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Review

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Plant chromosome engineering – past, present and future

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Contents

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Summary

Spontaneous chromosomal rearrangements (CRs) play an essential role in speciation, genome evolution and crop domestication. To be able to use the potential of CRs for breeding, plant chromosome engineering was initiated by fragmenting chromosomes by X-ray irradiation. With the rise of the CRISPR/Cas system, it became possible to induce double-strand breaks (DSBs) in a highly efficient manner at will at any chromosomal position. This has enabled a completely new level of predesigned chromosome engineering. The genetic linkage between specific genes can be broken by inducing chromosomal translocations. Natural inversions, which suppress genetic exchange, can be reverted for breeding. In addition, various approaches for constructing minichromosomes by downsizing regular standard A or supernumerary B chromosomes, which could serve as future vectors in plant biotechnology, have been developed. Recently, a functional synthetic centromere could be constructed. Also, different ways of genome haploidization have been set up, some based on centromere manipulations. In the future, we expect to see even more complex rearrangements, which can be combined with previously developed engineering technologies such as recombinases. Chromosome engineering might help to redefine genetic linkage groups, change the number of chromosomes, stack beneficial genes on mini cargo chromosomes, or set up genetic isolation to avoid outcrossing.

New Phytologist

I. Introduction

Agriculture needs a substantial increase in productivity if the human population wants to retain its standard of living (Gerland *et al.*, 2014). One way to achieve higher yields is to enhance genetic variation for crop breeding. This can be achieved by globally enhancing mutation rates via genotoxic agents or, more recently, by site-specfic induction of mutations via programmable nucleases, such as the CRISPR/Cas system (Pacher & Puchta, 2017). However, beside mutating single genes to obtain more beneficial traits, the right combination of traits is central for a breeding success. As genes are organized in chromosomes, the development of cutting-edge genetic methods to engineer and restructure chromosomes is an important contribution to address this enormous challenge. Along with the emergence of new technologies, plant chromosome engineering has evolved over time. Plant chromosome engineering was initiated by Sears (1956), who first used X-ray irradiation to incorporate foreign chromosome fragments into the chromosomes of wheat for the transfer of new traits. This technique is still being used in pre-breeding today. With the aid of endonucleases, precise DNA sequence-based chromosomal engineering is now conceivable. There are additional tools for plant genetics that enable the creation of minichromosomes and chromosome manipulation to alter the mechanism of inheritance. In this review, we concentrate on contemporary methods for modifying plant chromosomes to meet the requirements of green biotechnology. Based on this, we review recent advancements in the use of modified plant chromosomes in practice and discuss additional steps needed to establish them in widespread use. Aspects of engineered apomixis and meiotic recombination in plants are not included in our overview because they were the subject of excellent recent reviews (Underwood & Mercier, 2022; Mahlandt *et al.*, 2023; Xiong *et al.*, 2023).

II. Engineering of plant minichromosomes

A minichromosome is a small-sized chromosome, possessing telomeres, replication origins and a centromere, but little additional genetic information. Engineered minichromosomes that are stable throughout meiosis and mitosis have the potential to be used as vectors in plant biotechnology to stack many (*trans*/*cis*) genes needed for complex traits. Segregating independently of host chromosomes, they provide a platform for accelerating plant breeding. Recent research has provided proof of concept for crucial steps in the engineering of minichromosomes, such as truncating of endogenous chromosomes and *de novo* formation of centromeres by tethering a CENH3 fusion protein to a designed repeat array (Yu *et al.*, 2006; Dawe *et al.*, 2023).

Starting with the native chromosomes of the host plant, 'top-down' chromosome engineering entails maintaining a functional centromere while also removing as many gene-containing chromosome arms as possible to prevent gene dosage imbalances and, consequently, make the minichromosomes as phenotypically neutral as possible. There are numerous ways to shrink existing chromosomes to produce chromosome-based vectors. The integration of cloned telomeric repeats truncates distal regions of chromosomes by generating new telomeres at the integration locations, also called 'telomere seeding', as first demonstrated by Farr *et al.* (1991). The laboratory of J. Birchler was the first to employ this elegant *in vivo* approach in plants (Yu *et al.*, 2006; Vega *et al.*, 2008), reviewed in Birchler & Swyers (2020) (Fig. 1a). *Arabidopsis*-type telomere repeats were used to downsize maize chromosomes. Using a Cre/Locus of crossing-over on phage P1 (LoxP)-based site-specific recombination mechanism that was built

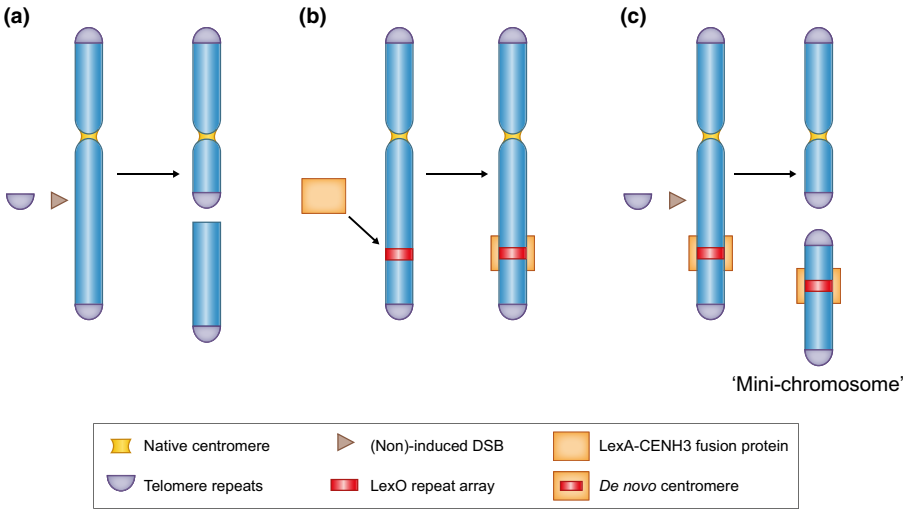

Fig. 1 Current and future strategies to engineer plant minichromosomes. (a) Truncation of an endogenous chromosome by telomere seeding. The site of chromosome truncation is random by default, but can potentially be targeted by the use of CRISPR/Cas9. The acentric fragment is lost in subsequent cell divisions. (b) *De novo* centromere formation via tethering of a LexA-CENH3 fusion protein to a chromosomal LexO repeat array. The presence of CENH3 at the ectopic site promotes the loading of additional CENH3 and other kinetochore proteins, creating a dicentric chromosome. (c) A combination of *de novo* centromere formation at LexO repeat array already integrated into a host chromosome with targeted telomere seeding proximal to the tandem repeats can release a prototype minichromosome. Alternatively, dicentric chromosomes can be broken during cell division.

into the transgenes, a subsequent reporter gene transfer from one constructed minichromosome to another was accomplished (Yu *et al.*, 2007b). Cre/LoxP is based on a recombinase enzyme isolated from the P1 bacteriophage that recombines DNA fragments (Sternberg & Hamilton, 1981). Besides using cloned telomere repeat arrays, another approach was successful in producing a minichromosome derived from a standard maize chromosome by combining free telomere repeats and the desired genes in a co-bombardment experiment (Gaeta *et al.*, 2013).

Apart from maize, minichromosomes were also created in *A*rabidopsis *thaliana* (Nelson *et al.*, 2011; Teo *et al.*, 2011), barley (Kapusi *et al.*, 2012), rice (Xu *et al.*, 2012), wheat (Yuan *et al.*, 2017) and rape seed (Yan *et al.*, 2017; Yin *et al.*, 2021) by telomere-mediated chromosomal truncation. The mechanism of truncation is not known in detail. The non-homologous end-joining (NHEJ) process, a natural repair mechanism for DNA lesions and used for transgene insertion, is also implicated in transgene-mediated telomere creation (reviewed in Birchler *et al.*, 2010). A step-wise natural increase of the telomere array length within few generations after telomere seeding might help to protect minichromosomes from deterioration. In *A. thaliana*, initial telomere lengths after transformation ranged from 1.0 to 2.3 kb in the first generation and were increased to 3–6 kb in the third plant generation (Teo *et al.*, 2011).

Supernumerary B chromosomes are the best targets for telomere seeding since they are added to the standard chromosome complement and non-essential by definition (reviewed in Houben *et al.*, 2014), which helps to prevent the adverse effects of chromosome truncation. In fact, numerous truncated B chromosome variants, which demonstrated significant sexual transmission and expression of inserted reporter genes, were obtained when B chromosome-containing maize was converted with a telomere seeding construct (reviewed in Birchler & Swyers, 2020). The fact that many B chromosomes show 'chromosome drive' (transmission is higher than 0.5), which promotes their preferred inheritance and, consequently, maintenance in the host plant population, is another potential advantage of starting with B chromosomes in chromosomal engineering (reviewed in Chen *et al.*, 2022). However, to take advantage of the B chromosome-specific drive, downsizing of the B chromosome should not result in the loss of chromosomal regions required for the drive process. On the contrary, the loss of drive might be viewed as an advantage because it could stabilize the transmission of truncated Bs. In fact, maize minichromosomes are drive-negative because a gene near the telomere of the B chromosome required for the drive has been lost (Masonbrink & Birchler, 2012).

Also, depending on the host species and the copy number of the B chromosomes, the B chromosome can express genes and have diverse effects on the expression of A chromosome-encoded genes (Boudichevskaia *et al.*, 2022; Shi *et al.*, 2022). Besides using B chromosome-based minichromosomes, the use of tetraploid or (telo)trisomic genotypes would minimize aneuploidy effects caused by the loss of genetic information due to chromosome downsizing (Teo *et al.*, 2011; Xu *et al.*, 2012).

The combination of the Cre/*LoxP* and the Activator (Ac)/Dissociation (Ds) transposable element (Ac/Ds) systems has successfully generated an *A. thaliana* ring minichromosome (Murata *et al.*, 2013). Minichromosome AtARC1 was derived from chromosome 2 and contained a 2.6 Mb pericentromeric region as well as a 250 kb centromeric repeat array. Surprisingly, *A. thaliana* ring minichromosomes were transmitted more stably than comparable linear minichromosomes. By contrast, in maize, the transmission of small ring chromosomes is impaired and results in chromosome instability (McClintock, 1932, 1938; Kaszas & Birchler, 1998).

As a by-product of haploid induction, a process resulting in *an organism* possessing only a single set of each homologous chromosome, using the CENH3-mediated genome elimination approach in *A. thaliana* (Tan *et al.*, 2015), 1–2% of phenotypically normal semi-haploids carried 3–10 Mb-sized linear or circular minichromosomes derived from (peri)centromeric regions of the haploid inducer genome. There were certain minichromosomes that were transmitted at a rate comparable to trisomic chromosomes. The laboratory of L. Comai successfully demonstrated the use of positive selection for a marker integrated in the (peri) centromeric region for the aim of preselection of stable minichromosomes (Tan *et al.*, 2023).

The minimum size for minichromosomes is unknown and likely species-specific; however, it may be determined by the specifications for appropriate sister chromatid cohesion or bivalent stability in meiosis (reviewed in Schubert, 2001). The meiotic transmission rate of engineered chromosomes is generally lower than that of endogenous chromosomes in plants and animals (reviewed in Irvine *et al.*, 2005; Birchler & Swyers, 2020). Therefore, the size of the minichromosomes must be balanced with enough mitotic and meiotic transmissibility for the creation of efficient minichromosome-based vectors (reviewed in Birchler & Han, 2013).

Other methods to cause random chromosome truncations include the use of gametocidal chromosomes (reviewed in Endo, 2007) and the breakage-fusion-bridge (BFB) cycles, which use dicentric chromosomes as intermediates (reviewed in Yu *et al.*, 2007a,b). For instance, the use of BFB cycles led to the generation of several minichromosomes from wheat chromosome 1B (Lukaszewski, 1997).

The main disadvantage of all hitherto performed plant chromosome truncation experiments is their non-targeted way of downsizing chromosomes. The targeted shortening of chromosomes will become an option in the future by using CRISPR/Cas for the targeted insertion of telomere repeats, provided that the sequencing data for the target chromosome is available. Alternatively, CRISPR/Cas-mediated large-scale deletions (Ordon *et al.*, 2017; Durr *et al.*, 2018) could be directly applied to chromosome downsizing in polyploids.

III. Engineering of plant centromeres

Being able to create centromeres in plants from scratch would be extremely helpful. In plants, centromeres cannot be produced by solely transforming centromeric sequences (Phan *et al.*, 2007; Liu *et al.*, 2023). In most eukaryotes, centromeres are epigenetically marked by the centromere-specific histone H3 variant (CENH3). To maintain a continuous CENH3 mark and, consequently,

centromere function, the presence of CENH3 before DNA replication acts as a signal for the loading of fresh CENH3 at the same site after DNA replication (reviewed in Talbert & Henikoff, 2020). In *Drosophila melanogaster*, *de novo* centromeres were produced at a predetermined position by artificially attaching a CENH3-fusion protein that had a sequence-specific DNA binding domain bearing the relevant target repeat sequence (Mendiburo *et al.*, 2011). In a comparable setup, the laboratory of I. Schubert harnessed the bacterial lactose repressor/lactose operator system to guide derivatives of CENH3 to LacO operator sequences in *A. thaliana* (Teo *et al.*, 2013). Using a LacO array-possessing *A. thaliana* line (Kato & Lam, 2001), tethering of the fluorescently labeled and nuclear-targeted fusion of the lac repressor with CENH3 (GFP-LacI-NLSCENH3) led to a *de novo* assembly of kinetochore proteins at LacO loci. Anaphase bridges, *a particular chromosome segregation error* that happens when chromosomes with two functioning centromeres exist, were formed along with the tethering of CENH3. The CENH3 tethering method for producing an engineered centromere was revisited in maize. Taking advantage of a maize line carrying engineered megabase repeat arrays that contain multiple binding sites for different DNA-binding proteins, the laboratory of K. Dawe applied the DNA-binding domains Gal4 and LexA fused to the N-terminus and CENH3 instead of using *lac* inhibitor protein (LacI; Zhang *et al.*, 2012; Dawe *et al.*, 2023; Fig. 1b,c). Chromatin immunoprecipitation demonstrated that LexA-CENH3 recruits native CENH3 to the engineered repeat array. Then, to replace native maize CENH3 and make a synthetic centromere that drives autonomous chromosome segregation across numerous generations in the absence of the original LexA-CENH3 activator protein, a LexA-CENH3 transgene expressing the oat CENH3 gene was employed. Hence, also in crop plants, the DNA binding protein-CENH3 tethering approach could be used for the creation of *de novo* engineered centromeres.

IV. Stacking genes of interest onto engineered chromosomes

Once minichromosomes can be created that satisfy all requirements for a viable vector, efficient ways for transferring expression cassettes for a variety of interesting genes will be required. Site-specific recombination, for instance using the Cre/loxP system (reviewed in Ow, 2016; Chen & Ow, 2017), is one potential solution. It has been demonstrated that it is possible to introduce gene expression cassettes into transgenic loxP sites in plant genomes (Louwerse *et al.*, 2007). This approach has been applied to modify maize and wheat minichromosomes (Gaeta *et al.*, 2013; Yuan *et al.*, 2017). Two loxP sites are required for recombination. Recombination between these sites leads to the excision of the sequence flanked by them if they are situated on a single linear DNA molecule and if they are in the same orientation. Alternatively, if they are in the inverse orientation, recombination results in the inversion of the sequence bordered by them. Parts of plant chromosomes flanked by transgenic loxP sites in the same orientation have been successfully deleted using Cre-mediated recombination (Stuurman *et al.*, 1996).

In light of the finding that *A. thaliana* ring minichromosomes may be more stable than linear ones, an approach of directed building of a plant artificial ring chromosome, utilizing the Cre/loxP system in *A. thaliana*, has been taken (reviewed in Murata, 2014). A large circular DNA fragment, including a sizable portion of the native centromere, was released through site-specific recombination between two loxP sites on the same chromosome. Although it did not participate in meiotic pairing, the resulting ring chromosome demonstrated significant mitotic and meiotic transmission (reviewed in Murata *et al.*, 2013; Murata, 2014). Newly available site-specific recombinases (reviewed in Dong & Ronald, 2021), also in combination with CRISPR-Cas9, will further improve the way how transgenes are stacked on minichromosomes. Recently, novel recombinases, instrumental for large DNA insertions, were developed. Durrant *et al.* (2023) and Yarnall *et al.* (2023) employed bioinformatic mining and protein engineering of phage-derived large serine recombinases to build tools for predictable genomic insertion of multi-kilobase DNA fragments in mammalian genomes. Also, the group of C. Gao was just able to insert a bigger fragment of DNA into the rice genome with a plant-optimized recombinase by using prime editing for the initial insertion of its recognition site (Sun *et al.*, 2023).

V. Engineering of centromeres to generate haploid inducers

The ability to produce (di)haploids has made it possible to significantly speed up the crop breeding process. A haploid technology is not yet available in many plants, though. Other than using *in vitro* techniques and gametophytic (haploid) cells, haploids have also been created by selectively losing a parental chromosome set during inter- or intraspecific hybridization (reviewed in Kalinowska *et al.*, 2018; Jacquier *et al.*, 2020).

The application of altered centromeres has become an emerging tool in haploid induction technology. For faithful chromosome segregation to occur during cell division, centromeres are necessary. In wide crosses, such as *Hordeum vulgare* and *H. bulbosum*, it was demonstrated that failure of CENH3 integration into centromeres preceded uniparental genome deletion (Sanei *et al.*, 2011). M. Ravi and S.W. Chan reported that haploid plants were formed when wild-type *A. thaliana* was crossed with an *A. thaliana* cenh3-1 mutant, supplemented with a green fluorescence protein (GFP)-tail swap construct (fusion of the N terminus of conventional H3 to the C terminus of CENH3) (Ravi & Chan, 2010) (Fig. 2). This combination resulted in haploids with wild-type parent genomes in as many as 25–45% of the progeny. When a wild-type female and a GFP-tail swap male were crossed, the proportion of haploid plants was significantly reduced. By introducing minor deletions or point mutations in CENH3 (Karimi-Ashtiyani *et al.*, 2015; Kuppu *et al.*, 2015, 2020; Wang & Ouyang, 2023), *A. thaliana* haploids were produced. Haploid induction was also possible after the application of functionally complemented *A. thaliana cenh3* null mutants with unmodified CENH3 variants from distant relatives, like different *Brassica* species for crossing with wild-type *A. thaliana* (Maheshwari *et al.*, 2015). In addition to haploidization, the CENH3-based

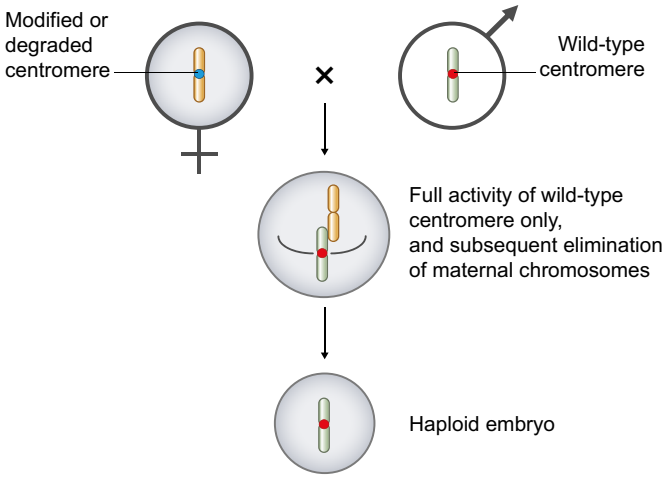

Fig. 2 Generation of haploids via application of CENH3 or KNL1 variants that lead to modified or degraded centromeres. Outcrossing between inducer and wild-type lines results in haploids due to uniparantel chromosome elimination.

approach has also been harnessed for forward mutagenesis screens, to reduce ploidy levels as well as to produce clonal seeds (reviewed in Ravi *et al.*, 2014).

To unravel the mechanism behind the selective elimination of the maternal chromosome complement in *A. thaliana* hybrids, Marimuthu *et al.* (2021) microscopically analyzed zygotes and the early stages of hybrid embryo development. The investigation showed that, whereas wild-type CENH3 remains, changes in CENH3 cause it to be selectively removed from the centromeres of mature eggs and early hybrid zygotes. The wild-type centromeres receive preferential CENH3 loading in the hybrid zygotes and embryos. Contrarily, CENH3-depleted centromeres typically disappear because they are unable to reassemble fresh CENH3 chromatin and the kinetochore. As a result, parental CENH3 variations lead to centromeres that are epigenetically distinct, creating a mating barrier and haploid offspring.

Intriguingly, when a point mutation in cenh3-4 was crossed with plants of the wild-type, the mutant produced a very low frequency (0.2%) of haploids. This point mutation caused inefficient CENH3 mRNA splicing and a significant drop in centromeric CENH3 (Capitao *et al.*, 2021). Thus, the suggested relationship between haploid formation and centromere size (Wang & Dawe, 2017) does not necessarily apply to the *cenh3-4* mutant. However, the application of a short heat stress treatment to the *cenh3-4* mutant resulted in an increase in haploidization frequency (4.1%) (Ahmadli *et al.*, 2022). An increased frequency of haploids was also found when the haploid inducer lines CENH3 GFP-tail swap and CENH3^{G83E} were used in combination with heat stress (Jin *et al.*, 2023). The endosperm failed to cellularize in seeds from heat-treated crosses. Many seeds also failed to germinate. Contrarily, *A. thaliana* CENH3 RNAi transformants could not produce haploidy when combined with heat stress (Zuo *et al.*, 2022), while showing a significant reduction of centromeric CENH3 (Lermontova *et al.*, 2011). Male sterility of the GFP-tail swap line makes maternal haploid induction challenging. However, lower temperatures dramatically enhanced the pollen quality of this line and the subsequent increase of temperature after crossing induced maternal haploids at *c.* 25% (Z. Wang *et al.*, 2023). A temperature effect on the frequency of haploids was also observed in wide crosses. When temperatures exceeded 20°C compared to temperatures below 17.5°C in *H. vulgare* × *H. bulbosum*, the elimination frequency of the *H. bulbosum* chromosomes was much higher (Pickering & Morgan, 1985). Therefore, the effect of temperature and, probably, other environmental factors could be considered to influence the haploidization frequency. On the contrary, an environmental impact on haploid frequency is disadvantageous if a uniparental genome elimination process is required for stable seed production.

A second centromeric protein was recently identified as a potential additional source of haploid inducers. The disruption of the CENH3 loading machinery via the inactivation of the centromere licensing factor KNL2 of *A. thaliana* resulted in the generation of haploids (1%) upon outcrossing with the wild-type (Lermontova *et al.*, 2013; Ahmadli *et al.*, 2022). Notably, when short-term temperature stress was applied, haploid induction effectiveness was 10 times higher in the knl2 mutant.

In addition to genetic modification, a parental genome has been eliminated using targeted *in vivo* degradation of the CENH3 protein. *Arabidopsis thaliana* haploids were produced by using degraded Green Fluorescent Protein (deGradFP) (Demidov *et al.*, 2022), a system that uses the ubiquitin-proteasome pathway in conjunction with a green fluorescent protein (GFP)-specific nanobody (VHHGFP4) fused to an E3 ligase (Caussinus & Affolter, 2016).

A haploid frequency of up to 7.6% was observed in pooled F1 seeds after outcrossing the CENH3-EYFP-complemented *cenh3.1* mother with plants expressing the GFP-nanobody-targeted E3 ubiquitin ligase. To prevent the production of deficient embryos due to endosperm abortion, CENH3 degradation should be limited to gametes and, preferably, egg cells for the future synthesis of a haploid inducer employing the targeted CENH3 degradation technique. Polyploids with numerous functioning CENH3 variants may benefit from the use of the targeted CENH3 *in vivo* degradation approach to generate haploids.

In crops, regrettably, the success of producing CENH3 variations causing haploidization has been limited, and the reported efficiencies are poor. An altered form of CENH3 was used to complement a homozygous *cenh3* mutant in maize, which led to an average haploid induction frequency below 1% (Kelliher *et al.*, 2016). The frequency of haploidization was raised to 5% by using a heterozygous CENH3 null mutation (Wang *et al.*, 2021). A haploid induction rate of up to 8% was obtained in wheat when a comparable haploid induction strategy was used (Lv *et al.*, 2020). According to Yoon *et al.* (2022), haploid induction rates between 0.5% and 1.4% were produced in switchgrass as a result of CENH3 misexpression. To develop a haploid inducer with a higher maternal haploid induction frequency in maize, different over-expressing maize CENH3 constructs were introduced into a Stock 6-derived haploid inducer (Meng *et al.*, 2022). The inbred line Stock 6 is known to cause haploidization when utilized as a pollen donor (Coe, 1959). An increase in maternal haploidization induction frequency of up to 16.3%, or an increase of *c.* 6.12% in comparison with the Stock 6-derived control lines, was seen

when a tail-altered CENH3 was substituted for the full-length CENH3 in the tagged expression cassette (Meng *et al.*, 2022). However, when native and modified CENH3 genes were coexpressed in wild-type plants, no haploids were produced. Therefore, the centromere function of Stock 6 could have certain flaws, which could have a comparable effect to that caused by a modified CENH3. Thus, overexpressing modified CENH3 in the lines descended from Stock 6 could result in an increased capacity for haploidization induction in the recently developed inducer lines (Meng *et al.*, 2022). Although there has been some progress made, the centromere-based haploidization method in crop breeding still requires further development.

VI. Inducing chromosomal inversions by CRISPR/Cas to redirect meiotic recombination

During the evolution of eukaryotic genomes, besides deletions, duplications and transposition events, the number, as well as the size of chromosomes changes over time due to consecutive translocations (Schubert & Vu, 2016; Mayrose & Lysak, 2021). Moreover, the positioning of genes on a specific chromosome might be altered by chromosome segment inversions (Huang & Rieseberg, 2020). Nevertheless, the collinearity of most genes is conserved in the face of these natural alterations. This indicates that changes in the chromosomal level can be best explained as the repositioning of large chromosomal segments of DNA, which arose due to the simultaneous occurrence of spontaneous double-strand breaks (DSBs) followed by consecutive misjoining of the DNA strands. With the advent of site-specific nucleases, it became clear that the controlled induction of DSBs could be the key to controlled plant genome engineering. If a single DSB is induced in the plant genome, it can be repaired either by homologous recombination (HR) or by non-homologous end joining (NHEJ). Whereas in meiosis, HR is efficient in repairing DSBs that are induced in a controlled manner by the topoisomerase-like enzyme Spo11, NHEJ represents the main mechanism of repair in somatic cells. Two independent pathways exist: classical (c) and alternative (a) NHEJ, which is also referred to as micro-homology mediated end joining (Puchta, 2005).

Early experiments using the homing endonuclease I-*Sce*I demonstrated not only that, through DSB induction, gene functions could be knocked out by NHEJ but also that foreign DNA can be integrated into the plant genome at the break site (Puchta *et al.*, 1996; Salomon & Puchta, 1998). By inducing two DSBs on the same chromosome, deletions could be generated (Siebert & Puchta, 2002) and even – although at very low frequency – heritable reciprocal translocations could be obtained by DSB induction on two different chromosomes in tobacco (Pacher *et al.*, 2007).

In principle, as demonstrated by the pioneering work of Ow (2016), the induction of CRs is also achievable by the application of site-specific recombinases such as Cre. The group was able to induce not only a heritable reciprocal exchange between two tobacco chromosomes (Qin *et al.*, 1994), but also larger chromosomal inversions and deletions between two lox sites using a transposase approach to insert one of the sites distant to the transgene on the same chromosome (Medberry *et al.*, 1995). However, due to the fact that there were no efficient means for the integration of lox sites at specific positions on plant chromosomes, the technology has not been applied in crops for chromosome engineering till now. As the site-specific integration of recombination sites by prime editing has just been demonstrated for the first time in plants, this situation might change soon (Sun *et al.*, 2023).

In the last 10 yr, biology in general and especially plant breeding has been transformed by applying the CRISPR/Cas system for different kinds of genome manipulations (Gao, 2021; Nasti & Voytas, 2021; Capdeville *et al.*, 2023; Wang & Doudna, 2023). Especially, the nucleases Cas9 and Cas12a have proven to be extremely efficient tools for cutting at almost any position in the plant genome. Thus, by inducing a single DSB, it became possible to knock out genes at will and with ease. By inducing two more or less closely adjacent DSBs, deletions can be obtained routinely (Durr *et al.*, 2018). With such a configuration, in addition to deletions, it is also possible to acquire an inversion of the sequence located between the DSBs. In contrast to deletions, which might be lethal if essential genes are lost, in the case of inversions, the genetic information is conserved besides small changes at the break sites. In a pilot study in *A. thaliana* using Cas9, it could be shown that the induction of kb-sized inversions is possible with efficiencies in the percent range, although at 2–3 times lower efficiencies compared with deletions at the same sites (Schmidt *et al.*, 2019). Recently, by applying long-read sequencing technologies, natural Mb-sized inversions have been found in many crop cultivars (Zuo *et al.*, 2017; Alonge *et al.*, 2020; Crow *et al.*, 2020; Jayakodi *et al.*, 2020). Their presence hinders the genetic exchange in the respective part of the chromosome with related varieties that do not harbour this particular inversion. The 1.1 Mb knob hkS4 inversion of *A. thaliana* chromosome 4 is a well-characterized example (Fransz *et al.*, 2000, 2016). The inversion occurred *c.* 5000 yr ago and is present in a number of ecotypes, such as Col-0, but not others, such as Ler-1. No genetic exchange is possible in this chromosome region if these ecotypes are crossed. However, it was unclear whether it would indeed be possible to induce Mb-sized inversions in plant chromosomes, as their occurrence might be extremely rare. Using the experience from having optimized the efficiency of *in planta* gene targeting experiments (Wolter *et al.*, 2018, 2021), it was possible to set up a feasible technology. By the egg-cell-specific expression of the highly active nuclease SaCas9 (Steinert *et al.*, 2015), together with the setup of an efficient screening protocol (Ronspies *et al.*, 2022a), it was possible to revert the 1.1 Mb-long knob inversion in Col-0. By screening *c.* 1600 seedings, the authors were able to identify seven independent recombinants carrying the respective reversion, three of them without loss of a single bp (Schmidt *et al.*, 2020). The inversion was confirmed cytologically by FISH analysis. By crossing the reversion line with Ler-1, it could be demonstrated that – after five millennia – genetic exchange occurred again in the formerly inverted region between the Col-0 and Ler-1 ecotypes (Fig. 3a). This approach has already been applied in crops: scientists from Corteva succeeded in reverting a 75.5 Mb inversion in maize that spans about a third of chromosome 2 (Schwartz *et al.*, 2020). Thus, this region could be unlocked for genetic exchanges with other maize cultivars.

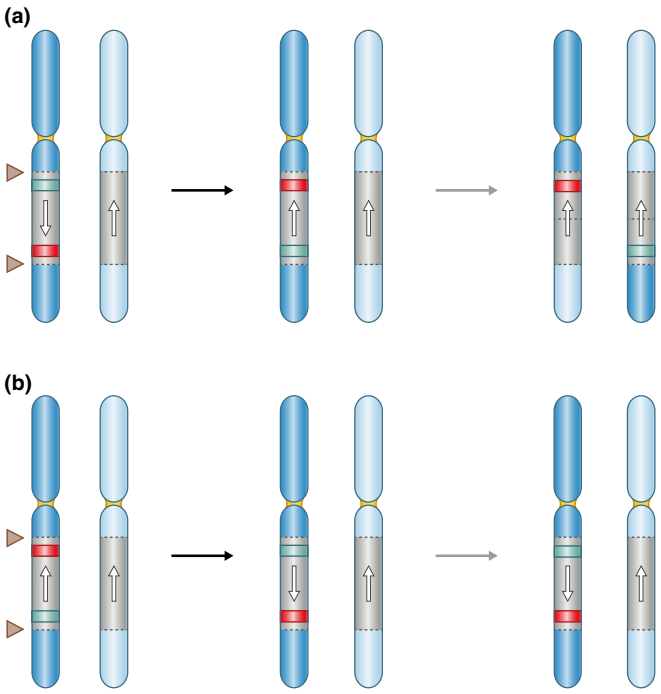

Fig. 3 Using DSB-induced chromosomal inversions as a mean to link or unlink traits. (a) Natural chromosomal inversions prevent that genes within the inversion (green and red) are segregated by meiotic recombination when crossed with individuals that do not harbor the inversion on the homolog. By reversing the inversion using CRISPR/Cas-mediated DSB induction, the respective part of the chromosome can be opened up for genetic exchange so that both traits can be segregated from each other by natural crossover formation. (b) To avoid segregation of traits, the respective chromosomal segments can be inversed at will by chromosome engineering. Thus, the linkage between the two genes is stabilized in crosses with other individuals carrying the respective segment in the original orientation. Black arrows represent CRISPR/Cas-mediated chromosome engineering and white arrows natural recombination.

Obviously, chromosome inversions could also be induced purposely to suppress genetic exchange (Fig. 3b). This was recently achieved in *A. thaliana*: a 17 Mb-long inversion within chromosome 2, covering 9/10 of its length, was generated in Col-0. After crossing with the ecotype Ler-1, marker analyses demonstrated that crossovers (COs) were reduced by more than an order of magnitude in the inverted region. It turned out that the few remaining exchanges were due to double COs in the inverted region. A single CO within the inversion would have resulted in chromosomes with identical sequences at both ends and, thus, inviable gametes (Ronspies *et al.*, 2022b). Thus, CRISPR/Cas-mediated inversion induction is a powerful tool to redirect genetic exchange on the chromosome level. Chromosomal inversions can also be used for other purposes: A 0.9 Mb inversion was induced to achieve a promoter swap in rice chromosome 1. Thus, the expression of a gene of interest could be massively increased with an approach the authors called 'knock up' (Lu *et al.*, 2021) (Fig. 4).

Besides inversions and deletions, duplication can also be obtained by break induction using the CRISPR/Cas system. Short tandem duplications of 100 bp and less, which might be useful for promoter manipulations, can be obtained by the induction of paired nicks (Schiml *et al.*, 2016; Wolter *et al.*, 2021). A 0.3 Mb

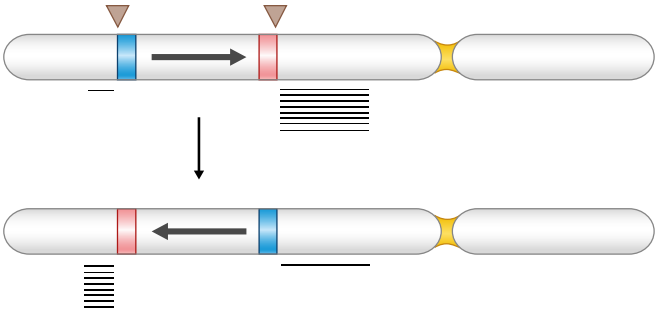

Fig. 4 Inducing a controlled chromosomal inversion for promoter swapping using CRISPR/Cas. With the 'knock-up' approach, the expression of mRNAs can be modulated without the use of foreign DNA. Thus, a weakly expressed gene can be upregulated by moving a strong promoter (pink box) into its close proximity. At the same time, the original weak promoter (blue box) now controls the expression of the other ORF. Black lines represent transcripts.

duplication could be obtained in rice by inducing DSBs at both borders of the duplicated region (Lu *et al.*, 2021). The easiest explanation for DSB-induced duplication events is the insertion of the cut-out chromosomal fragment of the sister chromatid into one of the DSBs induced in the other sister chromatid (Lynagh *et al.*, 2018).

VII. Breaking genetic linkages by CRISPR/Cas-mediated chromosomal translocations

Genetic linkages constitute a significant obstacle for breeders to combine desirable traits from wild and cultivated species. If genes are closely spaced on the same chromosome, it is not or hardly possible to separate them during meiosis by COs. Thus, different approaches have been taken to induce or enhance CO formation during meiosis in plants. However, a general increase of global COs by manipulation of the machinery processing meiotic recombination intermediates did not enhance genetic exchange in recombination-cold heterochromatic regions of the plant genome (for review, see Ronspies *et al.*, 2021). Also, the direct targeting of the meiotic DSB-inducing machinery by Cas9 to specific sites did not enhance local CO frequencies (Yelina *et al.*, 2022).

As DSBs can also be repaired by homologous recombination (HR) in somatic cells, a number of approaches have been taken to obtain COs in somatic cells. In a pioneering study, the group of Avi Levy was able to demonstrate that genetic exchange can be achieved in this way in tomato, although at a very low frequency (Filler Hayut *et al.*, 2017; Ben Shlush *et al.*, 2020). Later on, similar results were obtained in *A. thaliana* by the same group (Filler-Hayut *et al.*, 2021). In an independent study, scientists from Bayer were able to achieve a targeted crossover in hybrid maize after induction of DSBs by applying a Cas9 nuclease (Kouranov *et al.*, 2022). However, the resulting frequencies were too low for practical applications. A reason could be that HR is quite an inefficient mechanism of DSB repair in somatic plant cells. Moreover, recent results of the Levy group show that besides inducing COs, DSBs also trigger chromosome loss and chromothripsis-like rearrangements in tomato (Samach *et al.*, 2023). As these changes are deleterious, they are later on excluded from the gametophytes.

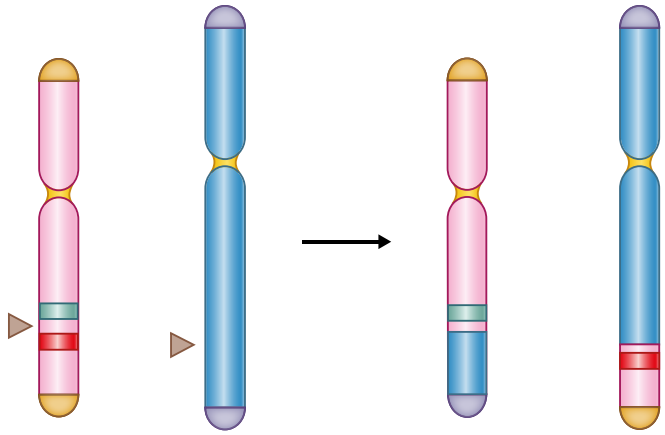

Fig. 5 Breaking of genetic linkages by DSB-induced translocations. Genes coding for agronomically attractive traits (green) are often positioned near genes coding for adverse traits (red) on the same chromosome. These genetic linkages cannot be broken by classical breeding. By inducing DSBs either in two heterologous chromosomes or in the respective homologs, it is possible to obtain a controlled chromosome arm exchange, after which the two genes get unlinked and the traits can be segregated.

By contrast, both pathways of NHEJ are highly efficient in repairing somatic DSBs in plants (Gehrke *et al.*, 2022). Thus, by inducing NHEJ-based translocations, genetic linkages could also be broken (Fig. 5). In a proof-of-concept experiment, the reciprocal exchange of parts of chromosome arms was demonstrated in *A. thaliana* (Beying *et al.*, 2020). The authors applied the same setup that had led to the successful induction of inversions (Ronspies *et al.*, 2022b) and were able to obtain exchanges between chromosomes 1 and 2 as well as 1 and 5. However, the frequencies were several-fold lower than the ones reported for intrachromosomal inversions. Interestingly, translocation frequencies could be enhanced by blocking the cNHEJ pathway (Beying *et al.*, 2020), a finding that had been reported for inversions before (Schmidt *et al.*, 2019). The explanation for this at-first-sight counterintuitive finding is the fact that the cNHEJ pathway is not only responsible for the joining of the broken ends but also, in contrast to the aNHEJ mechanism, keeps the two 'correct' ends of the DSB in close proximity. Thus, in the presence of several DSBs, the mis-joining of the 'wrong' ends is avoided as it might lead to genome instability due to the formation of acentric or bicentric chromosomes. Nevertheless, to increase the efficiency of chromosome engineering, it might well be worth considering blocking cNHEJ at the time of DSB induction. However, one has to keep in mind that the general loss of cNHEJ by itself is causing genetic instability. It will be only a matter of time before reciprocal chromosomal translocations will also be achievable in crops to break genetic linkages.

VIII. A bright future for plant chromosome engineering

In contrast to the mutation of genes, CRISPR/Cas-mediated chromosomal rearrangements have a completely different value for plant breeding and medical applications. In humans, spontaneous CRs are often correlated with malignancy in cancer. Therefore, the induction of CRs is of little interest for practical applications in mammals. They could be unintentionally induced due to off-site activity of the applied nuclease inducing further DSBs. To strictly avoid them during medical treatments, off-site activity has to be completely eliminated by the use of highly specific Cas enzymes. By contrast, the induction of CRs is a valuable novel tool for plant breeders to reorganize the genetic information of crops for a more efficient breeding process.

There are many possibilities we can envisage of how we might apply CRISPR/Cas for controlled changes on the chromosome level (Ronspies *et al.*, 2021). In principle, all changes we see during plant genome evolution (Mandakova & Lysak, 2018) could also be achievable in the future by using CRISPR/Cas. By consecutive translocations, we might be able to change the number of chromosomes and, thus, the number of linkage groups of a crop species. Moreover, we might be able to eliminate individual chromosomes in plant hybrids – as shown for mammals before (Zuo *et al.*, 2017) – by inducing multiple chromosome-specific DSBs. It has already been demonstrated that, by the induction of multiple breaks in centromeric or rDNA tandem repeats, cell death can be induced in specific organs and at specific time points in a controlled way in plants (Schindele *et al.*, 2022; Gehrke *et al.*, 2023).

KaryoCreate (karyotype CRISPR-engineered aneuploidy technology), a method that allows the production of chromosome-specific aneuploidies in human cells, was recently developed (Bosco *et al.*, 2023). Its foundation is the use of dCas9 coupled to mutant KNL1 to target chromosome-specific CENPA-binding satellite repeats. As a result, it was possible to create aneuploid chromosome sets with gains or losses of specific targeted chromosomes. It might be interesting to test whether such a technology could be beneficial for plant breeding.

Chromosome rearrangements are essential determinants in plant species evolution (Schubert & Vu, 2016). A combination of various consecutive CRs should allow us to achieve genetic isolation with respect to the originally used variety. Thus, crops might be obtained that, although almost identical to wild relatives on the gene level, cannot produce fertile progeny with them anymore. Thus, an uncontrolled spread of crops by outcrossing could be avoided.

Not only the applications of CRISPR/Cas alone but especially its combination with other tools might bring chromosome engineering to a new level. An attractive option is the combination of CRISPR/Cas approaches with site-specific recombinases. Just recently, using the CRISPR/Cas-based prime editing technology, it became possible to insert recombination sites into specific chromosomal positions with high efficiency, not only in mammals (Anzalone *et al.*, 2022; Yarnall *et al.*, 2023) but also in plants (Sun *et al.*, 2023). Although the technology has mainly been used to insert large segments of DNA so far, it should be applicable for the controlled induction of CRs. Such more complex rearrangements of plant genomes might be achievable much faster by combining these tools than by using Cas nucleases alone. We should be able to downsize regular chromosomes to minichromosomes by CRISPR/Cas. These could then be used as cargo chromosomes for gene stacking utilizing a recombinase and CRISPR/Cas-mediated integration of favorable traits simultaneously. On the contrary, the construction of artificial centromeres (Zhou *et al.*, 2022) might enable the build-up of synthetic chromosomes from scratch with

the help of CRISPR/Cas and recombinases. The minimum size required for a mitotically and meiotically stable synthetic chromosome is still unknown. Furthermore, small chromosomes can lose replication control not tied to the cell cycle, as has been demonstrated for B minichromosomes (Masonbrink *et al.*, 2013). However, at present, it is not possible to artificially synthesize large chromosomes of higher eukaryotes. The majority of efforts involving artificial chromosomes have created DNAs of less than 1 Mb size when no additional DNA sequences were added to existing chromosomes (reviewed in Venter *et al.*, 2022). Regardless of how one makes a minichromosome, likely, as observed in maize, the difficulty of finding pairing partners in meiosis and the precocious separation of sister chromatids at meiosis I exist (Han *et al.*, 2007). To overcome transmission issues, as suggested (Birchler & Swyers, 2020), it is conceivable that pollen selection genes could eventually be placed on minichromosomes, such that only the grains containing the selection genes would be viable.

Even though there is still much to learn about chromosomal engineering, current research is already able to generate knowledge that will advance science and help solve human problems. Being able to change the position of sequences on a chromosome in a targeted and predefined way will also enable us to explore a number of basic scientific questions that could not be addressed before. At the global level, the epigenetic state of sequences correlates with their chromosomal position. Often, chromosome arms are euchromatic, whereas centromeric and pericentromeric sequences are heterochromatic. Although we are able to change epigenetic states of very short sequence stretches in the plant genome by the use of dCas9-binding domains fused to epigenetic regulators (M. Wang *et al.*, 2023), chromosome engineering will allow us to bring megabases of sequences in a novel epigenetic context. Thus, it should be possible to discriminate whether the chromosomal position itself also has an influence on gene expression and on genetic exchange during meiosis or whether these are exclusively determined by the epigenetic state. Moreover, it will be interesting to test whether the epigenetic status might change over generations following chromosomal repositioning.

The plant nucleus is a dynamic three-dimensional (3D) structure with specific domains that occupy specific locations within the nucleus due to their epigenetic states and their chromosomal positions (Grob, 2020). Preliminary results indicate that undirected CRs can induce changes in the 3D organization of the nucleus (Picart-Picolo *et al.*, 2020). It will be interesting to analyze this phenomenon in a more systematic way using different kinds of CRs.

Taken together, one does not have to be a fortune-teller to realize that plant chromosome engineering will have an important impact not only on the future development of plant breeding but also on plant biology in general.

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Competing interests

None declared.

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