



CRISPR-Cas9 and beyond: what's next in plant genome engineering

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Received: 2 April 2021 / Accepted: 12 April 2021 / Published online: 16 July 2021 / Editor: Gary Bannon
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Abstract

Scientists have developed and deployed successive generations of genome engineering technologies for use in plants, including meganucleases, zinc finger nucleases, TAL effector nucleases, and CRISPR nucleases. Each of these tools has been hailed as potentially revolutionary, capable of providing more efficient and precise ways to modify plant genomes toward improving agronomic traits or making fundamental discoveries. The CRISPR nucleases, in particular, have accelerated the pace of innovation and expanded the boundaries of what is achievable within the plant research space. This review will take care to discuss current plant genome engineering technologies, covering both well-established and up-and-coming tools, as well as describe potential and real-world applications.

Keywords Genome Editing · CRISPR Cas

Room for improvement: generations of genome engineering tools

The process of introducing targeted modifications into a plant genome involves three common steps: recognition of a target DNA sequence, induction of a break, and repair. First, the sequence recognition module of the engineered nuclease recognizes the target DNA sequence. Next, the nuclease binds to the target DNA sequence and creates either a double-strand break (DSB) or single-strand break. Lastly, the DNA break is mended—either by the endogenous DNA repair pathways or by an engineered mechanism. The major DNA repair pathways include non-homologous end joining (NHEJ) and homology-directed repair (HDR) (Symington and Gautier 2011). A notable difference between these pathways is that while NHEJ is an error-prone repair process and often results in the introduction of mutations, such as small insertions and deletions (indels), HDR results in a precise repair. These basic principles underpin all of the genome-editing technologies currently in use, with the key differences between tools

distilling down to additional design features, such as modularity, specificity, efficiency, ease of delivery, and the interplay between these factors. There is no ‘best’ genome engineering tool, just the best tool for a specific job. This is especially true as it pertains to more recently discovered tools, *e.g.*, those under the ever-expanding CRISPR-Cas umbrella.

Meganucleases

Meganucleases, also referred to as homing endonucleases, were first described in the 1990s. In the organisms where they naturally occur, meganucleases are encoded by mobile genetic elements, either introns or inteins, and use double-strand breaks to propagate through the genome as part of selfish DNA elements (Voytas 2013; Smith *et al.* 2006; Paques and Duchateau 2007). Meganucleases are often small proteins that function as homodimer complexes, recognizing large stretches of DNA (20–40 nucleotides) through a site intrinsic to the protein, and subsequently creating a double-strand break (Voytas 2013; Puchta and Fauser 2014). Well-known meganucleases include the yeast mitochondria-derived protein I-SceI and the green algae chloroplast-derived protein I-CreI. Because existing meganuclease binding sites are rare in genomes of interest, meganucleases have been engineered to recognize new target sequences (Seligman *et al.* 2002; Sussman *et al.* 2004; Rosen *et al.* 2006). The relatively long recognition sequence results in higher specificity and lower off-target cutting. However, due to the difficulty of modulating meganucleases to accommodate

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new specificities, engineered meganucleases have had a more limited application compared to other sequence-specific nucleases (Daboussi *et al.* 2015).

Zinc finger nucleases

Zinc finger nucleases (ZFNs) are artificial sequence-specific nucleases that realized a breakthrough in programmable nucleases (Kim *et al.* 1996). ZFNs are engineered by fusing an array of zinc finger DNA-binding domains to the non-specific cleavage domain of the restriction endonuclease FokI (Chen *et al.* 2019). Within the zinc finger array, each domain recognizes a 3-nucleotide target sequence; thus, the makeup and order of the domains can be modified to match the desired site. In the early 2000s, ZFNs were used for sequence-specific mutagenesis in tobacco, which was the first time an engineered endonuclease recognized and cleaved chromosomal DNA (Lloyd *et al.* 2005; Wright *et al.* 2005). Despite these early advances, the application of ZFNs remained limited in plants due to the challenging nature of design and construction, as well as the restricted availability of targeting sites in genomes when compared to more recently developed genome engineering tools.

TAL effector nucleases

Similar to ZFNs, TAL effector nucleases (TALENs) are artificially engineered proteins that are composed of a modular array of DNA-binding domains, fused to the non-specific cleavage domain of FokI. The TALEN DNA-binding domain array design is derived from transcription activator-like (TAL) effectors, a family of proteins which uses such arrays to carry out their function. TAL effectors are secreted by the plant-pathogenic bacteria *Xanthomonas* spp. into plant host cells during infection, where they bind DNA sequences upstream of target genes in a sequence-specific manner, modulating expression of these genes to enhance infection (Mak *et al.* 2013). The DNA binding specificity of TAL effectors is encoded within tandem repeated amino acid sequences, with one repeat binding to a single DNA base (Mak *et al.* 2013). In TALENs, these repeats are arrayed to target specific sequences of interest, leveraging the natural modularity—and elegance—of TAL effectors to achieve genome engineering. Gene targeting reagents using TAL effector scaffolds have included not only TALENs, but also gene-specific activators and repressors (Geissler *et al.* 2011; Zhang *et al.* 2011; Mahfouz *et al.* 2012). Compared to meganucleases and ZFNs, TALENs are more programmable and have been more widely used in plant genome engineering. However, the high number of repeats makes the construction of TALENs and their delivery into plant cells challenging.

CRISPR-Cas nucleases

Similar to TALENs, the CRISPR-Cas system also borrows its elegance from nature. CRISPR-Cas nucleases were first characterized as part of the bacterial and archaeal adaptive immune system. Here, clustered regularly interspaced short palindromic repeats (CRISPR) encode for ‘spacer’ RNA molecules that form complexes with CRISPR-associated (Cas) nucleases and direct these proteins to degrade foreign nucleic acids. In these natural systems, the spacer sequences are derived from fragments of bacteriophages that had previously infected the prokaryote lineage, with the targeting specificity of the system dependent on the simple rules of nucleotide base-pairing. In order for a target DNA site to be recognized and cleaved, the sequence also needs to include a distal or proximal short sequence-specific motif, termed the protospacer adjacent motif (PAM); this requirement ensures that the prokaryote employing the CRISPR-Cas system will not target its own genome, as these motifs are not present between endogenous spacer sequences.

The CRISPR-Cas9 system from *Streptococcus pyogenes* (CRISPR-*SpCas9*) was the first to be developed for genome engineering (Barrangou *et al.* 2007; Bhaya *et al.* 2011; Terns and Terns 2011; Sorek *et al.* 2013; Koonin *et al.* 2017; Shmakov *et al.* 2017), and ‘CRISPR-Cas9’ often refers to this particular tool. To avoid confusion, herein, CRISPR-Cas9 is only used to refer to features common to CRISPR-*SpCas9* and its orthologs; in other cases, the origin species is noted. The engineered CRISPR-Cas9 system has two components: the (1) Cas9 nuclease and (2) a single guide RNA (sgRNA), consisting of a fusion of two RNA molecules—the spacer-containing CRISPR RNA (crRNA) and the transactivating crRNA (tracrRNA), which is required for maturation of the former moiety. The sgRNA directs the nuclease complex to a specific target DNA site, initiating the cleavage of the complementary DNA sequence (Chen *et al.* 2019). Cas9 possesses a bi-lobed architecture, with the smaller nuclease lobe containing two nuclease domains, RuvC and HNH, which cleave DNA strands that are complementary and non-complementary, respectively (Gasiunas *et al.* 2012; Jinek *et al.* 2012). In 2013, three independent groups established the CRISPR-*SpCas9* system for use in rice, wheat, tobacco (*Nicotiana benthamiana*), and Arabidopsis (Li *et al.* 2013; Nekrasov *et al.* 2013; Shan *et al.* 2013), and the development and application of this system has since progressed rapidly (Chen *et al.* 2019; Zhang *et al.* 2019; Wada *et al.* 2020). The CRISPR-Cas9 system has many advantages over other plant genome engineering tools (Andolfo *et al.* 2016)—including relative ease of design, cloning, and delivery into plant cells—which has led to the wide adoption of this technology and the development of myriad related technologies.

There are multiple strategies to deliver CRISPR-Cas reagents into plant cells, including stable expression, transient

expression, and DNA-free delivery. Genome engineering using stable expression of CRISPR-Cas DNA has been widely and successfully applied, through the tried-and-true methods of plant transformation and selection. However, some applications are incompatible with CRISPR constructs or marker genes integrated into the genome. In these cases, transgene-free derivatives can be obtained from stable transgenic plants through segregation over successive generations of plant propagation, or alternative methods for CRISPR-Cas reagent delivery can be used. In the transient CRISPR-Cas DNA delivery method, the normal selection steps are eliminated such that some regenerated plants are edited without any DNA integration, although this strategy can complicate downstream plant screening (Andersson *et al.* 2017; Lin *et al.* 2018; Chen *et al.* 2018). Additional methods involve delivering transcripts encoding CRISPR-Cas reagents directly into embryos to generate edited plants, albeit with lower editing efficiency (Zhang *et al.* 2011). Recently, an RNA virus-based vector system has been engineered for transgene-free delivery of the CRISPR-Cas cassette for efficient genome engineering in plants (Ma *et al.* 2020). Additionally, a DNA-free system has been developed in plants using Cas9-sgRNA ribonucleoprotein (RNP) complexes, which have been shown to be as efficient as stable delivery, as well as to exhibit a low off-target frequency (Woo *et al.* 2015; Svitashv *et al.* 2016; Liang *et al.* 2017, 2018).

Nothing was the same: the CRISPR-Cas revolution

Cas9 is not the only CRISPR-associated protein—it is just one family of proteins among many other CRISPR protein families. There are two major classes of CRISPR systems, differentiated by the method of CRISPR RNA processing: if the pre-CRISPR RNA processing and interference stages are accomplished by one single multifunctional protein, the CRISPR system is categorized as a class 2 system; otherwise, it is categorized as a class 1 system (Makarova *et al.* 2015). Each of these classes is divided into multiple types according to their signature proteins: type I, III, and IV belong to class 1, with Cas3, Cas10, and Csf1 as their respective signature proteins, while type II (Cas9), type V (Cas12a–e, Cas12g–i, and Cas14a–c), and type VI (Cas13a–d) belong to class 2 (Makarova *et al.* 2015). Recent research has expanded access to CRISPR-Cas systems beyond Cas9, elevating new systems with distinct advantages and disadvantages.

SpCas9 variants

The *SpCas9* protein has been extensively engineered to broaden the PAM compatibility, enhance specificity, and confer new functionality (Karvelis *et al.* 2017). *SpCas9* requires a 5'-NGG-3' PAM, where 'N' is any nucleotide, limiting which

genome positions can be targeted, especially in AT-rich species. So far, rational engineering of *SpCas9* has resulted in the generation of new Cas9 variants with four alternate PAM preferences (Kleinstiver *et al.* 2015; Nishimasu *et al.* 2018). One of these engineered Cas9 proteins has already been used to edit rice and Arabidopsis plants (Endo *et al.* 2019; Ge *et al.* 2019; Hua *et al.* 2019; Niu *et al.* 2020; Zhong *et al.* 2019), pointing to the promise of this strategy to expand the availability of target sites. In addition, there are a number of *SpCas9* proteins that have been rationally engineered to have enhanced specificity for their target sequence, thus reducing off-target activity (Zhang *et al.* 2017a, 2017b; Abudayyeh *et al.* 2016; Chen *et al.* 2017). Similar variants have been developed using directed evolution (Casini *et al.* 2018; Hu *et al.* 2018; Lee *et al.* 2018). Other engineered *SpCas9* variants include nuclease domain mutants, which result in new functional characteristics. Inactivation of a single nuclease domain results in a nuclease that only causes a single-strand break, termed a 'nickase' (nCas9), whereas inactivation of both domains produces a deactivated Cas9 protein (dCas9) (Jiang and Doudna 2017).

SpCas9 orthologs

Complementary to *SpCas9* engineering efforts, there has been a lot of research around discovering Cas9 orthologs from other species, as evolution has already worked to produce variants with diverse functionalities. So far, Cas9 orthologs from a number of bacterial species have been characterized, including *NmCas9* from *Neisseria meningitidis* (Hou *et al.* 2013), *SaCas9* from *Staphylococcus aureus* (Ran *et al.* 2015), *StCas9* from *Streptococcus thermophilus* (Müller *et al.* 2016), *FnCas9* from *Francisella novicida* (Hirano *et al.* 2016), and *CjCas9* from *Campylobacter jejuni* (Kim *et al.* 2017). These Cas9 orthologs have different PAM preferences, expanding the range of sequences that can be targeted (Wada *et al.* 2020). In addition, the genes encoding most of these proteins are smaller than *SpCas9*, providing an advantage for delivery into plant cells (Wada *et al.* 2020). Some of the Cas9 orthologs, such as *NmCas9*, bind longer target sequences, conferring additional specificity (Hou *et al.* 2013). Having a collection of Cas9 orthologs to choose from is also beneficial when designing orthogonal gene targeting experiments, as these nucleases can be used simultaneously to target different sites in the genome (Steinert *et al.* 2015 Puchta 2017).

Cas12a (Cpf1)

Cas12a—formerly known as Cpf1, *CRISPR from Prevotella and Francisella 1*—is a class 2 type V nuclease that has broad utility in genome engineering. Cas12a functions in a similar fashion to Cas9, forming a complex with a sgRNA to target specific DNA sequences to create double-stranded breaks.

Cas12a requires a T-rich PAM sequence, as compared to the G-rich PAM of Cas9, broadening the species and sites that can be targeted. The sgRNA architecture that is required for Cas12a is shorter than the Cas9 sgRNA architecture, making the Cas12a sgRNAs easier to synthesize, multiplex, and engineer. Moreover, Cas12a possesses RNase activity, meaning it can self-process a polycistronic CRISPR sgRNA array for multiplexed genome editing (Ran *et al.* 2015; Yamano *et al.* 2016). Cas12a also cuts DNA in a staggered fashion, creating an overhang which may promote repair and thus hypothesized to improve HDR efficiencies (Zetsche *et al.* 2015).

There are a number of Cas12a orthologs currently in use, including proteins from *Francisella novicida* (*FnCas12a*), *Acidaminococcus* sp. (*AsCas12a*), and *Lachnospiraceae bacterium* (*LbCas12a*) (Zetsche *et al.* 2015). Some of these Cas12a orthologs have been found to be more specific than *SpCas9* in several biological systems (Kim *et al.* 2016; Kleinstiver *et al.* 2016b; Tang *et al.* 2017; Zhong *et al.* 2018). Characterization of diverse Cas12a orthologs has identified nucleases with more varied PAMs beyond the standard site (Marshall *et al.* 2018). Similar to Cas9, Cas12a variants have been engineered to recognize different PAMs, with more than five distinct PAM sites currently available (Gao *et al.* 2017; Li *et al.* 2018; Zhong *et al.* 2018). Moreover, multiple versions of catalytically inactive Cas12a (dCas12a) have been engineered and repurposed for different applications (Zetsche *et al.* 2015; Yamano *et al.* 2016; Tang *et al.* 2017; Zhang *et al.* 2017b). So far, *LbCas12a* has been most widely deployed, having been used to successfully create edits in rice (Begemann *et al.* 2017a, 2017b; Xu *et al.* 2017; Yin *et al.* 2017). Continual advancements in the application of Cas12a for genome engineering will position this nuclease as complimentary to Cas9.

Cms1

In addition to Cas12a, another group of class 2 type V enzymes, termed Cms1s—CRISPR from *Microgenomates* and *Smithella 1*—efficiently generate indel mutations in rice (Begemann *et al.* 2017a, 2017b). Cms1 nucleases are smaller than both Cas9 and Cas12a nucleases and do not require a transactivating crRNA (Begemann *et al.* 2017a, 2017b). Cms1 nucleases also have an AT-rich PAM site requirement, which can offer obvious situational advantages as compared to other CRISPR-Cas nucleases (Begemann *et al.* 2017a, 2017b). Although Cms1 nucleases are still being developed for broader application, the differences in the function of these enzymes make them a potentially invaluable addition to the plant genome engineering toolbox.

Cas13a

Cas13a, formerly known as C2c2, has RNase activity and targets single-stranded RNA for degradation (Abudayyeh *et al.* 2016). Instead of a PAM, Cas13a requires a protospacer flanking site to induce a single-strand break. Interestingly, Cas13a also shows non-specific RNase activity that cleaves collateral RNA following initial binding to its target RNA *in vitro* and in bacteria (Abudayyeh *et al.* 2016; Gootenberg *et al.* 2017). This particular feature has led to the development of SHERLOCK, the specific high-sensitivity enzymatic reporting unlocking method, which can be used to detect specific RNA and DNA sequences (Abudayyeh *et al.* 2016). The CRISPR-Cas13a system has been used to decrease the expression of endogenous genes in rice and tobacco (*N. benthamiana*) (Abudayyeh *et al.* 2016; Aman *et al.* 2018). This system has also been engineered to interfere with RNA-guided immunity in Arabidopsis and tobacco (*N. benthamiana*) (Aman *et al.* 2018). These specific applications go beyond the possibilities presented by CRISPR-Cas9. Testing and developing more Cas13a orthologs and variants in plants will add additional RNA-targeting tools for transcriptional regulation, RNA base editing, RNA tracking, functional studies, pathogen detection, and disease control (Zhang *et al.* 2019).

Base editors

Base editing is a type of genome engineering that involves making specific nucleotide substitutions without reliance on the formation of a double-strand break or a donor template. To date, most base editing systems have involved linking base editing enzymes to nCas9, which is able to guide the enzyme to target sites where activity is then proximity-induced. The cytosine base editor (CBE) systems mediate the conversion of C to T in genomic DNA, with the base-editing enzymes including the combination of a cytidine deaminase and a uracil glycosylase inhibitor (Komor *et al.* 2016). Different CBE systems have leveraged different cytidine deaminase enzymes, with the rat APOBEC1 enzyme the most widely used in plants (Hess *et al.* 2017). C-to-G base editor systems have also been applied in plants, relying on nCas9 linked to CBE variants and uracil DNA *N*-glycosylase. The adenine base editor (ABE) systems mediate the conversion of A to G in genomic DNA (Gaudelli *et al.* 2017). This system links an nCas9 to an adenosine deaminase, with the *Escherichia coli* TadA enzyme the most commonly used in plants (Gaudelli *et al.* 2017). Base-editing systems offer several advantages over non-DSB-mediated genome editing in plants, including greater efficiency and precision. Moreover, multiplexed base editing is less likely to result in chromosomal rearrangements—which can happen with standard multiplexed editing. Additional research with a focus on the improvement of the efficiency and

specificity of these technologies, as well as their compatibility with multiplexing, will underpin more successful precision breeding efforts.

Prime editors

Prime editing is one of the latest additions to the CRISPR-Cas toolbox. This system performs RNA template-based DNA modifications using an engineered reverse transcriptase and is able to install small additions, deletions, and all 12 possible nucleotide conversions (Anzalone *et al.* 2019). A prime editor involves a fusion of nCas9 and reverse transcriptase, programmed with prime editing guide RNAs (pegRNAs) that encode the desired edit (Q. Lin *et al.* 2020). Prime editing was first applied in rice and wheat, with prime-edited rice plants obtained at high frequencies (Lin *et al.* 2020). Several subsequent studies have reported varying efficiencies in rice and other species (Butt *et al.* 2020; Hua *et al.* 2020; Jiang *et al.* 2020; Veillet *et al.* 2020). A vector system for prime editing was recently developed and validated for use in tobacco (*N. benthamiana*), rice, and Arabidopsis (Wang *et al.* 2021). The programmable and precise nature of prime editing makes it a powerful tool for plant genome engineering, and there will undoubtedly be an explosion of studies that apply and improve upon this tool.

Views: applications of genome editing in plants

The profusion of available genome engineering tools, as well as improvements in editing efficiency and precision, has led to an expansion of potential applications for these technologies. More recently developed tools have been used to induce homology-directed repair and edit multiple sites in parallel—approaches that stand to radically alter the feasibility of using genome engineering for basic research and crop improvement. In addition to discussing these advancements, this section provides a conceptual introduction to the types of edits that one could conceive of, using any number of the current technologies (Fig. 1).

Homology-directed repair

The homology-directed repair (HDR) pathway uses a template with homology to a double-strand break site to patch DNA damage. The template can be derived from the genome, such as from a sister chromatid, or from exogenously supplied DNA. For the type of HDR used in genome engineering, a repair template with desired sequence modifications is provided along with editing machinery, and the endogenous HDR pathway incorporates these changes into the break site (Salsman and Dellaire 2017). HDR can be used to introduce specific point mutations, as well as to insert, or replace,

desired sequences at a target DNA site. Precise gene modifications—such as knock-ins and replacements—facilitate breeding by introducing new alleles without linkage drag or generating allelic variants that do not exist naturally. However, it is still quite challenging to perform HDR-mediated gene targeting in plants due to the low efficiency of HDR and the limitations of donor template delivery in plant cells. One approach to increase HDR efficiency is to increase the amount of donor DNA delivered to the cell. Toward this end, a geminivirus-based DNA replicon has been used to increase the number of repair templates and improve efficiency across multiple plant species (Čermák *et al.* 2015; Butler *et al.* 2016; Wang *et al.* 2017; Dahan-Meir *et al.* 2018; Hummel *et al.* 2018). HDR efficiency can also be improved by changing the design or delivery method of the donor template. These modifications include increasing the length of homology arms or including a tag for targeting (Carlson-Stevermer *et al.* 2017; Ma *et al.* 2017; Aird *et al.* 2018; Ghanta *et al.* 2021).

Multiplex editing

Plant traits are rarely determined by a single gene but are dependent on the contributions of multiple genes. Thus, the goals of genome engineering go beyond editing single sites to editing multiple sites simultaneously, called multiplex editing. Multiplex editing has a number of exciting applications for genome engineering: it can be used to create multi-gene knockouts, chromosomal deletions and translocations, gene knock-ins, and quantitative variation across multiple sites (Salsman and Dellaire 2017). Of all of the available gene editing tools, the CRISPR-Cas system is by far the most amenable to multiplex editing, as multiple sgRNAs can be expressed along with a single nuclease to achieve editing at many distinct target sites (Hashimoto *et al.* 2018; Zsögön *et al.* 2018; Najera *et al.* 2019.) There are many methods to multiplex sgRNA expression, including using multiple tandem expression cassettes, or expressing sgRNAs as a polycistronic transcript under the control of a single promoter. In the latter case, the sgRNAs can be interspersed with ribozyme sites (Gao and Zhao 2014), Csy4 recognition sites (Čermák *et al.* 2017), or transfer RNA sequences (Xie *et al.* 2015)—all of which allow for processing in the plant cell to release mature sgRNAs for editing (Chen *et al.* 2019). In plants, CRISPR-Cas multiplex editing has been achieved in multiple species, with a recent publication reporting one-shot generation of 8× *N. benthamiana* and 12× Arabidopsis mutants (Stuttman *et al.* 2021).

Protein-coding edits

Genome engineering tools have been primarily used to make edits in coding regions. To create a gene knockout, an edit can

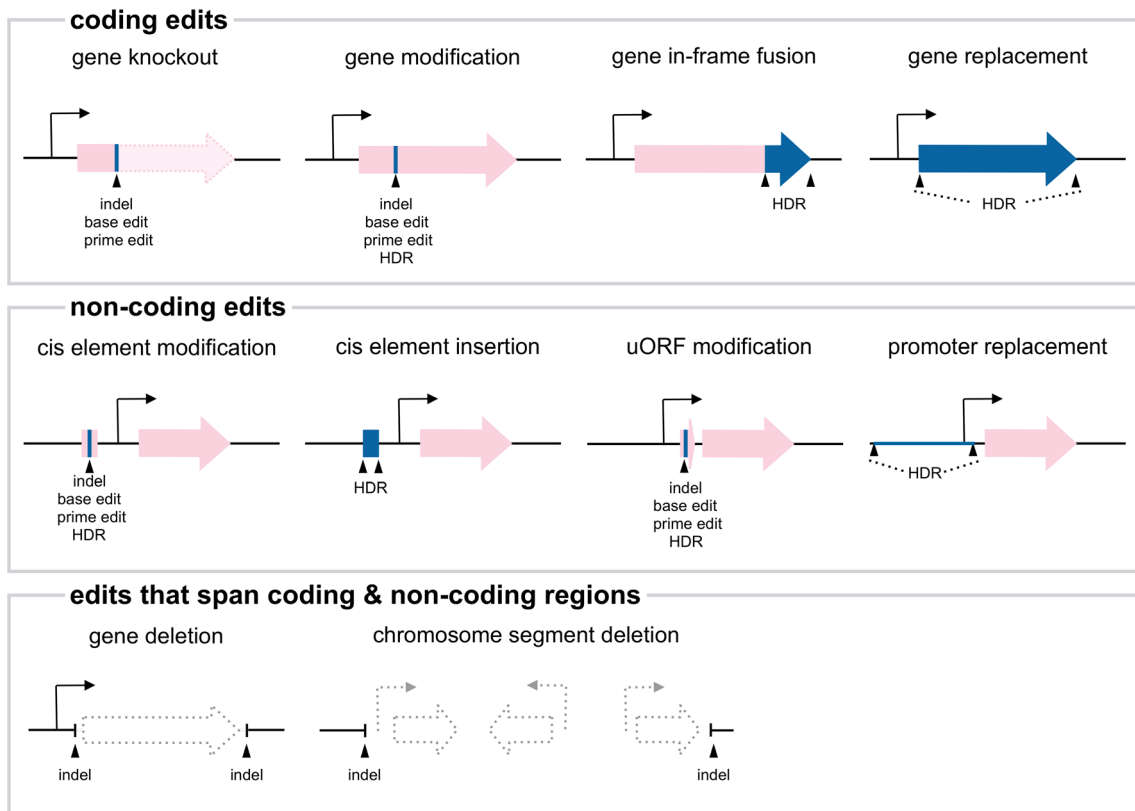


Figure 1. Types of edits that can be generated with genome engineering technologies. Range of edits that can be made using targeted insertions or deletions (indels), base edits, prime edits, or homology-directed repair (HDR). Edits can be generated in coding or genic regions (*top*), including gene knockouts, modifications, in-frame fusions, and replacements. Edits

can also be targeted to non-coding regions (*middle*); this includes cis element modifications and insertions, as well as modifications to upstream open reading frames (uORF) or whole promoter replacement. Edits can also span coding and non-coding regions (*bottom*), resulting in whole-gene deletion or even larger chromosome segment deletions.

be targeted to an exon upstream of the protein active site residues. In this case, small insertions, deletions, or base changes can cause a frameshift mutation, leading to an early stop codon and a non-functional protein (Fig. 1). In cases where the protein structure is flexible enough to allow for modification, targeted deletions or precise modifications within genes can also be used to alter protein activation or repression sites (Fig. 1). Targeted insertions via HDR can be used to generate in-frame fusion proteins at the endogenous locus, a creative strategy to study the biological function of a given protein (Wang *et al.* 2017) (Fig. 1). Similarly, HDR-mediated insertions can be used to make gene replacements, including allele swaps (Fig. 1). Although not strictly protein-coding edits, tandem edits can be used to make mutations that span coding and non-coding regions, leading to whole-gene deletions or chromosomal segment deletions (Fig. 1) (Zhou *et al.* 2014; Belhaj *et al.* 2015). In the latter case, larger deletions can target clusters of genes with related functions, an especially powerful approach where there is predicted functional redundancy. All of these types of edits can be used to create desired phenotypes or study the function of a gene of interest.

Non-coding edits

Genome-editing technologies can also be used to create modifications in cis-regulatory regions to alter gene regulation. This strategy has primarily focused on promoter sequences, such as replacing whole promoters via HDR or editing specific cis-regulatory elements (Piatek *et al.* 2015; Peng *et al.* 2017) (Fig. 1). In tomato, researchers edited the promoter regions of quantitative trait-related genes, creating a continuum of variation and leading to the selection of artificial alleles with improved traits (Rodríguez-Leal *et al.* 2017). Similar work on other agronomic genes of interest will allow for an exploration of dosage effects, leading to a more precise understanding of the relationship between gene expression and phenotypic variation. Cis-regulatory editing will prove instrumental for engineering plants with desirable characteristics, especially in cases where specific gene overexpression is the most promising route and natural variation is limiting. In these cases, it will be possible to insert enhancer sequences, or other cis-elements that lead to promoter upregulation, in order to increase transcription of a target gene (Fig. 1). In addition to

promoter engineering, gene regulation can also occur at the translation level, such as upstream open reading frames (uORFs) (Fig. 1). uORFs are well-characterized cis-elements, widespread among plant mRNAs, which often negatively regulate translation and mRNA decay (von Arnim *et al.* 2014). It has been shown that CRISPR-Cas targeting of a uORF in lettuce was able to significantly alter the metabolic profile of the leaf (Zhang *et al.* 2018). Additional research targeting uORFs is a promising strategy to alter phenotypes of interest and create plant varieties with desirable characteristics without making modifications to protein-coding regions.

Off-target edits

In addition to intended mutations, off-target mutations can also occur with the application of genome-editing tools. With all gene editing tools, there are known trade-offs between editing specificity and other priorities, such as ease of reagent design, cloning, delivery, and efficiency. Minimizing the likelihood of off-target mutations is one priority that must be balanced against other priorities and weighed accordingly based on research aims. Whereas off-target edits may not be such a concern when engineering plants to study the function of a particular protein in the lab, off-target edits are of critical importance when producing varieties that you may want to bring to market. Although off-target edits have often been discussed as a non-concern when applying CRISPR-Cas systems, studies that have performed whole-genome sequencing to detect off-target mutations resulting from the application of Cas9 or Cas12a nucleases have revealed that both of these nucleases have low incidence of off-targeting (Feng *et al.* 2014; Tang *et al.* 2018; Li *et al.* 2019).

Based on large-scale editing or binding experiments undertaken in non-plant systems, CRISPR-Cas off-target edits can be largely mitigated by prioritizing target specificity during sgRNA design by ensuring that your target sequence is unique and does not occur elsewhere in the genome, either as an exact match or with a low level of mismatches (Kim *et al.* 2016; Luo *et al.* 2019; Specht *et al.* 2020). Although this may be straightforward in species with small genomes, this may prove more challenging in species that have undergone whole-genome duplications. Moreover, specifically targeting edits to coding regions may be easier to accomplish than to non-coding regions, such as promoters, which can be highly repetitive.

Conclusions

With the benefit of hindsight, the pre-CRISPR-Cas9 part of the story of plant genome engineering can almost read like a prelude. However, it is worth considering that the principles

that underpin the use of CRISPR-Cas9 to engineer plants—delivery of reagents into the cell, the breadth and diversity of desired edits, responsible use of genome engineering, etc.—have been under development for decades, with invaluable input from other fields of study. That said, it is difficult to overestimate the impact that CRISPR-Cas9 has had, and will continue to have, on plant science, not least of which is the development and application of related CRISPR-Cas technologies. Cas12a, Cms1s, and Cas13a are all essential components of the growing genome-editing toolbox, with distinct use cases that transcend the possibilities of Cas9 techniques alone. The imagined and real-world applications for all of these tools, and the available tools themselves, seem to multiply every day, rendering review articles such as this one out-of-date by the time of publication. The pace and impact of these developments make this an exceedingly exciting time to be working in the plant genome engineering space.

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