Journal of Plant Biochemistry and Biotechnology (October–December 2023) 32(4):661–672
https://doi.org/10.1007/s13562-023-00860-2

REVIEW ARTICLE

Strategies to improve genome editing efficiency in crop plants

B. Aravind[1] · Kutubuddin Molla[2] · Satendra K. Mangrauthia[3] · Gireesha Mohannath[1]

Received: 31 July 2023 / Accepted: 25 October 2023 / Published online: 8 November 2023

Abstract

Genome editing technology comprises site-directed mutagenesis of genomes, involving alterations of few bases to precise replacement of a fragment or an entire gene sequence. Among multiple types of genome editing technologies developed, CRISPR-Cas9 and its latest variants have been revolutionizing the field of genetic engineering and plant biotechnology. Despite several advantages the CRISPR-Cas9 technology offers, it often suffers from low efficiency in creating desirable mutants in several crop plant species. In this review, we discuss various emerging strategies to improve genome editing efficiency in crop plants. The strategies include increased expression of genome editing components using high efficiency viral vectors, employment of inhibitors of chromatin modifiers, and using plant DNA viruses as donor DNA carriers. Additionally, we also discuss strategies to obtain transgene-free genome edited crops.

Keywords Genome editing · CRISPR-Cas · Transgene-free · Viral vectors · Histone deacetylase inhibitors · TRBO vector

Abbreviations

ABE	Adenine base editing
Alt-EJ	Alternative-end joining
BCTV	Beet curly top virus
BeYDV	Bean yellow dwarf virus
CBE	Cytosine base editing
CP	Coat protein
CRISPR	Clustered regularly interspaced short palindromic repeats
DMSO	Dimethyl sulfoxide
dsDNA	Double-stranded DNA
gRNA	Guide RNA
GVR	Geminivirus replicons
HATs	Histone acetyltransferases
HDACi	Histone deacetylase inhibitor(s)
HDACs	Histone deacetylases
HDR	Homology-directed repair
HR	Homologous recombination
MP	Movement protein
NA	Nicotinamide
NaBt	Sodium butyrate
NHEJ	Non-homologous end joining
PCR	Polymerase chain reaction
RNP	Ribonucleoprotein(s)
SDN	Site-directed nuclease
sgRNA	Single guide RNA
SSNs	Sequence-specific nucleases
TALENs	Transcription activator-like effector nucleases
TGMV	Tomato golden mosaic virus
Ti plasmid	Tumor-inducing plasmid
TMV	Tobacco mosaic virus
TRBO	Tobacco mosaic virus RNA-based overexpression
TRV	Tobacco rattle virus
TSA	Trichostatin
UGI	UNG inhibitor
UNG	Uracil DNA glycosylase
VIGE	Virus-induced genome editing
ZNFs	Zinc-finger nucleases

✉ Satendra K. Mangrauthia
Satendra.KM@icar.gov.in

✉ Gireesha Mohannath
gireesha@hyderabad.bits-pilani.ac.in

[1] Department of Biological Sciences, Birla Institute of Technology and Science, Pilani, Hyderabad Campus, Hyderabad, Telangana 500078, India

[2] ICAR-National Rice Research Institute, Cuttack, Odisha 753006, India

[3] ICAR-Indian Institute of Rice Research, Hyderabad, Telangana 500030, India

Introduction

Genome editing is a targeted nuclease-based method for introducing site-specific changes in the genome of an organism. The two most critical steps of genome editing are, DNA recognition, which is performed by either proteins or small RNA, and double-strand break (DSB) in DNA that is performed by nucleases (Gaj et al. 2016). Based on the first step, the genome editing tools have been categorized into two major groups. The first group includes meganucleases, transcription activator-like effector nucleases (TALENs), and zinc-finger nucleases (ZFNs), where DNA recognition is performed by specifically-designed protein molecules. Meganucleases or homing endonucleases are characterized by long recognition sites comprising 14–40 bp. These enzymes perform both DNA recognition and DSB generation (Silva et al. 2011). Limited number of naturally occurring recognition sites in genomes restrict the usage of meganuclease-based genome editing tools for mutating desired sites. Although protein engineering was successfully attempted in a few cases to alter the DNA recognition site specificity, overlapping of the DNA recognition domains with the nuclease domains in these enzymes often resulted in a loss of nuclease activity.

TALENs are produced by fusion of transcription activator-like effector (TALE) proteins and FokI nuclease from *Flavobacterium okeanokoites*. TALE proteins having specific DNA recognition ability are secreted by *Xanthomonos* bacteria (Joung and Sander 2013). On the other hand, ZFNs are created by fusing zinc fingers, the well-known DNA binding proteins, and FokI nuclease (Urnov et al. 2010; Zhang et al. 2010). Although TALENs and ZFNs were considered promising genome editing tools, their requirement of protein-based DNA recognition hampered the flexibility of these tools for editing the nucleotides of choice. Moreover, specific arrangement of zinc fingers or TALE proteins for recognition of precise nucleotides entails the expertise of protein engineering, further restricting their usage. The second and most recent group of genome editing tools includes clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein (Cas). Here, the DNA recognition is performed by a small RNA molecule of 19–23 nucleotides known as guide RNA (gRNA), and the nuclease function is performed by Cas proteins (Jinek et al. 2012). The gRNAs recognize specific nucleotides based on the sequence homology, and they can be chemically synthesized as oligomers. Straightforward gRNA/construct design and assembly as one of the major advantages, CRISPR/Cas-based genome editing lends enormous flexibility for introducing site-specific changes in any part of a gene or a genome (Aglawe et al. 2018; Biswal et al. 2019).

Next, the site-specific DNA breakage is followed by the activation of cellular DNA repair pathways, which largely include the non-homologous end joining (NHEJ) and the homology-directed repair (HDR) (Mao et al. 2019). The NHEJ does not require any donor template to repair the broken ends of DNA and often results in insertions/deletions (indels) and/or base substitutions at specific sites in the genome. The NHEJ is commonly utilized to create knockout mutants for a gene. The HDR is a DNA repair pathway that requires a donor template homologous to the targeted genomic region (Molla et al. 2022). It is commonly used for correction of bases or integration of whole genes or gene segments. Based on the DNA repair pathways utilized for nucleotide alteration, the genome editing has been classified into three categories namely, SDN1 (Site-Directed Nuclease1), SDN2, and SDN3 (Podevin et al. 2013) (Fig. 1). SDN1 employs NHEJ for creating mutations (indels or point mutations) while SDN2 employs HDR for replacing a shorter stretch of target nucleotides. Like SDN2, SDN3 also employs HDR but it is used to replace or introduce long stretches of DNA, often involving entire gene sequences. Several countries, including India have allowed SDN1 and SDN2 genome edited crop plants for commercial release with minimum biosafety regulations. However, the final product must be free from the exogenous DNA used for genome editing (e.g., CRISPR-Cas containing sequences) (Menz et al. 2020).

Strategies to improve genome editing efficiency

Increasing expression of Cas9 and sgRNA

In CRISPR-Cas-based genome editing, the expression levels of the recombinant Cas protein and the sgRNA determine the genome editing efficiency. The *Agrobacterium tumefaciens*-based transient delivery systems that utilize constitutive promoters, is constrained by limited expression of sgRNA that lacks 5′ and 3′ overhangs (Cong et al. 2013; Nekrasov et al. 2013). Interestingly, a recent study has shown that employing native U3/U6 and Ubiquitin promoters to drive expression of sgRNA and Cas9 could result in higher genome editing efficiency in white lupin (*Lupinus alba* L.). Over the decades, viral vectors have served as useful biotechnological tools owing to their potential to replicate independently and produce recombinant proteins in a variety of hosts (Scholthof et al. 1996, 2002). DNA (Baltes et al. 2014; Yin et al. 2015) and RNA (Ali et al. 2015) viral-based vectors have been experimented for delivering CRISPR/Cas components. Initially, transient gene editing by viral vectors had displayed relatively low efficiency (Ali et al. 2015; Yin et al. 2015), justifying a need to explore more efficient

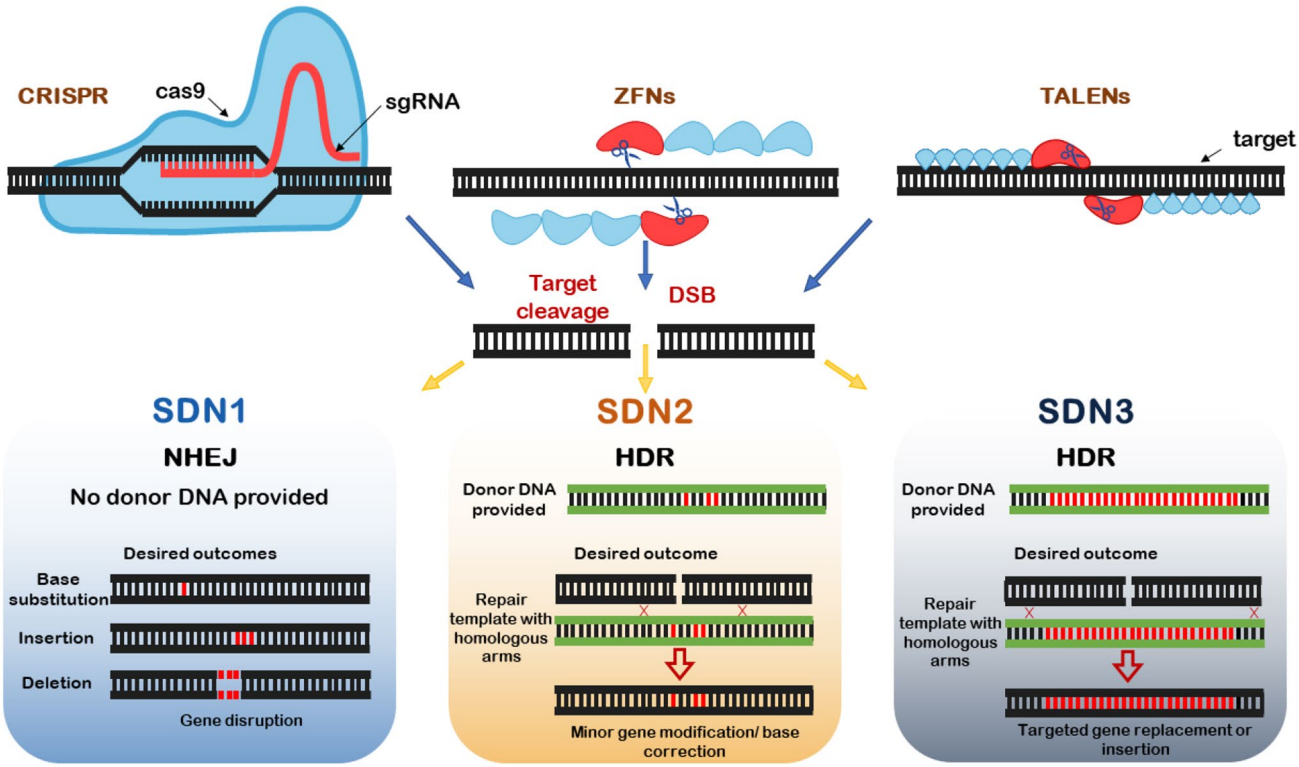

Fig. 1 Types of site-directed nucleases-based genome editing technologies. SDN1: DSB followed by NHEJ leading to insertion or deletion (indel) or base substitution, resulting in gene disruption; SDN2: DSB followed by HDR with the donor template (ssDNA/dsDNA/RNA) provided, resulting in small-scale mutations (modification of a few base pairs); SDN3: DSB followed by HDR with the donor DNA template with homologous ends containing the desired gene sequence resulting in targeted gene replacement and/or insertion of the sequence of interest. CRISPR: Clustered regularly interspaced short palindromic repeats; DSB: Double strand break; HDR: Homology-directed repair; NHEJ: Non-homologous end joining; SDN: Site-Directed Nuclease; sgRNA: Single guide RNA; TALENs: Transcription activator-like effector nucleases; ZNFs: Zinc-finger nucleases. Target: The targeted DNA sequence for editing; Donor DNA (green): The DNA provided as a template for the HDR; Red strokes: represent the newly inserted nucleotide(s); 'X': A mark denoting the place of recombination during HDR (color figure online)

vector systems. A study involving transient expression in *N. benthamiana* demonstrated utility of Tobacco Mosaic Virus RNA-based overexpression (TRBO), a breakthrough technology vector system (Lindbo 2007), in improving genome editing efficiency through enhanced expression of sgRNA (Cody et al. 2017). TRBO vector is an optimized Ti plasmid with a modified T-DNA region containing a coat protein (CP) deletion mutant of the Tobacco mosaic virus (TMV) U1 strain, which was originally created for the production of recombinant protein-coding sequences in host cells through agroinfiltration method (Lindbo 2007). The absence of the CP prevents the expressed recombinant proteins from systemically spreading throughout the plant, still permitting the cell-to-cell movement with the aid of the movement protein (MP). Moreover, CP seems to be a inducer of RNA silencing and therefore, it is hypothesized that its removal could let TRBO vector escape RNA silencing, resulting in very high levels of gene expression. (Lindbo 2007) has shown exorbitantly high levels of overexpressed recombinant protein (3 to 5 mg/g fresh weight of plant tissue) using TRBO vectors via transient agroinfiltration method in *N. benthamiana*. For its known ability to highly express the transgene, it was used to enhance the levels of the sgRNAs in *N. benthamiana* by (Cody et al. 2017). The study also demonstrates the potential of the TRBO vector for multiplexed sgRNA delivery, indicating its flexibility for gene expression and editing. Further, the study also reveals that removal of the 5′ and 3′ overhangs of sgRNA is not necessary for in vivo cleavage of the target genomic DNA by Cas9 although removal of 5′ overhangs is necessary for in vitro Cas9-mediated cleavage of target DNA. These results highlighted the flexibility of sgRNA under in vivo conditions. Similarly, another study showed that the presence of unrelated sequence in sgRNA as 5′ and 3′ overhangs does not impact Cas9 cleavage efficiency in vivo (Molla et al. 2022). Moreover, (Cody et al. 2017) also designed a new Cas9 construct called pHcoCas9, which resulted in higher rates of Cas9 protein expression than a previously used expression vector (pFGC-pcoCas9). The salient features of the pHcoCas9 construct include a human codon-optimized Cas9 nuclease gene, a 35S promoter, a 35S

terminator, 5′ TEV UTR and 3′ TEV UTR for increased translation efficiency (Cody et al. 2017). TRBO vector can also be explored for increased expression of Cas9. However, too much of Cas9 protein could potentially cause embryo lethality or higher frequencies of off-target mutations. Therefore, each vector type used for Cas9 overexpression should be carefully evaluated by measuring their genome editing efficiency vis-à-vis the frequency of off-target mutations.

Similar modifications have also been made to Tobacco rattle virus (TRV)-based viral vector systems, which enhanced CRISPR-Cas9-mediated gene editing efficiency in *N. benthamiana* (Ali et al. 2015). However, unlike TRBO vector system, multiplexed delivery of the gRNAs (individual agrobacterium cultures with a separate gRNA construct) through the modified TRV vector system lowered the efficiencies of genome editing compared to the delivery of single sgRNA (Ali et al. 2015). Interestingly, some recent studies have employed an engineered TRV-based vector for in planta delivery of sgRNAs fused to tRNA isoleucine ($tRNA^{Ileu}$) sequence to induce efficient multiplex somatic and biallelic heritable editing in a single generation in SpCas9-expressing transgenic Arabidopsis and *N. benthamiana* (Ellison et al. 2020; Nagalakshmi et al. 2022). It has been shown that tRNAs and other RNAs that promote cell-to-cell movement can increase virus-mediated mutagenesis when fused to sgRNAs (Ellison et al. 2020; Kujur et al. 2021; Lei et al. 2021; Nagalakshmi et al. 2022). Therefore, sgRNAs were fused to $tRNA^{Ileu}$ to facilitate efficient TRV movement in Arabidopsis and *N. benthamiana* (Ellison et al. 2020; Nagalakshmi et al. 2022). These TRV-based methods have also been successfully used for heritable base-editing in Arabidopsis (D. Liu et al. 2022a). Similarly, one more RNA virus (*Barley stripe mosaic virus*)-based vector has been used for efficient multiplexed heritable genome editing in wheat also bypasses tissue culture (Li et al. 2021).

Of late, several new virus-based tools spanning a wide host range have been developed to deliver CRISPR/Cas9 constructs to plants (Ariga et al. 2020; Kujur et al. 2021; Varanda et al. 2021; Zhang et al. 2019). Using these viral-based tools for genome editing is called virus-induced genome editing (VIGE) (Gentzel et al. 2022). The strategies to increase expression of Cas9 and sgRNA, as discussed above, are summarized in Fig. 2A.

DNA viruses as template carriers for SDN2 and SDN3 genome editing

Compared to SDN1, SDN2 and SDN3 types of genome editing are more challenging because they require additional component of a template DNA carrier for gene repair or gene replacement via the HR approach (Jang and Joung 2019). One of the commonly used approaches to provide a template DNA has been T-DNA-mediated integration of the donor DNA into the recipient's nuclear genome (Hiei et al. 1994; Kimura and Sinha 2008; Koncz et al. 1989). However, this approach suffers from low genome editing efficiency because only a limited copy number of donor DNA can be delivered using *Agrobacterium*-mediated transformation. Plant DNA viruses serve as ideal and more efficient alternative donor DNA carriers. Among the plant DNA viruses, geminiviruses have been explored both as carriers of donor DNA and as targets in CRISPR-Cas-based genome editing.

Geminiviruses constitute a large family of plant viruses with single-stranded circular DNA genomes of ~ 2.5 to 3.0 kb (Gutierrez 1999; Hanley-Bowdoin et al. 2000; King et al. 2011; Pilartz and Jeske 1992). They are either monopartite (entire genome exists as a single circular DNA) or bipartite (entire genome exists as two circular DNA) (Bisaro 1996; Jeske 2009). For example, *Beet Curly Top Virus* (*BCTV*) has a monopartite genome with a genome size of ~ 3 kb (Zerbini et al. 2017). On the other hand, *Tomato Golden Mosaic Virus* (*TGMV*) is a bipartite virus with two circular genomes (genome A: 2.58 kb and genome B: 2.52 kb) (Hamilton et al. 1984; MacDowell et al. 1986). These viruses serve as ideal donor DNA carriers because they replicate inside the nucleus and their genomes exist in hundreds to thousands of copies per cell. Therefore, geminivirus-based replicons are explored for transient expression of sequence-specific nucleases and to deliver donor DNA templates (Baltes et al. 2014). Moreover, replicons based on the *Bean yellow dwarf virus* (*BYDV*) have been found to increase gene targeting frequencies in tobacco (*Nicotiana tabacum*) by one to two orders of magnitude over traditional Agrobacterium-mediated transformation (Baltes et al. 2014). In the same study, nuclease-mediated DNA double-strand breaks, replication of the donor DNA template, and the pleiotropic activity of the geminivirus replication initiator proteins were found to aid the gene targeting. This study opened up new avenues to use geminivirus replicons (GVR) for generating plants with a desired DNA sequence modification under six weeks.

In a recent study, geminivirus replicons were used to induce high frequency, precise modification of the tomato genome through homologous recombination using TALENs and CRISPR-Cas9-based genome editing (Čermák et al. 2015). In this study, CaMV 35S promoter was inserted upstream of the endogenous anthocyanin mutant 1 (ANT1), resulting in overexpression and ectopic accumulation of pigments in tomato tissues. In another study, GVR was used to deliver sequence-specific nucleases (SSNs) to reduce sensitivity to herbicide Imazamox by targeting *ACETOLACTATE SYNTHASE1* (*ALS1*) gene (Butler et al. 2016). In this study, GVR-mediated genome editing yielded a higher number of desired point mutations compared to T-DNA-mediated genome editing, which produced no detectable mutations in the target gene (Butler et al. 2016). A study involving

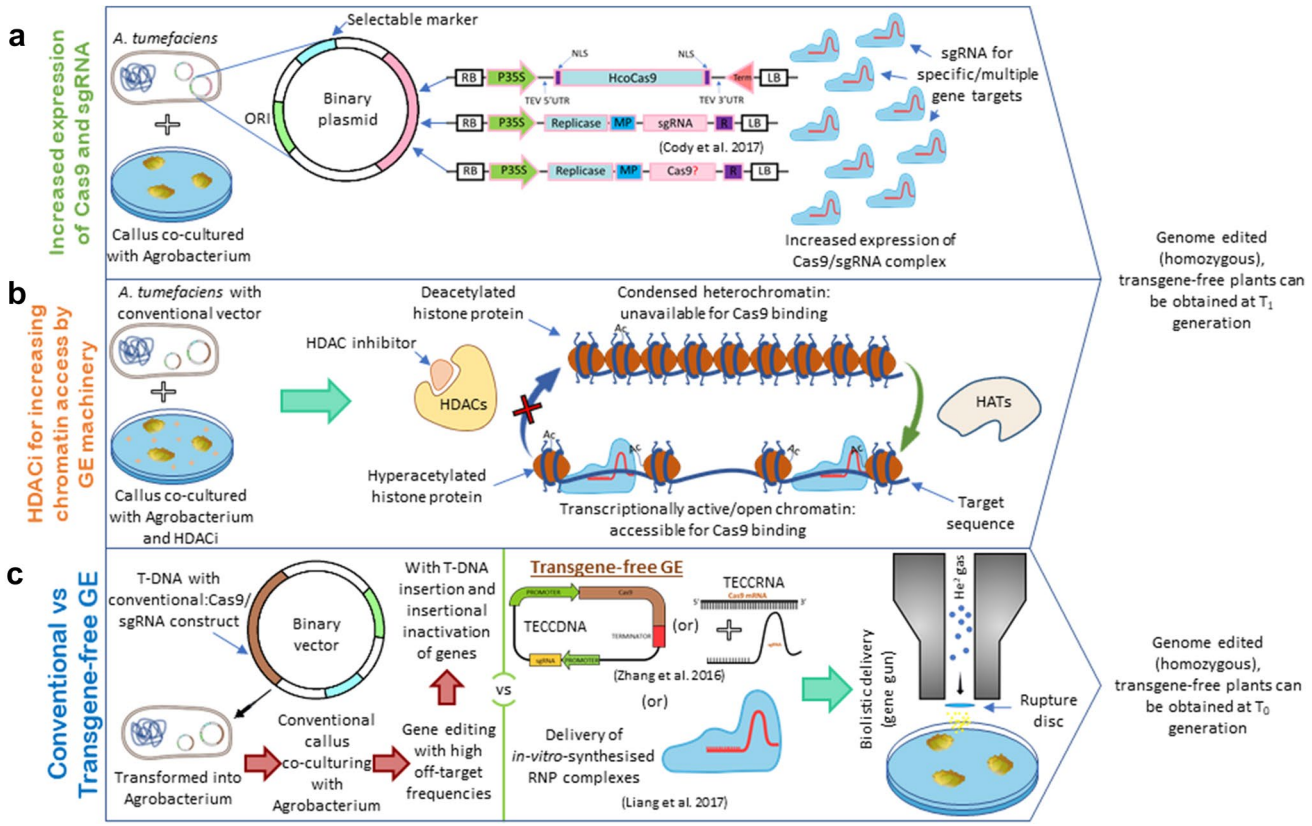

Fig. 2 Strategies for increasing genome editing efficiency. a: Increased expression of Cas9 and sgRNA: ORI: origin of replication, RB: right border, LB: left border, MP: movement protein, TEV: *Tobacco etch virus*, R: resistance gene, cas9?: can cas9 protein be overexpressed?, NLS: nuclear localization signal, UTR: untranslated region, Term: terminator, sgRNA: single guide RNA; b: HDACi for increasing chromatin access by GE machinery: HDACs: histone deacetylases, HATs: histone acetyltransferase; c: Conventional versus transgene-free GE: TECCDNA: transiently expressing CRISPR/Cas9 DNA, TECCRNA: transiently expressing CRISPR/Cas9 RNA, RNP: ribonucleoprotein complexes, GE: genome editing

genome editing of *Bean yellow dwarf virus (BeYDV)* demonstrates that CRISPR/Cas9 can efficiently access the geminivirus genome (Baltes et al. 2015). This feature provides an additional option of cleaving the donor DNA from a geminivirus replicon should one take such a modality for SDN2 or SDN3 type of genome editing. Although this study shows in principle that CRISPR-Cas9 technology can be successfully used to target geminiviruses or other DNA viruses, we are not highlighting it as an ideal anti-viral strategy. Because such a strategy may inadvertently lead to the development of more virulent strains of viruses due to random mutations caused by DNA repair subsequent to CRISPR-Cas9-mediated cleavage. Rather, our purpose to cite this study is to highlight the fact that CRISPR/Cas9 components can efficiently access the geminivirus chromatin.

Although geminiviruses could be useful as delivery agents, severe symptoms caused by geminivirus infections could pose a problem for plant survival. To circumvent this problem, mutated geminiviruses which cause reduced or no symptoms in secondary shoots but still replicate to reasonable levels can be used. For example, infections of *N. benthamiana* or Arabidopsis or sugarbeets with *BCTV* (wild-type) or *BCTV* null mutants for L2 (*BCTV L2*-) proceed similarly in primary infected tissue (Hormuzdi and Bisaro 1995; Raja et al. 2008). However, after removal of the primary infected shoots, secondary infected tissue (new growth) is symptomatic in wild-type *BCTV*-infected plants but exhibits recovery (no symptoms) in *BCTV L2*-mutant-infected plants (Hormuzdi and Bisaro 1995; Raja et al. 2008). Given the wide host range of GVRs, they can be used for SDN2- and SDN3-based plant genome editing in multiple crop plant species (Baltes et al. 2014). Lastly, GVRs offer one more advantage in being transgene-free compared to Agrobacterium-mediated transformation (Čermák et al. 2015).

Epigenetic strategies to increase chromatin access by genome editing machinery

In CRISPR-Cas9 genome editing, the Cas9 enzyme activity depends on the relaxation of the target site in the chromatin

to bind and nick the DNA strand. Chromatin relaxation is induced during various cellular processes like DNA replication, DNA repair, and transcription. Increased transcription and DNA replication rates have been shown to increase targeted gene repair frequencies in mammalian cells (Brachman and Kmiec 2005; Hu et al. 2005). Similarly, DNA replication and increased transcriptional activity of the target gene has been demonstrated to increase the likelihood of oligonucleotide-directed gene editing efficiency in bacteria as well (Huen et al. 2006, 2007).

The accessibility of the target DNA in the chromatin by DNA repair agents and genome-editing enzymes is substantially influenced by the open or closed state of the chromatin, impacting the genome editing efficiency (Chen et al. 2017; Isaac et al. 2016; Li et al. 2020; Liu et al. 2020; Verkuijl and Rots 2019; Yarrington et al. 2018). The regulation of gene expression relies largely on histone acetylation and deacetylation at lysine residues on the amino-terminal tails of histone proteins. Acetylation neutralizes the positive charge of the histone tails and weaken their affinity for DNA (Hong et al. 1993). Hyperacetylation of histones relaxes the chromatin structure, leading to transcriptional activation, while deacetylation of histones leads to chromatin condensation and gene repression. Histone acetyltransferases (HATs) and histone deacetylases (HDACs) catalyze chromatin histone acetylation and deacetylation reactions, respectively (Liu et al. 2016). HDAC inhibitors (HDACi) are frequently employed to understand the functions of these enzymes in chromatin remodeling and transcriptional reprogramming both in plant and animal model systems (Delcuve et al. 2012). These inhibitors lead to hyperacetylation of histones, resulting in chromatin loosening. Several classes of HDACi have been identified or designed, some of which were initially characterized for therapeutic cancer treatments. Few examples include sodium butyrate (NaBt, specific to HDAc Class I and IIa), Nicotinamide (NA, Class III), Trichostatin (TSA, Class I and II), MGCD-0103 (Class I and IV) and pan inhibitors like Vorinostat and Resminostat (Bond et al. 2009; Earley et al. 2007; Tandon et al. 2016).

A study in which maize cells were pretreated with HDACi sodium butyrate (5–10 mM) and nicotinamide (1–5 mM) for induction of relaxed chromatin, showed improved efficiency of oligonucleotide-directed mutagenesis (ODM) (Tiricz et al. 2018). In this study, maize cells expressing a non-functional GFP gene (bearing a premature stop codon TAG) was bombarded with the correcting oligonucleotides to restore GFP expression, either in the presence or absence of HDACi. Moreover, using the DNase I sensitivity assay, the authors (Tiricz et al. 2018) demonstrated the presence of a more relaxed chromatin in HDACi-pretreated maize cells compared to controls. Also, sodium butyrate and nicotinamide did not drastically reduce viability of maize cells (Tiricz et al. 2018).

In another study, treating animal cells with HDACi (TSA and PCI-24781) resulted in an increased efficacy of alternative end joining (alt-EJ) and homology-directed repair (HDR) upon CRISPR/Cas9-mediated DNA cleavage (Li et al. 2020). Similarly, the effects of HDAC inhibitor TSA on genome editing efficiency of Cas9 protein-gRNA ribonucleoprotein (RNP) in plant protoplasts were studied (Choi et al. 2021). In this study, a significant increase in the levels of histone H3 and H4 acetylation resulted in higher indel frequencies (3.3–3.8 times higher) compared to the DMSO control (Choi et al. 2021).

Prime editing and base editing, two of the latest variants of genome editing technologies, can introduce precise changes into the target genome at a single-base resolution (Molla et al. 2021). In a recent study in mammalian cells, HDACi have been employed to improve prime editing and base editing (N. Liu et al. 2022, b). In this study, HDACi enhanced cytosine base editing (CBE) and adenine base editing (ABE). Moreover, HDACi were also found to increase the purity of cytosine base editor products, accompanied by an enhanced acetylation of uracil DNA glycosylase (UNG) and UNG inhibitor (UGI) (N. Liu et al. 2022a, b). Similarly, HDACi can also be used in plants to increase prime editing and base editing efficiencies. However, we suggest that thorough phenotypic analysis of the genome edited plants should be carried out when HDACi are used to increase genome editing efficiency. Because HDACi may introduce some heritable epigenetic changes inadvertently even though the tissues for genome editing are exposed to HDACi only for a brief period of time. Other than using HADCi, a novel epigenetic strategy has recently been tested in human cells (Chen et al. 2022). It involves fusing Cas9 to PRDM9, a chromatin remodeling factor that deposits histone methylations H3K36me3 and H3K4me3 to mediate homologous recombination in human cells (Chen et al. 2022). This strategy resulted in enhanced genome editing compared to one that does not use any chromatin remodeling factor. Some strategies being used to induce chromatin opening for increased access of the target genomic region are summarized in Fig. 2B.

Transgene-free genome editing

Typically, introduction of genes into a plant nuclear genome either through agrobacterium-mediated transformation or any other method involve random integration of vector sequences into the target genome. This kind of gene integration results in generation of transgenic plants, which has two disadvantages. First, integration of gene sequences can occasionally happen in a gene sequence, potentially resulting in disruption of a gene function (also called insertional inactivation) (Lawrenson et al. 2015). Second, transgenic plants require regulatory clearance before they can be approved for

human consumption. Currently many countries including India have regulatory restrictions for commercial cultivation of transgenic crop plants. Given such restrictions, the development of transgene-free genome editing tools will be highly desirable. Through sexual segregation, transgene-free mutants could be obtained in sexually propagating plants (Molla et al. 2020), not in asexually propagated plants. In an effort to develop transgene-free genome editing tools for both sexually and asexually propagated plants, (Woo et al. 2015) delivered pre-assembled CRISPR-Cas9 ribonucleoproteins into protoplasts of lettuce and successfully generated transgene-free mutant plants. Since RNP do not integrate into the genome, RNP-mediated genome editing can generate transgene-free mutants with ease. Though this technology could be highly useful, it is either not feasible or highly challenging to isolate and culture protoplasts from all plant species, especially in most monocots that constitute the majority of cereal crop plants. Later, (Zhang et al. 2016) published a similar yet improved strategy to generate transgene-free genome-edited plants in T_0 generation. In this method, the CRISPR/Cas9 constructs as DNA or RNA transcripts were transiently expressed in callus cells of wheat using particle bombardment, followed by regeneration of plants in selection free media. Selection-free media allow the regeneration of calli lacking integrated CRISPR-Cas9 DNA cassettes. The study reports 43.8–61.9% of T_0 mutants free from CRISPR-Cas9 integration. In the same study, when in vitro-transcribed Cas9 mRNA and guide RNA were bombarded, editing efficiency of 1.1% was observed. Using this method, the authors were able to develop sufficient number of transgene-free gene-edited plants in the T_0 generation itself, substantially reducing the time and resource consumption compared to genome editing approaches that result in the generation of stable transgenic plants (Zhang et al. 2016). The same group reported another transgene-free strategy in wheat where in CRISPR/Cas9 ribonucleoproteins (RNPs) comprising the purified Cas9 protein and an in vitro-transcribed sgRNA were delivered into protoplasts or embryos using particle bombardment or polyethylene glycol (PEG)-mediated transfection (Liang et al. 2017). This method also yielded homozygous mutants in the T_0 generation itself with either no off-targets (when wheat embryos were used) or significantly reduced off-target mutations (fivefold lower) compared to plasmid-mediated expression of CRISPR-Cas9 components (Liang et al. 2017). Similar reduction in off-target mutations was also observed in human cells when CRISPR/Cas9 RNPs were delivered (Kim et al. 2014). One of the reasons for reduced off-targets in this method is that CRISPR/Cas9 RNP complex degrade quickly in vivo. Alternatively, modified Cas proteins such as Dead cas9 (dcas9) (Mali et al. 2013), SpCas9n (Cas9n) (Cong et al. 2013), and FokI Cas9 (fCas9) (Guilinger et al. 2014) have also been used to reduce the off-target mutations.

A recent study has demonstrated another transgene-free gene delivery method, which employs high aspect ratio nanomaterials (e.g., carbon nanotubes) to deliver genetic material into mature plants belonging to various species (Demirer et al. 2019). Of these, three (*Nicotiana benthamiana*, *Eruca sativa*, *and Gossypium hirsutum*) are dicots and one (*Triticum aestivum*) is a monocot. Nanomaterials used in this passive genetic material delivery method not only facilitated biomolecule transport into plant cells but also protected polynucleotides from nuclease degradation. This method can similarly be used to deliver genetic material for genome editing. The transgene-free genome editing strategies that we discussed above are summarized in Fig. 2C.

Induction of abiotic stress to improve genome editing efficiency

Temperature affects various biological processes, including enzyme kinetics, chromatin structure, and DNA repair pathways (Daniel et al. 2001; Oei et al. 2015; Pecinka et al. 2010). Therefore, it was hypothesized that modulating temperature at which plants are grown can potentially impact the efficiency of CRISPR/Cas9-medated in genome editing. In support of this hypothesis, (LeBlanc et al. 2018) demonstrated enhanced CRISPR-induced mutations in Arabidopsis by growing them at 37 °C instead of the standard temperature (22 °C). In their study, targeted mutagenesis was increased by ~ fivefold in somatic tissues and ~ 100-fold in the germline upon heat treatment. They also showed a ~ three-fold increase in sgRNA levels, which could enhance mutation rates if sgRNA levels are limiting in plants grown at 22 °C. As the optimal growth temperature for Streptococcus pyogenes, the source of SpCas9 used for the genome editing, is 40 °C, the authors speculated that the in vivo SpCas9 activity in plants could be higher at 37 °C, than 22 °C. The authors also found a similar increase in mutation rates in Citrus plants due to high temperature (LeBlanc et al. 2018).

In another study, a simplified heat-stress assay was used in Tobacco and Arabidopsis to increase frequencies of indels and base editing efficiency but not HDR (Blomme et al. 2022). Interestingly, similar enhanced rates of CRISPR-Cas9-mediated mutagenesis has also been observed in human cell lines, accompanied by increased sgRNA levels and higher Cas9 activity (Xiang et al. 2017). However, an increase in off-target mutation rates has also been observed with higher temperatures both in plants and human cell ines (LeBlanc et al. 2018; Xiang et al. 2017).

Although the underlying cause of increased mutagenesis with CRISPR nucleases at higher temperatures has mainly been explained by an increase in nuclease and sgRNA activity, the precise underlying mechanisms still remain to be understood.

Recently, other abiotic stresses such as salinity and osmotic stress have been explored to enhance genome editing efficiency. In one such study, (Ye et al. 2023) transformed GFP as a marker and edited it in the presence of sodium chloride and mannitol, which were used as abiotic stressors. The study found that NaCl and mannitol treatments increased the rate of genetic transformation and CRISPR/Cas9-mediated genome editing in potato, but inhibited root regeneration under higher concentrations of NaCl. However, all regenerated roots had mutations with no off-targets. Mechanistically, salt and osmotic stresses are known to cause ROS outburst, which in turn can cause DNA damage (Chiera et al. 2008; Raja et al. 2017). As a consequence, the cells could potentially upregulate NHEJ pathway to repair the damaged DNA. This potential upregulation in NHEJ pathway could play a role in increasing genome editing efficiecny (Ye et al. 2023). However, further investigations are required to understand the precise mechanisms underlying abiotic stress-enhanced genome editing efficiency.

Modulation of DSB repair pathways

RAD51 is a conserved eukaryotic protein that is responsible for an essential protein for homologous recombination-mediated DSB repair (Choi et al. 2020; Hong et al. 2019). A recent study in human cell lines has shown that constitutive expression of RAD51 could enhance the efficiency of CRISPR-Cas9-mediated gene knock-out and knock-in processes. Given the conservation of RAD51 gene family in plants (Lin et al. 2006; Suwaki et al. 2011), constitutive expression of RAD51 can be explored in plants to also enhance genome editing efficiency.

In another strategy, (Wimberger et al. 2023) inhibited the endogenous DNA-dependent protein kinase (DNA-PK) and DNA polymerase theta (PolΘ) to improve the integration efficiency and the precision of genome editing in human cell lines. DNA-PK is a critical component of NHEJ pathway, and PolΘ is a DNA polymerase enzyme involved in alternative end-joining (alt-EJ) mechanism to repair DSBs when non-homologous end joining (NHEJ) is unavailable. (Wimberger et al. 2023) identified inhibitors of DNA-PK and (PolΘ) as potent regulators of DSB repair pathway selection favoring HDR over EJ. DNA PK is conserved in plants and therefore, similar strategy of DNA PK inhibition can be explored in plants to enhance HDR efficiency.

Lastly, we highlight a reporter-based screening approach for high-throughput identification of small molecules (chemical compounds) that enhances efficiency of all three kinds (SDN1, SDN2, and SDN3) of genome editing (Yu et al. 2015). This study also found that a small molecule that inhibits HDR can enhance frame shift insertion and deletion (indel) mutations. Small molecules that modules SDN1, SDN2, and SDN3 in plants can also be identified through similar screening approaches.

Other strategies

Mutating the EFR receptor which detects the bacterial EF-Tu, a component of pathogen-associated molecular patterns (PAMPs), resulted in an enhanced Agrobacterium-mediated T-DNA transformation (Zipfel et al. 2006). Although several crop plant species, other than those belonging to Brassicaceae, appear to lack an ortholog for EFR gene in their genomes (Zipfel et al. 2006), their functional gene equivalent(s) may still be existing, which needs to be investigated.

To effectively deliver genetic material into monocot plant species, Ziemienowicz et al. (2012) designed Agrobacterium Transfer DNA-derived nano-complex. In this method, an in vitro-prepared nano-complex consisting of Agrobacterium-derived transfer DNA (T-DNA), its virulence protein D2 (VirD2), and recombination protein A (RecA from *E. coli*) were delivered to triticale microspores with the help of a Tat_2 cell-penetrating peptide (Tat_2 CPP). GE constructs can be delivered into monocots in a similar fashion.

Lately, the CRISPR/Cpf1 system from *Francisella novicida* has been emerging as a desirable alternative to CRISPR/Cas9 (Bin Moon et al. 2018). Cpf1 (also known as Cas12a) is a smaller endonuclease than Cas9, and it requires shorter CRISPR RNA (crRNA) to function effectively (Liu et al. 2017). Cpf1 is a single-stranded RNA-guided effector nuclease protein, which binds upstream of PAM and introduces 5 base pair (bp) staggered cuts into the DNA at the proximal end of the PAM, far away from the seed region. During the conversion of Cpf1-associated CRISPR repeats to mature crRNAs, the CRISPR/Cpf1 system does not require trans-activating crRNA (tracrRNA) (Zetsche et al. 2015). CRISPR/Cpf1 system successfully cleaves target DNA conveniently close to a short T-rich PAM, whereas Cas9 only works with a G-rich PAM.

Recently, a hyper-compact genome editor called CRISPR/Casϕ has been identified, which uses a single active site for both crRNA processing and crRNA-guided DNA cleavage for targeting foreign nucleic acids (Pausch et al. 2020). This strategy has been shown to be effective in in vitro, animal, and plant systems with greater target recognition potential than other CRISPR/Cas proteins. Moreover, the molecular weight of Casϕ protein is approximately half of the Cas9 and Cas12a, which makes it more convenient for delivery into host organisms (Pausch et al. 2020). CRISPR/Casϕ system can therefore serve as an effective alternative to CRISPR/Cas system for genome editing. Similarly efforts have been made to genetically engineer Cas nucleases to improve their cleavage efficiency. For example, (McGaw et al. 2022) used a high-throughput mutational scanning method to engineer

Cas12i2 (the type V CRISPR-Cas system), and found indel-enhancing single amino acid substitutions that enhanced on-target activity in immortalized and primary human cells while maintaining type V CRISPR-Cas systems' high specificity. ABR-001, an engineered Cas12i2 variant, can edit genomes with excellent efficiency and specificity, making it a promising in vivo gene editor (McGaw et al. 2022). ABR-001 could be explored in plant genome editing endeavors to for similar enhancement of editing efficiency.

Conclusions

Genome editing technologies comprise site-directed mutagenesis of genomes, involving alterations of few bases to replacement of one or more gene sequences. CRISPR-Cas9-based genome editing has been revolutionizing the field of plant biotechnology. We expect that several emerging strategies to improve CRISPR-Cas9-based genome editing efficiency that we discussed in this review would serve as a valuable source of information for genome editors. Given the minimal biosafety regulations for SDN1 and SDN2 genome edited crop plants in India and several other countries, genome editing-mediated crop plant improvement is expected to gain pace in the coming years.

Funding GM is thankful to the Birla Institute of Science and Technology (BITS) Pilani, Hyderabad campus, for the research grant (BITS/GAU/ACRG/2019/H0576) and the Science and Engineering Research Board (SERB), Government of India, for the Ramanujan Fellowship Research Grant (SB-S2-RJN-062-2017) and CRG grant (CRG/2020/002855). KM, SKM, and GM, are thankful to Indian Council of Agricultural Research (ICAR)-National Agricultural Science Fund (NASF) for the research grant (F. No. NASF/BGAM-9013/2022-23).

Data availability Not applicable.

Code availability Not applicable.

Declarations

Conflict of interest The authors declare no conflict of interest.

Ethics approval Not applicable.

Consent to participate Not applicable.

Consent for publication We hereby give our consent for publication of this manuscript.

References

Aglawe SB, Barbadikar KM, Mangrauthia SK, Madhav MS (2018) New breeding technique "genome editing" for crop improvement: applications, potentials and challenges. 3 Biotech 8:1–20

Ali Z, Abul-Faraj A, Li L, Ghosh N, Piatek M, Mahjoub A, Aouida M, Piatek A, Baltes NJ, Voytas DF (2015) Efficient virus-mediated genome editing in plants using the CRISPR/Cas9 system. Mol Plant 8(8):1288–1291

Ariga H, Toki S, Ishibashi K (2020) Potato virus X vector-mediated DNA-free genome editing in plants. Plant Cell Physiol 61(11):1946–1953. https://doi.org/10.1093/pcp/pcaa123

Baltes NJ, Gil-Humanes J, Cermak T, Atkins PA, Voytas DF (2014) DNA replicons for plant genome engineering. Plant Cell 26(1):151–163

Baltes NJ, Hummel AW, Konecna E, Cegan R, Bruns AN, Bisaro DM, Voytas DF (2015) Conferring resistance to geminiviruses with the CRISPR–Cas prokaryotic immune system. Nature Plants 1(10):1–4

Bin Moon S, Lee JM, Kang JG, Lee N-E, Ha D-I, Kim DY, Kim SH, Yoo K, Kim D, Ko J-H (2018) Highly efficient genome editing by CRISPR-Cpf1 using CRISPR RNA with a uridinylate-rich 3′-overhang. Nat Commun 9(1):3651

Bisaro DM (1996) Geminivirus DNA replication. DNA Replication Eukaryot Cells 33:833–854

Biswal AK, Mangrauthia SK, Reddy MR, Yugandhar P (2019) CRISPR mediated genome engineering to develop climate smart rice: challenges and opportunities. Semin Cell Dev Biol 96:100–106

Blomme J, Develtere W, Köse A, Arraiza Ribera J, Brugmans C, Jaraba-Wallace J, Decaestecker W, Rombaut D, Baekelandt A, Daniel Fernández Á, Van Breusegem F, Inzé D, Jacobs T (2022) The heat is on: a simple method to increase genome editing efficiency in plants. BMC Plant Biol 22(1):142. https://doi.org/10.1186/s12870-022-03519-7

Bond DM, Dennis ES, Pogson BJ, Finnegan EJ (2009) Histone acetylation, vernalization insensitive 3, flowering locus C, and the vernalization response. Mol Plant 2(4):724–737

Brachman EE, Kmiec EB (2005) Gene repair in mammalian cells is stimulated by the elongation of S phase and transient stalling of replication forks. DNA Repair 4(4):445–457

Butler NM, Baltes NJ, Voytas DF, Douches DS (2016) Geminivirus-mediated genome editing in potato (*Solanum tuberosum* L.) using sequence-specific nucleases. Front Plant Sci 7:1045

Čermák T, Baltes NJ, Čegan R, Zhang Y, Voytas DF (2015) High-frequency, precise modification of the tomato genome. Genome Biol 16:232. https://doi.org/10.1186/s13059-015-0796-9

Chen E, Lin-Shiao E, Trinidad M, Saffari Doost M, Colognori D, Doudna JA (2022) Decorating chromatin for enhanced genome editing using CRISPR-Cas9. Proc Natl Acad Sci U S A 119(49):e2204259119. https://doi.org/10.1073/pnas.2204259119

Chen X, Liu J, Janssen JM, Gonçalves MA (2017) The chromatin structure differentially impacts high-specificity CRISPR-Cas9 nuclease strategies. Mol Ther-Nucleic Acids 8:558–563

Chiera F, Meccia E, Degan P, Aquilina G, Pietraforte D, Minetti M, Lambeth D, Bignami M (2008) Overexpression of human NOX1 complex induces genome instability in mammalian cells. Free Radic Biol Med 44(3):332–342. https://doi.org/10.1016/j.freeradbiomed.2007.09.018

Choi E-H, Yoon S, Koh YE, Seo Y-J, Kim KP (2020) Maintenance of genome integrity and active homologous recombination in embryonic stem cells. Exp Mol Med 52(8):1220–1229

Choi SH, Lee MH, Jin DM, Ju SJ, Ahn WS, Jie EY, Lee JM, Lee J, Kim CY, Kim SW (2021) TSA promotes CRISPR/Cas9 editing efficiency and expression of cell division-related genes from plant protoplasts. Int J Mol Sci 22(15):7817

Cody WB, Scholthof HB, Mirkov TE (2017) Multiplexed gene editing and protein overexpression using a tobacco mosaic virus viral vector. Plant Physiol 175(1):23–35

Cong L, Ran FA, Cox D, Lin S, Barretto R, Habib N, Hsu PD, Wu X, Jiang W, Marraffini LA (2013) Multiplex genome engineering using CRISPR/Cas systems. Science 339(6121):819–823

Daniel RM, Danson MJ, Eisenthal R (2001) The temperature optima of enzymes: a new perspective on an old phenomenon. Trends Biochem Sci 26(4):223–225. https://doi.org/10.1016/s0968-0004(01)01803-5

Delcuve GP, Khan DH, Davie JR (2012) Roles of histone deacetylases in epigenetic regulation: emerging paradigms from studies with inhibitors. Clin Epigenetics 4:1–13

Demirer GS, Zhang H, Matos JL, Goh NS, Cunningham FJ, Sung Y, Chang R, Aditham AJ, Chio L, Cho MJ, Staskawicz B, Landry MP (2019) High aspect ratio nanomaterials enable delivery of functional genetic material without DNA integration in mature plants. Nat Nanotechnol 14(5):456–464. https://doi.org/10.1038/s41565-019-0382-5

Earley KW, Shook MS, Brower-Toland B, Hicks L, Pikaard CS (2007) In vitro specificities of Arabidopsis co-activator histone acetyltransferases: implications for histone hyperacetylation in gene activation. Plant J 52(4):615–626

Ellison EE, Nagalakshmi U, Gamo ME, Huang PJ, Dinesh-Kumar S, Voytas DF (2020) Multiplexed heritable gene editing using RNA viruses and mobile single guide RNAs. Nat Plants 6(6):620–624. https://doi.org/10.1038/s41477-020-0670-y

Gaj T, Sirk SJ, Shui S-L, Liu J (2016) Genome-editing technologies: principles and applications. Cold Spring Harb Perspect Biol 8(12):a023754

Gentzel IN, Ohlson EW, Redinbaugh MG, Wang G-L (2022) VIGE: virus-induced genome editing for improving abiotic and biotic stress traits in plants. Stress Biol 2(1):2. https://doi.org/10.1007/s44154-021-00026-x

Guilinger JP, Thompson DB, Liu DR (2014) Fusion of catalytically inactive Cas9 to FokI nuclease improves the specificity of genome modification. Nat Biotechnol 32(6):577–582

Gutierrez C (1999) Geminivirus DNA replication. Cell Mol Life Sci CMLS 56:313–329

Hamilton W, Stein V, Coutts R, Buck K (1984) Complete nucleotide sequence of the infectious cloned DNA components of tomato golden mosaic virus: potential coding regions and regulatory sequences. Embo J 3(9):2197–2205

Hanley-Bowdoin L, Settlage SB, Orozco BM, Nagar S, Robertson D (2000) Geminiviruses: models for plant DNA replication, transcription, and cell cycle regulation. Crit Rev Biochem Mol Biol 35(2):105–140

Hiei Y, Ohta S, Komari T, Kumashiro T (1994) Efficient transformation of rice (*Oryza sativa* L.) mediated by agrobacterium and sequence analysis of the boundaries of the T-DNA. Plant J. 6(2):271–282

Hong L, Schroth G, Matthews H, Yau P, Bradbury E (1993) Studies of the DNA binding properties of histone H4 amino terminus. Thermal denaturation studies reveal that acetylation markedly reduces the binding constant of the H4 tail to DNA. J Biol Chem 268(1):305–314

Hong S, Joo JH, Yun H, Kim K (2019) The nature of meiotic chromosome dynamics and recombination in budding yeast. J Microbiol 57:221–231

Hormuzdi SG, Bisaro DM (1995) Genetic analysis of beet curly top virus: examination of the roles of L2 and L3 genes in viral pathogenesis. Virology 206(2):1044–1054. https://doi.org/10.1006/viro.1995.1027

Hu Y, Parekh-Olmedo H, Drury M, Skogen M, Kmiec EB (2005) Reaction parameters of targeted gene repair in mammalian cells. Mol Biotechnol 29:197–210

Huen MS, Li X-T, Lu L-Y, Watt RM, Liu D-P, Huang J-D (2006) The involvement of replication in single stranded oligonucleotide-mediated gene repair. Nucleic Acids Res 34(21):6183–6194

Huen MS, Lu L-Y, Liu D-P, Huang J-D (2007) Active transcription promotes single-stranded oligonucleotide mediated gene repair. Biochem Biophys Res Commun 353(1):33–39

Isaac RS, Jiang F, Doudna JA, Lim WA, Narlikar GJ, Almeida R (2016) Nucleosome breathing and remodeling constrain CRISPR-Cas9 function. Elife 5:e13450

Jang G, Joung YH (2019) CRISPR/Cas-mediated genome editing for crop improvement: current applications and future prospects. Plant Biotechnol Rep 13:1–10

Jeske H (2009) Geminiviruses. TT viruses: the still elusive human pathogens. Springer, Berlin, pp 185–226

Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E (2012) A programmable dual-RNA–guided DNA endonuclease in adaptive bacterial immunity. Science 337(6096):816–821

Joung JK, Sander JD (2013) TALENs: a widely applicable technology for targeted genome editing. Nat Rev Mol Cell Biol 14(1):49–55

Kim S, Kim D, Cho SW, Kim J, Kim JS (2014) Highly efficient RNA-guided genome editing in human cells via delivery of purified Cas9 ribonucleoproteins. Genome Res 24(6):1012–1019. https://doi.org/10.1101/gr.171322.113

Kimura S, Sinha N (2008) Tomato transformation. Cold Spring Harb Protoc 2008(11):pdb. prot5084

King AM, Lefkowitz E, Adams MJ, Carstens EB (2011) Virus taxonomy: ninth report of the international committee on taxonomy of viruses. Elsevier, The Netherlands

Koncz C, Martini N, Mayerhofer R, Koncz-Kalman Z, Körber H, Redei GP, Schell J (1989) High-frequency T-DNA-mediated gene tagging in plants. Proc Natl Acad Sci 86(21):8467–8471

Kujur S, Senthil-Kumar M, Kumar R (2021) Plant viral vectors: expanding the possibilities of precise gene editing in plant genomes. Plant Cell Rep 40(6):931–934. https://doi.org/10.1007/s00299-021-02697-2

Lawrenson T, Shorinola O, Stacey N, Li C, Østergaard L, Patron N, Uauy C, Harwood W (2015) Induction of targeted, heritable mutations in barley and Brassica oleracea using RNA-guided Cas9 nuclease. Genome Biol 16:1–13

LeBlanc C, Zhang F, Mendez J, Lozano Y, Chatpar K, Irish VF, Jacob Y (2018) Increased efficiency of targeted mutagenesis by CRISPR/Cas9 in plants using heat stress. Plant J 93(2):377–386. https://doi.org/10.1111/tpj.13782

Lei J, Dai P, Li Y, Zhang W, Zhou G, Liu C, Liu X (2021) Heritable gene editing using FT mobile guide RNAs and DNA viruses. Plant Methods 17(1):20. https://doi.org/10.1186/s13007-021-00719-4

Li G, Zhang X, Wang H, Liu D, Li Z, Wu Z, Yang H (2020) Increasing CRISPR/Cas9-mediated homology-directed DNA repair by histone deacetylase inhibitors. Int J Biochem Cell Biol 125:105790

Li T, Hu J, Sun Y, Li B, Zhang D, Li W, Liu J, Li D, Gao C, Zhang Y, Wang Y (2021) Highly efficient heritable genome editing in wheat using an RNA virus and bypassing tissue culture. Mol Plant 14(11):1787–1798. https://doi.org/10.1016/j.molp.2021.07.010

Liang Z, Chen K, Li T, Zhang Y, Wang Y, Zhao Q, Liu J, Zhang H, Liu C, Ran Y, Gao C (2017) Efficient DNA-free genome editing of bread wheat using CRISPR/Cas9 ribonucleoprotein complexes. Nat Commun 8(1):14261. https://doi.org/10.1038/ncomms14261

Lin Z, Kong H, Nei M, Ma H (2006) Origins and evolution of the recA/RAD51 gene family: evidence for ancient gene duplication and endosymbiotic gene transfer. Proc Natl Acad Sci U S A 103(27):10328–10333. https://doi.org/10.1073/pnas.0604232103

Lindbo JA (2007) TRBO: a high-efficiency tobacco mosaic virus RNA-based overexpression vector. Plant Physiol 145(4):1232–1240

Liu B, Chen S, Rose AL, Chen D, Cao F, Zwinderman M, Kiemel D, Aïssi M, Dekker FJ, Haisma HJ (2020) Inhibition of histone deacetylase 1 (HDAC1) and HDAC2 enhances CRISPR/Cas9 genome editing. Nucleic Acids Res 48(2):517–532

Liu D, Xuan S, Prichard LE, Donahue LI, Pan C, Nagalakshmi U, Ellison EE, Starker CG, Dinesh-Kumar SP, Qi Y, Voytas DF (2022a) Heritable base-editing in *Arabidopsis* using RNA viral

vectors. Plant Physiol 189(4):1920–1924. https://doi.org/10.1093/plphys/kiac206
Liu N, Zhou L, Lin G, Hu Y, Jiao Y, Wang Y, Liu J, Yang S, Yao S (2022b) HDAC inhibitors improve CRISPR-Cas9 mediated prime editing and base editing. Mol Ther-Nucleic Acids 29:36–46
Liu X, Yang S, Yu C-W, Chen C-Y, Wu K (2016) Histone acetylation and plant development. Enzymes 40:173–199
Liu Y, Han J, Chen Z, Wu H, Dong H, Nie G (2017) Engineering cell signaling using tunable CRISPR–Cpf1-based transcription factors. Nat Commun 8(1):2095
MacDowell S, Coutts RH, Buck K (1986) Molecular characterisation of subgenomic single-stranded and double-stranded DNA forms isolated from plants infected with tomato golden mosaic virus. Nucleic Acids Res 14(20):7967–7984
Mali P, Aach J, Stranges PB, Esvelt KM, Moosburner M, Kosuri S, Yang L, Church GM (2013) CAS9 transcriptional activators for target specificity screening and paired nickases for cooperative genome engineering. Nat Biotechnol 31(9):833–838
Mao Y, Botella JR, Liu Y, Zhu J-K (2019) Gene editing in plants: progress and challenges. Natl Sci Rev 6(3):421–437
McGaw C, Garrity AJ, Munoz GZ, Haswell JR, Sengupta S, Keston-Smith E, Hunnewell P, Ornstein A, Bose M, Wessells Q, Jakimo N, Yan P, Zhang H, Alfonse LE, Ziblat R, Carte JM, Lu WC, Cerchione D, Hilbert B, Chong S (2022) Engineered Cas12i2 is a versatile high-efficiency platform for therapeutic genome editing. Nat Commun 13(1):2833. https://doi.org/10.1038/s41467-022-30465-7
Menz J, Modrzejewski D, Hartung F, Wilhelm R, Sprink T (2020) Genome edited crops touch the market: a view on the global development and regulatory environment. Front Plant Sci 11:586027
Molla KA, Shih J, Wheatley MS, Yang Y (2022) Predictable NHEJ insertion and assessment of HDR editing strategies in plants [brief research report]. Front Genome Editing. https://doi.org/10.3389/fgeed.2022.825236
Molla KA, Shih J, Yang Y (2020) Single-nucleotide editing for zebra3 and wsl5 phenotypes in rice using CRISPR/Cas9-mediated adenine base editors. aBIOTECH 1(2):106–118
Molla KA, Sretenovic S, Bansal KC, Qi Y (2021) Precise plant genome editing using base editors and prime editors. Nat Plants 7(9):1166–1187. https://doi.org/10.1038/s41477-021-00991-1
Nagalakshmi U, Meier N, Liu J-Y, Voytas DF, Dinesh-Kumar SP (2022) High-efficiency multiplex biallelic heritable editing in *Arabidopsis* using an RNA virus. Plant Physiol 189(3):1241–1245. https://doi.org/10.1093/plphys/kiac159
Nekrasov V, Staskawicz B, Weigel D, Jones JD, Kamoun S (2013) Targeted mutagenesis in the model plant *Nicotiana benthamiana* using Cas9 RNA-guided endonuclease. Nat Biotechnol 31(8):691–693
Oei AL, Vriend LE, Crezee J, Franken NA, Krawczyk PM (2015) Effects of hyperthermia on DNA repair pathways: one treatment to inhibit them all. Radiat Oncol 10:165. https://doi.org/10.1186/s13014-015-0462-0
Pausch P, Al-Shayeb B, Bisom-Rapp E, Tsuchida CA, Li Z, Cress BF, Knott GJ, Jacobsen SE, Banfield JF, Doudna JA (2020) CRISPR-CasΦ from huge phages is a hypercompact genome editor. Science 369(6501):333–337
Pecinka A, Dinh HQ, Baubec T, Rosa M, Lettner N, Mittelsten Scheid O (2010) Epigenetic regulation of repetitive elements is attenuated by prolonged heat stress in *Arabidopsis*. Plant Cell 22(9):3118–3129. https://doi.org/10.1105/tpc.110.078493
Pilartz M, Jeske H (1992) Abutilon mosaic geminivirus double-stranded DNA is packed into minichromosomes. Virology 189(2):800–802
Podevin N, Davies HV, Hartung F, Nogué F, Casacuberta JM (2013) Site-directed nucleases: a paradigm shift in predictable, knowledge-based plant breeding. Trends Biotechnol 31(6):375–383
Raja P, Sanville BC, Buchmann RC, Bisaro DM (2008) Viral genome methylation as an epigenetic defense against geminiviruses. J Virol 82(18):8997–9007. https://doi.org/10.1128/jvi.00719-08
Raja V, Majeed U, Kang H, Andrabi KI, John R (2017) Abiotic stress: Interplay between ROS, hormones and MAPKs. Environ Exp Bot 137:142–157. https://doi.org/10.1016/j.envexpbot.2017.02.010
Scholthof HB, Scholthof K-BG, Jackson AO (1996) Plant virus gene vectors for transient expression of foreign proteins in plants. Annu Rev Phytopathol 34(1):299–323
Scholthof K-BG, Mirkov TE, Scholthof HB (2002) Plant virus gene vectors: biotechnology applications in agriculture and medicine. Genetic engineering: principles and methods. Springer, Boston, pp 67–85
Silva G, Poirot L, Galetto R, Smith J, Montoya G, Duchateau P, Pâques F (2011) Meganucleases and other tools for targeted genome engineering: perspectives and challenges for gene therapy. Curr Gene Ther 11(1):11–27
Suwaki N, Klare K, Tarsounas M (2011) RAD51 paralogs: roles in DNA damage signalling, recombinational repair and tumorigenesis. Semin Cell Dev Biol 22(8):898–905. https://doi.org/10.1016/j.semcdb.2011.07.019
Tandon N, Ramakrishnan V, Kumar SK (2016) Clinical use and applications of histone deacetylase inhibitors in multiple myeloma. Clin Pharmacol: Adv Appl 8:35–44
Tiricz H, Nagy B, Ferenc G, Török K, Nagy I, Dudits D, Ayaydin F (2018) Relaxed chromatin induced by histone deacetylase inhibitors improves the oligonucleotide-directed gene editing in plant cells. J Plant Res 131:179–189
Urnov F, Rebar E, Holmes M, Zhang HS, Gregory PD (2010) Genome editing with engineered zinc finger nucleases. Nature Rev Genet 11:636–646
Varanda CMR, Félix MDR, Campos MD, Patanita M, Materatski P (2021) Plant viruses: from targets to tools for CRISPR. Viruses 13(1):141
Verkuijl SA, Rots MG (2019) The influence of eukaryotic chromatin state on CRISPR–Cas9 editing efficiencies. Curr Opin Biotechnol 55:68–73
Wimberger S, Akrap N, Firth M, Brengdahl J, Engberg S, Schwinn MK, Slater MR, Lundin A, Hsieh P-P, Li S (2023) Simultaneous inhibition of DNA-PK and Polϴ improves integration efficiency and precision of genome editing. Nat Commun 14(1):4761
Woo JW, Kim J, Kwon SI, Corvalán C, Cho SW, Kim H, Kim S-G, Kim S-T, Choe S, Kim J-S (2015) DNA-free genome editing in plants with preassembled CRISPR-Cas9 ribonucleoproteins. Nat Biotechnol 33(11):1162–1164
Xiang G, Zhang X, An C, Cheng C, Wang H (2017) Temperature effect on CRISPR-Cas9 mediated genome editing. J Genet Genom 44(4):199–205. https://doi.org/10.1016/j.jgg.2017.03.004
Yarrington RM, Verma S, Schwartz S, Trautman JK, Carroll D (2018) Nucleosomes inhibit target cleavage by CRISPR-Cas9 in vivo. Proc Natl Acad Sci 115(38):9351–9358
Ye M, Yao M, Li C, Gong M (2023) Salt and osmotic stress can improve the editing efficiency of CRISPR/Cas9-mediated genome editing system in potato. PeerJ 11:e15771. https://doi.org/10.7717/peerj.15771
Yin K, Han T, Liu G, Chen T, Wang Y, Yu AYL, Liu Y (2015) A geminivirus-based guide RNA delivery system for CRISPR/Cas9 mediated plant genome editing. Sci Rep 5(1):14926
Yu C, Liu Y, Ma T, Liu K, Xu S, Zhang Y, Liu H, La Russa M, Xie M, Ding S (2015) Small molecules enhance CRISPR genome editing in pluripotent stem cells. Cell Stem Cell 16(2):142–147
Zerbini FM, Briddon RW, Idris A, Martin DP, Moriones E, Navas-Castillo J, Rivera-Bustamante R, Roumagnac P, Varsani A

(2017) ICTV virus taxonomy profile: *Geminiviridae*. J Gen Virol 98(2):131

Zetsche B, Gootenberg JS, Abudayyeh OO, Slaymaker IM, Makarova KS, Essletzbichler P, Volz SE, Joung J, Van Der Oost J, Regev A (2015) Cpf1 is a single RNA-guided endonuclease of a class 2 CRISPR-Cas system. Cell 163(3):759–771

Zhang F, Maeder ML, Unger-Wallace E, Hoshaw JP, Reyon D, Christian M, Li X, Pierick CJ, Dobbs D, Peterson T (2010) High frequency targeted mutagenesis in *Arabidopsis thaliana* using zinc finger nucleases. Proc Natl Acad Sci 107(26):12028–12033

Zhang Y, Liang Z, Zong Y, Wang Y, Liu J, Chen K, Qiu J-L, Gao C (2016) Efficient and transgene-free genome editing in wheat through transient expression of CRISPR/Cas9 DNA or RNA. Nat Commun 7(1):12617

Zhang Y, Malzahn AA, Sretenovic S, Qi Y (2019) The emerging and uncultivated potential of CRISPR technology in plant science. Nat Plants 5(8):778–794. https://doi.org/10.1038/s41477-019-0461-5

Ziemienowicz A, Shim YS, Matsuoka A, Eudes F, Kovalchuk I (2012) A novel method of transgene delivery into triticale plants using the agrobacterium transferred DNA-derived nano-complex. Plant Physiol 158(4):1503–1513. https://doi.org/10.1104/pp.111.192856

Zipfel C, Kunze G, Chinchilla D, Caniard A, Jones JD, Boller T, Felix G (2006) Perception of the bacterial PAMP EF-Tu by the receptor EFR restricts agrobacterium-mediated transformation. Cell 125(4):749–760. https://doi.org/10.1016/j.cell.2006.03.037