

Applying gene editing to tailor precise genetic modifications in plants

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Joyce Van Eck*

From the Boyce Thompson Institute, Plant Breeding and Genetics Section, School of Integrative Plant Science, Cornell University, Ithaca, New York, USA

Edited by Joseph M. Jez

The ability to tailor alterations in genomes, including plant genomes, in a site-specific manner has been greatly advanced through approaches that reduced the complexity and time of genome sequencing along with development of gene editing technologies. These technologies provide a valuable foundation for studies of gene function, metabolic engineering, and trait modification for crop improvement. Development of genome editing methodologies began ~20 years ago, first with meganucleases and followed by zinc finger nucleases, transcriptional activator-like effector nucleases and, most recently, clustered regulatory interspaced short palindromic repeat (CRISPR)-associated protein (Cas) (CRISPR/Cas), which is by far the most utilized method. The premise of CRISPR/Cas centers on the cleaving of one or both DNA strands by a Cas protein, an endonuclease, followed by mending of the DNA by repair mechanisms inherent in cells. Its user-friendly construct design, greater flexibility in targeting genomic regions, and cost-effective attributes have resulted in it being widely adopted and revolutionizing precise modification of the genomes of many organisms. Indeed, the CRISPR/Cas system has been utilized for gene editing in many plant species, including important food crops, such as maize, wheat, rice, and potatoes. This review summarizes the various approaches, including the most recent designs being used to make modifications from as small as a single-base-pair change to insertion of DNA fragments. On the gene expression level, strategies are presented that make it possible to knock out or modulate through activation and repression. Also discussed are prerequisites necessary for CRISPR/Cas-mediated editing as well as the current challenges.

Genome or gene editing refers to a group of technologies that provide the ability to precisely modify the DNA of an organism through the addition, deletion, or alteration of genetic material. The development of gene editing technologies that made it possible to target specific genome regions was like finding the Holy Grail for plant genetic engineering and trait modification. Whereas gene delivery methods referred to as genetic engineering are also used to alter genetic material, there is no control over where integration occurs. However, genetic engineering has played a critical role for studying gene function and modifying traits at the molecular level. Site-directed genome modification has evolved along with advances in gene delivery methods and genome sequencing technologies. The evolution of editing methodologies began by targeting genes in mamma-

lian cell lines and in organisms such as bacteria and zebra fish; however, as methods became available in these systems, they were readily adapted for plant gene editing. The first approach available exploited meganucleases to achieve targeted double-strand DNA breaks in genomes of eukaryotes (1). Development of sequence-specific nuclease-based technologies that followed included zinc finger nucleases (ZFNs) (2), transcriptional activator-like effector nucleases (TALENs) (3), and, most recently, clustered regulatory interspaced short palindromic repeat (CRISPR)-associated protein (Cas), which was designated CRISPR/Cas (Fig. 1A) (4). Whereas each editing methodology has been used successfully in plants, the focus of this review will be on CRISPR/Cas, because it is by far the most widely used approach in plants.

The foundation of the CRISPR/Cas gene editing system was built based on the adaptive immune defense mechanism that bacteria deploys in response to viruses and plasmids (4). The CRISPR systems are categorized according to the Cas, resulting in two classes, Class 1 and Class 2 (5). Class 1 is composed of type I and type III Cas, found in *Archaea*, whereas Class 2 is a compilation of types II, IV, V, and VI from different bacteria, such as *Streptococcus pyogenes*, *Lachnospiraceae bacterium*, and *Francisella novicida* (5, 6). In brief, CRISPR/Cas-mediated editing is based on guidance of short RNA sequences, termed guide RNAs (gRNAs), designed to complementarily target DNA that is then cleaved by a Cas endonuclease (Fig. 1B). The endonuclease has a DNA cleavage recognition motif, referred to as a protospacer adjacent motif (PAM). The Cas protein cleaves DNA a few base pairs away from the PAM. Subsequent to the first reports of successful editing in plants through application of CRISPR/Cas, there have been many reviews that describe the basics and mechanisms of this powerful tool for precise genome modification (7–9).

CRISPR/Cas as with other methods of gene editing exploits the two different types of DNA repair mechanisms nonhomologous end-joining (NHEJ) and homologous recombination (HR) (Fig. 1B). NHEJ is the primary repair mechanism in plant cells, and its imprecise repair results in insertions or deletions (indels) within a gene sequence, resulting in disruption of gene expression (10). The knockout lines that are recovered are often exploited for a reverse genetics approach to decipher gene function. On the other hand, repair through HR is less frequent in plant cells because it is significantly inefficient compared with NHEJ (11). One of the advantages of using CRISPR/Cas, especially as a mutational tool in plants, is that through

* For correspondence: Joyce Van Eck, jv27@cornell.edu.
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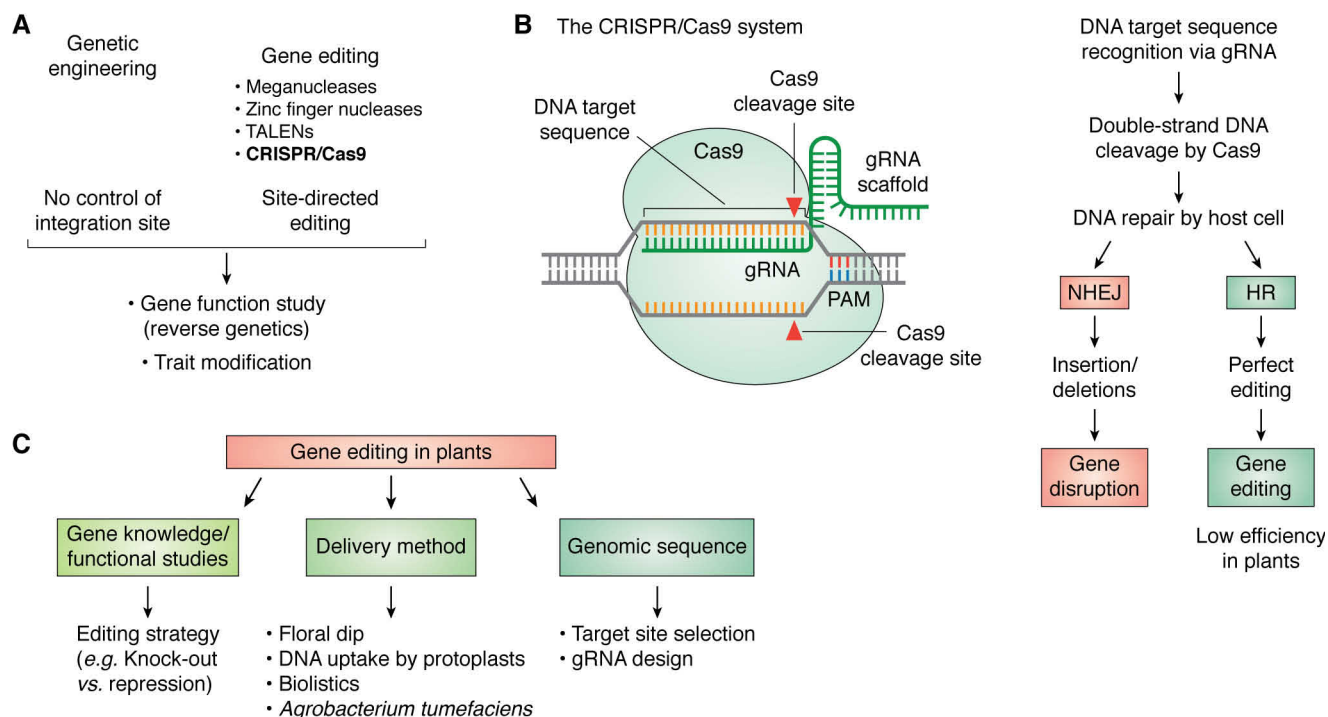


Figure 1. Gene editing approaches, the CRISPR/Cas9 complex, and prerequisites for editing in plants. A, gene editing approaches and outcomes compared with genetic engineering. B, the CRISPR/Cas9 complex showing target sequence and cleavage sites in the DNA. gRNA, guide RNA; PAM, protospacer adjacent motif. Comparison of the DNA repair pathways, nonhomologous end joining (NHEJ), and homologous recombination (HR), involved in gene editing. C, prerequisites that are needed before gene editing can be applied to plant species.

segregation in subsequent generations, progeny can be recovered that contain only the mutation without the Cas protein or other vector components integrated into the genome.

For genome modification with the CRISPR/Cas approach, the simplest method to accomplish at this point in plants is through the creation of indels that occur by the NHEJ repair system, but it is not as precise as HR. Achieving more precision to create a specific point mutation or insert a DNA sequence into the genome by exploiting HR, however, is much more challenging to achieve in plants than in other organisms (7). Insertion of a DNA sequence, referred to as homology-directed repair (HDR), requires the delivery of a DNA repair template along with the CRISPR/Cas components. However, the efficiency of HDR is low in plants, with only one in 10^5 – 10^4 transformation events, mainly because NHEJ is the primary repair mechanism in plants (12). The efficiency is not high enough for large-scale DNA insertion to plant genomes to be routine, as is the generation of indels to disrupt gene expression.

The early application of CRISPR/Cas was to induce indels to disrupt gene expression in different organisms, including plants, to facilitate gene function studies through a reverse genetics approach and to modify various plant traits. The number of genomes modified by the creation of indels, including some important food crops, such as maize (13), potato (14), rice (14), tomato (15), and wheat (16), has grown enormously since the very first reports of the application of CRISPR-mediated editing in plants. There are many reviews that provide information related to modified plant genomes with CRISPR/Cas-mediated editing in addition to the traits of interest (17–19).

Engineering efforts to expand the versatility of CRISPR/Cas makes it possible to tailor modifications beyond disruption of gene expression through the creation of indels, which is now considered routine. New designs make it possible to modulate gene expression, target RNA, modify the epigenome, and make changes down to the single-base-pair level. Whereas some of the most recent CRISPR/Cas tools and designs will be described here, a complementary overview of the expanded capabilities is presented by Adli (5).

Prerequisites for gene editing in plants

To accomplish gene editing in plants, knowledge of the genes of interest (function, expression control, and timing of expression), availability of efficient DNA delivery methodology, and genome sequence are all prerequisites (Fig. 1C). The knowledge of the genes and their function are key to determining the type of editing approach that would be best suited for the desired outcome. For example, would a knockout of expression be sufficient, or would transcriptional regulation of expression be required? Gene editing in plants would not be possible without established methodologies for DNA delivery into cells and the subsequent recovery of modified plants, which forms the basis of genetic engineering. Plant genetic engineering has been achieved through various methods, including floral dip (20), direct DNA uptake into protoplasts (21), delivery of DNA by biolistics (particle bombardment) (22), and *Agrobacterium*-mediated techniques (23). Each of these methods has advantages and disadvantages that need to be considered when choosing a delivery approach, because not all methods work, or at least work efficiently enough for all plant species. For

example, the floral dip method, which involves the dipping of flowers at immature stages into a solution of *Agrobacterium tumefaciens* that contains a gene construct and then harvesting mature seeds, results in recovery of transgenic plants of certain *Arabidopsis* ecotypes and its close relative, *Camelina sativa* (20, 24). However, the floral dip method is not applicable to many plant species, such as those clonally propagated, and it is not consistently reproducible to make it a realistic DNA delivery approach for other species (25).

A genome sequence provides the roadmap for selection of target sites for the gene or region of interest, such as a promoter and gRNA design based on PAM requirements for particular Cas proteins. Availability of plant genome sequences provides the foundation for application of the desired editing technologies described in this review. Depending on the gene or phenotype of interest, a genome sequence combined with the variety of editing approaches allows researchers to make precise modifications, ranging from a single base to insertion of DNA sequences. Having a genome sequence also facilitates gRNA design to target multiple gene copies with CRISPR/Cas. This is certainly needed for polyploid (more than two sets of chromosomes) species. There have been reports of successful CRISPR/Cas-mediated mutagenesis of multiple gene copies in several polyploid plant species, including oilseed rape, a tetraploid (26), and wheat, a hexaploid (27).

The first plant genome to be sequenced was *Arabidopsis*, which serves as a valuable model species, and it took 10 years to complete (28). However, as sequencing technologies have evolved and improved, the time and cost of sequencing large and complex genomes has decreased, making it more realistic to sequence a larger number of plant genomes (28, 29). Indeed, many of the genomes of major crop species have been sequenced (30). However, depending on the plant species, some are more difficult to sequence because of the large genome size, the level of heterozygosity and ploidy (more than two sets of chromosomes), and the amount and structure of repetitive DNA (28). These characteristics, which complicate sequencing efforts, set plant genomes apart from other organisms.

Utilization of CRISPR/Cas-mediated editing relies on both the availability of DNA delivery systems into plant cells as well as genomic resources. Whereas there might not be genome sequence available for every plant cultivar of interest, a reference genome sequence could be used for gRNA design and selection based on placement near the required PAM for cleavage by the Cas protein. gRNAs can be designed with this limited genomic information, but possible variations in DNA, such as single polymorphisms (SNPs) that might exist between a reference genome and a cultivar of interest, could lead to inefficient editing. Therefore, the sequence of the gRNA designed for a cultivar based on a reference genome should be analyzed to determine whether there is any variation between the sequences (31).

Expanding the CRISPR/Cas toolbox

There has been a remodeling of the Cas protein, specifically the protein designated SpCas9, or simply Cas9, which is from

Streptococcus pyogenes. Generation of these protein variants increased options beyond the ability to induce double-strand breaks to now having a variant that cleaves only one strand, referred to as a Cas9 nickase, and a variant that binds DNA but does not cleave, referred to as a “dead” Cas9 (dCas9) (19). These variants have been exploited for development of new designs that make it possible to achieve modifications that range from single-base-pair changes to exchanging of alleles (20, 21). These and other CRISPR/Cas-related tools and designs are described here (Fig. 2).

As the application of the CRISPR/Cas technology expands for various purposes in different organisms, it is driving the development of innovative designs that researchers can exploit to achieve desired outcomes for their investigations. For example, generation of new components has expanded the utility beyond disruption of gene expression to CRISPR/Cas versions that make it possible to modulate expression through activation and repression (5). To accomplish activation and repression, dCas9 is exploited for both applications, because it acts as a guide to a target sequence but does not cut DNA. The early designs for transcriptional activation fused the dCas9 with the VP64 transcriptional activator, which is a synthetic complex of four copies of the herpes simplex virus VP16 transactivation domain (32, 33). These reports demonstrated gene activation in human cell lines.

Depending on the function of a gene, a complete knockout of expression through the application of CRISPR/Cas can result in undesirable effects, such as lethality and abnormal development. In these situations, a decrease in expression level is more desirable than a total disruption. Repurposing the CRISPR/Cas system for knockdown or repression of gene expression led to two different approaches. CRISPR interference (CRISPRi) was first developed by Qi *et al.* (34) to knock down gene expression. They accomplished this through coexpression of dCas9 and a gRNA that resulted in a DNA recognition complex. This complex interfered with binding of transcription factors, transcriptional elongation, or RNA polymerase binding. In addition to demonstration of the effect on gene expression in mammalian cell lines, they also demonstrated that the effects were reversible. To improve upon the level of repression that could be achieved, Gilbert and colleagues (35) found that by fusing a repressor complex, such as the Kruppel-associated Box (KRAB), to dCas9, the level of repression was increased in yeast and human cell lines.

Utilization of the CRISPR/dCas9 approach has also been used for transcriptional regulation of plant gene expression. A recent review by Moradpour and Abdulah (36) provides a comprehensive overview of the plant species, targeted genes, and type of regulation achieved. In general, the CRISPR/dCas9 transcriptional regulation reported for plants was based on inclusion of plant-specific transcriptional effectors for activation or repression in combination with gRNAs and dCas9. The transcriptional effectors enable RNA polymerase and cofactor recruitment for regulation of gene expression (36). Lowder *et al.* (37) developed a protocol for application of VP64, as described above, for activation of plant gene expression and SRDX for repression.

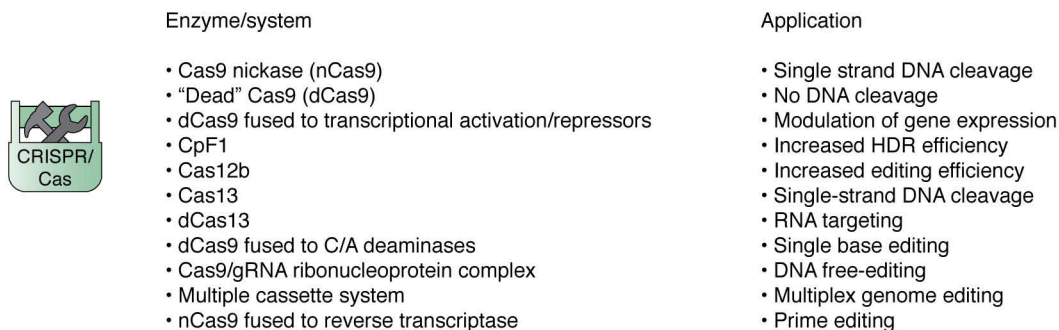
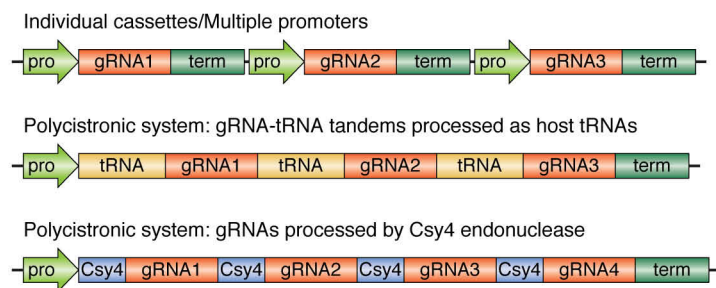
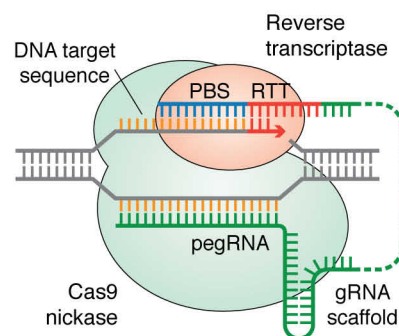
A Expanding the CRISPR/Cas toolbox**B** Multiplex genome editing**C** Prime editing complex

Figure 2. Examples of tools available for gene editing by CRISPR/Cas approaches. A, various Cas proteins including variants of CRISPR/Cas9. B, several vector systems available for targeting multiple genes. Top, based on individual cassettes for gRNA expression that each requires a promoter. Middle, polycistronic multiplex vector system based on tRNA-gRNA multiplex vector system all driven by one promoter. Bottom, Csy4 RNase-based excision system for processing gRNAs all driven by one promoter. pro, promoter; gRNA, guide RNA; term, terminator; tRNA, tRNA; Csy4, CRISPR system yersinia RNase 4. C, prime editing complex. PBS, primer binding site; RTT, reverse transcriptase template that contains the edit of interest; gRNA scaffold, contains the gRNA, CRISPR RNA, and transactivating CRISPR RNA; pegRNA, prime editing guide RNA; Cas9 nickase, engineered Cas9 variant that binds DNA but does not cleave.

The Cas protein collection

The initially available and subsequently most widely used Cas protein, Cas9, results in blunt-end cuts of the DNA a few base pairs upstream from the recognized PAM sequence, 5'-NGG-3' ($n = A, T, G, \text{ and } C$) (19). Cas9 has been shown to be effective in editing genomes of numerous organisms, including plants, with some groups developing plant codon-optimized versions depending on the species of interest. For example, there are codon-optimized versions available for monocots (38) and dicots (39). Monocots are plant species that have one cotyledon, which is the part of the germinating seed that will become a leaf, whereas dicots have two cotyledons. There are other distinguishing characteristics for each of these two groups, but the number of cotyledons is the primary one.

The number of Cas proteins available has increased following the reports of the first successful gene editing with CRISPR/Cas9 because of efforts to find proteins with different PAM requirements that translate into more flexibility for gRNA design (Fig. 2A). Some of these additional Cas proteins also have different types of DNA cleavage, resulting in either blunt or staggered ends (Table 1). The next Cas protein available after Cas9, Cpf1, which is also referred to as Cas12a, has properties different from those of Cas9. The PAM recognized by Cpf1 is 5'-TTTN-3', and it generates a staggered DNA cleavage that leaves four or five nucleotide overhangs (44). This type of cleavage has the potential to result in more efficient gene insertion via HDR. The sources of Cpf1 have been reported from several

organisms, including *Lachnospiraceae bacterium* ND2006 (LbCpf1) and *Francisella novicida* (FnCpf1) (44). To test their potential for higher-efficiency HDR in plants, Begemann *et al.* (6) combined these nucleases with gRNA and template repair DNA to demonstrate precise gene insertions in addition to indel mutations in rice. They found that when combined with gRNA and template-repair DNA, both LbCpf1 and FnCpf1 mediated both NHEJ and HDR gene editing. This was a significant contribution to the CRISPR/Cas toolbox, because being able to achieve more efficient HDR for DNA insertion broadens the capabilities to engineer genomes beyond the creation of indels.

In a recent report by Ming *et al.* (42), they presented their work on genome editing of rice with a more recently investigated Cas protein, Cas12b (Cas-C2c1). They compared the efficiency of Cas12b from different bacteria and found that the Cas12b from *Alicyclobacillus acidiphilus* (AaCas12b), which has a PAM site of 5'-TTN-3', was more efficient at genome editing in rice than Cas12b from *Bacillus thermoamylovorans* and *Bacillus hisashii*. Their results also support the need to be aware that although there are different sources of the various Cas proteins, they might not all be applicable for plant genome editing, and that some level of codon optimization might be needed (42, 45, 46).

In addition to Cas proteins that cleave DNA, there is an option, Cas13 (C2c2), which cleaves single-stranded RNA but not double-stranded DNA and has been engineered to work in

Table 1**Different types of CRISPR/Cas proteins that have been shown to be effective for plant genome editing**

Cas protein	Source	Nucleic acid cleavage	PAM sequence	Reference
spCas9	<i>Streptococcus pyogenes</i>	DNA blunt ends	NGG	40
FnCpf1 (Cas12a)	<i>Francisella novicida</i>	DNA staggered ends	TTTN	7
LbCpf1 (Cas12a)	<i>Lachnospiraceae bacterium</i>	DNA staggered ends	TTTN	7
AsCpf1 (Cas12a)	<i>Acidaminococcus</i> sp.	DNA staggered ends	TTTN	41
AaCas12b (C2c1)	<i>Alicyclobacillus acidiphilus</i>	DNA staggered ends	TTN	42
LwaCas13 (C2c2)	<i>Leptotrichia wadei</i>	Single stranded RNA		43

mammalian and plant cells (43, 47). There is also a catalytically inactive Cas13 available, termed dCas13, that binds RNA but does not cleave (48). One promising utility of CRISPR/Cas13-mediated editing of RNA is to modify noncoding RNAs (ncRNAs), which are highly relevant in plants because they are integral to a multitude of functions, such as gene expression and protein complex assembly (49). Application of CRISPR/Cas13 would expand the understanding of ncRNA involvement in various processes and provide additional strategies for plant-related research studies.

Base editing

To fine-tune modifications in genomes down to single-base-pair changes, David Liu's group at Harvard University modified the CRISPR/Cas9 design to facilitate the irreversible conversion of one DNA base to another, which was termed base editing (50, 51). An inactivated Cas9 (dCas9) that binds DNA but does not cleave was fused to a cytosine deaminase (cytosine base editor, CBE), which converts a cytosine to uracil, resulting in a C-to-T or G-to-A substitution within 5 bp of the dCas9 binding site. Adenine base editors (ABE) that also contained dCas9 were designed to convert A to G or T to C through use of the adenine deaminase APOBEC1 (51).

Base editing was first demonstrated in yeast and mammalian cell lines, with new designs under development to improve activity and allow more flexibility for single-nucleotide targeting (52). Through modification of the base editing complexes, several groups were able to achieve base editing in food crops such as rice (53), tomato (54), and wheat (55). Although base editing was achieved in these food crops, the incidence of off-targeting or causing mutations elsewhere in the genome was unknown. Therefore, Jin *et al.* (56) used whole-genome sequencing (WGS) to assess off-targeting in rice plants recovered after experiments that employed CBE and ABE designs. The WGS results revealed that CBE designs resulted in more off-targets than ABE, and they concluded that the CBEs need to be optimized to increase the fidelity in plants. The total number of off-targets categorized as C-to-T single-nucleotide variants were 504 and 632. To minimize the incidence of off-targeting, investigation would involve optimization of cytidine deaminase and possibly the uracil glycosylase inhibitor, which is a conserved DNA repair enzyme that removes uracil and initiates the base excision repair mechanism (57).

DNA-free gene editing

Food products that result from the application of genetic engineering methodologies are commonly referred to as GMOs, genetically modified organisms. Genetic engineering is based

on the introduction of DNA, often referred to as “foreign” DNA. The DNA introduced might not always be from a plant source, and this causes concern for some consumers. Gene editing, especially the approaches that involve the creation of indels, is expected to result in fewer concerns, because it is based on direct modification of a plant's DNA and not the introduction of foreign DNA. However, the current DNA delivery methods used for introduction of CRISPR/Cas reagents do involve the insertion of foreign DNA, including the Cas protein. This leads to the development of a DNA-free gene editing option driven by the need to have an approach where foreign DNA was not introduced because of consumer concern and multiple regulatory oversight requirements related to genetically engineered food crops (58). As a result, a system was designed that involved delivery of a ribonucleoprotein that contains the Cas9 protein together with a gRNA (CRISPR-Cas9 ribonucleoprotein complex) instead of DNA plasmids that harbor genes for these components (59). An additional component option is that a donor plant DNA sequence of interest can also be included for insertion or replacement in the genome.

However, while this method is attractive from the standpoint of assuaging consumer concern and decreasing regulatory scrutiny, one drawback is the amenability of this approach to the methods used for gene delivery into plant cells. The methods most amenable to this DNA-free approach are delivery into protoplasts or by biolistics (particle bombardment) and not *Agrobacterium* mediated, which is the method that works efficiently for the majority of crop species (60). It is difficult to regenerate plants from protoplasts for most species, and the biolistics method only works efficiently for a few species, such as maize and rice.

To investigate the effects of delivery methods on DNA-free editing, Banakar *et al.* (61) compared three approaches, biolistic RNP/DNA codelivery, biolistic delivery of CRISPR/Cas reagents as plasmid DNA, and *Agrobacterium*-mediated delivery of CRISPR/Cas reagents into mature-seed derived rice embryos that were cultured on medium for 7 days. All three approaches were successful at achieving the intended edits; however, both biolistic delivery approaches resulted in more than 14% random plasmid or chromosomal DNA fragment insertion at the intended target sites, whereas integration of random DNA was not observed with *Agrobacterium*-mediated delivery. These results demonstrated both the importance of the delivery method and the necessity for careful molecular assessment of recovered edited lines to expose unwanted modifications.

Multiplex genome editing

Multiplex genome editing involves the simultaneous delivery of multiple gRNAs within one CRISPR construct. The advantage

of providing multiple gRNAs is that modification of several related or unrelated genomic sites can be simultaneously targeted through delivery of one CRISPR construct into plant cells. This type of editing has been accomplished by designing constructs that are a string of connected gene cassettes (Fig. 2B) (62). However, there are more simplified vector construction systems available that were specifically developed to reduce the complexity of construct design to target multiple sites than those reported during the early days of CRISPR/Cas-mediated editing (63).

The simplified multiplex vector systems streamline the building of gene editing constructs to target multiple sites compared with CRISPR/Cas vector systems, which would require a series of cassettes, each containing a polymerase III promoter, such as U3 and U6, gRNA, and terminator (62). This is all in addition to separate cassettes needed for selectable marker and Cas genes. Depending on the number of selected target sites, this type of construction can be a challenge and lead to large plasmids that would not efficiently transfer into plant cells by gene delivery methods.

There was a reengineering of the design centered on individual gRNA expression cassettes by several groups that led to additional options for multiplex editing. Two of the early multiplex editing vector systems were reported by Xie *et al.* (64) and Cermak *et al.* (65). The design developed by Xie *et al.* was based on expression of a number of gRNAs from a single transcript. To accomplish this strategy, they relied on the endogenous tRNA (decodes mRNA into protein) processing that cuts both ends of tRNA precursors (Fig. 2B). They demonstrated that a tandem structure of multiple tRNA-gRNAs in a polycistronic expression cassette of a vector produced gRNAs with the intended *in vivo*-targeted sequences that resulted in the cleavage of multiple chromosomal targets by Cas9 in rice. They reported an efficiency of 100% in stable rice transgenic lines. The system developed by Cermak *et al.* also simplified construction for targeting multiples sites, because expression of both the gRNAs and Cas9 is driven by one type of polymerase promoter, polymerase II, which is a better characterized type than polymerase III options found in plants.

Since these early reports, other multiplex editing vector systems have been designed for simultaneously targeting multiple genes (65, 66). Cermak *et al.* (65) developed a toolkit that facilitates straightforward modular cloning for single or multiple gene knockouts, including large chromosomal deletions, homologous recombination, and regulation of transcription. This toolkit is readily available to groups interested in applying these various approaches for CRISPR/Cas mediated in plants. Included in this toolkit is a multiplex editing approach based on the CRISPR system *Yersinia* RNase 4 (Csy4) (Fig. 2B) and the tRNA system (Fig. 2B), described earlier. The Csy4 system relies on gRNAs flanked by Csy4 excision sites that are processed by the Csy4 endonuclease (67). The multiplex systems described to this point utilized Cas9 as part of the vector systems. However, a vector system designed by Zhang *et al.* (66) includes Cpf1 (Cas12a), which broadens the options for gRNA selection because of the different PAM requirements compared with those for Cas9. Their Cpf1 vector system is based on a user-friendly, modular-unit design to be easily adapted for vari-

ous editing applications, including knockouts, transcriptional repression, and multiplex editing.

Prime editing

The most recent CRISPR development, termed prime editing, makes it possible to delete or introduce DNA sequences with greater precision and flexibility in choosing target sites and has been referred to as “search-and-replace” genome editing (68). The prime editing design contains the Cas9 variant that nicks only one DNA strand and is referred to as a nickase. A reverse transcriptase (RT) is fused to the C terminus of the Cas9 nickase (Fig. 2C). This is combined with a gRNA called a prime editing guide RNA (pegRNA). Based on the template, the RT creates a new DNA strand that is inserted into the nicked site in the genome created by the Cas9 variant. Anzalone *et al.* (68) showed the efficacy of the search-and-replace approach they developed in human cell lines and predicted that, in theory, prime editing could be used to address approximately “89% of known pathogenic human genetic variants.”

As with other gene editing technologies that have been developed, the first reports were focused on nonplant organisms, but reports of successful application in plants shortly followed. The same is true for prime editing, with several groups reporting their results in rice soon after the report of this new editing approach (69–71). For these studies, prime editing constructs were introduced into rice protoplasts. Whereas they demonstrated editing of the target DNA with this new approach, the efficiencies were significantly lower than those reported for human cell lines, indicating that further optimization is needed for high-throughput editing of plant genomes. The efficiencies reported to date for prime editing in rice range from 0.05–26%; however, the largest number of experiments reported were in the range of 2% or lower, which overall is an order of magnitude lower than that reported for human cells (68–73). Based on these results, it is clear that modifications will need to be made to the prime editing design to improve the efficiency in plants.

Application of CRISPR/Cas editing for crop improvement—potential and challenges

The earliest reports of CRISPR/Cas gene editing in plants were for *Nicotiana benthamiana* and *Arabidopsis*, which are utilized as model systems for various areas of plant-related research (74). It was not surprising that the first reports were in models because of the availability of robust editing prerequisites: knowledge of genes from extensive functional studies, efficient genetic engineering methodologies, and genomic resources. Not long after these first reports in models, demonstrations of CRISPR/Cas-mediated editing in some major crops began to appear. The early reports in crop species focused on proof-of-concept experiments to generate indels to disrupt gene expression to demonstrate the feasibility of CRISPR/Cas-mediated gene editing in crops that included rice (75), tomato (76), and maize (77). Once there was demonstration that editing could be achieved at realistic levels, focus turned to editing genes to affect important traits. Traits in these subsequent studies included disease resistance in banana (78) and tomato

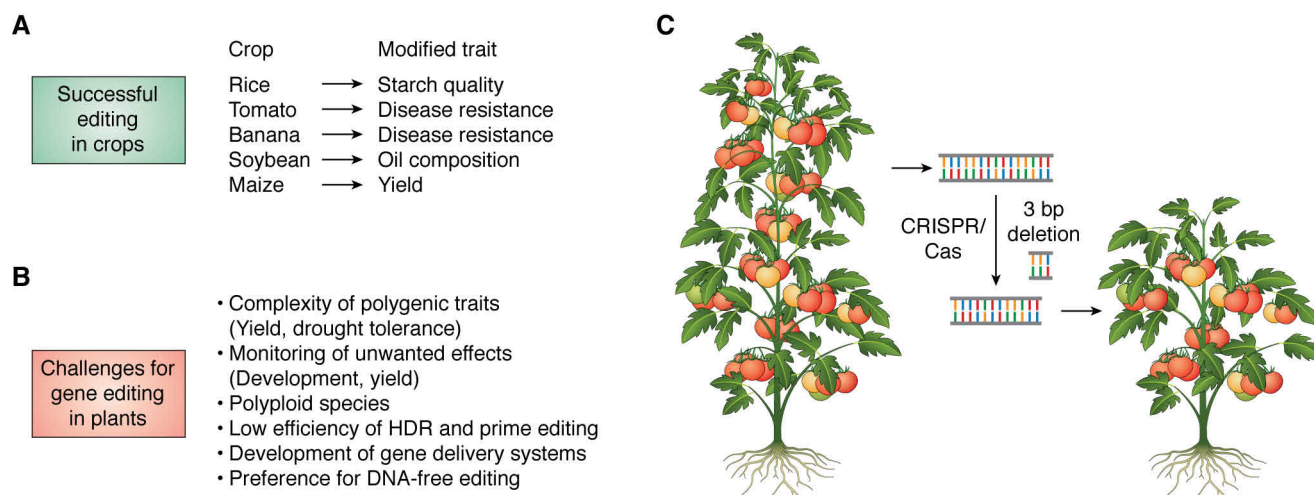


Figure 3. Application and challenges of CRISPR/Cas-mediated editing in plants. A, examples of gene editing in crop species and the corresponding trait that was modified. B, challenges that can affect the ability to efficiently edit plant genomes. C, schematic of CRISPR/Cas-mediated gene editing of tomato DNA for modification of plant height. Demonstration of how editing a gene responsible for plant growth can result in a deletion. The deletion knocks out the function of the gene resulting in a more compact growth habit.

(79), starch quality in rice (80), oil composition in soybean (81), and yield in maize (82) (Fig. 3A).

Although there is excitement about the utility of CRISPR/Cas as a tool to help advance crop improvement, there are some challenges (Fig. 3B). Desired phenotypes, such as modification of plant growth habit (Fig. 3C) (83) and fruit color (84), were easily achievable by generating expression knockouts of only one gene. However, traits such as yield and drought tolerance most likely would require more than one editing approach to achieve the desired effect because of the complex interplay of multiple genes, their expression levels, and timing of expression throughout growth and development.

Whereas mutagenesis with CRISPR/Cas to knock out gene expression is viewed as routine in some plant species, it is not as simple as the recovery of one ideal edited plant following the introduction of the CRISPR/Cas components into plant cells. Multiple edited plant lines need to be analyzed to first assess that editing has actually occurred. The second assessment is to determine that the intended phenotype alone was affected and that there were no unintended negative effects on other plant characteristics, such as development or yield. The number of plants that would need to be evaluated also depends on the ploidy level of the species because of the presence of multiple gene copies that would need to be edited to achieve the desired phenotype. In addition, the more complex genetic control involved in a trait and the number of editing approaches required to achieve the trait modification would increase the number of plants needed to be assessed.

A further complication is that several editing strategies, such as HDR and prime editing, are more challenging at this time than the creation of indels, which is now viewed as routine in many plant species. Further design modifications will be needed to bring these other approaches for editing to a level that will be realistic to affect more complex traits. These modifications include, but are not limited to, codon optimization for effective use in plants and strategies to overcome the predominant NHEJ DNA repair pathway to achieve HDR for DNA

replacement. In parallel, more efficient gene delivery systems are needed for application of the various editing technologies. As indicated in this review, several CRISPR/Cas approaches work more efficiently with delivery by biolistics or DNA uptake into protoplasts; however, these methodologies are not amenable to all plant species.

Despite the challenges, there have been a significant number of reports of CRISPR/Cas-mediated modifications of plant traits, and there are multiple reviews that outline the accomplishments in a vast array of plant species (18, 85, 86). CRISPR/Cas has also been shown to have potential to fast-track crop improvement, especially of underutilized orphan plant species, through what has been termed *de novo* domestication (83, 87, 88). Improvement of undesirable agronomic characteristics, such as wild, sprawling growth habit and small fruit size, that preclude adoption into agricultural production systems has the potential to broaden options for food crops and provide additional sources of income for farmers.

Summary

The advent of gene editing technologies, especially CRISPR/Cas, has significantly increased the possibilities for genome modification to advance functional genomics, metabolic engineering, biofortification, and crop improvement. There has been steady development of new tools, such as Cas proteins, and new approaches, such as base and prime editing, that broaden the options to precisely fine-tune gene expression. CRISPR/Cas has already proven to be a powerful tool for affecting various plant traits that have the potential to benefit plant-breeding programs focused on different areas of crop improvement. Whereas there have already been exciting developments in these areas related to CRISPR/Cas-mediated modification of traits, it is important to note that gene editing is not a replacement for traditional methods related to crop improvement; rather, it is simply one of many approaches available to achieve the desired outcomes. Edited plants, including the subsequent next generations, need to be assessed on a large scale in

multiple locations, as is done in plant breeding programs, to ensure the modified versions are not inferior in performance, namely, yield, to the nonedited counterparts. Plant breeders routinely assess their plant material across different environments to make certain the traits of interest are stable. Edited lines could also be used to enhance the variability in germplasm (different plant types) collections that plant breeders rely on for developing improved varieties. This can be thought of as expanding the number of paint colors available to an artist. Therefore, there will be a close interplay of plant breeding and gene editing that will broaden as the various editing technologies become more efficient in a wider range of plant species.

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Abbreviations—The abbreviations used are: ZFNs, zinc finger nucleases; TALENs, transcriptional activator-like effector nucleases; gRNAs, guide RNAs (gRNAs); PAM, protospacer adjacent motif; NHEJ, nonhomologous end-joining; HR, homologous recombination; HDR, homology-directed repair; CBE, cytosine base editor; ABE, adenine base editors; WGS, whole-genome sequencing.

References

- Daboussi, F., Stoddard, T. J., and Zhang, F. (2015) Engineering meganuclease for precise plant genome modification. in *Advances in New Technology for Targeted Modification of Plant Genomes* (Zhang, F., ed), pp. 21–38, Springer Science + Business Media, London, United Kingdom
- Kim, Y. G., Cha, J., and Chandrasegaran, S. (1996) Hybrid restriction enzymes: zinc finger fusions to Fok I cleavage domain. *Proc. Natl. Acad. Sci. U.S.A.* **93**, 1156–1160 [CrossRef Medline](#)
- Bogdanove, A. J., and Voytas, D. F. (2011) TAL effectors: customizable proteins for DNA targeting. *Science* **333**, 1843–1846 [CrossRef Medline](#)
- Doudna, J. A., and Charpentier, E. (2014) Genome editing. The new frontier of genome engineering with CRISPR-Cas9. *Science* **346**, 1258096–1258099 [CrossRef Medline](#)
- Adli, M. (2018) The CRISPR tool kit for genome editing and beyond. *Nat. Commun.* **9**, 1–13 [CrossRef](#)
- Begemann, M. B., Gray, B. N., January, E., Gordon, G. C., He, Y., Liu, H., Wu, X., Brutnell, T. P., Mockler, T. C., and Oufattole, M. (2017) Precise insertion and guided editing of higher plant genomes using Cpf1 CRISPR nucleases. *Sci. Rep.* **7**, 1–6 [CrossRef](#)
- Chen, K., Wang, Y., Zhang, R., Zhang, H., and Gao, C. (2019) CRISPR/Cas genome editing and precision plant breeding in agriculture. *Annu. Rev. Plant Biol.* **70**, 667–697 [CrossRef Medline](#)
- Bortesi, L., and Fischer, R. (2015) The CRISPR/Cas9 system for plant genome editing and beyond. *Biotechnol. Adv.* **33**, 41–52 [CrossRef Medline](#)
- Song, G. Y., Jia, M. L., Chen, K., Kong, X. C., Khattak, B., Xie, C. X., Li, A. L., and Mao, L. (2016) CRISPR/Cas9: a powerful tool for crop genome editing. *Crop J.* **4**, 75–82 [CrossRef](#)
- Puchta, H. (2017) Applying CRISPR/Cas for genome engineering in plants: the best is yet to come. *Curr. Opin. Plant Biol.* **36**, 1–8 [CrossRef Medline](#)
- Knoll, A., Fauser, F., and Puchta, H. (2014) DNA recombination in somatic plant cells: mechanisms and evolutionary consequences. *Chromosom. Res.* **22**, 191–201 [CrossRef Medline](#)
- Hilscher, J., Burstmayr, H., and Stoger, E. (2017) Targeted modification of plant genomes for precision crop breeding. *Biotechnol. J.* **12**, 1600173–1600174 [CrossRef](#)
- Feng, C., Yuan, J., Wang, R., Liu, Y., Birchler, J. A., and Han, F. (2016) Efficient targeted genome modification in maize using CRISPR/Cas9 system. *J. Genet. Genomics* **43**, 37–43 [CrossRef Medline](#)
- Das Dangol, S., Barakate, A., Stephens, J., Çaliskan, M. E., and Bakhsh, A. (2019) Genome editing of potato using CRISPR technologies: current development and future prospective. *Plant. Cell. Tiss. Org. Cult.* **139**, 403–416 [CrossRef](#)
- Kwon, C. T., Heo, J., Lemmon, Z. H., Capua, Y., Hutton, S. F., Van Eck, J., Park, S. J., and Lippman, Z. B. (2020) Rapid customization of Solanaceae fruit crops for urban agriculture. *Nat. Biotechnol.* **38**, 182–188 [CrossRef Medline](#)
- Wang, Y., Cheng, X., Shan, Q., Zhang, Y., Liu, J., Gao, C., and Qiu, J. L. (2014) Simultaneous editing of three homoeoalleles in hexaploid bread wheat confers heritable resistance to powdery mildew. *Nat. Biotechnol.* **32**, 947–951 [CrossRef Medline](#)
- Jaganathan, D., Ramasamy, K., Sellamuthu, G., Jayabalan, S., and Venkataraman, G. (2018) CRISPR for crop improvement: an update review. *Front. Plant Sci.* **9**, 985–987 [CrossRef Medline](#)
- Rojas-Vásquez, R., and Gatica-Arias, A. (2020) Use of genome editing technologies for genetic improvement of crops of tropical origin. *Plant Cell Tiss. Org. Cult.* **140**, 215–244 [CrossRef](#)
- Li, J., Li, Y., and Ma, L. G. (2019) CRISPR/Cas9-based genome editing and its applications for functional genomic analyses in plants. *Small Methods* **3**, 1800473–1800481 [CrossRef](#)
- Zhang, X., Henriques, R., Lin, S. S., Niu, Q. W., and Chua, N. H. (2006) *Agrobacterium*-mediated transformation of *Arabidopsis thaliana* using the floral dip method. *Nat. Protoc.* **1**, 641–646 [CrossRef Medline](#)
- Davey, M. R., Anthony, P., Power, J. B., and Lowe, K. C. (2005) Plant protoplasts: status and biotechnological perspectives. *Biotechnol. Adv.* **23**, 131–171 [CrossRef Medline](#)
- Kikkert, J. R., Vidal, J. R., and Reisch, B. I. (2004) Stable transformation of plant cells by particle bombardment/biolistics. in *Transgenic Plants* (Peña, L., ed.), pp. 61–78, Humana Press, Totowa, NJ
- Gelvin, S. B. (2017) Integration of *Agrobacterium* T-DNA into the plant genome. *Annu. Rev. Genet.* **51**, 195–217 [CrossRef Medline](#)
- Lu, C., and Kang, J. (2008) Generation of transgenic plants of a potential oilseed crop *Camelina sativa* by *Agrobacterium*-mediated transformation. *Plant Cell Rep.* **27**, 273–278 [CrossRef Medline](#)
- Altpeter, F., Springer, N. M., Bartley, L. E., Blechl, A. E., Brutnell, T. P., Citovsky, V., Conrad, L. J., Gelvin, S. B., Jackson, D. P., Kausch, A. P., Lemaux, P. G., Medford, J. I., Orozco-Cárdenas, M. L., Tricoli, D. M., Van Eck, J., et al. (2016) Advancing crop transformation in the era of genome editing. *Plant Cell* **28**, 1510–1520 [CrossRef Medline](#)
- Braatz, J., Harloff, H. J., Mascher, M., Stein, N., Himmelbach, A., and Jung, C. (2017) CRISPR-Cas9 targeted mutagenesis leads to simultaneous modification of different homoeologous gene copies in polyploid oilseed rape (*Brassica napus*). *Plant Physiol.* **174**, 935–942 [CrossRef Medline](#)
- Wang, W., Pan, Q., He, F., Akhunova, A., Chao, S., Trick, H., and Akhunov, E. (2018) Transgenerational CRISPR-Cas9 activity facilitates multiplex gene editing in allopolyploid wheat. *Cris. J.* **1**, 65–74 [CrossRef Medline](#)
- Li, F.-W., and Harkess, A. (2018) A guide to sequence your favorite plant genomes. *App. Plant Sci.* **6**, 1–7 [CrossRef](#)
- Michael, T. P., and Jackson, S. (2013) The first 50 plant genomes. *Plant Genome* **6**, 1–7 [CrossRef](#)
- Bolger, M. E., Weisshaar, B., Scholz, U., Stein, N., Usadel, B., and Mayer, K. F. X. (2014) Plant genome sequencing—applications for crop improvement. *Curr. Opin. Biotechnol.* **26**, 31–37 [CrossRef Medline](#)
- Schaeffer, S. M., and Nakata, P. A. (2015) CRISPR/Cas9-mediated genome editing and gene replacement in plants: transitioning from lab to field. *Plant Sci.* **240**, 130–142 [CrossRef Medline](#)

32. Perez-Pinera, P., Kocak, D. D., Vockley, C. M., Adler, A. F., Kabadi, A. M., Polstein, L. R., Thakore, P. I., Glass, K. A., Ousterout, D. G., Leong, K. W., Guilak, F., Crawford, G. E., Reddy, T. E., and Gersbach, C. A. (2013) RNA-guided gene activation by CRISPR-Cas9-based transcription factors. *Nat. Methods* **10**, 973–976 [CrossRef Medline](#)
33. Maeder, M. L., Linder, S. J., Cascio, V. M., Fu, Y., Ho, Q. H., and Joung, J. K. (2013) CRISPR RNA-guided activation of endogenous human genes. *Nat. Methods* **10**, 977–979 [CrossRef Medline](#)
34. Qi, L. S., Larson, M. H., Gilbert, L. A., Doudna, J. A., Weissman, J. S., Arkin, A. P., and Lim, W. A. (2013) Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression. *Cell* **152**, 1173–1183 [CrossRef Medline](#)
35. Gilbert, L. A., Larson, M. H., Morsut, L., Liu, Z., Brar, G. A., Torres, S. E., Stern-Ginossar, N., Brandman, O., Whitehead, E. H., Doudna, J. A., Lim, W. A., Weissman, J. S., and Qi, L. S. (2013) CRISPR-mediated modular RNA-guided regulation of transcription in eukaryotes. *Cell* **154**, 442–451 [CrossRef Medline](#)
36. Moradpour, M., and Abdulah, S. N. A. (2020) CRISPR/dCas9 platforms in plants: strategies and applications beyond genome editing. *Plant Biotechnol. J.* **18**, 32–44 [CrossRef Medline](#)
37. Lowder, L. G., Paul, J. W., and Qi, Y. (2017) Multiplexed transcriptional activation or repression in plants using CRISPR-dCas9-based systems. in *Methods in Molecular Biology* (Kaufmann, K., Mueller-Roeber, B. eds.), pp. 167–184, Humana Press Inc., Totowa, NJ
38. Xing, H. L., Dong, L., Wang, Z. P., Zhang, H. Y., Han, C. Y., Liu, B., Wang, X. C., and Chen, Q. J. (2014) A CRISPR/Cas9 toolkit for multiplex genome editing in plants. *BMC Plant Biol.* **14**, 1–12 [CrossRef](#)
39. Ma, X., and Liu, Y.-G. (2016) CRISPR/Cas9-based multiplex genome editing in monocot and dicot plants. *Curr. Protoc. Mol. Biol.* **115**, 1–21 [CrossRef](#)
40. Anders, C., Niewoehner, O., Duerst, A., and Jinek, M. (2014) Structural basis of PAM-dependent target DNA recognition by the Cas9 endonuclease. *Nature* **513**, 569–573 [CrossRef Medline](#)
41. Tang, X., Lowder, L. G., Zhang, T., Malzahn, A. A., Zheng, X., Voytas, D. F., Zhong, Z., Chen, Y., Ren, Q., Li, Q., Kirkland, E. R., Zhang, Y., and Qi, Y. (2017) A CRISPR-Cpf1 system for efficient genome editing and transcriptional repression in plants. *Nat. Plants* **3**, 1–5 [CrossRef](#)
42. Ming, M., Ren, Q., Pan, C., He, Y., Zhang, Y., Liu, S., Zhong, Z., Wang, J., Malzahn, A. A., Wu, J., Zheng, X., Zhang, Y., and Qi, Y. (2020) CRISPR-Cas12b enables efficient plant genome engineering. *Nat. Plants* **6**, 202–208 [CrossRef Medline](#)
43. Wolter, F., and Puchta, H. (2018) The CRISPR/Cas revolution reaches the RNA world: Cas13, a new Swiss Army knife for plant biologists. *Plant J.* **94**, 767–775 [CrossRef Medline](#)
44. Zaidi, S. S., Mahfouz, M. M., and Mansoor, S. (2017) CRISPR-Cpf1: a new tool for plant genome editing. *Trends Plant Sci.* **22**, 550–553 [CrossRef Medline](#)
45. Zetsche, B., Gootenberg, J. S., Abudayyeh, O. O., Slaymaker, I. M., Makarova, K. S., Essletzbichler, P., Volz, S. E., Joung, J., van der Oost, J., Regev, A., Koonin, E. V., and Zhang, F. (2015) Cpf1 is a single RNA-guided endonuclease of a class 2 CRISPR-Cas system. *Cell* **163**, 759–771 [CrossRef Medline](#)
46. Endo, A., Masafumi, M., Kaya, H., and Toki, S. (2016) Efficient targeted mutagenesis of rice and tobacco genomes using Cpf1 from *Francisella novicida*. *Sci. Rep.* **6**, 1–9 [CrossRef](#)
47. Abudayyeh, O. O., Gootenberg, J. S., Essletzbichler, P., Han, S., Joung, J., Belanto, J. J., Verdine, V., Cox, D. B. T., Kellner, M. J., Regev, A., Lander, E. S., Voytas, D. F., Ting, A. Y., and Zhang, F. (2017) RNA targeting with CRISPR-Cas13. *Nature* **550**, 280–284 [CrossRef Medline](#)
48. Cox, D. B. T., Gootenberg, J. S., Abudayyeh, O. O., Franklin, B., Kellner, M. J., Joung, J., and Zhang, F. (2017) RNA editing with CRISPR-Cas13. *Science* **358**, 1019–1027 [CrossRef Medline](#)
49. Schindele, P., Wolter, F., and Puchta, H. (2018) Transforming plant biology and breeding with CRISPR/Cas9, Cas12 and Cas13. *FEBS Lett.* **592**, 1954–1967 [CrossRef Medline](#)
50. Komor, A. C., Kim, Y. B., Packer, M. S., Zuris, J. A., and Liu, D. R. (2016) Programmable editing of a target base in genomic DNA without double-stranded DNA cleavage. *Nature* **533**, 420–424 [CrossRef Medline](#)
51. Gaudelli, N. M., Komor, A. C., Rees, H. A., Packer, M. S., Badran, A. H., Bryson, D. I., and Liu, D. R. (2017) Programmable base editing of A•T to G•C in genomic DNA without DNA cleavage. *Nature* **551**, 464–471 [CrossRef Medline](#)
52. Throny, B. W., Koblan, L. W., Levy, J. M., Yeh, W. H., Zheng, C., Newby, G. A., Wilson, C., Bhaumik, M., Shubina-Oleinik, O., Holt, J. R., and Liu, D. R. (2019) Continuous evolution of base editors with expanded target compatibility and improved activity. *Nat. Biotechnol.* **37**, 1070–1079 [CrossRef Medline](#)
53. Hao, L., Ruiying, Q., Xiaoshuang, L., Shengxiang, L., Rongfang, X., Jianbo, Y., and Pengcheng, W. (2019) CRISPR/Cas9-mediated adenine base editing in rice genome. *Rice Sci.* **26**, 125–128 [CrossRef](#)
54. Veillet, F., Perrot, L., Chauvin, L., Kermarrec, M. P., Guyon-Debast, A., Chauvin, J. E., Nogué, F., and Mazier, M. (2019) Transgene-free genome editing in tomato and potato plants using *Agrobacterium*-mediated delivery of a CRISPR/Cas9 cytidine base editor. *Int. J. Mol. Sci.* **20**, 1–10 [CrossRef](#)
55. Zhang, R., Liu, J., Chai, Z., Chen, S., Bai, Y., Zong, Y., Chen, K., Li, J., Jiang, L., and Gao, C. (2019) Generation of herbicide tolerance traits and a new selectable marker in wheat using base editing. *Nat. Plants* **5**, 480–485 [CrossRef Medline](#)
56. Jin, S., Zong, Y., Gao, Q., Zhu, Z., Wang, Y., Qin, P., Liang, C., Wang, D., Qiu, J. L., Zhang, F., and Gao, C. (2019) Cytosine, but not adenine, base editors induce genome-wide off-target mutations in rice. *Science* **364**, 292–295 [CrossRef](#)
57. Schormann, N., Ricciardi, R., and Chattopadhyay, D. (2014) Uracil-DNA glycosylases—structural and functional perspectives on an essential family of DNA repair enzymes. *Protein Sci.* **23**, 1667–1685 [CrossRef Medline](#)
58. Wolter, F., and Puchta, H. (2017) Knocking out consumer concerns and regulator's rules: efficient use of CRISPR/Cas ribonucleoprotein complexes for genome editing in cereals. *Genome Biol.* **18**, 1–3 [CrossRef](#)
59. DeWitt, M. A., Corn, J. E., and Carroll, D. (2017) Genome editing via delivery of Cas9 ribonucleoprotein. *Methods* **121–122**, 9–15 [CrossRef Medline](#)
60. Kanchiswamy, C. N. (2016) DNA-free genome editing methods for targeted crop improvement. *Plant Cell Rep.* **35**, 1469–1474 [CrossRef Medline](#)
61. Banakar, R., Eggenberger, A. L., Lee, K., Wright, D. A., Murugan, K., Zarecor, S., Lawrence-Dill, C. J., Sashital, D. G., and Wang, K. (2019) High-frequency random DNA insertions upon co-delivery of CRISPR-Cas9 ribonucleoprotein and selectable marker plasmid in rice. *Sci. Rep.* **9**, 1–13 [CrossRef](#)
62. Minkenberg, B., Wheatley, M., and Yang, Y. (2017) CRISPR/Cas9-enabled multiplex genome editing and its application. in *Progress in Molecular Biology and Translational Science* (Weeks, D. P., and Yang, B. eds.), pp. 111–132, Academic Press, Totowa, NJ
63. Armario Najera, V., Twyman, R. M., Christou, P., and Zhu, C. (2019) Applications of multiplex genome editing in higher plants. *Curr. Opin. Biotechnol.* **59**, 93–102 [CrossRef Medline](#)
64. Xie, K., Minkenberg, B., and Yang, Y. (2015) Boosting CRISPR/Cas9 multiplex editing capability with the endogenous tRNA-processing system. *Proc. Natl. Acad. Sci. U S A* **112**, 3570–3575 [CrossRef Medline](#)
65. Čermák, T., Curtin, S. J., Gil-Humanes, J., Čegan, R., Kono, T. J. Y., Konečná, E., Belanto, J. J., Starker, C. G., Mathre, J. W., Greenstein, R. L., and Voytas, D. F. (2017) A multipurpose toolkit to enable advanced genome engineering in plants. *Plant Cell* **29**, 1196–1217 [CrossRef Medline](#)
66. Zhang, Y., Zhang, Y., and Qi, Y. (2019) Plant gene knockout and knockdown by CRISPR-Cpf1 (Cas12a) systems. in *Methods in Molecular Biology* (Qi, Y. ed), pp. 245–256, Humana Press Inc., Totowa, NJ
67. Zhang, Y., Malzahn, A. A., Sretenovic, S., and Qi, Y. (2019) The emerging and uncultivated potential of CRISPR technology in plant science. *Nat. Plants* **5**, 778–794 [CrossRef Medline](#)
68. Anzalone, A. V., Randolph, P. B., Davis, J. R., Sousa, A. A., Koblan, L. W., Levy, J. M., Chen, P. J., Wilson, C., Newby, G. A., Raguram, A., and Liu, D. R. (2019) Search-and-replace genome editing without double-strand breaks or donor DNA. *Nature* **576**, 149–157 [CrossRef Medline](#)
69. Lin, Q., Zong, Y., Xue, C., Wang, S., Jin, S., Zhu, Z., Wang, Y., Anzalone, A. V., Raguram, A., Doman, J. L., Liu, D. R., and Gao, C. (2020) Prime genome editing in rice and wheat. *Nat. Biotechnol.* **38**, 582–585 [CrossRef Medline](#)

70. Xu, W., Zhang, C., Yang, Y., Zhao, S., Kang, G., He, X., Song, J., and Yang, J. (2020) Versatile nucleotides substitution in plant using an improved prime editing system. *Mol. Plant* **13**, 675–678 [CrossRef Medline](#)
71. Tang, X., Sretenovic, S., Ren, Q., Jia, X., Li, M., Fan, T., Yin, D., Xiang, S., Guo, Y., Liu, L., Zheng, X., Qi, Y., and Zhang, Y. (2020) Plant prime editors enable precise gene editing in rice cells. *Mol. Plant* **13**, 667–670 [CrossRef Medline](#)
72. Butt, H., Rao, G. S., Sedeeq, K., Aman, R., Kamel, R., and Mahfouz, M. (2020) Engineering herbicide resistance via prime editing in rice. *Plant Biotechnol. J.* [CrossRef](#)
73. Li, H., Li, J., Chen, J., Yan, L., and Xia, L. (2020) Precise modifications of both exogenous and endogenous genes in rice by prime editing. *Mol. Plant* **13**, 671–674 [CrossRef Medline](#)
74. Nekrasov, V., Staskawicz, B., Weigel, D., Jones, J. D., and Kamoun, S. (2013) Targeted mutagenesis in the model plant *Nicotiana benthamiana* using Cas9 RNA-guided endonuclease. *Nat. Biotechnol.* **31**, 691–693 [CrossRef Medline](#)
75. Feng, Z., Zhang, B., Ding, W., Liu, X., Yang, D. L., Wei, P., Cao, F., Zhu, S., Zhang, F., Mao, Y., and Zhu, J. K. (2013) Efficient genome editing in plants using a CRISPR/Cas system. *Cell Res.* **23**, 1229–1232 [CrossRef Medline](#)
76. Brooks, C., Nekrasov, V., Lippman, Z. B., and Van Eck, J. (2014) Efficient gene editing in tomato in the first generation using the clustered regularly interspaced short palindromic repeats/CRISPR-associated9 system. *Plant Physiol.* **166**, 1292–1297 [CrossRef Medline](#)
77. Liang, Z., Zhang, K., Chen, K., and Gao, C. (2014) Targeted mutagenesis in *Zea mays* using TALENs and the CRISPR/Cas system. *J. Genet. Genomics* **41**, 63–68 [CrossRef Medline](#)
78. Tripathi, J. N., Ntui, V. O., Ron, M., Muiruri, S. K., Britt, A., and Tripathi, L. (2019) CRISPR/Cas9 editing of endogenous banana streak virus in the B genome of *Musa* spp. overcomes a major challenge in banana breeding. *Commun. Biol.* **2**, 1–11 [CrossRef](#)
79. Nekrasov, V., Wang, C., Win, J., Lanz, C., Weigel, D., and Kamoun, S. (2017) Rapid generation of a transgene-free powdery mildew resistant tomato by genome deletion. *Sci. Rep.* **7**, 1–6 [CrossRef](#)
80. Sun, Y., Jiao, G., Liu, Z., Zhang, X., Li, J., Guo, X., Du, W., Du, J., Francis, F., Zhao, Y., and Xia, L. (2017) Generation of high-amylose rice through CRISPR/Cas9-mediated targeted mutagenesis of starch branching enzymes. *Front. Plant Sci.* **8**, 298–315 [CrossRef Medline](#)
81. Al Amin, N., Ahmad, N., Wu, N., Pu, X., Ma, T., Du, Y., Bo, X., Wang, N., Sharif, R., and Wang, P. (2019) CRISPR-Cas9 mediated targeted disruption of *FAD2-2 microsomal omega-6 desaturase* in soybean (*Glycine max*.L). *BMC Biotechnol.* **19**, 1–10 [CrossRef](#)
82. Shi, J., Gao, H., Wang, H., Lafitte, H. R., Archibald, R. L., Yang, M., Hakimi, S. M., Mo, H., and Habben, J. E. (2017) ARGOS8 variants generated by CRISPR-Cas9 improve maize grain yield under field drought stress conditions. *Plant Biotechnol. J.* **15**, 207–216 [CrossRef Medline](#)
83. Lemmon, Z. H., Reem, N. T., Dalrymple, J., Soyk, S., Swartwood, K. E., Rodriguez-Leal, D., Van Eck, J., and Lippman, Z. B. (2018) Rapid improvement of domestication traits in an orphan crop by genome editing. *Nat. Plants* **4**, 766–770 [CrossRef Medline](#)
84. Deng, L., Wang, H., Sun, C., Li, Q., Jiang, H., Du, M., Li, C. C. B., and Li, C. C. B. (2018) Efficient generation of pink-fruited tomatoes using CRISPR/Cas9 system. *J. Genet. Genomics* **45**, 51–54 [CrossRef Medline](#)
85. Mishra, R., and Zhao, K. J. (2018) Genome editing technologies and their applications in crop improvement. *Plant Biotechnol. Rep.* **12**, 57–68 [CrossRef](#)
86. Wang, T., Zhang, H., and Zhu, H. (2019) CRISPR technology is revolutionizing the improvement of tomato and other fruit crops. *Hortic. Res.* **6**, 1 [CrossRef](#)
87. Zsogon, A., Cermak, T., Voytas, D., and Peres, L. E. (2017) Genome editing as a tool to achieve the crop ideotype and *denovo* domestication of wild relatives: case study in tomato. *Plant Sci.* **256**, 120–130 [CrossRef Medline](#)
88. Li, T., Yang, X., Yu, Y., Si, X., Zhai, X., Zhang, H., Dong, W., Gao, C., and Xu, C. (2018) Domestication of wild tomato is accelerated by genome editing. *Nat. Biotechnol.* **36**, 1160–1163 [CrossRef](#)