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CRISPR/Cas Genome Editing and Precision Plant Breeding in Agriculture

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Abstract

Enhanced agricultural production through innovative breeding technology is urgently needed to increase access to nutritious foods worldwide. Recent advances in CRISPR/Cas genome editing enable efficient targeted modification in most crops, thus promising to accelerate crop improvement. Here, we review advances in CRISPR/Cas9 and its variants and examine their applications in plant genome editing and related manipulations. We highlight base-editing tools that enable targeted nucleotide substitutions and describe the various delivery systems, particularly DNA-free methods, that have linked genome editing with crop breeding. We summarize the applications of genome editing for trait improvement, development of techniques for fine-tuning gene regulation, strategies for breeding virus resistance, and the use of high-throughput mutant libraries. We outline future perspectives for genome editing in plant synthetic biology and domestication, advances in delivery systems, editing specificity, homology-directed repair, and gene drives. Finally, we discuss the challenges and opportunities for precision plant breeding and its bright future in agriculture.

Contents

1. INTRODUCTION

Crops provide food, feed, fuel, and other consumable resources for human life, thereby contributing enormously to society. The world population is predicted to reach 9.6 billion by 2050, and the global demand for crops will increase by 100–110% compared with 2005 (157). To feed and nourish a rapidly growing population in the face of climate change, decreased arable land, and shortage of available water resources, there is an urgent need for innovations in crop breeding technology to increase agricultural productivity and accelerate sustainable agricultural development.

Cross breeding, mutation breeding, and transgenic breeding are currently the main methodologies for crop improvement in modern agriculture (**Figure 1**). It takes many years to introduce desirable alleles by cross breeding and to increase variability by genetic recombination (138). Owing to thousands of years of directed evolution through breeding, large parts of the genomes of major crops are fixed, and genetic variability has been greatly reduced, limiting the potential of improving many traits. Mutation breeding has expanded genetic variation by introducing random mutations using chemical mutagens or physical irradiation (125). However, these

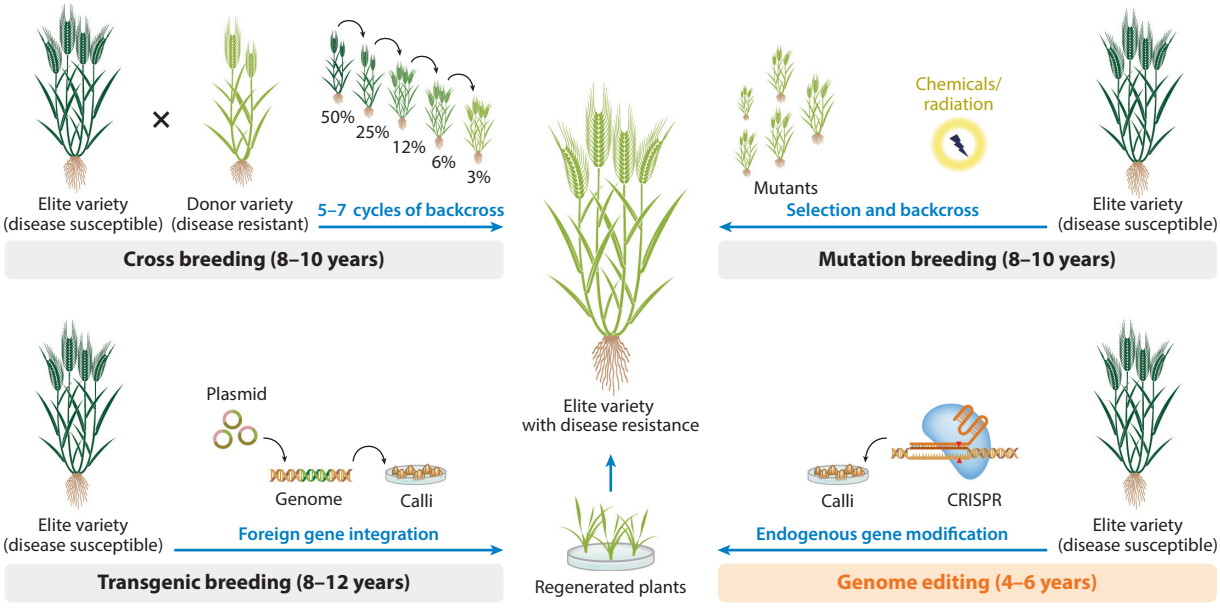


Figure 1

Comparison of breeding methods used in modern agriculture. Cross breeding: improving a trait (e.g., disease resistance) through crossing an elite recipient line with a donor line and selecting outstanding progeny with the desired trait. To introduce the desired trait from the donor line into the elite recipient line, the selected progeny must be backcrossed with the recipient line for several generations to eliminate unexpected linked traits. Mutation breeding: improving a trait using chemical or physical mutagens to treat plant materials (such as seeds) and generate mutants via random mutagenesis. Transgenic breeding: improving a trait by purposefully transferring exogenous genes into elite varieties. Genome editing: improving a trait by precisely modifying the target genes or regulatory elements or rearranging chromosomes in elite varieties.

procedures are restricted by their stochastic nature, and generating and screening large numbers of mutants are challenging. Such time-consuming, laborious, untargeted breeding programs cannot keep pace with the demands for increased crop production, even if marker-assisted breeding approaches are adopted to enhance selection efficiency (138). Transgenic breeding, which generates desired traits through the transfer of exogenous genes into elite background varieties, can break the bottleneck of reproductive isolation. However, commercialization of genetically modified crops is limited by long and costly regulatory evaluation processes as well as by public concerns (129).

Since the first gene-targeting experiment in tobacco (*Nicotiana tabacum*) protoplasts in 1988 (126) and the discovery that DNA double-strand breaks (DSBs) enhance gene-targeting efficiency in 1993 (131), scientists have sought to develop tools for targeted editing of plant genomes. In 2005, zinc finger nucleases were adapted in tobacco (172) and used in trait improvement in a few plants. In 2010, transcription activator-like effector nucleases (TALENs) were added to the plant genome-editing toolbox (19) (see the sidebar titled Zinc Finger Nucleases and Transcription Activator-Like Effector Nucleases). Although the use of these two platforms has led to important advances, each has unique limitations, and their use in plants is far from routine.

Gene targeting: precise gene replacement and knock-in via homology-directed repair

Protoplasts: plant cells lacking cell walls

ZINC FINGER NUCLEASES AND TRANSCRIPTION ACTIVATOR-LIKE EFFECTOR NUCLEASES

Zinc finger nucleases (ZFNs) are sequence-specific nucleases engineered by fusing the specific DNA binding domain of an artificial array of zinc fingers to the nonspecific cleavage domain of FokI. Each zinc finger recognizes a 3-base-pair (bp) target sequence. Because FokI functions as a dimer, typical ZFNs are designed as two ZFN monomers bound to an 18- or 24-bp sequence with a 5–7-nucleotide spacer. ZFNs function via protein-DNA binding, and new ZFNs must be constructed for each editing site. The limited availability of targeting sites in the genome, construction complexity, high but variable off-target rate, and high cost and skill needed for analysis have restricted their application.

Similar to ZFNs, transcription activator-like effector nucleases (TALENs) are artificially fused to a customized array of TALEs to the FokI cleavage domain. The amino acid sequences of the TALE repeat are highly identical except for di-residues at positions 12 and 13, which are known as repeat variable di-residues (RVDs). TALENs target sites in a one-RVD-to-one-nucleotide manner. Typically, a pair of TALE monomers binds to an approximately 50–60-bp target sequence within a 14–18-bp spacer, which is necessary for its function. Owing to the high repeat numbers of RVDs, the construction of TALENs remains challenging.

In 2013, three independent groups established the CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9) system for use in rice (*Oryza sativa*), wheat (*Triticum aestivum*), *Nicotiana benthamiana*, and *Arabidopsis thaliana* (73, 115, 140). For the first time in history, plant breeders had the widespread ability to control the specific introduction of targeted sequence variation, which provides a game-changing resource for rapid improvement of agricultural crops. Since then, continuous improvements in CRISPR/Cas systems, such as CRISPR/Cpf1 (183) and nucleotide substitution tools for base editing (144, 194), have made genome editing a widely adopted, low-cost, easy-to-use targeted genetic manipulation tool that has been applied to many crops (**Figure 1**). Traits that have been modified by genome editing include yield, quality, and biotic- and abiotic-stress resistance. This approach has also enhanced hybrid-breeding techniques, and eliminating unwanted traits or adding desired traits to elite varieties is now a straightforward process, allowing crop traits to be precisely modified, even within a single generation. CRISPR/Cas thus has the potential to enhance global food security and sustainable agriculture.

Hybrid breeding: breeding method in which two genetically different parents are crossbred and produce vigorous hybrids with better trait performance than either parent alone

Spacer: hypervariable sequence between DNA repeats of CRISPR loci derived from invasive nucleic acids

2. CRISPR/CAS SYSTEMS FOR PLANT GENOME EDITING

2.1. CRISPR/Cas Systems

The CRISPR/Cas system, comprising CRISPR repeat-spacer arrays and Cas proteins, is an RNA-mediated adaptive immune system in bacteria and archaea that provides defense against phages and other invasive genetic elements by cleaving the invader's nucleic acid genome. On the basis of their Cas genes and the nature of the interference complex, CRISPR/Cas systems have been divided into two classes that have been further subdivided into six types based on their signature Cas genes. Class 1 CRISPR/Cas systems (types I, III, and IV) employ multi-Cas protein complexes for interference, whereas class 2 systems (types II, V, and VI) accomplish interference with single effector proteins in complex with CRISPR RNAs (crRNAs). The CRISPR system that has been developed for genome editing is based on RNA-guided interference with DNA (64).

Type II CRISPR/Cas9 from *Streptococcus pyogenes* was the first system shown to specifically cleave DNA in vitro and in vivo (21, 36, 54, 103). After repurposing the CRISPR/Cas9

system for gene editing, the CRISPR/Cas9 system now has two components: the Cas9 nuclease and a single guide RNA (sgRNA) consisting of an artificial fusion of a crRNA and a fixed transactivating crRNA (**Figure 2*a***). The sgRNA and Cas9 protein form a Cas9/sgRNA complex, and 20 nucleotides at the 5′ end of the sgRNA direct this complex to a specific target DNA site using Watson-Crick base pairing. The sgRNA is loaded onto Cas9 to direct the cleavage of cognate DNA sequences adjacent to 5′-NGG-3′ protospacer-adjacent motifs (PAMs). Some studies reported that SpCas9 also cleaved target sites with the noncanonical NAG PAMs in mammalian cells (67) and rice (108). Cas9 proteins are characterized by two signature nuclease domains, RuvC and HNH, which cleave DNA strands that are complementary and noncomplementary, respectively (36, 54) (**Figure 2*a***). Ablation of catalytic residues in either domain produces a DNA nickase (nCas9), while, importantly, the inactivation of both domains produces a deactivated Cas9 protein (dCas9) that can still target specific genomic loci and serve as a scaffold for recruiting effector proteins (52). Cas9 enzymes derived from other bacteria, such as *Staphylococcus aureus* (SaCas9), *Streptococcus thermophilus* (StCas9), and *Neisseria meningitides* (NmCas9), have also been developed as tools for genome editing (12). To expand the scope of targeting, Cas9 has been engineered to recognize different PAMs, such as VQR-Cas9 (NGA PAM), EQR-Cas9 (NGAG PAM), VRER-Cas9 (NGCG PAM), SaKKH-Cas9 (NNNRRT PAM) (61), xCas9 (NG, GAA, and GTA PAM) (44), and SpCas9-NG (NG PAM) (120).

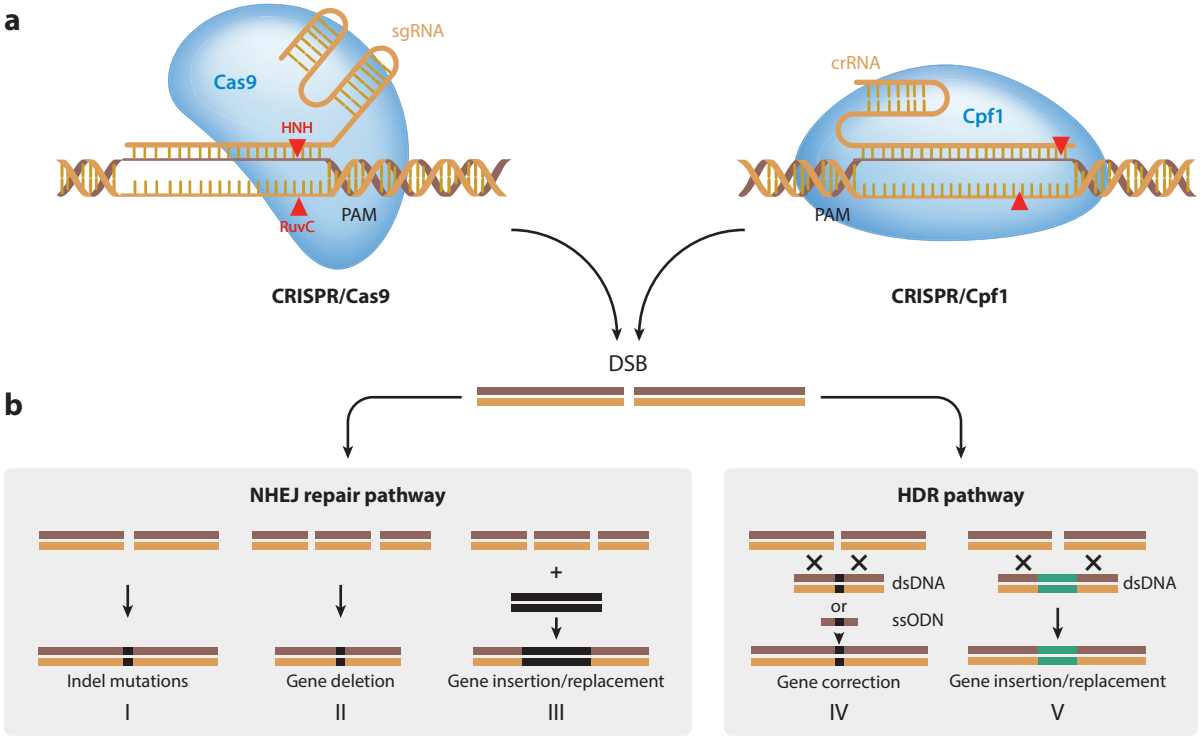
Protospacer-adjacent motif (PAM): conserved sequence adjacent to protospacers

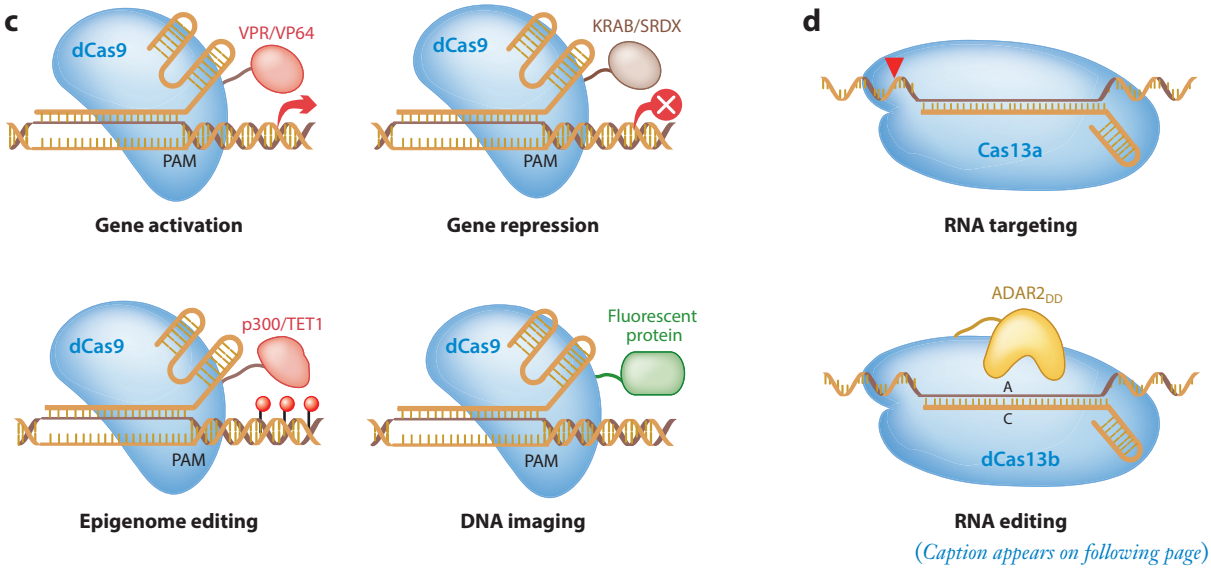
Like the type II CRISPR systems, the new class 2 type V-A Cas enzyme Cpf1 (now known as Cas12a), including variants from *Francisella novicida* (FnCpf1), *Acidaminococcus* sp. (AsCpf1), and *Lachnospiraceae bacterium* (LbCpf1), has also been used as genome-editing tools (183) (**Figure 2*a***). Cpf1 uses a T-rich PAM sequence for target DNA recognition, which expands the editing sites beyond those of G-rich PAM preferred by Cas9. The guide RNA of Cpf1 is shorter than that for Cas9 sgRNA (~43 versus ~80 nucleotides), and the Cpf1 cleavage site is located distal and downstream of the PAM sequence (**Figure 2*a***). Compared with the proximal and upstream sequences cleaved by Cas9, Cpf1 cleaves DNA in a staggered fashion, creating a 5-nucleotide 5′ overhang starting at 18 nucleotides 3′ of the PAM (183) (**Figure 2*a***). To address the limitations of the recognition of only TTTV PAM by Cpf1, Cpf1 variants have been engineered to recognize different PAMs, such as AsCpf1-RR (TYCV PAM), AsCpf1-RVR (TATV PAM), LbCpf1-RR (CCCC and TYCV PAM), and LbCpf1-RVR (TATG PAM) (33, 79, 189). In addition to Cpf1, another group of class 2 type V enzymes, termed Cms1 (CRISPR from *Microgenomates* and *Smithella*), efficiently generates indel mutations in rice. Cms1 nucleases are smaller than Cas9 and Cpf1 nucleases, do not require a transactivating crRNA, and have an AT-rich PAM site requirement (8). Recently, a CRISPR-Cas12b system from *Alicyclobacillus acidiphilus* (AaCas12b), a distinct type V-B system, has been characterized and repurposed to engineer mammalian genomes, and AaCas12b maintains optimal nuclease activity over a wide temperature range (31–59°C) (155).

To date, all Cas9 or Cpf1 systems target DNA. However, class 2 type VI systems, which are characterized by the presence of the single effector protein C2c2 (now known as Cas13), such as LwaCas13a (1) and PspCas13b (22), have been engineered to target RNA or to perform RNA editing (**Figure 2*d***). C2c2 is guided by a single crRNA and can cleave single-strand RNA targets carrying complementary protospacers via its HEPN domains. C2c2-mediated RNA targeting can be used for RNA knockdown and to study RNA biology (22).

2.2. Genome Editing via CRISPR-Induced DNA Double-Strand Breaks

A key characteristic of the CRISPR/Cas gene editing is the creation of DNA DSBs at target loci, which can be used to introduce a variety of genomic modifications by one of two main DNA repair pathways: nonhomologous end joining (NHEJ) and homology-directed repair (HDR) (151) (**Figure 2*b***).





(*Caption appears on following page*)

Figure 2 (*Figure appears on preceding page*)

CRISPR/Cas systems for genome editing and other manipulations. (*a*) Two CRISPR/Cas systems used for plant genome engineering: Cas9 and Cpf1. (*b*) Genome editing with CRISPR/Cas systems can have multiple outcomes, depending on the DSB repair pathways: I, II, and III are outcomes of the dominant NHEJ repair pathway; IV and V are outcomes of the HDR pathway using an available DNA donor template. (*c*) Overview of various applications of dCas9 fusion-based genome manipulations. dCas9 fused with other proteins including transcriptional activators or repressors, epigenetic effectors, and fluorescent proteins can be used for gene regulation, epigenome editing, and genomic labeling. (*d*) Cas13a can be used for RNA targeting, and dCas13b fused with ADAR can be used for RNA editing. Abbreviations: ADAR, adenosine deaminase acting on RNA; crRNA, CRISPR RNA; dCas, deactivated Cas; DSB, double-strand break; dsDNA, double-strand DNA; HDR, homology-directed repair; NHEJ, nonhomologous end joining; PAM, protospacer-adjacent motif; sgRNA, single guide RNA; ssODN, single-strand oligodeoxynucleotide; TET1, ten-eleven translocation.

2.2.1. Genome editing by nonhomologous end joining. The NHEJ repair pathway is preferred throughout much of the cell cycle and does not require a homologous repair template. It has therefore become a popular way to disrupt genes by creating small insertions or deletions at specific points in target genes (**Figure 2*b***). NHEJ can also be exploited to produce insertions of donor DNA sequences in a homology-independent manner and could thus be an efficient method for gene stacking for crop improvement.

A major advantage of CRISPR systems over TALENs and zinc finger nucleases is the ease with which multiple sites can be targeted simultaneously using multiple sgRNAs while expressing a single Cas9 or Cpf1 protein. Multiplex editing has sophisticated applications for genome engineering: It can be used to create multigene knockouts, chromosomal deletions and translocations, and gene knock-ins (135). Many approaches have been used to achieve multiplex gRNA expression from a single cassette in plants. One of the best approaches is the use of a single promoter to achieve uniform expression of each gRNA while fitting the system into a small vector to maintain editing efficiency. This has been achieved using a polycistronic gene construct in which the gRNA is interspersed with ribozyme sites (35), Csy4 recognition sites (14), or transfer RNA sequences (173), which are processed in the plant cell to release mature gRNAs for editing. In addition, the ability of Cpf1 to process its own crRNA provides an efficient method for multiplex genome editing in plants (168). Since it enables the simultaneous modification of multiple traits, the CRISPR system represents a highly efficient method for pyramid breeding.

2.2.2. Precision genome editing via the homology-directed repair pathway. Although NHEJ is highly efficient and well suited for large-scale knockout studies, it lacks the precision required for more sophisticated genome engineering. HDR-mediated genome editing can be used to precisely introduce specific point mutations and to insert or replace desired sequences into the target DNA (**Figure 2*b***). HDR is initiated in the S- and G2-phase of the cell cycle. Repair of the DSB requires a template with homology to the break site. The repair template can be the sister chromatid or an exogenous template, such as an exogenous DNA or single-strand DNA containing a desired sequence modification to be incorporated into the break site (135) (**Figure 2*b***).

Gene stacking: integration of genes into a predetermined locus via homology-directed repair or nonhomologous end joining that can then segregate as a single locus

Precise HDR-mediated genome modification has been widely used in many organisms. However, it is still quite challenging to perform HDR-mediated gene targeting in plants owing to the low efficiency of HDR and the limitations of donor template delivery in plant cells. Many strategies have been used to improve HDR-mediated gene targeting in plants. The use of positive-negative selection has led to successful gene targeting in rice using CRISPR/Cas9 (121), but this method is complex and limited to a few resistance genes. Another efficient strategy is to increase the amount of donor template in single cells to increase HDR efficiency. Geminivirus replicons based on bean yellow dwarf virus and wheat dwarf virus have been engineered to increase the number of copies of the donor template in many plant species, increasing the frequency of

HDR-mediated gene insertion (13, 40). Furthermore, the use of chimeric sgRNA molecules, including sgRNA and repair template sequences, also increases the efficiency of HDR in rice (11).

Cytidine deaminase: enzyme that forms a homotetramer, which catalyzes the irreversible hydrolytic deamination of cytidine and deoxycytidine to uridine and deoxyuridine, respectively

2.3. CRISPR/Cas9-Mediated Manipulations Beyond Double-Strand Breaks

CRISPR is not limited to creating DSBs; dCas9 can provide a unique platform for recruiting proteins for sequence-specific gene regulation, epigenome editing, and genome imaging.

Transcriptional repressor domains fused to dCas9 (e.g., the KRAB domain and SRDX domain) or transcriptional activators (e.g., VP64, p65AD, and VPR) can be used for gene regulation (26) (**Figure 2*c***). Targeted regulation of gene expression has provided interesting insights into the plant genome. Lowder et al. (93) recently evaluated simultaneous multigene activation and repression in plants. A synthetic repressor system (pCo-dCas9-3X-SRDX) was designed and tested on the *Arabidopsis CLEAVAGE STIMULATING FACTOR64* gene and on non-protein-coding genes (redundant microRNAs: miR159A and miR159B) (93). In addition, dCas9 fused to multiple TALEs (a potent dCas9-TV) conferred much stronger transcriptional activation of single or multiple target genes than the routinely used dCas9-VP64 activator in both plant and mammalian cells (87).

dCas9 recruits epigenetic effectors such as the histone demethylase LSD1, histone acetyltransferase p300, and ten-eleven translocation (TET) proteins to modify epigenetic marks at their DNA or histone targets (**Figure 2*c***); this can alter the status of chromatin modification and hence gene expression, cell differentiation, and other biological processes (65). The catalytic domain of the human demethylase TET1 fused with dCas9 and targeting the FWA promoter led to highly efficient targeted demethylation in *Arabidopsis* (32).

dCas9 fused with a fluorescent protein, such as GFP, can be used to visualize DNA loci harboring repetitive sequences and to label endogenous centromeres, pericentric regions, and telomeres with single or multiplex sgRNAs (16) (**Figure 2*c***). This type of genome imaging is used to examine features of plant genome architecture. For example, using dCas9 fused with eGFP/mRuby, researchers visualized telomere repeats in live *N. benthamiana* leaf cells and examined DNA-protein interactions in vivo (28).

3. BASE EDITING IN PLANTS

Beyond DSB-mediated genome editing, base-editing systems that can induce specific base changes that do not depend on HDR or donor DNA and do not involve the formation of DSBs provide a high-efficiency, simple, universal strategy for engineering nucleotide substitutions at target sites (**Figure 3**).

The cytosine base-editor (CBE) system, which consists of a cytidine deaminase fused with an nCas9 (D10A) and a uracil glycosylase inhibitor, converts targeted cytosine to uracil in genomic DNA (63) (**Figure 3*a***). Cytidine deaminases first convert the cytosine in DNA to uracil, and the uracil is then replaced by thymine during DNA replication. During this procedure, the fused uracil glycosylase inhibitor binds to and inhibits uracil DNA glycosylase, thus blocking uridine excision and the ensuing base excision repair pathway activity and increasing base-editing efficiency. The efficient base-editor 3 (BE3) system involving the fusion of the rat cytidine deaminase APOBEC1 (developed by the Liu group) has been widely used for gene editing in various animal and plant species (43). Modifications have been made to BE3 to expand its PAM requirements and to increase its editing efficiency and specificity (43). Similarly, three orthologs of cytidine deaminase, lamprey PmCDA1 (119), human AID (101), and human APOBEC3A (38, 169), have been combined with nCas9 to achieve efficient C-to-T substitution. The human APOBEC3A-based plant cytidine base editor has been used to efficiently convert Cs to Ts

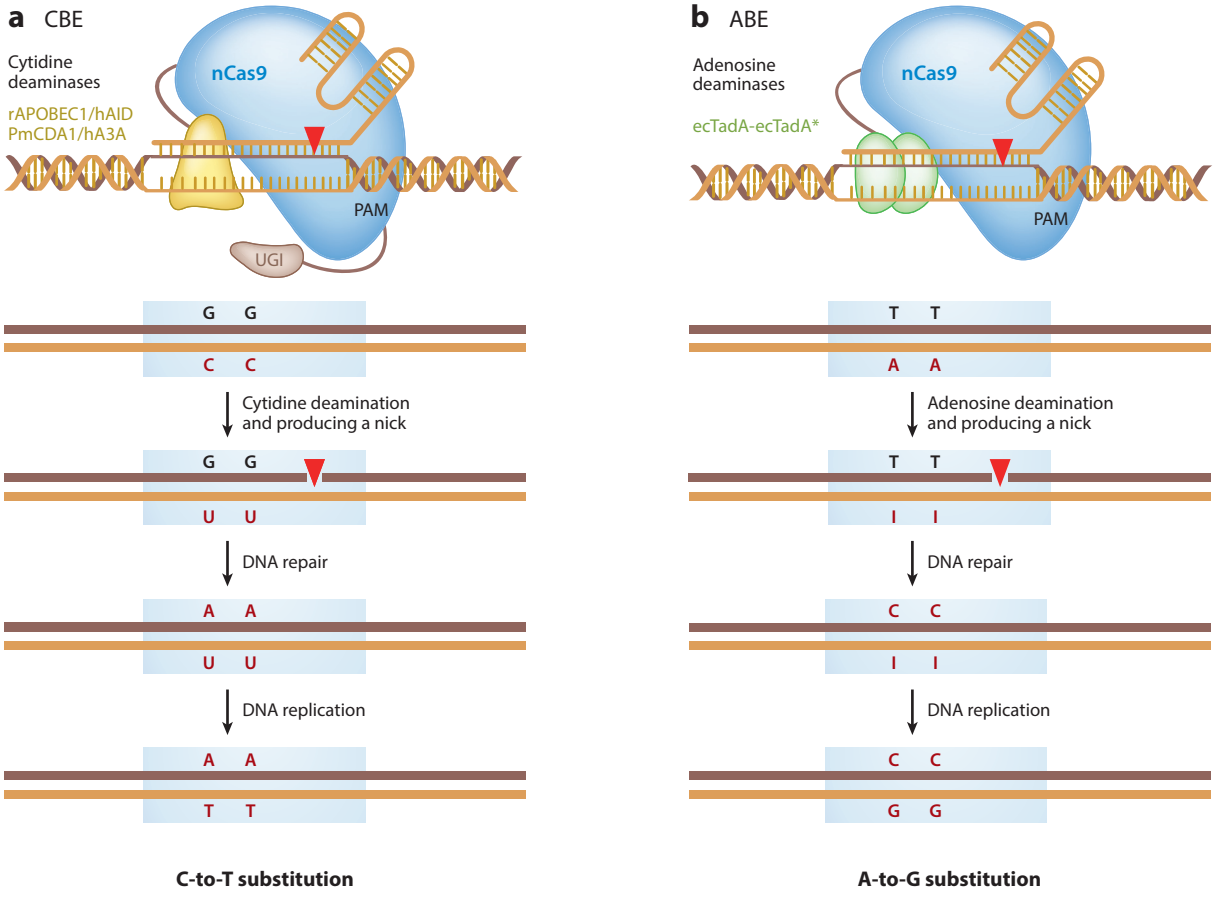


Figure 3

Mechanisms of base editing. (*a*) CBE-mediated C-to-T base-editing strategy. The deaminases include rAPOBEC1, hAID, PmCDA1, and hA3A. (*b*) ABE-mediated A-to-G base-editing strategy. The deaminase is the fusion protein ecTadA-ecTadA*. Abbreviations: ABE, adenine base editor; CBE, cytosine base editor; hAID, human activation-induced cytidine deaminase; nCas9, a DNA nickase; PAM, protospacer-adjacent motif; UGI, uracil glycosylase inhibitor.

in wheat, rice, and potato (*Solanum tuberosum*), with a 1–17-nucleotide editing window at all examined sites independent of sequence context (85, 193).

The Liu group subsequently developed adenine base editors (ABEs) that mediate the conversion of A to G in genomic DNA (37) (**Figure 3*b***). They used seven rounds of directed evolution and protein engineering to develop several versions of ABEs, consisting of *Escherichia coli* TadA (transfer RNA adenosine deaminase) and nCas9 (D10A). The seventh-generation ABEs (7.10) were used to convert A to G in a wide range of targets with high efficiency and product purity (37). ABE systems have also been optimized for wheat and rice, and our group has shown that the use of enhanced sgRNAs [sgRNA$^{(F+E)}$] combined with three copies of nuclear localization sequences at the C terminus of nCas9 achieved A-to-G conversion efficiencies of up to 60% in rice and wheat (69).

Base-editing systems offer several advantages over non-DSB-mediated genome editing in plants: (*a*) They are more efficient and generate far fewer undesired products than do DSB-mediated systems; (*b*) multiplex or whole-gene base editing is not likely to lead to chromosomal

rearrangements, such as large deletions and inversions; and (*c*) they can be used to create nonsense mutations to avoid DSB-induced in-frame indels. Although base-editing systems can be used to efficiently create the point mutation in the target site, they are unlikely to completely replace the strategies for DSB-mediated genome modification, such as gene insertion and gene replacement. Base-editing systems will be valuable tools for genetic research with various agricultural applications.

Ribonucleoprotein (RNP): complex composed of purified Cas protein and in vitro transcribed or artificially synthesized sgRNA

Transgene: segment of DNA isolated from one organism and introduced into a different organism

4. DELIVERY OF CRISPR/CAS REAGENTS TO PLANTS

The delivery of editing reagents to plant cells and the production of editing events are key steps in genome editing. CRISPR-mediated editing reagents, including DNA, RNA, and ribonucleoproteins (RNPs), can be delivered into plant cells by protoplast transfection, *Agrobacterium*-mediated transfer DNA (T-DNA) transformation, or particle bombardment. Protoplast transfection is normally used for transient expression, whereas *Agrobacterium*-mediated transformation and particle bombardment are the two major delivery methods for the production of edited plants.

4.1. Plant Genome Editing via CRISPR/Cas DNA

CRISPR/Cas DNA is the most commonly used genetic cargo for plant editing. DNA cassettes expressing Cas protein and sgRNA may be degraded or become randomly integrated into the plant genome.

4.1.1. Genome editing with stable expression of CRISPR/Cas DNA. On the basis of traditional DNA transformation methods, CRISPR/Cas DNA is delivered by *Agrobacterium*-mediated transformation or particle bombardment into recipient cells and, by selecting for a marker gene, the DNA is integrated into the plant genome and expressed to bring about genome editing (**Figure 4*a***). This strategy has been used for most types of plant genome editing. However, CRISPR constructs and marker genes can become integrated into the genome and cause side effects such as increased off-target changes, which could limit commercial applications. To avoid these problems, transgene-free derivatives can be obtained through genetic segregation by selfing and crossing. Gao et al. (34) included a fluorescent cassette as a marker for the presence of the CRISPR/Cas9 construct. Another interesting method is to use the suicide genes *CMS2* and *BARNASE* to kill transgene-containing pollen and embryos produced by the T0 plant (42). Although genetic segregation is an efficient method for obtaining transgene-free mutants, segregation cannot be used for asexually propagated crops such as potato, cassava (*Manihot esculenta*), and banana (*Musa* spp.). Moreover, a fragment of the DNA construct could become integrated into unknown sites.

4.1.2. Genome editing by transient expression of CRISPR/Cas DNA. Transient gene expression of CRISPR reagents represents an alternative delivery method for achieving transgene-free editing (**Figure 4*b***). In this method, the canonical selection steps using herbicides or antibiotics are eliminated so that some of the regenerated plants are edited without any integration of foreign DNA into the genome. This method was first reported in wheat (188). A CRISPR/Cas9 plasmid was delivered into immature wheat embryos by particle bombardment, and the resulting plants were regenerated without selection pressure, reducing the time required for regeneration via tissue culture by 3–4 weeks. The mutation frequencies were comparable to those of the conventional DNA-integration method in which selection pressure is applied during tissue culture. Importantly, transgenes were undetectable in up to 86.8% of the T0 mutants. The DNA of base

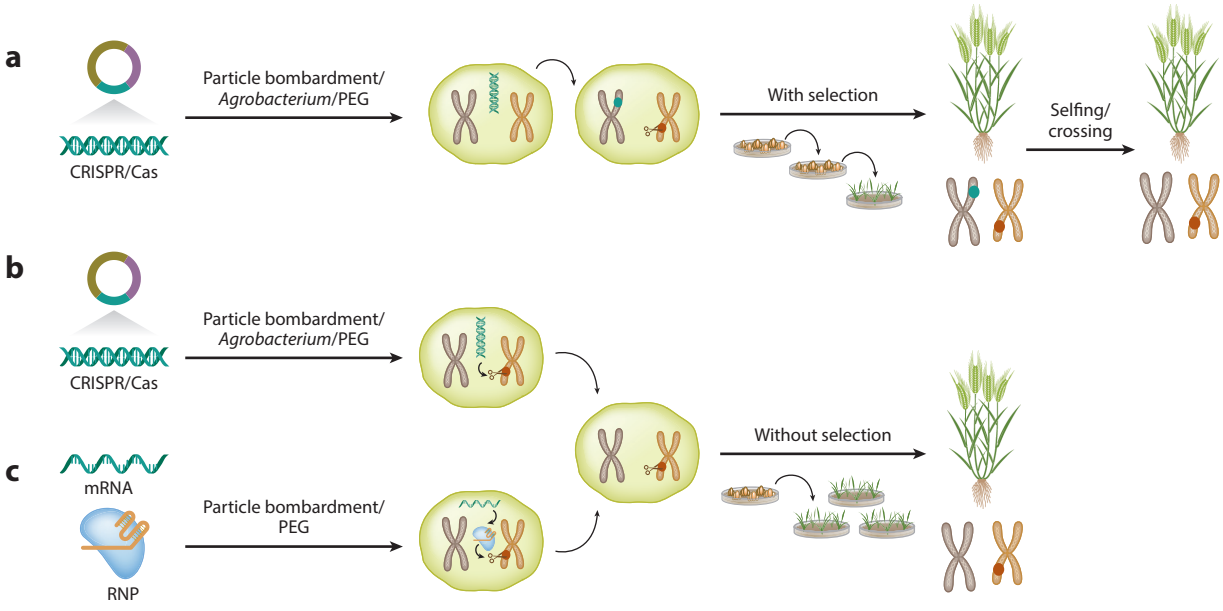


Figure 4

Delivery strategies for CRISPR/Cas systems to plants. (*a*) Traditional delivery methods for CRISPR/Cas DNA combined with herbicide or antibiotic selection. Transgene-free plants can be obtained through genetic segregation by selfing and crossing. (*b*,*c*) Transient delivery systems for transgene-free and DNA-free genome editing. CRISPR reagents include DNA, mRNA, and RNP. After transient expression, CRISPR/Cas DNA, mRNA, or RNP will be degraded, and the edited plants can be regenerated without selection pressure. Abbreviations: mRNA, messenger RNA; PEG, polyethylene glycol; RNP, ribonucleoprotein.

editors, such as plant cytidine base editors and plant adenine base editors, has also been delivered by the transient method, achieving transgene-free nucleotide substitutions (69, 193, 194). In addition to biolistic delivery, transgene-free editing has also been achieved by protoplast transformation in tetraploid potato and tobacco protoplasts (5, 91) and by *Agrobacterium*-mediated transient CRISPR/Cas expression in tobacco (17).

4.2. DNA-Free Genome Editing via CRISPR/Cas In Vitro Transcripts or Ribonucleoproteins

While the transient expression of CRISPR/Cas DNA succeeds in reducing transgene integration, it does not completely eradicate it; moreover, degraded DNA fragments may still be integrated into the plant genome. However, Zhang et al. (188) delivered in vitro transcripts of Cas9 and sgRNAs into immature wheat embryos by particle bombardment and generated DNA-free edited wheat (**Figure 4*c***). The editing efficiency was lower than that using the DNA expression system, perhaps owing to instability of the RNA. Nevertheless, this RNA delivery method reduces off-target effects.

To avoid the disadvantages of plasmid- and messenger RNA (mRNA)-based expression of Cas9/sgRNA, an efficient DNA-free genome-editing system has been developed using Cas9/sgRNA RNPs in plants (89, 90, 149, 171) (**Figure 4*c***). Cas9/sgRNA RNPs are as efficient as plasmid-based expression systems and have a low off-target frequency in cells. As the RNP can cleave the target immediately upon delivery without requiring the cellular transcription and translational machinery and is then degraded quickly, it has a lower propensity for off-target cleavage

than DNA-based expression. In 2015, Woo et al. (171) were the first to demonstrate genome editing in rice, *Arabidopsis*, tobacco, and lettuce (*Lactuca sativa*) protoplasts using polyethylene glycol–mediated RNP transfection. Regenerated lettuce mutants were produced at frequencies of up to 46%. Importantly, no off-target mutations were detected in *Arabidopsis* protoplasts or *bin2* lettuce mutants. CRISPR/Cas9 RNP has also been successfully delivered into grape (*Vitis vinifera*), apple, *Petunia* × *hybrida*, and potato protoplasts (6, 104, 146). Furthermore, Kim et al. (59) added Cpf1 to the RNP editing toolbox in plants, delivering LbCpf1/crRNA and AsCpf1/crRNA RNPs into soybean and wild tobacco protoplasts. However, regeneration from protoplasts is still challenging for most cereal crops, particularly monocots.

Therefore, RNP delivery by particle bombardment is the method of choice for most crops. CRISPR/Cas9 RNP delivery by particle bombardment has been reported in maize and wheat (89, 90, 149). Svitashev et al. (149) delivered preassembled Cas9/sgRNA RNPs into maize embryos and regenerated maize plants without selection, achieving editing frequencies ranging from 2.4% to 9.7%. Of these mutants, approximately 10% contained biallelic modifications. No mutations were detected at the off-target sites. Similarly, Liang et al. (89) delivered RNPs by particle bombardment into immature wheat embryos, achieving a comparable on-target editing frequency to that obtained in a parallel transient DNA expression experiment but with a lower off-target editing rate. As RNPs have been successfully used for BE3 and high-fidelity BE3 in animals (60, 133), the prospects for base editing with RNPs in plants are also good. Thus far, the A3A plant cytidine base editor has been used without uracil glycosylase inhibitor protein and assembled with in vitro–transcribed sgRNA for successful base editing in wheat protoplasts (193).

5. APPLICATIONS FOR PRECISION PLANT BREEDING

5.1. Knockout-Mediated Crop Trait Improvement

Eliminating negative elements is a promising strategy for genetic improvement. Therefore, knocking out genes that confer undesirable traits is the simplest and most common application of CRISPR/Cas9 (**Figure 5*a***). Traits that have been improved to date using CRISPR/Cas9 include yield, quality, and biotic- and abiotic-stress resistance. Hybrid-breeding techniques and many other important aspects of crop productivity have also been enhanced using this approach.

5.1.1. Increasing yields. The need for improved food security makes yield the primary target of gene editing for crop improvement. Yield is a complex trait that is dependent on many factors. Knocking out negative regulators known to affect yield-determining factors such as grain number (*OsGn1a*), grain size (*OsGS3*), grain weight (*TaGW2*, *OsGW5*, *OsGLW2*, or *TaGASR7*), panicle size (*OsDEP1*, *TaDEP1*), and tiller number (*OsAAP3*) created the expected phenotypes in plants with loss-of-function mutations in these genes, demonstrating that CRISPR/Cas9 is an effective tool for improving yield-related traits (74, 78, 92, 97, 187, 188). Simultaneous knockout of three grain weight–related genes (*GW2*, *GW5*, and *TGW6*) in rice led to trait pyramiding, which greatly increased grain weight (176). However, because most yield-related traits are quantitative and controlled by quantitative trait loci, simply knocking out individual factors may not be sufficient to increase yield in the field. Huang et al. (45) recently developed a method for the large-scale identification of genes that contribute to complex quantitative traits, such as yield, by combining pedigree analysis, whole-genome sequencing, and CRISPR/Cas9 technology. The authors sequenced 30 cultivars of the parents and descendants of the Green Revolution miracle rice variety IR8 and selected 57 genes retained in all high-yielding lines for gene editing via knockout or knockdown using Cas9 or dCas9. Phenotypic analysis revealed that many of these genes are

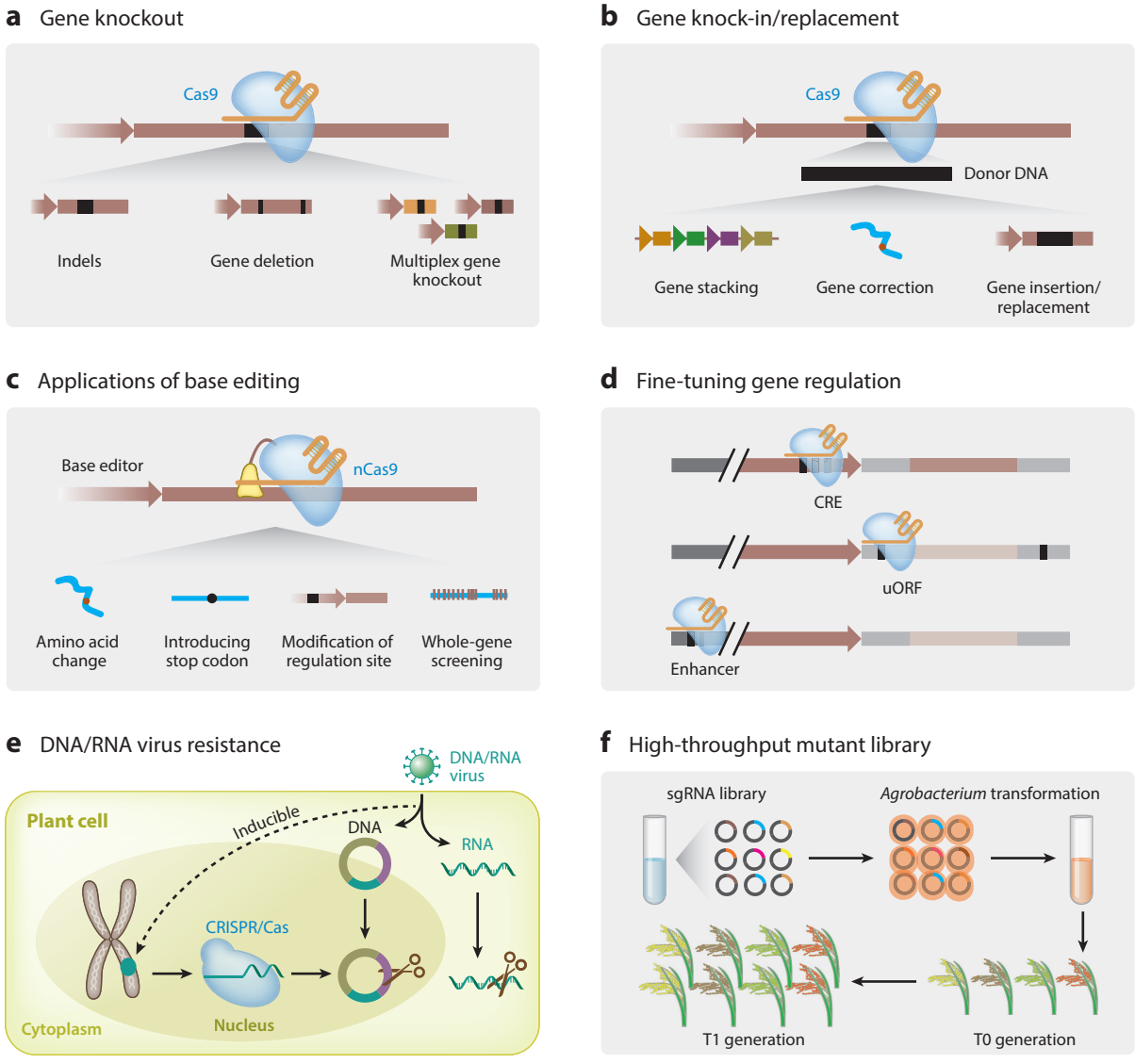


Figure 5

Overview of potential CRISPR/Cas-based applications for plant breeding. CRISPR/Cas-mediated crop trait improvement mainly focuses on yield, quality, and biotic and abiotic resistance. (*a*) CRISPR/Cas-mediated mutation can achieve indels, gene deletions, and multiplex gene knockout. (*b*) Gene insertion and replacement mediated by either homology-directed repair or nonhomologous end joining can achieve gene stacking for multiple traits, gene correction for gain-of-function, and gene insertion or replacement to produce new traits in breeding. (*c*) Applications of base editing for crop trait improvement, such as precise amino acid substitution, gene disruption by introducing a stop codon, gene regulation, and whole-gene screening. (*d*) CRISPR/Cas system-based gene regulation by editing the regulatory site in the untranslated region, promoter, or enhancer region. (*e*) CRISPR/Cas-based antiviral breeding strategies. The CRISPR system with a guide RNA targeting DNA or RNA viruses is integrated into the plant genome, conferring resistance to invading viruses. (*f*) CRISPR/Cas-based genome-wide screening, a valuable technique for functional genomics and genetic improvement. Abbreviations: CRE, *cis*-regulatory element; sgRNA, single guide RNA; uORF, upstream open reading frame.

important for rice yield. This work provided insight into the mechanism of yield development and may facilitate the molecular breeding of improved rice.

5.1.2. Improving quality. Quality traits vary depending on the specific breeding requirements. To date, quality improvements by genome editing have impacted starch content, fragrance, nutritional value, and storage quality in crops. Rice with low amylose content, and thus improved rice eating and cooking quality, was generated by knockout of *Waxy* via CRISPR/Cas9 (100, 185). DuPont Pioneer produced a CRISPR/Cas9 knockout waxy corn line with high yields for commercial use (162). CRISPR/Cas9 was also used to produce high-amylose and resistant starch rice by mutating the starch branching enzyme gene *SBEIIb*; consuming high-amylose foods should benefit patients with diet-related noninfectious chronic diseases (147).

Fragrance is an important quality trait in rice, and rice varieties with desirable fragrances when cooked have increased commercial value. A defect in the betaine aldehyde dehydrogenase 2 (*BADH2*) gene results in the biosynthesis of 2-acetyl-1-pyrroline, the major fragrance compound in fragrant rice. Using TALEN-targeted disruption of *OsBADH2*, our group has created a fragrant rice line with a similar 2-acetyl-1-pyrroline content (0.35–0.75 mg/kg) to that of the natural mutant fragrant rice variety (141). With the advent of convenient CRISPR/Cas9 techniques, we recently added the fragrance trait to more than 30 elite rice cultivars in major planting areas of China (C. Gao, unpublished data).

Gluten proteins from cereal crops trigger celiac disease in more than 7% of individuals in Western countries. The α-gliadin gene family, the major gluten-encoding gene family in wheat, consists of nearly 100 genes or pseudogenes. CRISPR/Cas9 editing offers a new way to alter traits controlled by large gene families with redundant functions. Indeed, by simultaneously knocking out most conserved domains of α-gliadin family members, researchers have created low-gluten wheat (136).

Other high-quality crops produced by CRISPR/Cas9 editing include seeds with high oleic acid oil in *Camelina sativa* (53, 112) and *Brassica napus* (123), tomatoes with a long shelf life (48, 76), high-value tomato with enhanced lycopene (82) or γ-aminobutyric acid content (77, 122), and potato (hairy roots) with reduced levels of toxic steroidal glycoalkaloids (113).

5.1.3. Biotic- and abiotic-stress resistance. Stresses are the main factors affecting crop yield and quality. Many plants with increased biotic-stress resistance, including resistance to fungal, bacterial, and viral diseases and insects, have been obtained via CRISPR/Cas9 knockout. For example, powdery mildew is a devastating fungal disease in crops. Using TALEN and CRISPR/Cas9, our laboratory knocked out all six *TaMLO* alleles in wheat and obtained plants with increased resistance to powdery mildew (170). Similarly, Nekrasov et al. (116) showed that CRISPR/Cas9-mediated knockout of *MLO* confers resistance to powdery mildew in tomato. Rice blast is a destructive fungal disease; blast-resistant rice was obtained via knockout of *OsERF922*, an ethylene-responsive factor transcription factor gene (164). Bacterial blight in rice is caused by *Xanthomonas oryzae* pv. *oryzae*. Deletion of the *OsSWEET13* promoter led to the production of plants resistant to this disease (191). With regard to viral diseases, use of CRISPR/Cas9 has also produced tungro disease–resistant *eif4g* rice (102), broad potyvirus–resistant *eif4e* cucumber (15), and cotton leaf curl disease–resistant *clcud* cotton (47). Recently, Lu et al. (96) found that disrupting *OsCYP71A1* blocked serotonin biosynthesis and greatly increased salicylic acid levels, thereby conferring resistance to plant hoppers and stem borers, the two most destructive pests of rice.

Among abiotic stresses, contamination of arable lands has created the need to prevent the accumulation of toxic heavy metals in crops. By knocking out *OsARM1*, *OsNramp5*, and *OsHAK1*, breeders have developed rice strains with low levels of cadmium, radioactive cesium, and arsenic,

respectively (118, 152, 165). In 2018, research on the *OsPYL* abscisic acid receptor gene family revealed that *pyl1/4/6* triple knockout rice created by CRISPR/Cas9 editing had increased grain yield, greater high-temperature tolerance, and reduced preharvest sprouting compared with wild type (110).

5.1.4. Speeding hybrid breeding. Hybrid breeding is a powerful approach for increasing crop productivity. A prerequisite for producing a high-quality hybrid variety is a male-sterile maternal line. Tremendous progress has been made in using CRISPR/Cas-mediated gene knockout to produce male-sterile lines, including thermosensitive male-sterile *tms5* lines in rice (190) and maize (72), photosensitive genic male-sterile *csa* rice (75), and *ms45* wheat (145). Hybrid sterility is the main obstacle to exploiting heterosis in breeding. To overcome the reproductive barriers in *japonica-indica* hybrids, *SaF/SaM* at the sterility locus *Sa* (174) and *OgTPR1* at the *S1* locus (175) were disrupted. Shen et al. (142) found that knockout of one or two copies of the *Sc* gene in the *indica* allele *Sc-I* also rescued male fertility in *japonica-indica* hybrids. Similarly, Yu et al. (181) showed that knockout of the toxin gene *ORF2*, which is responsible for the recently discovered selfish-gene suicide mechanism in rice, improved the fertility of *japonica-indica* hybrids. Very recently, genome editing was used to substitute mitosis for meiosis in rice by knocking out three key meiotic genes, *REC8*, *PAIR1*, and *OSD1*. Two independent groups developed asexual propagation lines either by simultaneous activation of BBM1 in the egg cell (57) or by knocking out *MTL* (163), enabling a fix of heterozygosity of hybrids through seed propagation.

Genome editing is also an effective approach for enhancing many other traits, such as improving haploid breeding (27, 178), shortening growth times (83), increasing silique shatter resistance (9), and overcoming self-incompatibility in diploid potato (179), to meet breeders' requirements.

5.2. Crop Trait Improvement via Knock-In and Replacement

Many agronomic traits are conferred by single-nucleotide substitutions, gene expression changes, or the addition of new gene functions. 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 (99) (**Figure 2*b***). Moreover, knock-in can be used to alter multiple elite traits by stacking genes in a single variety. Therefore, knock-ins and replacements have great value for crop trait improvement (**Figure 5*b***).

Unfortunately, because HDR is an infrequent DNA repair pathway, these techniques are far from routine, and their use in trait improvement has thus far been quite limited. Nevertheless, Shi et al. (143) used CRISPR/Cas9-mediated gene editing to improve drought tolerance in maize. *ARGOS8* encodes a negative regulator of ethylene responses and is expressed at low levels in most inbred maize lines. The authors increased *ARGOS8* expression by substituting or knocking in the *GOS2* promoter in place of the native *ARGOS8* promoter via HDR to drive *ARGOS8* expression (143). The edited *ARGOS8* variants had elevated *ARGOS8* transcript levels and increased yields under drought stress. Yu et al. (180) also created a tomato line with long shelf life by editing a T317A replacement into the *ALC* gene.

For HDR efficiency, a geminivirus-based DNA replicon has been used to increase the number of repair templates, which increased gene-targeting efficiency in potato (10), tomato (13, 24), rice (167), wheat (40), and cassava (46). For example, using geminivirus replicons, Čermák et al. (13) achieved a tenfold increase in the frequency of insertion of the cauliflower mosaic virus 35S promoter upstream of *ANT1* in tomato; constitutive expression of *ANT1* led to the generation of a purple tomato with increased anthocyanin content. Dahan-Meir et al. (24) developed a highly efficient selection- and reporter-free gene-targeting procedure using replicon-amplified donor

fragments and successfully repaired a fast-neutron-induced *crtiso* allele in tomato containing a 281-base-pair deletion with an efficiency rate as high as 25%.

Because herbicide selection is helpful for enriching gene-targeting events, the endogenous acetolactate synthase (*ALS*) and 5-enolpyruvylshikimate-3-phosphate synthase (*EPSPS*) genes are common targets of gene editing: Substitution of key amino acids in the conserved domains of *ALS* and *EPSPS* can confer resistance to sulfonylurea-based herbicides or glyphosate. Sulfonylurea-based herbicide-resistant soybean (84), maize (149, 150), and rice (11, 29, 148) plants were generated through HDR-introduced nucleotide changes in *ALS*. Similarly, T102I/P106S (TIPS) and T102I/P106A double amino acid substitutions of *EPSPS* were introduced into flax (*Linum usitatissimum*) (137) and cassava (46) by selecting for HDR-generated glyphosate resistance. Owing to the low rate of HDR, our laboratory collaborated with Li's laboratory to create TIPS *EPSPS* glyphosate-resistant rice via an intron-targeting strategy involving NHEJ-mediated gene replacement and insertion (71). Although indels may arise at the junctions of the recombination sites of the targeted intron, the final gene transcript is not affected. This method represents a higher-frequency alternative to HDR-mediated gene targeting in plants.

5.3. Applications of Base Editors in Plants

As many agriculturally important traits are conferred by single-nucleotide polymorphisms in either coding or noncoding regions, base editing is quite useful for plant breeding and crop improvement (**Figure 5*c***). One important application of base editing in coding regions is to confer herbicide resistance. Sulfonylurea- or imidazolinone-resistant rice (144), wheat (193; C. Gao, unpublished data), *Arabidopsis* (18), and watermelon (156) have been created by targeting *ALS* with a plant cytidine base editor, and haloxyfop-R-methyl resistant rice has been generated by targeting acetyl-coenzyme A carboxylase *ACCase* gene with a plant adenine base editor (69). Notably, the genotypes of base-edited polyploid wheat plants are extremely variable, especially when two sgRNAs are involved (C. Gao, unpublished data).

Alternative splicing is a regulatory process of gene expression that results in a single gene encoding multiple proteins, which can greatly increase the diversity of proteins capable of producing new traits. Base editing is also used to regulate RNA splicing pathways. Most eukaryotic mRNA splicing processes follow the canonical GU/AG rule. In these genes, introns contain a splice donor site (GU) at the 5′ end and a splice acceptor site (AG) at the 3′ end. Base editing can generate point mutations at these conserved nucleotides, leading to the loss of particular splice forms or mis-splicing. Kang et al. (56) disrupted the splicing acceptor site on an intron by converting A to G, which led to altered splicing of *AtPDS* mRNA. Xue et al. (177) converted G to A in the splice donor site, resulting in the constitutive retention of an intron of *AtHAB* and hypersensitivity to abscisic acid. In addition, Li et al. (86) created mRNA mis-splicing-induced null mutants of *AtMTA* and double mutants of *OsGL1* and *OsNAL1* in rice.

5.4. Fine-Tuning Gene Regulation in Plants

Besides creating mutations in coding sequences, modulating gene expression is a useful approach for examining gene function and can greatly facilitate plant breeding. Gene expression can be affected at several levels, including transcription, mRNA processing, and mRNA translation. These processes are under the control of a series of *cis*-regulatory elements, which can be modified by genome editing (**Figure 5*d***). To date, plant genome editing to alter gene expression has focused mainly on promoters, such as replacing promoters and deleting *cis*-regulatory elements (127, 128). Rodríguez-Leal et al. (134) edited the promoter regions of quantitative trait–related genes such as

SlCLV3, *SlS*, and *SlSP*, creating a continuum of variation and leading to the selection of mutated alleles with improved yields.

Gene regulation can also occur at the translational level. Upstream open reading frames (uORFs) are well-known *cis*-elements that often have negative effects on translation and nonsense-mediated mRNA decay (159). Bioinformatic analysis predicts that uORFs are widespread among plant mRNAs. For example, more than 35% of *Arabidopsis* mRNAs contain at least one putative uORF (159), and targeting uORFs with antisense oligonucleotides increases the translation efficiencies of primary ORFs (88). Zhang et al. (184) reported that deleting the translation start codon of a uORF enhanced translation of the downstream primary ORF. Also, using CRISPR/Cas9 to target a uORF in *LsGGP2*, the ascorbate content of lettuce (*L. sativa*) leaves increased by 80–140% (184).

Upstream open reading frame (uORF): protein-coding region with a translational start codon before the main coding region

In terms of regulation at the transcriptional level, enhancers also contain important *cis*-regulatory elements. Locating DNase I hypersensitive sites is an efficient way to identify putative enhancer elements (192). Many elements of mRNA 5′ leader sequences, such as RNA structures and internal ribosome entry sites, can affect translation (88). Some regulatory elements in 3′ untranslated regions also play important roles in gene regulation (81). These key elements all provide targets for genome editing.

5.5. Antiviral Plant Breeding Strategies

Viruses are thought to cause roughly half of all plant diseases, leading to massive losses in agricultural production worldwide (182). Because the CRISPR/Cas system provides a defense mechanism that cleaves plasmids, DNA viruses, and RNA viruses that invade archaea and bacteria, it can also be used to confer virus resistance in plants (**Figure 5*e***). For example, geminiviruses are single-strand DNA viruses with a double-stranded intermediate necessary for rolling-circle replication. Stable overexpression of Cas9 and sgRNAs that specifically target the geminivirus genome to inhibit its replication has been used for antiviral breeding in plants (3, 7, 51). However, indels caused by the NHEJ pathway are created at DSB sites, making it possible to generate virus variants that can escape Cas9/sgRNA cleavage (107). Because the stem-loop intergenic sequence is essential for geminivirus replication, and intergenic sequences that harbor indels generally lose replication initiation activity, these sequences are ideal targets for creating geminivirus-resistant plants (4). A drawback of the antiviral system is that constitutive expression of Cas9/sgRNA is apt to cause off-target mutations, but using a virus promoter to drive Cas9 expression can decrease off-target effects to an undetectable level (50).

Compared with DNA viruses, RNA viruses cause more losses to agricultural production (186). FnCas9 binds to RNA in a PAM-independent manner and inhibits translation and replication of hepatitis C virus in mammalian cells (130). FnCas9 also efficiently represses replication of cucumber mosaic virus and tobacco mosaic virus in plants (186). Unlike most Cas proteins, C2c2 can cleave single-strand RNA (2), and it interferes with turnip mosaic virus replication in plants (114).

5.6. High-Throughput Plant Mutant Libraries

Whole-genome-scale mutant libraries are valuable tools for functional genomics and genetic improvement (**Figure 5*f***). Traditional mutant libraries are based on random mutations induced by agents such as irradiation, T-DNA insertions, ethyl methanesulfonate (EMS) mutagenesis, and transposons. However, use of these methods requires many generations to stabilize loss-of-function mutations, and determining the relationship between phenotype and genotype among mutants is a time-consuming and laborious process. Two groups constructed large-scale

CRISPR/Cas9-generated knockout mutant libraries covering most rice genes (98, 109). Meng et al. (109) targeted nearly 13,000 genes that are highly expressed in rice shoot base tissue and obtained more than 14,000 independent T0 lines. Similarly, Lu et al. (98) targeted 34,234 genes in rice and generated more than 90,000 transgenic plants. Homozygous mutants were obtained in a single generation. Moreover, by locating the sgRNA, researchers can easily link phenotype with genotype. Finally, Jacobs et al. (49) produced mutant libraries of immunity-associated leucine-rich repeat genes of subfamily XII, comprising 54 members in tomato. The availability of high-quality, high-coverage, uniformly distributed knockout mutant libraries could facilitate the development of innovative germplasm strategies as well as crop trait improvement.

6. FUTURE PROSPECTS

6.1. Exploiting the Potential of Plant Synthetic Biology

Plant synthetic biology is an emerging field that combines plant biology with engineering principles to design and produce new devices that exhibit predictable behaviors. This field will play an important role in traditional crop improvement and will enable the development of novel bioproduction processes (117). Plants are the most important sources of the primary metabolites that feed the world (i.e., proteins, fatty acids, and carbohydrates), and they produce a diverse array of valuable secondary metabolites for medicinal and industrial purposes. The first transgenic plant was generated more than 30 years ago, marking the beginning of the age of designing plants with novel functions. The CRISPR/Cas system has great potential for improving plant design and synthetic biology. For example, artificial DNA sequences, including promoters, genes, transcriptional regulatory elements, and genome assemblies, can be inserted into plant genomes to alter cell or plant behavior to generate novel functions.

Nitrogen is a critical limiting element for crop growth and development. Most nitrogen fixation (*nif*) genes and their relative expression levels have been characterized (154). To reduce our dependence on inorganic fertilizers, the CRISPR/Cas system could be used to transfer the genetic elements of the Nod factor signaling pathway from legumes to cereals such as wheat, allowing the cereal to fix atmospheric nitrogen. In addition, an important goal of synthetic biology is to build regulatory circuits to manipulate plant behavior, producing novel traits that improve crop productivity (55). dCas9-mediated gene regulation via multiplex gene activation, repression, and epigenome editing offers unprecedented opportunities for designing synthetic transcription factors, which could be used to construct increasingly complex, programmable, efficient gene circuits. For example, in the C4 rice project (160), it is difficult to guarantee that the C4 photosynthesis pathway installed in rice can efficiently fix carbon; fine-tuning gene expression in the C4 pathway is required to optimize protein levels to increase the efficiency of carbon fixation. CRISPR/Cas-mediated multiplex gene regulation could serve as a tool for this synthetic biology project. In addition, dCas9-inducible systems could be combined with AND, OR, NAND, and NOR logic operators in cellular gene circuits.

6.2. Accelerating the Domestication of Wild Plants

Modern crops have been selectively bred for thousands of years, leading to the introduction of important characteristics that enable mechanical harvesting of high-quality, nutrient-rich food. However, this process has led to a loss of diversity that can affect fitness under certain environmental conditions (124). Key domestication events are mainly associated with mutations in so-called domestication genes with marked effects on key phenotypes, such as barley *vrs1*, responsible for

spike number (62); maize *tga1*, conferring naked kernels (166); and rice *Sh4*, *Rc*, *PROG1*, and *LABA1*, for nonshattering rachis, white pericarp, erect growth, and barbless awns, respectively (20). Given the increasing number of plant species that have been sequenced, genome editing provides an efficient approach to plant domestication and thus to expanding crop diversity and increasing the sustainability of agriculture. For example, CRISPR/Cas-enabled mimicry of domestication events in wild or semidomesticated plants could lead to the production of new crops and sources of diverse germplasm for breeding. Thus far, this technique has been used to manipulate monogenic domestication-related traits in wild relatives of crops with polygenic traits of interest. One attractive target for rapid domestication is the winter annual plant field pennycress (*Thlaspi arvense* L., Brassicaceae). Compared with many other oilseed plants, pennycress has a short growing season, extreme cold tolerance, high seed oil productivity, and distinct cover crop attributes and is related to other advanced mustard family members (139). Using genome-editing technologies to modify genes that control seed dormancy (*DOG1*), oil quality (*FAE1* and *FAE2*), glucosinolate accumulation (*HAG1* and *GTR2*), and oil content (*DGAT* genes) should greatly facilitate the development of elite domesticated varieties of pennycress.

Another example of crop domestication by genome editing is tomato. Modern tomato cultivars derived from intensive inbreeding cycles are suffering from increasing biotic and abiotic stresses. Wild tomato plants that are naturally stress tolerant can serve as ideal materials for de novo domestication via precisely engineering the domestication genes. Two independent studies have very recently implemented this strategy to accelerate the domestication of wild tomato in terms of growth habit, flower and fruit production, and nutritional traits in a matter of a few years without losing the stress tolerance of the original wild germplasm (80, 195). One group edited a wild relative of tomato called ground cherry (*Physalis pruinosa*) and produced plants with higher yield and bigger fruit (68). In the future, new domesticated crops with increased tolerance to a range of challenging environments, including deserts, maritime regions, low-nutrient soil, and cold climates, should promote agricultural diversity and help solve many of the problems associated with sustainable agriculture.

Zygote: single cell resulting from the fertilization of a female egg cell by a male sperm cell

Shoot apical meristem: a small population of stem cells located at the tip of the shoot axis, which continuously generates organs and tissues

Nanotechnology: field of study dealing with nanoscale objects

6.3. Improved Delivery Systems

The cell wall makes efficient delivery of genome-editing reagents to plant cells challenging. Current delivery systems are limited to specific plant species, genotypes, and tissues. In addition, almost all the current methods require tissue culture, a long and laborious process. Improving the existing delivery systems and developing new systems will be key in reducing barriers to inexpensive application of gene editing in plants (**Figure 6**). To expand the range of delivery systems, both *Agrobacterium* and plant genes could be manipulated to improve *Agrobacterium*-mediated transformation (39). Moreover, novel bacterial species could be extracted from nature or may even be rationally modified (66). In addition, fine-tuning the expression of developmental genes such as *Baby boom* and *Wuschel2* has dramatically increased the transformation efficiency of certain monocot crops, increased the range of elite genotypes that can be transformed, and substantially reduced the time required for plant regeneration (94, 95).

A further innovation will be achieving genotype-independent, tissue culture–free delivery via the plant germline or meristematic cells (132). Sperm cells, egg cells, and zygotes are emerging as realistic targets of delivery. For example, the use of pollen-mediated transformation would avoid the limitations of species specificity and regeneration using pollination or artificial hybridization. In addition, shoot apical meristems could be used for delivery, as stem cells are destined to differentiate into gametes (41). Novel delivery systems based on nanotechnology and virus particle–like structures also hold promise for crop improvement. For instance, carbon nanotubes have been

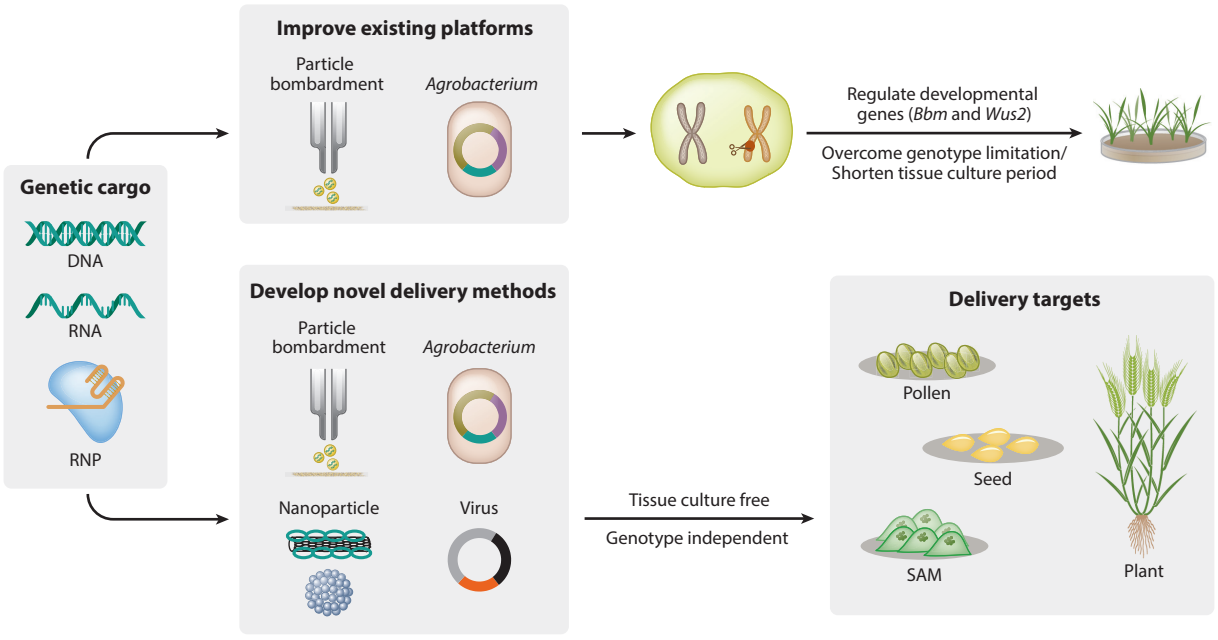


Figure 6

Ideal delivery strategies. (*Upper panels*) Improvements in existing delivery systems and the regulation of developmental genes to overcome species limitations and to speed tissue culture steps. (*Lower panels*) Tissue culture–free delivery systems, including delivery via plant germline or meristematic cells and nanotechnology-based delivery systems. Abbreviations: *Bbm*, *Baby boom*; RNP, ribonucleoprotein; SAM, shoot apical meristem; *Wus2*, *Wuschel2*.

used to deliver DNA into mature plant leaves, leading to successful protein expression (25). Other nanomaterials such as layered double hydroxides (111), mesoporous silica nanoparticles (23), and polyethylenimine (23) also have great potential for expanding the availability of delivery vehicles, as they may cause little cellular damage, have low toxicity, and yield high transformation efficiencies.

6.4. Improved Specificity of CRISPR/Cas Systems

Ongoing discussion concerns the degree of off-target changes that occur in plant genomes with CRISPR/Cas-mediated genome editors and whether this must be fully rectified prior to application for trait development. We argue that this is more of an academic concern, as outcrossing to different varieties is typical during commercial product development and seed multiplication, which enables exclusion of potential off-target effects with timelines that are still greatly reduced compared with standard cross-breeding crop development approaches. In some studies, whole-genome sequencing was performed to detect cleavage of off-target sites by Cas9 or Cpf1 nucleases in stably transgenic *Arabidopsis* (31), rice (153), and cotton plants (70), revealing that both Cas9 and Cpf1 activities are highly specific and that low-level off-targeting could be avoided by designing highly specific sgRNAs. Several strategies have been designed to improve the specificity of Cas9-linked base editors, such as extending sgRNA guide sequences, linking APOBEC1 with Cas9-HF1, and delivering base editors via RNP (58, 133).

6.5. Increased Efficiency of Precise Gene Editing Mediated by Homology-Directed Repair

A challenge of HDR-mediated gene editing is that it requires simultaneous induction of DSBs and delivery of a repair template to one location within the genome. There are several potential ways to increase the frequency of HDR in plant cells, for example, manipulation of DNA repair pathways. In mammalian cells, simultaneous knockout of *PolQ* and one of several genes that are essential for classical NHEJ (*Ligase 4*, *Ku70*, *Ku80*) resulted in 100% gene correction by HDR and no random integration of foreign DNA (105). *PolQ* is essential for T-DNA integration in plants (158); hence, this approach may eliminate the integration of donor DNA into the genome and increase the efficiency of HDR-mediated genome editing. In addition, heterologous expression of many critical proteins in the HDR pathway could increase HDR efficiency; examples include the homologous pairing and DNA strand exchange proteins RAD52, RAD54, and RPA; resection protein RecQL4; and Exo1 and Spo11 (106). Furthermore, new plant delivery methods that enhance donor delivery could increase the efficiency of HDR-mediated genome editing. *Agrobacterium* uses a type IV secretion system to deliver virulence effector proteins to plant cells (161), and VirD2 protein could be covalently linked to single-strand T-DNA, allowing T-DNA transfer through the protein transfer apparatus; this mechanism could co-deliver CRISPR DNA or RNP with donor templates to stimulate HDR-mediated genome editing. This system could also be combined with the nonintegrating geminivirus replicon system to increase the number of donor templates in cells.

6.6. Controlling Agricultural Invasive Species with CRISPR/Cas-Based Gene Drives

Gene drives based on the CRISPR/Cas gene-editing system provide a powerful method for efficiently spreading genetic elements through populations via sexual reproduction. This technique has great potential for public health and humanitarian purposes, such as reducing the burden of vector-borne diseases including malaria (30), although it does come with associated ethical and social concerns that cannot be avoided. In agriculture, CRISPR/Cas-based gene drives could be used to suppress or eliminate invasive species, such as pests and weeds, and could be used to alter pathogens and introduce new traits into existing populations. For example, pigweed (*Amaranthus*) could be engineered by gene drives to become susceptible to the widely used herbicide glyphosate. Because CRISPR/Cas-mediated gene drives have the capacity to influence entire agricultural ecosystems, any thoughts on potential application should go through extensive evaluation involving findings of both the natural and social sciences. With genome editing at an emerging age where careful explanation is needed for broader public acceptance, we argue that social responsibility should prevail and researchers should remain united on not allowing gene drives until regulatory and social frameworks are established and stabilized for current genome-editing methodologies.

7. CONCLUDING REMARKS

The unprecedented ability to generate targeted, sequence-defined, genome-wide genetic diversity in plants through genome editing has led to tremendous advances in basic plant research and crop breeding. The simplicity, versatility, and robustness of CRISPR/Cas systems make genome editing a powerful tool for precise crop improvement via gene knockout, knock-in, replacement, point mutations, fine-tuning of gene regulation, and other modifications at any gene locus in crops. It is also useful for antiviral breeding and high-throughput mutant library construction.

However, efficiently transferring technologies from the bench to the field requires rapid discovery of the genetic bases of important traits, enhanced efficiency of gene targeting (gene

insertion and replacement), effective delivery of CRISPR/Cas reagents to plant cells and subsequent plant regeneration with or without the need for tissue culture, and the availability of base editors with improved targeting range and frequency. Harnessing the concepts of synthetic biology and systems biology and advances in functional genomics, combined with the development of genome-editing technology, next-generation sequencing, and many other related techniques, will allow for the engineering of advanced crops with greatly improved qualities.

SUMMARY POINTS

1. CRISPR/Cas technology provides simple, efficient, and versatile tools for the study of plant biology and precision plant breeding.
2. Base-editing technologies enable direct conversion of one DNA nucleotide into another independent of double-strand break formation.
3. Genome editing via CRISPR/Cas RNPs provides a DNA-free and low off-target editing method in plants.
4. CRISPR/Cas editing of *cis*-regulatory elements, such as promoters and upstream open reading frames, enables the fine-tuning of gene expression, leading to quantitative trait variation.
5. CRISPR/Cas-mediated gene knockout, insertion, and replacement are being rapidly applied in attempts to enhance yield, quality, disease resistance, and stress tolerance traits in crops and to improve hybrid breeding and crop domestication.
6. Considerable technical hurdles still remain to be overcome for broader application of CRISPR/Cas technology in a precise and efficient manner across crop species. Chief among these improvements will be achieving effective nuclease reagent delivery and greatly increasing the frequency of homology-directed repair in plants.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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LITERATURE CITED

1. Abudayyeh OO, Gootenberg JS, Essletzbichler P, Han S, Joung J, et al. 2017. RNA targeting with CRISPR-Cas13. *Nature* 550:280–84

2. Abudayyeh OO, Gootenberg JS, Konermann S, Joung J, Slaymaker IM, et al. 2016. C2c2 is a single-component programmable RNA-guided RNA-targeting CRISPR effector. *Science* 353:aaf5573
3. Ali Z, Abulfaraj A, Idris A, Ali S, Tashkandi M, Mahfouz M. 2015. CRISPR/Cas9-mediated viral interference in plants. *Genome Biol*. 16:238
4. Ali Z, Ali S, Tashkandi M, Zaidi SS, Mahfouz MM. 2016. CRISPR/Cas9-mediated immunity to geminiviruses: differential interference and evasion. *Sci. Rep.* 6:26912
5. Andersson M, Turesson H, Nicolia A, Falt AS, Samuelsson M, Hofvander P. 2017. Efficient targeted multiallelic mutagenesis in tetraploid potato (*Solanum tuberosum*) by transient CRISPR-Cas9 expression in protoplasts. *Plant Cell Rep*. 36:117–28
6. Andersson M, Turesson H, Olsson N, Falt AS, Ohlsson P, et al. 2018. Genome editing in potato via CRISPR-Cas9 ribonucleoprotein delivery. *Physiol. Plant.* 164:378–84
7. Baltes NJ, Hummel AW, Konecna E, Cegan R, Bruns AN, et al. 2015. Conferring resistance to geminiviruses with the CRISPR–Cas prokaryotic immune system. *Nat. Plants* 1:15145
8. Begemann MB, Gray BN, January E, Singer A, Kelser DC, et al. 2017. Characterization and validation of a novel group of type V, class 2 nucleases for in vivo genome editing. bioRxiv 192799. **https://doi.org/10.1101/192799**
9. Braatz J, Harloff HJ, Mascher M, Stein N, Himmelbach A, Jung C. 2017. CRISPR-Cas9 targeted mutagenesis leads to simultaneous modification of different homoeologous gene copies in polyploid oilseed rape (*Brassica napus*). *Plant Physiol*. 174:935–42
10. Butler NM, Baltes NJ, Voytas DF, Douches DS. 2016. Geminivirus-mediated genome editing in potato (*Solanum tuberosum* L.) using sequence-specific nucleases. *Front. Plant Sci.* 7:1045
11. Butt H, Eid A, Ali Z, Atia MAM, Mokhtar MM, et al. 2017. Efficient CRISPR/Cas9-mediated genome editing using a chimeric single-guide RNA molecule. *Front. Plant Sci.* 8:1441
12. Cebrian-Serrano A, Davies B. 2017. CRISPR-Cas orthologues and variants: optimizing the repertoire, specificity and delivery of genome engineering tools. *Mamm. Genome*. 28:247–61
13. Čermák T, Baltes NJ, Čegan R, Zhang Y, Voytas DF. 2015. High-frequency, precise modification of the tomato genome. *Genome Biol*. 16:232
14. Čermák T, Curtin SJ, Gil-Humanes J, Čegan R, Kono TJY, et al. 2017. A multipurpose toolkit to enable advanced genome engineering in plants. *Plant Cell* 29:1196–217
15. Chandrasekaran J, Brumin M, Wolf D, Leibman D, Klap C, et al. 2016. Development of broad virus resistance in non-transgenic cucumber using CRISPR/Cas9 technology. *Mol. Plant Pathol.* 17:1140–53
16. Chen B, Gilbert Luke A, Cimini Beth A, Schnitzbauer J, Zhang W, et al. 2013. Dynamic imaging of genomic loci in living human cells by an optimized CRISPR/Cas system. *Cell* 155:1479–91
17. Chen L, Li W, Katin-Grazzini L, Ding J, Gu X, et al. 2018. A method for the production and expedient screening of CRISPR/Cas9-mediated non-transgenic mutant plants. *Hortic. Res.* 5:13
18. Chen YY, Wang ZP, Ni HW, Xu Y, Chen QJ, Jiang LJ. 2017. CRISPR/Cas9-mediated base-editing system efficiently generates gain-of-function mutations in *Arabidopsis*. *Sci. China Life Sci*. 60:520–23
19. Christian M, Čermák T, Doyle EL, Schmidt C, Zhang F, et al. 2010. Targeting DNA double-strand breaks with TAL effector nucleases. *Genetics* 186:757–61
20. Civáň P, Brown TA. 2017. Origin of rice (*Oryza sativa* L.) domestication genes. *Genet. Resour. Crop Evol.* 64:1125–32
21. Cong L, Ran FA, Cox D, Lin S, Barretto R, et al. 2013. Multiplex genome engineering using CRISPR/Cas systems. *Science* 339:819–23
22. Cox DBT, Gootenberg JS, Abudayyeh OO, Franklin B, Kellner MJ, et al. 2017. RNA editing with CRISPR-Cas13. *Science* 358:1019–27
23. Cunningham FJ, Goh NS, Demirer GS, Matos JL, Landry MP. 2018. Nanoparticle-mediated delivery towards advancing plant genetic engineering. *Trends Biotechnol*. 36:882–97
24. Dahan-Meir T, Filler-Hayut S, Melamed-Bessudo C, Bocobza S, Czosnek H, et al. 2018. Efficient *in planta* gene targeting in tomato using geminiviral replicons and the CRISPR/Cas9 system. *Plant J*. 95:5–16

25. Demirer GS, Zhang H, Matos JL, Goh N, Cunningham F, et al. 2019. High aspect ratio nanomaterials enable delivery of functional genetic material without DNA integration in mature plants. *Nat. Nanotechnol*. **https://doi.org/10.1038/s41565-019-0382-5**
26. Dominguez AA, Lim WA, Qi LS. 2015. Beyond editing: repurposing CRISPR-Cas9 for precision genome regulation and interrogation. *Nat. Rev. Mol. Cell Biol.* 17:5–15
27. Dong L, Li L, Liu C, Liu C, Shuaifeng G, et al. 2018. Genome editing and double fluorescence proteins enable robust maternal haploid induction and identification in maize. *Mol. Plant* 11:1214–17
28. Dreissig S, Schiml S, Schindele P, Weiss O, Rutten T, et al. 2017. Live-cell CRISPR imaging in plants reveals dynamic telomere movements. *Plant J.* 91:565–73
29. Endo M, Mikami M, Toki S. 2016. Biallelic gene targeting in rice. *Plant Physiol.* 170:667–77
30. Esvelt KM, Smidler AL, Catteruccia F, Church GM. 2014. Concerning RNA-guided gene drives for the alteration of wild populations. *eLife* 3:e03401
31. Feng Z, Mao Y, Xu N, Zhang B, Wei P, et al. 2014. Multigeneration analysis reveals the inheritance, specificity, and patterns of CRISPR/Cas-induced gene modifications in *Arabidopsis. PNAS* 111:4632–37
32. Gallego-Bartolomé J, Gardiner J, Liu W, Papikian A, Ghoshal B, et al. 2018. Targeted DNA demethylation of the *Arabidopsis* genome using the human TET1 catalytic domain. *PNAS* 115:E2125–34
33. Gao L, Cox DBT, Yan WX, Manteiga JC, Schneider MW, et al. 2017. Engineered Cpf1 variants with altered PAM specificities. *Nat. Biotechnol.* 35:789–92
34. Gao X, Chen J, Dai X, Zhang D, Zhao Y. 2016. An effective strategy for reliably isolating heritable and *Cas9*-free Arabidopsis mutants generated by CRISPR/Cas9-mediated genome editing. *Plant Physiol.* 171:1794–800
35. Gao Y, Zhao Y. 2013. Self-processing of ribozyme-flanked RNAs into guide RNAs in vitro and in vivo for CRISPR-mediated genome editing. *J. Integr. Plant Biol.* 56:343–49
36. Gasiunas G, Barrangou R, Horvath P, Siksnys V. 2012. Cas9-crRNA ribonucleoprotein complex mediates specific DNA cleavage for adaptive immunity in bacteria. *PNAS* 109:E2579–86
37. Gaudelli NM, Komor AC, Rees HA, Packer MS, Badran AH, et al. 2017. Programmable base editing of A•T to G•C in genomic DNA without DNA cleavage. *Nature* 551:464–71
38. Gehrke JM, Cervantes O, Clement MK, Wu Y, Zeng J, et al. 2018. An APOBEC3A-Cas9 base editor with minimized bystander and off-target activities. *Nat. Biotechnol.* 36:977–82
39. Gelvin SB. 2010. Plant proteins involved in *Agrobacterium*-mediated genetic transformation. *Annu. Rev. Phytopathol.* 48:45–68
40. Gil-Humanes J, Wang Y, Liang Z, Shan Q, Ozuna CV, et al. 2017. High-efficiency gene targeting in hexaploid wheat using DNA replicons and CRISPR/Cas9. *Plant J.* 89:1251–62
41. Hamada H, Linghu Q, Nagira Y, Miki R, Taoka N, Imai R. 2017. An *in planta* biolistic method for stable wheat transformation. *Sci. Rep.* 7:11443
42. He Y, Zhu M, Wang L, Wu J, Wang Q, et al. 2018. Programmed self-elimination of the CRISPR/Cas9 construct greatly accelerates the isolation of edited and transgene-free rice plants. *Mol. Plant* 11:P1210–13
43. Hess GT, Tycko J, Yao D, Bassik MC. 2017. Methods and applications of CRISPR-mediated base editing in eukaryotic genomes. *Mol. Cell* 68:26–43
44. Hu JH, Miller SM, Geurts MH, Tang W, Chen L, et al. 2018. Evolved Cas9 variants with broad PAM compatibility and high DNA specificity. *Nature* 556:57–63
45. Huang J, Li J, Zhou J, Wang L, Yang S, et al. 2018. Identifying a large number of high-yield genes in rice by pedigree analysis, whole-genome sequencing, and CRISPR-Cas9 gene knockout. *PNAS* 115:E7559–67
46. Hummel AW, Chauhan RD, Cermak T, Mutka AM, Vijayaraghavan A, et al. 2017. Allele exchange at the *EPSPS* locus confers glyphosate tolerance in cassava. *Plant Biotechnol. J.* 16:1275–82
47. Iqbal Z, Sattar MN, Shafiq M. 2016. CRISPR/Cas9: a tool to circumscribe cotton leaf curl disease. *Front. Plant Sci.* 7:475
48. Ito Y, Nishizawa-Yokoi A, Endo M, Mikami M, Toki S. 2015. CRISPR/Cas9-mediated mutagenesis of the *RIN* locus that regulates tomato fruit ripening. *Biochem. Biophys. Res. Commun.* 467:76–82

37. Explores an adenine base-editor (ABE) system that converts A to G in genomic DNA.

49. Jacobs TB, Zhang N, Patel D, Martin GB. 2017. Generation of a collection of mutant tomato lines using pooled CRISPR libraries. *Plant Physiol.* 174:2023–37
50. Ji X, Si X, Zhang Y, Zhang H, Zhang F, et al. 2018. Conferring DNA virus resistance with high specificity in plants using a virus-inducible genome editing system. *Genome Biol.* 19:197
51. Ji X, Zhang H, Zhang Y, Wang Y, Gao C. 2015. Establishing a CRISPR-Cas-like immune system conferring DNA virus resistance in plants. *Nat. Plants* 1:15144
52. Jiang F, Doudna JA. 2017. CRISPR-Cas9 structures and mechanisms. *Annu. Rev. Biophys.* 46:505–29
53. Jiang WZ, Henry IM, Lynagh PG, Comai L, Cahoon EB, Weeks DP. 2017. Significant enhancement of fatty acid composition in seeds of the allohexaploid, *Camelina sativa*, using CRISPR/Cas9 gene editing. *Plant Biotechnol. J.* 15:648–57
54. Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E. 2012. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* 337:816–21
55. Jusiak B, Cleto S, Perez-Piñera P, Lu TK. 2016. Engineering synthetic gene circuits in living cells with CRISPR technology. *Trends Biotechnol.* 34:535–47
56. Kang BC, Yun JY, Kim ST, Shin Y, Ryu J, et al. 2018. Precision genome engineering through adenine base editing in plants. *Nat. Plants* 4:427–431
57. Khanday I, Skinner D, Yang B, Mercier R, Sundaresan V. 2018. A male-expressed rice embryogenic trigger redirected for asexual propagation through seeds. *Nature* 565:91–95
58. Kim D, Lim K, Kim ST, Yoon SH, Kim K, et al. 2017. Genome-wide target specificities of CRISPR RNA-guided programmable deaminases. *Nat. Biotechnol.* 35:475–80
59. Kim H, Kim ST, Ryu J, Kang BC, Kim JS, Kim SG. 2017. CRISPR/Cpf1-mediated DNA-free plant genome editing. *Nat. Commun.* 8:14406
60. Kim K, Ryu SM, Kim ST, Baek G, Kim D, et al. 2017. Highly efficient RNA-guided base editing in mouse embryos. *Nat. Biotechnol.* 35:435–37
61. Kleinstiver BP, Prew MS, Tsai SQ, Topkar VV, Nguyen NT, et al. 2015. Engineered CRISPR-Cas9 nucleases with altered PAM specificities. *Nature* 523:481–85
62. Komatsuda T, Pourkheirandish M, He C, Azhaguvel P, Kanamori H, et al. 2007. Six-rowed barley originated from a mutation in a homeodomain-leucine zipper I-class homeobox gene. *PNAS* 104:1424–29
63. Komor AC, Kim YB, Packer MS, Zuris JA, Liu DR. 2016. Programmable editing of a target base in genomic DNA without double-stranded DNA cleavage. *Nature* 533:420–24
64. Koonin EV, Makarova KS, Zhang F. 2017. Diversity, classification and evolution of CRISPR-Cas systems. *Curr. Opin. Microbiol.* 37:67–78
65. Kungulovski G, Jeltsch A. 2016. Epigenome editing: state of the art, concepts, and perspectives. *Trends Genet.* 32:101–13
66. Lacroix B, Citovsky V. 2016. A functional bacterium-to-plant DNA transfer machinery of *Rhizobium etli*. *PLOS Pathog.* 12:e1005502
67. Leenay RT, Maksimchuk KR, Slotkowski RA, Agrawal RN, Gomaa AA, et al. 2016. Identifying and visualizing functional PAM diversity across CRISPR-Cas systems. *Mol. Cell* 62:137–47
68. Lemmon ZH, Reem NT, Dalrymple J, Soyk S, Swartwood KE, et al. 2018. Rapid improvement of domestication traits in an orphan crop by genome editing. *Nat. Plants* 4:766–70
69. Li C, Zong Y, Wang Y, Jin S, Zhang D, et al. 2018. Expanded base editing in rice and wheat using a Cas9-adenosine deaminase fusion. *Genome Biol.* 19:59
70. Li J, Manghwar H, Sun L, Wang P, Wang G, et al. 2018. Whole genome sequencing reveals rare off-target mutations and considerable inherent genetic or/and somaclonal variations in CRISPR/Cas9-edited cotton plants. *Plant Biotechnol. J.* **https://doi.org/10.1111/pbi.13020**
71. Li J, Meng X, Zong Y, Chen K, Zhang H, et al. 2016. Gene replacements and insertions in rice by intron targeting using CRISPR-Cas9. *Nat. Plants* 2:16139
72. Li J, Zhang H, Si X, Tian Y, Chen K, et al. 2017. Generation of thermosensitive male-sterile maize by targeted knockout of the *ZmTMS5* gene. *J. Genet. Genom.* 44:465–68
73. Li JF, Norville JE, Aach J, McCormack M, Zhang D, et al. 2013. Multiplex and homologous recombination-mediated genome editing in *Arabidopsis* and *Nicotiana benthamiana* using guide RNA and Cas9. *Nat. Biotechnol.* 31:688–91

54. Provides first demonstration that dual-transactivating crRNA:crRNA and a single RNA chimera can direct Cas9 to cleave double-strand DNA in vitro.

63. Explores base-editing technology that converts C to T in the genome without producing double-strand breaks.

74. Li M, Li X, Zhou Z, Wu P, Fang M, et al. 2016. Reassessment of the four yield-related genes *gn1a*, *dep1*, *gs3*, and *ipa1* in rice using a CRISPR/Cas9 system. *Front. Plant Sci.* 7:377
75. Li Q, Zhang D, Chen M, Liang W, Wei J, et al. 2016. Development of *japonica* photo-sensitive genic male sterile rice lines by editing *carbon starved anther* using CRISPR/Cas9. *J. Genet. Genom.* 43:415–19
76. Li R, Fu D, Zhu B, Luo Y, Zhu H. 2018. CRISPR/Cas9-mediated mutagenesis of *lncRNA1459* alters tomato fruit ripening. *Plant J.* 94:513–24
77. Li R, Li R, Li X, Fu D, Zhu B, et al. 2018. Multiplexed CRISPR/Cas9-mediated metabolic engineering of γ-aminobutyric acid levels in *Solanum lycopersicum*. *Plant Biotechnol. J.* 16:415–27
78. Li S, Gao F, Xie K, Zeng X, Cao Y, et al. 2016. The OsmiR396c-OsGRF4-OsGIF1 regulatory module determines grain size and yield in rice. *Plant Biotechnol. J.* 14:2134–46
79. Li S, Zhang X, Wang W, Guo X, Wu Z, et al. 2018. Expanding the scope of CRISPR/Cpf1-mediated genome editing in rice. *Mol. Plant* 11:995–98
80. Li T, Yang X, Yu Y, Si X, Zhai X, et al. 2018. Domestication of wild tomato is accelerated by genome editing. *Nat. Biotechnol.* 36:1160–63
81. Li W, Ma M, Feng Y, Li H, Wang Y, et al. 2015. EIN2-directed translational regulation of ethylene signaling in *Arabidopsis*. *Cell* 163:670–83
82. Li X, Wang Y, Chen S, Tian H, Fu D, et al. 2018. Lycopene is enriched in tomato fruit by CRISPR/Cas9-mediated multiplex genome editing. *Front. Plant Sci.* 9:559
83. Li X, Zhou W, Ren Y, Tian X, Lv T, et al. 2017. High-efficiency breeding of early-maturing rice cultivars via CRISPR/Cas9-mediated genome editing. *J. Genet. Genom.* 44:175–78
84. Li Z, Liu ZB, Xing A, Moon BP, Koellhoffer JP, et al. 2015. Cas9-guide RNA directed genome editing in soybean. *Plant Physiol.* 169:960–70
85. Li Z, Xiong X, Li J-F. 2018. New cytosine base editor for plant genome editing. *Sci. China Life Sci.* 61:1602–3
86. Li Z, Xiong X, Wang F, Li J-F. 2018. Gene disruption through base-editing-induced mRNA mis-splicing in plants. *New Phytol.* **https://doi.org/10.1111/nph.15647**
87. Li Z, Zhang D, Xiong X, Yan B, Xie W, et al. 2017. A potent Cas9-derived gene activator for plant and mammalian cells. *Nat. Plants* 3:930–36
88. Liang XH, Shen W, Sun H, Migawa MT, Vickers TA, Crooke ST. 2016. Translation efficiency of mRNAs is increased by antisense oligonucleotides targeting upstream open reading frames. *Nat. Biotechnol.* 34:875–80
89. Liang Z, Chen K, Li T, Zhang Y, Wang Y, et al. 2017. Efficient DNA-free genome editing of bread wheat using CRISPR/Cas9 ribonucleoprotein complexes. *Nat. Commun.* 8:14261
90. Liang Z, Chen K, Zhang Y, Liu J, Yin K, et al. 2018. Genome editing of bread wheat using biolistic delivery of CRISPR/Cas9 *in vitro* transcripts or ribonucleoproteins. *Nat. Protoc.* 13:413–30
91. Lin CS, Hsu CT, Yang LH, Lee LY, Fu JY, et al. 2017. Application of protoplast technology to CRISPR/Cas9 mutagenesis: from single cell mutation detection to mutant plant regeneration. *Plant Biotechnol. J.* 16:1295–310
92. Liu J, Chen J, Zheng X, Wu F, Lin Q, et al. 2017. GW5 acts in the brassinosteroid signalling pathway to regulate grain width and weight in rice. *Nat. Plants* 3:17043
93. Lowder LG, Zhang D, Baltes NJ, Paul JW, Tang X, et al. 2015. A CRISPR/Cas9 toolbox for multiplexed plant genome editing and transcriptional regulation. *Plant Physiol.* 169:971–85
94. Lowe K, La Rota M, Hoerster G, Hastings C, Wang N, et al. 2018. Rapid genotype "independent" *Zea mays* L. (maize) transformation via direct somatic embryogenesis. *In Vitro Cell. Dev. Biol.* 54:240–52
95. Lowe K, Wu E, Wang N, Hoerster G, Hastings C, et al. 2016. Morphogenic regulators *Baby boom* and *Wuschel* improve monocot transformation. *Plant Cell* 28:1998–2015
96. Lu HP, Luo T, Fu HW, Wang L, Tan YY, et al. 2018. Resistance of rice to insect pests mediated by suppression of serotonin biosynthesis. *Nat. Plants* 4:338–44
97. Lu K, Wu B, Wang J, Zhu W, Nie H, et al. 2018. Blocking amino acid transporter *OsAAP3* improves grain yield by promoting outgrowth buds and increasing tiller number in rice. *Plant Biotechnol. J.* 16:1710–22

98. Lu Y, Ye X, Guo R, Huang J, Wang W, et al. 2017. Genome-wide targeted mutagenesis in rice using the CRISPR/Cas9 system. *Mol. Plant* 10:1242–45
99. Luo M, Gilbert B, Ayliffe M. 2016. Applications of CRISPR/Cas9 technology for targeted mutagenesis, gene replacement and stacking of genes in higher plants. *Plant Cell Rep.* 35:1439–50
100. Ma X, Zhang Q, Zhu Q, Liu W, Chen Y, et al. 2015. A robust CRISPR/Cas9 system for convenient, high-efficiency multiplex genome editing in monocot and dicot plants. *Mol. Plant* 8:1274–84
101. Ma Y, Zhang J, Yin W, Zhang Z, Song Y, Chang X. 2016. Targeted AID-mediated mutagenesis (TAM) enables efficient genomic diversification in mammalian cells. *Nat. Methods* 13:1029–35
102. Macovei A, Sevilla NR, Cantos C, Jonson GB, Slamet-Loedin I, et al. 2018. Novel alleles of rice *eIF4G* generated by CRISPR/Cas9-targeted mutagenesis confer resistance to *Rice tungro spherical virus*. *Plant Biotechnol. J.* 16:1918–27
103. Mali P, Yang L, Esvelt KM, Aach J, Guell M, et al. 2013. RNA-guided human genome engineering via Cas9. *Science* 339:823–26
104. Malnoy M, Viola R, Jung MH, Koo OJ, Kim S, et al. 2016. DNA-free genetically edited grapevine and apple protoplast using CRISPR/Cas9 ribonucleoproteins. *Front. Plant Sci.* 7:1904
105. Mateos-Gomez PA, Gong F, Nair N, Miller KM, Lazzerini-Denchi E, Sfeir A. 2015. Mammalian polymerase θ promotes alternative-NHEJ and suppresses recombination. *Nature* 518:254–57
106. McVey M, Khodaverdian VY, Meyer D, Cerqueira PG, Heyer W-D. 2016. Eukaryotic DNA polymerases in homologous recombination. *Annu. Rev. Genet.* 50:393–421
107. Mehta D, Stürchler A, Hirsch-Hoffmann M, Gruissem W, Vanderschuren H. 2018. CRISPR-Cas9 interference in cassava linked to the evolution of editing-resistant geminiviruses. bioRxiv 314542. **https://doi.org/10.1101/314542**
108. Meng X, Hu X, Liu Q, Song X, Gao C, et al. 2018. Robust genome editing of CRISPR-Cas9 at NAG PAMs in rice. *Sci. China Life Sci.* 61:122–25
109. Meng X, Yu H, Zhang Y, Zhuang F, Song X, et al. 2017. Construction of a genome-wide mutant library in rice using CRISPR/Cas9. *Mol. Plant* 10:1238–41
110. Miao C, Xiao L, Hua K, Zou C, Zhao Y, et al. 2018. Mutations in a subfamily of abscisic acid receptor genes promote rice growth and productivity. *PNAS* 115:6058–63
111. Mitter N, Worrall EA, Robinson KE, Li P, Jain RG, et al. 2017. Clay nanosheets for topical delivery of RNAi for sustained protection against plant viruses. *Nat. Plants* 3:16207
112. Morineau C, Bellec Y, Tellier F, Gissot L, Kelemen Z, et al. 2017. Selective gene dosage by CRISPR-Cas9 genome editing in hexaploid *Camelina sativa*. *Plant Biotechnol. J.* 15:729–39
113. Nakayasu M, Akiyama R, Lee HJ, Osakabe K, Osakabe Y, et al. 2018. Generation of α-solanine-free hairy roots of potato by CRISPR/Cas9 mediated genome editing of the *St16DOX* gene. *Plant Physiol. Biochem.* 131:70–77
114. Nayak A, Tassetto M, Kunitomi M, Andino R. 2013. RNA interference-mediated intrinsic antiviral immunity in invertebrates. *Curr. Top. Microbiol. Immunol.* 371:183–200
115. Nekrasov V, Staskawicz B, Weigel D, Jones JD, Kamoun S. 2013. Targeted mutagenesis in the model plant *Nicotiana benthamiana* using Cas9 RNA-guided endonuclease. *Nat. Biotechnol.* 31:691–93
116. Nekrasov V, Wang C, Win J, Lanz C, Weigel D, Kamoun S. 2017. Rapid generation of a transgene-free powdery mildew resistant tomato by genome deletion. *Sci. Rep.* 7:482
117. Nemhauser JL, Torii KU. 2016. Plant synthetic biology for molecular engineering of signalling and development. *Nat. Plants* 2:16010
118. Nieves-Cordones M, Mohamed S, Tanoi K, Kobayashi NI, Takagi K, et al. 2017. Production of low-Cs^+ rice plants by inactivation of the K^+ transporter *OsHAK1* with the CRISPR-Cas system. *Plant J.* 92:43–56
119. Nishida K, Arazoe T, Yachie N, Banno S, Kakimoto M, et al. 2016. Targeted nucleotide editing using hybrid prokaryotic and vertebrate adaptive immune systems. *Science* 353:aaf8729
120. Nishimasu H, Shi X, Ishiguro S, Gao L, Hirano S, et al. 2018. Engineered CRISPR-Cas9 nuclease with expanded targeting space. *Science* 361:1259–62
121. Nishizawa-Yokoi A, Endo M, Ohtsuki N, Saika H, Toki S. 2014. Precision genome editing in plants via gene targeting and *piggyBac*-mediated marker excision. *Plant J.* 81:160–68

122. Nonaka S, Arai C, Takayama M, Matsukura C, Ezura H. 2017. Efficient increase of γ-aminobutyric acid (GABA) content in tomato fruits by targeted mutagenesis. *Sci Rep*. 7:7057
123. Okuzaki A, Ogawa T, Koizuka C, Kaneko K, Inaba M, et al. 2018. CRISPR/Cas9-mediated genome editing of the fatty acid desaturase 2 gene in *Brassica napus*. *Plant Physiol. Biochem*. 131:63–69
124. Østerberg JT, Xiang W, Olsen LI, Edenbrandt AK, Vedel SE, et al. 2017. Accelerating the domestication of new crops: feasibility and approaches. *Trends Plant Sci*. 22:373–84
125. Pacher M, Puchta H. 2017. From classical mutagenesis to nuclease-based breeding-directing natural DNA repair for a natural end-product. *Plant J*. 90:819–33
126. Paszkowski J, Baur M, Bogucki A, Potrykus I. 1988. Gene targeting in plants. *EMBO J*. 7:4021–26
127. Peng A, Chen S, Lei T, Xu L, He Y, et al. 2017. Engineering canker-resistant plants through CRISPR/Cas9-targeted editing of the susceptibility gene *CsLOB1* promoter in citrus. *Plant Biotechnol. J*. 15:1509–19
128. Piatek A, Ali Z, Baazim H, Li L, Abulfaraj A, et al. 2015. RNA-guided transcriptional regulation *in planta* via synthetic dCas9-based transcription factors. *Plant Biotechnol. J*. 13:578–89
129. Prado JR, Segers G, Voelker T, Carson D, Dobert R, et al. 2014. Genetically engineered crops: from idea to product. *Annu. Rev. Plant Biol*. 65:769–90
130. Price AA, Sampson TR, Ratner HK, Grakoui A, Weiss DS. 2015. Cas9-mediated targeting of viral RNA in eukaryotic cells. *PNAS* 112:6164–69
131. Puchta H, Dujon B, Hohn B. 1993. Homologous recombination in plant cells is enhanced by in vivo induction of double strand breaks into DNA by a site-specific endonuclease. *Nucleic Acids Res*. 21:5034–40
132. Ran Y, Liang Z, Gao C. 2017. Current and future editing reagent delivery systems for plant genome editing. *Sci. China Life Sci*. 60:490–505
133. Rees HA, Komor AC, Yeh WH, Caetano-Lopes J, Warman M, et al. 2017. Improving the DNA specificity and applicability of base editing through protein engineering and protein delivery. *Nat. Commun*. 8:15790

134. Demonstrates targeting *cis*-regulatory elements in promoters creates a continuum of variation in yield traits.

134. Rodríguez-Leal D, Lemmon ZH, Man J, Bartlett ME, Lippman ZB. 2017. Engineering quantitative trait variation for crop improvement by genome editing. *Cell* 171:470–80

135. Salsman J, Dellaire G. 2016. Precision genome editing in the CRISPR era. *Biochem. Cell Biol*. 95:187–201
136. Sanchez-Leon S, Gil-Humanes J, Ozuna CV, Gimenez MJ, Sousa C, et al. 2018. Low-gluten, nontransgenic wheat engineered with CRISPR/Cas9. *Plant Biotechnol. J*. 16:902–10
137. Sauer NJ, Narvaez-Vasquez J, Mozoruk J, Miller RB, Warburg ZJ, et al. 2016. Oligonucleotide-mediated genome editing provides precision and function to engineered nucleases and antibiotics in plants. *Plant Physiol*. 170:1917–28
138. Scheben A, Wolter F, Batley J, Puchta H, Edwards D. 2017. Towards CRISPR/Cas crops—bringing together genomics and genome editing. *New Phytol*. 216:682–98
139. Sedbrook JC, Phippen WB, Marks MD. 2014. New approaches to facilitate rapid domestication of a wild plant to an oilseed crop: example pennycress (*Thlaspi arvense* L.). *Plant Sci*. 227:122–32

140. Provides first demonstration of indel-inducing CRISPR/Cas9 system for plants.

140. Shan Q, Wang Y, Li J, Zhang Y, Chen K, et al. 2013. Targeted genome modification of crop plants using a CRISPR-Cas system. *Nat. Biotechnol*. 31:686–88

141. Shan Q, Zhang Y, Chen K, Zhang K, Gao C. 2015. Creation of fragrant rice by targeted knockout of the *OsBADH2* gene using TALEN technology. *Plant Biotechnol. J*. 13:791–800
142. Shen R, Wang L, Liu X, Wu J, Jin W, et al. 2017. Genomic structural variation-mediated allelic suppression causes hybrid male sterility in rice. *Nat. Commun*. 8:1310
143. Shi J, Gao H, Wang H, Lafitte HR, Archibald RL, et al. 2017. *ARGOS8* variants generated by CRISPR-Cas9 improve maize grain yield under field drought stress conditions. *Plant Biotechnol. J*. 15:207–16
144. Shimatani Z, Kashojiya S, Takayama M, Terada R, Arazoe T, et al. 2017. Targeted base editing in rice and tomato using a CRISPR-Cas9 cytidine deaminase fusion. *Nat. Biotechnol*. 35:441–43

145. Singh M, Kumar M, Albertsen MC, Young JK, Cigan AM. 2018. Concurrent modifications in the three homeologs of *Ms45* gene with CRISPR-Cas9 lead to rapid generation of male sterile bread wheat (*Triticum aestivum* L.). *Plant Mol. Biol.* 97:371–83
146. Subburaj S, Chung SJ, Lee C, Ryu SM, Kim DH, et al. 2016. Site-directed mutagenesis in *Petunia* × *hybrida* protoplast system using direct delivery of purified recombinant Cas9 ribonucleoproteins. *Plant Cell Rep*. 35:1535–44
147. Sun Y, Jiao G, Liu Z, Zhang X, Li J, et al. 2017. Generation of high-amylose rice through CRISPR/Cas9-mediated targeted mutagenesis of starch branching enzymes. *Front. Plant Sci.* 8:1298
148. Sun Y, Zhang X, Wu C, He Y, Ma Y, et al. 2016. Engineering herbicide-resistant rice plants through CRISPR/Cas9-mediated homologous recombination of acetolactate synthase. *Mol. Plant* 9:628–31
149. Svitashev S, Schwartz C, Lenderts B, Young JK, Mark Cigan A. 2016. Genome editing in maize directed by CRISPR-Cas9 ribonucleoprotein complexes. *Nat. Commun.* 7:13274
150. Svitashev S, Young JK, Schwartz C, Gao H, Falco SC, Cigan AM. 2015. Targeted mutagenesis, precise gene editing, and site-specific gene insertion in maize using Cas9 and guide RNA. *Plant Physiol.* 169:931–45
151. Symington LS, Gautier J. 2011. Double-strand break end resection and repair pathway choice. *Annu. Rev. Genet.* 45:247–71
152. Tang L, Mao B, Li Y, Lv Q, Zhang L, et al. 2017. Knockout of *OsNramp5* using the CRISPR/Cas9 system produces low Cd-accumulating *indica* rice without compromising yield. *Sci. Rep.* 7:14438
153. Tang X, Liu G, Zhou J, Ren Q, You Q, et al. 2018. A large-scale whole-genome sequencing analysis reveals highly specific genome editing by both Cas9 and Cpf1 (Cas12a) nucleases in rice. *Genome Biol.* 19:84
154. Temme K, Zhao D, Voigt CA. 2012. Refactoring the nitrogen fixation gene cluster from *Klebsiella oxytoca*. *PNAS* 109:7085–90
155. Teng F, Cui T, Feng G, Guo L, Xu K, et al. 2018. Repurposing CRISPR-Cas12b for mammalian genome engineering. *Cell Discov.* 4:63
156. Tian S, Jiang L, Cui X, Zhang J, Guo S, et al. 2018. Engineering herbicide-resistant watermelon variety through CRISPR/Cas9-mediated base-editing. *Plant Cell Rep.* 37:1353–56
157. Tilman D, Balzer C, Hill J, Befort BL. 2011. Global food demand and the sustainable intensification of agriculture. *PNAS* 108:20260–64
158. van Kregten M, de Pater S, Romeijn R, van Schendel R, Hooykaas PJJ, Tijsterman M. 2016. T-DNA integration in plants results from polymerase-θ-mediated DNA repair. *Nat. Plants* 2:16164
159. von Arnim AG, Jia Q, Vaughn JN. 2014. Regulation of plant translation by upstream open reading frames. *Plant Sci.* 214:1–12
160. von Caemmerer S, Quick WP, Furbank RT. 2012. The development of C_4 rice: current progress and future challenges. *Science* 336:1671–72
161. Voth DE, Broederdorf LJ, Graham JG. 2012. Bacterial type IV secretion systems: versatile virulence machines. *Future Microbiol.* 7:241–57
162. Waltz E. 2016. CRISPR-edited crops free to enter market, skip regulation. *Nat. Biotechnol.* 34:582
163. Wang C, Liu Q, Shen Y, Hua Y, Wang J, et al. 2019. Clonal seeds from hybrid rice by simultaneous genome engineering of meiosis and fertilization genes. *Nat. Biotechnol.* 37:283–86
164. Wang F, Wang C, Liu P, Lei C, Hao W, et al. 2016. Enhanced rice blast resistance by CRISPR/Cas9-targeted mutagenesis of the ERF transcription factor gene *OsERF922*. *PLOS ONE* 11:e0154027
165. Wang FZ, Chen MX, Yu LJ, Xie LJ, Yuan LB, et al. 2017. *OsARM1*, an R2R3 MYB transcription factor, is involved in regulation of the response to arsenic stress in rice. *Front. Plant Sci.* 8:1868
166. Wang H, Studer AJ, Zhao Q, Meeley R, Doebley JF. 2015. Evidence that the origin of naked kernels during maize domestication was caused by a single amino acid substitution in *tga1*. *Genetics* 200:965–74
167. Wang M, Lu Y, Botella JR, Mao Y, Hua K, Zhu JK. 2017. Gene targeting by homology-directed repair in rice using a geminivirus-based CRISPR/Cas9 system. *Mol. Plant* 10:1007–10

168. Wang M, Mao Y, Lu Y, Tao X, Zhu J-k. 2017. Multiplex gene editing in rice using the CRISPR-Cpf1 system. *Mol. Plant* 10:1011–13
169. Wang X, Li J, Wang Y, Yang B, Wei J, et al. 2018. Efficient base editing in methylated regions with a human APOBEC3A-Cas9 fusion. *Nat. Biotechnol.* 36:946–49
170. Wang Y, Cheng X, Shan Q, Zhang Y, Liu J, et al. 2014. Simultaneous editing of three homoeoalleles in hexaploid bread wheat confers heritable resistance to powdery mildew. *Nat. Biotechnol.* 32:947–51
171. Woo JW, Kim J, Kwon SI, Corvalan C, Cho SW, et al. 2015. DNA-free genome editing in plants with preassembled CRISPR-Cas9 ribonucleoproteins. *Nat. Biotechnol.* 33:1162–64
172. Wright DA, Townsend JA, Winfrey RJ Jr., Irwin PA, Rajagopal J, et al. 2005. High-frequency homologous recombination in plants mediated by zinc-finger nucleases. *Plant J.* 44:693–705
173. Xie K, Minkenberg B, Yang Y. 2015. Boosting CRISPR/Cas9 multiplex editing capability with the endogenous tRNA-processing system. *PNAS* 112:3570–75
174. Xie Y, Niu B, Long Y, Li G, Tang J, et al. 2017. Suppression or knockout of *SaF/SaM* overcomes the *Sa*-mediated hybrid male sterility in rice. *J. Integr. Plant Biol.* 59:669–79
175. Xie Y, Xu P, Huang J, Ma S, Xie X, et al. 2017. Interspecific hybrid sterility in rice is mediated by *OgTPR1* at the *S1* locus encoding a peptidase-like protein. *Mol. Plant* 10:1137–40
176. Xu R, Yang Y, Qin R, Li H, Qiu C, et al. 2016. Rapid improvement of grain weight *via* highly efficient CRISPR/Cas9-mediated multiplex genome editing in rice. *J. Genet. Genom.* 43:529–32
177. Xue C, Zhang H, Lin Q, Fan R, Gao C. 2018. Manipulating mRNA splicing by base editing in plants. *Sci. China Life Sci.* 61:1293–300
178. Yao L, Zhang Y, Liu C, Liu Y, Wang Y, et al. 2018. *OsMATL* mutation induces haploid seed formation in *indica* rice. *Nat. Plants* 4:530–33
179. Ye M, Peng Z, Tang D, Yang Z, Li D, et al. 2018. Generation of self-compatible diploid potato by knockout of *S-RNase*. *Nat. Plants* 4:651–54
180. Yu QH, Wang B, Li N, Tang Y, Yang S, et al. 2017. CRISPR/Cas9-induced targeted mutagenesis and gene replacement to generate long-shelf life tomato lines. *Sci. Rep.* 7:11874
181. Yu X, Zhao Z, Zheng X, Zhou J, Kong W, et al. 2018. A selfish genetic element confers non-Mendelian inheritance in rice. *Science* 360:1130–32
182. Zaidi SS, Tashkandi M, Mansoor S, Mahfouz MM. 2016. Engineering plant immunity: using CRISPR/Cas9 to generate virus resistance. *Front. Plant Sci.* 7:1673
183. Zetsche B, Gootenberg Jonathan S, Abudayyeh Omar O, Slaymaker Ian M, Makarova Kira S, et al. 2015. Cpf1 is a single RNA-guided endonuclease of a class 2 CRISPR-Cas system. *Cell* 163:759–71
184. Zhang H, Si X, Ji X, Fan R, Liu J, et al. 2018. Genome editing of upstream open reading frames enables translational control in plants. *Nat. Biotechnol.* 36:894–98
185. Zhang J, Zhang H, Botella JR, Zhu JK. 2018. Generation of new glutinous rice by CRISPR/Cas9-targeted mutagenesis of the *Waxy* gene in elite rice varieties. *J. Integr. Plant Biol.* 60:369–75
186. Zhang T, Zheng Q, Yi X, An H, Zhao Y, et al. 2018. Establishing RNA virus resistance in plants by harnessing CRISPR immune system. *Plant Biotechnol. J.* 16:1415–23
187. Zhang Y, Li D, Zhang D, Zhao X, Cao X, et al. 2018. Analysis of the functions of *TaGW2* homoeologs in wheat grain weight and protein content traits. *Plant J.* 94:857–66
188. Zhang Y, Liang Z, Zong Y, Wang Y, Liu J, et al. 2016. Efficient and transgene-free genome editing in wheat through transient expression of CRISPR/Cas9 DNA or RNA. *Nat. Commun.* 7:12617
189. Zhong Z, Zhang Y, You Q, Tang X, Ren Q, et al. 2018. Plant genome editing using FnCpf1 and LbCpf1 nucleases at redefined and altered PAM site. *Mol. Plant* 11:999–1002
190. Zhou H, He M, Li J, Chen L, Huang Z, et al. 2016. Development of commercial thermo-sensitive genic male sterile rice accelerates hybrid rice breeding using the CRISPR/Cas9-mediated *TMS5* editing system. *Sci. Rep.* 6:37395
191. Zhou J, Peng Z, Long J, Sosso D, Liu B, et al. 2015. Gene targeting by the TAL effector PthXo2 reveals cryptic resistance gene for bacterial blight of rice. *Plant J.* 82:632–43

170. Provides first use of genome editing in polyploid wheat to improve crop traits, providing a methodological framework for crop improvement.

183. Identifies Cpf1, a CRISPR effector that mediates robust DNA interference with features distinct from Cas9.

184. Describes how the disruption of uORFs by CRISPR/Cas9 efficiently enhances principal ORF expression at the translational level.

192. Zhu B, Zhang W, Zhang T, Liu B, Jiang J. 2015. Genome-wide prediction and validation of intergenic enhancers in Arabidopsis using open chromatin signatures. *Plant Cell* 27:2415–26

193. Zong Y, Song Q, Li C, Jin S, Zhang D, et al. 2018. Efficient C-to-T base editing in plants using a fusion of nCas9 and human APOBEC3A. *Nat. Biotechnol.* 36:950–53

194. Zong Y, Wang Y, Li C, Zhang R, Chen K, et al. 2017. Precise base editing in rice, wheat and maize with a Cas9-cytidine deaminase fusion. *Nat. Biotechnol.* 35:438–40

195. Zsögön A, Čermák T, Naves ER, Notini MM, Edel KH, et al. 2018. *De novo* domestication of wild tomato using genome editing. *Nat. Biotechnol.* 36:1211–16

193. Explores a new plant cytidine base editor containing a human cytidine deaminase that offers a larger editing window.

195. Demonstrates first use of genome editing for de novo domestication of wild species to create novel nutritious crops.