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Progress and prospects in plant genome editing

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The emergence of sequence-specific nucleases that enable genome editing is revolutionizing basic and applied biology. Since the introduction of CRISPR–Cas9, genome editing has become widely used in transformable plants for characterizing gene function and improving traits, mainly by inducing mutations through non-homologous end joining of double-stranded breaks generated by CRISPR–Cas9. However, it would be highly desirable to perform precision gene editing in plants, especially in transformation-recalcitrant species. Recently developed Cas9 variants, novel RNA-guided nucleases and base-editing systems, and DNA-free CRISPR–Cas9 delivery methods now provide great opportunities for plant genome engineering. In this Review Article, we describe the current status of plant genome editing, focusing on newly developed genome editing tools and methods and their potential applications in plants. We also discuss the specific challenges facing plant genome editing, and future prospects.

Genome editing that enables targeted genome modification in various organisms is revolutionizing biology. Genome editing is carried out using sequence-specific nucleases, including zinc-finger nucleases, transcription activator-like effector nucleases and the CRISPR–Cas system. Double-stranded breaks (DSBs) generated by these sequence-specific nucleases at targeted genome sites are generally repaired by either non-homologous end joining (NHEJ) or homologous recombination (HR), which lead to gene knockout or gene replacement, respectively. In the CRISPR–Cas9 system and the more recently developed CRISPR–Cpf1 system, which have been adapted from molecular components conferring bacterial immunity, the endonucleases Cas9 and Cpf1 are guided to specific genomic sites by single-guide RNAs (sgRNAs) that recognize the target DNA sequences through Watson-Crick base pairing. Owing to its low cost, ease of execution and efficiency, the CRISPR–Cas9 system has become the most widely adopted editing platform and is used in an expanding number of organisms, including various plant species—notably major crop species[1].

In plants, efficient CRISPR–Cas9-based genome editing generally comprises four steps (Fig. 1). First, the design and construction of a gene-specific sgRNA (step 1). Many online tools have been developed for computer-based design of sgRNAs[2]. However, the *in silico* design of sgRNAs has not been fully adapted for plants, and large-scale data collection and systematic study of sgRNA efficiencies in plant cells are needed to increase the accuracy of computational sgRNA selection. The activity of an sgRNA is best validated in protoplasts (step 2) before being used in genome editing. Thereafter, the components of the CRISPR–Cas9 system are delivered into plant cells (step 3), normally via *Agrobacterium*-mediated transformation or particle bombardment, and the Cas9 and sgRNA expression cassettes are stably integrated into the plant genome. Finally, transformed or regenerated plants with the desired modifications are identified by polymerase chain reaction (PCR) genotyping and confirmed by sequencing (step 4).

The unique features of plant genome editing raise particular concerns and provide particular challenges. Here we review the newly developed plant genome-editing tools and methods (mostly based on the CRISPR–Cas9 system), primarily from a plant-specific point of view. We also summarize the potential applications of the methods for plants and discuss the future directions of plant genome editing.

Delivery of genome-editing reagents into plant cells

To edit a plant genome, the CRISPR–Cas9 construct has to be transformed into plant cells, and whole plants have to be regenerated from the few transformed cells (Fig. 1). To date, genome editing has mostly been applied in transformable plants. Current transformation methods are normally genotype specific, and transformation procedures remain to be established for many plant species. Moreover, *Agrobacterium*, used for transforming most plants, raises regulatory concerns as it is considered a plant pathogen. Therefore, plant transformation is a major bottleneck for realizing the potential of plant genome editing. Experimental methods addressing DNA delivery and plant transformation issues deserve more intensive investigation. Advances that unify approaches across crops should be especially encouraged.

Recently, an elegant study demonstrated that overexpression of *Baby boom* (*Bbm*) and *Wuschel2* (*Wus2*) genes from maize (*Zea mays*) increases transformation frequencies in maize, sorghum (*Sorghum bicolor*), sugarcane (*Saccharum officinarum*), and indica rice (*Oryza sativa* ssp. *indica*)[3]. This approach appears to be genotype independent and may provide a method for improving transformation of recalcitrant crop species. Optimization of this approach and identification of more *Bbm*- and *Wus2*-like genes should enable more researchers to achieve efficient plant transformation and broaden the application of genome editing.

In most of the published plant genome-editing approaches, a CRISPR–Cas9 expression cassette is delivered into cells, integrates into the nuclear genome, becomes expressed, and cleaves the desired chromosomal site. However, only a small proportion of the delivered DNA becomes integrated into the plant genome. Interestingly, unintegrated transgenes can still be expressed and function for a short time, so transient gene expression of CRISPR–Cas9 may provide an alternative method of plant genome editing. Indeed, two simple and efficient genome-editing methods based on transient expression of CRISPR–Cas9 DNA or RNA have been recently developed in hard-to-transform wheat[4]. In these approaches, the canonical herbicide or antibiotic selection step during post-transformation

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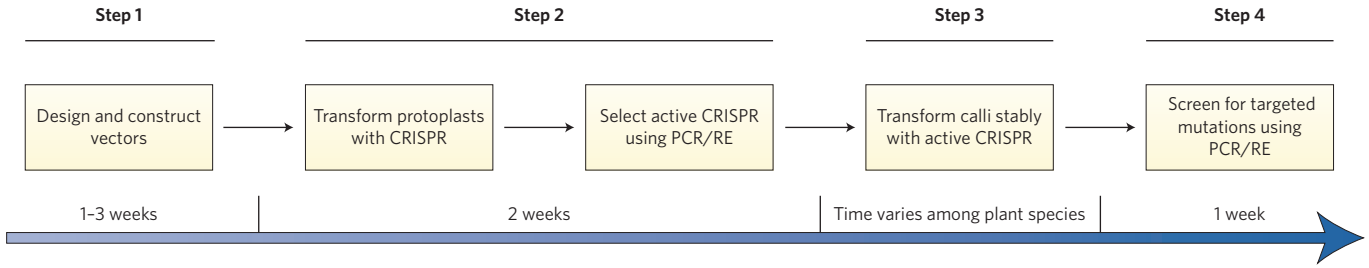

Figure 1 | General procedure of plant genome editing using CRISPR–Cas9. Plant genome editing can typically be divided into four continuous steps, and the estimated time needed for each step is indicated. PCR/RE, polymerase chain reaction/restriction enzyme digestion.

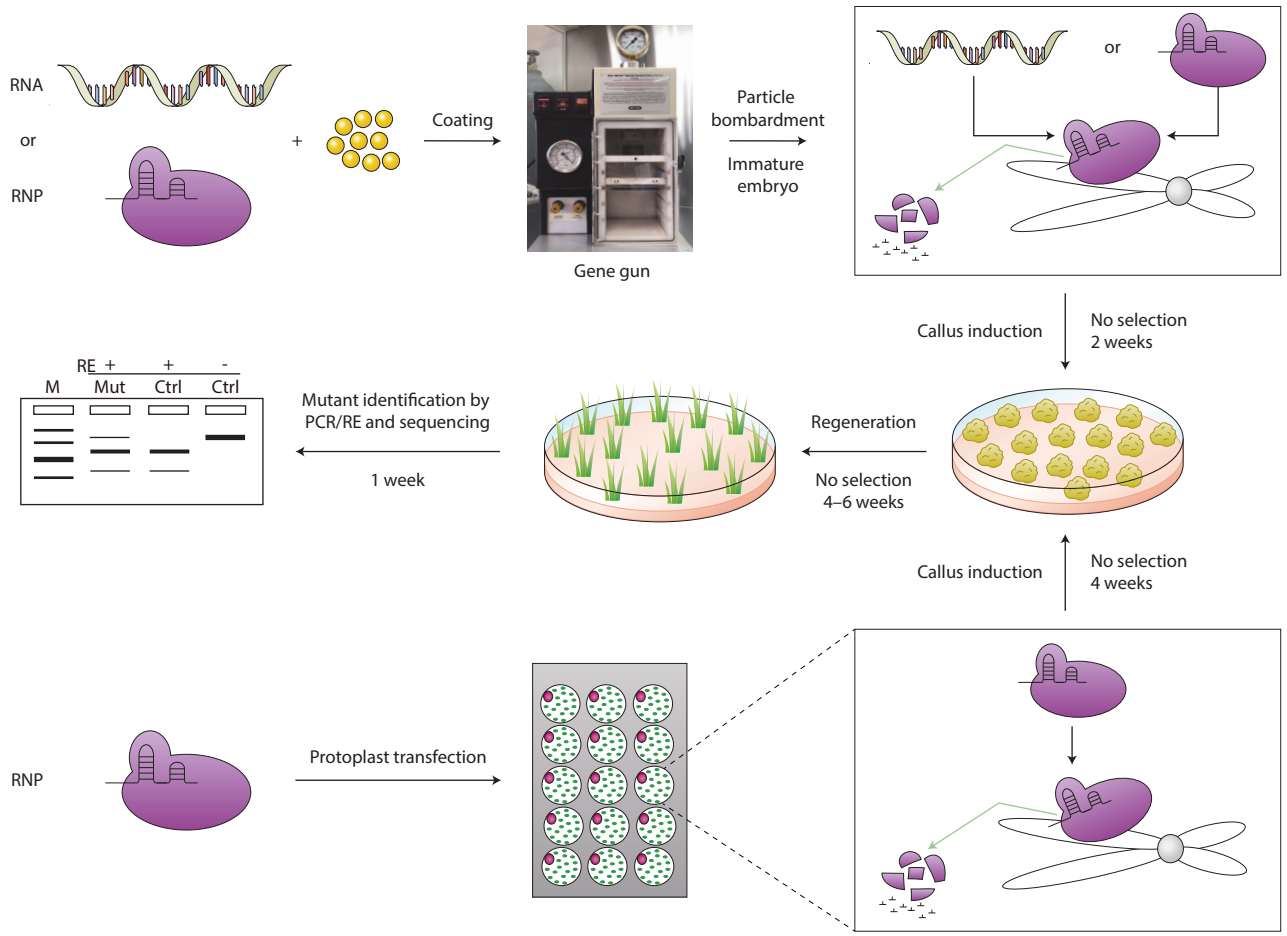

Figure 2 | DNA-free genome editing with CRISPR–Cas9 RNAs and RNP in plants. The CRISPR–Cas9 RNAs (*in vitro* synthesized Cas9 and sgRNA transcripts) or pre-assembled CRISPR–Cas9 RNP can be delivered into immature embryos via particle bombardment. Alternatively, pre-assembled CRISPR–Cas9 RNP can be transfected into plant protoplasts. Bombarded/transfected cells are induced to form calli, from which seedlings are regenerated under the selection-free conditions. Regenerated plants are screened for mutation via PCR/RE assay and sequencing. The estimated times needed are indicated for most steps. Delivering CRISPR–Cas9 reagents via RNP limits their temporal activity, thereby improving their precision. RE, restriction enzyme; M, DNA marker; mut, mutant; ctrl, control.

tissue culture was eliminated, and plants were regenerated from callus cells transiently expressing CRISPR–Cas9 (Fig. 2). As a result, the tissue culture procedures were shorter and less labour intensive. The mutation frequencies of target genes induced by the transiently expressed CRISPR–Cas9 DNA were comparable to those in conventional DNA-integration-based genome editing. Furthermore, transgene integration was significantly reduced in the transient expression systems[4] (Table 1). The CRISPR–Cas9 DNA or RNA transient expression-based genome-editing method was established in wheat callus cells[4], but is probably useful for many other plant species.

While harbouring insertions and/or deletions (indels) at the target site, plants stably transformed with CRISPR–Cas9 may contain unwanted insertions of plasmid DNA at both on-target and off-target sites[5]. These plants are often considered to be genetically modified organisms (GMOs) and may be tightly regulated in some countries, limiting the use of genome editing in plant biotechnology and sustainable agriculture. Although the foreign DNA can in principle be removed by genetic segregation, this is not feasible in plants that reproduce asexually. Even edited plants from which foreign DNA has been removed are not accepted by some local regulatory authorities because recombinant DNA constructs were used in their

Table 1 | Comparative analysis of CRISPR–Cas9 DNA-, RNA- and RNP-mediated genome editing in wheat.

	Conventional[85]	TECCDNA[4]	TECCRNA[4]	TECCRNP[8,9]
Mutation efficiency	++	++	+	++
Specificity	+	+	+	++
Total time	10–12 weeks	6–8 weeks	6–8 weeks	6–8 weeks
Cassette integration	Yes	Yes	No	No
Small DNA insertion	Yes	Yes	ND	No
Antibiotic selection	Yes	No	No	No

Conventional, conventional DNA-integration-based genome-editing methods; TECCDNA, transient expression of CRISPR–Cas9 DNA; TECCRNA, transient expression of CRISPR–Cas9 RNA; TECCRNP, transient expression of CRISPR–Cas9 ribonucleoproteins; +/++, level of mutation efficiency (or specificity), with ++ representing higher levels than +; ND, not determined.

production[6]. Two approaches involving DNA-free genome editing in plants have been described; these involve delivery of a mixture of Cas9-encoding mRNA and guide RNA[4] or pre-assembled ribonucleoproteins (RNPs)[7–9]. However, the efficiency of transient expression of CRISPR–Cas9 RNA is relatively low (Table 1), indicating that further optimization is needed. In this direction, one promising strategy would be to add some protectant to stabilize the RNA[10].

RNPs represent a promising approach for DNA-free genome editing in plants. Woo *et al.* transfected RNPs into protoplasts of four plant species and were able to induce targeted genome modifications in each case. However, only mutated lettuce plants could be regenerated from the RNP-transfected protoplasts[7]. This is not surprising, as currently only a very small number of plant species can be regenerated from protoplasts and few of these are monocot crops. Alternatively, RNPs have been delivered into maize and wheat embryos by particle bombardment to obtain edited plants[8,9]. As mutants generated in this way are in principle indistinguishable from those obtained by conventional breeding, they may not fall foul of current rules for GMOs.

Off-target effects of the CRISPR–Cas9 system in plants

Off-target effects, in which Cas9 cleaves genomic DNA sites that are imperfect complements of sgRNA, are one of the major disadvantages of the CRISPR–Cas9 system in human cells[11–13]. They impede potential applications of CRISPR, especially when precision genome editing is needed, such as in gene therapy. Off-target effects may not cause a serious problem for plant breeding based on genome editing as the physical and chemical mutagenesis used in conventional plant breeding generally produces many mutations in each plant[14], and the unwanted mutations can be removed by backcrossing. Nonetheless, backcrossing is time-consuming and would slow down progress in crop improvement. Moreover, the off-target effects of the CRISPR–Cas9 system could attract regulatory interest in genome-edited plants.

The specificity of CRISPR–Cas9 in plants has been evaluated by biased off-target detection[15]. Targeted sequencing of 13 putative off-target sites for 3 sgRNAs in rice only found mutations in 1 off-target site; that site harboured a single mismatch distal to the protospacer adjacent motif (PAM)[16]. Comparative analysis in wheat also revealed that the off-target frequency of CRISPR–Cas9 was very low[4]. Interestingly, when RNPs were used for editing, off-target mutations were barely detected by deep sequencing, supporting the view that this approach enhances the specificity of CRISPR–Cas9 in plants[7–9] (Table 1). However, to date, there have been no studies systematically characterizing CRISPR–Cas9 specificity in plants. Several unbiased approaches, such as BLESS, GUIDE-seq, HTGTS and Digenome-seq, have been developed to detect off-target changes in human cells[17–20], and these approaches need to be adapted to plants in order to systematically evaluate the specificity of Cas9 on a genome-wide scale. Nonetheless, increasing CRISPR–Cas9 specificity is a major challenge that requires attention in the plant-editing field. In fact, several strategies have been developed to enhance Cas9 specificity[21], including the Cas9 paired nickase strategy and recently identified high-fidelity Cas9 variants[22,23,24].

Orthogonal CRISPR systems for plant genome editing

Additional refinements of existing CRISPR–Cas9 methodology are needed, especially in relation to PAM specificity and efficiency. The most common Cas9 used in plants is from *Streptococcus pyogenes* (SpCas9) and recognizes NGG-type PAM. Although this PAM sequence is widely distributed across plant genomes, it does not cover the full genomes. New Cas9 variants and novel RNA-guided nucleases have expanded the diversity of PAM sequences[25–29]. Two of these SpCas9 variants were able to edit target sites with alternative non-canonical PAM sequences in rice[30]. More SpCas9 orthologues, such as StCas9 from *Streptococcus thermophilus* and SaCas9 from *Staphylococcus aureus*[31–33], were identified and were also able to induce targeted genome modifications in plants. Both StCas9 and SaCas9 require longer PAMs, which might increase specificity of the editing[31,32]. Recently, CRISPR–Cpf1 has emerged as a new tool for plant genome editing. Cpf1 cuts DNA in a different way; Cas9 cuts double-stranded DNA, generating blunt-end DSBs, whereas Cpf1 generates staggered cuts with 5′ overhangs[34]. The latter cleavage structure offers particular advantages for directed gene insertion into eukaryotic genomes using NHEJ[35]. The editing ability of Cpf1 has been tested in plants. Unlike Cas9, which usually creates small indels[36], Cpf1 generally generated larger indels (≥6 nucleotides)[37–40]. Remarkably, no off-target mutations were detected in these plants[37–39], underlining the high specificity of Cpf1. New CRISPR–Cas systems of this kind offer very attractive genome-editing tools. Furthermore, combination of different CRISPR systems can increase the capacity of multiplex genome editing[41].

Precision genome editing in plants

The main repair pathway in response to DSBs is NHEJ, which is error-prone and creates indels that normally lead to loss of gene function. In contrast, HR leads to sequence changes or sequence replacements (Fig. 3a)[42–44]. Gain-of-function point mutations, which can in principle be generated by HR, are useful in genetic studies and important for elucidating gene function. Many important agronomic traits are the result of point mutations in the coding regions of genes, such as the acetolactate synthase (*ALS*) gene, which confers herbicide resistance[45]. Although there are successful examples of gene replacement in rice and maize using CRISPR–Cas9 (refs 46,47), the extremely low efficiency of HR prevents its broader use in plant cells.

To achieve HR, a DNA repair template also needs to be delivered into plant cells, and the amount of template delivered into a cell could greatly affect HR efficiency. DNA replicons (deconstructed geminiviruses) have been used to deliver both the CRISPR–Cas9 reagents and DNA repair template and achieved greater than ten-fold enhancements of GT frequencies in tobacco, tomato, potato, hexaploid wheat and rice[48–52]. As geminiviruses are not integrated into host plant genomes, plants that are genome edited by DNA replicons may be free of GT reagents[51]. Furthermore, as geminiviruses

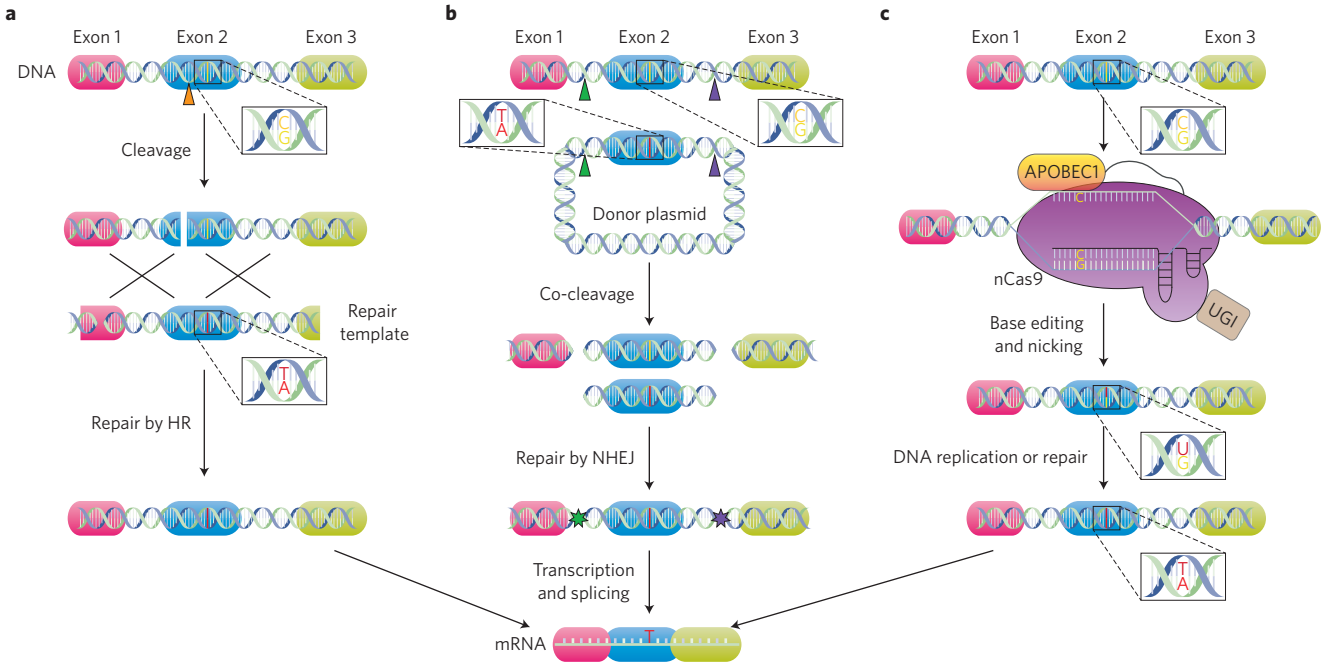

Figure 3 | Strategies for precision genome editing in plants. a, HR-mediated gene replacement. CRISPR–Cas9-induced DSBs can be repaired by HR in the presence of an exogenous repair template, leading to precise gene replacement. **b**, Intron-targeting-mediated gene replacement. Adjacent introns of a targeted exon and a donor DNA plasmid are targeted for cleavage by the same pair of sgRNAs. Exon replacement can be achieved via NHEJ. Indels (coloured asterisks) will be generated at the DSB sites. **c**, Base editing in plant cells. A fusion protein composed of rat cytidine deaminase APOBEC1 (orange), nCas9 (purple) and UGI (brown) is used as the plant base editor. DNA with a target C (yellow) recognized by a sgRNA (black) is bound by nCas9, which separates the DNA double-strand. The tethered APOBEC1 converts the target C to U (red) on the edited strand. Meanwhile, UGI inhibits the UDG-initiated base excision repair of the edited base. Although the gene replacement in **b** is accompanied by indels in introns, after transcription and splicing the final mRNAs contain the same specific change (C to T), similar to those obtained through **a** and **c**. The exons are marked by coloured cylinders. nCas9, Cas9–D10A nickase; UDG, uracil DNA glycosylase; UGI, UDG inhibitor.

used in DNA replicons have a large host range, many other plants, including some important crops, can be precisely modified through this approach.

Gene replacement can be achieved in plant cells by harnessing NHEJ[53]. A recent example is an efficient intron-mediated site-specific gene-replacement method using CRISPR–Cas9 (Fig. 3b)[54]. In this method, a pair of sgRNAs targeting adjacent introns of an exon and a donor DNA template with the sgRNA target sequence divided between its two ends were introduced together with a Cas9 construct into rice callus cells, and gene replacements in the exon were obtained in the regenerated plants at a frequency of 2%. Although indels are often generated in introns at the junctions between the endogenous gene and donor template, the final spliced gene product is generally acceptable because introns are tolerant of small changes provided that the splicing sites are not changed and the transcription is not greatly affected. Furthermore, the site-specific gene replacements were faithfully inherited and conferred the expected phenotypes[54]. Therefore, this approach may be a useful alternative for generating gene replacements in intron-containing genes.

Recently, based on the CRISPR–Cas9 system, an ingenious method called base editing has been developed for modifying single DNA bases at genomic target sites without forming DSBs or introducing a donor DNA template in animal, yeast and bacterial cells[55–59]. The most efficient version of base editor uses a protein fusion consisting of a Cas9 nickase (Cas9–D10A) and a cytidine deaminase that can convert C to T (or G to A), along with a uracil glycosylase inhibitor (UGI) to inhibit base excision repair of the base change[55,56]. Even though the present base editors can only convert C to T (or G to A), they provide an unprecedented tool for accurately modifying individual nucleotides in a genome. Base editors codon-optimized for plants are able to produce site-specific C to T conversions in a number of plant species (Fig. 3c)[60–64]. It appears that the deamination window of base editing in plant cells might be slightly wider than that in animal cells[62]. Furthermore, almost no indel mutations were found in the edited plants[62], suggesting that in plants, this tool is highly specific.

Base editing provides a powerful tool for generating point mutations in plants to improve traits and characterize gene function. However, usually, not only the desired C but also other Cs within the deamination window are converted by the deaminase[62], but future improvements may well succeed in narrowing down the deamination window to one base pair. Indeed, cytidine deaminase has recently been engineered to reduce its window from ~5 to 1–2 nucleotides[65]. In addition, several natural and engineered Cas9 variants with different PAM requirements have been employed to expand the target range of base editing[64]. To further overcome the limitations imposed by particular PAM sequences, it should be possible to combine any of a number of cytidine deaminases with other Cas9 orthologues or Cpf1. We can also envision the development of novel tools capable of editing the three other bases by engineering the fusion enzyme via approaches such as phage-assisted continuous evolution[66]. With the above improvements, the range of point mutations should be greatly expanded, eventually making it possible to target every single base in the genome.

Prospects and future directions

Further use of the CRISPR system will surely advance plant biology and crop breeding. New breeding technologies based on genome editing that allow simultaneous modification of multiple genetic loci in elite varieties will accelerate crop improvement and enhance global food security. However, information on genome sequences and gene functions is a precondition for effective genome editing,

and such information can also be provided in part by genome editing. It is probable that we will see increasing use of CRISPR for elucidating genome structure and gene functions in plants; for example, in repurposing Cas9 and Cpf1 for transcriptional regulation[40,67,68], visualizing gene loci[69–73], identifying epigenetic modification[74–76] and mechanisms regulating promoter activity, as well as in establishing causal relationships between single-nucleotide polymorphisms identified by genome-wide associate studies and genetic traits[77]. The development of novel Cas9-based applications, for example in genetic mapping[78] and the domestication of new crops[79], represents further major directions for plant genome editing. In addition to genome editing, the CRISPR–Cas9 system has been adapted to directly target plant-infecting geminiviruses, thereby increasing plant resistance to geminiviral diseases[80–82].

Genome editing represents a new breeding technology, which makes targeted or directional breeding possible. The CRISPR–Cas9 system has been used to improve a variety of crop traits, including yield level, nutritional value, stress tolerance, and pest and herbicide resistance[4,8,46,83–90]. Multiplex genome editing, which facilitates quick stacking of multiple traits in an elite variety background, will make a dramatic impact on efficiently improving complex agronomic traits in crop plants. Due to its low cost, precision and rapidness, genome editing provides an unprecedented possibility for plant breeding, and is being applied to an ever-increasing number of plant species. It is very probable that more and more plants bred with CRISPR technology will be ready for marketing in the near future.

Plant genome editing still faces challenges, primarily in terms of establishing a unified delivery method and making HR efficient, which will probably require innovative modifications of the CRISPR system and manipulation of DNA repair pathways. Furthermore, high-throughput CRISPR-based whole-genome functional screening of genes and DNA elements has not yet been demonstrated in plants. Nonetheless, because of its high efficiency, ease of use and relatively low cost, CRISPR-based genome editing has been quickly established as a powerful tool that is revolutionizing plant biology research and crop trait development, in a manner similar to that brought about by molecular cloning and PCR technologies. Although regulatory issues are not within the scope of this Review Article, it is clear that unreasonable scrutiny of genome-edited plants will reduce their ability to feed the fast-growing world population under our ever-evolving climatic conditions.

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Competing interests

The authors declare no competing financial interests.