



New breeding technique “genome editing” for crop improvement: applications, potentials and challenges

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Abstract

Crop improvement is a continuous process in agriculture which ensures ample supply of food, fodder and fiber to burgeoning world population. Despite tremendous success in plant breeding and transgenesis to improve the yield-related traits, there have been several limitations primarily with the specificity in genetic modifications and incompatibility of host species. Because of this, new breeding techniques (NBTs) are gaining worldwide attention for crop improvement programs. Among the NBTs, genome editing (GE) using site-directed nucleases (SDNs) is an important and potential technique that overcomes limitations associated with classical breeding and transgenesis. These SDNs specifically target a compatible region in the gene/genome. The meganucleases (MgN), zinc finger nucleases (ZFN), transcription activator-like effectors nucleases (TALENs), and clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated endonuclease (Cas) are being successfully employed for GE. These can be used for desired or targeted modifications of the native endogenous gene(s) or targeted insertion of *cis/trans* elements in the genomes of recipient organisms. Applications of these techniques appear to be endless ever since their discovery and several modifications in original technologies have further brought precision and accuracy in these methods. In this review, we present an overview of GE using SDNs with an emphasis on CRISPR/Cas system, their advantages, limitations and also practical considerations while designing experiments have been discussed. The review also emphasizes on the possible applications of CRISPR for improving economic traits in crop plants.

Keywords New breeding technique (NBT) · Site-directed nucleases (SDNs) · Zinc finger nucleases (ZFNs) · Transcription activator-like effectors nucleases (TALENs) · Cluster regularly interspaced short palindromic repeats (CRISPR/Cas)

Introduction

Plant breeding for crop improvement involves selection and hybridization, which are largely dependent on homologous recombination between chromosomes to generate the genetic diversity which has been in practice since the ancient time. To increase the range of natural variation for traits, plant breeders also have been exploring chemicals or irradiation

methods. Although, classical and mutational breeding have resulted in significant improvements in various agronomic traits, viz., yield, quality, nutrition, biotic and abiotic stress resistance, there are certain limitations in terms of labor, time and precise knowledge of selection. Recent advances in plant genetics and breeding approaches led to the development of marker-assisted selection or marker-aided breeding where traits are linked with specific DNA markers on the genome which facilitate in rapid and accurate selection for those traits. Further advancements in biotechnological methodologies enabled the researchers to add desirable traits in crops by inserting genes-of-interest from other organisms into the plant genome which is popularly known as transgenesis. The use of transgene (or foreign gene) and its non-specific integration in the host genome, the use of bacterial-origin selection markers and possibilities of somaclonal variations are the issues which raise concern for biosafety. A potential alternative to transgenic is cisgenics wherein

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variation is created at predetermined sites in the native gene(s) of an organism (creation of novel alleles).

Conversely, new breeding techniques (NBTs) offer additional options to replace conventional breeding and transgenic technology. NBTs which include a range of methods, viz., site-directed nucleases (SDNs), cisgenesis, RNA-dependent DNA methylation, grafting (non-genetically modified (GM) scion on GM rootstock), reverse breeding, agro-infiltration, etc. (Lusser et al. 2012), established for introduction of targeted changes in plant genome to modify the economic traits of crops. Among those, use of SDNs such as ZFNs (zinc finger nucleases), TALENs (transcription activator-like effectors nucleases) and CRISPR/Cas (cluster regularly interspaced short palindromic repeats/CRISPR-associated proteins) for genome editing (GE) has great potential for crop improvement. The SDNs create specific double-stranded breaks (DSBs) at desired locations in the genome and harness the cell's endogenous mechanisms to repair the DSBs by homologous recombination (HR) or non homologous end-joining (NHEJ). The NHEJ pathway repairs DSB by ligating broken ends without the help of a homologous template, often resulting in insertions or deletions (InDels) and single nucleotide polymorphism (SNP) at the cut site, thus causing mutations such as frameshift or nonsense mutations. On the other hand, HR allows gene replacement by replacing the DNA sequence using template at the break point. Both NHEJ and HR repair pathways are key processes for nuclease-based GE (Pardo et al. 2009). The field of GE has been witnessing considerable advances in terms of precision, efficiency and accuracy in methodology from meganucleases to ZFNs to TALENs till the most recent CRISPR/Cas nucleases which are revolutionizing the field of plant molecular biology.

The use of GE was pioneered by Paszkowski et al. (1988) for the integration of gene in the tobacco genome via homologous recombination. Later, Bibikova et al. (2003) reported targeted modifications using sequence-specific nucleases by direct gene transfer to *Nicotiana tabacum* protoplasts. Even after these early leads, it took more than 25 years to build up the information and experimental set-up required for successful application of GE in plants (Puchta and Fauser 2013). The historical timeline of GE in plants is provided in Fig. 1. In crop improvement, GE can be used for development of better phenotypes such as high yield, altering plant architecture, enhancing nutritional value and stress tolerance resistance, etc. The ease, low cost and speed of designing GE tools make it the most appropriate and feasible system for plant improvement in the present era of high-throughput technologies. Additionally, GE offers an advantage over traditional mutation breeding as it creates the variations in the selected target site with high frequency. It certainly transforms the transgenic technology as it integrate *cis*- or *trans*-genes without the extra burden of foreign genetic elements,

thus may reduce public concern, anxiety and regulatory costs of GM crops. An appreciation of the same is reflected by conferment as a method of the year for 2011 by Nature Methods.

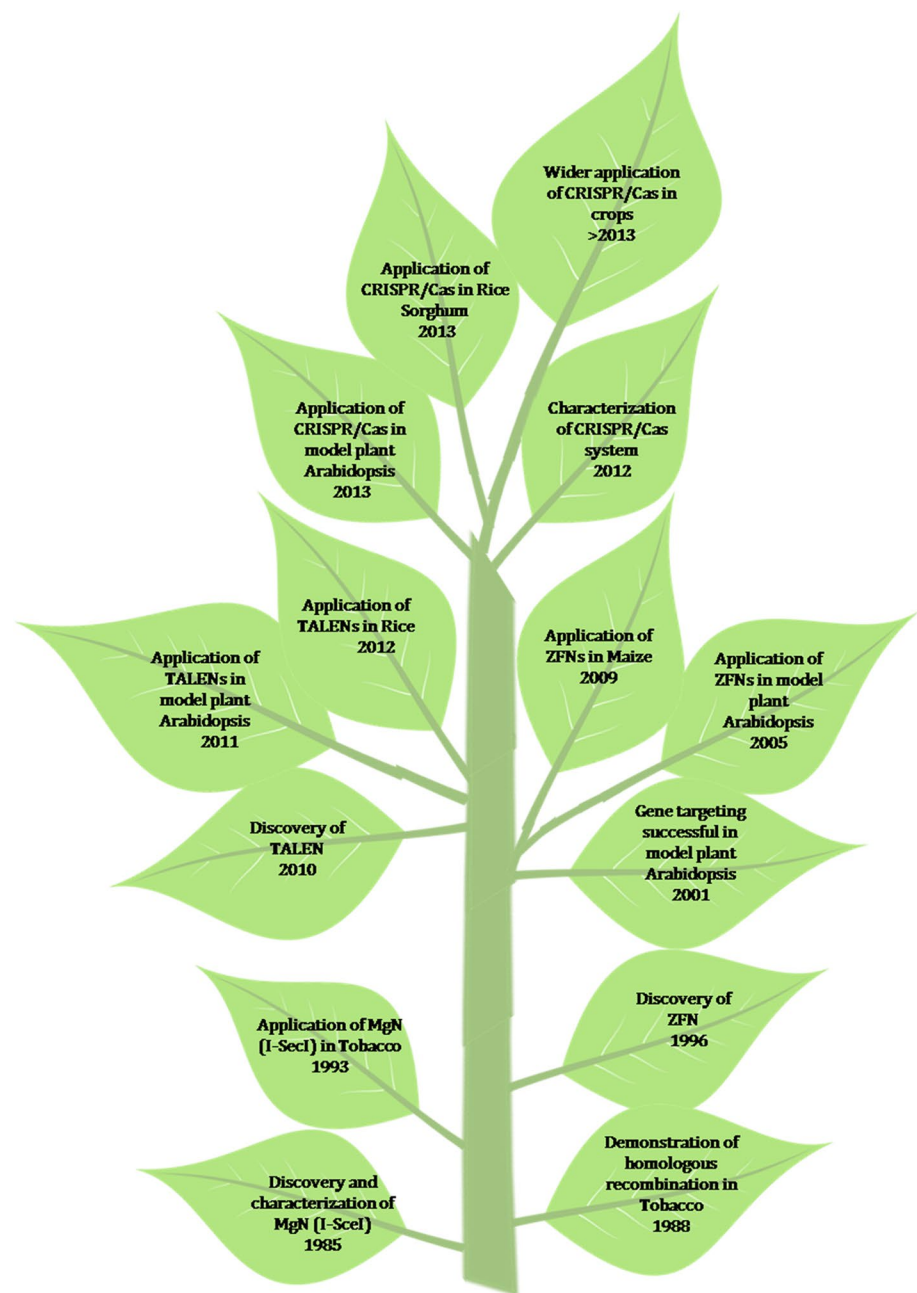
Genome editing tools

The meganucleases (MgNs) are naturally occurring unique enzymes having high activity and long recognition sequences known as the homing site (> 14 bp) and were employed as the first GE tool (Curtin et al. 2012) (Fig. 2a). There are about hundreds of MgNs identified in several organisms including bacteria, fungi and some of the plant species. Most commonly utilized nucleases include *I-SceI* and *I-CreI* which have copies of the LAGLIDADG motif. In spite of their greater specificity, use of MgNs in GE is restricted because of the presence of single homing site in genomes of many organisms and overlapping cleavage site and DNA binding domains, thus making it difficult to engineer DNA binding domain. Nonetheless, efforts have been made towards the development of MgN-TALE chimera (megaTAL) having greater flexibility as compared to native MgN. As MgN is an early tool of GE, we have not discussed this tool here further.

The limitations of MgNs were overcome by ZFNs which are created by linking zinc finger proteins to the cleavage domain of *FokI* endonuclease and were first reported in *Arabidopsis* by Lioyd et al. (2005). The ZFN DNA binding domain is generally composed of three to four zinc finger arrays each capable of recognizing 3-bp long sequence (Fig. 2b). Relative to the start point of the zinc finger α -helix, amino acids present at -1, +2, +3, and +6 position contribute to specificity for proper dimerization of *FokI* domain as it is critical to the functioning of ZFNs (Kim et al. 1996). The amino acids act as engineerable sites and can be customized to fit specific target sequences. The two ZFN monomers designed in such way, flank 6-bp long sequence within the DNA target sequence, allowing the *FokI* monomers to form an active dimer and to digest within that spacer sequence. Presence of sparse target sites, difficulties in engineering zinc finger arrays (Maeder et al. 2008) and low targeting efficiency leading to frequent off-target effects (DeFrancesco 2012) are the major constraints in the use of ZFNs. Several strategies have been reported to overcome these limitations such as nickases (ZFNickases) that take advantage of nicking at the single strand and emulate the HR rather the error-prone NHEJ pathway, ultimately resulting in reduced off-site targeting. Furthermore, an attempt to increase 4–6 zinc finger domains for each ZFN enhanced the activity and specificity of ZFNs (Sood et al. 2013).

Though ZFNs resolved some of the difficulties associated with MgNs, there was ample scope of further improvement

Fig. 1 Historical timeline of GE with respect to plants



in GE. Christian et al. (2010) for the first time suggested that zinc finger arrays could be substituted with the DNA recognition domain of TALEs (transcription activator-like effectors) to create TALENs that recognize and cleave DNA targets. These path-breaking experiments were done using two highly recognized TALENs, AvrBs3 and PthXo1 from the pepper pathogen, *Xanthomonas campestris* pv. vesicatoria and rice pathogen *X. oryzae* pv. *oryzae*, respectively. Like ZFNs, TALENs consists of TALE DNA binding domain having 33–35-long repeats of amino acids followed by 20 amino acids known as ‘half repeat’ and a nonspecific *FokI* cleavage domain (Fig. 2c). The conserved 12th and

13th positions of the TALE monomer impart specificity to nucleotide recognition and are thus called repeat-variable di-residues (RVDs). Research showed that first RVD involved in forming contact with RVD loop backbone whereas the second RVD involved in contact with DNA (Deng et al. 2012). The presence of thymine (T) before recognition site is essential to TALEN engineering (Voytas 2013). Although TALE DNA binding monomers are modular in nature, they still suffer from context-dependent specificity and their repetitive sequences incur high cost and labor for construction of novel TALE arrays (Juillerat et al. 2014). Nevertheless, new techniques such as GoldenGate and Platinum Gate

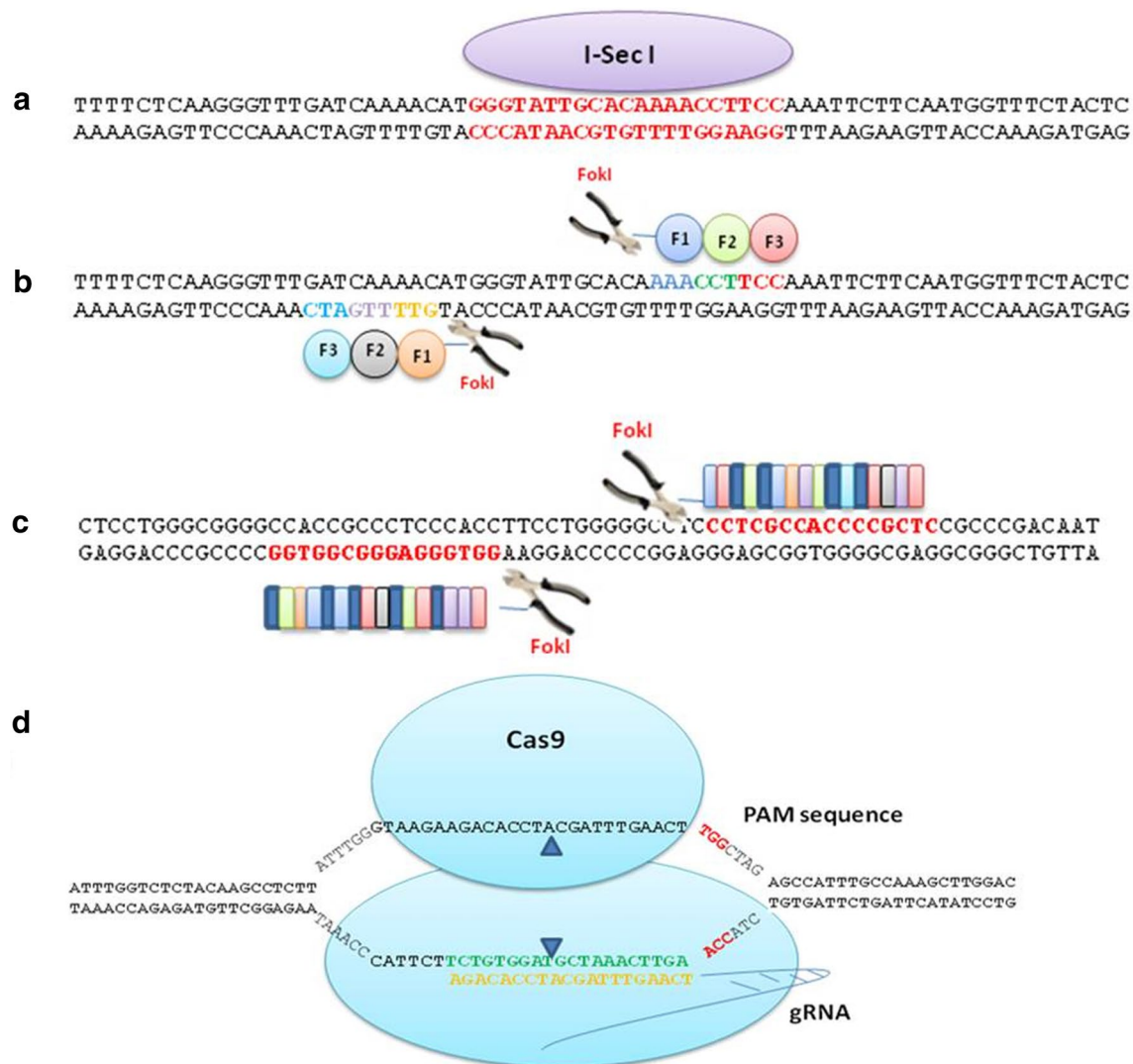


Fig. 2 Types of different SDNs used for GE. **a** MgNs: schematic representation of naturally occurring I-SecI MgNs. Homing site for I-SecI is 18 bp and it cleaves DNA within the homing site. DNA binding and cleavage domains are not clearly demarcated. **b** ZFNs: schematic representation of synthetic ZFNs, ZFNs are synthesized by fusion of zinc finger DNA binding domain and *FokI* cleavage domain. Zinc finger DNA binding domains are typically composed of three zinc finger arrays each capable of recognizing approximately 3 bp. **c** TALENs: schematic representation of synthetic TALENs, TALENs

are synthesized by fusion of TAL DNA binding domain and nonspecific *FokI* cleavage domain. Each TAL domain recognizes only one base. Binding specificity is manipulated by combining repeats that recognize individual bases in different orders. TALENs also work in dimer form. **d** CRISPR/Cas: schematic representation of CRISPR/Cas system. gRNA guides Cas9 protein for DNA DSB. gRNA form complex along with Cas9 protein and bind to seed sequence. Cas9 nucleases create DSB. Presence of PAM (NGG) sequence immediately downstream to target site is must for DSB

assays are in vogue for efficient construction of TALEN. The wide spectrum of engineered TALE for target binding makes it a more robust GE tool in plants (Zhang et al. 2013a, b).

The above three tools of GE described so far are based on recognition of specific DNA sequences by a protein molecule having DNA binding motif. However, engineering DNA binding motif according to the target sequence requires deep knowledge of protein biochemistry and protein engineering. This limitation has been resolved using small RNA sequences as DNA recognition molecules. To attain GE

through small RNAs, a prokaryotic immune system called CRISPR/Cas that provides a form of acquired immunity is being deployed (Marraffini and Sontheimer 2008). CRISPRs are often associated with Cas genes that code for proteins related to CRISPRs and three groups of eleven such systems have been reported. The Type II CRISPR/Cas system has been adapted for GE owing to the presence of protospacer adjacent motif (PAM) sequence and a second RNA, called trans-acting CRISPR RNA (tracrRNA). The tracrRNA teams up with CRISPR RNA (crRNA) to assist crRNA maturation

and recruit the Cas9 nuclease to DNA. To make this system more promising, the natural three-component system is further simplified by fusing together crRNA and tracrRNA, resulting in the creation of a single synthetic chimeric 'guide' RNA (sgRNA or gRNA) (Jinek et al. 2012) (Fig. 2d). For additional information about native CRISPR system of bacteria and archaea, readers are redirected to refer to a review by Bhaya et al. (2011).

A considerable advantage of the CRISPR/Cas system over other SDNs lies with the utilization of an RNA molecule to guide the nuclease to a specific nucleic acid target. RNA is easier and cheaper to synthesize than the protein domains used with ZFN and TALEN approaches. This makes CRISPR/Cas system simple, easy and highly effective (Kim et al. 2017, 2018; Sun et al. 2017). This system also has an advantage with reference to introducing mutations in multiple genes at the same time by introducing multiple gRNAs. However, availability of PAM sequence in adjacent to target site limits target site selection. To overcome such limitations, extensive research is being done especially in mammalian cells to establish proof of concept (Fu et al. 2013). Moreover, a plethora of publications appeared on successful applications of CRISPR/Cas system to engineer desired traits in plants which reflect that it is the most robust tool for crop improvement. A comparative account of GE tools has been summarized in Table 1.

Practical considerations

For successful GE in plants, four major steps are involved, viz., identification of target gene for desired trait (fully functionally characterized), construction of suitable vector/s, transformation and screening the transformants for intended mutation. The target gene should be unique in function and the mutation at particular site should either activate or repress its function. The pleiotropic effects and presence of multiple target sites should also be checked for avoiding the off-target effects. Additionally, for CRISPR/Cas system, the presence of PAM sequence at the 3' end of the target sequence is essential. If there are no PAM sequences for *S. pyogenes* Cas9 (i.e., NGG) within the desired sequence, Cas enzyme of different species or *S. pyogenes* Cas9 variants that bind other PAM sequences present in the desired target can be selected as well.

Next crucial step after selection of appropriate target sequence is the selection of a GE strategy. For engineering highly specific ZFNs, several methods such as modular assembly (MA), Oligomerized Pool Engineering (OPEN), Context-Dependent Assembly (CoDA), etc., are available for choice (Maeder et al. 2008). In case of TALEN, the GoldenGate assay is one of the most powerful tools for generating custom-made TALENs (Dahlem et al. 2012). Platinum

TALEN and Platinum Gate system are some of the recent systems to design highly efficient TALE repeat assembly (Sakuma et al. 2013). Online and offline tools such as TALEN-NT, idTALE and EENdb furnishes all the relevant information for engineering TALEN. Accurate selection of target site and designing of gRNA is the most critical step in CRISPR/Cas experiment. Sometimes, gRNA can recognize non-target sequences within genome which show partial homology called off-targets. Removal of off-target is an important criterion for designing a gRNA. In addition to "off-target activity", specific nucleotides within the target sequence should be carefully selected to maximize cleavage of the desired target sequence (on-target activity). Therefore, close examination of predicted on-target and off-target activity of each potential gRNA is necessary (Wolt et al. 2016a). Many softwares and online tools are available for locating potential PAM, target sequences, and ranking of the associated gRNAs based on their predicted on-target and off-target activity (Table 2). Some variants of gRNA are available commercially viz., truncated gRNA, ribozyme gRNA, polycistronic-tRNA-gRNA, which have improved the native CRISPR system for utilization in broader applicability (Khatodia et al. 2016).

Next step is to transform the GE vectors into the plant. Transformation vector possesses one or two nuclear localization signal (NLS), a suitable promoter and other regulatory elements. Generally, constitutive promoters such as CaMV35S, OsUBQ1 and Actin1 are used in constructs. With respect to CRISPR/Cas system, the use of appropriate RNA pol II promoters such as CaMV35s, AtUBQ, 35sPPDK, OsAct1, etc., is equally critical for expression of Cas9 gene (Mao et al. 2013; Li et al. 2013; Wang et al. 2016). Depending on the objectives of the experiment, variants of Cas9 can be selected (Table 3). For expression of target sequence along with gRNA, pol III promoters (mostly U6 and U3) are attached at 5' end. The 5'G is required for transcription initiation from the U6 promoter. The CRISPR/Cas system leverages the use of a single gene construct by combining all three components, viz., Cas gene, sgRNA and tracrRNA. These three components can be constructed separately in different vectors also. Vectors are available according to the objectives of the experiment such as to generate mutation (to create cut), for activation of gene expression or for generation of nick, etc. (Table 4).

After construction of suitable GE vector, it is transformed into plant genome to carry out the intended mutation. Unavailability of reliable transformation methods is a bottleneck for application of GE in plants as compared to animal transformation. Till now, *Agrobacterium*-mediated transformation and particle bombardment are the most successful transformation methods in plants. However, these methods remain inefficient for many crops due to some of the limitations such as: (1) longer tissue culture periods

Table 1 Summary of different genome editing tools

Features	MgNs	ZFNs	TALENs	CRISPR/Cas
Mode of action	Integrate construct at target site through HR	Protein pair assembled adjacent to target site and introduce DSB	Protein pair assembled adjacent to target site and introduce DSB	Hybridization of gRNA recruit Cas nuclease at hybridization site and introduce DSB (presence of PAM sequence facilitate cleavage)
Components	DNA recognition domain and nuclease domain	Zinc finger DNA binding domain and <i>FokI</i> nuclease domain	TAL DNA binding domain and <i>FokI</i> nuclease domain	gRNA, Cas9 protein
Nature	Synthetic	Synthetic	Synthetic	Synthetic
Length of recognition site (bp)	> 14	18–24	24–59	20–22
Specificity	High	High	High	Moderate
Off-target cleavage	Very low	Low	Low	Low (quite higher than ZFNs and TALENs)
Engineering of nuclease	Very difficult	Difficult	Less difficult	Simple
Flexibility	Very low	Low	Moderate	High
Availability of variants	Yes	Yes	Yes	Yes
Cost effectiveness	Costly	Costly	Costly	Reasonable
Time requirement	More	More	More	Less
Popularity	Less	Less	Moderate	Very high
Advantages	DSB is not required Allows various genome modifications	High efficiency Selectable marker not required DSB repaired by NHEJ or HDR Can target any sequence Lower chance of off-target cleavage than CRISPR	High efficiency Selectable marker not required DSB repaired by NHEJ or HDR Can target any sequence Lower chance of off-target cleavage than CRISPR	Very high efficient Selectable marker not required DSB repaired by NHEJ or HDR Can target any sequence Rapid construction, easy designing and easy delivery Multiplexing possible
Disadvantages	Difficult to construct novel MgN Few recognition sites in genome	More difficult to assemble than TALENs and CRISPR	More difficult to assemble than CRISPR	Requirement of PAM sequence may limit target site selection Off-target cleavage comparatively more than ZFNs and TALENs
No. of publications	Very few	Few	More	Much more

Table 2 List of available softwares and programmes for designing gRNA for plants

Software	Features	Support in plant species	Link
Cas-OFFinder	Identifies gRNA target sequence from an input sequence and checks off-target binding site	<i>Arabidopsis</i> , rice, tomato, corn	http://www.rgenome.net/cas-offinder/
Cas-Designer	Identifies gRNA target sequence from an input with low probability of off-target effect	<i>Arabidopsis</i> , rice, tomato, corn	http://www.rgenome.net/cas-designer/
Cas9 Design	Designs gRNA	<i>Arabidopsis</i> , rice	http://cas9.cbi.pku.edu.cn/database.jsp
E-CRISP	Designs gRNA	<i>Arabidopsis</i> , corn, rice	http://www.e-crisp.org/E-CRISP/designcrisp.html
CRISPR-P	Designs gRNA	Maize, rice, corn, <i>Sorghum</i> , Populus, grape vine, banana, groundnut, <i>Brassica</i> , citrus, coffee	http://cbi.hzau.edu.cn/crispr/
CHOP CHOP	Identifies target site	<i>Arabidopsis</i>	https://chopchop.rc.fas.harvard.edu/
CRISPR-PLANT	Designs gRNA	Rice, <i>Sorghum</i> , Brachypodium, tomato, <i>Medicago</i> , soybean	http://www.genome.arizona.edu/crispr/
CCTop	Identifies candidate gRNA target sites with reduced off-target quality	Tobacco, tomato, <i>Arabidopsis</i> , <i>Medicago</i>	http://crispr.cos.uni-heidelberg.de/
CRISPRdirect	Identifies candidate gRNA target sequences	<i>Arabidopsis</i> , rice, <i>Sorghum</i>	http://crispr.dbcls.jp/

obligatory to achieve transgenic plants from transformed cells and tissues, (2) low frequency of stably transformed events, (3) small DNA insert delivered by *Agrobacterium*-mediated gene transfer and (4) low precision of bombardment-mediated gene transfer. Moreover, transformation by particle bombardment has been standardized for certain staple food crops like wheat and maize and is expected to work efficiently for other crops which are difficult to regenerate from protoplast culture. The transformed plants are screened for desired mutations using several methods like restriction-based assays, viz., cell endonuclease, PCR-based assays, whole-genome re-sequencing, surveyor assays (Stoddard 2011) and target gene sequencing. Pre-engineered and customized ZFNs, TALENs, CRISPR/Cas are also available from commercial suppliers such as Collectis (<http://www.collectis.com>), Sigma-Aldrich (<https://www.sigmaaldrich.com/>), Bioresearch and Life Technologies (<https://www.biosearchtech.com>), etc., as per the specifications of the experiment. For more information readers are directed to refer latest reviews on practical considerations of efficient CRISPR/Cas experiment (Liang et al. 2016).

Strategies to overcome the challenges of off-site targeting

Binding and cleavage at nonspecific loci leads to unintended editing in genome called off-site effect and needs to be addressed for efficient utilization of SDNs. Sometimes, off-site targeting may result in cell toxicity. ZFNs recognize smaller sequence as compared to TALENs and as a result,

ZFNs shows more off-site targeting. There is evidence that 4–6 zinc finger domains for each ZFN half-enzyme significantly enhance activity and specificity of ZFN (Sood et al. 2013). Another important consideration is the length of spacer sequence, i.e., sequence separating two target sites. Specificity of ZFNs decreases as target spacer sequence increases more than 7 bp and more length of spacer sequence hinders dimerization of ZFN leading to off-site cleavage (Pattanayak et al. 2014). Zinc finger nickases (ZFNickases) have been developed to increase the specificity of ZFNs by inducing nick in to a single strand of DNA which stimulates HR without activating the error-prone NHEJ repair pathway (Ramirez et al. 2012). Similarly, optimizing spacer length is important in designing TALENs wherein longer binding site ensures increased specificity (Pattanayak et al. 2014). Although CRISPR/Cas system is effective in introducing mutations, it is more subjected to off-target effects than the other tools. To overcome the potential off-target effects of CRISPR/Cas system, Ran et al. (2013) developed a proof of concept in human cells by introducing a truncated version of Cas9 which induced nicks (SSBs) in the target sequence. Modified with less than 20 nucleotide target sequences, the gRNAs truncated at 5' end of their complementary sequences have been reported to decrease off-target effects in animals; use of these truncated gRNAs is an alternate approach to minimize off-site targeting (Fu et al. 2014; Pattanayak et al. 2014).

To predict specific gRNA spacers which are expected to have little or no off-target risk in RNA guided GE, Xie et al. (2014) developed CRISPR-PLANT database by assembling the genomic sequences of plants, viz., *Arabidopsis*,

Table 3 Different Cas9 variants

Species/variant of <i>Cas9</i>	Description	PAM sequence	Specific use	References
1. Variants of SpCas9 with altered PAM sequence				
SpCas9 D1135E variant	More selective for the NGG PAM over the NGA PAM	NGG	Altered PAM sequence	Kleinstiver et al. (2016), Jaskula-Ranga and Zack (2016), Slaymaker et al. (2016)
SpCas9 VRER variant	Cas9 variants developed by genetic engineering	NGCG	Altered PAM sequence	Jaskula-Ranga and Zack (2016), Kleinstiver et al. (2015)
SpCas9 EQR variant	Cas9 variants developed by genetic engineering	NGAG	Altered PAM sequence	Jaskula-Ranga and Zack (2016), Kleinstiver et al. (2015)
SpCas9 VQR variant	Cas9 variants developed by genetic engineering	NGAN or NGNG	Altered PAM sequence	Jaskula-Ranga and Zack (2016); Kleinstiver et al. (2015)
2. Cas9 from different bacteria				
SaCas9	<i>Staphylococcus aureus</i>	NNGRRT or NNRRR(N)	Different PAM sequence than PAM sequence of <i>S. Pyogenes</i>	Chen et al. (2017), Jaskula-Ranga and Zack (2016), Ran et al. (2015)
NmCas9	<i>Neisseria meningitidis</i>	NNNGATT	Different PAM sequence than PAM sequence of <i>S. Pyogenes</i>	Jaskula-Ranga and Zack (2016), Hou et al. (2013)
StCas9	<i>Streptococcus thermophilus</i>	NNAGAAW	Different PAM sequence than PAM sequence of <i>S. Pyogenes</i>	Jaskula-Ranga and Zack (2016), Mojica et al. (2009)
TdCas9	<i>Treponema denticola</i>	NAAAAC	Different PAM sequence than PAM sequence of <i>S. Pyogenes</i>	Eswelt et al. (2013)
3. Cas9 variants for increase on-target effect				
SpCas9-HF1	Variant created by genetic engineering to induce on-target effects		Lower off-target effect	Kleinstiver et al. (2016), Jaskula-Ranga and Zack (2016), Slaymaker et al. (2016)
SpCas9- HF2	Derivative of SpCas9-HF1 with additional D1135E substitution		Lower off-target effect	Kleinstiver et al. (2016), Slaymaker et al. (2016)
SpCas9- HF3	Derivative of SpCas9-HF1 with additional L169A substitution		Lower off-target effect	Kleinstiver et al. (2016), Slaymaker et al. 2016
SpCas9- HF4	Derivative of SpCas9-HF1 with additional Y450A substitution		Lower off-target effect	Kleinstiver et al. (2016), Slaymaker et al. (2016)
eSpCas9	Genetically engineered to induce on-target effects		Lower off-target effect	Kleinstiver et al. (2016), Jaskula-Ranga and Zack (2016), Slaymaker et al. (2016)
4. Cas9 variants for endonuclease activity				
Cas9 variant without endonuclease activity	A catalytically inactive Cas9 variant, carrying a mutation in both endonuclease domains (RuvC ⁻ HNH ⁻)		Epigenome and transcriptional modifications or regulation	Zentner and Henikoff (2015)
Cas9 nickases	The D10A mutation inactivates the RuvC endonuclease domain so that Cas9 can cleave only the DNA strand complementary to the gRNA		Cleavage of only one DNA strand complementary to the gRNA	Ramirez et al. (2012), Mali et al. (2013), Fauser et al. (2014)

Table 4 Representative CRISPR plasmid for plants

Function/objective	Description	Available vectors	References
Cut	Uses wild type Cas9 which efficiently generates double-strand breaks at sequences homologous to co-expressed gRNA	pFGC-pcoCas9, pK7WGF2::hCas9, pRGE31, HBT-pcoCas9, pRGE32, pICH47742::2 × 35S-5'UTR-hCas9(STOP)-NOST, pKSE401, Cas9 MDC123, pHSE401, pBUN411	Nekrasov et al. (2013), Belhaj et al. (2013), Li et al. (2013), Xie and Yang (2013), Xing et al. (2014)
Nick	Uses a mutated “nickase” version of the Cas9 enzyme which generates a single-strand DNA break (Nick), instead of a double-strand DNA break (Cut)	pHSN501, pBUN501	Xing et al. (2014)
Interfere	Uses a catalytically inactive Cas9 (dCas9) which can knockdown gene expression by interfering with transcription	pHSN6I01, pYPQ153, pBUN6I11, pEGB 35S:dCas9:Tnos (GB1191)	Xing et al. (2014), Sarrion-Perdigones et al. (2013)
Activate	Uses a catalytically inactive Cas9 (dCas9) fused to an activator peptide which can activate or increase gene expression	pYPQ152, pHSN6A01, pBUN6A11	Xing et al. (2014)

soybean, *Medicago*, tomato, *Brachypodium*, rice, *Sorghum*, and maize to access genome-wide predictions of specific gRNAs. Recently, Liang et al. (2017) and Svitashv et al. (2016) have demonstrated the efficiency of ribonucleoprotein-mediated GE in wheat and maize, wherein the off-target mutations were much lower than CRISPR/Cas9 DNA. In addition to that, efficiency of mutation was also higher in ribonucleoprotein-mediated GE.

Latest technological developments in CRISPR/Cas system

Advances have been made in native CRISPR/Cas system to improve its applications in plants. A new facet is being added regularly in the CRISPR technique. In this section technological development pertaining to CRISPR is discussed.

Vector construction There are two types of vector systems available for CRISPR namely binary vectors and single vector system. Binary vector system is an old one and has the advantage of rapid initial testing of CRISPR/Cas system. A vector containing different gRNA can be used for transformation of the plant which is already expressing the Cas9 protein. One more advantage associated with binary vector system is inclusion of different combinations of Cas proteins for specific gRNA, which gives more flexibility in designing the experiment with more targeted efficiency. A single vector containing Cas protein as well as gRNA is becoming more popular among researchers. In most of the single vector system, RNA polymerase II-based promoters such as CaMV35S, ubiquitin are used for expression of Cas9 gene whereas, RNA polymerase III-based promoters such as U6, U3 are used for expression of gRNA. Such type of

vector system exploits mixed dual promoters. Dual polymerase II promoter-based vectors use two different RNA polymerase II-based promoters to drive expression of Cas gene and gRNA whereas, single polymerase II promoter-based vectors uses only one RNA polymerase II-based promoters to drive expression of both *Cas* and gRNA. All these modifications help to reduce vector size which ultimately results in increased transformation efficiency (Lowder et al. 2015).

Delivery methods Transformation methods such as floral dip method, *Agrobacterium*-mediated *in planta* inoculation and regeneration of explants, particle bombardments, virus-mediated delivery, plasmid delivery, ribonucleotide protein complex delivery, RNA delivery, etc., are some of the recent advances in transformation methods for CRISPR in plants. Selection of viral delivery is limited, due to low editing efficiency in germline cells. Nevertheless, many reports are available for the virus-mediated delivery of CRISPR components in plants (Ali et al. 2015). Transformation of either Cas9 protein and gRNA complex (ribonucleotide protein) or Cas9 and gRNA alone through gene gun and PEG-mediated methods also reported the success (Wolter and Puchta 2017), but regeneration of protoplast still remains a major challenge associated with these types of methods.

Cpf1-an alternative to Cas9

The *cpf1* (also known as Cas12a) was identified by Schunder and colleagues (2013), in *Francisella* spp. Subsequently, Makarova et al. (2015), proposed a new classification for CRISPR/Cas systems, i.e., type V which is characterized by the Cpf1 ‘signature’ protein. Zetsche et al. (2015), cloned Cpf1 from the *Francisella novicida* (FnCpf1) and tested its function in *Escherichia coli*. The PAM requirement for

FnCpf1 is TTN and CTA (Zetsche et al. 2015). This system is also found in some of the bacteria like *Primoella*, *Acidaminococcus*, *Francisella*, *Lachnospiraceae*, etc.

The Cpf1 has some of the distinct characteristics as compared to Cas9 as it requires only a crRNA and does not utilize tracrRNA. The Cpf1 crRNAs are significantly shorter than ~100-nucleotide engineered sgRNAs required by Cas9, thereby offering cheaper and simpler guide RNA production. Small protein size and small gRNA (nearly half of the gRNA of Cas9) facilitate easy and efficient delivery of ribonucleoproteins to plant cell. Furthermore, the different sgRNA and crRNA requirements of Cas9 and Cpf1 allow both systems to be combined when multiplexing of different targets is desired. Although both Cas9 and Cpf1 make DSBs, Cas9 uses its RuvC- and HNH-like domains to make blunt-ended cuts within the seed sequence, whereas Cpf1 uses a RuvC-like domain to produce staggered cuts outside the seed sequence. Cpf1 produce staggered cuts which helps for directional cloning of the gene of interest. Different PAM sequence of Cpf1 gives increased possibilities to a target gene of interest at more than one place. One more advantage associated with the use of Cpf1 is that it cuts distal to PAM sequence and the targeted sequence may be susceptible for repeated cleavage. The publications on applications of Cpf1 in crop plants is on rise (Begemann et al. 2017; Kim et al. 2018; Liu and Wang 2017; Tang et al. 2017; Zaidi et al. 2017).

Screening assays Apart from the methods described in the section, companies like Applied Biological Materials (abm) (<https://www.abmgood.com>), TaKaRa (<http://www.clontech.com>), Thermo Fisher (<https://www.thermofisher.com>) are providing ready-to-use kits to check the efficiency of GE as well as monoallelic and biallelic mutations at target sites.

Various genetic modifications through genome editing in plants

Intervention in crop improvement using GE is increasingly becoming popular, widening its horizon from model plants to economically important cereals, legumes, fruits and vegetable crops. The successful applications of GE are described below under four broad sections.

Targeted mutagenesis

Deliberate changes, viz., addition/deletion/substitution intended at a specific site in the genome are termed as targeted mutagenesis. Zhang et al. (2010) reported an efficient method for targeted mutagenesis of *Arabidopsis ADH1* and *TT4* genes through regulated expression of ZFNs. In the same year, Osakabe et al. (2010) engineered ZFNs to target gene inactivation of a stress-response regulator

gene, *ABA-insensitive 4 (ABI4)* from *Arabidopsis*. Curtin et al. (2011) used CoDA method to engineer ZFNs and targeted mutagenesis of soybean Dicer-like (DCL) genes, RNA-dependent RNA polymerase (RDR), and HUA ENHANCER1 (HEN1) family members involved in RNA silencing. To achieve herbicide resistance, Pater et al. 2013, targeted *Arabidopsis polyphenol oxidase* gene (*PPO*) involved in heme and chlorophyll synthesis by designing specific ZFNs to create DSB in *PPO* and achieved butafenacil insensitive PPO. Christian et al. (2013) demonstrated that genome modification using TALENs can be efficiently transmitted to next generation by engineering TALENs which targeted *ADH1*, *MAPKKK1*, *TT4*, *NATA2* and *DSK2B* genes of *Arabidopsis*. Zhu's lab fused modified dHax3 DBD domain sequence to C terminal of a *FokI* cleavage domain and developed a hybrid TALEN (dHax3N) (Mahfouz et al. 2011). In vivo transient expression in tobacco leaves showed desired break using modified Hax3N in its artificial target sequence. Recently, four yield-related genes of rice, viz., *Gn1a*, *DEP1*, *GS3*, and *IPA1* were targeted using CRISPR/Cas9 for improving yield traits (Li et al. 2016).

Development of bacterial leaf blight (BLB)-resistant rice by high-efficiency TALE-based gene editing has been a landmark GE application in plants (Li et al. 2012). TALEN was used to edit a specific susceptibility gene, the sucrose-efflux transporter gene (*OsIIN3* aka *OsSWEET14*), by disrupting the effector-binding element in its promoter region without changing its expression. The resulting mutant lines displayed resistance to *AvrXa7* and *PthXo3* with morphologically normal phenotype. Recently, Blanvillain-Baufumé et al. (2017) used TALENs to target promoter of *OsSWEET14* to achieve resistance against bacterial blight. Shan et al. (2015) reported the mutation in the *OsBADH2* gene using specially designed TALENs resulting in increased aroma. Wang et al. (2016) used CRISPR/Cas system to achieve targeted mutations in *OsERF922*, ethylene responsive factor (ERF) which act as negative regulator of blast disease in rice (Liu et al. 2012) generating blast resistant rice lines.

Large-scale highly efficient targeted gene knockouts using TALENs were reported in rice (*OsDEP1*, *OsBADH2*, *OsCKX2*, and *OsSD1*) and *Brachypodium* (*BdABA1*, *BdCKX2*, *BdSMC6*, *BdSPL*, *BdSBP*, *BdCO11*, *BdRHT* and *BdHTA1*) with mutation rates reaching > 30% (Shan et al. 2013a). Liang et al. (2014), compared the mutagenesis efficiency using TALENs as well as CRISPR/Cas system in maize by targeting genes, viz., *ZmIPK1A*, *ZmPDS*, *ZmMRP4* and *ZmIPK* and obtained 23.1% efficiency in protoplasts and up to 13.3–39.1% efficiency in somatic mutations. Anderson et al. (2014), delineated that ZFNs can be effectively used for creating mutations in somatic as well as germline cells in polyploidy genome like soybean. High oleic soybean varieties were developed by targeted mutagenesis in fatty acid desaturase genes, *FAD2-1A* and *FAD2-1B* gene using

TALENs (Haun et al. 2014). Lor et al. (2014) reported the successful use of TALENs in tomato for targeted mutagenesis of PROCERA (PRO), which is a negative regulator of gibberellin signaling and successfully created new PRO allele. Wendt et al. (2013), reported the assembly of several TALENs for editing an anti-nutritional factor gene, phytase, *HvPAPHy* in barley and were able reduced its content in grain/seed. Another interesting study by Clasen et al. (2016) demonstrated the use of TALENs to knockout the vacuolar invertase gene (*VInv*) in potato, making its quality desirable for processing.

The CRISPR/Cas system has been used for targeted mutagenesis and was exploited in *Arabidopsis*, tobacco, rice and wheat. Zhang et al. (2014) tested 11 target genes in two rice sub-species for their amenability to CRISPR/Cas9-induced editing and determined the patterns, specificity and heritability of the gene modifications. Another study by Fauser et al. (2014), showed that only the nuclease, but not the nickase is an efficient tool for NHEJ-mediated mutagenesis in plants while targeting *ADH1* and *TT4* genes of *Arabidopsis*. Highly efficient site-specific modifications in rice and wheat using codon-optimized Cas9 of *Streptococcus pyogenes* and tailored gRNAs were reported by Shan et al. (2013b). Li et al. (2013), successfully achieved mutagenesis in the *phytoene desaturase* gene of *Arabidopsis* and tobacco using CRISPR/Cas9 with 2.7–4.8% frequency. A CRISPR/Cas construct containing two different cassettes of gRNA was successfully used to achieve mutations in two genes viz. *LAZY1* and *CHLOROPHYLL A OXYGENASE1* in *Arabidopsis* (Mao et al. 2013). Interestingly, CRISPR/Cas9 has been using in polyploid crop like wheat wherein only one homeoallele of *MLO-A1* gene was mutated to achieve resistance to powdery mildew (Wang et al. 2014). Jia and Wang (2014), for the first time reported the editing *phytoene desaturase* gene (*CsPDS*) in sweet orange and achieved mutation rate of approximately 3.2–3.9%. Zhang et al. (2016), demonstrated that transient expression of CRISPR/Cas9 DNA in wheat callus cells efficiently induced targeted and transgene-free mutants. Homozygous mutants with no detectable transgenes for *TaGASR7*, *TaGW2* and *TaLOX2* in hexaploid bread wheat and *TdGASR7* in tetraploid durum wheat were generated. Osakabe et al. (2016) used truncated gRNA (tru-gRNA)/Cas9 combination for GE in *Arabidopsis* to generate new alleles for *OST2* gene, a proton pump, with no off-target effects and high average mutation rates (up to 32.8%). The new mutant alleles for *OST2* exhibited altered stomata closing in response to environmental conditions which is a highly desirable trait for abiotic stress tolerance. Woo et al. (2015), demonstrated the potential of CRISPR/Cas in targeted mutagenesis by protoplasts transformation in *Arabidopsis thaliana*, tobacco, lettuce and rice and achieved efficiency up to 46% with small insertions or deletions indistinguishable from naturally occurring genetic variation.

Svitashev et al. (2016), also reported targeted mutagenesis in maize using the CRISPR/Cas9 ribonucleoprotein complexes. Ueta et al. (2017) optimized the CRISPR/Cas9 system to introduce somatic mutations selectively into a gene controlling parthenocarpy, *SIIAA9* and achieved 100% mutation rates in the T_0 generation. Kim et al. (2018), targeted wheat dehydration responsive element binding protein 2 (*TaDREB2*) and wheat ethylene responsive factor 3 (*TaERF3*) using CRISPR.

Targeted gene insertion/replacement

Gene replacement differs with targeted gene insertion, in which particular endogenous gene(s) of an organism is replaced with a new version of the same gene. Shukla et al. (2009) selectively targeted *IPK1* gene of maize (*ZmIPK1*) using specifically designed ZFNs to alter phytate biosynthesis in maize seeds. Further, this optimized method was used for site-specific insertion of a gene conferring tolerance to the herbicide bialaphos, *phosphinothricin acetyl transferase* (*PAT*). Zhang et al. (2013a, b) developed targeted insertion of herbicide resistance *acetolactate synthase* gene (*ALS*) gene in tobacco using TALENs. In another such study by Townsend et al. (2009), successful gene replacement for *SurA* and *SurB* genes in tobacco was achieved using ZFN to achieve resistance to imidazolone and sulphonyl urea with the maximum gene replacement frequency of 4%. Ainley et al. (2013), reported sequential stacking of two herbicide resistance genes '*pat*' and '*aad1*' using ZFNs with modular trait landing pads into the maize genome and demonstrated co-segregation of traits in subsequent generations. Recently, van de Wiel et al. (2017) discussed various aspects of GE in the presence of oligonucleotide to assist the repair of the DSB.

Targeted gene excision

The intended gene can be deleted or excised from the genomic region using GE. Petolino et al. (2010) demonstrated the excision of GUS by transforming transgene flanked by ZFN cleavage sites and crossing the transformants with the plant expressing a corresponding ZFN gene. Evidence for complete deletion of a 4.3 kb sequence comprising the *GUS* gene was shown. Antunes et al. (2012) demonstrated the excision of DNA segment from *Arabidopsis* genome using synthetic homing endonuclease, viz., PB1 which excised unwanted transgenic DNA from the genome. Such capacity of removal of undesired DNA segment may play a very important role in the development of marker free-transgenic plants (Curtin et al. 2012). This will be very much useful to excise antibiotic markers from already developed transgenic events which face biosafety hurdles due to the presence of antibiotic or any other marker genes. It can

also be extended to delete highly repetitive DNA to generate new loci or to eliminate undesired loci.

Targeted structural changes of genome

Targeted structural changes include large-scale addition, deletion, inversion, duplication or translocation of DNA at intended site in the selected genome. There are few examples which exploit SDNs for large targeted structural changes in plant species. Zhou et al. (2014) reported large chromosomal segment deletions through Cas9/sgRNA which were inherited in multiple generations in rice. The promoters of rice susceptibility genes, viz., *SWEET11* and *SWEET14* were edited at specific sites using Cas9/sgRNA resulting into heritable large chromosomal deletions (> 100 kb).

Potential target genes for GE for crop improvement

Though various economically important genes have been modified by GE, there is huge scope to exploit these tools to modify many useful genes in important food crops such as rice, wheat, maize, legumes, etc. Well-characterized genes present in single copy and governing qualitative traits can be the ideal choice for GE. Nevertheless, there are many examples where genes involved in quantitative traits are also targeted. Here, we have provided a list of some well-characterized genes from important crop plants which are highly potential targets for GE (Table 5). Practical considerations mentioned in section “Practical considerations” can be applied for both structural as well as regulatory genes and to target noncoding RNAs such as microRNAs (miRNAs). In case of miRNA, it is essential to understand the role of particular miRNA in the regulation of stress or development. The miRNAs being master regulators of biological pathways, need to be carefully picked for genome editing.

Table 5 Potential target genes for GE in some important crop plants

Crop	Trait	Gene	References
Rice	Rice blast resistance	WRKY gene 45	Akagi et al. (2014)
Rice	Yield/grain productivity	<i>OsSPL14</i> (squamosa promoter binding protein-Like 14, also known as IPA1)/ <i>OsEBS</i>	Dong et al. (2013)
Rice	Phosphorous use efficiency	<i>Pup-1</i>	Wissuwa et al. (1998, 2002)
Rice	Brown plant hopper resistance	<i>OsLecRK1</i> (lectin receptor kinase)	Ji et al. (2016)
Rice	Cold tolerance	Rice carbon catabolite repressor 4(<i>CCR4</i>)-associated factor 1B	Chou et al. (2014)
Rice	Drought tolerance	<i>osa-miR162</i> , <i>osa-miR164</i>	Fang et al. (2014), Tian et al. (2015)
Rice	Grain size, grain number, grain yield	<i>osa-miR397</i>	Zhang et al. (2013a, b)
Rice	Phosphate starvation response	<i>osa-miR399</i>	Hu et al. (2011, 2015)
Rice	Photoperiod-sensitive male sterility	<i>osa-miR2118</i>	Fan et al. (2016)
Wheat	Gluten content	<i>RabD</i>	Tyler et al. (2015)
Wheat	Disease resistance	Lr34	Krattinger et al. (2016)
Maize	Quality protein maize (QPM)	<i>opaque 2</i>	Mertz et al. (1964)
Maize	Disease resistance	<i>Htn1</i>	Hurni et al. (2015)
Maize	Heat tolerance	Peroxisomal ascorbate peroxidase (<i>TapAPX</i>)	Padaria et al. (2014)
Pearl Millet	Grain quality enhancement/reduction of grain anti-nutritional factors	Phytate, polyphenols, tannins	Vinoth and Ravindran (2017)
Groundnut	Increased content of poly unsaturated fatty acids	<i>FAD2</i>	Janila et al. (2016)
Tomato	Tomato yellow leaf curl virus resistance	<i>Ty-1</i> resistance gene	Butterbach et al. (2014)
Potato	Disease resistance	Potato late blight resistance genes	Jo et al. (2015)
Potato	Quality enhancement	Asparagine synthetase-1 gene	Chawla et al. (2012)
Soybean	Pod shattering resistance	NAC (NAM, ATAF1/2 and CUC2) family genes, SHATTERING1-5 (<i>SHAT1-5</i>)	Dong et al. (2014)
Pegionpea	Sterility Mosaic Disease Resistance	<i>SVI</i>	Daspute et al. (2014)
Pea	Disease resistance	<i>er1</i> (<i>E. pisi</i> resistance)	Fondevilla et al. (2006)
Chickpea	Drought tolerance	<i>ERECTA</i> -like kinase	Varshney et al. (2014)

It is important to understand the networking of miRNAs in the regulation of important biological processes. In a recent review, Mangrauthia et al. (2017a), have described the possibilities and challenges of miRNA editing for crop improvement.

Editing of quantitative trait nucleotide (QTN)

Quantitative trait locus (QTL) mapping is frequently used to identify genomic regions associated with a complex phenotypic trait of interest. Most of the QTL studies are unable to decipher how multiple genetic factors influence a particular phenotype. The QTLs can act through combined action of multiple sites within a gene or across multiple genes acting in the same gene set. Quantitative trait nucleotide (QTN) is a set of SNPs (single nucleotide polymorphism) in a gene or multiple genes of the same set that act together for expression of a particular trait. Till date, many QTLs have been identified in various crop plants governing agronomically important traits for tolerance/resistance to drought, salinity, cold, heat, disease and insect, etc., but, only a few QTLs are exploited for crop improvement. QTN can facilitate the use of QTLs in crop improvement more effectively (Lee et al. 2014). Identification of QTNs has become easier since the advent of several genome sequence information as well as rapid developments in SNP genotyping techniques including whole-genome association studies, but, the functional validation of these QTNs remains the biggest challenge for the successful application of QTNs in crop improvement. GE would be highly useful for functional analysis of QTN since it allows precise genome modifications at intended multiple genomic regions with ease and cost-effective way.

Introgression of the agronomically important QTLs from wild species or landraces into elite cultivars is one of the widely used practices by plant breeders. Functional analysis of QTN can facilitate the introduction of these QTLs in popular elite cultivars without affecting their original characteristics. GE can help in the creation of the desired allele at an intended locus which can replace marker-assisted introgression of genes and QTLs. This will not only solve the problem of linkage drag, but also would be less laborious and time-consuming. Novel alleles of important genes can also be created and examined for their phenotypic effects which can serve as new source of variation for plant breeding.

With respect to efficient use of QTNs in plant breeding, promotion of alleles by GE (PAGE) is gaining importance in recent times. Variants controlling the quantitative traits should be known to improve the trait using GE. Generally, as compared to small-effect QTNs, large-effect QTNs are easier to detect which is the reason why GE has concentrated more on large-effect QTNs. A large number of QTNs need to be edited for understanding the complex QTLs. Recently,

Jenko et al. (2015) has put forth the idea for use of PAGE in association with genomic selection (GS) in livestock breeding program. The potential of PAGE for improvement of quantitative traits has been checked by different strategies. It has been established that GS complemented by PAGE is more useful for selection due to the ability to increase favorable alleles at intended QTNs. Such studies are very much needed in plants to facilitate plant breeding for accurate improvement of quantitative traits.

Potential applications of genome editing for understanding plant genome

Targeted transcriptional regulation

Regulation of gene expression includes a wide range of mechanisms that are used by cells to increase/decrease/maintain level of specific gene products (protein or RNA). Plants are confronted continuously by challenges of surrounding environment and respond accordingly. The transcription factors, activators, enhancers and suppressors are key players in regulating genes at the transcriptional level. The nucleases can be potentially used to target transcriptional regulation of endogenous genes. By engineering the regulatory element binding sites by SDNs, the regulation of endogenous gene can be altered (Fig. 3a). In another approach, binding of SDNs to particular sequences blocks regulatory element to bind to those sequences which in turn attenuate its further regulatory function (Fig. 3b). The role of SDNs in targeted transcriptional regulation is quite new and needs to be explored further. Recent research in animal cells has shown SDNs in the targeted reprogramming of endogenous genes (Dominguez et al. 2015; Braun et al. 2016). Such studies have potential outcomes in plants as well. Reviews by Katayama et al. (2016) and Piatek and Mahfouz (2017) provide an update on programmable transcriptional regulation.

One of the interesting targets in plants is the noncoding miRNAs involved in gene regulation of various metabolic activities (Mangrauthia et al. 2017b). The expression of miRNAs can be altered suitably not only to understand their precise function, but also to engineer the trait under regulation. Fine tuning the expression of transcription factors and miRNAs would be highly useful to improve complex or quantitative traits such as drought stress, salt stress, and temperature stress tolerance. This has immense significance in developing resilient plants for combating the challenges of climate change. In future, there lies an expectant scope for plant scientists to exploit SDNs for functional genomic studies and crop improvement through modulation of key transcription factors or regulatory molecules such as miRNAs (Sailaja et al. 2014; Mangrauthia et al. 2017a). Thus, the known microRNA can be an ideal target for regulating

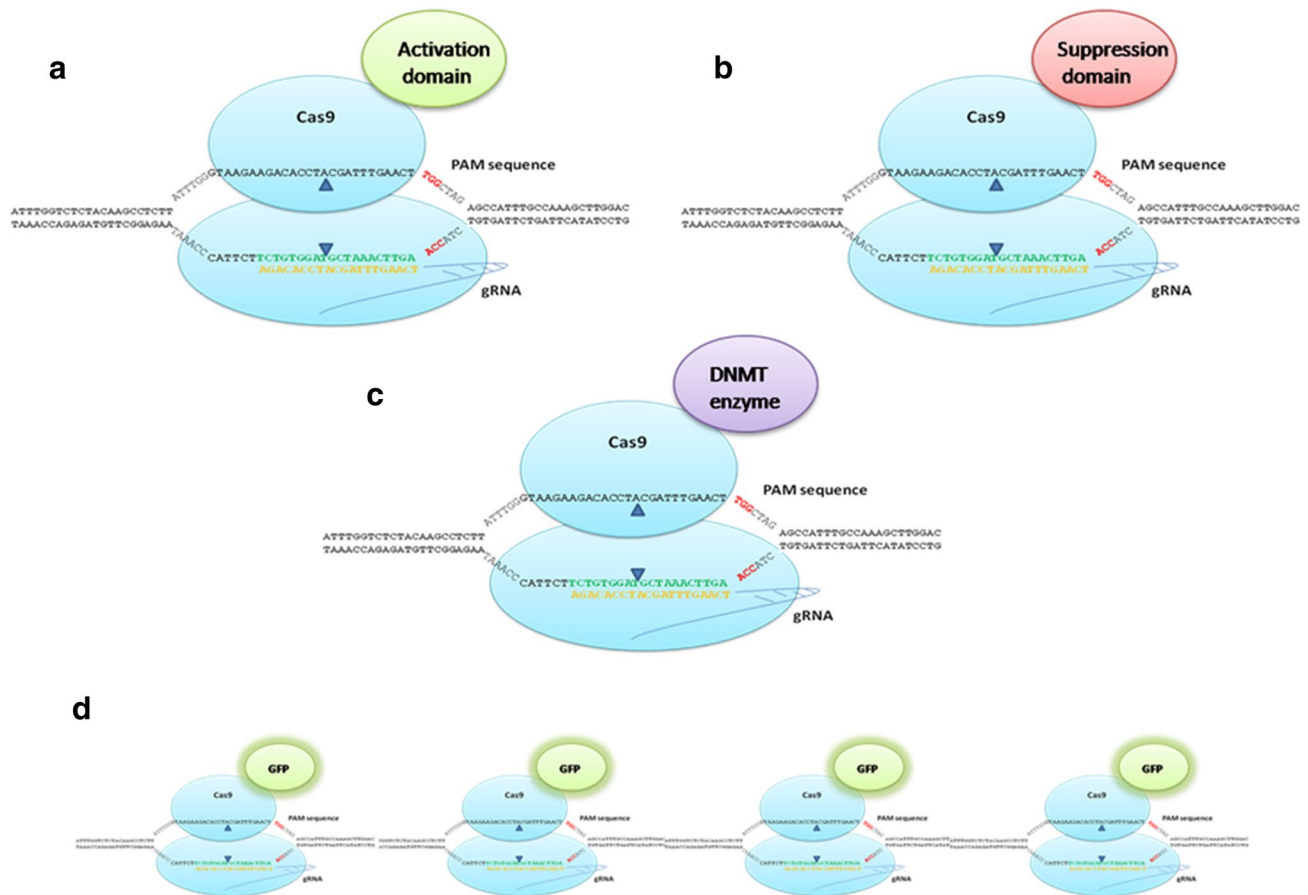


Fig. 3 Overview of various CRISPR/Cas 9-based applications. **a** Cas9 nucleases fused with activation domain can be used for transcriptional activation of targeted gene. **b** Cas9 nucleases fused with suppression domain can be used for transcriptional suppression of targeted gene. **c** Cas9 nucleases fused with chromatin modification

enzyme DNMT (DNA methyl transferase) domain can be used for epigenetic modifications of DNA or histone. **d** Cas9 nucleases fused with GFP (green fluorescent protein) can be used to enable imaging of specific genomic locus

the gene expression. Similarly, the roles of unknown miRNA can also be exploited using GE for the desirable trait of interest (Zhou et al. 2017).

Epigenome modifications

The biological code of life lies in the genome as well as in epigenome which has a profound effect on expression and regulation. The role of epigenome has been very well documented in case of stress tolerance, yield, heterosis, etc., in plants (Springer 2013). Engineering the regulators of DNA, histone methylation and other epigenetic modifications in plants gives new directions towards achieving desired phenotypes (Fig. 3c). Chromatin modification regulators such as HAT (histone acetyl transferase), DNMTs (DNA methyl transferase), HDAC (histone deacetyl transferase), IDM1 (increased DNA methylation 1), ROS1 (repressor of silencing 1), KYP3 (Kryptonite 3), DNA methyltransferases (MET1, CMT3, and DRM2), IBM1 (increase in

bonsai methylation 1), MBD7 (methylation binding domain 7), AS11 (Ant-silencing 1), etc., can be recruited at specific site of DNA for altering gene expression. As some epigenetic marks can be transmitted to offspring, epigenetic mechanisms may provide plasticity for the dynamic control of various agronomically important traits such as drought, heat, salinity, cold tolerance, etc., without the generation of genomic lesions. Thus, GE can be employed to create a novel source of epigenetic variation for trait improvement. Efforts have been made for epigenetic modifications in animal cells by targeting enzymes involved in histone modification or chromatin remodeling (Zentner and Henikoff 2015). Vora et al. (2016) have reviewed various aspects of epigenome modifications with CRISPR/Cas9 system.

Understanding plant–pathogen interactions

Generally, in compatible plant–pathogen interactions, the pathogen invades plant tissues and diverts its nutrition for

its growth by recognizing specific binding sites of nutrient transporters. The pathogen can also inactivate or alter the resistance-associated genes to make plant susceptible, for example, *Arabidopsis MPK3* and *MPK6* (mitogen-activated protein kinases) genes are inactivated by the HopAI1 effectors of *Pseudomonas syringae*, resulting in suppression of pathogen-associated molecular pattern-triggered immunity (PTI). The recognition sequences of such plant genes can be altered using SDNs without losing their primary function. Another approach involves knocking out of host susceptibility genes whose products are utilized by the pathogen for its own survival and growth and for negative regulation of plant defense responses. Many susceptible genes like *Mlo* in barley and *Arabidopsis* (negative regulator of *PEN* gene-associated disease resistance to powdery mildews) (Humphry et al. 2006; Piffanelli et al. 2004), tomato recessive allele *SIMlo2* and *SIMlo1* (Pavan et al. 2008), *Arabidopsis LOV1* gene (Sweat et al. 2008) and rice *HDT701* gene (Ding et al. 2010) can be targeted for achieving biotic stress resistance. Modifications of such genes using suitable engineered nuclease have brighter prospects for developing next-generation disease/insect-resistant crops (Liu et al. 2017).

GE can be used very effectively to understand the functional genomics of plant pathogenic fungi. Such efforts are being made to annotate the function of fungal elicitors and studying the plant–fungus interaction (Selin et al. 2016). Information generated from such study will be a valuable resource for plant resistance breeding. In a recent review, Barakate and Stephens, (2016) have discussed in depth the potential role of CRISPR/Cas9 system in understanding plant–pathogen interaction.

Functional genomics

Reverse genetics plays an important role in deciphering the function of a gene(s) by analyzing their phenotypic effects (Gilchrist and Haughn 2010). Several techniques of functional genomics like RNA interference (RNAi), Target Induced Local Lesions In Genome (TILLING) and Virus-Induced Gene Silencing (VIGS) have been extensively used in crop plants for deciphering the function of genes. A large number of genes need to be functionally characterized for improving agronomically important traits in various plants. Due to the advent of high-throughput sequencing technologies like next-generation whole-genome sequencing, transcriptome sequencing (RNA-Seq), metagenomics etc., a large amount of nucleic acid data of several plants and microbial species is available in public databases. In spite of the voluminous data, there has been very slow progress in determining the functions of genes (Zimin et al. 2014). Moreover, lack of functional annotation has been a major bottleneck in utilizing the information on genomics in crop improvement through breeding or transgenic approaches. GE

has an advantage in analyzing gene function as it mutates either one gene/allele or multiple genes depending on the study. Tools used for GE can target more than one gene at a time which is highly useful in the functional analysis of quantitative traits governed by several genes as against other techniques targeting only one or a couple of genes at a time (Jao et al. 2013). Furthermore, it will also allow understanding the gene/protein interactions and the network of genes involved in several interconnected biological pathways as well as that of repetitive elements. It is also possible to modify a particular domain/motif or individual amino acid of the protein without changing its structure or confirmation which is highly desirable in functional analysis of genes.

Precision plant breeding

Traditional breeding exploits the existing diversity in the gene pool. Lack of required diversity for traits is a bottleneck in present-day plant breeding. GE is one of the effective tools to create diversity in given plant species/germplasm which can be ultimately exploited for breeding (Rani et al. 2016). The genome edited plant can be used as a donor parent in conventional plant breeding to improve the desired trait. This is the fastest way to improve local varieties for desired traits. Cross-incompatibility and hybrid sterility are some of the major limitations of traditional plant breeding which affects exploitation of available allelic and genetic diversity. Knock out of genes involved in cross compatibility and hybrid sterility by GE can help to overcome these problems. Targeting genes involved in cell division and replication can be used to obtain true-to-type plants through apomixes in crop species where maintenance of purity is difficult, e.g., cross-pollinating plants. Development of haploid plant also can be achieved with the help GE by targeting genes involved in spindle fiber formation and cell division. Development of male sterile plants by targeting genes involved in maintaining pollen fertility is one of the interesting and emerging areas to hasten hybrid development (Chang et al. 2016; Zhou et al. 2016). These applications hold a golden promise for future generation crops.

Genome editing and regulatory considerations

The regulatory considerations in case of GE differ from that for genetically modified crops. Regulations can be drafted in a case dependent manner to handle genome edited crop plants. The changes brought through GE are very similar to natural mutations and may be put outside the purview of biosafety regulations. Worldwide reforms are made for regulations governing GM crops to accommodate the latest advances. Many researchers have opinion that GE is

likely to be less controversial than GM because of its precision. Additionally, such plants are free from any selectable marker. Although this technology offers a better alternative to GM that could be much more acceptable to consumers, there are issues to be solved. As GE is liable to off-site targeting, its probable side-effects need to be analyzed. Some off-targets can cause unknown nucleotide changes associated with unknown phenotypes. It is imperative to create awareness regarding the genome edited plants, better known to be NBT plants, among the consumers for facilitating its rational discourse in markets. Regulatory agencies need to consider how best to foster responsible use of GE without inhibiting research and development. Further, licensing of technology and issues of intellectual property rights (IPRs) need to be taken care in case of commercial applications of GE in crop plants. For more details regarding regulations, readers are directed to read review by Wolt et al. (2016b).

Challenges

The improvements in GE and their applications are ever expanding in the field of plant science. Although success has been witnessed in the specificity and ease of genome engineering in the last few years, challenges still exist. To achieve the full potentiality of this technology, major challenges must be addressed. Probability of getting DSBs and its further repair at both locus of a targeted region is less in diploid plant species. In case of polyploid plants, the targeted locus is present in more than two copies, in such condition to get a homozygous plant for a modified locus is more difficult. To achieve homozygous plant for all targeted loci, one needs to screen a large number of plant population after GE experiment. There is a demand to develop facilities like high-throughput phenotyping to evaluate the phenotypes of genome edited lines.

To boost applications of GE in crop plants, there is need to improve recombination frequency in several folds. The limitations associated with polyploid plants can be overcome by targeting a specific gene from the gene family by proper selection of target sequence (Anderson et al. 2014; Sánchez-León et al. 2017; Wang et al. 2017). Selection of unique gene sequence and avoidance of conserved sequence is the key to success for editing member of multigenes family.

Unavailability of highly efficient and sophisticated transformation methods for some important crops also poses a challenge. Nano-particle (gold or tungsten) delivery by gene gun, liposome-mediated transformation, macro-injection and WHISKR transformation methods should be validated for its efficiency. These methods can be used for better and effective transformation. There is need to develop well-standardized regeneration protocol for major food crops, vegetables,

and horticultural crops. Another challenge pertains in context to toxicity to cells caused by off-target DSBs, which prevent widespread use of these nuclease targeting systems in plants. Some of the possible strategies to overcome off-site targeting of SDNs have already been discussed. A better understanding of homology-directed endogenous DNA repair mechanisms that follow nuclease-mediated DNA cleavage will help to increase specificity and accuracy of an experiment by reducing off-site targeting and also help to develop new tools of GE. High-throughput sequencing platforms facilitate de novo sequencing and re-sequencing of genomes of many plant species which can generate vast data of SNPs. Most of the available SNPs are not phenotypically characterized. Phenotypically well-characterized SNPs can be good candidates for GE for crop improvements. So, there is need to initiate HapMap projects in important food crops which will provide a phenotypic characterization of SNPs.

Availability of pooled CRISPR libraries is one of the latest advancements. Addgene (<http://www.addgene.org>) provides such CRISPR-pooled libraries for different organisms. Such pooled libraries consist of thousands of plasmids, each plasmid containing a gRNA for a different target gene. Target cell should be treated with the pooled library to create a mutant population of cells, which can be phenotyped for the desired trait. To date, such pooled CRISPR libraries are not available for plant species. Availability of such libraries at least for model plants will be definitely a valuable resource for functional genomics and ultimately for crop improvement. Besides the coding region, libraries designed for the noncoding genomic region DNA will help us for functional characterization.

Conclusions

In the last few years, there has been an enormous acceleration in the development of GE tools and its applications for trait improvement. CRISPR/Cas is the most flexible and versatile version with tremendous potential for crop improvement. Biosafety rules and regulations for GE crops may not be much stringent as for transgenics because of its accuracy, specificity, and efficiency in the mode of action. GE not only helps in improving crops by altering traits, but also has been playing a tremendous role in functional genomics. Till date, GE is employed for improvement of single trait, but there is a great scope for improving multiple traits in a way to develop designer crops. There is a need to put more concerted efforts to address concerns like minimizing the off-site targeting, increasing rate of recombination frequency and developing high-throughput protocols for commercial crops. There is a need to formulate an international consortium to develop genome edited lines of important crops (e.g., rice, maize, wheat) covering the whole genome. Huge data

of SNPs can be used in defining the QTNs, and their use in crop improvement by GE. Overall, GE possesses the ability to boost next-generation plant breeding and plant genomic research.

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Compliance with ethical standards

Conflict of interest Authors declare that there is no conflict of interests.

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