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REVIEW

# Genome editing technologies and their applications in crop improvement

**Rukmini Mishra**[1] · **Kaijun Zhao**[1]

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**Abstract**
Crop improvement is very essential to meet the increasing global food demands and enhance food nutrition. Conventional crop-breeding methods have certain limitations such as taking lot of time and resources, and causing biosafety concerns. These limitations could be overcome by the recently emerged-genome editing technologies that can precisely modify DNA sequences at the genomic level using sequence-specific nucleases (SSNs). Among the artificially engineered SSNs, the CRISPR/Cas9 is the most recently developed targeted genome modification system and seems to be more efficient, inexpensive, easy, user-friendly and rapidly adopted genome-editing tool. Large-scale genome editing has not only improved the yield and quality but also has enhanced the disease resistance ability in several model and other major crops. Increasing case studies suggest that genome editing is an efficient, precise and powerful technology that can accelerate basic and applied research towards crop improvement. In this review, we briefly overviewed the structure and mechanism of genome editing tools and then emphatically reviewed the advances in the application of genome editing tools for crop improvement, including the most recent case studies with CRISPR/Cpf1 and base-editing technologies. We have also discussed the future prospects towards the improvement of agronomic traits in crops.

**Keywords** Crop improvement · Genome editing · ZFN · TALENs · CRISPR/Cas9

## Introduction

Rapid growth in population, climate change and environmental hazards are certain important issues that have threatened the global food security (Sundström et al. 2014). Increasing agricultural productivity is crucial for attaining food sufficiency. Hence, developing high yield, disease resistant and climate resilient crops is very essential to enhance the productivity of crops. All these years, conventional breeding methods like hybridization and mutation breeding have played a vital role in increasing crop productivity. But the gradual declination in natural genetic diversity in crop plants has hugely affected the crop production (Govindaraj et al. 2015). In last few decades, molecular breeding technology such as making use of transgenic or genetically modified (GM) crops has held great promise in overcoming the problems of conventional breeding approach and has strengthened the agricultural productivity. However, it is still labour-intensive and time consuming. Moreover, public acceptance of these GM crops is a great challenging due to social, environmental and political issues (Lusser et al. 2012).

Recently, genome editing has emerged as a novel technology that make use of sequence-specific nucleases (SSNs) to introduce targeted mutations in crops with high efficiency and precision and has been successful in overcoming the limitations of conventional breeding approach (Georges and Ray 2017). The artificially engineered SSNs such as zinc finger nucleases (ZFNs), transcriptional activator-like effector nucleases (TALENs) and clustered regularly interspaced short palindromic repeats (CRISPR)-associated endonuclease Cas9 (CRISPR/Cas9) have proven to be highly efficient in targeted mutagenesis in a wide range of crops and model plants (Schiml and Puchta 2016; Miglani 2017). The engineered nucleases can create a double-stranded breaks (DSBs) in the target region of the DNA, which is subsequently repaired by cell's own natural repair mechanism of

✉ Kaijun Zhao
zhaokaijun@caas.cn

[1] National Key Facility for Crop Gene Resources and Genetic Improvement (NFCRI), Institute of Crop Science, Chinese Academy of Agriculture Sciences (CAAS), Beijing 100081, China

homologous recombination (HR) or non-homologous end joining (NHEJ). The non-homologous end joining pathway is basically error prone, as it creates random mutations leading to frameshift mutations and target gene knockout, whereas in HR pathway the repair mechanism is more precise where a donor DNA containing sequences homologous to those flanking the DSBs site leading to gene replacement or foreign gene cassette knock in as intended (Voytas and Gao 2014; Baltes et al. 2015).

Although HR is more precise as compared to NHEJ, the frequency of illegitimate recombination is higher in NHEJ as compared to homologous recombination (Steinert et al. 2016). Hence, the use of gene replacement or gene targeting via HR is very limited in comparison to NHEJ. Gene knockouts and loss-of-function mutations help in understanding gene functions, but their applications in crop improvement are still limited as many of the important traits are conferred by the random point mutations, indels or insertion of a new gene (Lawrenson et al. 2015). Recently, CRISPR–Cpf1 (CRISPR from *Prevotella* and *Francisella* 1) is identified as a new tool for efficient genome editing, with higher efficiency, specificity, and potentially wider applications than CRISPR–Cas9 (Zaidi et al. 2017). Most recently, Base editing has emerged as an advanced approach which enables conversion of G–C base pairs to A–C base pairs without the requirement of a DSB or donor template (Komor et al. 2016; Shimatani et al. 2017). These advances in the field of genome editing can help in creating novel traits with high efficiency and precision and accelerate crop improvement.

There are several informative reviews on genome editing tools and their applications in crop improvement (Abdallah et al. 2015; Kumar et al. 2015; Petolino et al. 2016; Song et al. 2016; Khandagale and Nadaf 2016; Miglani 2017; Sharma et al. 2017). A thorough review of SDN-1 (site-directed nuclease-1) by (van de Wiel 2017) has mentioned the improvements and new variants of SDN-1, which is the generation of small deletions or insertions (indels) at a desired target by genome editing tools. In this review, we emphatically reviewed the advances in application of genome editing tools for crop improvement, including the most recent case studies with CRISPR/Cpf1 and base-editing technologies. We also address the challenges and future prospects of the genome editing tools towards improvement of crops.

## Genome editing tools: an overview

All genome editing tools rely on the SSNs which introduce double-stranded breaks (DSBs) at the specific target locus (Carroll 2014). The breaks are then subsequently repaired by cell's own endogenous repair mechanisms either by non-homologous end joining (NHEJ) or homologous recombination (HR) (Fig. 1) (Sander and Joung 2014). These repair mechanisms introduce point mutations in the target DNA sequences, thereby altering the reading frames and generating gene knockouts, insertions, replacements and chromosomal rearrangements (Chen and Gao 2014).

## Mega nucleases

Mega nuclease enzymes were the first to be used for genome editing purposes (Paques and Duchateau 2007). The mega nucleases are mainly classified into four families: LAGLIDADG, His-Cys box, GIY-YIG, and the HNH family and the LAGLIDADG family consists of popular mega nucleases I-CreI and I-SceI. They usually recognise and cut large DNA sequences (12–40 bps). Initially developed for the animal and human system, it has also been used for genome editing

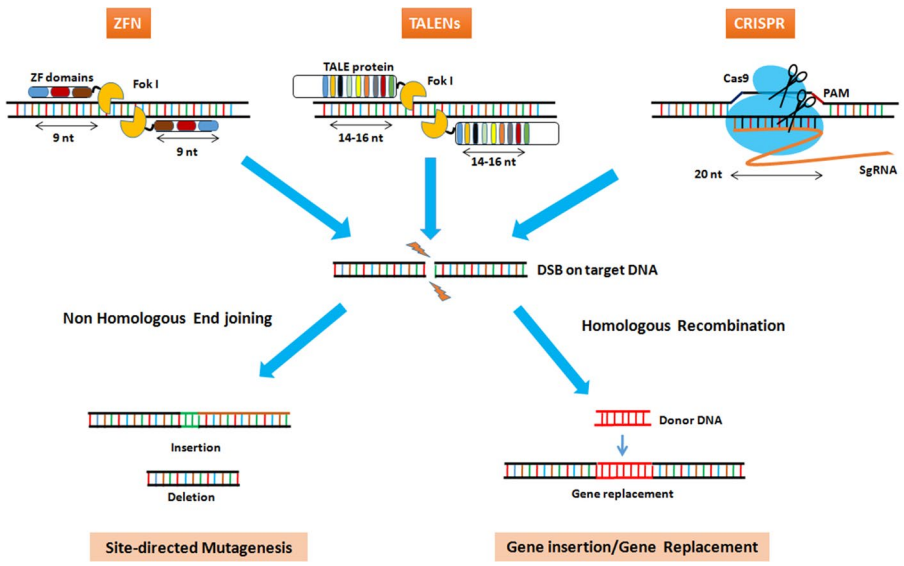


**Fig. 1** The basic mechanism of genome editing systems: the sequence-specific nucleases (ZFNs, TALENs and CRISPR/Cas9) create a double-stranded breaks (DSBs) at the target site which is subsequently repaired either by non-homologous end joining method (NHEJ) or homologous recombination (HR) by cellular system which results in gene disruption by indels or gene addition or replacement, respectively

purposes in plants, including *Arabidopsis thaliana* and *Zea mays* (Gao et al. 2010; Antunes et al. 2012). A modified mega nuclease, derived from the I-CreI homing nuclease was used to target *MS26* gene of maize and create plants that were male sterile when homozygous for the gene knockout (Djukanovic et al. 2013). However, the overlapping nature of the DNA-binding and nuclease domains of homing endonucleases makes it difficult to engineer them to target different DNA site(s) (Paques and Duchateau 2007) and their use was also limited due to patent issues.

## Zinc finger nucleases (ZFNs)

Zinc finger nucleases are thought to be the first generation-engineered endonucleases that uses designed zinc finger domains to recognise a specific DNA sequence in the genome (Carroll 2014; Lloyd et al. 2005). A typical Cys2-His2 zinc finger protein is composed of 3–6 zinc finger domains (ZFPs) and each is capable of recognising a 3 bp long target DNA sequence. The zinc finger protein is linked to a non-specific nuclease domain named *Fok*I. A pair of ZFNs bind to their respective DNA target sequences and align with each other in reverse fashion. Dimerization of the *Fok*I nuclease domain makes it active and enable it to create DSBs between the recognised sequences (Fig. 1) (Durai et al. 2005; Weeks et al. 2016). The DSBs are usually repaired by error-prone NHEJ pathway.

Sangamo Biosciences is the first company to develop ZFNs (Scott 2005). Although vectors containing corresponding DNA were available commercially from Sigma-Aldrich, the licensing agreements with Sangamo Biosciences and Dow Agro sciences have limited the use of ZFNs in major public funded molecular genetic laboratories. Oligomerized pool engineering, has been developed for use in plant genome modification (Townsend et al. 2009; Zhang et al. 2010). Context-dependent assembly (CoDA), which uses pre-selected two-finger units and bacterial one-hybrid screening of zinc finger libraries has been used as alternative methods to generate ZFNs (Sander et al. 2011; Curtin et al. 2012; Joung et al. 2000; Durai et al. 2005). Certain improvements in the generation of ZFNs can help simplify the editing tool, however, the non-specific binding of the zinc-finger motifs often results in off-target cleavage with ZFNs (Carroll 2011; Voytas 2013).

The use of ZFN for plant genome editing was first time reported in tobacco (Wright et al. 2005) and *Arabidopsis* (Lloyd et al. 2005). Till date, there are numerous successful reports of genome editing in tobacco, maize, *Arabidopsis*, soybean, canola and other plants (Shukla et al. 2009; Osakabe et al. 2010; Townsend et al. 2009; Curtin et al. 2011). An endochitinase-*50* (*CHN50*) gene sequence was successfully targeted by ZFNs for site-directed DNA integration and herbicide resistance in tobacco (Cai et al. 2009). The donor plasmid consisting of both ZFNs and a donor DNA construct comprising a pat herbicide resistance gene cassette flanked by short stretches of homology to the *CHN50* gene was successfully delivered by *Agrobacterium* and yielded up to 10% targeted homology-directed transgene integration. Similarly, Shukla et al. (2009) reported the targeted cleavage of the *Inositol-1, 3, 4, 5, 6-Pentakisphosphate kinase 1* (*IPK1*) gene, one of the phytic acid biosynthesis gene in maize. The donor template DNA containing a promoter less *PAT* gene along with a 2A 'stutter' sequence flanked by 815 bp of sequence homologous to *IPK1* gene was co-delivered by *Agrobacterium*. The *ABA Insensitive*-4 (*ABI4*) gene, driven by a heat-shock protein promoter in *Arabidopsis* (Osakabe et al. 2010), Dicer-*like* genes, *DCL4a* and *DCL4b*, under the control of an estrogen-inducible promoter in soybean (Curtin et al. 2011) and *Alcohol dehydrogenase* and *chalcone* synthase genes in *Arabidopsis* (Zhang et al. 2010) were successfully targeted by ZFNs for site targeted mutagenesis. Furthermore, Ainley et al. (2013) reported multiple trait stacking at a specific locus using ZFNs.

## TAL effector nucleases (TALENs)

Transcription activator-like effector (TALE) is a class of secreted proteins of plant pathogenic bacterial genus *Xanthomonas*, tend to be injected into plant cell through the type III secretion system to activate transcription of specific target genes that promote disease in plants (Boch and Bonas 2010). Each TALE contains a central DNA binding domain consisting of a variable number of 33–35 amino acid repeats. The repeats are nearly identical except for the two variable amino acids at the positions 12 and 13, designated as repeat variable di-residues (RVDs). Each RVD specifically recognizes one nucleotide, and the DNA target sequence of a given TALE is determined by combination of repeat number and composition of the RVDs in the repeats (Boch et al. 2009; Moscou and Bogdanove 2009). TALEN is a chimeric protein composed of a DNA binding domain of TALE and a *Fok*I nuclease domain. Like ZFNs, a pair of TALENs together make a cut in desired DNA region (Weeks et al. 2016). The DSBs are then repaired either by NHEJ or HR pathway. TALENs are more efficient than ZFNs in terms of low off-target effects.

The first successful use of TALENs for gene editing was reported in yeast (Christian et al. 2010; Li et al. 2011). Till date, TALENs have been successfully used for genome editing in almost all major model and crop plants like rice (Shan et al. 2015), wheat (Wang et al. 2014), maize (Char et al. 2015), tomato (Lor et al. 2014), potato (Clasen et al. 2016), barley (Wendt et al. 2013), Arabidopsis (Cermak et al. 2011), tobacco (Zhang et al. 2013) etc. TALEN system was used to induce mutations in the target gene, *Acetolactate synthase* (*ALS*), in potato (Nicolia et al. 2015) and tobacco

(Zhang et al. 2013). The *g*lossy2 (gl2) of maize and *PROCERA* genes of tomato have been modified with TALENs to create stable and heritable mutations (Lor et al. 2014; Char et al. 2015). TALEN technology has been efficiently used in creating fragrant rice (Shan et al. 2015), for disease resistance against bacteria blight (Li et al. 2012) and blast disease (Wang et al. 2017a, b). These studies indicate the importance of TALEN system in improving traits of crops.

### CRISPR–Cas9 system

The type II CRISPR/Cas9 system from *Streptococcus pyogenes* is the most recently developed genome editing tool, widely accepted because of its simplicity, efficiency, easy to use and amicability. CRISPR is a bacterial defence mechanism against bacteriophages (Barrangou et al. 2007; Jinek et al. 2012; Cong et al. 2013). CRISPR story began in 1980s with the discovery of 29-nucleotide repeat sequences in the *E*. coli genome by Ishino and co-workers while working on the *iap* gene (Ishino et al. 1987). The initial findings stimulated the interest in CRISPR research and then the pace got accelerated with a huge number of publications unravelling different aspects of CRISPR system. The CRISPR/Cas9 system contains mainly three components: the Cas9 endonuclease, a crRNA (CRISPR RNA) and a tracrRNA (trans-activating crRNA). The role of Cas9 endonuclease is to cut the invading phage DNA into small pieces, which get integrated into the CRISPR array as a spacer. Subsequently, crRNA and a complementary tracrRNA are transcribed from the CRISPR array and form a double-stranded RNA structure that recruits Cas proteins for cleavage (Datsenko et al. 2012; Jinek et al. 2012). A protospacer adjacent motif (PAM) sequence (5′-NGG-3′) present in the downstream of the target DNA is a pre-requisite for the binding and cleavage of target DNA (Jinek et al. 2012).

There are two nucleic acid binding grooves namely REC lobe and NUC lobe in the Cas9 endonuclease (Gasiunas et al. 2012; Jinek et al. 2012). The REC lobe is a Cas9-specific functional domain, whereas the NUC lobe consists of three components-RuvC, HNH and PAM-interacting domains (Nishimasu et al. 2014).The homology of RuvC and HNH helps in predicting their nuclease domain structures. The Cas9 enzyme becomes active when it comes in contact with sgRNA at its REC lobe. Then Cas9-sgRNA complex scans the double-stranded DNA for PAM sites, once encountered the proper PAM, the HNH nuclease domain cleaves the RNA–DNA hybrid, while RuvC cleaves the other strand. Subsequently, the DSBs is repaired either by the error-prone NHEJ or highly precise HR pathways. NHEJ usually introduce indel mutations, whereas HR repairs the DSBs more precisely with gene insertion or replacement methods (Shukla et al. 2009).

Unlike ZFNs and TALENs, the construction of CRISPR/Cas system doesn't require any complex protein engineering steps. CRISPR/Cas system also allows us to edit multiple genes at the same time with simultaneous introduction of DSBs at multiple sites (Mao et al. 2013). These advantages has helped the system in superseding its predecessors and get wide acceptance in scientific community. The first group of studies that demonstrated the applications of CRISPR/Cas system in plants was reported in the model species *A. thaliana* and *N. benthamiana* as well as in rice crop (Feng et al. 2013; Nekrasov et al. 2013; Shan et al. 2013a, b; Li et al. 2013). Subsequently, numerous articles were published indicating the potentiality of the CRISPR system in a wide range of crops like rice (Wang et al. 2016), wheat (Wang et al. 2014), maize (Char et al. 2017) and tomato (Pan et al. 2016). Genome editing with CRISPR/Cas9 has been demonstrated in rice for disease resistance (Wang et al. 2016), enhancing the grain weight (Xu et al. 2016), and herbicide resistance (Xu et al. 2014).

## Applications of genome editing tools in crop improvement

Genome editing technologies have been efficiently used in targeting genes in a wide range of crops and improve the varieties for higher yield, better quality, enhanced nutritional value and disease resistance (Table 1). The genome editing tools have shown great potentials in enhancing crop resistance to several biotic and abiotic stresses by targeting the traits mainly controlled by negatively regulatory genes (Wang et al. 2016). For example, the DuPont scientists have successfully employed CRISPR–Cas9 method to generate novel variants of ARGOS8, a negative regulator of ethylene responses in maize crop (Shi et al. 2017). They have tried to insert the native maize GOS2 promoter, into the 5′-untranslated region of the native *ARGOS8* gene or replace it via homology-directed DNA repair. The study has helped in generating novel variants of *ARGOS8* for breeding drought-tolerant crops in maize. Similarly, the targeted mutation of the *ERF* transcription factor gene *OsERF922* in rice enhanced resistance to the rice blast fungal pathogen (Wang et al. 2016). In a recent study, CRISPR/Cas9 technology was used to modify the canker susceptibility gene *CsLOB1* in citrus varieties (Jia et al. 2017). This study will provide a promising pathway to generate disease-resistant citrus varieties. The CRISPR/Cas system was successfully used to edit three homeoalleles (*TaMLO-A, TaMLO-B* and *TaMLO-D*) of the *MLO* gene that confers resistance to powdery mildew in bread wheat (Wang et al. 2014). Recently, Zhang et al. (2017), reported enhanced disease resistance to powdery mildew disease by modifying of three homoeologs of *TaEDR1* gene simultaneously in wheat by CRISPR/

**Table 1** List of genes targeted by genome editing tools for crop improvement

| Crop | Target gene | Genome editing platform | Function | References |
|---|---|---|---|---|
| Rice | *OsSWEET13* | TALENs | Enhanced resistance to bacterial blight | Zhou et al. (2015a) |
| | *OsERF922* | CRISPR/Cas 9 | Enhanced resistance to blast disease | Wang et al. (2016) |
| | *GW2, GW5* and *TGW6* | CRISPR/Cas 9 | Improvement of grain weight | Xu et al. (2016) |
| | *CSA* | CRISPR/Cas 9 | Photoperiod controlled male sterile lines | Li et al. (2016) |
| | *ALS* | CRISPR/Cas 9 | Enhanced herbicide resistance | Sun et al. (2016) |
| | *SBEIIb* and *SBEI* | CRISPR/Cas 9 | Generation of high amylose rice | Sun et al. (2017) |
| | *Os09g29100* | TALENs | Enhanced resistance to bacterial leaf streak | Cai et al. (2017) |
| | *Hd 2, Hd 4* and *Hd 5* | CRISPR/Cas 9 | Early maturity of rice varieties | Li et al. (2017a, b) |
| Wheat | *MLO* | CRISPR/Cas 9 | Resistance to powdery mildew | Wang et al. (2014) |
| | *TaEDR1* | CRISPR/Cas 9 | Resistance to powdery mildew | Zhang et al. 2017 |
| Maize | *ARGOS8* | CRISPR/Cas 9 | Novel variants of *ARGOS8* for drought-tolerance | Shi et al. (2017) |
| | *ZmIPK* | | Reduction of anti-nutritional compound phytic acid | Liang et al. (2014) |
| Barley | *HvPM19* | CRISPR/Cas 9 | Positive regulation of grain dormancy | Lawrenson et al. (2015) |
| Soybean | *FAD2-1A* and *FAD2-1B* | TALENs | Improvement of oil quality | Haun et al. (2014) |
| Tomato | *SlMlo1* | CRISPR/Cas 9 | Resistant to powdery mildew | Nekrasov et al. (2017) |
| | *SlWUS* | CRISPR/Cas 9 | Increased fruit size | Rodríguez-Leal et al. 2017 |
| | *SP5G* | CRISPR/Cas 9 | Day neutrality and early flowering | Soyk et al. (2016) |
| | SlAGL6 | CRISPR/Cas 9 | Facultative parthenocarpy | Klap et al. (2017) |
| Potato | *VInv* | TALENs | Reduction of sugar accumulation | Clasen et al. (2016) |
| Citrus | *CsLOB1* | CRISPR/Cas 9 | Resistance to citrus canker | Jia et al. (2017) |

Cas genome editing method. Most recently, a study demonstrated genetic modification of the *EBEtal7* binding site in the *Os09g29100* gene promoter via TALEN editing could be deployed to reduce Tal7 binding, which could potentially reduce disease severity (Cai et al. 2017). This approach is quite promising in engineering rice cultivars with reduced susceptibility to *X. oryzae*. Kamoun's group efficiently targeted *SlMlo1* gene, one of the major contributor to powdery mildew susceptibility, to generate transgene-free tomato named Tomelo, resistant to powdery mildew disease using CRISPR/Cas9 technology (Nekrasov et al. 2017). The transgene-free genetically edited crops like Tomelo can be widely adopted to meet the growing demands for food worldwide.

Plant viruses cause physiological changes in plants and are a major threat to global food security. Many researchers have demonstrated the application of CRISPR/Cas9 system to confer resistance against plant viruses (Ali et al. 2015b; Ji et al. 2015; Baltes et al. 2015; Iqbal et al. 2016). CRISPR/Cas9 system can be used to target multiple viral strains by co-infiltration of sgRNA targeting *Tomato yellow leaf curl virus* (TYLCV), *Beet curly top virus* (BCTV), and *Merremia mosaic virus* (MeMV) in *N. benthamiana* plants (Ali et al. 2015a, b). Similarly, virus interference activities have been demonstrated in *N. benthamiana* against *Bean yellow dwarf virus* (BeYDV) and BSCTV, respectively (Baltes et al. 2015; Ji et al. 2015). Cas9/sgRNA delivery through gemini virus replicons (GVRs) can be used to confer broad spectrum gemini virus resistance by targeting *Cotton leaf curl Kokhran virus* (CLCuKoV) and also showed that targeting the conserved mononucleotide sequence can simultaneously target multiple begomovirus (CLCuKoV, TYLCV, TYLCSV, MeMV, BCTV-Worland, and BCTV-Logan) (Ali et al. 2016). A multiplexed CRISPR/Cas9 system was used to target the whole cotton leaf curl disease (CLCuD)-associated begomovirus complex along with its associated satellite molecules for broad-spectrum resistance against begomovirus in cotton (Iqbal et al. 2016). CRISPR/Cas9 technology was used to develop virus resistance in cucumber (*Cucumis sativus* L.) by targeting the eIF4E (eukaryotic translation initiation factor 4E) gene (Chandrasekaran et al. 2016). The non-transgenic mutated lines exhibited broad resistance to viruses like Cucumber vein yellowing virus (CVYV), Zucchini yellow mosaic virus (ZYMV) and Papaya ringspot mosaic virus-W (PRSV-W). These studies suggest that CRISPR/Cas9 genome editing is an efficient technique for targeting multiple sites for multiple viruses to confer broad-spectrum resistance in major crops and model plants.

While most of the genome editing tools have so far being aimed at creating null alleles through engineering mutations in the coding sequences, recent development also suggested that various elements of CRISPR/Cas9 system could be incorporated at creating novel allelic variations through engineering diverse types of cis-regulatory mutations (Čermák et al. 2017). Scientists at Cold Spring Harbor Laboratory (CSHL) have successfully used CRISPR/

Cas9 technology to overcome yield barriers in crops and accelerate crop improvement. Lippman and his group have reported a seminal work on the development of novel variations in fruit size of tomato through targeted editing of a *cis* regulatory element (CRE) in the CLAVATA-WUSCHEL stem cell circuit (Rodríguez-Leal et al. 2017). Wild tomato species *Solanum pimpinellifolium* carrying a CRISPR/Cas9 mediated 4-bp deletion in the CArG promoter element of the putative tomato WUS (*SlWUS*) gene resulted in increased locule number and thus the fruit size. A similar experiment in a domesticated tomato (*S. lycoperson*) increased the fruit size by upto 70% (Rodríguez-Leal et al. 2017). As the *fascinated* (*fas*) and *locule number* (*ls*) QTLs that are generated through mutation in the promoter regions of *SlWUS* are considered as major contributors to fruit size in domesticated tomato (Xu et al. 2015; Somssich et al. 2016), it is tangible to believe that CRISPR/Cas9 technology could be used to engineer QTLs by mutating CREs with distinct functions.

Grain weight is one of the most important quantitative traits in rice production. Improving the grain weight in rice helps in improving rice production. CRISPR/Cas9-mediated multiplex gene editing was used for rapid pyramiding to improve grain weight in LH422, by targeting three major genes (*GW2, GW5* and *TGW6*) that negatively regulate rice grain weight (Xu et al. 2016). Pyramiding null mutations of major genes using CRSIPR/Cas9 has not only improved the grain weight in rice but also facilitates the study of quantitative traits and their applications in crop breeding. Similarly, rice heading date is also an important agronomic trait that determines rice distribution and production. Traditional breeding methods are time consuming and laborious. Thus, Chinese scientists have efficiently used the CRISPR/Cas9 mediated multiplex genome editing to target three major genes (*Hd2, Hd4* and *Hd5*) that negatively regulate the heading date of rice varieties to develop early maturity rice varieties (Li et al. 2017a, b).

The enhancement in nutritional properties of starch in rice grain has been possible due to the generation of high amylose rice plants by targeting starch-associated genes (*SBEII b* and *SBEI*) through CRISPR/Cas9-mediated genome editing (Sun et al. 2017). Liang et al. (2014) discussed the presence of anti-nutritional compound Phytic acid (PA), myo-inositol 1, 2, 3, 4, 5, 6-hexakisphosphate in maize. PA is poorly digested in humans and possesses a threat to the environment, thus, PA content of maize seeds was reduced by designing two gRNAs targeting the *ZmIPK* (inositol phosphate kinase) gene that catalyses a key step in PA biosynthetic pathway. The highly efficient ISU Maize CRISPR system developed by Bing Yang and his group is simpler, easy to construct and has increased frequencies of mutagenesis as compared to other systems. As the mutant lines generated through this system does not contain any foreign DNA sequences, it can be readily used for trait improvement in crops and widely accepted by research community (Char et al. 2017). Multicopy genes were targeted in *Hordeum vulgare* investigating the use and target specificity requirements of Cas9 editing (Lawrenson et al. 2015). They targeted two copies of *HvPM19* gene encoding an ABA-inducible plasma membrane protein, which in wheat acts as a positive regulator of grain dormancy, an important agronomic trait in cereals.

Oils with high oleic acid are considered to have a longer shelf life, enhanced oxidative stability and does not require hydrogenation process. In soybean, the oil quality was improved by targeting *FAD2-1A* and *FAD2-1B* genes that convert oleic acid to linoleic acid using TALENs (Haun et al. 2014). The mutated lines showed almost four times more oleic acid than the wild-type parents. Further, the mutated soybean lines were also found to be transgene free. This study indicates the potential application of genome editing technology in improving the oil quality in soybean. Cold storage of potatoes enhances their postharvest shelf life and make them available throughout the year. But long-term storage leads to accumulation of reducing sugars due to cold-induced sweetening (CIS) and further leads to the formation of acrylamide, a potential carcinogen (Clasen et al. 2016). Zhang and group have effectively knocked out the *vacuolar invertase* gene (*VInv*) using TALENs to reduce the accumulation of reducing sugars (Clasen et al. 2016). *VInv* is a gene which encodes a protein that breaks down sucrose into glucose and fructose. The knockout lines resulted in lower levels of reducing sugars and lacked foreign DNA.

## Recent advances in genome editing

### CRISPR–Cpf1: a new system for genome editing

CRISPR–Cpf1 (CRISPR from *Prevotella* and *Francisella* 1) is identified as a new system for targeted genome editing in mammalian systems (Zetsche et al. 2015). Cpf1 is class II type V endonuclease, that recognizes a T-rich PAM (5′-TTTN-3′) present in the 5′ end of the target site and requires a single crRNA of ~44-nucleotide (nt) length for cleavage. Cpf1 generates sticky or cohesive ends unlike blunt ends in case of SpCas9, which enhances the efficiency of gene insertion at a precise genome location. Cpf1 enzymes have also been shown to have lower rates of off-target activity as compared to Cas9 nucleases (Kim et al. 2016). These advanced features make it a more desirable editing system in plants as compared to SpCas9 (Zaidi et al. 2017). Several reports have demonstrated this new system to be an effective DNA-free genome-editing tool for plant genome editing (Kim et al. 2017; Xu et al. 2017). Cpf1 system was used for multiplexed gene editing in rice by editing four *OsBEL* genes (Wang et al. 2017a, b). Most recently, Yang and colleagues have

successfully generated stable and heritable mutations in rice by selecting two genome targets in the *OsPDS* and *OsBEL* genes (Xu et al. 2017).

Begemann and his colleagues screened the Cpf1 nucleases from *Francisella novicida* (FnCpf1) and *Lachnospiraceae* bacterium ND2006 (LbCpf1) for their ability to induce targeted gene insertions via homology-directed repair (Begemann et al. 2017). *Chlorophyllide-aoxygenase* gene of rice (*CAO1*) was selected as the target gene. Scientists have successfully demonstrated that both FnCpf1 and LbCpf1, has the capability to generate precise gene insertions as well as indel mutations in rice genome when used together with crRNA and repairing template DNA. This study indicates the wide adoption of Cpf1 genome editing technology can make a huge impact on plant biotechnology. CRISPR–Cpf1 system has wide applications in plant genome editing like functional screening based on gene knockouts, transcriptional repression using catalytically inactivated Cpf1 (dCpf1), or transcriptional activation using dCpf1 fused with a transcription activator domain, epigenome editing with dCpf1 fused to epigenetic modifiers, and the tracking of cell lineages with DNA-barcoding techniques. These advanced applications will enhance the improvement of yield and quality of crops and help in attaining food security and sustainability (Zaidi et al. 2017).

Yiping Qi and his colleagues demonstrated the efficacy of CRISPR/Cpf1 system (AsCpf1 and LbCpf1) as transcriptional repressors and their role in targeted gene repression in vivo (Tang et al. 2017). In the study, they demonstrated that when LbCpf1 is coupled with Pol II-promoter and a double ribozyme system to express and mature the crRNAs, acts as an effective mutagen in rice. CRISPR–Cpf1 technology was used to knock out an early developmental gene *EPFL9* (epidermal patterning factor like-9) in rice to study the loss of function of genes (Yin et al. 2017a, b). *OsEPFL9, also known as stomagen, is a* developmental gene in rice which regulates leaf stomatal density. The mutated plants showed eightfold reduction in stomatal density without having any off-target activity. The study not only helps in understanding the loss of functions of the gene but also helps in understanding the early development of plant.

## DNA-free genome editing in plants

The critical objectives to be considered during the escalation of CRISPR/Cas9 method are the prevention of transgene assimilation and minimization of off-target activity of Cas9. Accordingly, it is a goal to design a CRISPR/Cas9 Ribonucleoproteins (RNP)-mediated genome editing technology to generate adequate and definitive genome editing. The assessment of CRISPR/Cas9 RNP demonstrated strong editing activity and strong decrease in off-target activity in-vitro. Reserchers have succesfully edited a wide range of plants like *A. thaliana*, tobacco, lettuce and rice using CRISPR/Cas9RNPs (Woo et al. 2015). Similar studies have also been reported in maize (Svitashev et al. 2016). Recently, Korean scientists have efficiently delivered CRISPR/Cas9 RNPs to the protoplast system of grape and apple to generate transgene-free plants (Malnoy et al. 2016). Researchers have targeted a susceptible gene named *MLO-7*, to enhance disease resistance against powdery mildew in grape cultivar and *DIPM-1, DIPM-2* and *DIPM-4 genes* to enhance fire blight disease resistance in apple. Gao and group from China have effectively demonstrated CRISPR/Cas9RNP mediated genome editing in immature embryo cells of bread wheat (Liang et al. 2017).

## Base editing in cereal crops

Single-base changes generates elite trait variations in crop plants which helps in accelerating crop improvement (Zhao et al. 2011; Voytas and Gao 2014). Genome editing in plants using CRISPR–Cas9 system via homology directed repair (HDR) is quite challenging due to low frequency and efficiency of delivery of template DNA. Base editing which enables single base change in the genome without the requirement of a DSB or foreign DNA donor template could be an efficient and advanced approach for crop improvement (Komor et al. 2016; Nishida et al. 2016). A catalytically inactive CRISPR–Cas9 domain (Cas9 variant) is fused with a cytosine deaminase domain to form base editors which converts G–C base pairs to A–T base pairs (Nishida et al. 2016). There are few publications which demonstrates the optimization of base-editing in cereal crops (Zong et al. 2017; Shimatani et al. 2017; Lu and Zhu 2017).

Caixia Gao and group from China have successfully demonstrated base editing in cereal crops like rice, wheat and maize plants using a plant base editor named nCas9-PBE, composed of rat cytidine deaminase APOBEC1 and a Cas9 variant [Cas9-D10A nickase (nCas9)] (Zong et al. 2017). Researchers have designed a sgRNA for each of three rice genes (*OsCDC48, OsNRT1.1B* and *OsSPL14*), three different sgRNAs (S1, S2 and S3) for wheat *TaLOX2* and one sgRNA for maize *ZmCENH3*. The rice gene *OsCDC48* which regulates senescence and cell death was targeted using *Agrobacterium*-mediated transformation and the mutated plants revealed a mutation efficiency up to 43.48%. In case of wheat, pnCas9-PBE and pTaU6-LOX2-S1-sgRNA constructs were delivered into immature wheat embryos by particle bombardment. Sequencing results revealed C to T substitutions at positions 3, 6 and 9 in T0–3 mutated plant and one C to T substitution at position 3 in T0–7 mutated plants. T0 transgenic maize lines also revealed C–T mutations. The results indicated that nCas9-PBE can be used as a potential tool for producing point mutations useful in the precision breeding of wheat, rice and maize. Targeted

point mutations was also reported in rice by targeting two genes *OsPDS*, which encodes a *phytoene desaturase*, and *OsSBEIIb*, which encodes a starch branching enzyme IIb using nCas9 fused with a cytidine deaminase enzyme and the uracil glycosylase inhibitor (UGI) (Li et al. 2017a, b). The results revealed single base mutations with more than 40% mutagenesis efficiency.

Japanese scientists have successfully demonstrated targeted base editing in tomato and rice plants using Target-AID (target-activation induced cytidine deaminase) with a construct comprising nuclease-deficient Cas9 (*dCas9*) or nickase CRISPR/Cas9 (*nCas9*) fused to *Petromyzon marinus* cytidine deaminase (*PmCDA1*)1 and sgRNAs (Shimatani et al. 2017). Researchers have induced multiple herbicide-resistance point mutations in rice plants by multiplexed editing using herbicide selection. *Acetolactate synthase* (ALS) enzyme confers herbicide tolerance when mutated. Target-AID was used to mutate C287 in rice using an appropriate sgRNA and then the sgRNA together with $dCas9^{Os}$-$PmCDA1^{At}$or $nCas9^{Os}$-$PmCDA1^{At}$ was transformed into rice calli by the *Agrobacterium* method. Results revealed spontaneous resistance mutations in W548C/L regardless of Target-AID treatment at a frequency of 1.56%, but $nCas9^{Os}$-$PmCDA1^{At}$ induced 3.41% IMZ tolerance. In case of tomato, marker-free plants were generated harbouring stable DNA substitutions. Two endogeneous tomato genes, *DELLA* and *ETRI* that regulate plant hormone signalling were targeted using CRISPR/Cas9 system *Cas9At*. D10A mutant *nCas9At* was fused with either a human codon-optimized *PmCDA1* (*nCas9At-PmCDA1Hs*) or a version codon-optimized for *Arabidopsis* (*nCas9At-PmCDA1At*). Indels or C-to-T or C-to-G substitutions were observed in transformed lines indicating gain or loss of function in genomes. The above studies indicate that precise base editing is a useful and efficient approach to generate point mutations and accelerate crop improvement.

### Off-target activity: a major concern

The off-target activity of Cas9 is a major issue in CRISPR–Cas9 system (Fu et al. 2013). Improper concentration in the Cas9: sgRNA ratio, the presence of promiscuous PAM sites and insufficient Cas9 codon optimization are some of the factors responsible for undesired cleavage of the DNA regions (Song et al. 2016). Off-target effects may not be a serious problem in plants as the unwanted mutations can be removed by repeated backcrossing. But backcrossing is time-consuming and affects the progress in crop improvement. In case of plants with higher genome size but without genome sequence information, three different strategies like double nicking strategy, SpCas9-FokI strategy and SpCas9 strategy can be used for minimizing off-target activity (Ran et al. 2015; Tsai et al. 2014). Truncated sgRNA strategy can be used in plants with fully sequenced genomes to reduce off-target effects (Fu et al. 2014). Moreover, several computational algorithms such as Cas-OFFinder, CRISPR-P, CRISPR-Plant and CRISPR Primer Designer have been used to create gene-specific sgRNAs with minimal off-target effects (Bae et al. 2014; Yan et al. 2015b). Lawrenson et al. (2015) reported that a reduction in the expression levels of Cas9/sgRNA reagents could significantly decrease the off-target effects. Furthermore, next-generation sequencing methods like GUIDE-seq, Digenome-seq and ChIP-seq can also identify the off-target sites for Cas9/gRNA (Tsai et al. 2015; Kim et al. 2015). Shortening the gRNA spacer sequence to 17–18 nt increases targeting fidelity (Fu et al. 2014). Recently, high-fidelity Cas9 variants have been engineered by substituting 3–4 amino acids. These Cas9 variants have been quite effective in addressing off-target issue in plants (Kleinstiver et al. 2016).

## Future prospects and research opportunities

Recent advances in genome editing techniques have revolutionized the field of basic and applied biology. However, there are certain issues and challenges like the SSN/DNA delivery methods, off-target effects and the balance between HR/NHEJ pathways that has to be addressed for better efficiency and output. Although off-target activity is negligible in plants as compared to animal systems, still there is a need to address the off-target mutations systematically in major crops and other plant species. Moreover, some aspects of the editing system are still unclear, including the catalytic activity of Cas9, target sites identification and the importance of PAM sites. A thorough understanding of these aspects will help solving the problems associated with it and enhance the efficiency of the editing system. Several approaches like Digenome-seq and GUIDE-seq have been developed to detect off-target activities in human cells (Kim et al. 2015; Tsai et al. 2015) and need to be adapted to plants too for better efficiency and specificity of Cas9.

High-precision genome editing in plants, especially in transformation-recalcitrant species has been a major challenge in current times. Transformation methods are usually genotype specific and also raise regulatory concerns due *to Agrobacterium*-mediated transformation in plants (Yin et al. 2017a, b). Therefore, the DNA delivery methods and plant transformation issues have to be addressed. Recently, Lowe demonstrated that over-expression of *Baby boom* (*Bbm*) and *Wuschel2* (*Wus2*) genes from maize (*Zea mays*) increases transformation frequencies in different crops such as rice, maize and sorghum. (Lowe 2016). This approach is genotype independent and may be useful in improving transformation of recalcitrant crop species. Identification of *boom* and *wuschel* like genes can improve

the transformation efficiencies in recalcitrant crop species and broaden the genome editing applications for more crops in future.

Till date, the genome editing systems are mainly used to destroy genes in plants by inducing DSBs following NHEJ repair that introduces Indels at the target site. Precision editing by sequence replacement and fragment knock-in via homologous recombination (HR) has more important implications for crop improvement because more desired traits relay on gain of function mutation. However, the precision editing is still difficult because the efficiency of HR is very low in plants. Base editing has emerged as an advanced and precise tool in generating point mutations in crops. However, there is still a lot of scope for improvisation and its application in major cereal crops and plants. Plant cytidine deaminase homologs may also be tested for possible improvements in the efficiency of base-editing in near future.

## Conclusion

In the last 5 years, genome editing has flourished as a technology and revolutionized the field of functional genomics and crop improvement in plants. CRISPR/Cas9 has emerged as the most promising approach due to its simplicity, ease of use, versatility and tolerable off-target effects. The genome editing system holds great promise in generating crop varieties with enhanced disease resistance, improved yield and quality and novel agronomic traits which will be beneficial for farmers and consumers. The technology has been successfully used for targeted mutagenesis in various model and other crops. Most recently, CRISPR–Cpf1 has been used as a new and alternative method for plant genome editing which can overcome some limitations of CRISPR/Cas9such as the PAM site requirement, and therefore, widen the scope of genome editing in crops. Nevertheless, increasing case-studies suggest that the CRISPR/Cas9 is an efficient and frequently-used technology that can accelerate basic and applied research towards crop improvement.

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### Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

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