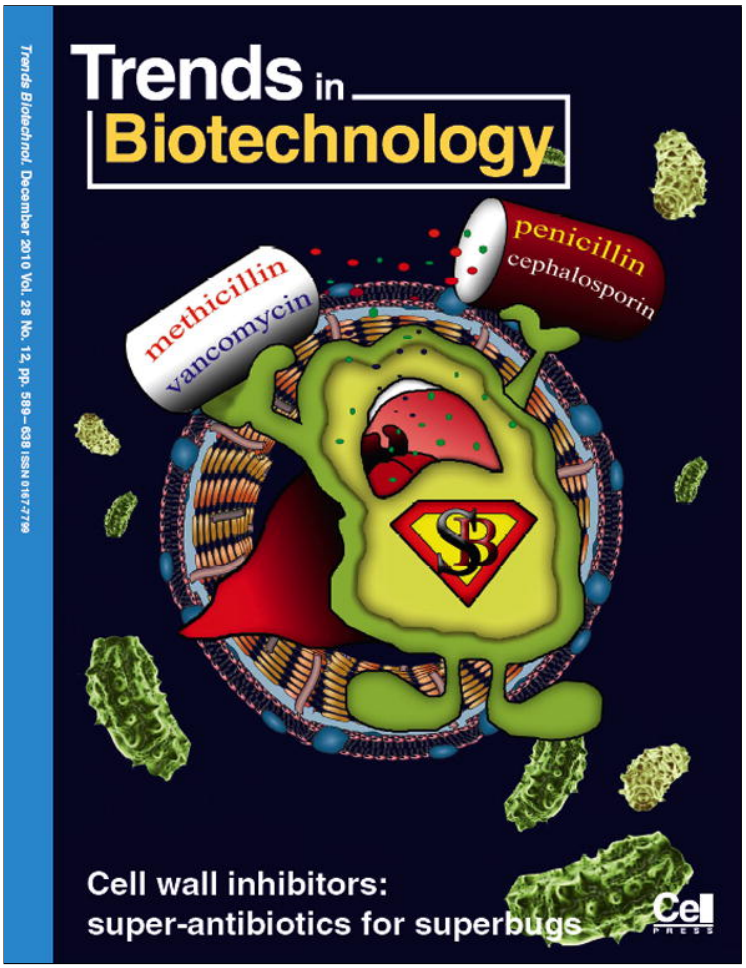

Review

Chromosome engineering: power tools for plant genetics

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The term ''chromosome engineering'' describes technologies in which chromosomes are manipulated to change their mode of genetic inheritance. This review examines recent innovations in chromosome engineering that promise to greatly increase the efficiency of plant breeding. Haploid *Arabidopsis thaliana* have been produced by altering the kinetochore protein CENH3, yielding instant homozygous lines. Haploid production will facilitate reverse breeding, a method that downregulates recombination to ensure progeny contain intact parental chromosomes. Another chromosome engineering success is the conversion of meiosis into mitosis, which produces diploid gametes that are clones of the parent plant. This is a key step in apomixis (asexual reproduction through seeds) and could help to preserve hybrid vigor in the future. New homologous recombination methods in plants will potentiate many chromosome engineering applications.

What is chromosome engineering?

Plant biotechnology uses genetic modification to create many useful traits. This innovation is layered on a constant background of conventional plant breeding, which will grow in importance as global climate change raises new challenges for agriculture [1]. Molecular markers generated by high-throughput sequencing will increase the efficiency of plant breeding [2]. However, the inherent slowness of combining favorable traits through genetic crosses and subsequent selection cannot be overcome by genomics alone. Chromosome engineering aims to create artificial chromosomes *de novo* or to change basic genetic processes by manipulating chromosome proteins. Tools created by chromosome engineering can greatly accelerate plant breeding. Artificial chromosome construction in plants has been summarized recently [3,4], thus this review focuses on methods that modify features of existing chromosomes to change their inheritance properties. Future applications in plant chromosome engineering that utilize homologous recombination technology are also proposed.

Engineering centromeres to produce haploid plants

A fundamental difficulty in plant breeding is the need to produce functionally homozygous lines with consistent phenotypes (Figure 1). Molecular markers reduce the number of progeny that must be screened to recover useful trait combinations. However, several generations of selfing or backcrossing are required to create a new inbred. Once spontaneously arising haploid plants were discovered, geneticists realized that they offered a shortcut [5]. By producing haploids from a heterozygous parent, then converting them back into diploids (termed "doubled haploids"), breeders could rapidly make homozygous lines (Figure 1). Haploid production has revolutionized breeding in crops where it can be efficiently performed [5–7]. For example, hundreds of thousands of doubled haploid maize lines are produced each year. Haploids can accelerate genetic mapping and are beneficial for genomics because they remove heterozygosity. If haploid production is so useful, why has it not been universally adopted? To understand this question, we must explore barriers to haploid production by standard methods.

Regeneration of cultured haploid cells to yield adult plants is a widely practiced haploid production method [5,7]. Microspores (pollen precursors) are the most common starting material because of their higher number per flower, but ovules have also been cultured. These methods are efficient for a few species (e.g. canola or *Brassica rapa*) but have not worked in many important crops. Development of tissue culture protocols is largely empirical. In some species, phenotypic variation arising from tissue culture (termed "somaclonal variation") can be deleterious. Furthermore, regeneration is frequently too inefficient for production breeding and protocols are usually limited to a few genotypes.

A more biologically interesting haploid production method involves crossing a crop to a distant relative in an interspecific or intergeneric cross [5,7]. In a fraction of progeny, the genome from one parent is selectively eliminated after fertilization, yielding a haploid with chromosomes from the desired parent only. A classic example is the cross between cultivated barley (*Hordeum vulgare*) and *Hordeum bulbosum*, in which the *H. bulbosum* chromosomes are missegregated and lost during embryogenesis [8]. In many wide crosses, the seed is inviable and embryo rescue is needed to regenerate an adult plant. Maize haploid inducers, many derived from the classic "Stock6" line, are rare examples of an intraspecies cross that produces genome elimination [9]. Mapping of loci responsible for genome elimination in Stock6 has not yet identified genes that control the trait, although these efforts are narrowing down the genomic regions responsible for the phenotype [9,10]. As the mechanism underlying genome elimination in wide crosses is currently unknown, the phenomenon cannot be recreated in a new species.

A recent discovery in *Arabidopsis thaliana* suggests a completely new strategy for creating haploid plants [11]

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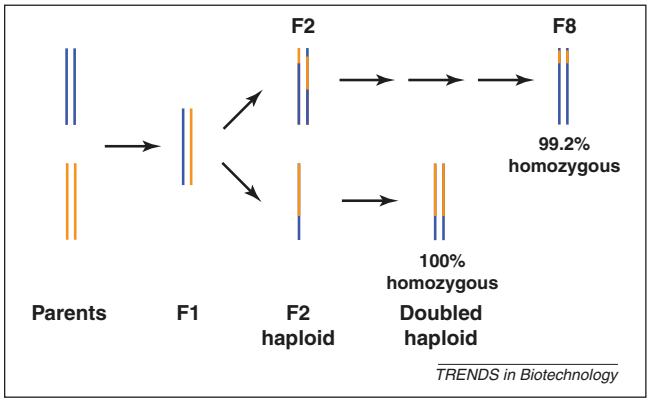

Figure 1. Haploid production accelerates conventional plant breeding. Traits from two different parents are combined in an F1 hybrid via crossing. Generations of inbreeding (e.g. F8 signifies the eighth generation since the original cross) are needed to produce functionally homozygous lines. Haploids have only one allele of every gene, thus if they can be converted back into diploids they can produce homozygous lines in a single step.

(Figure 2). Centromeres are loci that nucleate kinetochores, the protein complexes that bind to spindle microtubules and mediate chromosome segregation during cell division. In the novel method, centromeres are subtly disabled by mutating a kinetochore protein (such mutants must maintain chromosome segregation function to be viable). Crossing this centromere mutant to wild-type mixes two sets of chromosomes in the fertilized zygote. Chromosomes from the mutant parent (the "haploid inducer") have defective kinetochores and can be lost by missegregation during zygotic mitosis. Resulting adult plants are haploids with only chromosomes from their wild-type parent. This method mimics the genome elimination seen in wide crosses and potentially allows the process to be engineered into any plant.

In the published study, a haploid inducer was created by altering the essential kinetochore protein CENH3, a variant of histone H3 that replaces conventional H3 in centromeric nucleosomes [12]. Similar to conventional histone H3s, CENH3 has a C-terminal histone fold domain that complexes with other histones to form the nucleosome core and an N-terminal tail domain that protrudes from the nucleosome [13]. Unlike conventional histones, CENH3s evolve rapidly, particularly in their N-terminal tail. In *A. thaliana* haploid inducers, endogenous CENH3 was replaced by introducing transgenic proteins into a *cenh3* null mutant. The most efficient haploid inducer adds an N-terminal GFP tag to the protein and replaces the hypervariable tail of CENH3 with the tail of conventional H3 (termed "GFP-tailswap"). When *cenh3 GFP-tailswap* plants were crossed to wild-type, up to 50% of F1 progeny were haploid. All wide crosses described above produce a mixture of haploid progeny and diploid hybrids, in which chromosomes from both mutant and wild-type parents are kept (Figure 2). The frequency of genome elimination produced by GFP-tailswap in *A. thaliana* is higher than any previously reported wide cross. This suggests that centromere-mediated genome elimination might improve the efficiency of haploid production, even in crops such as maize and canola.

A key feature of the *A. thaliana* GFP-tailswap line is the ability to make either maternal or paternal haploids by crossing the mutant with female or male wild-type plants, respectively. Microspore culture produces haploids with paternal chromosomes and paternal cytoplasm. Crossing a CENH3-based haploid inducer (as the female) with a wild-type male shifts paternal chromosomes into the maternal cytoplasm. Cytoplasmic male sterility is useful for producing hybrid seed and facile cytoplasm exchange is likely to be one of the major applications of haploid inducers based on CENH3 alterations [14].

How can CENH3 engineering create a haploid inducer in crops?

Endogenous *CENH3* must be inactivated or chromosomes from the inducer will not be outcompeted by those from the wild-type parent [11]. TILLING or insertional mutagenesis could create a *cenh3* mutation (such methods will be greatly aided by advances in high-throughput sequencing) [15]. Without a *cenh3* mutant, gene silencing methods,

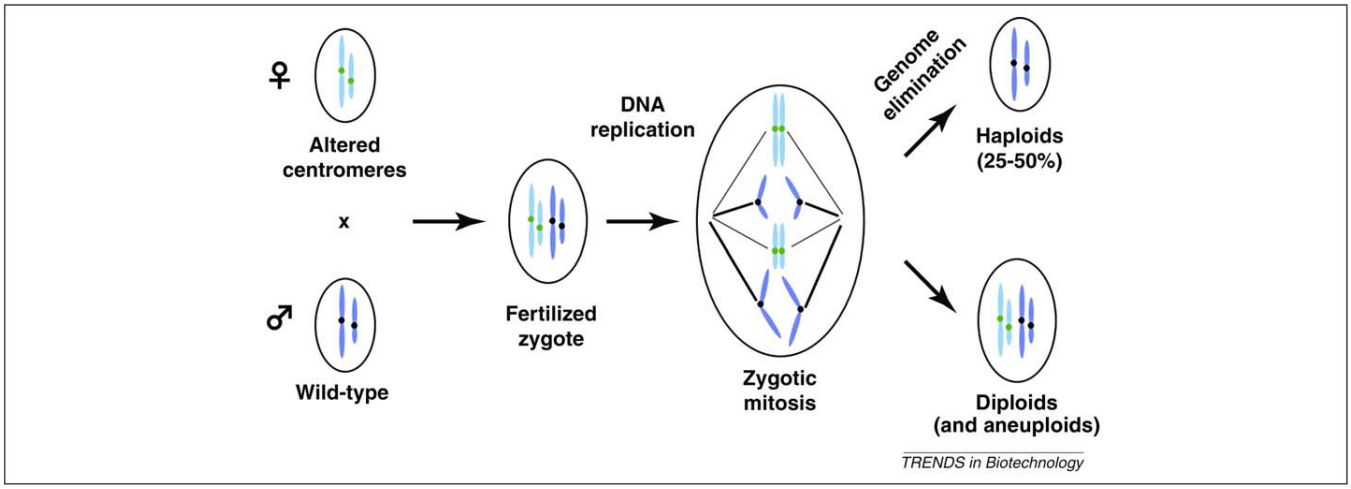

Figure 2. Altering centromeres to produce haploid plants. When a plant expressing an altered CENH3 protein (altered centromeres) is crossed to wild-type, its chromosomes (light blue) compete poorly during zygotic mitosis and are lost through missegregation in a process termed ''genome elimination''. A substantial fraction (25–50%) of adult plants can be haploids, with chromosomes from only their wild-type parent (dark blue). Note that the identity of the mutant and wild-type chromosomes must be maintained through DNA replication, presumably because pre-existing CENH3 remains associated with the kinetochore.

such as RNAi, should inactivate the endogene in any plant. The promoter for the RNAi transgene might need to be expressed in gametophytes, to ensure that endogenous CENH3 is absent from pollen or egg cell chromosomes (the commonly used 35S promoter is often poorly expressed in gametophytes [16]). Mutant CENH3 transgenes could be synthesized with altered codon usage to evade RNAi and should probably be expressed from the native *CENH3* promoter. CENH3 is a small protein, thus a single transgene can contain an RNAi transgene as well as a transgene expressing a mutant variant. Thus, a haploid inducer could conceivably be made in a single transformation. Haploids in *A. thaliana* were produced through seeds; as such, CENH3 engineering might avoid the need for tissue culture and, in some crops, potential somaclonal variation. Ideally, the method could offer haploid technology to breeders without access to highly standardized tissue culture facilities.

Can other centromere alterations create a haploid inducer?

GFP-tailswap is not the only CENH3 variant that induces genome elimination. GFP-tagged full-length CENH3 also induces haploids, at a lower frequency, and many other alterations to CENH3 might cause missegregation in a cross. It has been suggested that alterations to the CENP-C protein could also cause genome elimination [17]. The potential for engineering other kinetochore proteins to produce haploids will depend on their behavior during DNA replication. After fertilization, both mutant and wild-type chromosomes are replicated during S phase, prior to the first zygotic mitosis. If a kinetochore protein is removed during DNA replication and reloaded onto both chromosome sets from a common pool, there will be no difference between chromosomes from the two parents and therefore no genome elimination. Pre-existing CENH3 at kinetochores is probably retained during DNA replication and partitioned equally between the two replicated sisters [18]. This explains why chromosomes from the mutant and wild-type retain their different behaviors, even if additional CENH3, presumably a mixture of mutant and wild-type protein, is loaded after S phase. CENP-C binds to centromere DNA directly, which might increase the chance that it remains associated with replicated chromosomes [19,20].

Manipulating meiotic recombination frequency

A high meiotic recombination rate is useful for introgressing traits controlled by a small number of genes into another genetic background. Regions of the genome with suppressed recombination, often correlating with a high percentage of heterochromatin, pose particular difficulties. Forward genetic screens for mutants with elevated recombination are feasible in maize and *A. thaliana* using elegant genetic marker systems established to study local recombination. Kernel pigment phenotypes or fluorescent proteins expressed in pollen allow high-throughput scoring [21,22]. Reverse genetic approaches to increase recombination draw on meiosis research from yeast, mammals and plants. Meiotic recombination is initiated by double-stranded breaks catalyzed by the nuclease Spo11, which is broadly conserved in eukaryotes [23,24]. Processing of the double-stranded break can yield a crossover outcome (resulting in recombination) or a non-crossover repair event. Molecular understanding of this process is deepening, suggesting opportunities for engineering elevated recombination rates.

Two types of engineering could, in principle, elevate meiotic recombination. First, chromosome structure might be altered to allow easier access by recombination factors. Mutations that disrupt heterochromatin in the fission yeast *Schizosaccharomyces pombe* increase meiotic recombination near centromeres [25]. Although this is a promising approach, radical changes in chromatin structure might affect gene expression in unwanted ways. A note of caution is warranted based on comparing *A. thaliana rna-dependent rna polymerase2* (*rdr2*) mutants to maize *mop1* mutants in the orthologous enzyme. Both mutations reduce DNA methylation in the non-CG sequence context, yet *rdr2* has a very subtle phenotype, whereas *mop1* causes severe developmental defects [26–29]. This could result from the fact that the maize genome contains many more repeats, which could have evolved to play a larger role in gene regulation. In the future, it might be possible to produce local changes in chromatin structure, perhaps with engineered sequence-specific DNA binding proteins fused to enzymes that modify epigenetic marks (see discussion on engineered DNA binding proteins, below).

A second strategy for increasing meiotic recombination is to focus on recombination proteins themselves. In addition to Spo11, several other proteins that help to initiate recombination have been discovered through forward genetic screens (a majority of which were conducted in *A. thaliana*) and reverse genetic approaches using gene expression profiling to identify candidates [23,24]. Further explorations into meiotic crossover control are likely to yield practical insights. A recent study has discovered a *Caenorhabditis elegans* protein that regulates the crossover/non-crossover choice [30]. Furthermore, DNA helicases are key controllers of recombination rate in yeast [31–33] and manipulating such proteins in plants might increase meiotic recombination.

A related problem for plant breeders is introgressing traits from wild relatives that are so distantly related that chromosome pairing in meiosis I is difficult. Such homeologous pairing (between related chromosomes from different species) can be genetically controlled, as shown by the wheat Ph1 locus, which prevents recombination between homeologs [34]. The recent discovery that Ph1 downregulates cyclin-dependent kinases offers hope that the meiotic cell cycle machinery can be manipulated to allow homeologous recombination [35].

Reverse breeding

A radically different method, termed “reverse breeding”, takes the opposite approach to the methods described above [36]. Reverse breeding suppresses meiotic recombination completely, resulting in the formation of gametes with various combinations of the intact chromosomes from either parent (Figure 3). Although meiotic recombination ensures accurate chromosome segregation during meiosis I, meiosis in the complete or near-complete absence of recombination will still yield rare, viable gametes. If these can be turned into adult plants by producing haploids (and

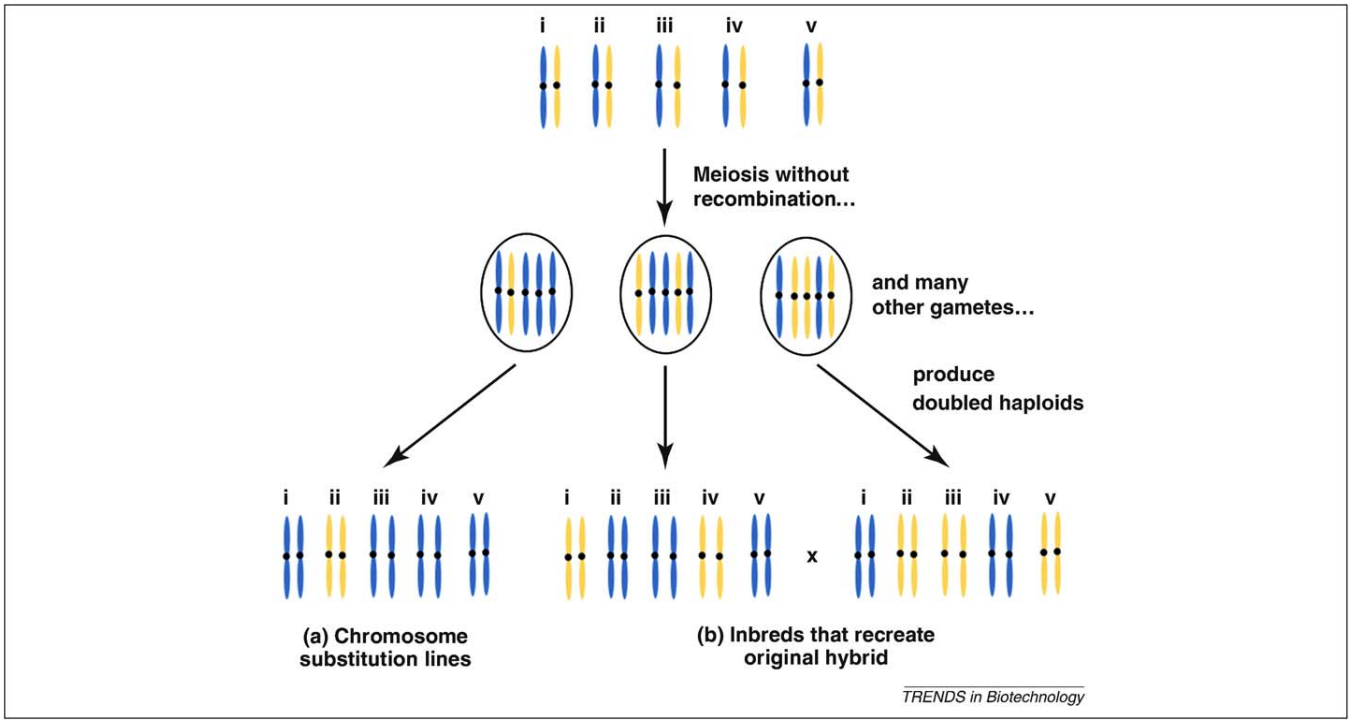

Figure 3. Reverse breeding can produce lines containing intact parental chromosomes, which allows heterozygous genotypes to be recreated. In reverse breeding, meiotic recombination is suppressed. Intact parental chromosomes (i–v) segregate into gametes, which can be converted into fertile plants by producing doubled haploids. **(a)** Single chromosomes can be transferred into an otherwise different genetic background. **(b)** Appropriate inbred lines can be crossed together to recreate the heterozygous genotype of the original parent.

subsequently doubled haploids), it is possible to create chromosome substitution lines in which a single chromosome from one inbred is transferred into the background of a different inbred parent (Figure 3). Such lines can be hugely valuable for trait mapping and introgression.

Heterosis (hybrid vigor) is a cornerstone of plant breeding [37]. Another application of reverse breeding is to start with an elite hybrid and create two inbred lines that will recreate the vigorous hybrid genotype when crossed (Figure 3). Reverse breeding to fix hybrid vigor is applicable to species with <12 chromosomes, because it is mathematically realistic to create complementary combinations of parental chromosomes for such plants [36]. Reducing meiotic recombination is straightforward given the large number of meiosis-specific proteins involved in this process. RNAi is an appealing method for downregulating recombination because it can be controlled by a conditional promoter. The combination of such methods with CENH3-based haploid inducers could make reverse breeding feasible for many crops in the near future.

CENH3-based haploid induction, reverse breeding and future methods to elevate recombination rate share an interesting feature: they are likely to involve transgenic plants in a breeding step but can produce completely non-transgenic progeny. It will be interesting to see whether such lines are classified as genetically modified organisms (GMOs), because they are indistinguishable from organisms that never had a transgenic parent. If doubled-haploid lines made using CENH3-based inducers or reverse breeding are not regulated as GMOs, it will be easier to market them where public resistance to transgenic foods is high, for example in Europe.

Chromosome engineering for apomixis

Hybrid seeds have greatly increased agricultural productivity, but their genotype cannot be propagated through sexual reproduction. Asexual reproduction through seeds (apomixis) occurs in many plant species [38]. It is thought that apomixis alternates with sexual reproduction, allowing such plants to multiply favorable genotypes yet still create variation when necessary. Apomixis is often described as a potentially revolutionary technology for agriculture, because it could perpetuate vigorous hybrids indefinitely [38,39]; however, attempts to introgress the trait into crops have not succeeded. Furthermore, map-based cloning of genes that control apomixis has not yet identified individual loci responsible for the trait [40].

Although there are many ways for apomixis to occur in nature, a common route for scientists seeking to engineer it is to divide the process into three steps [39]. First, meiosis must be bypassed or altered so that the plant produces diploid gametes without recombination. The *dyad* mutant of *A. thaliana* was the first genetic lesion found to produce clonal diploid gametes, but the precise function of the DYAD/SWI1 protein in meiosis is not known [41,42]. Second, embryogenesis should begin without fertilization. Third, endosperm development must also be triggered without fertilization. Chromosome engineering has had notable recent success in achieving the first step [43].

A complex but efficient solution for creating clonal diploid gametes is to combine three mutations that affect meiotic chromosomes and meiotic cell cycle progression [43]. Removing the SPO11 nuclease prevents meiotic recombination. Chromosomes in *spo11* mutants segregate randomly in meiosis I, because they cannot pair with their homolog. In

meiosis I, sister chromatids normally segregate to the same side of the spindle, because their centromeres are held together by the meiosis specific cohesin protein REC8 [44,45]. When *spo11* and *rec8* mutations are combined, sister chromatids segregate to opposite sides of the spindle in meiosis I, effectively turning this division into mitosis. The final mutation, *osd1*, prevents the onset of meiosis II, leaving two diploid gametes with the same genotype as the parent plant (see Ref. [43] for diagrams illustrating the full process). *spo11 rec8 osd1* mutants are termed "MiMe", because they convert meiosis into mitosis. In MiMe plants, an astonishing 85% of female gametophytes and 100% of the pollen have the diploid genotype of the parent plant. The challenge of engineering apomixis now shifts to coaxing the diploid embryo sac to form a seed without fertilization. Prospects for solving this problem through developmental genetics have been reviewed elsewhere [46].

Homologous recombination for chromosome engineering

Precise chromosome engineering using homologous recombination has tremendous potential for basic research and for biotechnology applications. A classic example is the engineering of balancer chromosomes in mouse [47]. Balancer chromosomes have an inversion that contains a recessive lethal mutation (Figure 4). They prevent recombination within the inverted interval and cannot be homozygous and are therefore very useful for maintaining mutations in a heterozygous state. In some crops, hybrid vigor can depend on a very small number of loci or even on single heterozygous genes [48]. If a counterselection against homozygotes can be achieved, engineering plant balancer chromosomes could be a way to preserve the advantages of heterosis without full apomixis. Balancer chromosomes inevitably result in partial sterility and this property will limit their application in crops where the seed is the product. However, balancers can be valuable research tools for plant genetics, even in these species.

Engineered translocations are another potential application in plants. The evolutionary history of karyotype rearrangement can be reconstructed, for example in the study that revealed how the base chromosome number of eight in the *Brassicaceae* was converted to the *A. thaliana* karyotype of five [49]. Normally, karyotype differences would prevent genetic exchange between two species; however, engineered translocations might restore sufficient synteny to allow productive recombination if the problem of homeologous pairing can be overcome. The converse approach is to create novel translocations to reproductively isolate a plant, a potentially useful application in crops where intercrossing with wild relatives is a concern.

Site-specific recombinases, such as Cre–Lox or FLP/FRT, can create precise chromosome insertions, deletions, translocations and inversions, and work well in plants [50]. A powerful use of site-specific recombination is to target transgenes to specific genomic locations [51]. This can be repeated through several rounds to allow "transgene stacking" or the insertion of multiple transgenes at the same locus [52]. Maize homologous recombination has been achieved recently by cutting the desired locus with a sequence-specific zinc-finger endonuclease and thereby enhancing recombination frequency [53,54]. This suggests that we can now integrate Lox or FRT sites in precise locations, bringing single-nucleotide accuracy to plant chromosome engineering and allowing the precise engineering of chromosome rearrangements (Figure 4). One limitation of homologous recombination is the cost of designing custom zinc fingers, but this is likely to decrease as the method is more widely adopted. The transcription-activator-like (TAL) class of plant pathogen effectors represents an entirely different class of modular sequence-specific DNA binding proteins [55,56]. TAL proteins contain short tandemly repeated domains, each of which recognizes a single base pair of DNA. Homologous recombination in *Drosophila melanogaster* has evolved from a method used by only a handful of laboratories to a routine technique [57]. A similar trajectory will allow chromosome engineers to create a new set of power tools for plant genetics.

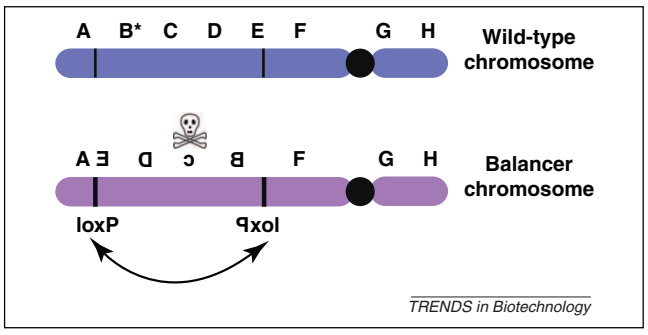

Figure 4. Homologous recombination allows precise engineering of balancer chromosomes. Balancer chromosomes contain an inversion relative to wild-type that prevents recombination in this interval (because recombination in this region creates dicentric and acentric chromosomes that are generally fatal to gametes). The letters A–H indicate loci on the chromosomes to show the position of the inversion. Balancer chromosomes can be constructed by integrating site-specific recombinase recognition sites (dark lines indicate loxP sites) in precise locations using homologous recombination. The balancer chromosome cannot be homozygous owing to a lethal mutation ('c') within the inversion. If B/B* comprise a pair of alleles that confers heterosis (hybrid vigor), balancers allow them to be maintained in the heterozygous state if the B*/B* homozygote can be selected against.

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