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Review

Recent progress in plant genome engineering: from large insertions to chromosome number changes

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The adaptation of the CRISPR/Cas system as a biotechnological tool has enabled a wide spectrum of targeted genome modifications. Whereas earlier approaches focused on small sequence changes, recent years have seen a shift toward larger-scale alterations. Advances in homology-directed gene targeting now enable efficient, scar-free kilobase insertions, while combining nuclease-deficient Cas effectors with recombinases or transposases allows the integration of much larger sequences. Prime editing further expands this scope, enabling inversions, replacements, and deletions spanning hundreds of kilobases to several megabases. More recently, genome engineering has reached a new stage with chromosome fission and fusion, demonstrating the feasibility of controlled karyotype restructuring. Together, these advances open new opportunities for crop improvement, from establishing reproductive barriers and mimicking evolutionary processes to trait stacking on Plant Artificial Chromosomes.

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Introduction

Global climate change and the projected rise of the human population to nearly 10 billion by mid-century threaten global food security, requiring about 50% more food production than in 2010 [1,2]. Meeting this demand will require enhancing productivity and resource-use efficiency on existing agricultural land through sustainable production systems. Future breeding strategies must rapidly adapt crops to shifting environmental conditions, including tolerance to abiotic stresses such as drought, salinity, and extreme weather, as well as resilience to emerging pathogens. Conventional approaches like selection and mutation breeding have delivered substantial improvements but are constrained by limited precision, high off-target variation, and long development times. The deeper understanding of complex genomic structures (for details, see article by Zhu et al. in this issue) and the advent of sequence-specific nucleases — particularly Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated (CRISPR/Cas) — has transformed molecular breeding, enabling targeted genome modifications with unprecedented efficiency. Since their first use in plants just over a decade ago, CRISPR/Cas tools have been applied to ~120 crop and model species [3–5], accelerating the development of improved varieties. Beyond introducing sequence-specific double-strand breaks (DSBs) repaired by often unpredictable endogenous pathways, recent advances now enable precise and user-defined sequence changes. These capabilities extend genome engineering from single-nucleotide edits to large insertions and complex chromosomal rearrangements (Table 1), offering transformative potential for refining existing cultivars and domesticating new crops from wild species.

Recent advances in large DNA insertions

In plants, DSBs are predominantly repaired through the error-prone non-homologous end-joining (NHEJ) pathway, which typically generates small insertions or deletions (indels) within the target locus [6]. Consequently, the precise, site-specific, and scar-free integration of sequence alterations spanning several kilobases for the refinement of traits or the incorporation of defined allelic variants remains technically challenging. To overcome these limitations, recent strategies either exploit the cell's DSB repair pathways to achieve targeted sequence integration or rely on DSB-independent systems capable of introducing precise genetic changes, each offering distinct advantages and limitations that have shaped their application in plant genome engineering.

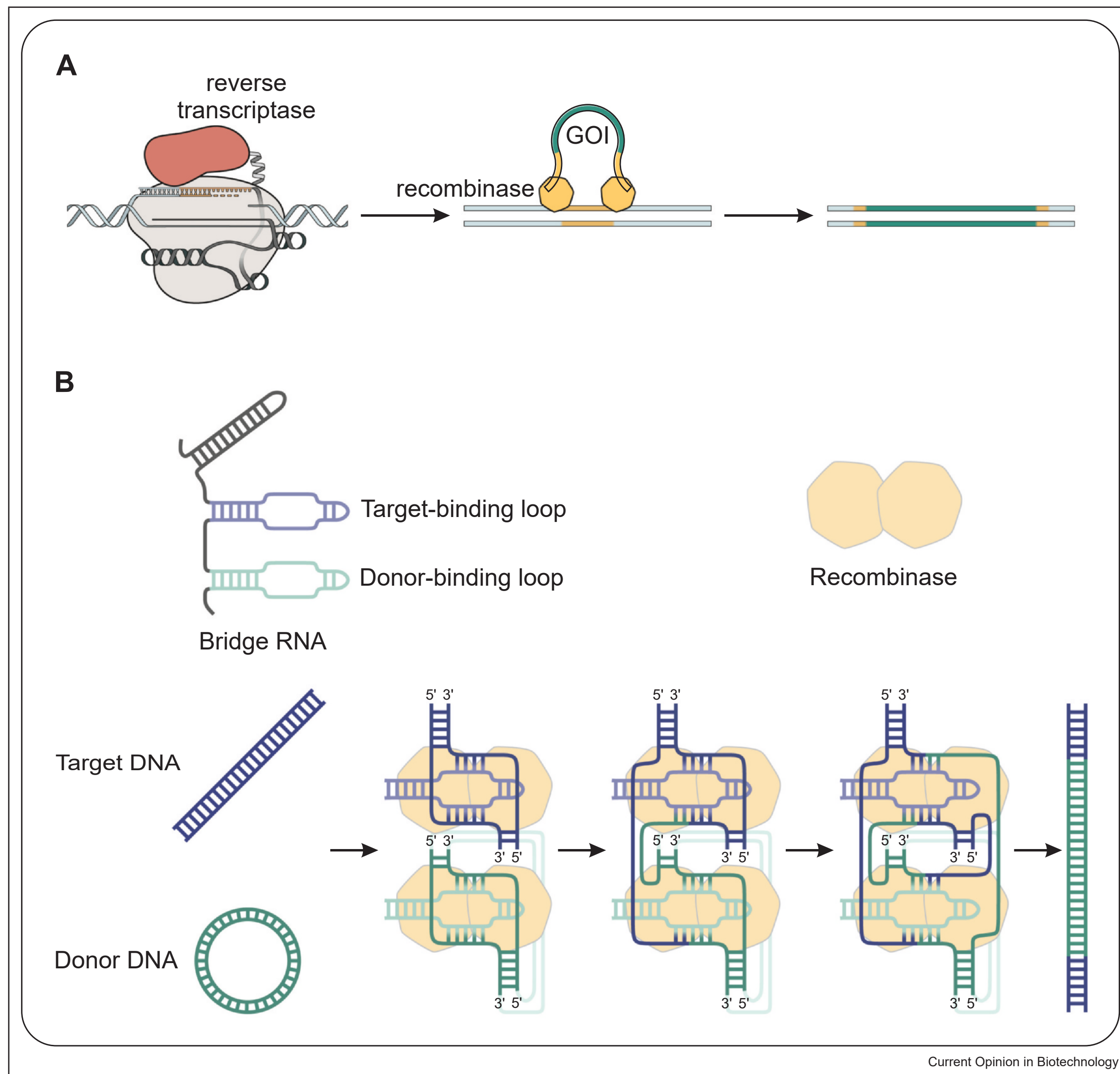
Leveraging the predominant DSB repair pathway in plants, an approach combining chemically modified donor DNA with CRISPR/SpCas9 enables targeted insertion of

Table 1

Overview of recent advances in large-scale genome engineering.

System category	System name	Main feature	Validated organisms	Ref.
Recent advances in large DNA insertions				
NHEJ-mediated insertion	CRISPR/Cas9 in combination with chemically modified donor DNA (blunt phosphorylated dsODN and terminal phosphorothioates)	Targeted insertion of sequences up to 2 kb	*Oryza sativa*	[7]
	DOTI	Insertion of transcription activator-like effector binding elements into the promoter region	*Setaria viridis*, *Oryza sativa*	[8]
HDR-mediated insertion	Cas nuclease fused to 5′ exonucleases (UL12, T7)	Precise and scar-free insertion of several kb, increased knock-in efficiency	*Arabidopsis thaliana*, *Nicotiana benthamiana*, *Triticum aestivum*	[13]
RT-based editing	Engineered Plant Prime Editor (ePPE)	Enhanced editing efficiency compared with original PPE, particularly for small edits	*Oryza sativa*	[16]
Recombinase-based integration	PASTE	Targeted integration of large DNA cargos up to 36 kb without reliance on DNA repair pathways	Human cells	[17]
	PASSIGE	Efficient integration of large cargos (> 10 kb), outperforming PASTE in efficiency	Human cells	[18]
	PrimeRoot	Integration of DNA fragments up to 11.1 kb	*Oryza sativa*, *Zea mays*	[19]
	PCE	Precise insertions up to 18.8 kb; outperforms PrimeRoot	Human cells, *Oryza sativa*, *Zea mays*, *Triticum aestivum*	[20]
Transposase-based integration	CASTs	Integration of cargo DNA up to 10 kb at ~100% efficiency	*Escherichia coli*	[21, 22]
	evoCAST	> 200-fold activity increase; insertion efficiencies up to 30%; integration of DNA > 10 kb	Human cells	[23]
	TATSI	Targeted integration with ~27% efficiency for small cargos; 8.3% for fragments up to 9 kb	*Arabidopsis thaliana*, *Glycine max*	[24]
Bridge RNA/IS elements	IS110 mobile genetic elements	Mediate insertion, excision, and inversion of DNA	*Escherichia coli*	[25]
	IS110-derived bridge RNA systems	Mobilization of up to 0.93 Mb DNA	Human cells	[26]
Large-scale chromosomal rearrangements				
Genetic recombination engineering	CRISPR/Cas-mediated inversion	Disruption and stabilization of allelic combinations with minimal epigenetic effects across generations	*Arabidopsis thaliana*	[27, 30, 31]
Chromosomal deletions	CRISPR/Cas-mediated deletions	Removal of genomic segments up to 684 kb	*Arabidopsis thaliana*	[28]
Chromosome restructuring	CRISPR-mediated translocation	Reciprocal translocations up to 1 Mb with minimal phenotypic consequences	*Arabidopsis thaliana*	[34, 35]
Large-scale chromosomal engineering	DualPE	Precise deletions up to 2 Mb; gene replacements up to 258 kb; inversions up to 3.6 Mb	*Arabidopsis thaliana*, *Triticum aestivum*, *Nicotiana benthamiana*, *Solanum lycopersicum*	[29,32,33]
Changing chromosome numbers				
Karyotype reduction	CRISPR/Cas-mediated karyotype reduction	Reduction from 10 to 8 chromosomes with a stable phenotype and transcriptome	*Arabidopsis thaliana*	[36]
Karyotype increase/ neocentromere induction	CRISPR-mediated *de novo* centromere seeding	Lines with 22 chromosomes display normal growth, reproduction, and phenotype	*Zea mays*	[38–40]

Figure 1



Overview of DSB-free methods for targeted insertion of large sequences. **(a)** Based on its high precision and efficiency for small insertions, PE can be used to install short recognition sequences for SSRs (yellow), which subsequently can mediate the targeted integration of large DNA fragments. **(b)** The bridge RNA system is based on a mobile genetic element that excises from the genome to form a circular DNA intermediate. A structured non-coding RNA guides sequence-specific recombination by directing the cognate recombinase through two internal loops, one recognizing the genomic target site and the other the donor sequence.

sequences up to 2 kb into the rice genome with efficiencies of up to 25% by using a blunt, 5′-phosphorylated double-stranded oligodeoxynucleotide (dsODN) bearing phosphorothioate linkages at both ends to increase oligonucleotide stability and provide ligatable ends for direct NHEJ-mediated integration [7]. To improve precision, orientation, and efficiency, the method was refined into directional oligonucleotide-based targeted insertion (DOTI), which exploits the ability of SpCas9 to generate 1-nucleotide 5′ overhangs. Matching these with complementary overhangs on the ends of chemically protected dsODNs containing the intended insert markedly increased insertion accuracy and directionality across all tested target sites, reaching efficiencies of up to 60.9% and enabling seamless insertions in *Setaria viridis* and *Oryza sativa* [8]. Besides NHEJ, the homology-directed repair (HDR) pathway can be utilized to replace or insert sequences at predetermined genomic locations without leaving any genomic scar. However, its application in plants has been limited by inherently low rates of homologous recombination. As HDR-based gene targeting requires extended 3′ single-stranded overhangs [9,10], the predominantly blunt ends and 5′ overhangs produced by Cas9 and Cas12a, respectively, may limit its efficiency.

Based on the premise that fusing 5′ exonucleases to intronized version of SpCas9 [11] or temperature-tolerant LbCas12a [12] could promote the generation of free 3′ ends, a recent study identified two exonuclease families — herpesvirus UL12 and bacteriophage T7 — that increased HDR frequencies by up to 38-fold in *Nicotiana benthamiana*. In *Arabidopsis thaliana*, a Cas9-UL12 fusion increased knock-in frequencies tenfold, while in wheat, stable and heritable knock-ins exceeding 2 kb were achieved in 1% of primary transformants [13]. These findings open perspectives for routine production of heritable knock-in and gene replacement events in plants, with exonuclease–Cas9 fusions enabling recovery of desired edits from screening only 50–100 transformants.

Beyond such DSB-dependent strategies, emerging methods such as reverse transcriptase (RT)-, recombinase-, or transposase-based systems employ DSB-free mechanisms to reduce off-target editing and improve the efficiency and precision of targeted insertions [14]. As an RT-mediated genome-editing approach, prime editing (PE) uses a CRISPR/SpCas9 nickase-RT fusion. A PE guide RNA (pegRNA), extended at its 3′ end, directs the complex to the target site, anneals to the cleaved DNA strand, and serves as a template for the RT to incorporate the sequence changes encoded in the pegRNA into the genomic DNA [15]. Although PE is a versatile genome-editing technology, its use in plant cells is limited by low efficiency, driving efforts to enhance its performance. Modifying the RT and adding a viral nucleocapsid protein increased editing by up to 5.8-fold in rice protoplasts, yet insertion efficiency dropped as the insert size increased up to 34 bp, indicating its current potential, especially for small edits [16]. However, given the high precision of PE, this capability can be leveraged to install short recognition sequences for site-specific recombinases (SSRs), enabling the targeted integration of larger DNA cargos (Figure 1a). The Programmable Addition via Site-specific Targeting Elements (PASTE) system exemplifies this approach by fusing a CRISPR/SpCas9 nickase to both an RT and a serine integrase, allowing PE-mediated installation of short *att* landing sites that are subsequently recognized for site-specific insertion of DNA fragments up to 36 kb at efficiencies of up to 50% in human cells [17]. More recently, the Bxb1 recombinase-based Prime-editing-Assisted Site-Specific Integrase Gene Editing (PASSIGE) strategy was developed, enabling efficient integration of large DNA cargos (> 10 kb) and outperforming PASTE by up to 16-fold in human cell lines with pre-installed landing sites [18]. Meanwhile, in plants, the Prime editing-mediated Recombination Of Opportune Targets (PrimeRoot) system combines engineered plant prime editors with optimized Cre and FLP recombinases to enable efficient integration of DNA fragments up to 11.1 kb in rice. PrimeRoot was further validated in maize, demonstrating cross-species applicability [19]. As editing scale and recombination site scars remain limiting factors, a scarless chromosome editing platform was recently developed for kilobase- to megabase-scale manipulations in plants and human cells. The Programmable Chromosome Engineering (PCE) system uses PE to insert optimized Cre-Lox recombination sites with 10-fold reduced reversibility, followed by targeted genomic recombination mediated by an AI-assisted engineered Cre recombinase, enabling precise insertions up to 18.8 kb and efficiencies up to 4.7-, 5.3-, and 11.8-fold higher than PrimeRoot in rice, maize, and wheat protoplasts, respectively [20].

Beyond recombinase-based systems, the discovery of Tn7-like CRISPR/Cas-associated transposons (CASTs) has introduced a distinct class of genome engineering tools that couple RNA-guided DNA recognition with transposase-mediated integration. In this system, a nuclease-deficient Cas-effector alongside a TnsD/TniQ-like protein recruits a TnsABC transposase complex to integrate its cargo DNA downstream of a guide RNA-specified target. The first programmed CAST-mediated transposition in *E. coli* was based on a nuclease-deficient type I-F Cascade from *Vibrio cholerae* Tn6677 and allowed integration of cargo DNA ranging in size from 290 bp to 10.1 kb at a fixed distance downstream of the target site [21]. Combining high efficiency, specificity, and directionality with minimal tandem-insertion byproducts, CASTs are rapidly emerging as powerful bacterial genome engineering tools [22]. To address the minimal activity of type I-F CASTs in human cells, activity-enhancing mutations were introduced into *Pseudoalteromonas* sp. S983 CAST components, yielding an evolved CAST (evoCAST) system that exhibits over 200-fold higher integration activity, achieves insertion efficiencies of up to 30%, and is capable of integrating DNA fragments exceeding 10 kb [23]. In plants, CASTs have served as the conceptual basis for developing the transposase-assisted target-site integration (TATSI) system, which co-expresses catalytically active SpCas9 or LbCas12a with the rice *Pong* DNA transposon machinery. Following excision from the donor site, the *mPing* transposable element carrying a DNA cargo integrates into induced DSBs, enabling sequence-specific insertion in *A. thaliana* with efficiencies of 27% for small cargo and 8.3% for fragments up to 9 kb, and has also been adapted for soybean, a major crop lacking efficient targeted insertion tools [24].

Recently, the repertoire of nucleic acid-guided systems was expanded through the discovery of IS110 insertion sequences, a family of minimal, autonomous mobile genetic elements. These ‘cut-and-paste’ elements excise from the genome without leaving scars, forming a circular DNA intermediate as part of their transposition cycle. A structured, non-coding bridge RNA encoded by these elements guides sequence-specific recombination, directing the cognate recombinase through two internal

loops, recognizing the genomic target site and the donor sequence, respectively (Figure 1b). Recently demonstrated to be biotechnologically applicable in *E. coli*, these target- and donor-binding loops can be independently reprogrammed to mediate insertion, excision, or inversion of DNA in a programmable manner [25]. Most recently, the related IS*622* element was adapted for targeted induction of inversions and deletions in human cells, mobilizing up to 0.93 Mb of DNA [26]. Given their versatility and ability to mediate complex genome rearrangements without DSB, bridge RNA systems hold considerable promise for future applications.

Large-scale chromosomal rearrangements

Besides the targeted integration of new DNA sequences, the precise rearrangement of existing genomic regions provides an additional layer of genome engineering potential. Such rearrangements can be achieved by inducing two DSBs on the same chromosome, which enables the generation of defined deletions, duplications, or inversions [27–29].

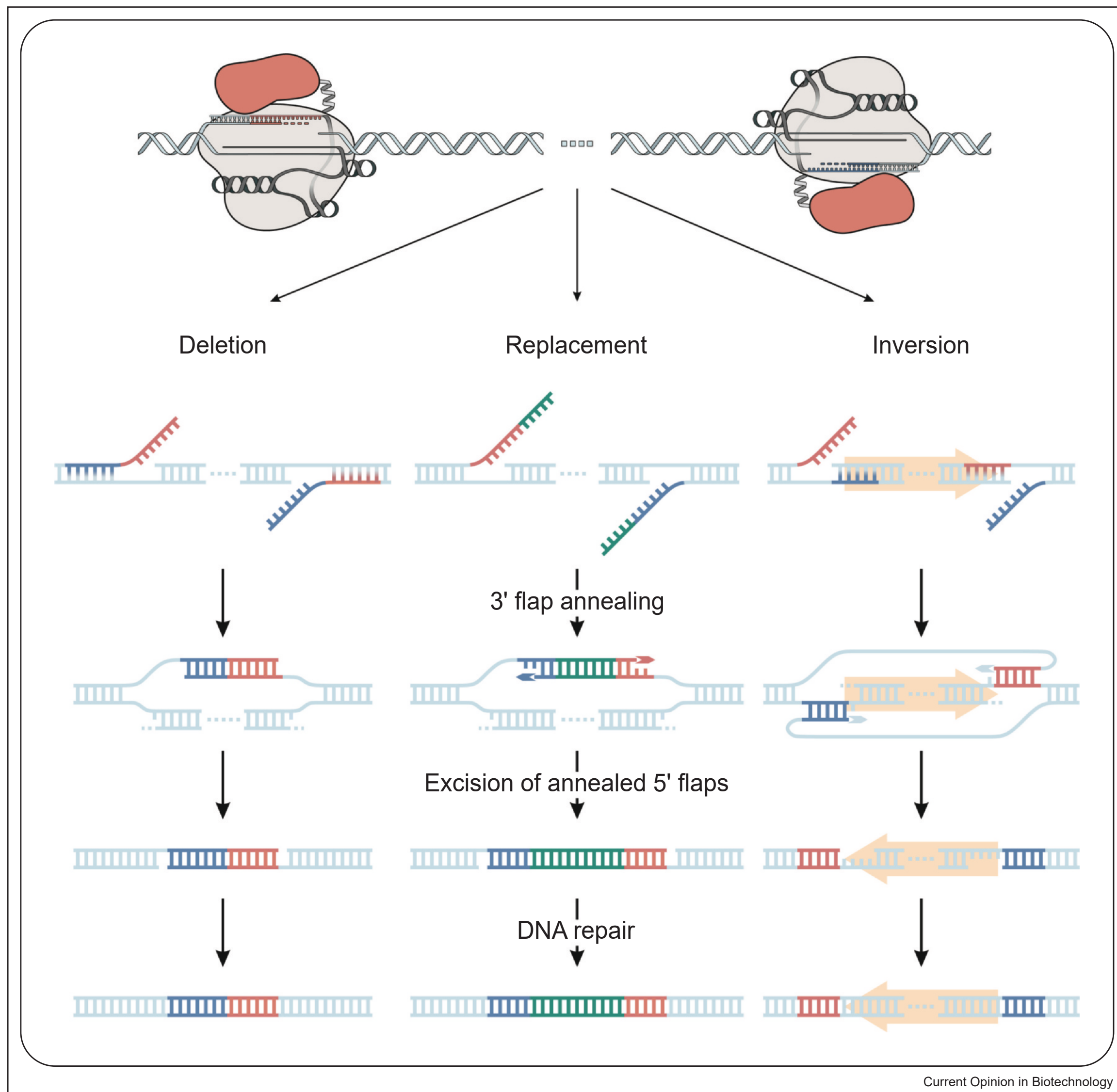
Inversions are especially interesting for plant breeding as they permit the controlled manipulation of genetic recombination to both disrupt and stabilize allelic combinations, a strategy successfully demonstrated in Arabidopsis [27,30]. Interestingly, a recent study on the epigenetic landscape of peri- and paracentric inversions in Arabidopsis suggested that relocating heterochromatic pericentric sequences into euchromatic regions has only a minimal effect on epigenetic states and gene expression across subsequent generations [31]. Besides inversions, CRISPR/Cas-based approaches have enabled targeted deletions in multiple plant species, although their size is often limited by the substantial loss of genetic information. In *A. thaliana*, a recent study demonstrated the targeted removal of retained syntenic blocks of up to 684 kb using SpCas9, encompassing up to 60 genes and 112 transposable elements. The resulting plants remained viable, with some exhibiting distinct phenotypes and widespread transcriptomic changes, pointing to the potential of large chromosomal deletions as tools for genome minimization and allele replacement in plants [28]. In addition to multiplex CRISPR-based strategies for inducing large deletions, a dual prime editing (DualPE) system has recently been established to enable not only large-scale deletions but also gene replacements and inversions with high precision (Figure 2). DualPE combines a plant-optimized prime editor with dual engineered pegRNAs positioned at sites flanking the target DNA fragment to generate complementary 3′ DNA flaps for deletions or inversions, or partially complementary flaps containing the desired sequence changes for replacements. In wheat, DualPE mediated deletions up to 2 Mb, replacements of up to 258 kb, and inversions of up to 205 kb with efficiencies of up to 51%. In *N. benthamiana* and tomato, large-fragment edits reached efficiencies of up to 72%, establishing DualPE as a powerful approach for large-scale chromosomal engineering and precision crop improvement [32]. While DualPE enables precise sequence modifications, megabase-scale inversions are often more efficiently achieved with standard CRISPR/Cas approaches. Thus, this strategy was recently implemented in Arabidopsis to invert a 3.6-Mb genomic segment, which exchanged the promoters of *FLOWERING LOCUS T* and *HTA3*, thereby demonstrating that inversions can also be exploited to alter gene expression by inducing a designed promoter swap between two genes, previously achievable only through transgenic approaches [29,33].

Changing chromosome numbers

Extending the principle of inducing two DSBs to engineer defined rearrangements within a chromosome, inducing two DSBs on separate chromosomes enables the generation of reciprocal translocations, similarly allowing for breaking genetic linkages for plant breeding. Interestingly, *A. thaliana* lines carrying such SaCas9-induced reciprocal translocations of up to 1 Mb between chromosomes I and II or I and IV displayed wild-type-like morphology, minimal and dispersed transcriptional changes, unchanged histone mark profiles, and stable telomere lengths across generations, highlighting the genomic and phenotypic robustness of *A. thaliana* to large-scale chromosome restructuring [34,35].

Most recently, CRISPR/Cas-mediated translocations were successfully harnessed not only to transfer entire chromosome arms but also to fuse independent chromosomes, thereby reducing the karyotype of *Arabidopsis thaliana* from 10 to 8 chromosomes (Figure 3a). Using CRISPR/Cas-mediated breaks at subcentromeric and subtelomeric sites, entire arms of chromosome 3 were fused either to chromosome 1 or redistributed to chromosomes 1 and 5. Despite this substantial karyotype reduction, the plants displayed a wild-type-like phenotype and remarkable transcriptomic stability. However, crosses with wild-type plants resulted in reduced fertility, suggesting that directed chromosome fusions may not only reshape recombination landscapes but also offer new breeding strategies by redefining linkage groups and establishing reproductive barriers to wild relatives. Interestingly, minichromosomes generated through arm transfer were only transiently detected across generations, likely due to the absence of essential genes [36], indicating that minimal requirements for stable inheritance are not yet fully defined. Their reduced meiotic transmission likely reflects species-specific constraints on chromosome size, chromatid cohesion, and bivalent stability [37], making the definition of configurations that ensure faithful segregation a key outstanding challenge.

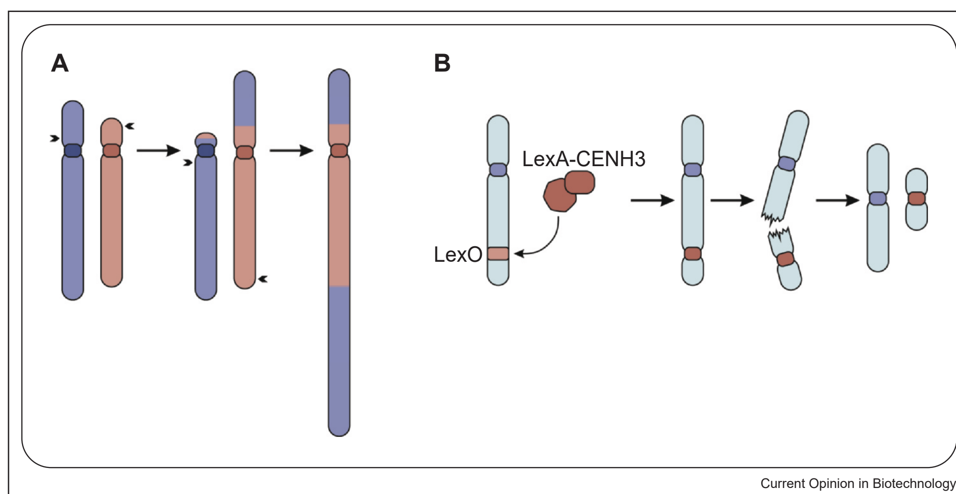
Figure 2



Overview of DualPE-mediated induction of targeted large-scale deletions, replacements, and inversions. DualPE uses two pegRNA flanking the targeted DNA fragment to generate 3′ overhangs, which are designed according to the desired edit. Targeted deletions can be achieved through complementary 3′ flaps, while replacements are induced using partially complementary overhangs harboring the edited sequence. For the precise inversion of a target sequence, the 3′ flaps are complementary to the opposing inversion junction.

While CRISPR/Cas-mediated translocations have demonstrated the possibility of reducing chromosome numbers, complementary approaches have achieved chromosome number increase through *de novo* centromere seeding. For this purpose, LacI-fused CENH3 derivatives were tethered to LacO arrays at non-centromeric sites, reconstituting functional centromeres in maize [38]. Building on this concept, a recent synthetic centromere tethering strategy in maize employed a LexA–CENH3 fusion protein to recruit native CENH3 to LexO repeat arrays on a chromosome arm, enabling kinetochore assembly and inducing random breakage of dicentric chromosomes into self-sustaining, heritable neochromosomes (Figure 3b) [39]. The derived lines carrying 22 chromosomes instead of the typical 20 displayed normal growth, reproduction, and phenotype [40]. Coupled with CRISPR/Cas-mediated engineering, such tethering strategies offer a powerful means to induce targeted chromosome breakage, paving the way for precise karyotype restructuring and *de novo* chromosome construction. The resulting additional chromosomes provide expandable genomic space suitable for high-capacity gene stacking to support structurally complex trait assemblies.

Figure 3



Overview of approaches for targeted karyotype modifications. **(a)** Sequential translocations of entire chromosome arms can fuse independent chromosomes, resulting in one enlarged chromosome and one minichromosome. If — as shown — the minichromosome is lost during segregation, the karyotype is reduced; alternatively, a stably inherited minichromosome can serve as a vector for a PAC. **(b)** Conversely, centromere seeding enables an increase in chromosome number. Here, CENH3 derivatives are recruited to non-centromeric regions via DNA-binding domains (LacI or LexA) tethered to arrays of their recognition sequences (LacO or LexO), thereby reconstituting functional centromeres. During cell division, spindle-induced tension can cause chromosome fission, ultimately producing two distinct chromosomes.

Given their potential as modular platforms for trait stacking, engineered minichromosomes and neochromosomes highlight the growing feasibility of creating synthetic chromosomal platforms suitable for high-capacity applications, including Plant Artificial Chromosomes (PAC) systems. Building on advances in large-DNA integration, PACs provide modular vectors for installing sizeable genomic segments and coordinating multigene traits or entire biosynthetic pathways — such as those underlying stress resilience, metabolic engineering, or quality improvement — while maintaining these assemblies as recombination-insulated, heritable units [41]. Especially in polyploid crops, where genome redundancy complicates conventional trait stacking, synthetic chromosomes could offer orthogonal integration sites with defined copy number and predictable inheritance. Realizing these prospects will require better insight into the minimal gene content compatible with stable minichromosome maintenance, robust design principles for synthetic centromeres across diverse genomic backgrounds, and improved control over segregation fidelity during meiosis. Together, these advances could enable PAC systems to substantially expand the scope of plant genome engineering.

Current practical limitations and bottlenecks

Although chromosome-scale engineering is advancing rapidly, its practical deployment remains limited by challenges in transformation and DNA delivery, which continue to restrict the efficient introduction of large DNA fragments across diverse genotypes. Precise, marker-free integration of sizeable payloads is similarly hindered by low locus-specific efficiencies, though ongoing improvements in editor performance, donor design, and delivery approaches are steadily expanding feasible insertion capacities [37,41,42]. As progress in genotype-flexible delivery platforms and targeted integration technologies continues, these bottlenecks are expected to diminish, thereby broadening the utility of chromosome engineering for plant breeding.

Conclusion

The advent of CRISPR/Cas has expanded plant genome engineering far beyond single-gene alteration, enabling precise interventions across the scale of chromosomes. Among these modifications, large insertions allow the introduction of complex traits directly into native chromosomes. Likewise, CRISPR/Cas-mediated deletions, inversions, and translocations provide tools to restructure chromosomes and control gene dosage, while large-scale deletions could also be applied to chromosome downsizing in polyploids. Such approaches open possibilities to mimic evolutionary processes, explore fundamental limits of chromosome length, and generate artificial reproductive isolation. With the recent breakthroughs in targeted chromosome fission and fusions, even karyotype-scale reprogramming is now within reach. Together, these advances point toward a new era of plant breeding that operates at the chromosomal level, integrating precise editing with synthetic chromosome platforms to create resilient crops and novel genetic diversity.

CRediT authorship contribution statement

Seda Yaşar: Conceptualization, Writing – original draft. **Fabienne Gehrke:** Conceptualization, Writing – original draft, Writing – review & editing. **Niklas Capdeville:** Conceptualization, Writing – review & editing, Visualization. **Holger Puchta:** Conceptualization, Writing – review & editing.

Data Availability

No data were used for the research described in the article.

Declaration of Competing Interest

The authors declare no conflict of interest.

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- • of special interest
- •• of outstanding interest

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