. In a subsequent step, meiotic recombination of the formerly recombination-cold region was tested between the accession Ler-1, which is devoid of the hk4S knob, and a homozygous hk4S knob reversion line. As expected, CO events could be detected in the hybrid lines, which were equally distributed over the inverted area. As many crop plants carry natural inversions,

this approach will be very helpful for breeders to reactivate recombination-dead regions.

Recently, a 75.5-Mb pericentric inversion on chromosome II has been inverted in a maize inbred line by scientists of Corteva Agriscience. Using pre-assembled gRNA and ribonucleoprotein complexes, DSBs flanking the large inversion were induced in 2,000 maize embryos (Schwartz et al., 2020). After selection and analysis, two T0 plants showed a full 75.5-Mb long pericentric reinversion on chromosome II. This is a major advance as it shows that chromosomal rearrangements can also be induced in crop plants with more complex genomes.

NHEJ-mediated translocation

While chromosome translocations in mammals are often associated with the occurrence of various genetic diseases and cancer (Rowley, 2001; Bunting and Nussenzweig, 2013), in plants these types of genome rearrangements are important for trait diversity, speciation, and evolution (Lysak et al., 2006; Gabur et al., 2019; Schmidt et al., 2019b). Since stabilizing of trait linkages or breaking linkage drags is essential for crop optimization, chromosome engineering has a huge potential for breeding. Based on the first evidence that simultaneous DSB induction on heterologous chromosomes may lead to reciprocal translocations (Pacher et al., 2007), Beying et al. (2020) induced DSBs in intergenic regions on the long arm of chromosomes I and II of Arabidopsis, using the Cas9 nuclease. Here, a reciprocal translocation of both 0.5-Mb chromosome ends could be detected in 0.01% of the samples via ddPCR. To determine which repair pathway was used to form the translocations, NGS analysis was performed and revealed error-free ligation in 60%, while the remaining samples often showed small deletions at the junction. This suggests that cNHEJ is the main pathway for forming the chromosomal translocation. Furthermore, a knockout of KU70 resulted in a five times higher occurrence of translocations, demonstrating that cNHEJ suppresses the joining of unlinked DSB ends, as has been shown for inversions before.

For the induction of heritable translocations, an egg cell-specific expression of Cas9 was used. Translocations between chromosomes I and II as well as chromosomes I and IV were induced successfully in independent approaches. The translocation between chromosome I and II stood out in particular with translocation frequencies of up to 2.5% in individual T2 lines. Here, independent translocation events were identified in four plants in a Col-0 background. A FISH-based microscopic analysis confirmed the successful translocation between chromosomes I and II. Sequencing of homozygous translocation-bearing offspring revealed cNHEJ-mediated repair of the junction sites, whereby three of the four lines carried a perfect ligation of both junctions, while the remaining line showed a 44-bp deletion at one junction. Translocation induction in the *ku70*-mutant further improved translocation frequency. Here, successful translocation events were increased to 3.75% in individual T2 lines. In total, eight individual plants were identified carrying the reciprocal translocation between chromosomes I and II. As expected, all analyzed junctions showed larger deletions and inversions, indicating repair via aNHEJ. While higher translocation frequencies in *ku70* mutants appear attractive for further applications, the precision of the approach suffers due to the high mutation ratio, making the outcome of CRISPR/Cas-based chromosome rearrangement less predictable (Beying et al., 2020).

Is HR a valuable alterative to NHEJ for plant chromosome engineering?

For an unbiased evaluation of the potential of NHEJ in chromosome engineering, one has to view results in relation to what has been achieved using HR-based approaches. Indeed, most heritable genetic changes are based on the repair of DSBs by HR, which occurs in a temporally controlled manner in meiotic cells leading to an exchange of parental genetic material between two homologous chromosomes. The nonreciprocal transfer of genetic information leads to a noncrossover product (NCO), while the reciprocal exchange of homologous fragments leads to allelic shuffling and is referred to as crossovers (COs; Mercier et al., 2015). For the initiation of meiotic recombination, a programmed DSB is induced by the highly conserved SPORULATION11 (SPO11) topoisomerase-like protein (Bergerat et al., 1997). After processing of the DSB, the arising 3′-single-stranded overhang can invade in the double helix of the paired homolog and form a displacement loop (D-loop). If the invading strand of the D-loop is elongated via SDSA, the structure can be resolved and the break can be repaired using the elongated 3′-single-strand overhang. SDSA-based repair results in NCO products. Alternatively, the D-loop can be transformed into a double holiday junction, extending the D-loop so that the invading strand can anneal to the remaining DSB end (Beying et al., 2021). Depending on the resolution of this repair intermediate, both CO and NCO products can arise. These CO events can generate new allelic combinations. Thus, two favorable traits might be combined or an unfavorable one might be eliminated from an elite cultivar (Figure 4A). Although genetic exchange is highly desired for breeding, CO events are rare and limited to the euchromatic parts of chromosomes, which often hinders the segregation of linked favorable and unfavorable traits, especially if coded in between a short distance on the same chromosome.

A comprehensive study in yeast (*Saccharomyces cerevisiae*) reported targeted COs by fusion of the natural meiotic DSB inductor SPO11 to DNA-binding domains, such as zinc fingers, transcription activator-like effector modules and the CRISPR/Cas9 system, showing that overcoming this limitation is possible. Depending on the DNA recognition domain and the targeted sequences, an increased CO frequency and a SPO11-mediated DSB induction could be detected. However, the effect was quite small and restricted to euchromatic regions (Sarno et al., 2017). Recently, published data suggest that recruitment of the natural DSB-inducing

machinery is not sufficient to affect CO induction in plants using a similar approach (Yelina et al., 2021). Here, the SPO11 complex partner, meiotic topoisomerase VIB, which is essential for SPO11-mediated DSB induction, was fused with a deadCas9 and guided to CO-accessible regions in *A. thaliana*. However, no improvement in CO frequency or distribution was obtained.

In a pioneering study, recombination between homologous chromosomes in somatic cells could be demonstrated after targeted DSB induction by Cas9 in tomato (Filler Hayut et al., 2017). The experimental setup is based on two genetically distinct tomato accessions, which carry different mutations in the PHYTOENE SYNTHASE (*PSY1*) gene. Using Cas9, a DSB between these mutations was induced, followed by a fruit color assay and single-nucleotide polymorphisms sequencing to analyze genomic reshuffling events in hybrid plants. The analysis revealed somatic HR events, including gene conversions and one putative CO, which unfortunately was not transmitted through the germline. Nevertheless, this demonstrates that targeted somatic HR can be used for precise chromosomal rearrangements. Recently, extended to another tomato locus, called CAROTENOID ISOMERASE, the occurrence of two targeted COs was detected through whole-genome sequencing and it was confirmed that these COs can be transmitted through the germline (Ben Shlush et al., 2020). Furthermore, a recent study demonstrated targeted recombination in somatic maize cells. In two independent approaches, Kouranov et al. (2021) induced DSBs in chromosome III of both parental homologs in F1 hybrid maize using the LbCas12a nuclease. Genotypic analyses were able to identify targeted CO events, where in one case the respective junction contained a deletion and in the other no mutation. Therefore, cNHEJ as well as HR might be responsible for somatic CO formation. Furthermore, it was shown that these targeted COs can be inherited (Kouranov et al., 2021). These studies show that, despite their low efficiency, there is potential for CRISPR/Cas applications in CO induction to improve biodiversity in commercial crops.

Another approach to influence trait heritage is implemented by the gene-drive concept (Figure 4B). The selective inheritance of target genes from only one parent was established first in insects and mice and is used to convert heterozygous traits into homozygous traits (Kyrou et al., 2018; Grunwald et al., 2019). A study by Zhang et al. (2021) demonstrated the establishment of a gene-drive system in *A. thaliana*. This system is based on the prior integration of a gene-drive cassette into the CRYPTOCHROME 1 (*CRY1*) gene via HR, resulting in *cry1* drive lines. The gene-drive cassette consisted of a Cas9 coding sequence and a gRNA for DSB induction in the natural *CRY1* locus. After crossing the homozygous *cry1* lines with wild-type plants, heterozygous F1 progeny were generated in which expression of the gene-drive cassette resulted in targeted DSB induction in the wild-type *CRY1* locus. Repairing this break via the HR-based mechanism, the gene-drive cassette-bearing *cry* locus can be used as a template. This leads to a conversion of the wild-type *CRY1* locus to the *cry* locus and thus to a transfer of the gene-drive cassette. As a result, homozygous *cry1* loci could be detected in up to 8% of the F1 plants. Additionally, in another approach, a nonautonomous trans-acting gene drive was performed, whereby the gene-drive unit and the target locus were located on different chromosomes. Here, gene drive-based conversion of a heterozygous to a homozygous locus could be identified in 1.25% of the analyzed F1 plants. To improve efficiency of gene drive in plants, the use of transformation boosters that enhance HR efficiencies in somatic cells, as it has been shown for gene targeting in maize (Peterson et al., 2021), might be an option.

Thus, despite various attempts, HR-based chromosome engineering is currently, in contrast to yeast (Sarno et al., 2017), not a feasible technology for plants.

Conclusion

Taken together, all these results demonstrate a key role of NHEJ not only in efficient mutation induction but also for various kinds of chromosome engineering. Thus, deletions, inversions, and duplications within a chromosome and also translocations between chromosomes are achievable. Interestingly, the knockout of one or the other pathway might have decisive consequences on the efficiency as well as product classes. While cNHEJ suppresses all kinds of chromosomal restructuring in which previously unlinked DSBs are joined, it is essential for SSB-induced formation of tandem duplications. In contrast, aNHEJ, a backup mechanism for joining of any DSB ends in a more complex way, is also involved in the formation of patch insertions. By manipulating these pathways, the occurrence of specific product classes might be enhanced as shown for the knockout of cNHEJ in HR gene targeting (Qi et al., 2013; Endo et al., 2016). As a new level of CRISPR/Cas applications has been achieved in the case of plants (Lee and Wang, 2020), exciting novel question arise (see Outstanding Questions; Rönspies et al., 2021): Are we going to be able to change the number of chromosomes by fusion or fission? Can we reconstruct

Outstanding Questions

- Can we further improve the efficiency of chromosome engineering by the manipulation of DNA repair pathways?
- Can we develop chromosome engineering in all important crops?
- Will we be able to change chromosome numbers in plants?
- Is the induction of chromosomal rearrangements possible in polyploid crops with multiple homologous chromosomes?
- Will it be possible to establish genetic isolation and, thus, new plant species by induced NHEJ-based chromosomal rearrangements?

genome evolution? Can we create novel plant species by making individuals genetically incompatible by genome restructuring? Only the future will tell how fast we will be able to answer these questions and how far we can go, but 8 years after the start of the CRISPR/Cas revolution, we have already seen a number of dreams materializing.

Acknowledgment

We thank Michelle Rönspies for critical reading of the manuscript.

Funding

This work is funded by a European Research Council ERC grant (Grant number ERC-2016-AdG_741306 CRISBREED) to H.P.

Conflict of interest statement. None declared.

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