Review Article

Approaches of Chromosomal Engineering ~~through~~ including CRISPR–Cas Technology: A way forward

ABSTRACT

The breeding of crops is dependent on the potential to ~~disrupt~~interrupt or maintain genetic links between characteristics, and the availability of genetic variability. CRISPR ~~and~~ Cas, is a ~~two new~~ genome-editing techniques, have made it possible for breeders to introduce regulated and site-specific genetic diversity while simultaneously improving qualities with high efficacy. The existence of genomic linkage, ~~on the other hand,~~ is a ~~serious~~ barrier in transferring desirable features among domesticated species from their wild counterparts. Creating variants ~~that have defects inside~~within the meiotic recombination mechanism homologous chromosomes which results in increased global crossover rates among homologous parental chromosomes that are ~~homologous~~, is one approach to dealing with this problem. While this initially seemed to be a viable technique, ~~no~~ but crossover rates have been improved in recombination-cold areas of the genetic structure ~~thus far~~is difficult. Consequently, attempts have been made to induce site-specific DSBs in both somatic and meiotic plant cells by utilizing CRISPR–Cas techniques to achieve preset crossovers among homologs. Nonetheless, this method has not yielded significant heritable homologous crossings which were recombination-based. Lately, CRISPR–Cas has been used to achieve hereditary chromosomal rearrangements (CRs), including translocations and inversions, in plants. This method allows for the development of megabase CRs by DSB repair through non-homologous end-joining after insertion of DSBs in somatic plant cells. This technique may potentially make it possible to restructure genomes on a more global scale, culminating in the creation not just of synthetic plant chromosomes, but also that of new plant species.

Keywords: CRISPR-Cas; genetic linkage; crossover induction; chromosome engineering; reciprocal translocations; synthetic chromosomes; CRISPR-associated protein

1. INTRODUCTION

The world's population continues to grow at an exponential rate and it is becoming increasingly difficult to meet future food demands due to restrictions in food production resulting from limited farmland availability or unpredictable yields. The progression of innovative and rapid methods to enhance prevailing crops is therefore critical to fulfilling upcoming food requirements. Such enhancements include greater productivity, improved nutritional content, and better resistance to both biological and environmental stresses [1]. When using traditional breeding strategies, this procedure may need years to complete because of various factors, for example, the linkage-drag, i.e., linked inheritance of unfavorable genetic

material coupled with desirable traits, or diminished genetic variation owing to the cultivation approach [2]. ~~A case of~~An example of linkage-drag in plants is the connection ~~–~~of TMV tolerance to ~~–TMV with~~ lower tobacco production [3,4]. A major shift in genetic enhancement has begun with the introduction of "clustered regularly interspaced short palindromic repeats–CRISPR-associated protein" (CRISPR–Cas) systems as a gene-editing tool [5–9]. This technology uses an endonuclease that can able to cause double-strand breaks (DSBs) practically anywhere in the genetic structure, and a synthetic sgRNA to direct the endonuclease towards its intended cut site [10]. DNA repair systems, including "homology-directed repair" (HDR) and "non-homologous end-joining" (NHEJ), are used to repair the DSBs induced by the endonuclease [11]. Although the NHEJ mechanism, which is prevalent in somatic plant cells, is prone to errors and, has been shown to result in minor deletions or insertions at the DSB site [11]. The NHEJ repairing may also result in CRs, including translocations, inversions, or deletions, when multiple DSBs are triggered concurrently [12,13]. Rearrangements of chromosomes that occur naturally in mammals are mostly linked to cancer and other genetic diseases [14–16]. Cancer may be caused by these rearrangements that alter the function of a tumor suppressor gene or trigger an oncogene [17]. Hemophilia A, EDMD, and Hunter syndrome are a few of the conditions that may be induced by CRs [18–21]. CRs, on the other hand, play an essential role in speciation and adaptation [22], particularly in crops. For example, heterozygous rearrangements may result in the creation of imbalanced gametes during the process of meiotic recombination, which can induce reproductive isolation or impair fertility [22,23]. These may also adversely affect gene expression regulation across the entire genome [24]. As the activity of meiotic recombination is hindered inside the rearranged region in heterozygotes, the genomic information stored in chromosomal translocations and inversions cannot be used for gene shuffles [25,26]. Thus, breeding operations can become complicated with the suppression of genetic exchange among two varieties with the occurrence of these arrangements. CRISPR–Cas has so far been used to make relatively minor genetic modifications in plants. Nevertheless, in numerous modern experiments in both *Zea mays* and *Arabidopsis thaliana*, the introduction of megabase-scale CRs has been reported [27–29]. In this study, we discuss chromosomal engineering, for the breeding of plants, as a unique technique for stabilizing or breaking genetic markers. Later on, we also explore the possibility for alternate strategies of generating recombination across homologous chromosomes.

2. MANIPULATING DNA REPAIR PATHWAYS TO BREAK GENETIC LINKAGES IN PLANT BREEDING

Allelic combinations in plants are generated by crossovers or the mutual interchange of genetic information, among homologous chromosomes during meiosis. These Crossovers help for the combination of beneficial traits in elite crops while also allowing for the elimination of detrimental traits. Nonetheless, the majority of chromosomes do not exchange genetic information because distribution and crossover rates are extremely restricted and unregulated in natural processes [30]. As a result, the number of suitable recombination events is quite restricted, with linkage drag being mostly unavoidable. During meiotic division, the parental genomic sets are split throughout two successive nuclear divisions, with no interstitial replication, resulting in the generation of haploid reproductive cells [31]. Crossovers are required for the precise homologous chromosome segregations during the initial meiotic division because they allow the physical linkage of the coupled homologous chromosomes, known as bivalents [32]. This is guaranteed in all living things by the preservation of one mandatory crossover per chromosomal pair, also considered as the smallest crossover number required for recombination events

to take place. Despite the fact that meiotic recombination is the primary source of genetic variation in plant progeny, crossover counts hardly surpass three per bivalent.

The production of DSBs by SPO11 homologs, which are highly conserved, sets the foundation for meiotic recombination [33]. A crossover's likelihood depends on the details of the succeeding meiotic DSB repair processes [31]. To facilitate the establishment of the D-loop, i.e., initial repair phase leading to the formation of the crossover, DSB terminals are removed and strand infiltration inside homologous sequences takes place. For the development of non-crossovers in which the invading strand gets lengthened, SDSA is an essential process. The D-loop is subsequently discarded and the DSB is spanned for repair assembly. Alternatively, in a second-end capture, the D-loop may be lengthened such that the rejected strand can anneal with the contrary side of the DSB. The creation of the second key intermediate, the double Holliday junction, is enabled as a result. This is the only process in crossover production that uses endonucleolytic cleavage to disintegrate the double Holliday junction. According to research, there are a variety of conditions in crops that might cause the balance of crossover origination to move in different directions. It seems that using this information by overexpressing crossover-promoting or crossover-limiting elements is a potential strategy for boosting genetic diversity in crops.

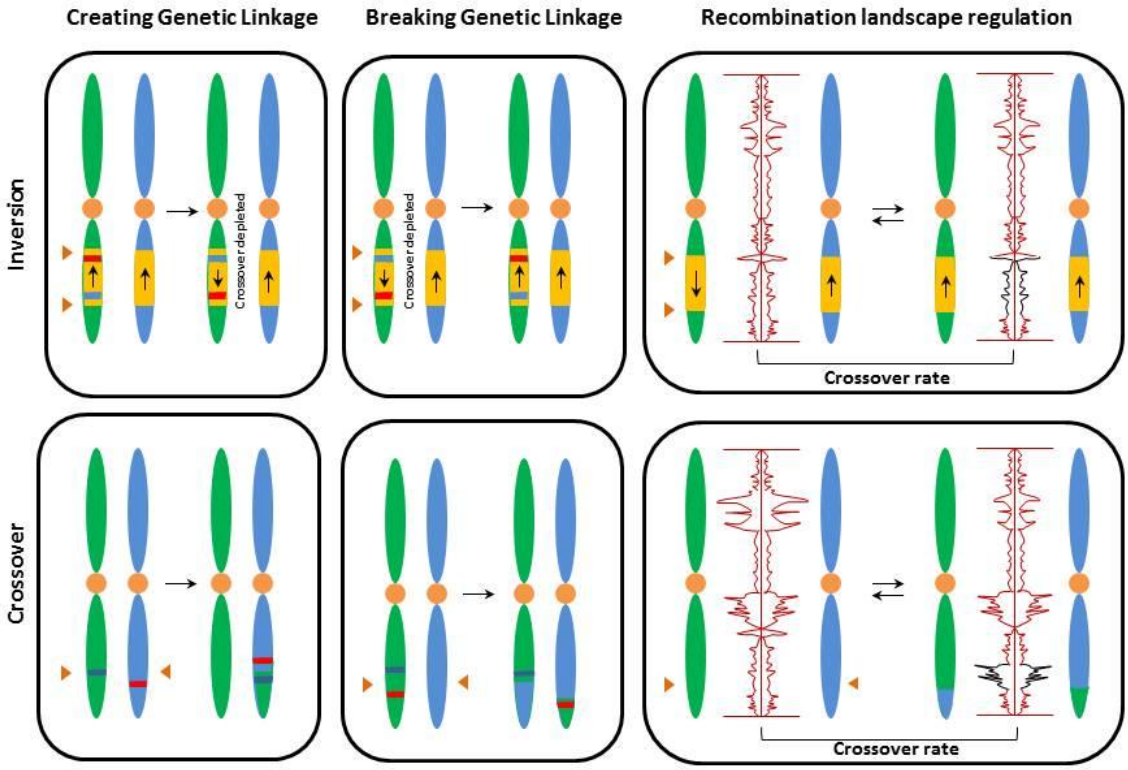

Fig. 1. Control of genetic exchange by targeted induction of crossovers and inversions

The protein-coding gene FANCM was the first factor discovered that limited crossover in plants. When *fancm* mutations were introduced into *A. thaliana*, its crossover frequency tripled [34]. The enzyme BLM homolog RECQ4 has been identified as another crossover inhibiting factor. *Arabidopsis* has two homologs, RECQ4A and RECQ4B, that are closely linked to each other [35,36]. The crossover frequency of the *recq4a recq4b* double mutant has been shown to increase by 6.2 times [36]. The SDSA-promoting destabilization of D-loops is facilitated by the actions of FANCM and RECQ4 [37,38]. As a result, it was hypothesized that the combined mutation of these enzymes represented an almost total lack of SDSA [39]. These factors suggest that every DSB results in a double Holiday junction-like structure that is resolved by resolvases, with crossovers and NCOs prevailing in equal amounts. Other elements also play a role in the regulation of crossover control in a plant system. The unique AAA-ATPase FIGL-1, in conjunction with its partner FLIP, is essential for the regulation of recombinases that initiates the strand invasion [40,41]. It has been shown that mutations taking place in *Arabidopsis figl1* enhance the number of crossovers in both hybrid and inbred lines. When utilizing these mutations in crops, the drawback is their sterility, as has been shown in tomato, pea, and rice mutants. This leads to a breeding dead end which is undesirable [42,43]. As an alternative to utilizing anti-crossover factor mutations, it is possible to overexpress crossover-promoting factors, for instance, the E3 ligase HEI10 [44]. *HEI10* artificial overexpression in *Arabidopsis* results in a spike in crossover frequencies, particularly in the domain of subtelomeric euchromatin [45]. The reported crossover increase, however, was again confined to the chromosomal arms when the results were paired with a *recq4a recq4b* mutant background, with the greatest impact found in subtelomeres and no effect observed in centromeres [46]. Surprisingly, the rice synaptonemal complex protein ZEP1 had a comparable effect on the number of crossings as HEI10 overexpression, suggesting that other factors may be at play [47]. As numerous mechanisms have an impact on crossover abundance, combining several approaches has the potential to further increase crossover frequency. As an example, the combined mutation of *recq4a recq4b* and *figl1*, *or recq4a recq4b* and *fancm*, in *Arabidopsis* inbred strains led to a tenfold rise in crossovers. Triple mutants of the *recq4a recq4b figl1* gene showed an increase of 7.8 times. Since a quadruple mutant with *fancm* showed no further increase, a threshold was hypothesized to have been achieved [48]. RECQ4 homologs were shown to have a major role in the frequency of crossovers in a 2018 research, demonstrating that these results might be applied to a variety of crops. Tomato, pea, and rice hybrids that had RECQ4 homologs inactivated had crossover frequencies that were increased by 2.7-, 4.7-, and 3.2-fold, respectively, indicating the possibility of employing mutants to speed up breeding procedures [43]. While modifying crossover routes to enhance meiotic recombination is highly promising, care should be used when modulating DNA repair components as the most effective technique for modifying *RECQ4* homologs is not without its drawbacks. RECQ4, a component of the RTR (RecQ/Top3/Rmi1) complex, not only plays a critical role in crossover regulation but also maintains somatic genome integrity [35,49]. Such as, in *A. thaliana*, when utilizing recq4 mutants, it is possible that secondary mutations may accumulate and that the overall fitness of the plant would decline [50,51]. Similar findings were made in the case of FANCM, which plays a critical role in the preservation of genomic integrity in crops [52]. Finally, the methods for increasing crossover frequencies that have been presented have only been effective in increasing crossover frequency and had no effect over crossover positioning, resulting in recombination-depleted regions like the pericentromeric regions, unaffected by these changes.

3. CRISPR–CAS-MEDIATED CROSSOVER ~~INDUCTION~~

Meiotic mutants, as described above, only increase crossover frequencies, but do not alter the distribution of crossovers which results in, a large portion of the genome that cannot be triggered for recombination [30]. Therefore, a simple technique that can target these areas directly during meiosis to induce DSBs and promote homologous recombination (HR) should be adopted [53]. In theory, two separate techniques can be employed to accomplish this objective: either utilizing a programmable DNA nuclease for DSB creation or utilizing its DNA-binding capabilities to lead the innate DSB-inducing mechanism to the desired target position in the genome.

SPO11, a naturally occurring regulator of meiotic DSB induction, was first revealed to be adaptable to genome-editing tools in a comprehensive yeast research by employing ZFNs, TALENs, and the CRISPR–Cas network as DNA binding proteins [54]. A considerable rise was seen in crossover frequency when these SPO11 fusions were directed to regions where DSB induction is normally low during meiosis. Nonetheless, natural constraints on the SPO11-mediated DSB induction mechanism were evident in parts of the genome where DSBs are inhibited during meiosis (e.g., the centromeric and pericentromeric regions). Recently, the same strategy was applied in A. thaliana [55] by fusing the SPO11 complex partner MTOPVIB, which is essential for SPO11-mediated DSB induction during meiosis, to a dead Cas9 and then guiding it to a crossover hotspot previously demonstrated to be accessible for crossover regulation. Similarly, the production of DSBs in yeast had been accomplished by directing SPO11 to hotspots. As a result, a rise in the crossover rate was generally anticipated. However, there was no discernible effect on crossover frequency or distribution from dCas9–MTOPVIB. Following these findings, it is possible that only recruiting endogenous DSB-inducing machinery will not be adequate to influence crossover incidence in plants.

The direct generation of meiotic site-specific DSBs has not yet been reported, however, Cas9-generated DSBs have effectively triggered recombination among homologous chromosomes in tomato somatic cells [56]. To induce an allele-specific DSB in the *PHYTOENE SYNTHASE 1* gene in hybrid plants, two genetically unique tomato accessions were used. The use of a fruit-color test allowed the researchers to distinguish between NHEJ and homologous recombination repair results. Additionally, somatic HR events were also discovered in the study of SNP redistribution data. Gene conversions and a possible crossover were among the results of the study. This work showed that CRISPR–Cas production of targeted DSBs may be used to control HR in plants, a previously unknown possibility.

Recently, a similar strategy was undertaken by the same researchers, who used hybrids of genetically diverse tomato accessions—which had heterozygous mutations in the *CAROTENOID ISOMERASE* gene—to serve as a selection system to identify HR-mediated somatic recombination [57]. According to the findings of this research, one crossover incidence with at least 1Mbp transitions in the two directions was reported.

Given the low frequency of HR in higher eukaryotic organisms, recombination across homologous chromosomes through NHEJ may be preferred to recombination facilitated by HR. For example, if the homologous chromosomes are subjected to DSB induction at the same time, NHEJ may result in the exchange of genetic material. HR has recently been shown in human cells by end-joining and targeting the *CD44* gene [58]. By using DSB induction and compound heterozygous mutations in the interval across these mutations, it was possible to identify future reciprocal recombination by the straightforward revival of genomic function. There was reciprocal recombination for both double-stranded breaks

and nicks when they were directed towards each homologous chromosome. There was a ten time increase in the frequency of recombination when DSBs were induced compared to nicks, reaching frequencies of roughly 0.1%. An interesting finding was the failure to induce recombination when double-strand breaks and nicks were steered to a single homologous chromosome. This suggests that Non-homologous end joining is preferred to homologous recombination when it comes to the focused recombination stimulation in higher eukaryotic organisms.

4. CRISPR–CAS-MEDIATED CHROMOSOME ENGINEERING IN YEAST AND MAMMALS

Among the first approaches for designing ways to disrupt genetic linkages is the global enhancement or site-specific induction of crossovers, as stated above. The CRISPR–Cas technology, on the other hand, might be utilized to reorganize and reorder chromosomes by the production of massive CRs. Using this method, significant progress has been achieved in various organisms, including mammals and yeast, and it may be possible to draw useful lessons from this experience for the use of identical strategies in crops.

For instance, in animal cells, Using the CRISPR–Cas approach, researchers have been able to mimic oncogenic CRs to understand cancer onset [59,60]. CRISPR–Cas has been used successfully by a number of researchers to reproduce chromosomal abnormalities in animal cells [60–62]. Several translocations, such as the t(11;22) and t(8;21), which induce acute myeloid leukemia and Ewing's sarcoma, respectively, have been successfully replicated [61]. A translocation on human chromosome 2 is another instance of a cancerous CR, which results in the creation of a defective fusion protein owing to the alignment of the two genes *ALK* and *EML4*. It is related to a specific form of cancer in the lungs and has been effectively induced in mice and human cells using the CRISPR–Cas system [63]. So far, the CRISPR–Cas system has been demonstrated to be useful for modifying animal chromosomes in multiple different ways.

CRISPR–Cas techniques for reshaping chromosomes and genomic characteristics in yeast have also made significant development over the past few years. In *Saccharomyces cerevisiae*, scientists were able to reduce its 16 chromosomes to just 1 and 2 in two separate experiments [64,65]. This was made possible by inducing a variety of CRISPR–Cas-mediated fusions and translocations. Since the wild type having 16 chromosomes could not be backcrossed, it was hypothesized that the strain having 2 chromosomes may be considered a separate species under the conventional biological species concept [64]. Moreover, cases of chromosomal shuffling in yeast have been documented as a result of the CRISPR–Cas-facilitated production of CRs [66, 67].

5. REVERSION OF NATURAL CHROMOSOMAL INVERSIONS CAN RESTORE GENETIC EXCHANGE IN RECOMBINATION-DEAD REGIONS IN PLANTS

The presence of CRs in a wide spectrum of crops has been shown [29,68–70]. It is possible to discover sequence variation across a crop plant by performing sequence assemblies on a chromosome-scale in order to capture the supragenome [68]. Greater than 5Mb-long inversion polymorphisms in genome arrangement of contemporary elite barley germplasm were found [68]. Thus, a significant amount of genomic material is now unavailable for breeding purposes, implying that an effective technology to

generate or negate evolutionary-derived CRs in a targeted way would be very beneficial to crop breeders. A few years ago, the use of Cas9 from a gram-positive bacterium Staphylococcus aureus allowed researchers to demonstrate that precise inversions of approximately 18 kb can be generated in Arabidopsis [71]. Following their initial findings, they went further another step and reverted an inversion based on the evolution of the popular heterochromatic knob on the short arm of chromosome 4 (hk4S), which would be carried by numerous accessions and has a 1.17Mb size [23,28,72]. In the 38 main transformants, the authors found 7 distinct inversion incidences. Crop plants may also be able to trigger or revert inversions in the Mb range, according to this study. Another goal of the researchers was to determine if the meiotic recombination process could be reintroduced in hybrids harboring the reversed knob in a heterozygous condition.

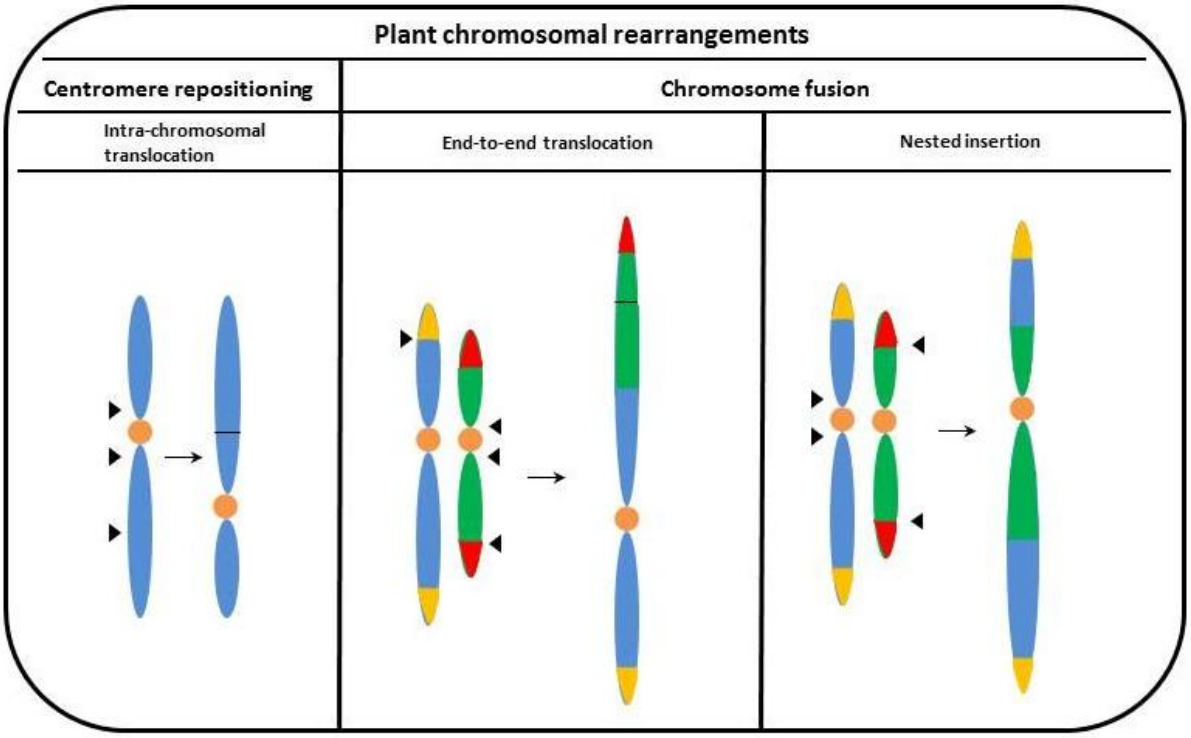

Fig. 2. Future perspective on chromosome engineering in plants

Previously, no evidence of gene flow between Columbia and Landsberg *erecta*, the former of which had a knob, has been found inside the rearranged region [73]. Schmidt et al. used a recombination assay based on SNP to see whether their theory was correct and discovered that hybrids' crossover rates could be restored [28]. This significant analysis implies that undoing evolutionary-derived CRs may liberate the genetic information contained inside the CR, allowing for meiotic crossover to occur. With regards to this work, the first crop plant inversion facilitated by CRISPR–Cas, in *Zea mays*, was recently procured [29]. This suggested that chromosomal engineering may be viable in other crops that are also sensitive to transformation similar to maize crops. In crop improvement, the restoration or production of meiotic chromosomal inversions may be utilized to replace crossovers in previously altered parts of the genome or to disrupt linkage groups by physical separation (Figure 1). However, inversions might also be

utilized to maintain genetic links between desirable characteristics (Figure 1). These artificial inversions prevent HR between chromosomes during meiosis.

6. INDUCTION OF RECIPROCAL TRANSLOCATIONS IN PLANTS

Large translocations, like inversions, are widely observed in crop plants and may result in a decrease in meiotic recombination [25,69]. Semi-sterility may be caused by reciprocal translocations in plants, which can also be linked to male and female sterility [74]. As a result, the controlled production of these translocations will almost certainly become a valuable tool for crop breeders in the process of maintaining or breaking maintaining genetic linkages. A linkage may be established and disrupted by joining desirable genes from non-identical chromosomes inside the same chromosome and by physical separation, respectively (Figure 2). Recently, in plants, the first directed mutual translocations have been successfully induced. Translocations between *A. thaliana* 1 and 2 as well as 1 and 5 chromosomes were successfully achieved by Beying et al. The translocated segments were about 1Mb and 0.5Mb in size. There were translocation rates of about 2.5% and 3.75% in the natural background and in the NHEJ variant ku70, respectively, in discrete T2 lines. This hard evidence research gives reason to be optimistic that the similar would be feasible in crops in the near future.

7. POTENTIALS OF RESTRUCTURING THE PLANT CHROMOSOME

It's intriguing to imagine what more could be conceivable in the future now that it's possible to rearrange the order of genes inside and across chromosomes. However, one should remember that chromosomal engineering in crop plants is still very much in its infancy despite recent advances. CRs are very rare compared to the development of mutations based on DSBs. In fact, a quantitative examination of mutual translocations found that only 1 out of 10,000 cells had translocation events, meaning that very few repair responses result in the formation of CRs [27]. Wild-type host proteins owned by the cNHEJ pathway preserve the matching damaged DNA ends in immediate contact after a DSB to ensure their re-ligation, hence preventing the development of CRs. Because of this, it may be advantageous to induce CRs within a cNHEJ-genetic background in certain cases. In *A. Thaliana*, translocation and inversion rates are both increased, the former by approximately to five times, when the Ku70 enzyme, a key participant in cNHEJ, is absent [27]. DSB repair is taken over by the secondary NHEJ pathway when Ku70 is absent. This NHEJ pathway has no affinity for retaining the damaged DNA ends together. Hence the usage of DSB repairing mutants may increase the likelihood of acquiring CRs. Nonetheless, the disadvantage of such a strategy is that a general repair defect may lead to additional, undesirable modifications in the genome. Furthermore, Enhancing CR frequencies may also be achieved by using sgRNAs and Cas nucleases that have been designed for effective DSB generation in the relevant plant species [75,76]. Moreover, detecting unusual CRs requires an effective screening protocol. A. thaliana has successfully used a mass screening methodology that was initially designed to find infrequent gene targeting occasions [77].

A variety of CRs have occurred throughout evolution and could perhaps set a standard for the types of genomic modifications that CRISPR–Cas-mediated chromosomal engineering may be able to achieve. CRs are common during polyploidization mechanisms that result in dysploid changes, which in turn lead to a diploid chromosome pair [78]. The decrease in the number of chromosomes should be conceivable,

either by end-to-end translocation or nested insertions, both of which have been recorded in natural CRs. Polyploids like potatoes and wheat might be suitable candidates for chromosomal editing techniques like these. The recent in-vivo observation of centromere relocation in maize following gamma irradiation further supports the theory that centromere repositioning arises regularly throughout plant genomic evolution [78,79]. It may be conceivable in the near future to imitate nature by altering centromere potions and chromosomal numbers in the same way that it has been feasible to cause translocations and inversions, which take place with great frequency upon an evolutionary level (Figure 2). Nevertheless, it is important to remember that if numerous DSBs are induced concurrently, additional unexpected genetic alterations may result [80]. Along with present techniques, this would enable fundamental issues to be answered about how gene expression and chromatin state are affected by different locations of a chromosome. Additionally, chromosomal engineering may enable the reconstruction of the chromosome sets of contemporary plant species' ancestors.

8. THE CREATION OF SYNTHETIC CHROMOSOMES AND NEW PLANT SPECIES

Since the turn of the millennium, a unique method of modifying genomes has surfaced, i.e., the fabrication of artificial chromosomes. Owing to their tiny size, the earliest manufactured chromosomes were viral in origin. Following this achievement, artificial bacterial chromosomes of several strains and artificial yeast chromosomes were constructed [81]. Although theoretically feasible, the introduction of completely synthesized chromosomes from other creatures into crop plants has been hampered by a number of technological challenges, such as limits throughout the transformation procedure. Conventional plant transformation techniques, including *Agrobacterium* and biolistics, can only transfer so much DNA at a time until they run out of efficiency. The ability of an *Agrobacterium* to transfer is reliant on the normal *E. coli* vector capacity [82] of around 150 kb. Biolistic transformation, on the other hand, is capable of transferring up to 1,050 kb, although it may cause substantial degradation to the host genome and transgene and is typically incapable of delivering intact molecules exceeding a few kilobases [83,84]. Furthermore, synthetic chromosomes require a certain size in order to accommodate at least a single crossover throughout meiosis. In doing so, we may avoid the early separation of sister chromatids, which might lead to deterioration and the removal of genetic information [82]. Given the existing limitations of constructing synthetic crop plant chromosomes from the beginning, the next phase in the synthesis of these synthetic chromosomes will most likely be accomplished using CRISPR–Cas-mediated chromosomal engineering, in conjunction with the insertion of shorter lengths of artificial DNA.

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