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Feature Review

Construct design for CRISPR/Cas-based genome editing in plants

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CRISPR construct design is a key step in the practice of genome editing, which includes identification of appropriate Cas proteins, design and selection of guide RNAs (gRNAs), and selection of regulatory elements to express gRNAs and Cas proteins. Here, we review the choices of CRISPR-based genome editors suited for different needs in plant genome editing applications. We consider the technical aspects of gRNA design and the associated computational tools. We also discuss strategies for the design of multiplex CRISPR constructs for high-throughput manipulation of complex biological processes or polygenic traits. We provide recommendations for different elements of CRISPR constructs and discuss the remaining challenges of CRISPR construct optimization in plant genome editing.

Highlights

Many Cas nucleases (e.g., SpCas9-NRRH, SpG, SpCas9-NG) that can target non-canonical protospacer adjacent motifs (PAMs) have been developed for plant genome editing.

Near-PAMless Cas nuclease SpRY has been optimized for plant genome editing to increase the flexibility of gRNA design.

A next-generation genome editing technology, prime editing, has been tested in many plants, including *Arabidopsis*, rice, maize, potato, and tomato.

Multiplex clustered regularly interspaced short palindromic repeat (CRISPR) systems based on tRNA/gRNA or Csy4 work better for Cas9 and a hammerhead and hepatitis delta virus (HH-HDV)-based system works better for Cas12a.

A multiplex CRISPR system expressing up to 24 gRNAs has been tested in plants.

Use of multiple introns in the Cas gene dramatically improves editing efficacy.

Improved pegRNA design significantly improves the efficiency of the prime editor.

Genome editing and associated technologies

Genome editing can be defined as a targeted intervention of genetic materials (i.e., DNA or RNA) in living organisms to deliberately alter their sequences. Although genome editing can target both DNA and RNA, here we only review DNA editing. DNA editing mainly relies on the introduction of *in vivo* DNA double-stranded breaks (DSBs) induced by the engineered sequence-specific nucleases (SSNs) programmed to recognize predefined sites in a genome. The induced DSBs are then repaired by cellular DNA repair mechanisms, namely non-homologous end-joining (NHEJ) and homology-directed repair (HDR) (Figure 1). The repair of DSBs by NHEJ results in mutation at the break site, largely via imprecise sequence insertions or deletions (indels), disrupting the native structure and function of the targeted sequences (e.g., genes, promoters). In addition, NHEJ can mediate targeted sequence insertion or replacement when a suitable DNA fragment is provided [1]. By contrast, repair by HDR can precisely introduce predefined sequences carried by a donor DNA template (Figure 1).

The SSNs, with the capacity to introduce DSB in DNA, are referred to as the key elements in genome editing technologies and include meganucleases [2], zinc finger nucleases (ZFNs) [3], transcription activator-like effector nucleases (TALENs) [4], and clustered regularly interspaced short palindromic repeat (CRISPR) systems [5–8]. Unlike ZFNs and TALENs, which rely on protein–DNA interaction to define target specificity, CRISPR systems use RNA–DNA interaction to guide the DNA targeting and cleavage, making it a simple, efficient, and inexpensive technology for genetic manipulation. CRISPR systems have now become the leading genome editing technology and have been applied in a wide variety of plant species. Efficient genome editing has been achieved in many dicot and monocot species using diverse CRISPR-Cas systems for fundamental research and crop improvement and the application of CRISPR-Cas technology in plants has been increased dramatically over the past few years [9–12].

Three classes of CRISPR technology are currently available for editing plant genomes [10,13]. These are CRISPR-Cas nucleases, base editors, and prime editors. CRISPR-Cas nucleases

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require inducing DSB, whereas base editors and primer editors do not require DSB to edit genomes. Over the past few years, there has been tremendous progress in the development of CRISPR-based technologies. The rapid discovery and development of diverse CRISPR toolboxes thus can make the prospect of selecting a tool for desired application daunting, particularly for researchers new to the genome editing technology. Besides selection of the right CRISPR tools, delivery of CRISPR reagents to plant cells is challenging. In some systems such as mammalian cells, purified protein or mRNA of a Cas protein, as well as the **gRNA** (see Glossary), can be simultaneously delivered to a zygotic cell. In this way, targeting possibility can be improved by controlling the dosage of Cas proteins and gRNAs. This approach has also been shown to work in plants, but there are still some significant challenges to be overcome. Thus, most frequently, CRISPR reagents are delivered into plants via a construct harboring a *Cas* gene and at least one gRNA along with the components required for their expression (e.g., promoter, terminator) through *Agrobacterium*-mediated transformation or particle bombardment. Hence, construct design is a critical step to conduct the CRISPR experiment. Different elements of a CRISPR construct can significantly influence the editing outcome and optimization of *Cas* gene and gRNA expression are often required to achieve efficient editing [14–19]. Specifically, the following three factors need to be considered to design CRISPR genome editing constructs: (i) Cas proteins, (ii) gRNAs, and (iii) **gene regulatory elements (GREs)** used to express Cas protein and gRNAs. Here, we review different CRISPR-based genome editing technologies and their technical aspects with the aim to guide users in selecting the appropriate editing technologies and optimizing construct design for various applications. We restrict our discussion to the targeted editing of DNA sequence and refer readers to excellent reviews for other CRISPR applications in plants such as transcriptional regulation [20,21] and epigenetic editing [22].

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Different types of CRISPR-based genome editors

In this section, we discuss different CRISPR reagents and recent developments that progressively increased the applicability and effectiveness of genome editing technologies in plants. This will help identify and select the appropriate technologies and Cas proteins for different applications.

CRISPR-Cas nucleases

Cas9 is currently the most widely used nuclease in CRISPR studies, particularly one isolated from *Streptococcus pyogenes* (SpCas9). It complexes with a **single guide RNA (sgRNA)** for DNA targeting and requires a short stretch of nucleotides known as **protospacer adjacent motif (PAM)** downstream of its target sequence for DNA recognition (Figure 1A). The PAM sequence for SpCas9 is 5′-NGG-3′ (N = A, T, C, G). Once Cas9 recognizes its PAM sequence, the Cas9-sgRNA complex binds to the target sequence and generates a DSB at the target site (Figure 1D). DNA cleavage activity of Cas9 is achieved by the combined effort of two parts of the protein called the recognition domain and the nuclease domains (RuvC and HNH). The recognition domain senses the complementary DNA sequence and the nuclease domains cleave the DNA [23].

Despite the widespread use and proven efficacy of SpCas9 for genome editing purpose across a wide range of organisms, it does have certain limitations. Firstly, it often recognizes DNA sequences that share high sequence identity with the target site, resulting in off-target editing. Secondly, the stringent NGG PAM requirement limits the target DNA that can be manipulated with SpCas9. Thirdly, delivery of SpCas9 into plant cell via a viral-based vector is difficult due to its relatively large size that exceeds the cargo capacity of the **virus-based vector**. To overcome these limitations, several natural and engineered variants of SpCas9 have been developed that recognize alternative PAMs (Table 1). Among them, *Staphylococcus aureus* Cas9 (SaCa9) is a

natural variant and notable one [24]. It recognizes 5′-NNGRRT and its coding sequence is ~1.0 kb shorter than that of SpCas9, thus being suitable to use with virus-based vectors [25,26]. Many engineered SpCas9 variants have also been applied in plant genome editing, including Cas9-NG and xCas9 [27–35], as well as iSpyMacCas9 [36]. A remarkable engineered variant of SpCas9 is SpRY, which is capable of targeting almost all PAM sites (NRN>NYN) [37], and has also been applied in plant genome editing [38,39]. A high-fidelity variant of SpCas9 that has low off-target activity has also been developed (Table 1). Off-target issues can also be reduced by using paired Cas9 nickase [40]. Recently, a number of Cas9 variants that recognize non-canonical PAM (e.g., SpCas9-NRRH) have also been applied in plants [41].

The development of engineered Cas9 proteins with relaxed PAM requirements has broadened the targeting capacity of Cas9; however, there are some potential pitfalls. Some preliminary reports suggest that Cas9 that has very relaxed PAM requirement can have reduced activity at the canonical PAM site. This may be due to the presence of several more putative PAM sequences in the genome, which could cause the proper binding to the target to be delayed [9,42]. A recent report has shown that SpCas9-NG can mutate gRNA residing in the T-DNA, which generates new variants of gRNA and increases the risk of potential off-target editing [43]. The same vector self-editing problem has also been reported for SpRY in plants [38]. This problem, however, could be partially overcome by changing gRNA scaffold sequences [43].

Cas12 nucleases are the second most widely used Cas proteins in plants; particularly, Cas12a (formerly Cpf1) has been applied in many plant species. It recognizes AT-rich PAMs (Table 1) and thus is suitable to edit AT-rich genomic regions. It produces staggered DSBs, which makes it better for experiments relying on HDR. Cas12a only requires one short CRISPR RNA (crRNA, ~42 nt), making it more economical to synthesize and easier to use for multiplex editing. Moreover, the RNase activity of Cas12a can process one CRISPR array into individual crRNAs for multiplex genome editing. Since the cleavage site of Cas12a is distal from the PAM sequence, Cas12a can continuously cut DNA until the edits prevent the crRNA binding, potentially resulting in higher editing efficiency and larger deletions [5,44]. To broaden the PAM recognition range, Cas12a orthologs and engineered variants have been investigated in plants (Table 1). Although nickase activity of Cas12a has been reported *in vitro* with mismatched [45] and shortened crRNAs [46], it has not been demonstrated in plants and reliably used in base editing and prime editing applications in plants. The other Cas12 nuclease that has been applied in plant genome editing is Cas12b. Like Cas12a, it creates a staggered DSB at the target site. It requires, however, both crRNA and transactivating crRNA (tracrRNA) for its activity, which is different to Cas12a. Interestingly, Cas12b is smaller than the widely used SpCas9 and Cas12a [47] and thus more suitable for delivery into plant cells via a virus-based vector.

Base editors

Base editors can enable targeted base changes without DSBs and donor DNA templates. Base editors are created by fusing an engineered base modification enzyme, such as deaminase, to a catalytically inactive Cas9 (dCas9) or partially active Cas9 known as Cas9 nickase (nCas9), which can cut only a strand of DNA (Figure 1B). It should be noted here that although both dCas9 and nCas9 can be used in a base editor, a modern base editor mainly uses nCas9 because of its high performance over dCas9-based base editors. nCas9 (Cas9D10A) nicks the nonedited strand to promote DNA repair using the edited strand as the template, resulting in higher editing efficiencies [48,49]. Current base editing tools include cytosine base editors (CBEs) and adenine base editors (ABEs), which can achieve C/G to T/A and A/T to G/C, respectively. Recently, a DNA base editor capable of C/G to G/C DNA base transversion has been reported [50–53]. This new class of base editors is termed C to G base editors (CGBEs). CGBE has not been tested in plants yet, whereas

Glossary

Codon optimization: a process to change codon composition of a recombinant gene to improve gene expression and increase translation efficiency by accommodating codon bias of the host organism.
Csy4: a 21.4 kDa protein that recognizes its RNA substrate via sequence- and structure-specific contacts. It cleaves cognate RNAs at the 3′ end of a 5-bp stem-loop, generating crRNAs comprising a unique spacer sequence flanked by 8 and 20 repeat-derived nucleotides on the 5′ and 3′ ends, respectively.
Directed protein evolution: a laboratory process by which biological entities with desired traits are created through iterative rounds of genetic diversification and library screening or selection.
Gene regulatory elements (GREs): noncoding DNA that regulates the transcription of a gene. Examples of GRE include promoter, terminator, enhancer, and intron.
Guide RNA (gRNA): a specific RNA sequence that is used as a guide for Cas nuclease to target the DNA region of interest.
Hammerhead (HH) and hepatitis delta virus (HDV) dual ribozyme: a small RNA molecule that can mediate sequence-specific intramolar RNA cleavage.
Prime editing guide RNA (pegRNA): a specialized guide RNA that simultaneously contains guide sequence, a primer binding site sequence, and a template containing the desired edit. pegRNA is longer than the conventional gRNA.
Primer binding site (PBS): a short DNA sequence (~13 nt) used in pegRNA, which allows the 3′ end of the nicked DNA to hybridize to the pegRNA strand upon cutting by the Cas9 nickase.
Protospacer adjacent motif (PAM): a short sequence (2–6 bp) next to the DNA sequence targeted by the Cas nuclease. The PAM is required for a Cas nuclease to cut. It is generally found 3–4 nt downstream from the cut site of Cas9.
RT template: a DNA sequence that contains the desired edit sequence in the prime editing system. Because editing is performed by RT using the RT template sequence, this sequence is called the RT template.

both ABEs and CBEs have been applied in various plants with high success. Over the past few years, remarkable progress has been made and different versions of base editors have been developed to improve the efficacy and specificity. Different versions of CBE include base editor 1 (BE1), base editor 2 (BE2), base editor 3 (BE3), and base editor 4 (BE4) (Box 1). Efficient C/G to T/A base editing has been widely achieved in many plant species and the most used system is BE3 [54–56]. Further improved CBEs, such as PmCDA1-CBE_V04 and A3A/Y130F-CBE_V04, were recently developed with high editing activity and specificity as well as reduced indel byproducts [57]. TadA8e and TadA9 are the most active ABEs, with the widest sequence compatibility among the ABE series developed and recommended for converting A/T to G/C in a variety of targets with improved performance and product purity [38,58]. Notably, CBEs can induce Cas-independent genome-wide off-target mutations in plants and mammalian systems, while ABEs have minimal off-target effects [59–61]. However, CBEs can be engineered to reduce off-target editing while maintaining comparable on-target editing [57,59,62] (Box 1). Therefore, we recommend using the improved version of CBEs.

Prime editors

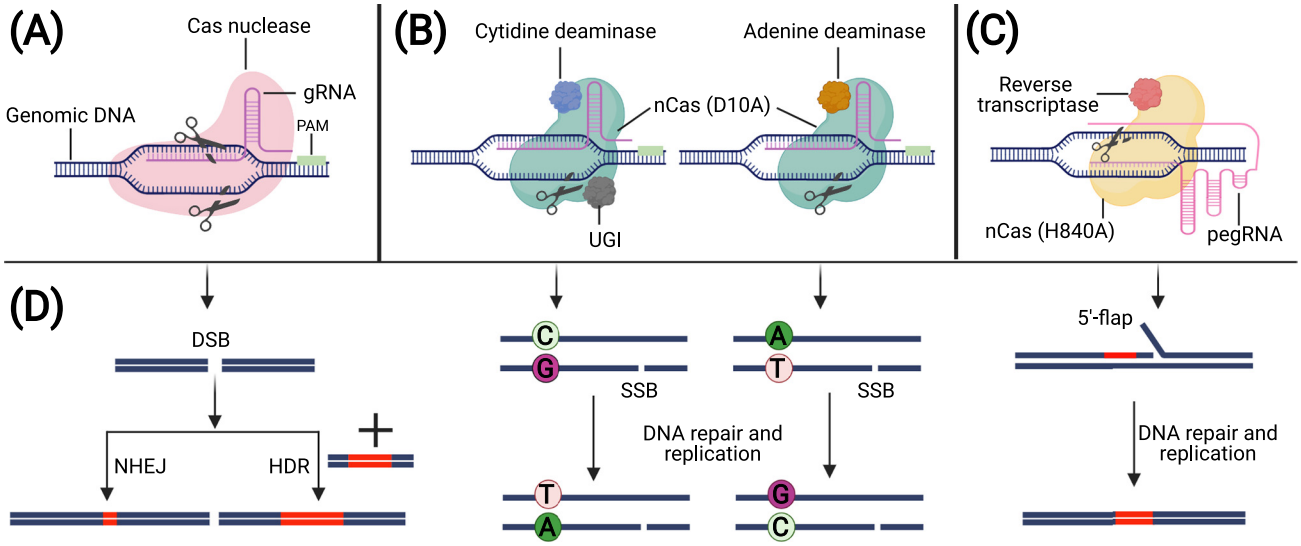
Current base editors used in plants can only achieve six out of 12 possible base changes [53,63]. The use of base editors is also limited if there are no PAM sequences close to the editing site or undesired editable bases fall into the editing window. To overcome these limitations, a versatile precise genome editing approach, prime editing, has been developed, which can achieve all 12 possible base conversions as well as generate small indels without the introduction of DSBs or DNA donors [64]. A standard prime editor contains an engineered reverse transcriptase enzyme along with a Cas9 nickase and a **prime editing guide RNA (pegRNA)** (Figure 1C). Unlike in base editors, where Cas9D10A nickase is used to increase editing efficiency, a Cas9H840 nickase is used in prime editors. Apparently, use of Cas9 nickase instead of dCas9 stimulates the DNA repair system and improves the outcome of base editors and primer editors [54,64]. Different versions of prime editors, such as primer editor 1 (PE1), prime editor 2 (PE2), and prime editor 3 (PE3), have been developed. Prime editing has been demonstrated both in mammalian cells and in different plants such as rice (*Oryza sativa*) [65–71], wheat (*Triticum aestivum*) [66], maize (*Zea mays*) [72], tomato (*Solanum lycopersicum*) [73], and potato (*Solanum tuberosum*) [74]. Editing efficiency, however, is low in plant systems compared with mammalian cells and it is difficult to obtain homozygous and biallelic edits. Further optimization is thus required to broaden its application in plants. A recent study in rice has, however, shown that designing **primer binding site (PBS)** with a melting temperature of 30°C and the use of two pegRNAs *in trans* encoding the same edits enhanced the editing efficiency up to 17.4-fold [75]. Because there is no Cas12a nickase that can reliably work in plants, the application of Cas12a to base editing and prime editing in plants is limited.

Different types of outcomes produced by various types of CRISPR editor are listed in Figure 1E. Figure 2 shows a decision tree explaining different editing purposes and the appropriate editors required to achieve the desired outcomes.

Design and selection of gRNAs

The success of a CRISPR experiment largely depends on the selection of an appropriate target site and the design of an effective gRNA against that target. Each gRNA consists of two parts: a CRISPR RNA, which contains a spacer, and a scaffold sequence known as trans-activating CRISPR RNA (tracrRNA). The spacer sequence is replaced for every new target. Part of the spacer sequence close to the PAM site is regarded as the seed sequence, which is very important for target recognition and binding because this region is first bound to the target DNA following the PAM recognition [76,77]. Mismatch in the **seed region** of gRNA is less tolerated (i.e., a mismatch between the crRNA and the target site in the seed region might abolish

Seed region: the portion of the gRNA close to the PAM site (i.e., 3′ region of gRNA) is called the seed region. The length of the seed region for the CRISPR/Cas9 system is ~10–12 bp. Any mismatch in this region might completely abolish the editing.
Single guide RNA (sgRNA): in the natural CRISPR/Cas9 system, the editing is performed by two RNA molecules: one is crRNA and the other one is trans-activating crRNA (tracrRNA). To make the editing system easier, crRNA and tracrRNA are fused together and this fused product is known as sgRNA.
Virus-based vector: viral vectors are usually derived from parental wild type viruses whose viral genes (essential for replication and virulence) have been replaced with the heterologous genes intended for cell manipulation. They can be used to deliver DNA into plant cells, including CRISPR constructs.



(E)

Types of mutation	Starting sequence	Desired product	Editing reagents
Stochastic indel	PAM	Indel Indel PAM	Cas nucleases
Small insertion (e.g., 1-30 bp)	PAM	1 bp PAM	Cas nucleases-HDR Prime editor
Small deletion (e.g., 1-80 bp)	3 bp PAM	PAM	Cas nucleases-HDR Prime editor
Large insertion (e.g., >30 bp)	PAM	1 kb PAM	Cas nucleases-HDR Cas nucleases-NHEJ
Large deletion (e.g., >40 bp)	PAM 500 bp PAM	PAM	Cas nucleases-HDR Cas nucleases-NHEJ
Base transition point mutation	C G	T A	Base editor (CBE, ABE) Cas nucleases-HDR
Base transition and transversion point mutation	C G	A T	Cas nucleases-HDR Prime editor
Chromosomal inversion			Cas nucleases-HDR Cas nucleases-NHEJ

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the CRISPR activity). The length of the seed sequence varies between different Cas family proteins. For example, the length of seed sequence for Cas9 family proteins is ~8–12 nt, whereas it is 5–6 nt for Cas12a nucleases. Some studies further narrowed down the seed sequence of Cas9 family proteins to 5 nt [78].

The site that is selected for gRNA design within a targeted region depends on the editing purpose. For instance, targets located at earlier exons of a gene have a higher chance of generating knockouts based on premature termination codon (PTC) induced by NHEJ-generated indels. However, exons very close to an ATG or intron–exon junction should be avoided as it is common that PTC near an ATG or intron–exon junction do not lead to loss of function. This is because non-sense mediated decay, which destroys PTC-bearing transcript, is more effective when the PTC is located ≥50–55 nt of intron–exon junction than near the ATG or intron–exon junction [79]. In base editing and prime editing, target sites should fall inside the editing window. The base editing window can be shifted by changing deaminases and Cas proteins [80], to optimize desired base changes and limit bystander mutations. The nucleotide features of a gRNA and its associated secondary structure are the two main parameters that affect gRNA efficacy. Generally, an effective gRNA has GC content of 30–80%, no mismatch to the intended target, especially in the seed region targeting the nontranscribed strand [78,81–86]. For prime editing, the recommended design of pegRNA includes a PBS of approximately 13 nt and a reverse transcriptase (RT) template of 10–16 nt, while avoiding an adjacent C at the 3′-end of the gRNA. In addition, synonymous mutations can be introduced to disrupt the PAM sequence to prevent further editing of the targeted strand. In PE3, it is recommended to design the gRNA that is used to nick the nonedited DNA strand ~50 bp away from the initial pegRNA-mediated nick on the edited DNA strand to decrease the formation of indels [64,68].

gRNAs can be designed using various web-based software. Nearly 30 web-based tools exist to design gRNAs [87,88]. Therefore, selecting a website for gRNA design can be complicated. There are several criteria users need to consider when selecting a website for designing gRNAs. The first criterion is what kind of input the program allows. Some websites only support uploaded sequences, whereas others (e.g., CHOPCHOP) allow users to provide the transcript identifier (from RefSeq or Ensemble), which can avoid manually entering exon sequences of protein-coding genes. Several tools design gRNAs that cover multiple transcripts, whereas others cover multiple exons of the same gene, which is useful for designing gRNA libraries, as it decreases the likelihood of all chosen gRNAs hitting a weakly expressed exon. Some websites encourage users to enter several targets in a single batch, which is useful for creating gRNA libraries [87]. The diversity of genomes supported by the web tools is another criterion users should consider, as a vast majority of websites do not support designing gRNAs for plants. Fortunately, some websites, such as CRISPy-Web [89], allow users to design gRNAs using user provided genomes. The diversity of supported Cas enzymes is another important factor. Not all websites support designing gRNAs for Cas proteins that recognize alternate PAMs. Prediction of on-target and off-target activity is also an important factor to consider. Some tools such as CRISPOR allow users to determine genome-wide off-target mutations, whereas others do not.

Figure 1. Different types of genetic modifications generated by CRISPR-based genome editors. (A) CRISPR-Cas nucleases. (B) Base editors. (C) Prime editor. (D) Editing mechanisms. (E) Different types of editing outcomes generated by various genome editors. This figure was created using BioRender (https://biorender.com/). Abbreviations: ABE, adenine base editor; CBE, cytidine base editor; CRISPR, clustered regularly interspaced short palindromic repeats; DSB, double-strand break; gRNA, guide RNA; HDR, homology-dependent repair; nCas, Cas nickase; NHEJ, non-homologous end-joining; PAM, protospacer adjacent motif; pegRNA, prime editing guide RNA; SSB, single-strand break; UGI, uracil DNA glycosylase.

Table 1. CRISPR-Cas nucleases used in plant genome editing

Cas nuclease	PAM	Mutation	Key features	Refs
SpCas9	NGG	WT	Highly efficient	[10,143]
SpCas9-VQR	NGA	D1135V/R1335Q/T1337R	Alternate PAM	[19,108,144]
SpCas9-EQR	NGAG	D1135E/R1335Q/T1337R	Alternate PAM	[19]
SpCas9-VRER	NGCG	D1135V/G1218R/R1335E/T1337R	Alternate PAM	[144]
SpCas9-NG	NG	R1335V/L1111R/D1135V/G1218R/E1219F/A1322R/T1337R	Highly relaxed PAM	[28,34,35,43]
iSpymacCas9	NAA	R221K/N394K	Good for A-rich site	[36]
SpCas9-HF1	NGG	N497A/R661A/Q695A/Q926A	Low off-target	[145–147]
eSpCas9	NGG	K810A/K1003A/R1060A	Low off-target	[145–147]
HypaCas9	NGG	N692A/M694A/Q695A/H698A	Low off-target	[147,148]
eHF1-Cas9	NGG	N497A/R661A/Q695A/K848A/Q926A/K1003A/R1060A	Low off-target	[148]
eHypa-Cas9	NGG	N692A/M694A/Q695A/H698A/K848A/K1003A/R1060A	Low off-target	[148]
HiFi Cas9	NGG	R691A	Low off-target	[149]
xCas9	NG, GAA GAT	A262T/R324L/S409I/E480K/E543D/M694I/E1219V	Low off-target Flexible PAM	[27,29,33,150]
SaCas9	NNGRRT	Natural variant	Low off-target High efficiency	[26,110]
SaCas9-KKH	NNNRRT	E782K/N968K/R1015H	Flexible PAM	[151]
St1Cas9	NNAGAAW	Natural variant	Alternate PAM	[26]
ScCas9	NNG	Natural variant	Flexible PAM	[152]
XNG-Cas9		R1335V/A262T/R324L/S409I/E480K/E543D/M694I/L1111R/ D1135V/G1218R/E1219V/E1219F/A1322R/T1337R	Highly relaxed PAM	[153]
SpRY	NGD, NAN	D1135L/S1136W/G1218K/E1219Q/R1335Q/T1337R	Highly flexible PAM	[38,39,41]
SpG	NG	D1135L/S1136W/G1218K/E1219Q/R1335Q/T1337R	Highly flexible PAM	
SpCas9-NRRH	NRRH	I322V/S409I/E427G/R654L/R753G/R1114G/D1135N/V1139A/ D1180G/E1219V/Q1221H/A1320V/R1333K	Flexible PAM	[41]
SpCas9-NRCH	NRCH	I322V/S409I/E427G/R654L/R753G/R1114G/D1135N/E1219V/ D1332N/R1335Q/T1337N/S1338T/H1349R	Flexible PAM	[41]
SpCas9-NRTH	NRTH	I322V/S409I/E427G/R654L/R753G/R1114G/D1135N/D1180G/ G1218S/E1219V/Q1221H/P1249S/E1253K/P1321S/D1322G/R1335L	Flexible PAM	[41]
AsCas12a	TTTV	Natural variant	T-rich PAM	[154,155]
LbCas12a	TTTV	Natural variant	T-rich PAM	[154,156]
LbCas12a-RR	TYCV, CCCC	G532R/K595R	Alternate PAM	[157,158]
LbCas12a-RVR	TATV	G532R/K538V/Y542R	Alternate PAM	[157,158]
FnCas12a-RVR	TATG	N607R/K613V/N617R	Alternate PAM	[158]
enLbCas12a	TTTV	D156R/G532R/K538R	Temperature tolerant	[156]
ttLbCas12a	TTTV	D156R	Temperature tolerant	[156,159]
AacCas12b	VTTV	Natural variant	Temperature tolerant	[160,161]
AaCas12b	VTTV	Natural variant	High efficiency	[160]
BthCas12b	ATTN	Natural variant	T-rich PAM	[160]
BhCas12b v4	ATTN	Natural variant	T-rich PAM	[162]
BvCas12b	ATTN	Natural variant	T-rich PAM	[162]
Lb5Cas12a	TTTV	Natural variant	T-rich PAM	[127]
BsCas12a	TTTV	Natural variant	T-rich PAM	[127]
Mb2Cas12a	TTV	Natural variant	T-rich PAM	[127]

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Table 1. (continued)

Cas nuclease	PAM	Mutation	Key features	Refs
TsCas12a	TTTV	Natural variant	T-rich PAM	[127]
MlCas12a	TTTV	Natural variant	T-rich PAM	[127]
BoCas12a	TTTV	Natural variant	T-rich PAM	[127]
MbCas12a	TTTV	Natural variant	T-rich PAM	[127]
Mb2Cas12a-RVR	TATV	N563R/ K569V/ N573R	Alternate PAM	[127]
Mb2Cas12a-RVRR	TTTV, TTV, TATV, TYCV, CCCV, CTCV	N563R/ K569V/ N573R/K625R	Flexible PAM	[127]

The planned downstream experiments are also an important factor when designing and/or selecting gRNAs. For example, when a gRNA is expressed using a *U6* and *U3* promoter, transcription is greatly enhanced if 'G' or 'A' is the first base of the gRNAs, respectively. Some tools (e.g., CRISPR-P) automatically design gRNAs starting with 'G' for the *U6* promoter or 'A' for the *U3* promoter. Some software also allows users to design gRNAs that either destroy or generate a restriction enzyme following editing, which is useful for rapid screening of editing events. Considering these features, we recommend different design programs, depending on editing purposes: CRISPOR [90], CRISPR-P [91], RGEN Cas designer [92], or CHOPCHOP [93] for CRISPR-Cas nucleases, RGEN BE-Designer [94] or PnB designer [95] for base editors, and PrimeDesign [96], pegFinder [97], or PlantPegDesigner [75] for prime editors. Interestingly, pegRNA efficiency can now be predicted in human cells using the Deep-PE [98] program. No such program, however, is available for plants yet. Although gRNAs are usually designed using a software, it is not uncommon that an experienced user may design gRNAs manually, tailored

Box 1. Base editor and prime editor: structure and mode of action

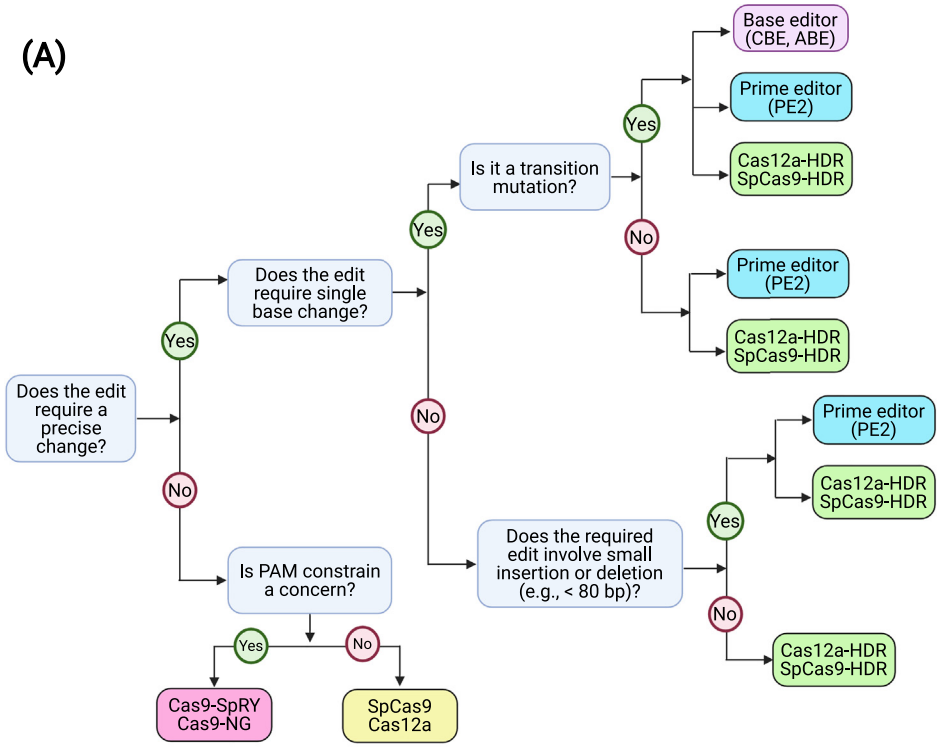
Base editors

CBEs were first developed by fusing a deaminase of rat apolipoprotein B mRNA editing enzyme (rAPOBEC1) to the N terminus of a catalytically dead Cas9 (D10A and H840A, dCas9) to create C to U conversions, and U is later recognized as T during DNA repair or replication. This system is referred to as BE1. BE2 incorporates a uracil-DNA glycosylase inhibitor (UGI) domain to the C terminus of dCas9 to inhibit the activity of uracil DNA glycosylase (UDG) in the base excision repair pathway, thus preventing the deaminated bases being converted back to the original bases. BE3 further replaces the dCas9 with a Cas9 nickase (D10A, nCas9) to nick the nonedited strand and promote DNA repair using the edited strand as the template [48]. By fusing an extra UGI in BE4, the product purity has been improved [138,139]. To optimize base editing efficiency in plants, several deaminases have been investigated. Higher editing efficiency has been observed using *Petromyzon marinus* cytosine deaminase 1 (PmCDA1) than with rAPOBEC1 in rice [126]. In addition, efficient base editing has been demonstrated in rice and tomato using PmCDA1 fused to the C terminus of nCas9 [140]. Other deaminases that have been used to achieve high editing efficiency include APOBEC3A in wheat, rice, and potato (*Solanum tuberosum*) [141], as well as hAID*Δ (a human AID variant lacking a nuclear export signal) in rice [142].

Prime editors

The first generation of prime editor (PE1) consists of: (i) a Moloney murine leukemia virus reverse transcriptase (RT) fused to the C terminus of a Cas9 (H840A) nickase with a flexible linker; (ii) a prime editing guide RNA (pegRNA) containing a gRNA, a PBS, and an **RT template** harboring the desired edit sequence. When Cas9 nickase generates a nick at the target site, the 3′-end of the nicked DNA strand will hybridize with the PBS and initiate reverse transcription of the RT template, resulting in equilibration between the 3′ flap containing edits and the unedited 5′ flap. DNA edits can be incorporated by removing the 5′ flap and ligating the 3′ flap, followed by repair of the heteroduplex DNA [64]. To improve the editing efficiency of PE1, PE2 has been developed by introducing five mutations into RT. Furthermore, one gRNA is included in PE3 to nick the nonedited strand so that the edited strand can be used as the template for repair of the heteroduplex DNA. Editing efficiency is further improved using PE3 in mammalian cells. To decrease the formation of DSBs due to the double nicks in PE3, gRNA can be designed to only match the non-edited strand instead of the original sequence. This approach is termed PE3b [64].

(A)



(B)

Transformed cell and/or tissue type	Types of plant	Promoter	Terminator
Callus or somatic	Monocotyledonous	Rice or maize *UBQ*, rice *ACT*	Pea *rbcS-E9*
	Dicotyledonous	Arabidopsis or parsley UBQ	Pea *rbcS-E9*
Germline or gametic	Monocotyledonous Dicotyledonous	*RPS5a, YAO, EC1, DMC1*	Pea *rbcS-E9*

Trends in Plant Science

Figure 2. Guidance on the selection of appropriate genome editors (A) and their regulatory parts (B) for various genome editing needs. This figure was created using BioRender (https://biorender.com/). Abbreviations: ABE, adenine base editor; ACT, actin; CBE, cytidine base editor; HDR, homology-dependent repair; PAM, protospacer adjacent motif; PE2, prime editor 2; *UBQ*, ubiquitin.

for specific needs, such as easy detection of edits by restriction fragment length polymorphism analysis. A guideline on gRNA design for different types of CRISPR-based genome editors and selection of appropriate promoters to express them is outlined in Figure 3. A comparison of the most used gRNA design software for different types of CRISPR editor is given in Table 2.

Choosing GREs to express Cas proteins and gRNAs

The expression levels of Cas proteins and gRNAs significantly influence the outcome of CRISPR/Cas-mediated genome editing. High-level expression improves editing efficiency, whereas lower level reduces efficacy [14–16,19,99–101]. Therefore, promoters with strong and constitutive expression patterns are usually used to express the *Cas* gene and gRNA(s) in plants. The most widely used promoters to express the *Cas* gene in plants are promoters isolated from plant

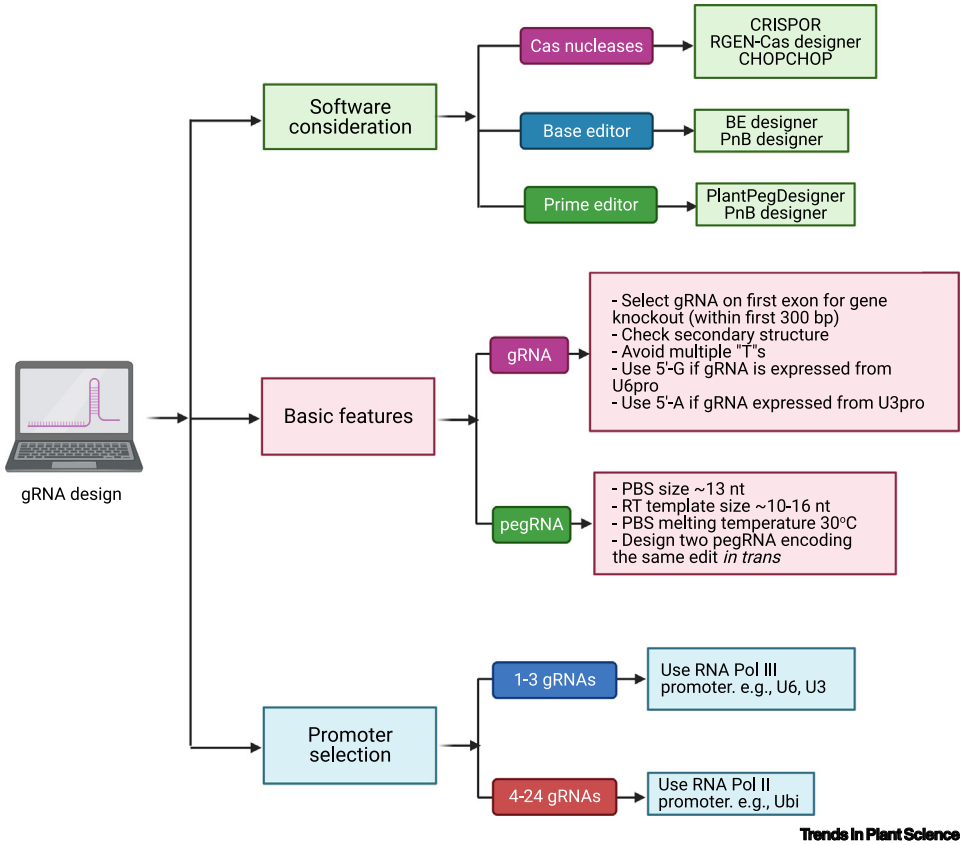


Figure 3. Guidance on the design of gRNAs for different types of CRISPR-based genome editors. This figure was created using BioRender (https://biorender.com/). Abbreviations: CRISPR, clustered regularly interspaced short palindromic repeats; gRNA, guide RNA; nt, nucleotide; PBS, primer binding site; pegRNA, prime editing guide RNA; pro, promoter; RT, reverse transcriptase.

microbes (e.g., *CaMV*, *NOS*) or housekeeping genes (e.g., *UBIQUITIN, ACTIN*). Some studies reported that Cas protein expressed from constitutive promoters isolated from the plant housekeeping genes such as *UBIQUITIN* or *ACTIN* results in higher mutation rates in both monocots and dicots in comparison with the viral constitutive promoters, such as *CaMV35S* [19,101–108]. Constitutive promoters, however, are not always a good choice. In some cases, tissue-specific, inducible, or developmentally regulated promoters provide a better option. For example, egg cells in arabidopsis (*Arabidopsis thaliana*) can be transformed via floral dip. Widely used *CaMV* or *UBIQUITIN* promoters do not perform well in egg cells. In these cases, the use of egg cell, ovule, or meiotic cell-specific promoter, such as *YAO* or *EC1* [103,109,110], significantly improves editing efficiency in germline cells. Tissue-specific, inducible, or developmentally regulated promoters have also been highly effective when CRISPR reagents are delivered to plant cells via an *Agrobacterium*-mediated tissue culture system. One recent report [101] showed that maize *DMC1* promoter was highly active in the callus tissue and expression of the *Cas* gene under this promoter produced ~66% biallelic or homozygous mutants. Other studies [111,112] have also reported significant improvement in editing outcomes when a specific rather than a constitutive promoter was used.

Unlike Cas proteins, choosing a promoter to express gRNAs is more straightforward. Most frequently, RNA polymerase III promoters, such as promoters of small nuclear RNA (snRNA) genes

Table 2. Comparison of various gRNA design software for different types of CRISPR/Cas-based genome editors

Software	Supported enzyme	Supported editor	Input	Off-TAS[a]	On-TAS	Allow automated primer design for			URL	Refs
						gRNA cloning	On target MS	Off-target MS		
CRISPOR	Almost all Cas9, Cas12a, and their variants	CRISPR-Cas nucleases	DNA sequence,genomic coordinates	Yes	Yes	Yes	Yes	Yes	http://crispor.tefor.net/	[90]
CHOPCHOP	Cas9, Cas12a, Cas13	CRISPR-Cas nucleases	DNA sequence,genomic coordinatesgene IDRefSeq	Yes	Yes	No	Yes	No	https://chopchop.cbu.uib.no/	[93]
CRISPR-P	Almost all Cas9, Cas12a, and their variants	CRISPR-Cas nucleases	DNA sequence,genomic coordinatesgene ID	No	Yes	Yes	No	No	http://crispr.hzau.edu.cn/cgi-bin/CRISPR2/CRISPR	[91]
RGEN-Cas designer	Almost all Cas9, Cas12a, and their variants	CRISPR-Cas nucleases	DNA sequence	Yes	Yes	No	No	No	http://www.rgenome.net/cas-designer/	[92]
PE-Designer	Cas9-NGG Cas9-NG Cas9-VQR Cas9-VRER	Primer editor	DNA sequence	Yes	No	No	No	No	http://www.rgenome.net/pe-designer/	[163]
pegFinder	Cas9-NGG Cas9-NG Cas9-SpRY	Prime editor	DNA sequence	No	No	Yes	No	No	http://pegfinder.sidichenlab.org/	[164]
PnB Designer	Cas9-NGG	Base editorPrime editor	DNA sequence,genomic coordinates	No	No	No	No	No	https://fgcz-shiny.uzh.ch/PnBDesigner/	[95]
BE Designer	Cas9 and its variants	Base editor	DNA sequence,genomic coordinates	Yes	No	No	No	No	genome.net/be-designer/	[94]
PlantPegDesigner	Cas9-NGGCas9-NG	Prime editor	DNA sequence	No	No	Yes	No	No	http://www.plantgenomeediting.net/	[75]

[a]Abbreviations: MS, mutation screening; TAS, target activity scoring.

U6/U3, are used to express gRNAs in plants. Occasionally, when multiple gRNAs are expressed from one promoter, an RNA polymerase II promoter such as *CmYLCB* [113] can also be used. When choosing *U6/U3* promoters, priority should be given to endogenous *U6/U3* promoters because this may result in better editing outcomes, as demonstrated in different plants [18,114]. It should be noted that multiple versions of *U6/U3* promoters are available. Their expression patterns might be different and therefore users need to identify the appropriate variants for their experiments.

In addition to promoters, terminators can also influence Cas and gRNA transcripts' stability, thus affecting the editing efficiency of CRISPR/Cas systems [104,109]. The gRNA stability is an important factor affecting the efficiency of CRISPR systems [8,115]. In arabidopsis, it has been found that terminators are a key factor that stabilizes the Cas9 mRNA in plant germline cells (e.g., egg cells) [102,104,109]. Variation in the stability of the Cas9 mRNA results from the use of different terminators [109]. It is thought that a weak terminator in the Cas9 transcription unit allows RNA Pol II readthrough, which could interfere with RNA Pol III-mediated transcription of gRNAs when both expression cassettes are oriented in the same direction [104]. There are two ways to overcome this problem: (i) use of a strong terminator for the Cas9 expression cassette, so that the possibility of a RNA Pol II readthrough is minimized; and (ii) placing the gRNA and Cas9 expression cassettes in the opposite (head-to-head) direction [104]. Several studies have systematically evaluated the effect of different terminators on the efficiency of the CRISPR/Cas9 system in plants and concluded that the best terminator for Cas9 expression cassette in plants is the *rbcS-E9* terminator from *Pisum sativum* [102,104,109]. A list of promoters and terminators that have been shown to improve the editing efficiency in plants are listed in Table 3.

Codon optimization of *Cas* genes and the use of intron

The different variants of the same *Cas* gene with **codon optimization** for the target plant species have been found to generate high mutation rates compared with the non-codon optimized control [14,116,117]. Codon optimization affects the stability and/or splicing pattern of Cas mRNA and, consequently, the amount of functional Cas protein in cells. Codon optimization of *Cas* genes might be needed for each host species. Genes in some genomes might consist of protein-coding sequences that are either AT- or GC-rich, based on codon preference. For example, plant genes in the Gramineae family usually have higher GC content at the 5′ region of their open reading frames [118]. This occurrence suggests that heterologous expression in the host of Gramineae family may require codon optimization (i.e., mimicking the natural codon preference of endogenous genes). Such an approach was applied in rice [106], which showed significant improvement in editing efficiency resulting from codon-optimization of Cas9 according to the average codon frequencies in a large number of monocot plant species. While many *Cas* genes used in plants do not have introns, introducing introns may help improve *Cas* gene expression. For example, the insertion of multiple introns into a maize codon-optimized Cas9 (zCas9) drastically improved genome editing efficiency in arabidopsis [119].

Design of multiplex CRISPR constructs

Many applications, such as genome engineering for rewiring metabolic pathways and promoter editing for introducing quantitative trait or **directed evolution**, often require editing multiple sites in one targeted region or multiple targeted regions in a genome simultaneously. When different members of a gene family are targeted, it might be possible to design one gRNA targeting multiple conserved sites. Genome editing, however, often deals with multiple genes from different families that do not share highly conserved regions. This requires an alternative approach to simultaneously edit multiple target sites using multiple gRNAs, which is known as multiplex CRISPR-based genome editing. The straightforward way to achieve multiplex genome editing is stacking individual gRNA transcription units driven by either *U6* or *U3* promoter together in one construct, which

Table 3. A list of gene regulatory elements (GREs) commonly used in plant CRISPR constructs

Name of GRE	Source	Type of GRE	Function/uses	Tested plant	Refs
GhU6.3	Cotton	Promoter	To express sgRNA	Cotton	[18]
Ghu6.7	Cotton	Promoter	To express sgRNA	Cotton	[161]
StU6	Potato	Promoter	To express sgRNA	Potato	[165]
OsU6a	Rice	Promoter	To express sgRNA	Rice and many other monocot plants	[106]
OsU6b	Rice	Promoter	To express sgRNA	Rice	[106]
OsU6c	Rice	Promoter	To express sgRNA	Rice	[106]
TaU6	Wheat	Promoter	To express sgRNA	Wheat	[166]
TaU3	Wheat	Promoter	To express sgRNA	Wheat, maize	[167,168]
AtU6-26	Arabidopsis	Promoter	To express sgRNA	Arabidopsis and many other dicot plants	[104]
AtU6-29	Arabidopsis	Promoter	To express sgRNA	Arabidopsis	[168]
AtU6-1	Arabidopsis	Promoter	To express sgRNA	Arabidopsis	[168]
Sl-U6	Tomato	Promoter	To express sgRNA	Tobacco	[128]
Sl-U3	Tomato	Promoter	To express sgRNA	Tobacco	[128]
CsVMV	Plant virus	Promoter	To express Cas9	Barley	[169]
AtM24	Arabidopsis	Promoter	To express Cas9	Tomato, wheat, barley, *Medicago*	[113]
FMV 34S	Plant virus	Promoter	To express Cas9	Tomato, wheat, barley, *Medicago*	[113]
NOS	*Agrobacterium*	Promoter	To express Cas9	Tomato, wheat, barley, *Medicago*	[113]
AtUbi10	Arabidopsis	Promoter	To express Cas9	Tomato, wheat, barley, *Medicago*	[113]
PvUbi1	Switchgrass	Promoter	To express Cas9	Tomato, wheat, barley, *Medicago*	[113]
CmYLCV	Plant virus	Promoter	To express both Cas9 and sgRNA	Tomato, wheat, barley, *Medicago*	[113]
PvUbi2	Switchgrass	Promoter	To express Cas9	Tomato, wheat, barley, *Medicago*	[113]
ZmUbi	Maize	Promoter	To express Cas9	Maize and many other monocot plants	[154]
AtMGE1	Arabidopsis	Promoter	To express Cas9 in meiotic cell	Arabidopsis	[104]
AtAG	Arabidopsis	Promoter	To express Cas9 in floral meristem cell	Arabidopsis	[104]
AtICU2	Arabidopsis	Promoter	To express Cas9 in meiotic cell	Arabidopsis	[104]
CsVMV	Plant virus	Promoter	To express Cas9	Arabidopsis	[104]
AtRPS5A	Arabidopsis	Promoter	To express Cas9 in meristem cell	Arabidopsis	[104]
AtU6-26	Arabidopsis	Terminator	To terminate sgRNA transcription	Arabidopsis and many other dicot plants	[104]
AtU6-29	Arabidopsis	Terminator	To terminate sgRNA transcription	Arabidopsis	[168]
AtU6-1	Arabidopsis	Terminator	To terminate sgRNA transcription	Arabidopsis	[168]
AtCLV3	Arabidopsis	Promoter	To express Cas9 in stem cell	Arabidopsis	[103]
AtYAO	Arabidopsis	Promoter	To express Cas9 in stem cell	Arabidopsis	[103]
AtEC1.1	Arabidopsis	Promoter	To express Cas9 in egg cell	Arabidopsis	[103]
AtEC1.2	Arabidopsis	Promoter	To express Cas9 in egg cell	Arabidopsis	[103]
rbcSE9	Pea	Terminator	To terminate *Cas* gene transcription	Arabidopsis, rice	[104,109]
CaMV 35S	Plant virus	Terminator	To terminate *Cas* gene transcription	Arabidopsis	[102]
Atug7	*Agrobacterium*	Terminator	To terminate *Cas* gene transcription	Arabidopsis	[102]
NOS	*Agrobacterium*	Terminator	To terminate *Cas* gene transcription	Arabidopsis	[102]
Act2	Arabidopsis	Terminator	To terminate *Cas* gene transcription	Arabidopsis	[102]

(continued on next page)

Table 3. (continued)

Name of GRE	Source	Type of GRE	Function/uses	Tested plant	Refs
MAS	*Agrobacterium*	Terminator	To terminate *Cas* gene transcription	Arabidopsis	[102]
ATPase	Tomato	Terminator	To terminate *Cas* gene transcription	Arabidopsis	[102]
rbcSC3	Tomato	Terminator	To terminate *Cas* gene transcription	Arabidopsis	[102]
H4	Potato	Terminator	To terminate *Cas* gene transcription	Arabidopsis	[102]
rbcSE9	Pea	Terminator	To terminate *Cas* gene transcription	Arabidopsis	[102]
GILT	Arabidopsis	Terminator	To terminate *Cas* gene transcription	Arabidopsis	[102]
ALB	Arabidopsis	Terminator	To terminate *Cas* gene transcription	Arabidopsis	[102]
API	Arabidopsis	Terminator	To terminate *Cas* gene transcription	Arabidopsis	[102]
HSP	Tomato	Terminator	To terminate *Cas* gene transcription	Rice	[170]
OCS	*Agrobacterium*	Terminator	To terminate *Cas* gene transcription	Rice	[170]
OsBiP1	Rice	Promoter	Bidirectional expression of both sgRNA and *Cas* gene	Rice	[170]
DMC1	Maize	Promoter	To express Cas9 in reproductive tissue	Maize	[101]
OsUbi1	Rice	Promoter	To express Cas9	Rice	[108]
OsAct1	Rice	Promoter	To express Cas9	Rice, cotton	[108,161]
PcUbi4-2	Parsley	Promoter	To express Cas12a	Arabidopsis	[156]
OsUbi	Cotton	Promoter	To express Cas12a	Cotton	[161]
OsAct1	Rice	Promoter	To express Cas9	Rice	[113]
StIV2	Potato	Intron	Multiplexing sgRNA and *Cas* gene	Rice	[123]
OsCDPK2_1	Rice	Intron	Multiplexing sgRNA and *Cas* gene	Rice	[123]
RcCAT_1	Castor bean	Intron	Multiplexing sgRNA and *Cas* gene	Rice	[123]
OsUBI10	Rice	Intron	Multiplexing sgRNA and *Cas* gene	Rice	[171]

has been routinely used in plants [106,120]. Repeated use of a *U6* or *U3* promoter in the same construct, however, may cause variation in gRNA expression level and transgene silencing in plants [106]. Besides, increasing the number of gRNA transcription units may cause cloning difficulty due to the repetitive use of the same promoters and terminators. Further, when virus-based vectors are used to deliver the constructs, it becomes challenging to package all the components due to cargo limit of the vector [121,122]. Therefore, more compact multiplex strategies are usually preferred.

Current compact multiplex CRISPR systems can be divided into two broad categories: (i) two transcriptional unit (TTU) multiplex system (Figure 4A), and (ii) single transcriptional unit (STU) multiplex system (Figure 4B). The TTU multiplex system can be further divided into: (i) mixed dual promoter system, where the *Cas* gene is expressed from an RNA Pol II promoter and the gRNAs from RNA Pol III promoters (Figure 4Ai); (ii) dual promoter system, where both the *Cas* gene and gRNAs are expressed from two independent RNA Pol II promoters (Figure 4Aii); and (iii) a single promoter system that relies on an RNA Pol II capable of transcribing both the *Cas* gene and the gRNAs at the same time (Figure 4Aiii). In the STU multiplex CRISPR system, both the *Cas* gene and the gRNAs are under the control of one promoter and one terminator. The STU system can further be divided into two groups: (i) RNA processing enzyme-based STU (rpeSTU) and (ii) intron-based STU (iSTU) system. The STU systems offer advantages for applications that require inducible or tissue-specific expression, as well as for CRISPR-based transcriptional regulations in plants [120,123].

Precise processing and release of individual gRNAs from a polycistronic transcript is the key to the success of a multiplex CRISPR system. The polycistronic mRNA containing multiple

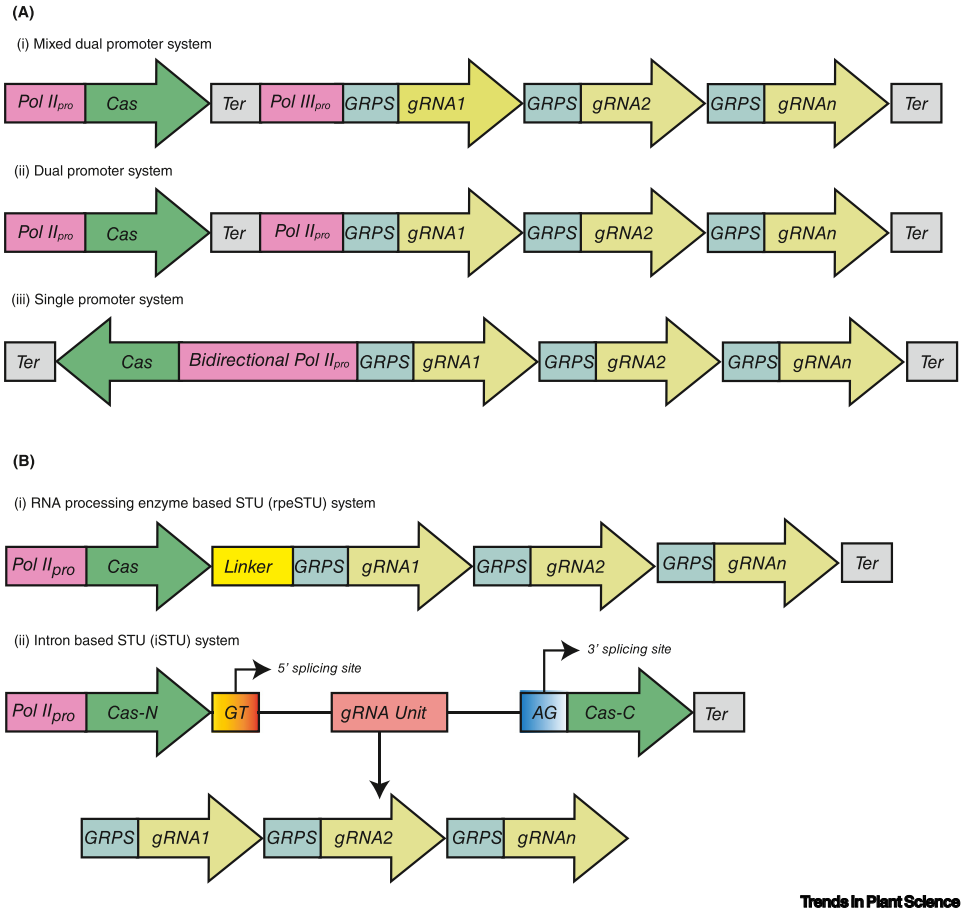


Figure 4. Different architectures of multiplex CRISPR constructs. (A) Two transcriptional unit (TTU) multiplex system. (B) Single transcriptional unit (STU) multiplex system. Here, Pol II means RNA Polymerase II promoter and Pol III means RNA polymerase III promoter; linkers mean nonfunctional DNA sequence used to connect two adjacent functional DNA elements. Abbreviations: Cas, CRISPR associated protein; Cas-C, C terminal region of Cas protein; Cas-N, N terminal region of Cas protein; CRISPR, clustered regularly interspaced short palindromic repeats; gRNA, single guide RNA; GRPS, guide RNA processing system, such as tRNA, HH-HDV, and Csy4; pro, promoter; ter, terminator.

gRNAs can be processed by RNA-cleaving enzymes post-transcriptionally into single gRNAs. Different RNA-cleaving enzymes have been shown to work for multiplex genome editing, including **HH and HDV dual ribozyme** (HH-HDV), CRISPR associated RNA endoribonuclease **Csy4** from *Pseudomonas aeruginosa*, and tRNA processing enzymes [123–125]. Among these gRNA processing systems, tRNA and Csy4 systems appear more effective for Cas9 and HH-HDV for Cas12a [113,126,127].

To date, up to 24 gRNAs have been expressed in plants from one construct, albeit at reduced efficiency [128]. It appears that the stoichiometry of the gRNA:Cas complex in the cell is important for efficient gene editing using multiplex CRISPR systems because an inappropriate concentration of each gRNA:Cas complex can lead to reduced editing events [127,128]. Although it is easy to express gRNAs at high levels using a strong ubiquitous promoter, coexistence of multiple gRNAs in a cell at the same time dilutes the concentration of each gRNA:Cas complex harboring a specific target sequence [109,129]. Thus, although the overall gRNA:Cas complex concentration may remain stable, the functional concentration of each gRNA:Cas complex variant would be

reduced in inverse proportion to the numbers of gRNA variants, each targeting a different site [109,130]. Therefore, strong ubiquitous expression of *Cas* gene is necessary to increase the functional concentration of each gRNA:Cas complex in plant cells to improve the efficiency of multiplex editing [109,131,132]. Using a dual Pol II prompter system with HH-HDV-based processing of crRNAs, 16 target sites can be simultaneously edited by Cas12a within one generation in rice [127]. A general guidance on the design of multiplex CRISPR constructs includes choosing the right: (i) Cas protein, such as SpCas9 or Cas12a for multiplex editing (Figure 1A); (ii) RNA polymerase II-based promoter to express multiplex gRNAs; and (iii) gRNA processing system based on either tRNA, Csy4, or HH-HDV enzyme.

Concluding remarks and recommendations

Over the past few years, there has been tremendous progress in CRISPR-based genome editing technologies. To date, at least 30 Cas proteins (Table 1) have been reported to edit plant genomes. The availability of the large number of Cas proteins can make it difficult to choose the best one for a desired application. The factors one should consider when choosing a CRISPR nuclease enzyme are: (i) type of edits desired, (ii) PAM requirements, (iii) target specificity, and (iv) editing efficiency. These features dictate the ease with which a researcher will be able to deliver the nuclease to plant cells and the available genomic sites that the nuclease will target (GC- versus AT-rich sites). Moreover, it is advisable to test multiple nuclease orthologs to identify the most efficient variants, particularly when this technology is applied to new plant species. gRNA design is another critical factor for CRISPR experiments. Many web tools are available for designing gRNAs. One important note here is that different web tools serve different purposes and a single tool might not fit for all desired applications; users might need to combine different tools for their intended experiments [87,133,134]. Choice of the right promoter and terminator to express the *Cas* gene and gRNAs is another critical factor that one should consider when designing CRISPR constructs. This can be challenging for researchers new to the CRISPR genome editing technology and this review is meant to provide the necessary details for the decision-making process. Apart from construct-specific factors, the delivery of genome editing constructs into plant genome is a key challenge for plant genome editing. Most plants require complicated tissue culture systems to deliver CRISPR constructs into their genomes. Tissue culture-based plant transformation is laborious and inefficient. Recent breakthroughs in plant transformation have developed some systems such as *de novo* meristem induction with plant growth factors [135], or enhanced plant regeneration with morphogenic factors such as Growth-Regulating Factor (GRF)-GRF-Interacting Factor (GIF) [136] and BABY BOOM (BBM)/WUSCHEL2 (WUS2) [137]. Further application and development of these technologies can largely overcome many of the bottlenecks in plant transformation (see Outstanding questions). Based on our analyses, we recommend the following:

- SpCas9 with NGG PAM and PAMless SpRY are the preferred nucleases for plant genome editing. Since base editing and prime editing have been predominantly demonstrated in plants with Cas9, SpCas9 and SpRY are also the primary choices over other Cas systems. Other SpCas9 variants such as Cas9-NG and SpG may have higher editing efficiencies at some NG PAM sites in plants and hence may also be considered. In plants, targeted mutagenesis using Cas9 nucleases has shown high specificity, indicating that it might not be necessary to use high-fidelity Cas9, such as SpCas9-HF1 and HypaCas9, since they usually result in low editing efficiency.
- A3A-BE3, A3A/Y130F-BE3, PmCDA1-BE3, PmCDA1-CBE_V04, and A3A/Y130F-CBE_V04 are the preferred CBEs.
- ABE8e and ABE9.0 are the preferred ABEs.
- Use of paired pegRNAs with the melting temperature of PBS at ~30°C for prime editing.

Outstanding questions

Can the development of tissue culture-independent transformation systems be improved to deliver CRISPR reagents to plant cells, particularly for non-model crop plants and tree species?

Can the prime editing systems in plants be improved?

Can transversion type base editing systems be developed?

Can the HDR-based genome editing in plants be improved?

Can the CRISPR construct design be automated?

Can the efficiency and precision of CRISPR/Cas systems be improved by using artificial intelligence and machine learning?

Can insertion or replacement of large DNA sequences in plant genomes be more effective?

Can editing of plant organelle (e.g., mitochondrion, chloroplast) genomes be more efficient?

Can technologies for tissue- or cell type-specific gene editing in plants be improved?

- *Arabidopsis thaliana UBIQUITIN 10 (UBI10)* or *35S* promoter for expressing the Cas protein in most dicotyledonous plants and maize or rice Ubi promoter to express the Cas protein in monocotyledonous plants.
- LbCas12a and Mb2Cas12a for large deletions or homologous recombination-based experiments.
- RNA polymerase II promoter that has strong and ubiquitous expression pattern should be used when multiple gRNAs are expressed from a single promoter. In this case, we do not recommend using RNA polymerase III promoters such as *U6* and *U3* promoters.
- We highly recommend testing and comparing the genome editing constructs and different gRNAs in a protoplast system as a prescreen step, especially in a plant species that is difficult to transform or requires a complicated tissue culture process for transformation.
- We also suggest considering testing the improved plant transformation systems with the use of effective morphogenic factors to deliver the genome editing constructs into plant genomes.

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Author contributions

M.M.H. and X.Y. conceived the idea. M.M.H. and Y.Z. led the writing and revision of the manuscript. G.Y., K.D., W.M., J.G. C., G.A.T., Y.Q., and X.Y. contributed to the manuscript revision. All authors accepted the final version of the manuscript.

Declaration of interests

Y.Q. is a consultant for Inari Agriculture and CTC Genomics. The remaining authors declare no conflict of interests.

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