Biotechnol Lett (2020) 42:1611–1632
https://doi.org/10.1007/s10529-020-02950-w

REVIEW

A review of CRISPR associated genome engineering: application, advances and future prospects of genome targeting tool for crop improvement

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Received: 14 October 2019 / Accepted: 25 June 2020 / Published online: 8 July 2020

Abstract The Cas9 nuclease initiates double-stranded breaks at the target position in DNA, which are repaired by the intracellular restoration pathways to eliminate or insert pieces of DNA. CRISPR-Cas9 is proficient and cost-effective since cutting is guided by a piece of RNA instead of protein. Emphasis on this technology, in contrast with two recognized genome editing platforms (i.e., ZFNs and TALENs), is provided. This review evaluates the benefits of chemically synthesized gRNAs as well as the integration of chemical amendments to improve gene editing efficiencies. CRISPR is an indispensable means in biological investigations and is now as well transforming varied fields of biotechnology and agriculture. Recent advancement in targetable epigenomic-editing tools allows researchers to dispense direct functional and transcriptional significance to locus-explicit chromatin adjustments encompassing gene regulation and editing. An account of diverse sgRNA design tools is provided, principally on their target competence prediction model, off-target recognition algorithm, and generation of instructive annotations. The modern systems that have been utilized to deliver CRISPR-Cas9 in vivo and in vitro for crop improvement viz. nutritional enhancement, production of drought-tolerant and disease-resistant plants, are also highlighted. The conclusion is focused on upcoming directions, biosafety concerns, and expansive prospects of CRISPR technologies.

Keywords Cas9 nucleases · CRISPR-Cas9 · sgRNA · ZFNs · TALENs · Biotechnology · Epigenomic editing

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Introduction

Genome editing is a kind of genetic engineering mechanism wherein DNA is introduced, obliterated, modified or substituted in the genome of a living individual. Homologous recombination is the foundation of genome engineering, but its occurrence at low frequencies limits the editing efficiency (Chen et al. 2019). To improve editing frequency, researchers took over the utility of enzyme endonucleases that intricate to restore DNA double-stranded breaks (DSB). There are various genome altering technologies like zinc finger nuclease (ZFN) and transcription activator-like effector nuclease (TALEN) already been discussed for targeted modifications of the genome (Zhang et al. 2010; Adli 2018).

Clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated9 (Cas9) technology is being widely used to incorporate high

specificity and activity, at the preferred target locus. As endonucleases, Cas proteins are known to use a single guide RNA (sgRNA) to make complementary base pairs with target DNA followed by cutting the DNA at explicit sites (Agrotis and Ketteler 2015). Supposedly, using CRISPR a method can be developed to engineer just about any DNA sequence in the genome as it offers flexibility, easy multiplexing, and scaling. Nowadays, its applications have reached a variety of fields, counting biotechnology, biological investigation, human medicinal application, and agricultural research (Veres et al. 2014; Chen et al. 2019).

The guide RNA (gRNA) of the CRISPR-Cas9 system is the RNA element that possibly comprises either the chimeric sgRNA or the dual RNAs (crRNA:tracrRNA) (Arroyo et al. 2016). The gRNAs can be swiftly created by the use of chemical synthesis methods and offer correspondent characteristics and advantages, such as integration of chemical modifications to improve on-target precision, gene editing proficiencies, and genome-scale high throughput range analysis for practical genomic studies (Ryan et al. 2018). The CRISPR-Cas9 genome complex and epigenome editing can be introduced into living cells for precise and dynamic manipulation of an epigenetic state that would facilitate its employment in plants.

Abiotic stresses such as drought, salinity, etc., can decrease crop yield up to 50% (Afzal et al. 2019). CRISPR technology has been used to study some significant drought stress-related genes such as AREB1 and OsSAPK2 in Arabidopsis and rice, respectively (Shinwari et al. 2020). It has been documented that CRISPR is used to manipulate the genome of different plant species, including *Arabidopsis*, *Medicago truncatula,* tomato, potato, wheat, corn, rice, and mushroom (Khatodia et al. 2016; Gong et al. 2002; Papikian et al. 2019). Some mainstream problems allied with nucleic acid-based application analysis are off-target effects, ethical concerns, and a need for safe and proficient delivery systems. Although several methods have been developed to detect the off-target mutations such as SITE-seq, Digenome-seq, GUIDE-seq, and DISCOVER-seq, etc. (Wada et al. 2020) yet these major bottlenecks exist in plant system. Hence the emphasis is given on the current modern systems developed to transport and consequently deliver CRISPR in vivo and in vitro for a variety of advantageous applications.

In the present communication, the salient features of the CRISPR-Cas9 system, a comprehensive comparison, as well as chemical synthesis and modifications of the sgRNA elements are discussed. A brief description of the bioinformatics tools used to design sgRNA is also mentioned. Epigenetic changes, regulation mechanisms, and their possible implications in the plants are highlighted. Finally, the focus is laid on possible delivery strategies and genome editing applications in plants.

A comparative mechanism of genome editing by CRISPR-Cas9, ZFNs, and TALENs

Technologies for the introduction of site-specific alterations and amendments in the genome of cells and individuals remain exclusive. Supplementary examples of programmable genome editing machinery consist of TALENs and ZFNs (Fig. 1). TALENs and ZFNs function as dimers and only the protein components are required.

A ZFN is a heterodimer in which every subunit comprises a zinc finger domain and a FokI endonuclease domain (Urna et al. 2010). Genome editing by ZFNs has been demonstrated in plants, including rice and Arabidopsis (Ainley et al. 2013; Gallego-Bartolome et al. 2019). ZFNs are effectual genome editing elements; however, they were not extensively adopted because of the complexity in nature of the contact between zinc fingers and DNA. Other limitations include the inherent difficulty in designing, interest-dependent specificity, and difficulty in authenticating such proteins for a particular DNA locus of context (Sander et al. 2011).

TALENs are dimeric transcription nucleases or factor built from arrays of 33 to 35 amino acid modules, each one of which is targeted to a single nucleotide. Researchers can easily design TALENs because there is a one-to-one recognition convention among protein repeats and nucleotide sequences; hence it can target nearly any sequence of interest presently by assembling the arrays (Luo et al. 2019). TALENs has been used to edit the genomes of a wide variety of plants, including barley (Budhagatapalli et al. 2015), rice (Shan et al. 2015), soybean (Du et al. 2016), sugarcane (Jung and Altpeter 2016), maize (Char et al. 2015), and potato (Clasen et al. 2016). TALENs were simpler to construct and authenticate,

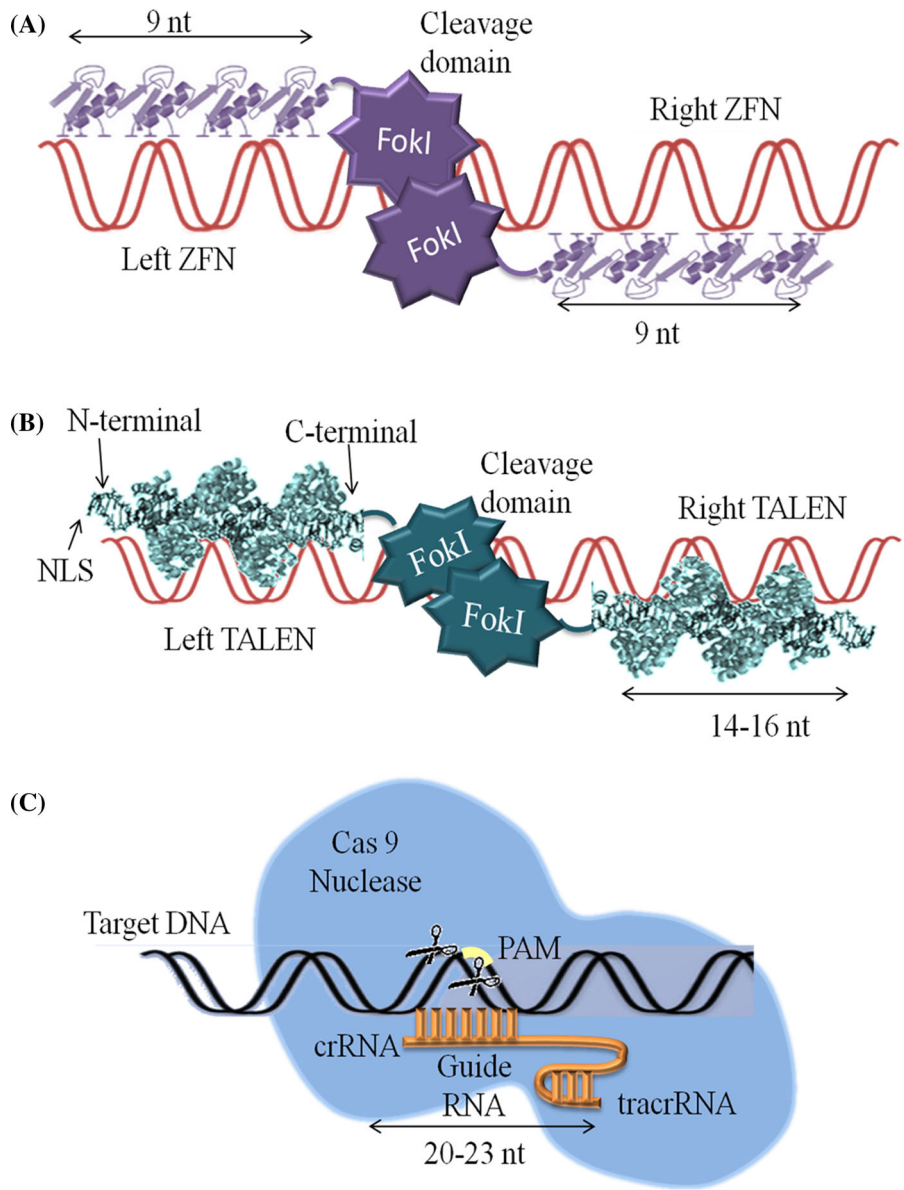


Fig. 1 A diagrammatic evaluation of various pliable sequence explicit genome editing nucleases that cleaves adjoining DNA sequences to generate nicks on corresponding strands: **A** Zinc-finger nucleases (ZFNs) are dimer, with every monomer comprising of DNA binding domain (3–6 zinc finger recurs identifying 9–18 nucleotides) and type II restriction endonuclease Fok1 domain. **B** Transcription activator-like nucleases (TALENs) are dimers with every subunit consisting of DNA binding domain (conserved, 23–28 amino acid sequence explicit for each nucleotide) and Fok1 nuclease domain. **C** CRISPR/Cas9: Cas9 naturally evolved, RNA-guided endonuclease directed by sgRNA, (crRNA and tracrRNA) for precise objective cleavage. It recognizes about 20 nucleotide recognition spot upstream of protospacer adjacent motif (PAM) of its DNA target

facilitating an inexpensive, faster method of genome editing; however, the difficulties in synthesis, protein designing, and corroboration remained an obstruction to its extensive adoption in genome editing applications.

CRISPR-Cas consists of a single distinct monomeric protein and a chimeric RNA unit. Unlike ZFNs or TALENs, CRISPR-Cas is like a DNA-targeted form of RNA interference. CRISPR-Cas has revolutionized the genome-editing field as it is simple, inexpensive,

easily programmed. It is also well efficient, as only 20 nucleotides in the gRNA need to be customized to identify a diverse target. The targeting of endonucleases to a specific locus results in DNA cleavage and induces the cell to undertake homology-directed repair (HDR), microhomology-mediated end joining (MMEJ) or non-homologous end joining (NHEJ). HDR occurs as a repair template-specific desired genomic modification that enables precise editing (Bassett et al. 2013). MMEJ is an error-prone repair system that involves the arrangement of microhomologous sequences internal to broken ends prior to joining and is coupled with insertions and deletions (Yanik et al. 2018). In the case of NHEJ, no DNA repair template is provided, and its error-prone nature often leads to inactivating mutations (Chen et al. 2019) (Fig. 2). Some other repair mechanisms also exist like single-stranded annealing (SSA) pathway of HDR, which requires only a single DNA duplex and uses the repeat sequences as the identical sequences as in HDR (Yanik et al. 2018). A specialized form of MMEJ is known as polymerase theta-mediated end joining (TMEJ) and can repair breaks using ≥ 1 bp of homology (Schimmel et al. 2019).

Chemical synthesis of the guide RNA

The RNA unit of the CRISPR-Cas9 complex can be created enzymatically or via the chemical synthesis process (Helm et al. 1999). Enzymatic synthesis is a cost-effective method, and the process of in vitro transcription requires a DNA template, T7, T3, or SP6RNA polymerases and ribonucleoside triphosphates. A 5′-triphosphate remains on the gRNA after transcription that necessitates elimination by phosphatase enzyme following purification (Cho et al. 2013). Solid-phase synthesis chemistry is used to create synthetic gRNAs. There is greater flexibility in time consumption, yield, length, and higher precision in the synthesized RNAs with no obligation for several cloning and sequencing steps. Chemical production of gRNAs employs amalgamation (solid phase) through nucleoside phosphoramidite structure blocks for constructing gRNA (Kelly et al. 2016). 2′-Silyl (2′-TBDMS, 2′-TOM), 2′-*O*-thionocarbamate (TC) (Cullot et al. 2019) and 2′-bis(acetoxyethoxy)-methyl ether (2′-ACE) (Scaringe et al. 1998) are some of the RNA synthesis chemistries offered.

Researchers successfully generated gRNA sequences intended for *Streptococcus pyogenes* Cas9 structures using a chemical synthesis approach (Anderson et al. 2015). A two-RNA approach with a crRNA and tracrRNA to program Cas9 or a sgRNA approach can be used. Conventional chemistries such as 2′-TOM or TBDMS (Pitsch et al. 2001) are capable of synthesizing RNA > 70 bases whereas long RNA ($\sim$ 150 nucleotides) are characteristically synthesized employing TC or 2′-ACE chemistries (Cullot et al. 2019). Jinek et al. (2012) reported that in *S. pyogenes*, the crRNA, tracrRNA, and sgRNA are in the order of 40, 70, and 100 nucleotides in length, respectively. Hence use of 2′-ACE or TC chemistries is ideal for synthesis providing high throughput, greater purity, rapid coupling rates, and higher production than any other RNA chemistries.

The tools used for CRISPR/Cas9 designing

The precision of the CRISPR-Cas system depends on well-designed sgRNA as it is a critical aspect of the successful editing of target genes. The design tools differ in parameters and design specifications, predominantly highlighting the on-target efficacy calculation models and off-target calculation algorithms to ultimately improve sgRNA specificity (Zhu 2015).

Various computational tools have been created to design sgRNA with improved specificity and efficiency. Certain representatives are described (Table 1) that has been developed to assemble information and offer useful purpose to study CRISPR-Cas organization. Wong et al. (2015) developed WU-CRISPR; the program is suggested for its ease of use and proficient sgRNA design using a machine learning technique. The tool recognizes several sequences and structural arrangements from Doench's dataset (Doench et al. 2014) and constructs a sgRNA effective estimate model with SVM. Chari dataset was used to assess and compare the tool for superlative performance (Chari et al. 2015). The preceding off-target scoring process cannot be quickly contained through organisms, so the researchers wished for a novel procedure to evaluate the off-target action and termed it CASPER (Mendoza and Trinh 2017). The model was derived from Hsu–Zhang matrix and appraised for its off-target activity even in the absence of adequate experimental statistics (Hsu et al. 2013).

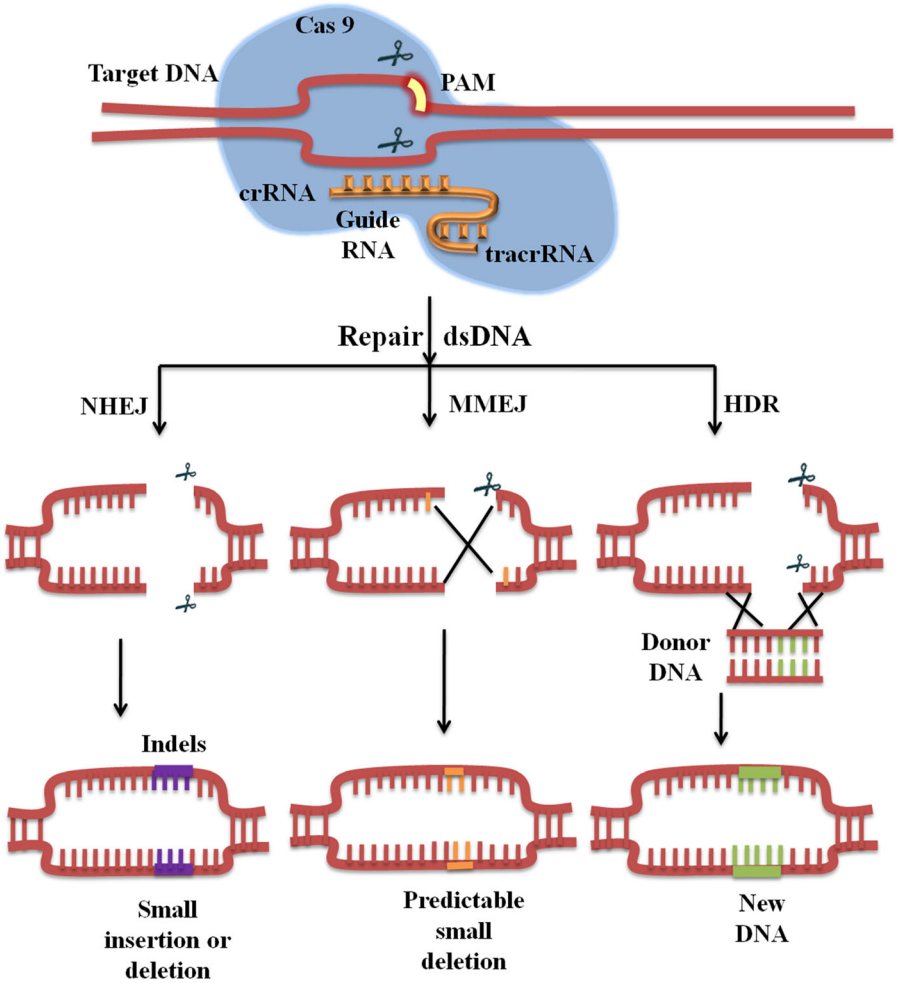


Fig. 2 A diagrammatic representation of Cas9 in genomic editing with endogenous cellular site-specific nucleases: The double stranded breaks (DSBs) generated by CRISPR/Cas9 system be repaired by non-homologous end joining (NHEJ), microhomology-mediated end joining (MMEJ) or homologous recombination (HR) pathways. NHEJ produces random insertions or deletions (indels) of random base pairs as a result of homozygous, heterozygous or biallelic mutations. Diminutive microhomologies (~ 5–25 bp) bordering DSB recombine through MMEJ, resulting in deletion amid homology arms. HDR can produce desired precise nucleotide substitution mutations or indels by homologous recombination guided through donor DNA digested with the identical endonuclease following related overhangs

Additionally, several other tools are also reported, such as Cas-OFFinder (Baltes et al. 2014), SSFinder (Upadhyay and Sharma 2014), CRISPR-P (Lei et al. 2015), and Cas OT (Xiao et al. 2014) that eases the sgRNA designing process. The assembly of expression vectors and delivery of those vectors in plant systems involves the use of diverse methodologies essential for amplifying editing efficiencies. There is still a great deal to be experimented and optimized for the exploitation of CRISPR-Cas9 in plant systems.

CRISPR mediated epigenetic regulations in plants

Examining the usage of the Cas9 system to inspect regulatory sequences that can transform gene expression through epigenetic mechanisms, and chromatin modifications are the outcomes of the discovery of a versatile RNA-guided DNA-targeting platform (Naito et al. 2015). Canver et al. (2017) showed that the majority of the gRNAs did not affect gene expression regulation when aimed to create indel mutations in recognized enhancer regions. This led to the understanding that only a few critical domains are

Table 1 CRISPR design tools used in different systems

S.No	Analytical tools	Genome specificity	Link/ web address	Main features of tools	Species/ cell line tested	User input sequence	Cas 9 source	Protospacer adjacent motif (PAM) available	Basis for results targeting	Provider	References
1.	Optimized CRISPR design	Yes	https://crispr.mit.edu	• Genome-wide possible off-targets analysis • Highlight guides having high aim specificity • Flagging guides having frequent or genic off-targets	Humans	DNA sequence	*Streptococcus pyogenes* Cas9	NGG and NAG	• Guide the assortment validation of aimed sequences • Off-target analysis	Zhang Lab, MIT	Hsu et al. (2013)
2.	CCTop	Yes	https://crispr.cos.uni-heidelberg.de	• Experimental authentication intended for gene inactivation, HDR and NJEH methods	Medaka (*Oryzias latipes* oryLat2), *Xenopus tropicalis* (JGI4.2/xenTro3), zebrafish (*Danio rerio* Zv9/danRer7), cavefish (*Astyanax mexicanus* AstMex102), stickleback (*Gasterosteus aculeatus* BROADS1/gasAcu1)	DNA sequence	11 PAMs	NGG, NRG, NNGRRT, NNNNGATT, NNAGAAW, NAAAAC	• Identifies positions and ranks the entire entrant sgRNA target sites concurrent to their off-target assessments • Presents complete documentation	University of Heidelberg	Stemmer et al. (2015)
3.	ChopChop	Yes	https://chopchop.cbu.uib.no/	• Allow broad range of inputs including genomic regions, gene identifiers and pasted sequences • Presents an array of sophisticated alternatives for target choice • Dynamic graphical output demonstration including an interactive illustration of the gene • Integration of TALEN and CRISPR-Cas9 design into one tool		RefSeq Gene ID Genomic region	*Streptococcus pyogenes* Cas9, Custom PAM	NGG, NNAGAA, NNNNGANN	• Efficient sequence alignment algorithms to decrease exploration time • Precise prediction of off-target hits	Harvard University	Montague et al. (2014)

Table 1 continued

S.No	Analytical tools	Genome specificity	Link/ web address	Main features of tools	Species/ cell line tested	User input sequence	Cas 9 source	Protospacer adjacent motif (PAM) available	Basis for results targeting	Provider	References
4.	CRISPOR	Yes	https://crispor.tefor.net/	• Optimal on-target efficacy prediction model • Dependent on gRNA expression from either a U6 promoter or transcribed in vitro		DNA Sequence Genomic Region	9 PAM	NGG, NGA, NGCG, NNAGAA, NGGNG, NNGRRT, NNNRRT, NNNNGMTT, NNNNACA, TTTN	• Predicts off-targets • Chooses and clones competent guide sequences • Use of eight efficient scoring systems	University of California, Santa Cruz	Haeussler et al. (2016)
5.	sgRNA designer	No	https://portals.broadinstitute.org/gpp/public/analysis tools/sgrna-design	• Off-target spots are estimated via the CFD (Cutting Frequency Determination) score count		DNA sequence Transcript ID Gene ID Gene Symbol	*S. pyogenes* and *S. aureus*	NGG	• Recent Microsoft execution of this scoring representation is Azimuth 2.0	Broad Institute	Doench et al. (2014)
6.	Off-Spotter		https://cm.jefferson.edu/Off-Spotter/	• Lacks a firm definition of the seed • User assertion of seed's position and extent on-the-fly				NGG, NAG, NNNNACA, NNGRRT (R is A or G)	• Output is represented as a histogram depicting the number of possible off-targets as a purpose of the mismatches number	Thomas Jefferson University	Pliatsika and Rigoutsos (2015)
7.	ChopChop v2 web tool	Yes	https://chopchop.rc.fas.harvard.edu/	• Support for custom length sgRNAs, to amplify targeting array as well as specificity • Assesses the sequence composition of sgRNA along with adjacent region by utilizing models assembled from manifold studies		RefSeq Gene ID Genomic region	Cpf1 and Cas9 nickases	User customizable	• Proficient sequence alignment algorithms to reduce search instant • Strictly predicts off-target binding	University of Bergen	Labun et al. (2016)

Table 1 continued

S.No	Analytical tools	Genome specificity	Link/ web address	Main features of tools	Species/ cell line tested	User input sequence	Cas 9 source	Protospacer adjacent motif (PAM) available	Basis for results targeting	Provider	References
8.	E-CRISP	Yes	https://www.ecrisp.org/ECRISP/	• Confirm target specificity of the presumed designs • Evaluation of genomic outlook viz. exons, transcripts and CpG islands		Gene ID Gene Symbol DNA sequence	Cas9		• S-score off-target scoring		Heigwer et al. (2014)
9.	CRISPR Finder				Human and mouse cell surface markers			User customizable		Geneious	Doench et al. (2014)
10.	CRISPRdirect	Yes	https://crispr.dbcls.jp/	• User can surf the detailed list of probable off-target sites having limited complementarity with the preferred sequence	*Arabidopsis*, *Sorghum*, *Oryza sativa* and budding yeast	DNA sequence Genomic region Accession number	Custom PAM	NNN, NGG, NRG	• Program investigates the complete genome for ideal matches of input target sequence (20 mer) along with the seed sequence (8 or 12 mer) neighboring the PAM	Database Center for Life Science (DBCLS)	Naito et al. (2015)

significant for enhancer function (Oldridge et al. 2015; Maurano et al. 2012). Sumoylation has majorly been linked to transcriptional repression mechanism (Decque et al. 2016). However, its functional roles are focused on proteasome-dependent proteolysis, activation of DNA damage signaling cascades (Wu et al. 2014), cellular localization, and assembly (Deshaies and Joazeiro 2009) fitting in the general principle of the dCas9-KRAS system.

Numerous reports simultaneously established the concept for up- or down-regulation of target genes drawn out by dCas9 fusions to catalytic domains, directing methylation and demethylation of CpG islands that span promoter regions (McDonald et al. 2016; Vojta et al. 2016). Similarly, evidence of the production of synergistic effects leading to increased methylation of the promoter by targeting multiple promoter sites and by co-expression of diverse sgRNAs is provided. The study was demonstrated using a dCas9-DNMT3A fusion (with a Gly_4Ser flexible linker). Which showed competent and precise CpG island methylation of the *BACH2* and *IL6ST* promoters (Vojta et al. 2016). In a Transgenic *Arabidopsis* plant overexpression of a chromatin remodeling gene *AtCHR12* shows evidence of growth termination in stem and bud. On the contrary, the response under unfavorable conditions was fewer in the AtCHR12-knockout mutant than in the wild type plants (Mlynarova et al. 2007). The current advancement in understanding various epigenetic control mechanisms and in developing effective and flexible tools to study these procedures makes it easy to exploit it for crop management and improvement (Fig. 3).

Gene/ genome editing applications using guide RNA for crop plant improvement

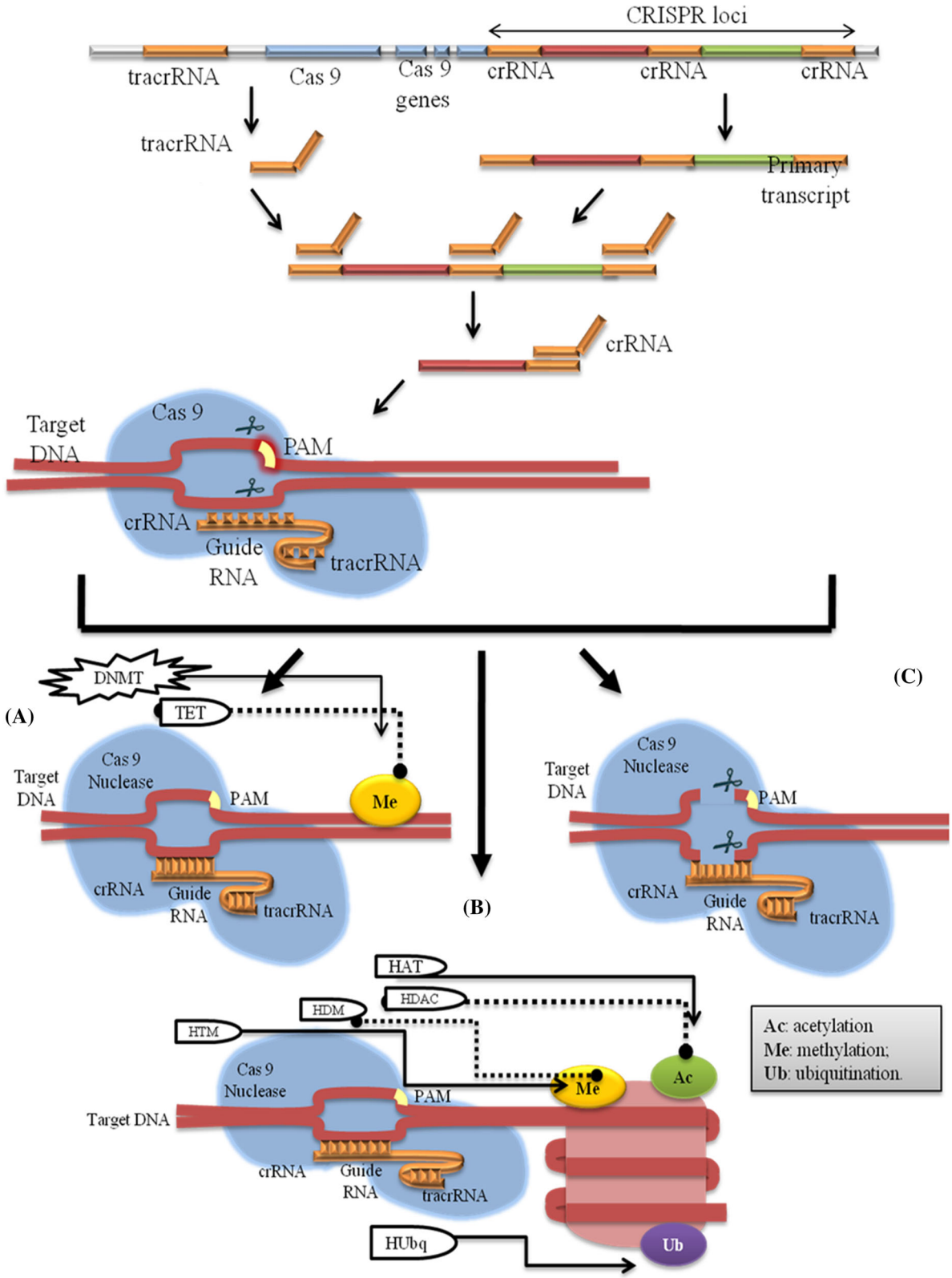
Genome editing by CRISPR is adaptable to edit any gene in any monocot or dicot plant species. CRISPR-Cas9 has already been used to improve tolerance to biotic pathogens (fungal, viral or bacterial), or abiotic stresses (cold, heat, drought, salt), enhance metabolic pathways, improve nutritional value, grain quality, increase shelf life, obtain haploid seeds, and upsurge agricultural yield (Wang et al. 2014, 2016). Decline of phytic acid content in maize (Liang et al. 2008) and the formation of acrylamide free potatoes (Halterman et al. 2016) has also been reported. Representative applications of CRISPR-Cas in plant improvement have been discussed in the subsequent section (Table 2).

In dicotyledons

Characteristic and evident research work has been done for the production of non-browning apples, and potatoes employing Polyphenol oxidase (PPO) gene mutant (Halterman et al. 2016). In a recent study, Ortigosa et al. (2018) reported the creation of a tomato variety resistant to the bacterial speck disease caused by *Pseudomonas syringae* pv. *tomato* (PtoDC3000) without reducing resistance to necrotrophs. The functional ortholog of AtJAZ2 in tomato favorably aggregates in stomata showing that SlJAZ2 is a key co-receptor of coronatine (COR) in stomatal guard cells. Using CRISPR-Cas9 SlJAZ2 was modified to create dominant JAZ2 repressors that lacked the C-terminal Jas domain (SlJAZ2Δjas) and disallowed stomatal reopening by COR providing resistance to PtoDC3000. Furthermore, it also established a novel CRISPR-Cas-built tactic for crop protection that could be employed in the field.

Li et al. (2017a) worked on a Chinese herb *Salvia miltiorrhiza* with documented vasorelaxation and antiarrhythmic properties. The researchers targeted the diterpene synthase gene (SmCPS1) concerned in the biosynthesis of tanshinone that utilizes geranylgeranyl diphosphate (GGPP) as substrate. The tanshinone biosynthesis metabolic flux was switched to the taxol synthesis pathway by using SmCPS1 knockout (post-GGPP synthesis step) mutants as GGPP is also a substrate for taxol biosynthesis. Three homozygous mutants with zero tanshinone accumulation and a decreased proportion of eight chimeric mutants were produced. Using CRISPR/Cas9-*Agrobacterium rhizogenes* mediated alteration from twenty-six independent transgenic hairy root lines of *Salvia*. Malzahn et al. (2019) demonstrated CRISPR-Cas12a mediated genome editing in two target genes (TT4 and GL2) in transgenic *Arabidopsis*. Cas12a was also used for targeted genome editing in *Nicotiana benthamiana, Solanum lycopersicum,* and *Arabidopsis thaliana* (Bernabé-Orts et al. 2019).

In an experiment, researchers demonstrated Cpf1-mediated gene targeting in protoplasts isolated from wild tobacco and soybean. The result led to effective mutational induction in AOC in wild tobacco and


CRISPR loci
tracrRNA
Cas 9
Cas 9 genes
crRNA
crRNA
crRNA
tracrRNA
Primary transcript
crRNA
Target DNA
Cas 9
PAM
crRNA
Guide RNA
tracrRNA
(A)
DNMT
TET
Target DNA
Cas 9 Nuclease
PAM
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crRNA
Guide RNA
tracrRNA
(B)
(C)
Target DNA
Cas 9 Nuclease
PAM
crRNA
Guide RNA
tracrRNA
HAT
HDAC
HDM
HTM
Cas 9 Nuclease
PAM
Me
Ac
Target DNA
crRNA
Guide RNA
tracrRNA
HUbq
Ub
Ac: acetylation
Me: methylation;
Ub: ubiquitination.

◀**Fig. 3** Method of CRISPR/Cas9 action and epigenetic manipulation based on the probability to allocate chromatin modifiers: CRISPR loci after incorporation of foreign DNA is transcribed into prime transcript and progressed into crRNA by aid of tracrRNA, later Cas9 intricate with a crRNA, cleaves foreign DNA. **a** Targeted relocation of transcriptional regulator-enzymes accountable for modification in the DNA methylation; DNMT, DNA methyltransferase; TET, ten-eleven translocation enzymes. **b** Targeted relocation of transcriptional regulator-histone modifiers; HDM, histone demethylase; HAT, histone acetyltransferase; HMT, histone methyltransferase; HDAC, histone deacyetylase; HUbq, histone ubiquitin ligase. **c** Gene knockout modification

FAD_2 paralogues in soybean (Kim et al. 2017). In *Solanum tuberosum* CRISPR-Cas9 was used to knock out the gene encoding granule-bound starch synthase (GBSS) by a single transfection. It resulted in the generation of amylopectin producing potato, which is an extremely required marketable trait (Andersson et al. 2017). An experimental study on CRISPR-Cas9 targeted modification in *Citrus sinensis* for disease resistance against *Xanthomonas citri* causing citrus canker was conducted. Deletion of the intact EBE_{PthA4} sequence series from susceptibility gene Lateral organ boundaries 1 (CsLOB1) alleles examined the intensity of resistance to wanjincheng orange as CsLOB1 promoter augments disease resistance (Peng et al. 2017). Chandrasekaran et al. (2016) created non-transgenic homozygotic mutant cucumber plants that were resistant to several viruses such as cucumber vein yellowing virus, papaya ringspot mosaic virus, etc. The researchers inactivated elF4E (eukaryotic translation initiation factor gene) using the CRISPR-Cas9 system.

In monocotyledons

Li et al. (2016) evidenced that multiple regulators of significant traits can be edited in a single rice cultivar Zhonghua 11 by CRISPR-Cas9. They used the CRISPR system to mutate the genes controlling grain number, grain size, panicle, and plant architecture, i.e., Gn1a, GS3, DEP1, and IPA1, respectively. The second generation of the gn1a, dep1, and gs3 mutants showed a higher grain number, dense, panicles, and large grain size, respectively. Besides, semi-dwarf and grain with a lengthy-awn phenotype were also detected in dep1 and gs3 mutants, correspondingly. The ipa1 mutants presented two distinct phenotypes, having either fewer or more tillers. Such studies facilitate the separation of complex gene regulatory systems in the same genomic background and the assembling of vital traits in cultivated varieties. Another study was conducted in rice plants using three engineered gRNAs with a 20–22 nucleotide seed region customized to pair with distinctive rice genomic locations. The experimental analysis led to the conclusion that the mismatch site involving target DNA and gRNA seed is a substantial determinant of the Cas9 targeting exactitude. The resulting mutational proficiency of the target site was expected to be 3–8% (Khlestkina and Shumny 2016).

Lawrenson et al. (2015) targeted two copies of HvPM19 using Cas9 genome editing in barley (*Hordeum vulgare*). The researchers observed Cas9-induced mutations in the first generation of the lines. Wang et al. (2014) have utilized CRISPR-Cas9 technology to generate transgenic *Triticum aestivum* plants conferring resistance to powdery mildew. This report has provided a methodological framework to improve polyploid crops. The researchers have showed that TaMLO-A1 allele (TALEN-induced mutation in MILDEW-RESISTANCE LOCUS (MLO) proteins) in barley plant confers heritable broad-spectrum resistance to powdery mildew. Zhou et al. (2014) reported large chromosomal segment deletions (115–245 kb) induced by Cas9 as well as the inheritance of genome edits in multiple generations, by targeting four sugar efflux transporter (OsSWEET) genes in rice. Up to 87–100% editing efficiency was observed in T_0 transgenic plants, all with di-allelic edits.

Wang et al. (2016) indicated that gene modification via CRISPR-Cas9 is a useful approach for enhancing blast resistance in rice. The researchers reported the improvement of rice blast resistance by targeting the OsERF922 gene in rice. Among 50 T_0 transgenic plants twenty-one mutant plants were identified, and several Indel mutations at the target site were revealed by Sanger sequencing. Moreover, six second generation homozygous mutant lines were additionally studied for a blast resistance phenotype and various agronomic traits viz. plant height, panicle length, number of grains per panicle, flag leaf length, and width etc. It was also observed that the number of blast lesions formed after pathogen infection was decreased in mutant lines as compared to wild-type plants. Some other noteworthy reports include the enhanced resistance to herbicides (Endo et al. 2016) and

Table 2 Exploration of CRISPR-Cas9 system technology in plants/crop improvement

S.No.	Plant species	Group	Target gene	Target traits	DSB repair Pathways utilized	Cas9 codon optimization	Promoters (Cas9, gRNA)	Transformation technique	Mutation frequency	Detection Technique	Reference
1.	*Arabidopsis thaliana*	Dicotyledon	eIF(iso)4E (eIF transcription factor)	Turnip mosaic virus disease; mutants show generation of genetic resistance.	NHEJ	Plant	*PcUbi4-2, AtU6- 26*	*Agrobacterium* - mediated transformation (floral dipping)	-	RT-PCR	Pyott et al. (2016)
2.	*A. thaliana*	Dicotyledon	FLS2	Utilization of mesophyll protoplasts	NHEJ in transient transfection	Arabidopsis (including intron)	CaMV35SP DK, AtU6	PEG-protoplast transfection	1.1–5.6%	PCR, Sequencing	Li et al. (2013)
3.	*A. thaliana*	Dicotyledon	GUUS, UGUS	Stable inheritance of induced targeted mutagenesis.	HR	Arabidopsis	PcUBI4-2, AtU6	Stable agro-transformation	-	GUS staining	Fauser et al. (2014)
4.	*A. thaliana*	Dicotyledon	PDS3	Utilization of mesophyll protoplasts	NHEJ in transient transfection	Arabidopsis (including intron)	CaMV35SP DK, AtU6	Leaf agro-infiltration	2.7%	PCR, sequencing	Li et al. (2013)
5.	*A. thaliana*	Dicotyledon	ADH1	Identification of heritable endogenous gene targeting events	HR	Arabidopsis	PcUBI4-2, AtU6	Stable agro-transformation	0.14%.	PCR, phenotype, sequencing	Schiml et al. (2014)
6.	*A. thaliana*	Dicotyledon	ADH1, TT4, RTEL1	Demonstration of stable inheritance of targeted mutagenesis	NHEJ in stable transfection	Arabidopsis	PcUBI4-2	Agro-transformation by floral dip	26.7%	PCR, deep sequencing	Fauser et al. (2014)
7.	*Brassica oleracea*	Dicotyledon	BolC.GA4; (Arabidopsis ortholog)	Characterization of gene utility for crop enhancement	NHEJ in stable transfection	GA4a CsVMW; humans	At-U6-26	Agro-transformation of cotyledonary petioles	10%	Pre-digested PCR, TA cloning and Sanger sequencing	Lawrenson et al. (2015)
8.	*Oryza sativa*	Monocotyledon	PDS, BADH2, MPK2, Os02g23823	Induction of gene knockouts in rice callus	NHEJ in transient transfection	Rice	2xCaMV35S, OsU3	PEG-protoplast transfection	14.5–38.0%	PCR, RE	Shan et al. (2013)
9.	*O. sativa*	Monocotyledon	OsSWEET	Disease susceptibility; to recognize and optimize the transport and reserve of carbohydrates for enhancing yield.	NHEJ in stable transfection	OsUbi; rice	OsU6.1, OsU6.2	*Agrobacterium* mediated transformation	12.5%	Sequential cloning	Zhou et al. (2014)
10.	*O. sativa*	Monocotyledon	PTG1	Enhancing multiplex editing capability via endogenous tRNA processing system	NJEH	Rice ubiquitin	U3 snoRNA (*U3p*)	*Agrobacterium* -mediated transformation	44-60% (indels); 13-20% (biallelic mutations)	PCR	Xie et al. (2015)

Table 2 continued

S.No.	Plant species	Group	Target gene	Target traits	DSB repair Pathways utilized	Cas9 codon optimization	Promoters (Cas9, gRNA)	Transformation technique	Mutation frequency	Detection Technique	Reference
11.	*O. sativa*	Monocot yledon	GUUS		HR	Rice	ZmUbi, OsU3	Transient particle bombardment of callus	-	GUS staining	Miao et al. (2013)
12.	*O. sativa*	Monocot yledon	OsERF922	Enhanced rice blast disease resistance	NJEH	Maize ubiquitin promoter (Ubi)	OsU6a; 2 CaMV35S promoters (2 × 35S)	Electroporation	42%	Sanger sequencing	Wang et al. (2016)
13.	*O. sativa*	Monocot yledon	OsBADH2	Induction of gene knockouts in rice callus	NHEJ in stable transfection	Rice	2xCaMV35S, OsU3	Particle bombardment of callus	7.1% (9/97)–9.4% (7/98)	PCR, RE	Shan et al. (2013)
14.	*O. sativa*	Monocot yledon	OsWaxy	Mutational regulation of amylose synthase	NHEJ in stable transfection	ZmUbi; rice	OsU3, OsU6a, OsU6b, OsU6c	Gibson assembly method	85.4% (biallelic, homozygous, heterozygous)	Degenerate Sequence Decoding method	Ma et al. (2015)
15.	*O. sativa*	Monocot yledon	SWEET14	Rice bacterial blight susceptibility genes	NHEJ in transient transfection	Rice	CaMV35S, OsU6	PEG-protoplast transfection	-	Pre-digested PCR, RE	Jiang et al. (2013)
16.	*O. sativa*	Monocot yledon	Gn1a, DEP1, GS3, IPA1	Enhanced grain number, dense erect panicles, and larger grain size, respectively	NJEH	Maize ubiquitin promoter	U6a	Agro-transformation in embryogenic calli	42.5% (Gn1a), 67.5% (DEP1), 57.5% (GS3), 27.5% (IPA1)	RE, PCR	Li et al. (2016)
17.	*O. sativa*	Monocot yledon	OsPDS	Induction of gene knockouts in rice callus	HR	Rice	2xCaMV35S, OsU3	Transient PEG-protoplast transfection	7%	RE, PCR, RE	Shan et al. (2013)
18.	*Zea mays*	Monocot yledon	LIG1, Ms26 and Ms45, and ALS1 and ALS2	Male fertility genes, acetolactate synthase	NHEJ	ZmUbi; maize	ZmU6	Biolistic transformation and co-delivery	77–100% (biallelic, heterozygous)	PCR, Sequencing	Svitashev et al. (2015)
19.	*Z. mays*	Monocot yledon	IPK	Targeted mutagenesis of key enzyme in phytic acid biosynthetic pathway for decreased synthesis	NHEJ in transient transfection	Rice	2xCaMV35S, ZmU3	PEG-protoplast transfection	16.4–19.1%	PCR, RE	Liang et al. (2014)
20.	*Triticum aestivum*	Monocot yledon	MLO-A1	Repress resistance pathway of powdery mildew	NHEJ in transient transfection	Plant	ZmUbi, TaU6	PEG-protoplast transfection	36%	T7E1	Wang et al. (2014)
21.	*T. aestivum*	Monocot yledon	MLO-A1		NHEJ in stable transfection	Plant	ZmUbi, TaU6	Particle bombardment of embryo	5.6% (4/72)	T7E1	Brooks et al. (2014)
22.	*T. aestivum*	Monocot yledon	PDS, INOX	Phytoene desaturase	NHEJ in transient transfection	Human	CaMV35S, CaMV35S	Agro-transfection of cells from immature embryos	18–22%	PCR, sequencing	Upadhyay et al. (2013)

Table 2 continued

S.No.	Plant species	Group	Target gene	Target traits	DSB repair Pathways utilized	Cas9 codon optimization	Promoters (Cas9, gRNA)	Transformation technique	Mutation frequency	Detection Technique	Reference
23.	*T. aestivum*	Monocotyledon	MLO		NHEJ in transient transfection	Rice	2xCaMV35S, TaU6	PEG-protoplast transfection	28.5%	PCR, RE	Shan et al. (2013)
24.	*Glycine max*	Dicotyledon	ALS1	Encode acetolactate synthase involved in amino acid biosynthesis		EF1A2; soybean	U6-9-1	Particle bombardment	59–76%		Li et al., (2015)
25.	*Nicotiana benthamiana*	Dicotyledon	NbPDS3	Utilization of mesophyll protoplasts	HR	Arabidopsis (with intron)	CaMV35SPDK, AtU6	Transient PEG-protoplast transfection	10.7%	PCR, RE	Li et al., (2013)
26.	*N. benthamiana*	Dicotyledon	PDS	Phytoene desaturase	NHEJ in transient transfection	Human	CaMV35S, AtU6	Leaf agroinfiltration	1.8–2.4%	PCR, RE	Nekrasov et al. (2013)
27.	*N. benthamiana*	Dicotyledon	BeYDV (short intergenic region, trans acting replication initiation protein)	Leaf thickening, chlorosis and curling	NJEH in transient expression			*Agrobacterium* mediated transformation	87%	-	Baltes et al. (2014)
28.	*N. benthamiana*	Dicotyledon	PDS	Phytoene Desaturase	NHEJ in transient transfection	Human	CaMV35S, CaMV35S	Leaf agroinfiltration	12.7–13.8%	-	Upadhyay et al. (2013)
29.	*N. benthamiana*	Dicotyledon	TYLCV-IR (intergenic regions), RCA regions	Leaf curl disease; mutants showed delayed and reduced viral DNA accumulation							Ali et al., 2015
30.	*Nicotiana tabacum*	Dicotyledon	PDS, PDR6	Phytoene desaturase	NHEJ in stable transfection	Tobacco	2xCaMV35S, AtU6	Agro-transformation of leaf discs	82% (9/11)–88% (14/16)	Phenotype, PCR, RE Sequencing	Gao and Zhao (2014)
31.	*N. tabacum*	Dicotyledon	PDS, PDR6	Phytoene desaturase	NHEJ in transient transfection	Tobacco	2xCaMV35S, AtU6	PEG-protoplast transfection	16.2–20.3%	PCR, RE	Gao and Zhao (2014)
32.	*Solanum tuberosum*	Dicotyledon	GBSS	Increased amylopectin content	-	CaMV 35S	AtU6, StU6	PEG-mediated protoplast transfection	67%	Restriction cloning	Andersson et al. (2017)
33.	*Solanum lycopersicum*	Dicotyledon	GFP, SHR	-	NHEJ in stable transfection	Nicotiana	CaMV35S, AtU6	Hairy root transformation by *Agrobacterium rhizogenes*	-	RE, PCR; phenotype	Ron et al. (2014)
34.	*S. lycopersicum*	Dicotyledon	ANT1	Anthocyanin biosynthesis enhancement	-	35S	ANT1; AtU6	*Agrobacterium* mediated transformation	57.1% heterozygous; 13.1% homozygous	Golden gate	Cermak et al. (2015)

Table 2 continued

S.No.	Plant species	Group	Target gene	Target traits	DSB repair Pathways utilized	Cas9 codon optimization	Promoters (Cas9, gRNA)	Transformation technique	Mutation frequency	Detection Technique	Reference
35.	*S. lycopersicum*	Dicotyledon	SlAGO7, Solyc08g041770, Solyc07g021170, Solyc12g044760	Biogenesis of trans-acting short interfering RNAs	NHEJ in stable transfection	Human	2xCaMV35S, AtU6	Agro-transformation of cotyledons	75 (6/8)–100% (29/29, 8/8)	Phenotype, PCR, sequencing	Brooks et al. (2014)
36.	*S. lycopersicum*	Dicotyledon	SlJAZ2	Bacterial speck resistance	HR	Ubiquitin promoter		Agro-transformation		RT-PCR	Ortigosa et al. (2018)
37.	*Sorghum bicolor*	Monocotyledon	DsRed;			CaMV 35S	AtU6	Agro-transformation of immature embryos	28%		Jiang et al. (2013)
38.	*S. bicolor*	Monocotyledon	Co-transfected DsRed		NHEJ in stable transfection	Monocot	OsActin1, OsU6	Agro-transformation of immature embryos	28% (5/18)	DsRed fluorescence	Jiang et al. (2013)
39.	*Cucumis sativus*	Dicotyledon	eIF4E (eukaryotic translation initiation factor 4E)	Cucumber vein yellowing virus, zucchini yellow mosaic virus and papaya ring spot mosaic virus resistance					Heterozygous non-transgenic mutants		Chandrasekaran et al. (2016)
40.	*Hordeum vulgare*	Monocotyledon	HvPM19	Characterization of gene utility for crop enhancement	NHEJ in stable transfection	GA4a CsVMW; humans	At-U6-26	Agro-transformation of cotyledonary petioles	23% and 10%	Pre-digested PCR, TA cloning and Sanger sequencing	Lawrenson et al. (2015)

thermosensitive genic male sterility in maize and wheat (Li et al. 2017b; Okada et al. 2019). Producing genetic resistance to viruses has huge potential to manage diseases for which no natural resistance has been reported, such as maize lethal necrosis disease (Luria et al. 2017). These results infer advance aspects in molecular breeding to enhance plant function utilizing optimized CRISPR/Cas9-plant systems.

Delivery strategies with special emphasis on plants

One of the critical challenges in targeting cells in plant systems is the secure and competent transfer of CRISPR-Cas9 genome-editing complex (Joung et al. 2017). Hence, an emphasis is given on the modern systems developed to transport CRISPR-Cas9 in-vivo and in-vitro (Han et al. 2017). Genome editing using CRISPR-Cas9 is performed by three strategies. The primary and foremost approach utilizes a simple and suitable plasmid-based CRISPR-Cas system, programming the Cas protein with sgRNA from the identical vector (Ran et al. 2013). Cas9 protein can be delivered using electroporation, microinjection, and lipid nanoparticle strategies (Qin et al. 2015). The next approach is based on carrying the fusion of the sgRNA and Cas9 mRNA. It offers improvements in off-target effects and limits the time of gene-editing (Niu et al. 2014). Delivery strategies such as microinjection, electroporation, and lipid nanoparticles (Zuris et al. 2015) can be classified under this strategy. The third approach is based on delivering the combination of the

Cas protein and the sgRNA. It is used widely due to several advantages such as elevated editing efficiency, quick action, and no requirement of promoter choice or codon optimization (Kim et al. 2014). This combination of Cas protein and sgRNA can be delivered using electroporation, cell-penetrating peptide (CPP), and gold nanoparticles (Zuris et al. 2015).

Physical delivery approaches

Physical delivery strategies employ temporary disruption of physical barriers and allow cargo to reach its targeted location. Electroporation is an extensively used strategy, and it offers high transfection efficiency and usage in the in-vitro and in-vivo analysis (Tebas et al. 2014). The inadequacy of electroporation is that the plasmid DNA is barely assimilated into approximately 0.01% of target cells. Moreover, it induces substantial cell death and also leads to nonspecific transfection. Microinjection is another physical delivery approach where cargoes are injected to the target site using a 0.5–5.0 µm diameter needle (Raveux et al. 2017). Protoplast transformation has been confirmed advantageous for the evaluation of the efficiency of CRISPR/Cas9 designs where plasmids can be delivered into protoplasts using electroporation and microinjection (Malnoy et al. 2016). Using particle bombardment technique that offers high transformation efficiency researchers have succeeded in delivering exotic DNA into scutellar tissues of maize, epidermal tissues of *Allium cepa*, and leaf and cell culture of several other crops (Maggio et al. 2014).

Non-viral delivery approaches

The non-viral vectors offer advantages of availability, safety, lack of size limitation, and cost-effectiveness (Glass et al. 2018). *Agrobacterium*-mediated plant transformation is an extremely multifaceted, evolved, and widely used method that utilizes genetic determinants of bacterium and host plant cells mutually (Gelvin 2003). Vector ZH11 was transformed via *A. tumefaciens*-mediated callus transformation. Additionally, *A. rhizogenes* mediated-hairy roots are an excellent transformation model system for species of fabaceae. The transient assay can be implemented to test the CRISPR genome editing ability (Hiei et al. 1994). PEG mediated transformation is a simple reproducible and highly competent strategy for the transformation of plant protoplasts (Liu and Vidali 2011). Nanoparticles composed of mesoporous silica (Cunningham et al. 2018), gold, layered double hydroxides (Mitter et al. 2017), and polyethylenemine (Cunningham et al. 2018) are widely used as carriers. Carbon nanotubes have been used as a delivery vehicle to transfer DNA for successful protein expression in mature plant leaves (Demirer et al. 2018). The commonly used explants in plant transformations include calli, i.e., unorganized cell mass (monocots and eudicot), leaf cuttings (eudicot), and zygotic embryos (monocots) (Ikeuchi et al. 2016).

Viral delivery approaches

Despite the safety distress and the chances of introduction of undesirable mutations, viral delivery systems are the most proficient method to carry plasmid-based nucleic acids to cells in the in-vitro and in-vivo analysis (Koike-Yusa et al. 2014). Virus mediated genome editing has been reported in both inoculated and non-inoculated leaves. In a recent report, the authors developed a *tobacco rattle RNA virus*-mediated genome transduction method for *N. benthamiana* (Mahas et al. 2019). *Bean yellow dwarf virus, begomovirus, cabbage leaf curl virus,* and *wheat dwarf virus* are some of the most widely used DNA viruses for gene transduction. *Bean yellow dwarf virus* has been used to target *stALS1, stALS2* (*Solanum tuberosum* acetolactate synthase1) and *P-GUS:NPTII* (Promoter of GUS and neomycin phosphotransferase) gene in *S. tuberosum* and *Nicotiana tabacum* respectively (Butler et al. 2015; Baltes et al. 2014). *The wheat dwarf virus* has also been used as a viral vector to target *Ubi, MLO, GFP* (β-glucuronidase [GUS] reporter controlling gene, MildewLocusO, green fluorescent protein) in *T. aestivum* (Gil-Humanes et al. 2017). These methods provide several advantages such as immense infection efficiency, broad cell tropism, and long-term gene expression (Zaidi and Mansoor 2017). However, there are disadvantages of difficulty in production, limited packaging size, and potential for insertional mutagenesis.

Conclusion and future prospective

It is improbable for traditional plant breeding to meet the growing food demands as well as other ecological

challenges. On the contrary, CRISPR-Cas technology is removing genome editing barriers and has the potential to revolutionize plant breeding. What has been achieved so far with this technology is just the tip of the iceberg. The CRISPR system can be used for several futuristic applications in plant systems, such as for studying abiotic stress responses or adaptation pathways. Likewise, activation or suppression of genes can be regulated by utilizing CRISPR as a binding tool to stimulate repressors or activators to induce traits. CRISPR also has the capability for gene shuffling, i.e., assembling desirable traits in the genome that would group even in traditional cross-breeding. This technology will allow the emerging genomic and systems biological data to be exploited more comprehensively in gene discovery as well as novel trait development in countless plant species. CRISPR has been used for improved screening for genes and traits in human health via guide molecule libraries. This could be potentially used in plants to screen for characters contributing to crop yield, pest, and disease resistance. Application in orthogonal gene targeting is another aspect which is so far not been tested in plant systems. Hence, it is crucial to present parallel studies in plants to guarantee the adaptability to different species.

Bioinformatic gRNA design tools can be used to increase efficiency and decrease off-target effects. The tools depend on the activity prediction models and off-target detection algorithms; therefore, there is a need for additional CRISPR-Cas datasets for the development of new design tools. A substantial bottleneck to the implementation of CRISPR tools in agriculture is the effective packaging and delivery of CRISPR-Cas complex to the targeted plant cells. Novel delivery methods need to be established to achieve high-efficiency genome editing in plants. Thus, the outlook for improvement in reducing the size of presented Cas proteins or the innovation of smaller Cas9 proteins is needed.

Genome editing is a promising technology with the ability to contribute to food generation for the use of the rising population. However, the biosafety, social and ethical concerns remain about the usage of genome editing in plants. The major concern is the risk of creating undesirable genetic changes in plants due to off-target mutations. Fragments of the CRISPR-Cas9 might be inserted into expected or unexpected sites during the DNA repair mechanism or degraded into filler DNA. Substantial work is being required including improving gRNA design strategies, protein engineering, ribonucleoprotein delivery, using spatiotemporally controlled Cas9, or gRNAs through chemical or environmental inducers, that can modify CRISPR function. The human population has been subjected to Cas9 protein homologs long before the utilization of CRISPR-Cas9 in genome editing. The amino acid sequence of the Cas9 protein from *S. pyogenes* has $\sim$ 58%, 35%, and $\geq$ 80% similarity to Cas9 protein from *S. thermophilus* (probiotic), *Lactobacillus plantarum* (probiotic and in food production) and human commensal and pathogenic bacteria such as *S. dysgalactiae* subsp. *equisimilis*, *Staphylococcus aureus, Klebsiella pneumonia,* respectively (El-Mounadi et al. 2020). Nevertheless, there is a need to revise the regulations of genome-edited plants and to enlighten the general community about their characteristics. A sustainable future for agriculture can now be imagined along with the responsibility of continuously resolving both scientific and public concerns about its usage.

Author contributions NKS wrote the initial draft of the manuscript; SA and PS helped with the literature review; SA prepared the manuscript; SA and PS contributed to the several revisions of the manuscript.

Funding This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Compliance with ethical standards

Conflict of interest The authors declare no conflicts of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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