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

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One Sentence Summary: Heritable plant chromosome engineering can be achieved in somatic cells using CRISPR/Cas to induce non-homologous double strand break repair pathways.

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28 **Abstract**

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

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

56 For a long time, high-yielding traits had been selected by classical breeding methods.
57 However, this field has been revolutionized by the application of site-specific nucleases for
58 the induction of targeted genetic change (Atkins and Voytas, 2020; Gao, 2021; Schindele et
59 al., 2020; Zhang et al., 2019). The targeted induction of double-strand breaks (DSB) enables
60 the recruitment of the cell's own repair machinery. In eukaryotes, two main repair pathways
61 exist for DSB repair: non-homologous end-joining (NHEJ) and homologous recombination
62 (HR). Which of the two mechanisms occurs is determined by the cell cycle phase and the cell
63 type (Trenner and Sartori, 2019). In somatic plant cells, DSBs are mainly repaired via NHEJ,
64 whereas DSBs induced during meiosis are repaired by HR.

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

75 CRISPR/Cas-based genome engineering was not only rapidly applied in the model
76 organism Arabidopsis (*Arabidopsis thaliana*) (Fauser et al., 2014; Li et al., 2013), but also in
77 crops, such as rice (*Oryza sativa*), maize (*Zea mays*), tomato (*Solanum lycopersicum* L.) and
78 even cotton (*Gossypium hirsutum* L.) and banana (*Musa acuminata*) (Jaganathan et al.,
79 2018), to induce mutations based on erroneous NHEJ. Pioneering results have been
80 achieved by simultaneous editing of multiple loci, e.g. in the *de novo* domestication of the
81 wild tomato *Solanum pimpinellifolium* (Zsögön et al., 2018) and various salt-tolerant or
82 disease-resistant accessions (Li et al., 2018). A Cas9-based multiplexing approach was used
83 to target different genes whose knockout is responsible for improved traits and yield of crops.
84 Altered morphology, increased fruit number and size as well as an optimized nutritional
85 content could be achieved in a remarkably short time.

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86 **The mechanisms of non-homologous end-joining and their application in modifying**
87 **individual genes**

88 Since most genome engineering methods rely on targeted DSB induction and
89 subsequent cellular repair, it is of enormous importance to understand these repair pathways
90 in order to assess the outcome of the intended modification. DSB repair is highly conserved
91 between plants and mammals, with different pathways competing for successful repair
92 (Ceccaldi et al., 2016; Zhao et al., 2020). Thus, a DSB can be either repaired via HR, which
93 mainly acts in the late S and G2 phase of the cell cycle as sister chromatids can be used as
94 a repair template, or by the error-prone NHEJ, which dominates in somatic plant cells
95 (Beying et al., 2021; Puchta, 2005). In plants, as in mammals, two NHEJ-based DSB repair
96 sub-pathways are known (Figure 1) (Zhao et al., 2020). In classical NHEJ (cNHEJ) (Figure
97 1A), the break ends are rapidly bound by the abundant, ring-shaped heterodimer KU70/KU80
98 (Walker et al., 2001) which recruits a wide variety of other repair factors and subsequently
99 facilitates the break to be re-ligated by DNA LIGASE 4 (LIG4) (Grawunder et al., 1997). A
100 cNHEJ repair may result in small deletions or insertions next to perfect ligations.

101 In contrast, repair via the alternative NHEJ (aNHEJ) pathway results in larger
102 deletions since microhomologies, present at the break sites, are used for annealing (Figure
103 1B). This leads to the loss of the intermediate sequence. Here, the break ends can be bound
104 by poly(ADP-ribose)-polymerase 1 (PARP1) (Audebert et al., 2004; Robert et al., 2009), a
105 polymerase competing with the KU heterodimer (Wang et al., 2006). Recruited by PARP1,
106 the 5’-3' resection of the DSB can occur, creating short single-strand overhangs (Truong et
107 al., 2013). The exposed microhomologies can anneal, with the resulting repair intermediate
108 being stabilized by polymerase Q (PolQ) (Black et al., 2016; Seol et al., 2018; Wyatt et al.,
109 2016; Zahn et al., 2015). After the protruding 3' ends have been degraded by nucleases
110 (Bennardo et al., 2008), PolQ-initiated fill-in synthesis can begin (Ahmad et al., 2008; Hogg
111 et al., 2012). Finally, the break can be ligated by a Xrcc1/Ligase III complex or Ligase I
112 (Liang et al., 2008; Masani et al., 2016). Moreover, POLQ is essential for the integration of
113 T-DNA, following *Agrobacterium tumefaciens*-mediated floral dip transformation of
114 Arabidopsis (Nishizawa-Yokoi et al., 2021; van Kregten et al., 2016).

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115

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

121 The integration of a target sequence at a defined site can be achieved by inducing a
122 single DSB in the target sequence (Salomon and Puchta, 1998). Insertion efficiencies of
123 2.2% were achieved by an intron targeting-based method in rice (Li et al., 2016). Another
124 approach enabled efficient integration in up to 25% of the analyzed samples by modifying
125 DNA ends of the donor with a phosphorothioate linkage and 5' phosphorylation. Thus, it is
126 now feasible to integrate regulatory elements upstream of agronomically important genes to
127 manipulate the expression pattern in crops (Lu et al., 2020). Next to NHEJ-based strategies,
128 HR-based approaches can be pursued for error-free and predictable modification of target
129 sequences. In recent years, some promising approaches lead to the optimization of gene
130 targeting efficiencies, creating another attractive tool for plant breeding (Dong and Ronald,
131 2021; Huang and Puchta, 2019)

132 The induction of two DSBs can lead to the deletion of the intervening sequence
133 (Figure 2) (Siebert and Puchta, 2002). Targeted formation of deletions can be used in basic
134 research for functional analyses (Durr et al., 2018), the induction of smaller deletions is also
135 an attractive approach for practical applications in molecular breeding. For example, by using
136 CRISPR/Cas9 in a multiplex approach in tomato, deletions within regulatory elements of
137 promoters could be induced, altering tomato yield and fruit quality (Rodríguez-Leal et al.,
138 2017). Also, large deletions have been successfully induced in crops, ranging from 245 kb in
139 rice (Zhou et al., 2014) to 1 Mb in soybean (*Glycine max* (L.) Merr.) (Duan et al., 2021).
140 Moreover, deletions can be combined with the integration of a defined sequence at the break
141 sites. To do so, a suitable donor is introduced into the cell that can be integrated in place of
142 the deleted region. To ensure that the NHEJ-based sequence mutations do not affect coding
143 regions, CRISPR/Cas-based intron targeting was used in rice to exchange exon sequences
144 (Li et al., 2016).

145 In addition to these modifications, induction of two breaks can lead to different
146 chromosomal rearrangements (Figure 2) (Rönspies et al., 2021). Thus, the induction of
147 staggered single strand breaks (SSB) enables the formation of duplications (Schiml et al.,
148 2016). Simultaneous induction of two DSBs on the same chromosome can result in the
149 integration of the intervening sequence in the reverse orientation, leading to an inversion
150 (Schmidt, Pacher, Puchta, 2019), whereas the induction of two DSBs on different

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151 chromosomes can lead to reciprocal translocations (Beying et al., 2020). In the subsequent
152 sections we will take a closer look at these kinds of induced changes.
153

154 **NHEJ-mediated duplications**

155 Effective adaptation to changing environmental conditions over many generations can
156 be achieved by the evolution of plant genomes through chromosomal restructuring and gene
157 copy variation. A particularly rapid change in genome structure was observed in
158 CHROMATIN ASSEMBLY FACTOR 1 Arabidopsis mutants which resulted in large tandem
159 duplications in addition to a significant reduction of ribosomal genes up to 20 % compared to
160 the wild type. The duplication of more than one hundred genes resulted in an increased
161 transcript number which lead to, among other things, an increased resistance to pathogens
162 (Picart-Picolo et al., 2020). Most likely, these duplications are due to induction of random
163 DSBs in the mutant, resulting from its defect in chromatin organization. It is tempting to
164 speculate that duplication of these segments originates from translocations between sister
165 chromatids or homologues. The duplicated region might be excised from one sister
166 chromatid and re-integrated in the other one via NHEJ-based repair. Thus, the formation of
167 duplications could be achieved by inducing DSBs at both ends of the target region. Evidence
168 for the feasibility of this approach was recently demonstrated in a study in Arabidopsis. Here,
169 DSBs were induced flanking a segment of 2.3 kb or 8.5 kb (Lynagh et al., 2018). Both
170 approaches indicated a successful duplication of the segment in somatic tissue.
171 Furthermore, the smaller fragment of 2.3 kb was successfully transmitted to the next
172 generation in one line.

173 Bioinformatic analysis of natural DNA insertions revealed that short tandem
174 duplications are overrepresented in rice (Vaughn and Bennetzen, 2014). Sometimes, DSB
175 repair is associated with insertions which can arise by an SDSA-like mechanism. During this
176 process, sequences from distant parts of the genome can be copied into the break site
177 (Gorbunova and Levy, 1997; Salomon and Puchta, 1998). If copying of these regions occurs
178 discontinuously, patch insertion patterns can be formed at the repaired site (Figure 3A). In
179 contrast, the formation of tandem duplication could not be explained by such a mechanism.
180 Therefore, an alternative model was proposed in which the formation of tandem duplications
181 results from defective repair of adjacent SSBs in opposite strands (Vaughn and Bennetzen,
182 2014). Indeed, a paired Cas9 nickase approach, which was used to induce neighboring
183 genomic SSBs on opposite strands, showed that the majority of observed insertions were
184 simple tandem duplications between nicks (Schiml et al., 2014, 2016). Figure 3B shows the
185 mechanism explaining these duplications. Starting from the SSB, DNA is synthesized from
186 both ends, resulting in a duplication of the sequence between the staggered nicks. It can
187 differ in length, depending on the extent of the 5' end resection.

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188 In a recent study, Wolter *et al.* defined the role of different DSB repair pathways in
189 insertion formation (Wolter et al., 2021). The paired nickase approach was applied to a
190 variety of mutants in different repair proteins to induce 5' staggered ends with a nick distance
191 of 50 bp. In the wild type, tandem duplications and, to a lesser extent, patch insertions were
192 mainly detected, in addition to deletions. Mutation patters of representative members of the
193 cNHEJ pathway (KU70 and LIG4), the aNHEJ pathway (X-Ray Repair Cross Complementing
194 1 (XRCC1) and PARP1) and the HR pathway (Radiation Sensitive 51 (RAD51) and
195 Radiation Sensitive 54 (RAD54)) were analyzed. In the case of both cNHEJ mutants, *ku70*
196 and *lig4,* NGS analysis revealed a drastically different mutation pattern, with a distinct
197 reduction in insertions and an increased number of deletions compared to the wild type and
198 all other tested mutants. Detailed analysis of these insertions showed that, in contrast to the
199 wild type, in which around 90% of all insertions were tandem duplications, in both cNHEJ
200 mutants the occurrence of tandem duplications was dramatically reduced in comparison to
201 patched insertions. In contrast, analysis of mutants devoid of the aNHEJ factors XRCC1 and
202 POLQ, showed almost exclusively tandem duplications with a complete lack of patch
203 insertions. Thus, the presence of the cNHEJ pathway is a prerequisite for tandem duplication
204 formation, whereas aNHEJ plays no role in this process. The authors suggest that the
205 KU70/KU80 heterodimer either directly protects longer single-stranded overlaps from
206 nucleolytic degradation and/or promotes the fill-in reaction. On the other hand, aNHEJ, and
207 here POLQ as a central factor, are required for the formation of patch insertions, whereas the
208 binding of the KU heterodimer might even hinder the formation of this class of insertions. As
209 patch insertions are a regular outcome of DSB repair, in contrast to tandem duplications,
210 which were only detected after the induction of paired nicks, it is likely that POLQ is generally
211 required for their formation in plants.
212 The fact that the presence of cNHEJ is essential for the formation of tandem
213 duplications is consistent with recently published data on mammalian cells (Schimmel et al.,
214 2021). Tandem duplications preferentially arise at DSBs with 3′ protruding ends in a Ku80-
215 dependent manner (Schimmel et al., 2017). Subsequently, it was shown that
216 DNA polymerase α (Pol α)-primase can be activated near DSBs with 3′ overhangs and
217 initiate the fill-in synthesis, generating blunt ends that can be repaired via the cNHEJ
218 (Schimmel et al., 2021). Unfortunately, it has not yet been elucidated which polymerases are
219 required for tandem duplication formation at 5' overhangs, but there are some indications that
220 the DNA repair polymerase λ and μ are involved in mammals (Schimmel et al., 2017).
221 In terms of practical applications, an interesting question is, how far apart the paired
222 nicks can be induced on opposite DNA strands, so that duplications arise at a reasonable
223 frequency. Whereas distances of 50 and 100 bps turned out to be efficient, there was a steep
224 reduction in their occurrence in the case of 250 and 600 bp (Schiml et al., 2016; Wolter et al.,

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225 2021). Despite this limitation to about 100 nucleotides, the controlled induction of tandem
226 duplications by a paired-nick approach is a promising tool for applications in genome
227 engineering. Also, this methods appears to be particularly suitable for the manipulation of
228 promoter regions (Rodríguez-Leal et al., 2017; Wolter et al., 2019). Duplication of
229 transcription factor binding sites could help to enhance gene expression for crop
230 improvement.
231

232 **NHEJ-mediated inversions**

233 Large genomic changes play a substantial role in plant biodiversity. Especially
234 inversions are associated with environmental adaptation and niche specification (Schubert
235 and Vu, 2016). A consequence of large-scale inversions in different plant species is hybrid
236 sterility, centromere shifting as well as the formation of new open reading frames (ORFs), but
237 also disruption of already existing genes, resulting in alteration of expression profiles, and, in
238 some cases, the formation or breakage of genetic linkages. The most prominent inversion of
239 *A. thaliana* is the heterochromatic knob hk4S inversion on the short arm of chromosome IV
240 (Fransz et al., 2016). This inversion with a size of 1.17 Mb is found in the Columbia
241 accession, but not in the Landsberg accession and is associated with a pericentromeric shift.
242 To demonstrate the feasibility of inversions for chromosome engineering purposes, Schmidt
243 *et al.* established a CRISPR/Cas9-based system to generate targeted heritable inversions
244 (Schmidt, Pacher, Puchta, 2019). In a proof-of-concept experiment, the induction of two
245 DSBs, 3 kb apart, within a single chromosome was tested, resulting in up to 7% deletions
246 and up to 2% inversions. Molecular analysis of the newly formed junctions of the inverted
247 sequences revealed that most of the inversions were devoid of deletions or other mutations,
248 indicating that cNHEJ plays a key role. To verify this finding, the same approach was
249 performed in the DNA repair mutant *ku70*. Surprisingly, digital droplet (dd)PCR showed a
250 twofold increase in the formation of inversions at the two tested loci, indicating that the
251 KU70/KU80 heterodimer is also required for tethering the broken ends during the repair
252 process. Consistently, in animals, a single XRCC4-like factor (XLF) dimer recruited by the
253 KU70/KU80 heterodimer has been shown to promote tight alignment of DNA ends. A
254 mutation of the KU binding site for XLF affected end-joining efficiency and accuracy (Graham
255 et al., 2018; Nemoz et al., 2018). In the absence of KU70, the chance of a ligation of the
256 unlinked broken ends is increased, resulting in more inversions. However, this improvement
257 comes with a price: due to the lack of protection of the broken DNA ends by the KU70/KU80
258 heterodimer, the majority of inversions contained deletions within the newly formed junctions.
259 To obtain heritable inversions, a strategy was used that was first developed to obtain
260 rare gene targeting events in Arabidopsis. Here, the use of the egg cell-specific EGG
261 CELL1.1 (EC1.1) promoter, fused to the EC1.2 enhancer for tissue-specific expression of the

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262 Cas9 nuclease, resulted in a three-fold increase in gene targeting frequency and, thus,
263 heritable gene targeting events (Wolter et al., 2018). Indeed, replacement of the constitutive
264 promotor with the egg cell-specific promotor restricting the expression of the Cas9-nuclease
265 to the early stage of plant development, allowed more efficient and heritable induction of
266 inversions in wild type plants. In the experimental setup, inversions of defined sequences
267 comprising up to 18 kb were induced at two different loci. Thus, inversion events were
268 detected in up to 10 % of the tested progeny of individual T1 plants. A total of 25 plants with
269 a fully inverted sequence were identified. Sequencing of six junctions indicated error-free
270 repair of the break sites. Hence, inversions can now be induced precisely and more
271 efficiently in wild-type plants. (Schmidt, Pacher, Puchta, 2019). Apart from the replacement of
272 the Cas-driving promotor, prior testing of the nuclease cutting efficiency on the target
273 sequence turned out to be of great importance for the success of the approach.

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

286 Recently, a 75.5 Mb pericentric inversion on chromosome II has been inverted in a
287 maize inbred line by scientists of Corteva Agriscience. Using pre-assembled gRNA and
288 ribonucleoprotein (RNP) complexes, DSBs flanking the large inversion were induced in 2000
289 maize embryos (Schwartz et al., 2020). After selection and analysis, two T0 plants showed a
290 full 75.5 Mb-long pericentric reinversion on chromosome II. This is a major advance as it
291 shows that chromosomal rearrangements can also be induced in crop plants with more
292 complex genomes.
293

294 **NHEJ-mediated translocation**

295 While chromosome translocations in mammals are often associated with the
296 occurrence of various genetic diseases and cancer (Bunting and Nussenzweig, 2013;
297 Rowley, 2001), in plants these types of genome rearrangements are important for trait
298 diversity, speciation and evolution (Gabur et al., 2019; Lysak et al., 2006; Schmidt,

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299 Schindele, Puchta, 2019). Since stabilizing of trait linkages or breaking linkage drags is
300 essential for crop optimization, chromosome engineering has a huge potential for breeding.
301 Based on first evidence that simultaneous DSB induction on heterologous chromosomes
302 may lead to reciprocal translocations (Pacher et al., 2007), Beying *et al.* induced DSBs in
303 intergenic regions on the long arm of chromosome I and II of Arabidopsis, using the Cas9
304 nuclease (Beying et al., 2020). Here, a reciprocal translocation of both 0.5 Mb chromosome
305 ends could be detected in 0.01% of the samples via ddPCR. To determine which repair
306 pathway was used to form the translocations, NGS analysis was performed and revealed
307 error-free ligation in 60%, while the remaining samples often showed small deletions at the
308 junction. This suggests that cNHEJ is the main pathway for forming the chromosomal
309 translocation. Furthermore, a knockout of KU70 resulted in a five times higher occurrence of
310 translocations, demonstrating that cNHEJ suppresses the joining of unlinked DSB ends, as
311 has been shown for inversions before.

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

329

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

331 For an unbiased evaluation of the potential of NHEJ in chromosome engineering, one
332 has to view results in relation to what has been achieved using HR-based approaches.
333 Indeed, most heritable genetic changes are based on the repair of DSBs by HR which occurs
334 in a temporally controlled manner in meiotic cells leading to an exchange of parental genetic
335 material between two homologous chromosomes. The non-reciprocal transfer of genetic

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336 information leads to a non-crossover product (NCO), while the reciprocal exchange of
337 homologous fragments leads to allelic shuffling and is referred to as crossovers (CO)
338 (Mercier et al., 2015). For the initiation of meiotic recombination, a programmed DSB is
339 induced by the highly conserved SPORULATION11 (SPO11) topoisomerase-like protein
340 (Bergerat et al., 1997). After processing of the DSB, the arising 3' single-stranded overhang
341 can invade in the double helix of the paired homologue and form a displacement loop (D-
342 loop). If the invading strand of the D-loop is elongated via synthesis-dependent strand
343 annealing (SDSA), the structure can be resolved and the break can be repaired using the
344 elongated 3' single-strand overhang. SDSA-based repair results in NCO products.
345 Alternatively, the D-loop can be transformed into a double holiday junction (dHJ), extending
346 the D-loop so that the invading strand can anneal to the remaining DSB end (Beying et al.,
347 2021). Depending on the resolution of this repair intermediate, both CO and NCO products
348 can arise. These CO events can generate new allelic combinations. Thus, two favorable
349 traits might be combined or an unfavorable one might be eliminated from an elite cultivar
350 (Figure 4A). Although genetic exchange is highly desired for breeding, CO events are rare
351 and limited to the euchromatic parts of chromosomes which often hinders the segregation of
352 linked favorable and unfavorable traits, especially if coded in between a short distance on the
353 same chromosome.

354 A comprehensive study in yeast (*Saccharomyces cerevisiae*) reported targeted COs
355 by fusion of the natural meiotic DSB inductor SPO11 to DNA-binding domains, such as zinc
356 fingers (ZFs), transcription activator-like effector (TALE) modules and the CRISPR/Cas9
357 system, showing that overcoming this limitation is possible. Depending on the DNA
358 recognition domain and the targeted sequences, an increased CO frequency and a SPO11-
359 mediated DSB induction could be detected. However, the effect was quite small and
360 restricted to euchromatic regions (Sarno et al., 2017). Recently published data suggest that
361 recruitment of the natural DSB-inducing machinery is not sufficient to affect CO induction in
362 plants using a similar approach (Yelina et al., 2021). Here, the SPO11 complex partner,
363 meiotic topoisomerase VIB (MTOPVIB), which is essential for SPO11-mediated DSB
364 induction, was fused with a deadCas9 and guided to CO-accessible regions in *A. thaliana*.
365 However, no improvement in CO frequency or distribution was obtained.

366 In a pioneering study, recombination between homologous chromosomes in somatic
367 cells could be demonstrated after targeted DSB induction by Cas9 in tomato (Filler Hayut et
368 al., 2017). The experimental setup is based on two genetically distinct tomato accessions
369 which carry different mutations in the PHYTOENE SYNTHASE (*PSY1*) gene. Using Cas9, a
370 DSB between these mutations was induced, followed by a fruit color assay and single-
371 nucleotide polymorphisms (SNPs) sequencing to analyze genomic reshuffling events in
372 hybrid plants. The analysis revealed somatic HR events, including gene conversions and one

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373 putative crossover which unfortunately was not transmitted through the germline.
374 Nevertheless, this demonstrates that targeted somatic HR can be used for precise
375 chromosomal rearrangements. Recently, extended to another tomato locus, called
376 CAROTENOID ISOMERASE (*CRTISO*), the occurrence of two targeted COs was detected
377 through whole genome sequencing and it was confirmed that these COs can be transmitted
378 through the germline (Ben Shlush et al., 2020). Furthermore, a recent study demonstrated
379 targeted recombination in somatic maize cells. In two independent approaches, Kouranov *et*
380 *al.* induced DSBs in chromosome III of both parental homologues in F1 hybrid maize using
381 the LbCas12a nuclease (Kouranov et al., 2021). Genotypic analyses were able to identify
382 targeted CO events, where in one case the respective junction contained a deletion and in
383 the other no mutation. Therefore, cNHEJ as well as HR might be responsible for somatic CO
384 formation. Furthermore, is was shown that these targeted COs can be inherited (Kouranov et
385 al., 2021). These studies show that, despite their low efficiency, there is potential for
386 CRISPR/Cas applications in CO induction to improve biodiversity in commercial crops.
387 Another approach to influence trait heritage is implemented by the gene drive concept
388 (Figure 4B). The selective inheritance of target genes from only one parent was established
389 first in insects and mice and is used to convert heterozygous traits into homozygous traits
390 (Grunwald et al., 2019; Kyrou et al., 2018). A study by Zhang *et al.* demonstrated the
391 establishment of a gene drive system in *A. thaliana* (Zhang et al., 2021). This system is
392 based on the prior integration of a gene drive cassette into the CRYPTOCHROME 1 (*CRY1*)
393 gene via HR, resulting in *cry1* drive lines. The gene drive cassette consisted of a Cas9
394 coding sequence and a gRNA for DSB induction in the natural *CRY1* locus. After crossing
395 the homozygous *cry1* lines with wild type plants, heterozygous F1 progeny were generated in
396 which expression of the gene drive cassette resulted in targeted DSB induction in the wild-
397 type *CRY1* locus. Repairing this break via the HR-based mechanism, the gene drive
398 cassette-bearing *cry* locus can be used as a template. This leads to a conversion of the wild-
399 type *CRY1* locus to the *cry* locus and thus to a transfer of the gene drive cassette. As a
400 result, homozygous *cry1* loci could be detected in up to 8% of the F1 plants. Additionally, in
401 another approach, a non-autonomous trans-acting gene drive was performed, whereby the
402 gene drive unit and the target locus were located on different chromosomes. Here, gene
403 drive-based conversion of a heterozygous to a homozygous locus could be identified in
404 1.25% of the analyzed F1 plants. To improve efficiency of gene drive in plants, the use of
405 transformation boosters that enhance HR efficiencies in somatic cells, as it has been shown
406 for gene targeting in maize (Peterson et al., 2021), might be an option.
407 Thus, despite various attempts, HR-based chromosome engineering is currently, in
408 contrast to yeast (Sarno et al., 2017), not a feasible technology for plants.
409

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410 **Conclusion**

411 Taken together, all these results demonstrate a key role of NHEJ not only in efficient
412 mutation induction but also for various kinds of chromosome engineering. Thus, deletions,
413 inversions and duplications within a chromosome and also translocations between
414 chromosomes are achievable. Interestingly, the knockout of one or the other pathway might
415 have decisive consequences on the efficiency as well as product classes. While cNHEJ
416 suppresses all kinds of chromosomal restructuring in which previously unlinked DSBs are
417 joined, it is essential for SSB-induced formation of tandem duplications. In contrast, aNHEJ,
418 a backup mechanism for joining of any DSB ends in a more complex way, is also involved in
419 the formation of patch insertions. By manipulating these pathways, the occurrence of specific
420 product classes might be enhanced as shown for the knockout of cNHEJ in HR gene
421 targeting (Endo et al., 2016; Qi et al., 2013). As a new level of CRISPR/Cas applications has
422 been achieved in the case of plants (Lee and Wang, 2020), exciting novel question arise
423 (see Outstanding Questions) (Rönspies et al., 2021): Are we going to be able to change the
424 number of chromosomes by fusion or fission? Can we reconstruct genome evolution? Can
425 we create novel plant species by making individuals genetically incompatible by genome
426 restructuring? Only the future will tell how fast we will be able to answer these questions and
427 how far we can go, but eight years after the start of the CRISPR/Cas revolution we have
428 already seen a number of dreams materializing.
429

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430

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.

431

432

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?

433

434

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439

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440 ## Figure Legends:

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

451

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

457

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

466

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

477

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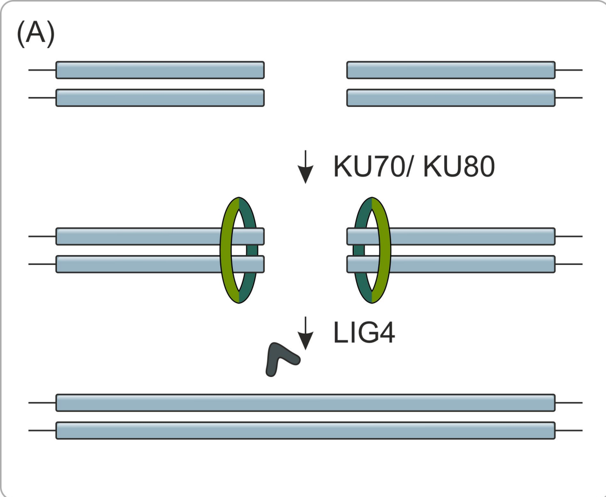

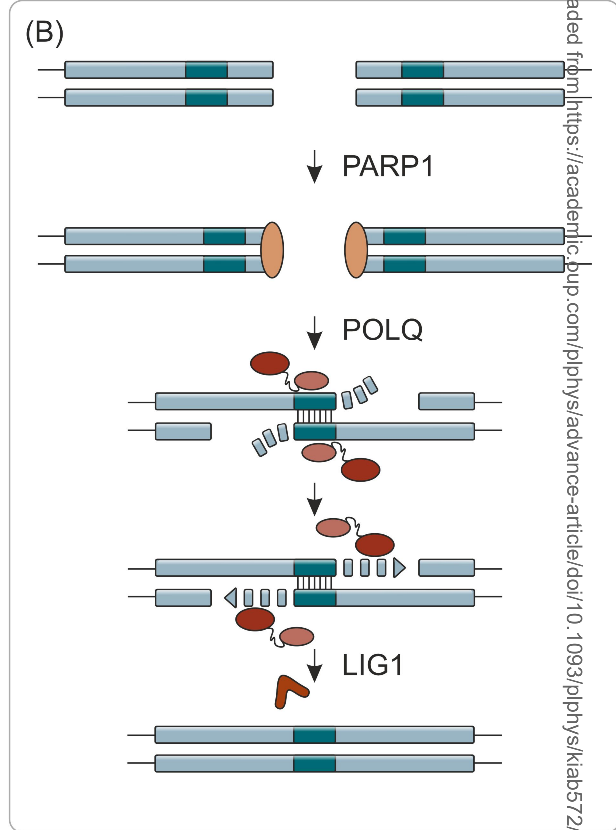

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Figure 1: DSB repair via NHEJ.

A DSB can be repaired via cNHEJ (A) or the aNHEJ (B). In cNHEJ-mediated repair, the broken ends are bound by the KU70/KU80 heterodimer and re-ligated by LIG4. 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, initiating the 5' to 3' resection of the ends. The annealing of the exposed microhomologies takes place, stabilized by the polymerase POLQ, 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.

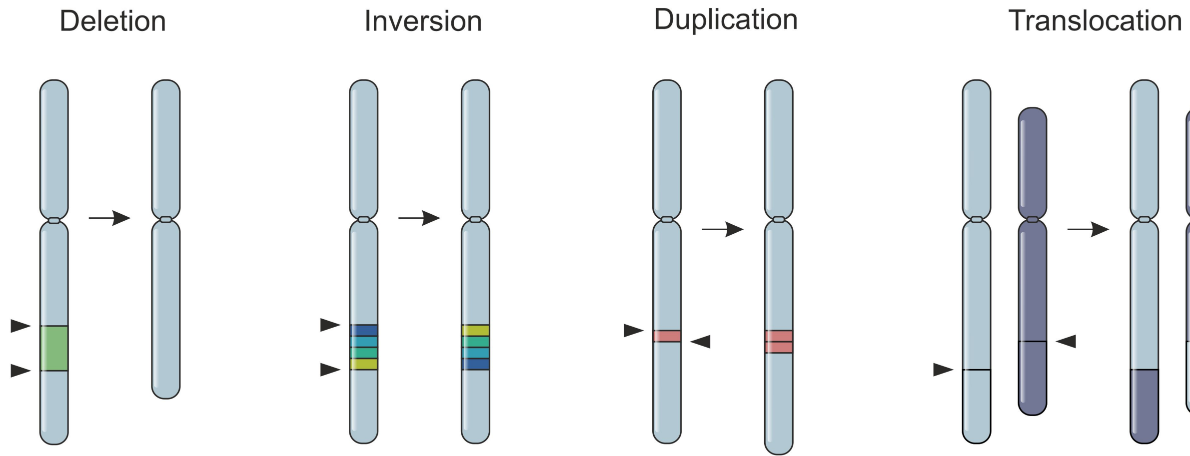

Figure 2: Possible chromosomal rearrangements after targeted break induction.

If two DSBs 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 non-homologous chromosomes can result in a translocation by exchanging the ends of the chromosomes.

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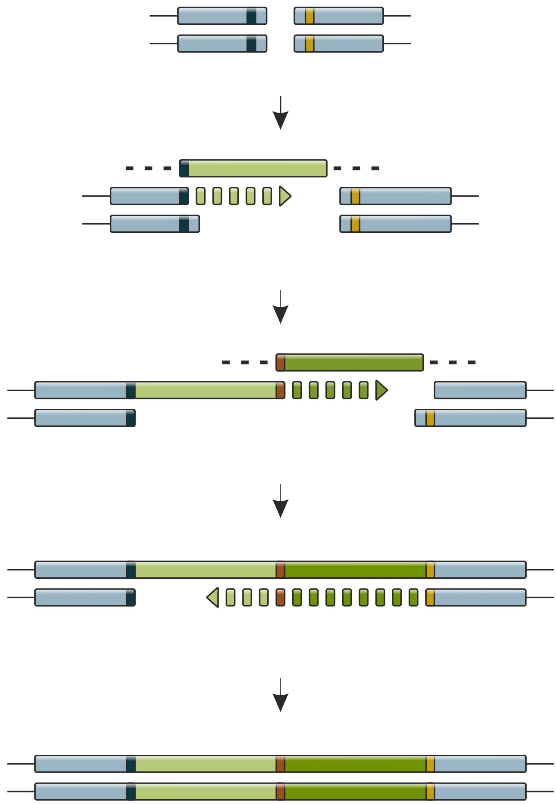

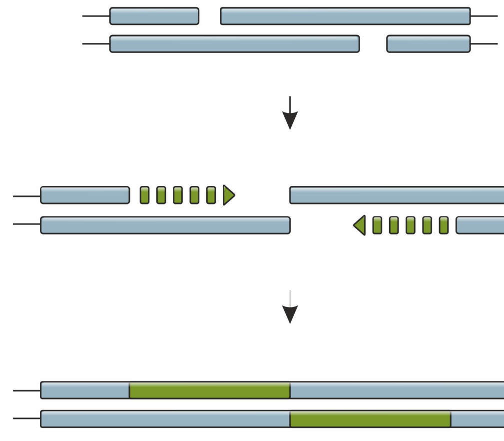

Figure 3: Possible mechanisms for the formation of insertions.

(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 may allow hybridization with distant sequences 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.

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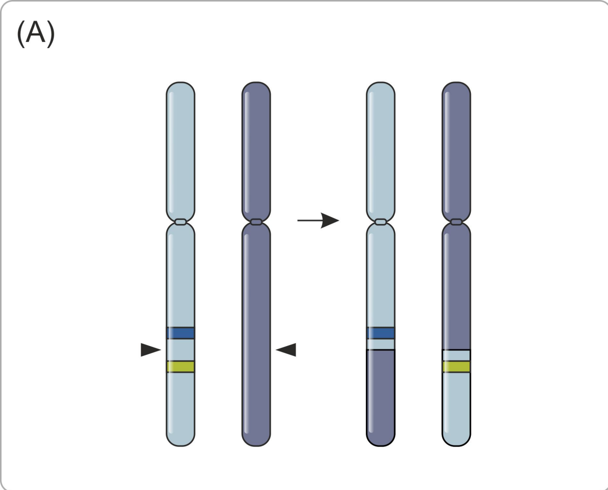

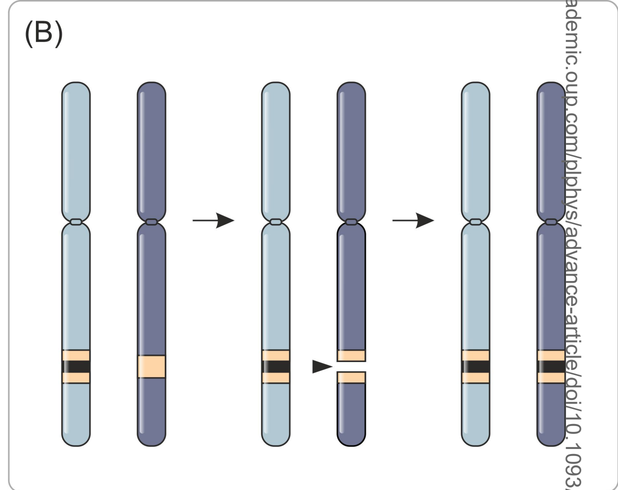

Figure 4: HR-based applications for plant chromosome engineering.

(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 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.

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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.

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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?

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