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SIGNIFICANCE OF NEW PLANT BREEDING TECHNOLOGIES FOR SUSTAINABLE AGRICULTURE AND FOOD SECURITY

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Abstract

In the scenario of a new agriculture, breeding techniques are expected to play a significant role in order to increase the sustainability of productive processes from an environmental, economic, and social point of view. Over the past 50 years, new breeding strategies have been developed alongside the integration of various techniques, the development of in vitro techniques, and the development of molecular strategies. The use of New Breeding Techniques (NBTs) based on thorough understanding of the genome of species and varieties will enable the development of new results that overcome the constraints of conventional breeding techniques and their length while minimizing the risks of the first generation of molecular breeding tools. The use of high-throughput mutant libraries, the development of methods for fine-tuning gene regulation, strategies for breeding virus resistance, and applications of genome editing for trait improvement are all summarized here. We discuss potential applications of genome editing in domestication and synthetic biology of plants, as well as developments in delivery techniques, editing specificity, homolog directed repair, and gene drives. Our discussion of precision plant breeding's promising future in agriculture concludes with a discussion of the challenges and opportunities facing this field.

Key words: *New Breeding Techniques (NBTs), genome editing, CRISPR/Cas, precision plant breeding, trait improvement.*

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Introduction

Crops provide food, feed, fuel, and other consumable resources for human life, thereby con- tributing 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. 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 method- ologies for crop improvement in modern agriculture. It takes many years to introduce desirable alleles by cross breeding and to increase variability by genetic recombination (1). 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 (2). However, these procedures are restricted by their stochastic nature, and generating and screening large numbers of mutants are challenging. Such time-consuming, laborious, untargeted breeding programs can- not keep pace with the demands for increased crop production, even if marker-assisted breeding approaches are adopted to enhance selection efficiency (3). Transgenic breeding, which gen- erates 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 (1; 2). Since the first gene-targeting experiment in tobacco (*Nicotiana tabacum*) protoplasts in 1988 (4) and the discovery that DNA double-strand breaks (DSBs) enhance gene-targeting ef- ficiency in 1993 (131), scientists have sought to develop tools for targeted editing of plant genomes. In 2005, zinc finger nucleases were adapted in tobacco (5) and used in trait im- provement in a few plants. In 2010, transcription activator-like effector nucleases (TALENs) were added to the plant genome-editing toolbox (6) (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.

Zinc finger nucleases and transcription activator-like effector nucleases

Zinc finger nucleases (ZFNs) are sequence-specific nucleases engineered by fusing the specific DNA binding do- main 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 inter- spaced short palindromic repeats/CRISPR-associated protein 9) system for use in rice (*Oryza sativa*), wheat (*Triticum aestivum*), *Nicotiana benthamiana*, and *Arabidopsis thaliana (7; 8; 9)*. 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 (13) and nucleotide substitution tools for base editing (10;11; 12) have made genome editing a widely adopted, low-cost, easy-to-use targeted genetic manipulation tool that has been applied to many crops. 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.

CRISPR/CAS systems for plant genome editing 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 Type II CRISPR/Cas9 from *Streptococcus pyogenes* was the first system shown to specifically cleave DNA in vitro and in vivo (13; 14; 25, 16). 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. 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 (17; 18) and rice (108). Cas9 proteins are characterized by two signature nuclease domains, RuvC and HNH, which cleave DNA strands that are complementary and noncomplementary, respec- tively (36, 54). 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 (19). 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) (20; 21), xCas9 (NG, GAA, and GTA PAM) (22; 23), and SpCas9-NG (NG PAM) (24).

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 (25; 26) . 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 the for Cas9 sgRNA (~43 versus ~80 nucleotides), and the Cpf1 cleavage site is located distal and down stream of the PAM sequence (. 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) . To address the limitations of the recog- nition 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 (16, 27; 28). 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) (29; 30).

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) (31; 32).

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. 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 knockins (33; 34; 35). 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 (36; 37), 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 (38). Since it enables the simultaneous modification of multiple traits, the CRISPR system represents a highly efficient method for pyramid breeding.

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 re- quired 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. 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 sistering a desired sequence modification to be incorporated into the break site (39; 40) .

Precise HDR-mediated genome modification has been widely used in many organisms. How- ever, 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 strate- gies 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 (41; 42; 43), 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, in- cluding sgRNA and repair template sequences, also increases the efficiency of HDR in rice (11).

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). 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) (44; 45). In addition, dCas9 fused to multiple TALEs (a potent dCas9-TV) conferred much stronger transcriptional activation of single or multiple tar- get genes than the routinely used dCas9-VP64 activator in both plant and mammalian cells (46). dCas9 recruits epigenetic effectors such as the histone demethylase LSD1, histone acetyltrans- ferase p300, and ten-eleven translocation (TET) proteins to modify epigenetic marks at their DNA or histone targets; this can alter the status of chromatin modification and hence gene expression, cell differentiation, and other biological processes (47). The catalytic domain of the human demethylase TET1 fused with dCas9 and targeting the FWA promoter led to highly efficient targeted demethylation in *Arabidopsis* 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) . 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).

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.

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 (48). 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) in wheat, rice, and potato (*Solanum tuberosum*), with a 1–17-nucleotide editing window at all examined sites independent of sequence context (49; 50).

The Liu group subsequently developed adenine base editors (ABEs) that mediate the conversion of A to G in genomic DNA(37) . 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 (11; 51). 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 (52). 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.

Similarly, three orthologs of cytidine deaminase, lamprey PmCDA1 (11), human AID (53; 54), and human APOBEC3A (55; 56) 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 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.

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.

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.

Genome editing with stable expression of CRISPR/Cas DNA.

On the basis of tradi-tional 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. 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 self- ing and crossing. Gao et al. (34; 57) 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 (58). Although genetic segregation is an efficient method for obtaining transgene-free mutants, segrega- tion 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.

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. (59; 60) delivered in vitro transcripts of Cas9 and sgRNAs into immature wheat embryos by particle bombardment and generated DNA-free edited wheat. 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 (61; 62). 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 trans- lational machinery and is then degraded quickly, it has a lower propensity for off-target cleavage than DNA-based expression. In 2015, Woo et al. (63) were the first to demonstrate genome edit- ing 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 (64; 65; 66). 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 (67; 68; 69). Svitashev et al. (70) delivered preassembled Cas9/sgRNA RNPs into maize em- bryos 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 muta- tions were detected at the off target sites. Similarly, Liang et al. (71) 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 (72).

Applications for precision plant breeding

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. 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.

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 fac- tors. 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 (73, 74; 75; 76; 77). Simultaneous knock- out 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 quan- titative 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 areimportant for rice yield. This work provided insight into the mechanism of yield development and may facilitate the molecular breeding of improved rice.

Improving quality

Quality traits vary depending on the specific breeding requirements. To date, quality improvements by genome editing have impacted starch content, fragrance, nu-tritional 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 (78; 79). DuPont Pioneer produced a CRISPR/Cas9 knockout waxy corn line with high yields for com- mercial use (80). 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 (81; 82). 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 fra- grant 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 (83). 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 (84; 85). Other high-quality crops produced by CRISPR/Cas9 editing include seeds with high oleic acid oil in *Camelina sativa* (86; 87) and *Brassica napus* (88), tomatoes with a long shelf life (48, 76), high-value tomato with enhanced lycopene (82) or γ-aminobutyric acid content (77, 89), and potato (hairy roots) with reduced levels of toxic steroidal glycoalkaloids (90).

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, bac- terial, and viral diseases and insects, have been obtained via CRISPR/Cas9 knockout. For exam- ple, 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 (91). Similarly, Nekrasov et al. (92) showed that CRISPR/Cas9- mediated knockout of *MLO* confers resistance to powdery mildew in tomato. Rice blast is a de- structive fungal disease; blast-resistant rice was obtained via knockout of *OsERF922,* an ethylene- responsive factor transcription factor gene (93; 94). 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 (95). With regard to viral diseases, use of CRISPR/Cas9 has also produced tungro disease–resistant *eif4g* rice (102), broad potyvirus–resistant *eif4e* cucumber (96), and cotton leaf curl disease–resistant *clcud* cotton (97; 98). Recently, Lu et al. (99) 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 ac- cumulation 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 (100; 101; 102) 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 (103).

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 mater- nal 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 (104; 105) and maize (72), photosensitive genic male-sterile *csa* rice (104), and *ms45* wheat (106). 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*(108) and *OgTPR1* at the *S1* locus (109) were disrupted. Shen et al. (108) found that knockout of one or two copies of the *Sc* gene in the *in- dica* allele *Sc-I* also rescued male fertility in *japonica-indica* hybrids. Similarly, Yu et al. (109) 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 (110) or by knocking out *MTL* (111), enabling a fix of heterozygosity of hybrids through seed propagation. Genome editing is also an effective approach for enhancing many other traits, such as improv- ing haploid breeding (112; 113) shortening growth times (113), increasing silique shatter resistance (10), and overcoming self-incompatibility in diploid potato (115), to meet breeders' requirements.

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 replace- ments facilitate breeding by introducing new alleles without linkage drag or generating allelic variants that do not exist naturally (116) . Moreover, knock-in can be used to alter mul- tiple elite traits by stacking genes in a single variety. Therefore, knock-ins and replacements have great value for crop trait improvement.

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. (117) 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 num-ber of repair templates, which increased gene-targeting efficiency in potato (118), tomato (119; 120) rice (120), wheat (122), and cassava (122). For example, using geminivirus replicons, Cˇ ermák et al. (123) 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. (123) developed a highly efficient selection- and reporter-free gene-targeting procedure using replicon-amplified donorfragments 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 (89), maize (124; 125), and rice (126; 125) 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*) (127) and cassava (128) 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 replace ment and insertion (129). 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.

Applications of Base Editors in Plants

As many agriculturally important traits are conferred by single-nucleotide polymorphisms in ei- ther coding or noncoding regions, base editing is quite useful for plant breeding and crop improvement. One important application of base editing in coding regions is to confer herbicide resistance. Sulfonylurea- or imidazolinone-resistant rice (130), wheat (131; 132).C. Gao, unpublished data), *Arabidopsis* (133), and watermelon (134) have been created by targeting *ALS* with a plant cytidine base editor, and haloxyfop-R-methyl resistant rice has been generated by target- ing acetyl-coenzyme A carboxylase *ACCase* gene with a plant adenine base editor (77; 135). Notably, the genotypes of base-edited polyploid wheat plants are extremely variable, especially when two sgRNAs are involved.

Alternative splicing is a regulatory process of gene expression that results in a single gene en- coding 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 missplicing. Kang et al. (136) 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. (137) created mRNA mis-splicing-induced null mutants of *AtMTA* and double mutants of *OsGL1* and *OsNAL1* in rice.

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 af- fected 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. To date, plant genome editing to alter gene expression has focused mainly on promoters, such as replacing promoters and deleting *cis*-regulatory elements (137¸138) Rodríguez-Leal et

al. (139) edited the promoter regions of quantitative trait–related genes such *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 (140). 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 effi- ciencies of primary ORFs (141). Zhang et al. (142) 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% (143).

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 (144). Many elements of mRNA 5∗ leader sequences, such as RNA structures and internal ribosome entry sites, can affect translation (145). Some regulatory elements in 3∗ un- translated regions also play important roles in gene regulation (146). These key elements all provide targets for genome editing.

Antiviral Plant Breeding Strategies

Viruses are thought to cause roughly half of all plant diseases, leading to massive losses in agricul- tural production worldwide (147). 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. 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 in- hibit its replication has been used for antiviral breeding in plants (50¸147). 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 (148). 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 (149; 150).

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 (151). FnCas9 also efficiently represses replication of cu- cumber mosaic virus and tobacco mosaic virus

in plants (152; 153). Unlike most Cas proteins, C2c2 can cleave single-strand RNA (2), and it interferes with turnip mosaic virus replication in plants (154).

High-Throughput Plant Mutant Libraries

Whole-genome-scale mutant libraries are valuable tools for functional genomics and genetic improvement. 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-scaleCRISPR/Cas9-generated knockout mutant libraries covering most rice genes (155; 156) Meng et al. (157) 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. (158) 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. (160) 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.

Future Perspectives of Considering New Breeding Techniques (NBTS)

Exploiting the Potential of Plant Synthetic Biology

Plant synthetic biology is an emerging field that combines plant biology with engineering prin- ciples 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 bio- production processes (161). 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 fixa- tion (*nif*) genes and their relative expression levels have been characterized (162; 163). 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 (164). 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 (165), 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.

Accelerating the Domestication of Wild Plant

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 (65; 166). 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 (67); maize *tga1,* conferring naked kernels (167); and rice *Sh4*, *Rc*, *PROG1*, and *LABA1*, for nonshattering rachis, white pericarp, erect growth, and barbless awns, respectively (25). 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 in- creasing the sustainability of agriculture. For example, CRISPR/Cas-enabled mimicry of domes- tication 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 (168). Using genome-editing technologies to modify genes that control seed dormancy (*DOG1*), oil quality (*FAE1* and *FAE2*), glucosinolate accumulation (*HAG1* and *GTR*2), 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 cul- tivars 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 (169; 170) 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 associ- ated with sustainable agriculture.

Improved Delivery Systems

The cell wall makes efficient delivery of genome-editing reagents to plant cells challenging. Cur- rent delivery systems are limited to specific plant species, genotypes, and tissues. In addition, al- most 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 inex- pensive application of gene editing in plant. To expand the range of delivery systems, both *Agrobacterium* and plant genes could be manipulated to improve *Agrobacterium*-mediated transformation (49). 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 (95; 135).

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 differ- entiate into gametes (51). Novel delivery systems based on nanotechnology and virus particle–like structures also hold promise for crop improvement. For instance, carbon nanotubes have used to deliver DNA into mature plant leaves, leading to successful protein expression (34). Other nanomaterials such as layered double hydroxides (77) mesoporous silica nanoparticles (28), and polyethylenimine (39) 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.

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 appli- cation 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* (131), rice (157), and cotton plants (80), 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 (89; 159).

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 (145). *PolQ* is essential for T-DNA integration in plants (133); hence, this approach may eliminate the integration of donor DNA into the genome and in- crease 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 pro- tein RecQL4; and Exo1 and Spo11 (108). 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.

Gene drives based on the CRISPR/Cas gene-editing system provide a powerful method for effi- ciently 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, 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.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 sub- sequent 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 biol-ogy 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.

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