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The future of genome editing in plants

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The future of genome editing in plants differs from how it is used today. For both research and product development, we need to think beyond the creation of simple single-nucleotide polymorphisms and short deletions in genes. We believe that the future of genome editing in plants involves mimicking the natural evolutionary processes that have shaped plant genomes and been the target of artificial selection during crop domestication and improvement. This includes programming large structural variations (insertions, duplications, deletions, inversions and translocations) and controlling plant recombination and endogenous transposable elements that naturally reshape plant genomes. The key is that genome editing will be used to reshape plant genomes in a manner that could have happened naturally, but now these changes can be directed rapidly in the laboratory.

Methods and tools for precision engineering of plant genomes have existed for over 30 years, starting with the introduction of site-specific recombinases (such as Cre and Flp)[1], through the development of sequence-specific programmable nucleases, such as meganucleases, zinc-finger nucleases and transcription activator-like effector (TALE)-nucleases[2], and now the almost exclusive use of CRISPR–Cas[3]. There are three key properties that make CRISPR–Cas the current premiere tool for genome editing: first, a high degree of sequence specificity, with recognition sequences large enough to work on unique sites, even in large plant genomes; second, the relative ease of programmability; and third, the ability to add diverse functionalities, including deaminases (base editors), reverse transcriptases (prime editors), transcriptional enhancers or repressors and several chromatin remodelling functionalities.

How editing is used today

Most of the CRISPR-based applications being used in plant sciences today involve targeting a single gene either using the nuclease function of a Cas-related protein to create a DNA double-strand break (DSB) or using Cas-linked base editors to change bases in an untemplated manner. The DSB is repaired by non-homologous end joining (NHEJ), generating small insertion–deletion (indel) edits at or near genes that in some cases may appear as single-nucleotide polymorphisms (SNPs) (Fig. 1a). NHEJ and base editing generate polymorphisms, typically targeted to a protein-coding gene, microRNA[4], long non-coding RNA[5] or promoter regulatory unit (such as an enhancer). Complexity can be layered by multiplexing the production of guide RNAs (gRNAs) and thus simultaneously targeting multiple genes[6] (Fig. 1b). In addition, multiplexing of gRNAs that target the same promoter has been used to generate sequence variation in the form of SNPs, small indels and larger deletions at promoters (Fig. 1c), which generate expression diversity[7]. The point of targeting these polymorphisms is to generate mutations that affect a target gene, genetic pathway or trait[8]. Yet the majority of genome editing performed today involves targeting a single gene or a small number of genes to induce small indel mutations. Although these small indels are not the subject of this Perspective, they will continue to have a prominent role in both plant research and product development.

How we envision editing technologies will be used in the future

Structural variation (SV) in plant genomes has been naturally and artificially selected to create many of the most important agricultural innovations[9]. Naturally occurring SV is the basis of much of the phenotypic variation on which breeding is based[10–12]. These are classically referred to as intrinsic traits, in the sense that the plant germplasm already has this sought-after ability, and incremental modulation of these traits (such as flowering time and plant architecture) was first selected for, then bred, and now can be directly engineered by genome editing. A common criticism of genome editing is that it can only modulate intrinsic traits, while extrinsic traits are classically defined as those that the plant does not already possess and must therefore be added

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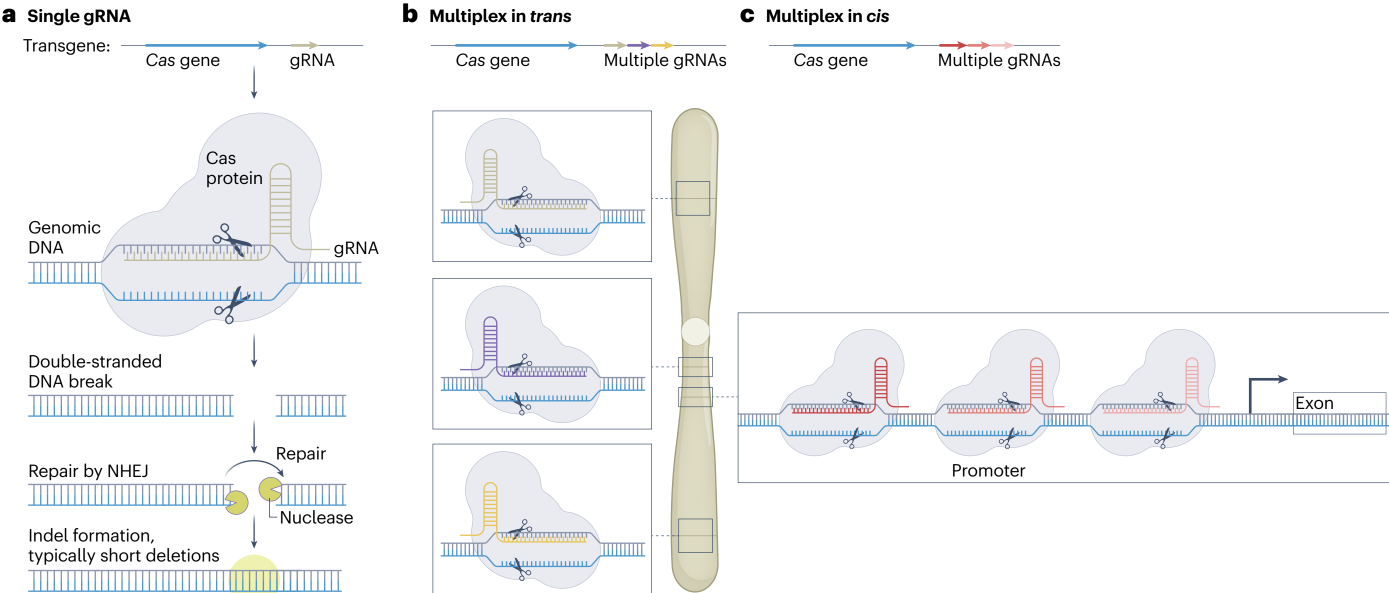

Fig. 1 | Today's use of CRISPR–Cas to generate small-scale indels at or near genes. a, Genome cleavage by a Cas protein directed by the CRISPR gRNA. In plants, the DSB that is generated is most often repaired by NHEJ and results in a short untemplated deletion of 1–10 bp. **b**, Multiplex genome editing in *trans*, where two or more gRNAs direct Cas activity simultaneously to multiple sites of activity. **c**, Multiplex genome editing in *cis*, where two or more gRNAs direct Cas activity to the same region of the genome (such as a gene's promoter), often resulting in larger deletions between gRNA target sites.

via transgenes. The key example of a classic extrinsic trait is the one that is the most valuable to industry: herbicide tolerance. However, key examples exist where plants naturally evolved this 'extrinsic' trait intrinsically from SV in their own genome, completely blurring the lines between the classically defined intrinsic and extrinsic traits. Under heavy selective pressure, agricultural weeds rapidly evolve resistance to herbicides by intrinsically boosting the copy number of the target genes via gene duplication[13] (Fig. 2a). The increased copy number leads to protein levels that are too high for the herbicide to inhibit. SV has therefore been used through artificial selection to improve a classically extrinsic trait, and this suggests that in the future SV could be engineered to generate any trait using a mixture of genome editing and cisgenic (within a breeding pool) gene movement (Fig. 2b).

Multiple recent review articles have summarized the state of the art for producing small edits at the gene level, including highly multiplexed edits, to produce specific phenotypic traits[14,15]. Beyond the generation of SNPs and indels, we believe that the future of genome editing involves mimicking the larger SV cound naturally in plant genomes. SV includes larger deletions (generally defined as changes greater than 50 base pairs (bp)), insertions, duplications, inversions and translocations that rapidly evolve in plant genomes and are observed between strains of the same species[11,12,16]. Genome editing can be used to trigger these processes in a targeted manner through the formation of DSBs, while repair pathways can be manipulated and/or recruited, and specific outcomes beyond SNPs and short deletions screened for. In this Perspective, we focus on the strengths and limitations of novel editing methods and tools to create precise SV, presence–absence variation and chromosomal recombination. Although naturally occurring SV is the basis of much of the phenotypic variation on which crop breeding and domestication are based, the tools and methods to precisely engineer SV are just starting to emerge.

Current technologies to program SV

The efficiency and precision of DSBs created by Cas nucleases can be applied to engineering a wide range of SV. The ends generated by two or more simultaneous DSBs can be repaired by NHEJ (or by homology-directed repair (HDR) if the sequences that flank the DSBs are similar) to create new junctions in any configuration, resulting in all possible inter- and intra-chromosomal SVs. As with any new large genome change that occurs naturally or is induced by a mutagen, not all engineered SVs will be viable or stable, but they can be precisely designed to produce the desired outcome.

Programming chromosomal deletions

In plant genomes, DSBs by a Cas-related protein at single sites typically generate short (<10 bp) deletions, but larger deletions have been observed when gRNAs are multiplexed, resulting in two or more DSBs on the same chromosome (Fig. 2b). This method of generating SV has been used to produce ~1-kb deletions in a tomato promoter that create a range of expression patterns[7], the removal of large transposable-element-rich regions from the *Physcomitrium patens* genome[17] and deletions of >100 kb that remove multiple genes in rice[18]. These deletions can be used to simplify the genome (as in ref. 17), remove tandemly repeated genes and remove entire pathways encoded by biosynthetic gene clusters.

Methods of targeted insertion

After deletions, the simplest case of programming SV is the insertion of transgenes, which historically has been achieved through random integration. The technology to program the target site of DNA insertion is sought after for the integration of epitope tags for research on plant proteins, and in industry for the placement of transgene-encoded traits, such as herbicide resistance genes (Fig. 2c). Targeting transgene insertion can be performed today using recombinases, HDR, PrimeRoot editing, transposon insertion and NHEJ knock-in[19–22].

Targeting trait insertion is beneficial because transgene cargo can be placed into 'SafeHarbor' regions of genomes that do not generate mutations that interrupt an endogenous gene and are permissive to predictable and durable gene expression (high expression is typically desired). In addition, traits can be stacked adjacent or linked to each other (Fig. 2d), reducing the complexity of downstream breeding. Using a combination of Cas9-mediated insertions of *frt* recombination sites, followed by Flp-recombinase-mediated cassette exchange,

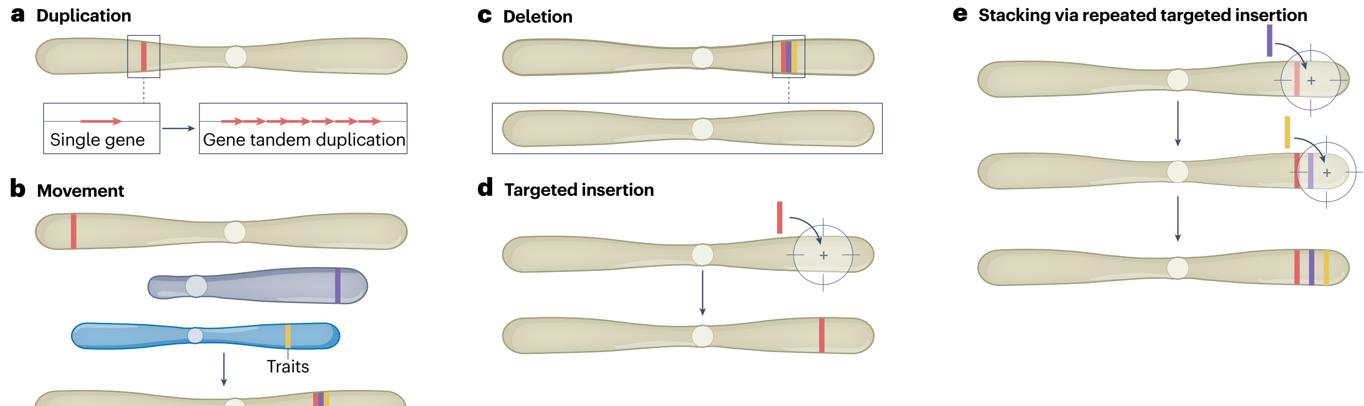

Fig. 2 | Key examples of medium-scale SV induced by genome editing. **a**, Programming tandem duplications of genes or regulatory units. The duplicated region increases in copy number and can result in boosted protein levels and traits. **b**, Movement and insertion of different genes or traits (coloured lines) from different chromosomes to one locus, which is highly beneficial for breeding pipelines. **c**, Programming large deletions using gRNAs at the deletion boundaries. **d**, Targeted insertion of either transgenes or cisgenes can be accomplished using several approaches that combine CRISPR–Cas with recombinase, reverse transcriptase or transposase proteins. **e**, Repeated targeted insertion can be used to stack genes and traits. Targeted insertion is currently being used to cluster beneficial traits and could be used in the future to create custom chromosomes latent with traits moved from across the genome. These new linkage groups are useful in breeding pipelines to speed the introgression of these traits into new germplasms.

a system of 'complex trait loci' has been developed[19]. This enables the stacking of multiple genes into genetically linked arrays, facilitating the introgression of multiple traits that would otherwise be unlinked if plant transformation used standard random integration.

The same technologies that are used for targeted insertion of transgenes can be applied to the cisgenic insertion (or repositioning) of endogenous genes or regulatory regions as the delivered cargo. For example, in rice, Cas9-mediated DSBs were used to produce the precise insertion of transcriptional enhancers ranging from 26 to 64 bp, as well as the insertion of promoters up to 2 kb (ref. 23). The insertion of centromeric repeats that direct chromosome segregation and the formation of artificial chromosomes also represents a desired cisgenic inserted cargo[24].

In addition to simple insertions, regions of the endogenous genome can be replaced. For example, as the expression of the *ARGOS8* gene in maize is relatively weak, the endogenous promoter was deleted and a stronger promoter simultaneously inserted[25]. This insertion occurred via HDR using *ARGOS8* homology arms that surrounded the strong promoter sequence. One can start to envision the future of using gene insertion technologies to cisgenically reconfigure genomes such that important endogenous trait genes are clustered together for breeding efficiency (as in ref. 26).

Programming duplications that alter copy number variation

Gene duplication can result in a gene taking on a new function (neofunctionalization), or the genes can retain their original purpose at a higher dosage and expression level[27]. Copy number variations (CNVs) are structural variants that include duplications and amplifications of genes. CNVs have been described in most major crop species and are thought to have played a key role in the selection of important agronomic traits, including disease resistance[28]. For example, CNVs are common in nucleotide-binding leucine-rich repeat (NB-LRR) genes involved in plant defence. NB-LRR disease resistance genes are often clustered in highly variable tandem duplicated gene islands, which enables the frequent generation of rearrangements, presence–absence variation and natural translocations[29]. It is possible to mimic this natural CNV in a precise manner by creating targeted Cas DSBs in conserved sequences of the genes in the tandem arrays, resulting in changes in the copy number, along with novel alleles[30]. Breeding programmes can also take advantage of NB-LRR translocations to create linkage group clusters of disease resistance genes that bring loci together in the genome so they are inherited as a single locus (Fig. 2b).

The evolutionary process of gene duplication has generated large gene and protein families in plant genomes and can lead to examples of trait boosting and rapid evolution such as the example of herbicide resistance in agricultural weeds described above. Gene duplication leads to CNV, expression increases and natural trait boosting to overcome stresses, such as the repeated convergent evolution of gene duplications that overcome drought tolerance in grasses[31]. Duplicated gene copies can be located adjacent to the originating gene (as in Fig. 2a), on the same chromosome but at a distance, on a different chromosome or even off the chromosome on an episome[32]. Transposable elements are natural gene duplicators[33] that could be used to engineer CNVs in the future.

In addition to genes, larger chromosome segments, entire chromosomes or entire genomes (autopolyploidy) can be naturally duplicated. When CRISPR–Cas is used to program a chromosome deletion, the by-product can be a sister or homologous chromosome that has the corresponding region duplicated[34]. For example, a 300-kb chromosomal duplication was obtained in rice by inducing DSBs at both borders of the duplicated region[35]. However, compared with the other SV that has been programmed by genome engineering, duplication has hardly been taken advantage of and represents a future target of engineering.

Programming chromosomal recombination

The major sources of individual variation on which breeding is based are generated in meiosis through independent assortment of chromosomes and meiotic recombination, especially crossing over. The ability to precisely engineer the positions of crossovers would allow breeders to design and produce the desired variation more precisely (Fig. 3a). The positions of crossovers are determined by the DSBs, the position and frequency of which vary throughout the genome. The fusion of Spo11 to sequence-specific nucleases, including Cas9, has been used to target the positions of meiotic crossovers in yeast[36]. It will be interesting to see whether such an approach will work in plants.

An alternative to targeting meiotic recombination is to create the crossovers in mitotic (somatic) cells during the process of plant transformation. Plants regenerated from mitotic cells in which a reciprocal

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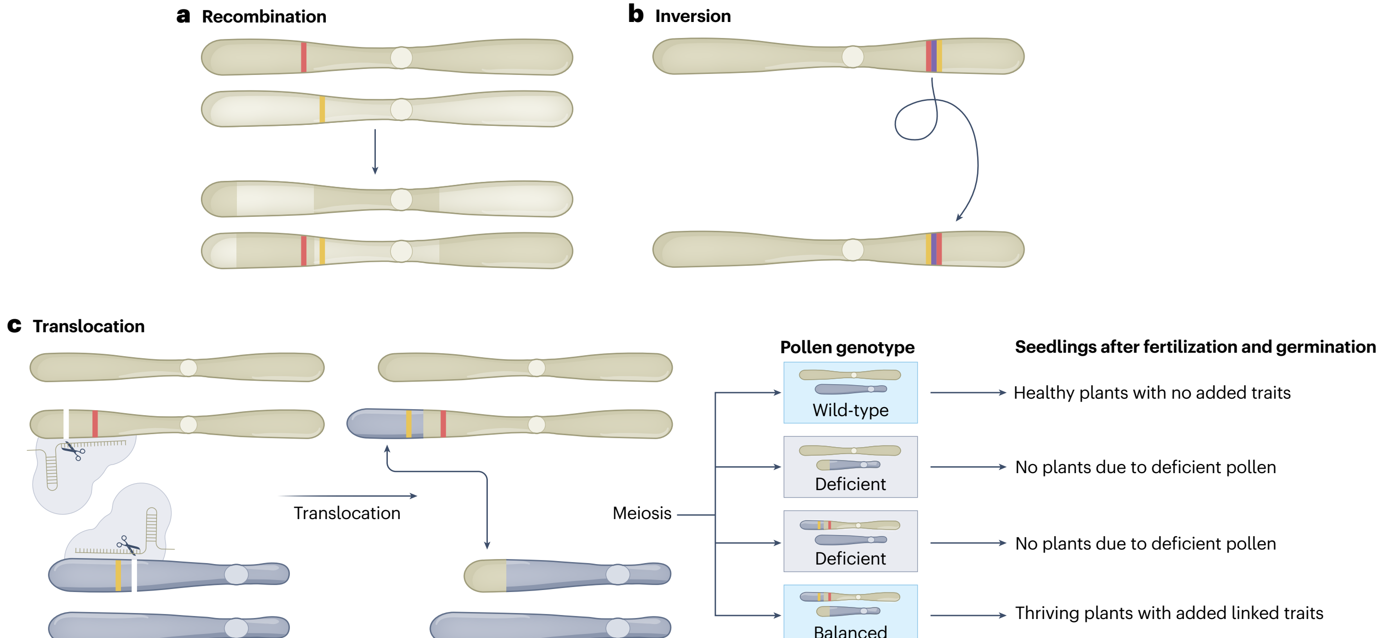

Fig. 3 | Examples of large-scale SV induced by genome editing. a, Programming the sites of recombination events selected by CRISPR–Cas genome editing can be used to break unwanted trait linkage (not shown) or to cluster beneficial alleles on a single chromosome. **b**, Inversions of large segments of chromosomes can be used to suppress recombination or to re-enable recombination in a region that underwent an inversion in the past. **c**, Similar to recombination (**a**), translocations can be used to break genetic linkage between two or more factors, or to group beneficial traits (as shown). In contrast to recombination with homologous chromosomes, translocation occurs between different chromosomes (shown in different colours).

recombination between homologous chromosomes has occurred transmit the recombination event to the next generation through the germline. Moreover, targeted crossovers created in mitotic cells are likely to not be subject to the meiotic phenomenon of crossover interference, opening the possibility of relatively precise double crossovers to pinpoint the targeted transfer of beneficial haplotypes during breeding. Using a single gRNA targeting a specific position on a pair of homologous chromosomes, it was possible to create simultaneous DSBs on both chromosomes in somatic cells, resulting in a reciprocal recombination in maize and in tomato, mimicking meiotic crossovers[37,38].

Generating inversions that block recombination

The application of long-read sequencing technologies to plant genomes revealed that natural Mb-sized inversions are surprisingly abundant in crop cultivars[39]. This might be because inversions do not lead to loss of genetic information and are thus much less detrimental than deletions. Inversions can be programmed with two CRISPR target sites similarly to a large deletion, but with increased screening of the resulting population of the inversion of the central DNA fragment (Fig. 3b) rather than deletion. In one example from rice, a promoter swap that enhanced the expression of a gene of interest was achieved by a 0.9-Mb inversion[35]. In addition to boosting the expression of a single gene, inversions can be used to affect recombination and thus genetic exchange in the respective part of the chromosome if the mating partner lacks the respective inversion. By reverting natural inversions, it is possible to reopen recombination-dead regions for genetic exchange and breeding, as demonstrated in a proof-of-concept experiment in *Arabidopsis*. By combining DSBs induced by Cas9 and an efficient screening protocol, it was possible to obtain a number of recombinants carrying a reversion of the 1.1-Mb knob inversion in Col-0. By crossing the reversion line with a strain that lacked the inversion, genetic exchange was restored after five millennia[40]. This approach has also been applied in crops: a natural 75.5-Mb inversion in maize that spans about a third of chromosome 2 was reverted[41], enabling genetic exchange with other maize cultivars.

In contrast to enabling recombination, it is also possible to use genome engineering to hinder genetic exchange. This was recently demonstrated by inducing a 17-Mb inversion in chromosome 2 of Col-0 *Arabidopsis*, covering 9/10 of its length. After crossing with the ecotype Ler-1, marker analyses demonstrated that the recovery of progeny with crossovers was suppressed in the inverted region[42]. The induction of inversions is thus a powerful tool to redirect genetic exchange on the chromosomal level.

Translocations between chromosomes

Translocations also play a major role during plant genome evolution[43] and can be engineered via genome editing (Fig. 3c). Using Cas9, parts of chromosome arms were exchanged in *Arabidopsis*, including reciprocal exchanges between chromosomes in almost the Mb size range[44]. This has utility if genes are closely linked, as it may be beneficial to separate them to break their genetic linkage. One way to achieve this goal is to induce NHEJ-based reciprocal translocations to localize the genes on different chromosomes. Although the reported frequencies are lower than for inversions, translocations are the most effective means for restructuring plant genomes in a global manner[45]. By consecutive translocations of chromosome arms, it may be possible to change the number of chromosomes and thus the number of linkage groups in a crop species. Translocations are also expected to be an effective means to shrink individual chromosomes to transform them into mini or artificial chromosomes. In contrast, chromosomes might be enlarged to combine multiple beneficial traits (as in Fig. 3c). In either case, consideration needs to be taken to ensure that significant changes in chromosome architecture produce varieties that exhibit meiotic stability during outcrosses and are therefore compatible with downstream breeding strategies.

The new technologies that are needed

Routine programming of plant SV will require both important incremental improvements in the technologies described above and new technologies that do not exist today. Key editing characteristics such

as cargo size, rate of the edit and reduction of off-target unintended mutations are currently the focus of many of these technologies. This includes improving homologous recombination in plants, possibly by influencing the repair pathways to avoid NHEJ after DSB. In addition, now that recombinases, integrases, DNA transposases and reverse transcriptases have all been successfully used with CRISPR–Cas to engineer plant SV, a notable, potentially highly useful protein that has not been domesticated (altered for our use) yet is the replicase/helicase function generated by rolling-circle helitron transposable elements. Helitrons are known to duplicate plant genes at a high rate and leave a very small footprint[33], and they are natural genome engineers that have currently untapped potential for future engineering.

Another technological improvement will be programmable recombinases. The current version of recombinases act on specific recombination sequences that first need to be added to specific sites in the genome and then can participate in DNA removal, DNA addition and/or mediating chromosomal translocations[46]. At first these recombination sites were added to the genome through traditional transgenesis[19], but now PrimeRoot, Twin Prime, PASTE and other approaches can be used to insert these short sequences[20,47,48]. In the future, we may be able to direct the recombinase proteins to work on endogenous sequences (without first integrating recombination sites) in a programmable manner similar to how Cas-related proteins can be programmed with a gRNA, which would be a valuable tool to engineer plant genomes because of their ability to avoid the natural plant preference for NHEJ.

Adding large pieces of DNA to plant cells is still a major obstacle for genome engineering in plants, in contrast to other organisms. Such a technology would be especially attractive for integrating or moving quantitative trait loci or positive haplotypes. Whereas DNA of Mb size can be transferred by yeast cell fusion in mammalian cells[49], such a technology is still lacking for plants. Only in the model moss *Physcomitrium patens* has it been possible to replace a 155-kb chromosomal fragment by synthetic DNA using PEG transformation of shorter fragments and their assembly by homologous recombination[17].

An indirect improvement that will aid all plant genome engineering is the generation of a more high-throughput method to screen for rare editing events. Long-read sequencing is ideal for the detection of SV[50], but de novo genome assembly at the level at which it can be applied to a screening population to identify a rare edit is currently not feasible. The added benefit of large-scale long-read screening will be the simultaneous detection of unintended off-target changes, which is currently performed as a secondary screen only after the primary edit is detected.

A key innovation will be programming plant SV without the integration of the machinery (CRISPR–Cas and other) required to generate the edit. Typically, this machinery is added transgenically to the plant genome to generate the edit but then needs to be removed via another edit, recombination or segregation. This is especially cumbersome for clonally propagated plants where segregation is not an option or extremely difficult. Non-integrating transient transformation is sought after using several different approaches including nanomaterial-based transient plant transformation[51], the use of non-integrating *Agrobacterium* strains[52] and viruses that infect the plant but are not transmitted to the next generation[53]. The use of viruses to encode the machinery needed for plant gene editing is particularly attractive because it avoids laborious and expensive tissue-culture-based plant transformation, but to date these CRISPR–Cas systems are limited to creating small indels[54]. Programming germinal SV without tissue culture or the integration of a transgene into the plant genome is a highly desired future technology.

Before any of the above SVs can be programmed, we need to understand which SV will provide the desired outcome. There is a need now more than ever for basic research on genomics and trait discovery to understand what SV changes to target. The identity of beneficial SVs will probably come from the long-read sequencing of crop germplasm pools alongside screens for beneficial traits. These naturally occurring SVs (or even induced SVs) can then be regenerated in a targeted manner in a germplasm of choice. Trait discovery pipelines will need to adjust to the mindset of programming large SVs in addition to mutations in single genes.

Last, regulatory agencies will have to develop science-based policies to deal with a wide range of SV edits that are currently becoming possible. These SV edits extend beyond where regulatory agencies are today with one-at-a-time indel mutations in single genes. There have been recent positive recommendations towards accepting induced SV and cisgenic approaches in both North America and Europe[55]. Since natural SV has been the basis of traditional crop improvement through screening and breeding, we believe that inducing and programming SV is the next step and the future of genome editing in plants.

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Author contributions

R.K.S. conceived the project. R.K.S., H.P. and L.G. wrote the manuscript.

Competing interests

The authors declare no competing interests.

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