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https://doi.org/10.1007/s42994-019-00010-0

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REVIEW

CRISPR-Cas nucleases and base editors for plant genome editing

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Received: 4 June 2019 / Accepted: 2 November 2019

Abstract Clustered regularly interspaced short palindromic repeats (CRISPR)—CRISPR-associated protein (Cas) and base editors are fundamental tools in plant genome editing. Cas9 from *Streptococcus pyogenes* (SpCas9), recognizing an NGG protospacer adjacent motif (PAM), is a widely used nuclease for genome editing in living cells. Cas12a nucleases, targeting T-rich PAMs, have also been recently demonstrated in several plant species. Furthermore, multiple Cas9 and Cas12a engineered variants and orthologs, with different PAM recognition sites, editing efficiencies and fidelity, have been explored in plants. These RNA-guided sequence-specific nucleases (SSN) generate double-stranded breaks (DSBs) in DNA, which trigger non-homologous end-joining (NHEJ) repair or homology-directed repair (HDR), resulting in insertion and deletion (indel) mutations or precise gene replacement, respectively. Alternatively, genome editing can be achieved by base editors without introducing DSBs. So far, several base editors have been applied in plants to introduce C-to-T or A-to-G transitions, but they are still undergoing improvement in editing window size, targeting scope, off-target effects in DNA and RNA, product purity and overall activity. Here, we summarize recent progress on the application of Cas nucleases, engineered Cas variants and base editors in plants.

Keywords CRISPR, SpCas9, Cas12a, Cas12b, PAM, Cytidine/adenine base editors

INTRODUCTION

CRISPR (Clustered regularly interspaced short palindromic repeats)—Cas (CRISPR associated protein), derived from a bacterial innate immune system, is a leading sequence-specific nuclease (SSN) used for genome editing. The CRISPR locus is characterized by direct repeats of varying sizes (21–48 bp), separated by non-repetitive spacer sequences of defined sizes (26–72 bp), in bacterial and archaeal genomes (Jansen et al. 2002; Haft et al. 2005; Rath et al. 2015). Spacer sequences can be transcribed and processed into individual CRISPR-RNAs (crRNAs), which represent the basic component of the archaeal and bacterial adaptive immune response against invading genetic elements (e.g. phage DNA and plasmids). The second key component of CRISPR-mediated adaptive immunity is Cas proteins, encoded by *Cas* genes (Jansen et al. 2002). Cas endonucleases are expressed from a locus adjacent to the CRISPR arrays. Even though Cas proteins exhibit polymorphism within genomes, they are all known to have the ability to interact with nucleic acids. *Cas* core genes have been well characterized and shown to mainly encode nucleases, helicases or RecB-family exonucleases (Haft et al. 2005). Based on the signature *Cas* genes and the organization of the CRISPR loci, the CRISPR-Cas system is

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Published online: 30 November 2019

classified into two classes and six types (Type I to Type VI), each of which has several subtypes (~ 20 subtypes) (McGinn and Marraffini 2019). While CRISPR systems of types I, III and IV (Class 1) involve multiple Cas proteins to degrade DNA/RNA, Type II, V and VI (Class 2) require single multifunctional proteins to achieve nucleic acid degradation (Jiang and Doudna 2015).

Streptococcus pyogenes Cas9 (SpCas9), the first well-characterized endonuclease from Class 2 Type II-A CRISPR system, has been widely used for genome editing in various organisms, including plants. SpCas9 assembles with a single-guide RNA (sgRNA), which is engineered by fusing crRNA and trans-activating crRNA (tracrRNA) (Jinek et al. 2012). Ribonucleoprotein complex then recognizes and binds targeted DNA sequences followed by a 5′-NGG-3′ PAM. SpCas9 cleaves DNA, resulting in blunt-ended double-stranded breaks (DSBs). SpCas9 is a multidomain protein that forms two lobes, a recognition (REC) lobe and a nuclease (NUC) lobe (Nishimasu et al. 2014). The REC lobe is responsible for sgRNA and DNA binding, while the HNH and RuvC nuclease domains of the NUC lobe cleave the complementary (targeting) and non-complementary (non-targeting) strands of DNA, respectively. The PI domain (PAM-interacting domain) of NUC lobe is essential for the recognition of the PAM sequence. But PAM requirement also reduces the number of potential target sites in a genome. To address this limitation, Cas9 orthologs or engineered Cas9 proteins recognizing non-canonical PAMs have been explored.

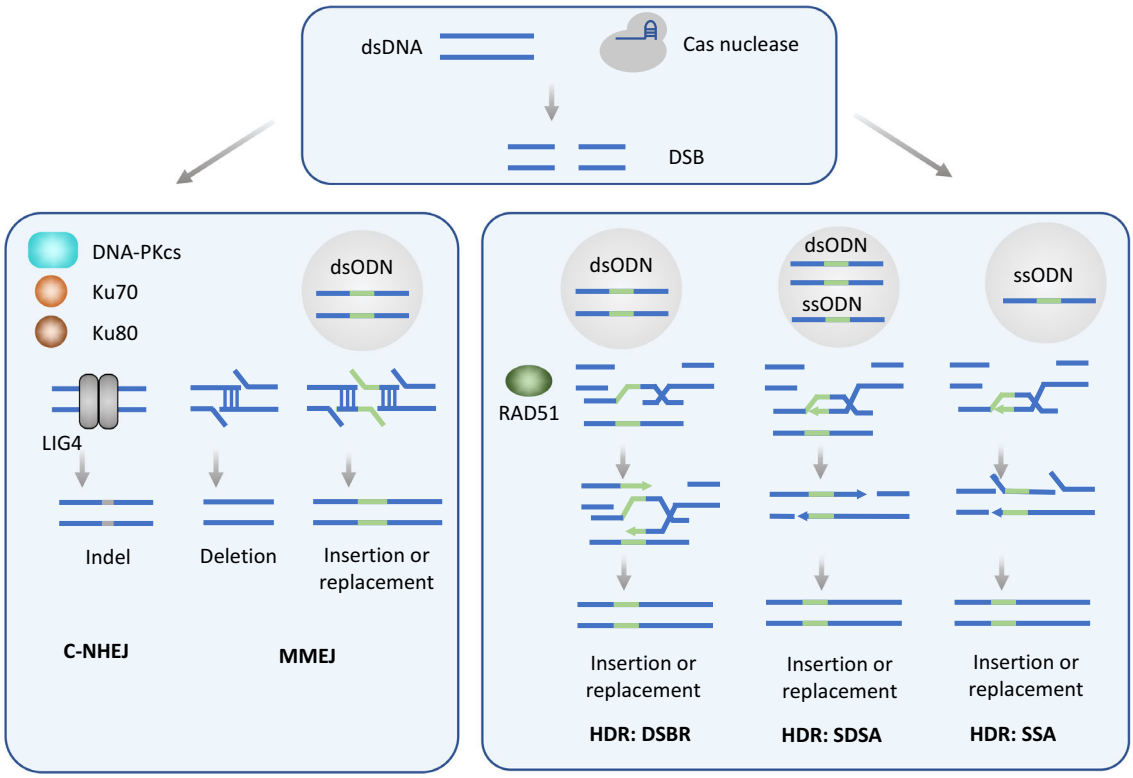
DNA repair pathways are triggered upon the formation of DSBs induced by Cas endonucleases (Fig. 1). Two major DSB repair pathways are the non-homologous end joining (NHEJ) pathway and the homology directed repair (HDR) pathway. The classical (or canonical) NHEJ (C-NHEJ) pathway is the predominant mechanism, which joins two broken DNA ends (Lieber 2010). Repair by C-NHEJ is error-prone and can result in small deletions or insertions at the junction, often causing a reading frame shift and the disruption of gene function. In the alternative NHEJ pathway, also known as the microhomology-mediated end joining (MMEJ) pathway, 5–25 bp microhomology sequences at DSB sites lead to the annealing of broken DNA ends before the ligation. MMEJ may also allow the insertion of desired double-stranded oligodeoxynucleotides at the DSB site (Fig. 1A) (Waterworth et al. 2011). In contrast to NHEJ, HDR pathway requires a template with homology arms for the repair process (Puchta 2004). Homologous recombination is restricted to the S and G2 phases of the eukaryotic cell cycle and is essential for sister chromatid exchange, while NHEJ remains active throughout the cell cycle. At the beginning of HDR (pre-synapsis stage) end resection of DNA surrounding the DSB is initiated by MRN (Mre11–Rad50–Nbs1) complex including RAD50 protein to produce 3′ ssDNA (single-stranded DNA) overhangs, subsequently covered by proteins for stabilization (Waterworth et al. 2011). RAD51 and its paralogs are essential in the HDR repair. Assembly of the RAD51 nucleoprotein searches for DNA homology and mediates DNA strand invasion, which leads to DNA synthesis from the 3′-end of the invading strand followed by DNA ligation (Holthausen et al. 2010; Dexheimer 2013). Although multiple mechanisms for HDR repair have been proposed, three of them are well-known: DSBR (Double-Strand Break Repair), SDSA (Synthesis-Dependent Strand Annealing) and SSA (Single-strand annealing) (Fig. 1A) (Waterworth et al. 2011). HDR allows precise genome editing, including gene insertions. Genome editing is a consequence of the SSN (e.g. CRISPR-Cas) cleavage and DNA repair. While we acknowledge the importance of DNA repair pathways in genome editing (Fig. 1), few studies have explored DNA repair pathways for enhancing genome editing efficiencies and manipulating editing outcomes in plants (Qi et al. 2013; Endo et al. 2016a) and thus this topic will not be a focus.

Base editing is another CRISPR-mediated genome engineering method that enables direct, irreversible conversion of one base pair to another without the need for introducing DSBs or template DNA (Komor et al. 2016; Nishida et al. 2016; Gaudelli et al. 2017). Base editors, such as Cytosine base editors (CBEs) and Adenine base editors (ABEs), are mainly composed of a deaminase fused to a Cas9 variant [nickase (nCas9) or catalytically deactivated Cas9 (dCas9)]. Base editors allow the generation of precise mutations, including the introduction of single nucleotide polymorphisms (SNPs), alternative splicing sites and stop codons. The application of base editing in plants will undoubtedly facilitate basic research and plant breeding. In this mini-review, we will summarize recent developments of CRISPR-Cas technology in plant genome editing with a focus on Cas nucleases and base editors.

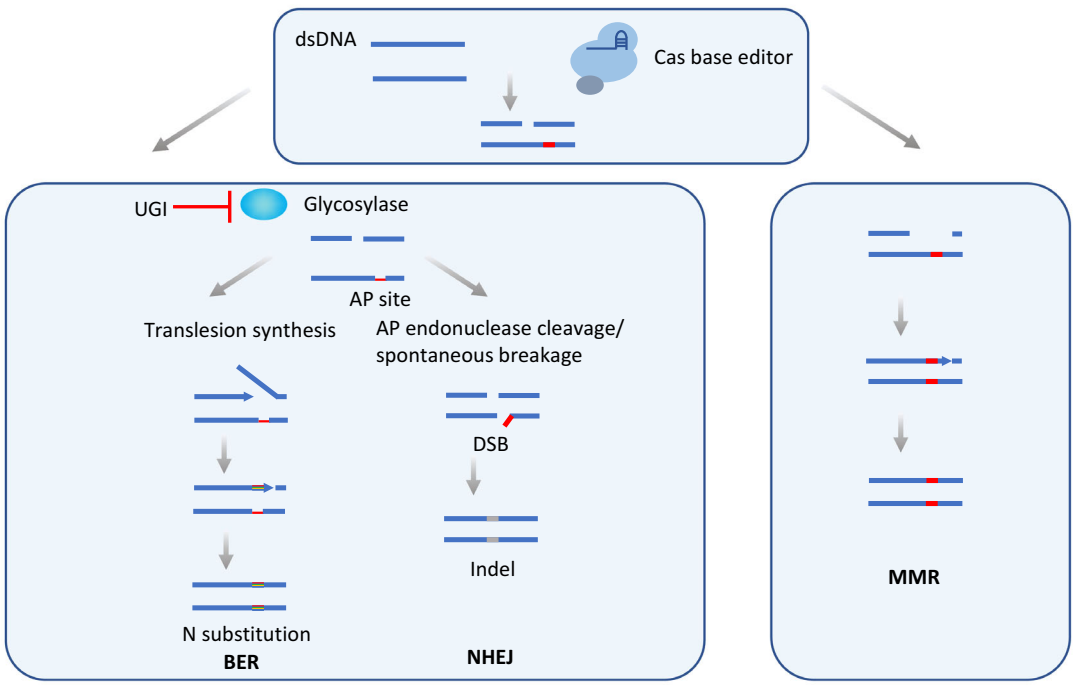
TARGETING ALTERED PAMS WITH CAS9 ORTHOLOGS AND ENGINEERED VARIANTS

The PAM (5′-NGG-3′) requirement is a limitation in the utility of SpCas9 endonuclease. Interestingly, it was demonstrated in rice that SpCas9 could also recognize the 5′-NAG-3′ PAM (Meng et al. 2018). To enable targeting of alternative PAMs, the PI domain of wild-type SpCas9 has been mutated, resulting in the generation of

A DNA DSB repair pathways



B DNA mismatch and nick repair pathways



◀**Fig. 1** DNA repair pathways involved in CRISPR-mediated genome editing. **A** DNA DSB (double-strand break) repair pathways. Two major repair pathways after a DSB formation induced by Cas nuclease: Classical (canonical) NHEJ (C-NHEJ) pathway is mediated by Ku70/Ku80 heterodimer, DNA-PKcs (DNA protein kinase catalytic subunit), holoenzyme and LIG4/XRRC4/XLF complex. NHEJ may result in small deletions or insertions (indels). Alternatively, MMEJ (microhomology-mediated end joining) can result in longer deletions, as well as gene replacement or insertion in the presence of a donor. HDR pathway occurs at low frequency and needs templates with homology arms. dsODNs (double-stranded oligodeoxynucleotides) or ssODNs (single-stranded oligodeoxynucleotides) may be used as templates to insert or replace DNA fragments using three types of HDR repair, including DSBR (Double-Strand Break Repair), SDSA (Synthesis-Dependent Strand Annealing) or SSA (Single-strand annealing). **B** DNA mismatch and nick repair pathways. The third generation of cytidine base editors (BE3) generates base changes at one strand and a nick in another strand of the DNA. To repair DNA mismatch, the changed base is cleaved and leaves an abasic (AP) site. AP site formation by DNA glycosylases will lead to BER (Base excision repair) or NHEJ. BER uses the strand harboring the AP site as the template to repair the nick, leading to random single nucleotide (N) substitutions. DSBs can be generated through AP endonuclease cleavage or spontaneous breakage, thus triggering the NHEJ repair pathway. The use of uridine glycosylase inhibitor (UGI) is favorable to block AP site formation and shift to MMR (Mismatch repair) pathway, which is a conserved post-replicative repair mechanism that uses the edited strand as the template to repair the nick (on the right)

PAM-altered SpCas9 variants with amino acid substitutions, namely VQR, EQR and VRER (Kleinstiver et al. 2015a). VQR, EQR and VRER recognize 5′-NGAN-3′, 5′-NGAG-3′ and 5′-NGCG-3′ PAMs, respectively. In rice, the SpCas9 variants VQR and VRER have been demonstrated to recognize 5′-NGA-3′ and 5′-NGCG-3 PAM sequences, respectively (Hu et al. 2016, 2018a).

Recently, xCas9 was developed by directed evolution to expand the range of PAM recognition and to reduce off-target binding in mammalian cells (Hu et al. 2018b). xCas9 recognizes 5′-NG-3′, 5′-GAA-3′ and 5′-GAT-3′ PAM sequences. To evaluate xCas9 in plants, Wang et al. (2019) generated two variants (xCas9 3.6 and xCas9 3.7) of xCas9 using the rice codon optimized SpCas9 and tested them in rice, targeting 63 genomic sites. xCas9 recognized 5′-GAA-3′ and 5′-NG-3′ PAMs in the rice genome with a relatively low targeting efficiency compared to the canonical 5′-NGG-3′ PAM. According to recent studies in rice, xCas9 had weak or no targeting activity at 5′-NGH-3′ (H = A, T and C) PAM sites (Ren et al. 2019). Similarly, xCas9 was found to have lower editing efficiency at 5′-NGH-3′ sites, while possessing similar activity to SpCas9 at the canonical 5′-NGG-3′ PAM (Zhong et al. 2019). Hua et al. (2019a) also showed induced mutations at 5′-NG-3′ and 5′-GAT-3′ PAM sites in rice by xCas9, albeit with low activity.

Nishimasu et al. (2018) reported another engineered variant of SpCas9 (SpCas9-NG) recognizing 5′-NG-3′ PAMs, which showed higher cleavage kinetics than xCas9 by in vitro assays and higher editing activity in human cells. SpCas9-NGv1 has been demonstrated to target 5′-NG-3′ PAMs in *Arabidopsis* and rice with enhanced specificity (Endo et al. 2019). SpCas9-NG was shown to successfully edit rice genomic sites with 5′-NGN-3′ PAMs, for which wild-type SpCas9 had little activity (Ren et al. 2019; Hua et al. 2019a; Zhong et al. 2019). However, both SpCas9-NG and its variant SpCas9-NGv1 had lower activity targeting 5′-NGG-3′ PAM sites than wild-type SpCas9 (Zhong et al. 2019). Furthermore, SpCas9-NG showed better editing rates at AT-rich PAM sites (5′-GAT-3′, 5′-GAA-3′, 5′-CAA-3′) than xCas9 (Zhong et al. 2019). More recently, genome editing with xCas9 and Cas9-NG, albeit with low efficiency, was also reported in *Arabidopsis* (Ge et al. 2019).

Cas9 endonucleases obtained from different bacterial species (e.g. *Staphylococcus, Francisella, Brevibacillus*) likewise recognize different PAMs. Ran et al. (2015) characterized the Cas9 ortholog from *Staphylococcus aureus* (SaCas9) and defined its 5′-NNGRRT-3′ PAM site. A SaCas9 variant, referred to as KKH SaCas9, was engineered to recognize the 5′-NNNRRT-3′ PAM (Kleinstiver et al. 2015b). SaCas9 showed a comparable editing efficiency to SpCas9 in *Arabidopsis*, rice and tobacco (Steinert et al. 2015; Kaya et al. 2016). Both SaCas9 and its KKH variant have been shown to induce high mutagenesis frequencies in rice (Qin et al. 2019). In another study, a 1092 aa Cas9 ortholog was identified in *Brevibacillus laterosporus* (BlatCas9), with the PAM preference of 5′-NNNNCNDD-3′ (D = A, G or T) (Karvelis et al. 2015). BlatCas9 was able to achieve genome editing in maize cells (Karvelis et al. 2015). St1Cas9 from *Streptococcus thermophilus* was identified with a PAM requirement of 5′-NNAGAAW-3′ (W = A or T) and was shown to efficiently induce mutations in *Arabidopsis* at frequencies comparable to SpCas9 (Esvelt et al. 2013; Steinert et al. 2015).

In addition, multiple Cas9 orthologs have been used for genome editing in mammalian systems, but not in plants. The Cas9 from *Neisseria meningitidis* (NmCas9), recognizing a 5′-NNNNGATT-3′ PAM, was shown to mediate efficient genome editing in human cells (Hou et al. 2013). Hirano et al. (2016) characterized the high-resolution structure of the largest Cas9 protein (FnCas9; ∼ 1629 aa) from *Francisella novicida* with the PAM of 5′-NGG-3′. A triple mutant of FnCas9 referred to as RHA was obtained in the same study, which has a PAM requirement of 5′-YG-3′. In addition, Cas9 from *Streptococcus canis* (ScCas9) can target a 5′-NNG-3′ PAM in both bacterial and human cells (Chatterjee et al.

2018). More recently, the PAM recognition domain of *Streptococcus macacae* Cas9 (Smac Cas9) and the nuclease domain of *Streptococcus pyogenes* (Spy Cas9) were fused to generate SpyMacCas9, which had high editing activity at 5′-NAA-3′ PAM sites (Jakimo et al. 2018). These Cas9 orthologs and engineered variants are likely to further expand targeting scope in plants (Fig. 2).

SPCAS9 VARIANTS WITH IMPROVED TARGETING SPECIFICITY

Cas9 may have off-target effects when used in genome editing and such off-target activities could be reduced by protein engineering. A number of single amino acid mutants of SpCas9 were generated by Slaymaker et al. (2016) and two of them, SpCas9(K855A) and eSpCas9(1.1), have been shown to reduce genome-wide off-target activity. The authors observed that neutralization of positively charged groups in SpCas9 led to a dramatic reduction in unwanted indel formation. It was proposed that wild-type Cas9 possesses more Gibbs free energy than is needed for optimal recognition of the target site and thus will be prone to off-target activity. Similarly, a triple mutant referred to as SpCas9-HF1 (R661A/Q695A/Q926A) has been engineered, which retained its on-target activities (90–140% editing efficiencies of wild type Cas9), while dramatically reducing off-target effects (Kleinstiver et al. 2016). It was found that both SpCas9-HF1 and eSpCas9(1.1) were very sensitive to a mismatch at the 5′ end of the protospacer, and only perfectly matched protospacers (e.g. through precise ribozyme processing) resulted in high on-target activities (Kim et al. 2017a).

Both eSpCas9 and SpCas9-HF1 have been tested in plants. In rice, it was found that optimal on-target activity of these high-fidelity Cas9 variants required a perfectly matched 20-nucleotide protospacer, which was achieved using a tRNA-sgRNA scaffold (Zhang et al. 2017). In *Arabidopsis*, it was also shown that a tRNA-sgRNA(m) (e.g. modified sgRNA) scaffold aided eSpCas9(1.1) mediated editing, while SpCas9-HF1 barely worked (Zhang et al. 2018). Recently, it was demonstrated in rice that xCas9 possesses higher targeting specificity than the wild-type SpCas9. However, the generally low editing activities of these high-fidelity SpCas9 variants in plants may prevent their wide use by the plant research community. It will be interesting to see whether other high-fidelity Cas9 proteins, such as

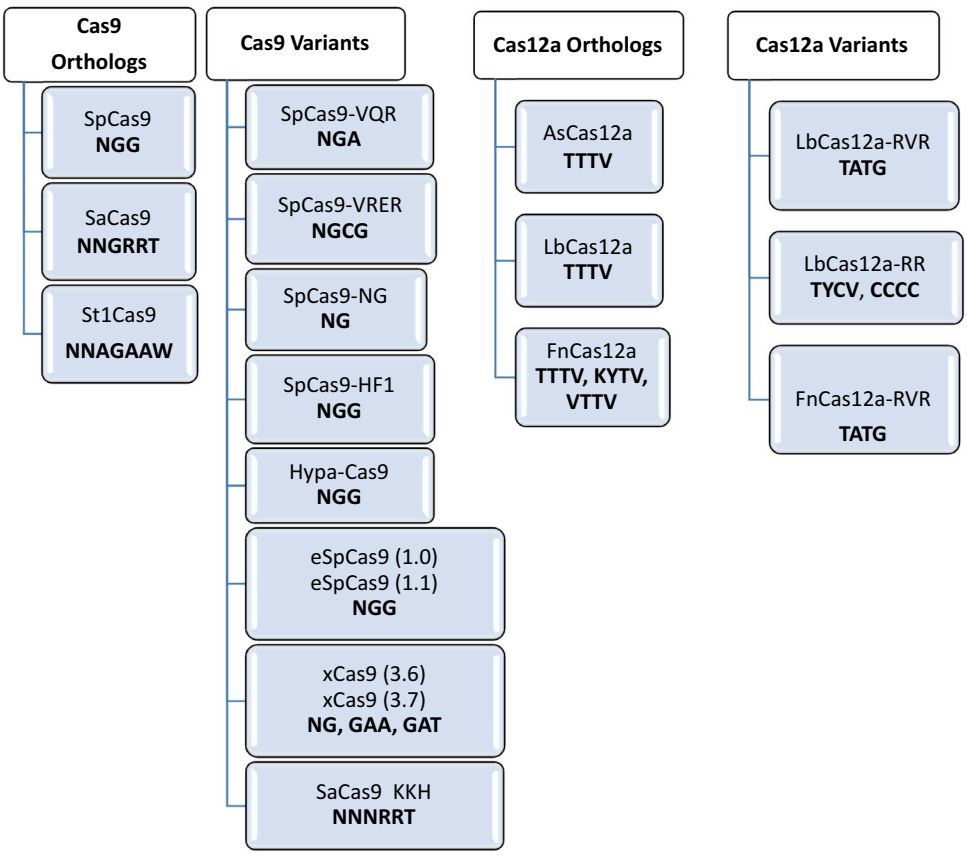


Fig. 2 PAM recognition by Cas9 and Cas12a orthologs and engineered variants used for plant genome editing. V = A, C, G; Y = T or C; R = A or G; K = G or T. *Sp*: *Streptococcus pyogenes; Sa: Staphylococcus aureus*; *St*: *Streptococcus thermophilus*; *As*: *Acidaminococcus spp. BV3L6; Fn: Francisella novicida* U112, *Lb: Lachnospiraceae bacterium* ND2006

HiFi Cas9 (Vakulskas et al. 2018) and Sniper-Cas9 (Lee et al. 2018), could enable highly efficient genome editing in plants.

CAS12A ORTHOLOGS AND VARIANTS

In 2015, a Class 2 Type V-A enzyme, referred to as Cas12a (formerly Cpf1), was discovered and characterized in detail (Zetsche et al. 2015). There are several differences between Cas12a and SpCas9. First, Cas12a-mediated genome editing only requires a crRNA (~ 43 nt), which is significantly shorter than the sgRNA of SpCas9 (~ 100 nt). Second, Cas12a requires a T-rich (5′-TTTV-3′ or 5′-TTV-3′) PAM, which is suitable for targeting AT-rich genomic regions. Third, Cas12a produces staggered ends of 4–5 bp, which may facilitate NHEJ-mediated gene replacement. Fourth, a single RuvC-like domain of Cas12a is responsible for DNA cleavage (Zetsche et al. 2015), unlike SpCas9 that has two functional catalytic domains for DNA strand cleavage. This feature makes it a challenge to generate a Cas12a nickase. Finally, Cas12a cleaves DNA in distant locations from the seed sequence, which may allow continuous cleavage of the target DNA. This feature makes Cas12a more suitable for donor insertions, as proven in Zebrafish embryos, in which a higher HDR frequency has been obtained by LbCas12a than by SpCas9 (Moreno-Mateos et al. 2017).

To date, three orthologs of Cas12a, FnCas12a (from *Francisella tularensis* subsp. *novicida U112)*, AsCas12a (from *Acidaminococcus* sp. BV3L6) and LbCas12a (from *Lachnospiraceae* bacterium ND2006), have been used in genome editing in plants, including rice, *Arabidopsis*, soybean, tobacco, maize, tomato, citrus and cotton (Endo et al. 2016b; Kim et al. 2017b; Li et al. 2018a, 2019a; Hu et al. 2017; Xu et al. 2017; Tang et al. 2017; Wang et al. 2017; Yin et al. 2017; Malzahn et al. 2019; Lee et al. 2019; Bernabé-Orts et al. 2019; Jia et al. 2019). Generally, LbCas12a and FnCas12a were shown to be more reliable in plant genome editing than AsCas12a. Elevated temperatures (e.g. 32 °C) improved AsCas12a's enzymatic activity and resulted in up to 92.8% gene editing frequency in T0 rice lines (Malzahn et al. 2019). The editing frequency is also influenced by the design of the crRNA expression cassette and has reached 100% with the use of the double ribozyme system in T0 rice plants (Tang et al. 2017; Zhong et al. 2018). Furthermore, a tRNA-based processing system and a Cas12a CRISPR array-based processing system were both successfully used for multiplexed plant genome editing (Wang et al. 2017; Ding et al. 2018).

While AsCas12a, FnCas12a and LbCas12a all prefer a 5′-TTTV-3′ PAM, a recent study showed that FnCas12a can target most of 5′-VTTV-3′ PAM sites in rice (Zhong et al. 2018). Relaxed PAM recognition can also be achieved with engineered Cas12a variants (Fig. 2). Altered PAM recognition was previously demonstrated by engineered variants of AsCas12a, namely RR and RVR (Gao et al. 2017), which recognize non-canonical 5′-TYCV-3′ and 5′-CCCC-3′, as well as 5′-TATV-3′ PAMs in human cells, respectively. Recently, Li et al. (2018a) showed that the LbCas12a-RR variant was able to edit 5′-TYCV-3′ PAMs in rice. Zhong et al. (2018) reported that LbCas12a-RR and -RVR variants, but not FnCas12a variants, were able to recognize these altered PAMs in rice.

CAS12B ENDONUCLEASES

Cas12b (formerly C2c1) is a Class II Type V-B endonuclease that targets double-stranded DNA (Strecker et al. 2019). Cas12b specifically cuts DNA with its single RuvC domain and produces staggered ends with ~ 7-nt overhangs (Wu et al. 2017; Liu et al. 2017). Cas12b recognizes a distal 5′-T rich (5′-TTN-3′) PAM and its catalytic activity depends on both the presence of tracrRNA and crRNA (dual-RNA-guided), similar to SpCas9 (Shmakov et al. 2015). Structural analysis of a Cas12b from *Bacillus thermoamylovorans* (BthCas12b) showed that recognition of sgRNA and PAM-containing duplex by Cas12b was different from Cas9 and Cas12a (Wu et al. 2017). Liu et al. (2017) reported the crystal structure of AacCas12b isolated from *Alicyclobacillus acidoterrestris,* a Gram-positive soil bacterium that grows in high temperatures (35–55 °C).

Teng et al. (2018) identified a Cas12b ortholog from *Alicylobacillus acidiphilus* (AaCas12b) that kept its nuclease activity between the temperatures of 31 °C and 59 °C, enabling genome editing in mammalian cell cultures. Recently, a Cas12b ortholog from *Bacillus hisashii* (BhCas12b) was characterized in detail and its engineered mutant, BhCas12b v4 was tested for cleavage activity in human cells under lower temperatures than the wild-type form (Strecker et al. 2019). Artificial crRNAs have also been successfully engineered to work with multiple Cas12b orthologs for genome editing in human cells (Teng et al. 2019). Cas12b represents an ideal enzyme by its small size and potential low off-target activity and could be adapted for plant genome editing in the near future.

BASE EDITORS

Base editing is a genome editing approach utilizing specific "base editors" to convert a base into another in a targeted manner (Komor et al. 2016; Nishida et al. 2016; Gaudelli et al. 2017). Precise C-to-T or A-to-G changes could be achieved on a desired DNA sequence by base editors, without the formation of DSBs and -induced NHEJ or MMEJ events that eliminates further re-arrangements and without the need for HDR that is restricted to S- and G2-phases of cells. Thus, base-editing represents a powerful and promising tool for point-mutagenesis that can be exploited in crop development, as seen in the manipulation of genes associated with agronomical traits (Hua et al. 2018; Yan et al. 2018; Kang et al. 2018; Endo et al. 2019; Zhang et al. 2019).

At present, there are three major groups of base editors: cytidine base editors (CBEs), adenine base editors (ABEs) and RNA base editors (adenosine deaminases act on RNA: ADARs) (reviewed in Molla and Yang 2019). CBEs catalyze the conversion of C-G to T-A through an initial deamination of cytidine to uracil, which results in a U-G mismatch. U is recognized as T during repair or replication, thus forming a T-A pair. The first generation of CBEs (BE1) comprised of cytidine deaminase rAPOBEC1 fused to dCas9 carrying the D10A and H840A mutations (Komor et al. 2016). BE1 can achieve efficient and targeted deamination in vitro, but not in vivo. This is due to the cellular base excision repair (BER) pathway that usually converts the edited U-G pairs back to C-G pairs. In BER, DNA glycosylases remove the mismatched base by cleaving the *N*-glycosidic bond between the target base and deoxyribose, creating apurinic or apyrimidinic (AP) sites (also known as abasic sites) on DNA (Schormann et al. 2014). Abasic sites are resolved by an AP endonuclease, followed by gap filling and ligation (Fig. 1B). Uracil DNA Glycosylase (UDG) removes U from DNA in cells and leads to the BER pathway (Komor et al. 2016). To improve base editing efficiency in vivo, a UGI (Uracil-DNA glycosylase inhibitor) domain from *Bacillus subtilis* bacteriophage PBS1 was fused to the C terminus of a cytidine deaminase to prevent base editor-induced BER, thus the mismatch can be repaired through mismatch repair (MMR) pathway, an alternative route and important mechanism for post-replication repair of bases that are mis-incorporated to DNA. This base editor architecture is referred to as BE2. In BE3 and BE4, dSpCas9 is replaced with nSpCas9 nickase, harboring the D10A mutation abolishing the catalytic activity of the RuvC-like domain, thus inducing cellular repair on non-edited strand using edited DNA as a template. BE3 increases the base editing frequency substantially; however, it generally also results in insertions and deletions (indels), due to the ultimate production of double-stranded breaks. When a nick and an AP site are generated at the same target site, DSBs may occur due to AP endonuclease cleavage or spontaneous breakage, which tend to be repaired by NHEJ. Increasing the number of UGIs that are available at the editing sites can increase the purity of edits (Komor et al. 2017). Fourth-generation base editors (BE4, SaBE4, BE4-Gam, SaBE4-Gam) can boost the C:G to T:A base editing efficiency (approximately 50% in human cells) with reduced indel formation (Komor et al. 2017). Recently, C-to-T base editing purity was improved in yeast by reducing the width of editing window by removing non-essential deaminase sequences and optimizing linker sequences (Tan et al. 2019). Improvement of DNA base editors for PAM compatibility, editing specificity and their current applications can be found in two recent reviews (Rees and Liu 2018; Molla and Yang 2019).

Cytidine deaminases have been used to edit genomes of rice, wheat, maize, tomato, potato and watermelon (Zong et al. 2017; Li et al. 2017; Lu and Zhu 2017; Ren et al. 2017, 2018, 2019; Shimatani et al. 2017; Zong et al. 2018, 2019; Tian et al. 2018; Endo et al. 2019; Hua et al. 2019b; Veillet et al. 2019; Zhang et al. 2019). Optimizations for increasing targeting flexibility, improving editing purity and expanding PAM recognition are provided in Table 1. Target-AID, a base editor utilizing the cytidine deaminase domain of *Petromyzon marinus* (PmCDA1) has shown a C-to-T editing efficiency of 53% with off-target activity of 0.38% in tomato, suggesting that it is one of the most optimal deaminases for plants (Shimatani et al. 2017). rAPOBEC1 and PmCDA1, hAID and human APOBEC3A (A3A) have been used in C-to-T base editing in plants (Ren et al. 2018; Zong et al. 2018). PmCDA1 has also been fused to Sp nCas9-NGv1 and successfully used to edit the rice genome (Endo et al. 2019). In addition, a combination of cytidine base editing and DNA-free genome editing has been demonstrated in plants (Zong et al. 2018; Veillet et al. 2019).

Deamination of adenine by adenine base editors produces an inosine (I) base, which is read as guanine by DNA replication or repair machinery. As there are no known natural enzymes that deaminate adenine in dsDNA, Gaudelli et al. (2017) developed deaminase variants based on *Escherichia coli* tRNA adenine deaminase (TadA), which catalyze the deamination of adenine on ssDNA. The group used directed evolution and protein engineering to produce an improved version of a mutated TadA-dCas9 heterodimeric protein that has high base editing efficiency in mammalian cells. Among adenine base editors, ABE7.10 showed the highest base

Table 1 Compositions of modified base editors and their demonstrations in plants

DNA base editor	References (first description)	Composition	Feature	Plants	References
C to T conversion					
BE2	Komor et al. (2016)	rAPOBEC1-Sp dCas9 (D10A, H840A)-UGI	Inhibition of Uracil *N*-glycosylase; circumventing cellular DNA repair processes (BER)	Rice, wheat, maize	Zong et al. (2017)
BE3	Komor et al. (2016)	rAPOBEC1-Sp nCas9 (D10A)-UGI	Nicking non-edited strand; favoring U to A over U to G outcome	Rice, wheat, maize	Hua et al. (2018); Li et al. (2017); Lu and Zhu (2017); Ren et al. (2017); Zong et al. (2017); Zhang et al. (2019)
BE3	Komor et al. (2016)	Sp nCas9 NGv1(D10A)-rAPOBEC-UGI	NGv1 nCas9 recognizing NG PAM; increase targeting flexibility	Rice	Endo et al. (2019)
NGv1-BE3	Nishida et al. (2016)	Sp nCas9 NGv1(D10A)-AID-UGI	NGv1 nCas9 recognizing NG PAM; increase targeting flexibility	Rice	Endo et al. (2019)
BE4	Komor et al. (2017)	rAPOBEC1-Sp nCas9 (D10A)-UGI-UGI	Addition of second UGI domain, improving editing purity	Rice	Ren et al. (2017)
rBE5	Ren et al. (2018)	hAID*Δ-Sp nCas9 (D10A)	hAID deaminase; increase in editing efficiency	Rice	Ren et al. (2018)
rBE9	Ren et al. (2018)	hAID*Δ-Sp nCas9 (D10A)-UGI	Addition of the UGI domain, improving editing purity compared to rBE5	Rice	Ren et al. (2018)
VQR-BE3	Kim et al. (2017d)	rAPOBEC1-Sp nCas9 VQR (D10A)-UGI	VQR nCas9 recognizing NGA PAM; increasing targeting flexibility	Rice	Hua et al. (2019b)
Sa (KKH)-BE3	Kim et al. (2017d)	rAPOBEC1-Sa nCas9 KKH (D10A)-UGI	Sa nCas9 recognizing NNNRRT PAM, increasing targeting flexibility	Rice	Hua et al. (2019b)
Target-AID	Nishida et al. (2016)	Sp nCas9 (D10A)-CDA1-UGI	CDA1 provides slightly shifted editing activity window compared to APOBEC1	Rice, tomato	Shimatani et al. (2017)
Target-AID	Nishimasu et al. (2018)	Sp nCas9 NGv1(D10A)-CDA1-UGI	CDA1 provides slightly shifted editing activity window compared to APOBEC1, Sp nCas9-NGv1 recognizing NG PAM; increasing targeting flexibility	Rice	Endo et al. (2019)
BE3	Komor et al. (2016)	rAPOBEC1-Sp n xCas9 (D10A)-UGI	xCas9 was used for recognizing NGN, GAA, GAT, CAA PAMs; increasing targeting flexibility	Rice	Zhong et al. (2019); Hua et al. (2019a)
Target AID	Nishida et al. (2016)	Sp nCas9 NG (D10A)-PmCDA1-UGI	Target AID was upgraded to reduce Indels by fusion of UGI to PmCDA1	Rice	Zhong et al. (2019)
rBE9	Ren et al. (2018)	hAID*Δ-Sp nCas9 NG (D10A)-UGI	Expanding the scope of gene editing by recognition of NG PAM	Rice	Ren et al. (2019)
A3A-PBE	Zong et al. (2018)	APOBEC3A-Sp nCas9 (D10A)-UGI	Replacing rAPOBEC1 with APOBEC3A for increased editing efficiency, specificity, and expanded deamination window	Rice, wheat, potato	Zong et al. (2018)

Table 1 continued

DNA base editor	References (first description)	Composition	Feature	Plants	References
A3A-Gam	Zong et al. (2018)	Gam-APOBEC3A-Sp nCas9 (D10A)-2 × UGI	Addition of Gam to A3A-PBE to increase editing efficiency and product purity	Rice, wheat	Zong et al. (2018)
A to G conversion					
ABE6.3 ABE7.8 ABE7.9	Gaudelli et al. (2017)	TadA-TadA mutant-Sp nCas9 (D10A)	Offer higher editing efficiency at position closer to PAM compared to ABE7.10	*Arabidopsis thaliana, Brassica napus*	Kang et al. (2018)
ABE7.10	Gaudelli et al. (2017)	TadA-TadA mutant-Sp nCas9 (D10A)	Most efficient and sequence context-independent ABE, activity window further from PAM compared to ABE6.3, 7.8 and 7.9	*Arabidopsis thaliana, Brassica napus,* rice, wheat	Kang et al. (2018); Hua et al. (2019b); Li et al. (2018b)
ABE7.10	Gaudelli et al. (2017)	TadA-TadA mutant-Sp n xCas9 (D10A)	xCas9 was used for recognizing NGN, GAA, GAT, CAA PAMs; increasing targeting flexibility	Rice	Hua et al. (2019a)
ABE7.10	Gaudelli et al. (2017)	TadA-TadA mutant-Sp nCas9 NGv1 (D10A)	NGv1 nCas9 recognizing NG PAM; increase targeting flexibility	Rice	Negishi et al. (2019)
ABE7.10*	Yan et al. (2018)	TadA-TadA mutant-Sp nCas9 (D10A)	Broader activity window compared to ABE7.10	Rice	Yan et al. (2018)
VQR-ABE	Hua et al. (2018)	TadA-TadA mutant-Sp nCas9 (D10A) VQR	VQR nCas9 recognizing NGA PAM; increasing targeting flexibility	Rice	Hua et al. (2019b)
VRER-ABE	Hua et al. (2018)	TadA-TadA mutant-Sp nCas9 (D10A) VRER	VRER nCas9 recognizing NGCG PAM; increasing targeting flexibility	Rice	Hua et al. (2019b)
Sa (KKH)-ABE	Hua et al. (2018)	TadA-TadA mutant-Sa nCas9 (D10A) KKH	Sa nCas9 recognizing NNNRRT PAM, increasing targeting flexibility	Rice	Hua et al. (2019b)
rBE15	Yan et al. (2018)	TadA-TadA*7.10-Sp dCas9 (D10A, H840A)	Employs catalytically inactive dCas9	Rice	Yan et al. (2018)
rBE18	Yan et al. (2018)	TadA-TadA*7.8-Sp dCas9 (D10A, H840A)	Employs catalytically inactive dCas9	Rice	Yan et al. (2018)
rBE14	Yan et al. (2018)	TadA-TadA*7.8-Sp nCas9 (D10A) NG	Expanding the scope of gene editing by recognition of NG PAM	Rice	Ren et al. (2019)
PABE7	Li et al. (2018b)	TadA-TadA*-Sp nCas9 (D10A)-3xNLS	Optimizes location and number of NLS to increase base editing efficiency	Rice, wheat	Li et al. (2018b)

editing efficiency, of up to 4.1% in *Arabidopsis* and 8.8% in *Brassica* protoplasts, and with low undesirable mutation rates (< 0.1%) (Kang et al. 2018). The ABE7.10 architecture was further optimized for base editing of crop plants with respect to the number and locations of nuclear localization signal sequences (Li et al. 2018b).

Recently, a series of adenine and cytosine base editors were developed using both SpCas9 and SaCas9 variants to expand the targeting scope in rice (Hua et al. 2019b; Qin et al. 2019). Similarly, adenine and cytosine base editors based on Cas9-NG were also reported to edit relaxed PAM sites in plants (Negishi et al. 2019; Ren et al. 2019; Hua et al. 2019a).

Either loss-of-function or gain-of-function phenotypes can be achieved with base editing. It is possible to introduce a nonsense mutation in the gene of interest by C-to-T base editors. Loss-of-function phenotypes can also be achieved by introduced missense mutations or mutations that alter RNA splicing sites as demonstrated in *Arabidopsis* (Kang et al. 2018; Li et al. 2019b). Gain-of-function phenotypes were reported such as dominant semi-dwarf phenotype by editing *SLR1* in rice (Lu and Zhu 2017) and herbicide resistance by editing *ALS* in rice and wheat (Shimatani et al. 2017; Zong et al. 2018; Zhang et al. 2019) or editing *ACC* in rice (Li et al. 2018b). In principle, base editors could also be applied to target upstream open reading frames (uORFs) for improved protein translation, as well as to target promoter regions to introduce quantitative traits, as recently demonstrated with the use of Cas9 nuclease (Rodríguez-Leal et al. 2017; Zhang et al. 2018).

OFF-TARGET EFFECTS OF CRISPR-CAS SYSTEMS

Off-target effects of CRISPR-Cas systems are potential concerns in many of their applications. Whole-genome sequencing (WGS) conducted in *Arabidopsis*, tomato and cotton showed that off-target activity induced by SpCas9 has rarely occurred (Feng et al. 2014; Nekrasov et al. 2017; Li et al. 2018c; Tang et al. 2018). High targeting specificity was also recently reported for Cas12a in rice and cotton (Tang et al. 2018; Li et al. 2019a). SpCas9 has some tolerance to 1-nt mismatches in protospacers (Wu et al. 2014; Tang et al. 2018). Such tolerance was drastically diminished with high-fidelity SpCas9 variants. For example, xCas9 was more sensitive to 1-nucleotide mismatch than the wild-type SpCas9 in both human cells and rice (Hu et al. 2018b; Zhong et al. 2019). The simultaneous introduction of 2-nucleotide mismatches in protospacers virtually abolished the editing activities of LbCas12a and FnCas12a (Tang et al. 2017; Zhong et al. 2018). The specificity of wild-type Cas9 and Cas12a can be further improved with engineered variants, making off-target edits by Cas proteins less of a concern in plants. However, lack of high-quality reference genomes for many plant species and cultivars is a major limitation for off-target assessments. Unbiased methods such as WGS and Digenome-seq provide comprehensive analysis (e.g. from small indels to structural variations) of the genome but are costly (Zischewski et al. 2017).

Base editors were recently found to exhibit a tendency to cause genome-wide off-target effects. Similar to nucleases, putative off-target sites for base editing can be identified by tailored methods such as Digenome-seq (Kim et al. 2017c). However, a comprehensive assessment of off-targeting requires WGS. For example, BE3 led to elevated genome-wide C to T base changes in mouse and rice (Zuo et al. 2019; Jin et al. 2019). Such an off-target effect appears to be independent of Cas9, but due to the ectopic expression of the cytidine deaminase. ABE does not seem to cause genome-wide off-target mutations (Jin et al. 2019). Interestingly, both BE3 and ABE were also shown to cause transcriptome-wide off-target effects by editing RNA transcripts (Grünewald et al. 2019; Rees et al. 2019). Fortunately, these off-target effects at DNA and RNA levels can be greatly reduced with engineered deaminase variants (Grünewald et al. 2019; Rees et al. 2019; Zhou et al. 2019).

CONCLUSION

The scope of genome editing is expanded widely using novel Cas9 and Cas12a engineered variants and orthologs, which recognize a broader range of PAM sequences. To date, only some Cas9 and Cas12a variants and orthologs have been adapted for plant genome editing indicating great potential of further expanding genome editing scope in plants in the near future. Base editing is a powerful technique to generate point mutations in vivo. Given the high number of SNPs associated with agronomic traits in plants, gene editing by base editors could play a significant role in generating favorable allelic combinations. However, improved base editors with reduced off-target effects are needed to allow precise genome editing in plants. The continued evolution of these genome editing tools will further enhance fundamental and translational plant research.

Acknowledgements We apologize to the authors whose studies were not cited due to limited space. Our plant genome editing research is supported by the National Science Foundation Plant Genome Research Program (IOS-1758745) and USDA-NIFA Biotechnology Risk Assessment Research Program (2018-33522-

28789), Foundation for Food and Agriculture Research (593603), and Syngenta Biotechnology. We thank Aimee Malzahn and Dr. Brian Iaffaldano for proofreading this manuscript.

Author contributions FG, YZ, SS and YQ wrote the manuscript. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest The authors have no conflict of interests to declare.

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