CRISPR/Cas for Crop Improvement*

A Brief Review

Arvinder Singh and Muskan Bokolia

CRISPR/Cas technology, among the other gene editing systems like TALENs, ZFNs and homing endonucleases, is the preferred choice for genome modification in all types of organisms—from microbes, plants to animals—and has countless applications in various disciplines as diverse as industries, basic research and medicines. In the recent past, this gene editing technology has been used for targeting multiple genes in various crops including *Arabidopsis*, rice, maize, soybean and tobacco for producing new varieties with improved traits like increased yield, biotic and abiotic stress tolerance, improved food quality. The advantages of this technology over genetic engineering for producing elite plants (non-transgenics) will avoid the stringent regulatory tests and ethical issues related to these plants being accepted by the public.

Since ages, plants have been an inseparable part of human life. These include horticultural crops, floricultural and ornamental plants which are cultivated for food, nutritional and medical uses, and also for aesthetic purposes. In the present scenario of the world's increasing population, ensuring food security (in terms of food availability with balanced nutritional quality) is a challenge to the human race. Other factors adding-on to this challenge includes reducing agricultural land, extreme weather fluctuations, biotic and abiotic stresses like drought, metals, pesticides, insects, and viruses which are constraining farming and food production at the pace with increasing population. Therefore, developing plants with diverse and desired characteristics will be of great value in solving the problems associated with agricultural practices for yield enhancement and improved nutritional qualities.

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Hundreds of years ago, man started improving crops by introducing new traits into plants from their crossable relatives through hybridization. Till the early 20th century, the sole method of producing genetic variations in plants remained the natural or spontaneous processes. Subsequently, breeders learned to create plant mutants using chemical and physical mutagens [1]. Although hybridization and mutagenesis techniques showed some promising results, the time required, the laborious nature of the former, and the randomness of the latter in getting the desired plant variety gave rise to the need of developing improved and advanced techniques for the purpose. With the advent of molecular biology technique—rDNA technology—DNA sequence-specific manipulations became possible by transferring the gene of interest under the tissue-specific promoters in the target plant using vectors. Several methods viz., physical (gene gun, sonication, electroporation), chemical (calcium phosphate precipitation), and biological (using Ti-plasmid) have been contributing since the last three decades to understanding basic plant biology and producing genetically modified/transgenic plants with improved qualities. Gene transfer using these methods usually leads to transient transformation or sometimes silencing of foreign genes; moreover, the release of these GM crops commercially has also been a matter of public concern.

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In the recent years, the emergence of highly versatile techniques based on targeted or site-specific nucleases (engineered/ bacterial) has opened up the possibilities of direct modification of the gene sequence of all the cell types in eukaryotic organisms [2]. Unlike transgenic approach, which generally results in the random insertion of a foreign gene into the host genome, gene editing technologies produce defined mutants and hence could serve as a potential tool in functional genomics and crop improvement. The basic principle behind the use of targeted nucleases involves the generation of double-stranded cut in the target-DNA followed by repair using homologous-recombination (HR) or non-homologous end-joining (NHEJ) methods, which results in substitutions and insertions/deletions (INDELs) at the target sites. These genome

editing techniques evolved from the use of homing endonucleases (HE) followed by protein-based nucleases. HEs, also known as meganucleases, are naturally occurring endonucleases in microbes with recognition sequence of 14 bp (in contrast to other restriction enzymes with 4–8 bp) and producing two segments of target DNA by site-specific cleavage [3]. However, these suffered from several drawbacks like lack of absolute sequence specificity and possible host genome mutations. This technique was further improved by incorporating DNA–binding domain (proteins) and an effector domain (nucleases, transcriptional activators and repressors, transposases, etc.) which provides novel characteristics of recognizing unique restriction sequences and specific cleavage at that place by the special restriction endonuclease like *Fok1*. Such gene editing tools included ZFNs and TALENs [4]. The ZFNs are composed of a zinc-finger binding domain (Cys2–His2-Zn finger protein) and a DNA cleavage domain (*Fok1* nuclease). TALENs comprise TAL protein which has a central DNA-binding domain composed of 13–28 repeat monomers of 34 amino acids and an endonuclease like ZFN. Another difference between these tools is that whereas TALENs can target 3 nt (nucleotides) in one go, ZFNs can address only 1 nt making the former more site-specific in its action than latter. Since TALENs have been shown to possess several advantages over ZFNs, especially the large range of engineered TALENs for target binding, it has been used as an effective genome editing tool in various organisms and cells viz., *Arabidopsis*, tobacco, human cells, etc [5]. Recently, a novel method alternative to *Fok1*- based methods for site-directed mutagenesis has been derived from an ancient immunity system adopted in nature by some prokaryotic cells like bacteria and is known as CRISPR. The latest discovery of CRISPR/Cas based technology for gene editing seems encouraging in terms of better efficiency, feasibility, and multirole clinical applications.

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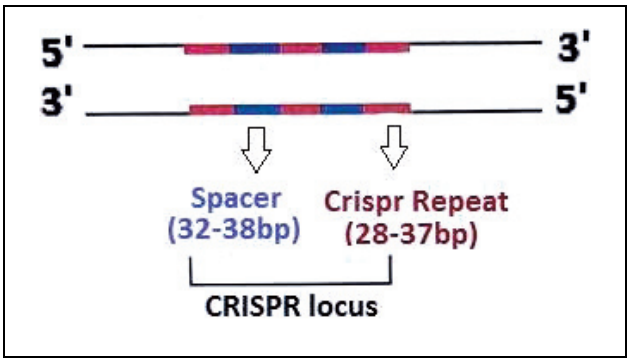

Figure 1. A CRISPR locus representing spacer (blue) and short DNA repeats (red).

What Is CRISPR/Cas Technology?

[1] See CRISPR/Cas9 System: 2020 Nobel Prize in Chemistry, Rajesh Ramachandran, Vol.25, No.12, pp.1669–1680, 2020.

CRISPR[1] stands for 'Clustered Regularly Interspaced Short Palindromic Repeats'. These are specialized stretches of DNA (28–37 bp) in the CRISPR locus present mainly in the bacterial systems to protect themselves from invading viruses. Between these repeats are present the spacer DNA which are acquired by the bacteria from the DNA of the phages that has attacked it previously (*Figure* 1). These spacers act as a genetic memory of the previous infection. If the same virus attacks again, the bacterial cell could defend itself by cutting off any of the attacking viral DNA sequence matching the spacer sequence. If bacterial cell counters any new virus, a new spacer is made and added to the chain of existing spacers and repeats. The enzymes (endonucleases, helicases and polymerases), which are the products of *Cas* genes help in the spacer acquisition. According to the bacterial cell types, the CRISPR system can be of three different kinds and help in protecting the bacteria from the viral attacks through the following steps (*Figure* 2):

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(A) **Adaptation** (spacer acquisition): It involves the cutting, processing and insertion of the DNA of the invading virus into the CRISPR sequences as new spacers (protospacers). It requires the use of Cas1 enzymes bearing nucleases activities.

(B) **CrRNA production**: The transcription of the CRISPR loci

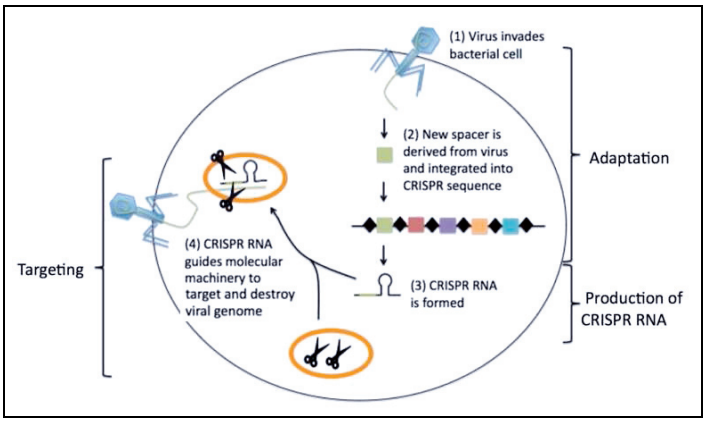


Figure 2. Three steps of the CRISPR/Cas system in the defense system of bacteria. Here, the black diamonds represent the short DNA repeats of a bacterial genome and the coloured boxes are spacers derived from the previously unseen virus that had infected a bacterium. The CRISPR sequence undergoes transcription followed by RNA processing to generate short crRNA molecules. The crRNA associates with and guides the bacterial molecular machinery to a matching target sequence in the invading virus. The molecular machinery cuts up and destroys the invading viral genome. Figure adapted from *Molecular Cell*, 54(2), pp.234–44, 2014.

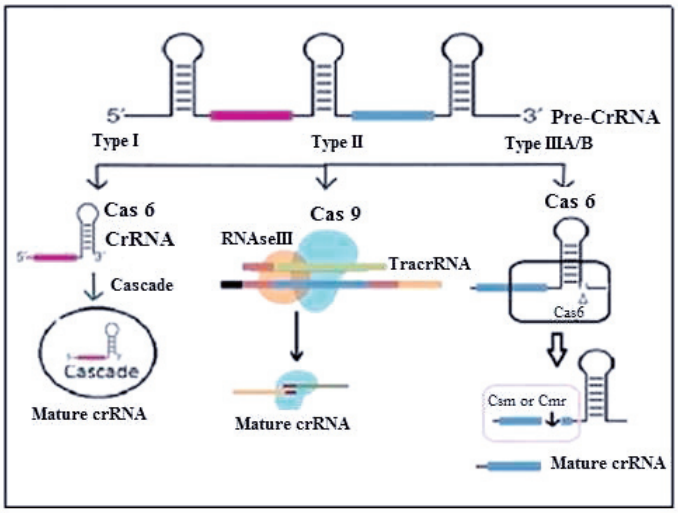
and the inserted spacers takes place in this step to form the pre-CRISPR RNA, which further undergoes processing to form mature crRNA. The processing of pre-crRNA is of three types involving different types of *Cas* gene products as depicted in *Figure* 3.

(C) **Targeting**: Here, crRNA acts as guide RNA and helps the bacterial molecular machinery (Cas enzymes, mainly endonucleases) to recognize the target, i.e., invading viruses and destroy it. The target region on the viral genome is associated with the 2–6 bp long DNA sequence adjacent to it and known as PAM (protospacer adjacent motif). It is recognized by Cas nucleases to cut the target DNA; however, the different Cas nucleases recognize specific PAMs. The most commonly used Cas in CRISPR based technology is Cas9 from *Streptococcus pyogenes* whose PAM sequence is 5′-NGG-3′.

Today, CRISPR is being used in all types of organisms and has countless applications in disciplines as diverse as industries, basic research, and medicine.

Transformation of CRISPR/Cas Reagents in Plants

It is well-known that the gene targeting involves the use of genetic engineering methods, either to insert a new DNA sequence at a



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specific locus or to substitute one gene for other desired foreign gene, in the host genome. Genetic engineering in case of plants to produce transgenics from single transformed cell requires various tissue-culture based transformation methods which mainly include protoplast transfection (transient expression), *Agrobacterium*-mediated gene transfer, and particle bombardment (stable expression). The latter two have been proved to be major delivery methods for CRISPR-mediated editing reagents including DNA, RNA and ribonucleoproteins (RNPs) in plants for the production of the edited plants. Despite their common applications, these methods have some disadvantages and limitations viz., random nature of gene insertion, labour intensity, time-consumption and applicability to limited plant species. Further, it is challenging to create mutation using CRISPR/Cas technology in some transformation recalcitrant species such as wheat, cotton, soybean, and woody plants using these *in-vitro* generation methods. Following the revolutionary progress of CRISPR/Cas9 mediated plant genome editing, researchers have focused on the development of more efficient delivery platforms, preferably into the germline cells to bypass the need for tissue culture and regeneration after editing [6]. Besides this, the focus has also been to generate

genome-edited plants that are free of foreign DNA. The various delivery methods viz., floral-dip, nanoparticle-mediated delivery and magnetofection-mediated delivery methods have been employed successfully in some plants for producing edited plants without requiring a plant tissue culture facility [7]. The other advantage of using these delivery methods is that these are not limited to protoplast but can directly deliver CRISPR/Cas reagents into plant regenerative tissues.

Further, in the case of many allopolyploid plant species like wheat and cotton, it would be difficult to target multiple homologous genes at a time using classical plant transformation methods. With the advancement of CRISPR/Cas technology, different strategies have also been developed to target multiple genes at a time, i.e., multiplexing. This multiplex genome engineering can target various genes within the genome or distinct targets within one gene to increase mutation efficiency [8, 9]. This technique involves the expression of multiple gRNAs under different promoters or single promoters using the polycistronic gRNA unit. This CRISPR/Cas based multiplexed genome editing has been demonstrated and applied in various crops such as potato, *Arabidopsis* and banana, and has been proved as a more relaxed approach in comparison to the other known genome modification tools [10–12]. Recently, three homeologous of TaGASR7 (*TaGASR7-A1*, *-B1* and *-D1*) has been targeted in wheat to study the frequency of mutations and heritability generated through multiplexing genome editing [13]. The five sgRNAs were combined in tRNA spaced polycistronic cassettes under the transcriptional control of single TaU6 promoter and bombarded into the mature embryo of hexaploid wheat. Analysis of T_0 generation resulted in inducing the targeted transgene-free mutants for all the six alleles. Another study on wheat has also shown this approach as an efficient method for genome editing in complex polyploid crops [14].

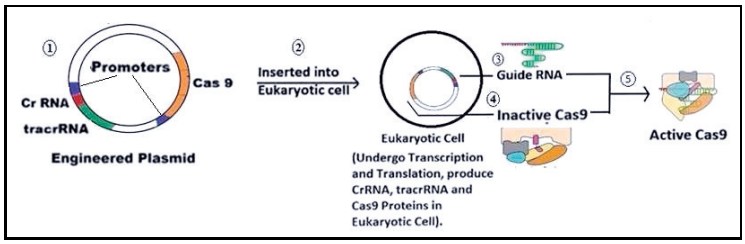

Figure 4. CRISPR/Cas9 cassette engineering and its insertion into the host species/cell lines. (1) Engineering of the CRISPR/Cas9 system involves the ligation of single guide-RNA in place of the complementary crRNAs and tracrRNAs of the natural CRISPR system and the Cas9 protein with the promoters specific for gRNA and Cas9. (2) Transfection into the host cell using a suitable transformation method. (3) Transcription of guide RNA as a single sequence. (4) Transcription and translation of Cas9 nuclease. (5) Binding of guide RNA to Cas9 and activation of Cas9.

Applications of CRISPR/Cas Technology in Crop Improvement

The natural ability of bacteria to defend itself from the attack of bacteriophages could be harnessed by delivering the gene editing reagents in the cell for the targeted gene. The CRISPR methodology requires (i) the synthesis of single guide RNA (gRNA), (ii) identification of PAM sequences in the target site and (iii) Cas nuclease enzymes. A complete gene construct, involving gRNA along with *Cas* gene sequence specific for particular Cas enzyme generation, is cloned into a suitable vector and then introduced into the host cells followed by the screening of edited lines for the desired trait (*Figure* 4). In the last half decade, the CRISPR/Cas9 technique has been used extensively to edit plant genome with ease compared to ZFNs and TALENs methods. This gene editing technology has also been used for targeting multiple genes in various crops including *Arabidopsis*, rice, maize, soybean and tobacco for producing new varieties with improved traits like increased yield, biotic and abiotic stress tolerance, improved food quality, etc. The use of CRISPR for genome editing in various crops for trait improvement has been presented briefly as below.

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Yield and Quality Improvement

Rice, being a model plant in monocots as well as having an abundance of PAM, has been the target for using the CRISPR/Cas9 technology for improving any trait of interest. Several genes viz., *GW2*, *GW5*, and *TGW6* have been knocked out using this technique with the result of 30% increase in the thousand-kernel

weight [15]. Similarly, in hexaploid wheat, the knock-out of the *GaSR7* gene also exhibited an increase in thousand-grain weight [16]. In maize seeds, the phytic acid has been known as an anti-nutritional component as well as an environmental pollutant. The targeted knock-out of the genes viz., *ZmIPK1A*, *ZmIPK*, and *ZmMRP4* involved in phytic acid synthesis had resulted in improving the nutritional quality of the *Zea mays* [17]. For enhancing the oil composition in *Camelina sativa*, CRISPR/Cas9 technique has shown better results than TALENs in knocking out all the three *FAD2* genes–whose products were responsible for decreasing the oil properties [18]. Similarly, knock-out of the RNA recognition motif-containing gene *SlORRM4* delayed the tomato fruit ripening by lowering the respiratory rate and ethylene production [19]. In rice, starch nutritional properties have also been improved after targeting and mutating the *SBEIIb* gene for modifications in the long chains of amylopectin using CRISPR/Cas9 technology [20]. Many of the food applications of potato rely on its starch quality and improving it has been an important area of research. Knock-out of four alleles of *GBSS* gene in hexaploid potatoes has resulted in producing waxy genotype with complete lack of amylose counterpart [21].

Biotic and Abiotic Stress Resistance

Gene editing has also been applied to increase stress resistance by editing stress-related genes in different plants. The canker-resistant citrus cultivars were produced by CRISPR/Cas9-targeted modification of the *CsLOB1* promoter [22]. Using the same approach, CRISPR/Cas9-mediated gene disruption of the tomato *SIMLO1* gene has resulted in rapid generation of tomato, fully resistant to powdery mildew [23]. Recently, through gene editing of *Gh14-3-3d* , resistance to *Verticillium dahliae* infestation has been reported in cotton [24]; this cotton could be a potential source in breeding programmes. Similarly, the knockout of *VvWRKY52* gene in grape increased disease resistance to fungal infection (*Botrytis cinerea*) [25]. CRISPR/Cas9 genome editing system has also been reported in wheat protoplasts for two

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abiotic stress-related genes namely, wheat dehydration responsive element-binding protein 2 (*TaDREB2*) and wheat ethylene-responsive factor 3 (*TaERF3*). Herbicide tolerance is a very important trait in agriculture worldwide. So far, only four glyphosate-tolerant GM crops (transformed with bacterial *EPSPS* gene) are grown on a large scale: corn, soybean, rapeseed, and sugar beet. CRISPR/Cas9 has also been used successfully in rice to generate herbicide resistance through HDR-based genome changes and by gene knock-out respectively [26]. In addition to producing herbicide-resistant crops, CRISPR/ Cas9-mediated gene insertion and replacement methods have created drought-resistant properties in maize [27].

Improvement in Horticultural Crops

To combat the burden of fulfilling the food security, there is also a dire need to improve the horticultural crop production along with quality traits linked aligned to consumer preferences. Comprising an essential part of agriculture production, most genome editing (Ca. 72%) with horticultural crops has been performed in vegetables followed by some floral and medicinal plants. Many vegetable crops, viz. cabbage, carrot, cucumber, potato, etc., have undergone genome editing using CRISPR/Cas technology for modifications in their plant height, albino character, resistance against viruses and increased amylopectin/amylose ratio respectively [28–30]. Seedless fruits is an attractive attribute nowadays. Parthenocarpic tomatoes have also been tried by introducing mutations into *SlIAA9* gene using CRISPR/Cas9 strategy. To promote early flowering and fruit development in plants, reducing the long juvenile phase could be a good strategy. Utilizing CRISPR technology in apple and pear, *TFLI* gene was edited with the help of two sgRNAs, resulting in early flowering [31]. Similarly, GABA is regarded as a health-promoting functional compound. A 19-fold increase in its content has been demonstrated in genome-edited tomato plant [32]. In numerous ornamental plants, like *Lilium*, *Chrysanthemum*, *Petunia*, *Torenia*, etc., this strategy has also proved beneficial in improving the fragrance, colour, and

shelf-life of flowers.

CRISPR/Cas Versus Genetic Engineering

During the last 20 years, rDNA technology is serving as an important tool for producing plants with improved traits related to nutritional quality, stress resistance, disease resistance, etc. However, it has certain limitations. Here, the whole gene constructs with selectable marker genes are permanently introduced into the targeted plant. It is an expensive and time-consuming affair with the chances of non-targeted integration of transgene in the genome where it may also interrupt endogenous gene expression. Moreover, after the creation of GMOs using this technology, there are still several obstacles in their commercialization as these have to pass through the regulatory testing and ethical issues which may take 8–10 years for the release of the improved variety. In contrast to genetic engineering, CRISPR/Cas system has been proving as a promising and revolutionary technology for producing directed and controlled changes in the genome. This system is delivered as a protein-RNA complex which gets degraded after edition. So, these non-transgenic improved plants may not be subjected to the existing regulations on genetic modifications. Further, this technology will save the high investments of both time and resources spent on producing GMOs using rDNA technology. In addition, it can be convincible for public acceptance since these plants will not be carrying any foreign gene.

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Future Prospects

New technology, viz. ZFN, TALENs and CRISPR described above do not fall under the definition of GMOs as per regulatory regimes in many countries. The genome editing technology, mainly the CRISP/Cas9 system, has emerged as a modernized tool for genome modifications not only in plants but also in animals and microbes. Newly modified CRISP/Cas systems like xCas9, Cpf1, Cas13, Cas14 are also being used for genome editing purposes nowadays. As no technology develops without limitation, CRISPR/Cas sys-

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tem also need to overcome some obstacles. This system depends upon designing the guide RNA, so it would be required to sequence the whole genome of the plant to prevent off-target gene editing. To ensure higher chances of genome modification, selection of suitable promoter for both gRNA and *Cas* gene must be highly addressed. Another issue which also needs to be resolved is the large size of the CRISPR/Cas system which is unsuitable for its packing into the viral genome. Moreover, to expedite the genome editing process, tissue culture free systems must be developed as *Agrobacterium*-mediated gene transfer cannot be carried out with ease in all plants, and it takes a long time to select and characterized the mutants in the system.

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