CRISPR/Cas9: A Revolutionary Tool for Recent Advances in Crop Improvement: A Review

Abhishek K. Gowda*, S. B. Mishra, Mainak Barman and M. Saikumar

Department of Plant Breeding and Genetics, Dr. Rajendra Prasad Central Agricultural University, Pusa, Bihar, India

*Corresponding author

Abstract

The recent advances in agricultural biotechnology and genetic engineering have brought numerous benefits to the food and agricultural sector by improving the essential characteristics of plant agronomic traits. Targeted genome editing using sequence specific nucleases (SSNs) provides a general method for inducing targeted deletions, insertions and precise sequence changes in a broad range of organisms and cell types. Genome editing tools, such as siRNA-mediated RNA interference, transcription activator-like effector nucleases (TALENs) and zinc-finger nucleases (ZFNs) for DNA repair has been widely used for commercial purposes. However, the discovery of the CRISPR/Cas9 system as genome editing tool it has revolutionized the broad field of life sciences. Clustered regularly interspaced short palindromic repeats (CRISPR) was discovered for the first time in bacteria and archaea as a virological defensive DNA segment. CRISPR-Cas9 as an advanced molecular biological technique can produce precisely targeted modifications in any crop species. CRISPR/Cas9 owing to its efficiency, specificity and reproducibility, this system was said to be the “breakthrough” in the field of biotechnology. Apart from its application in the field of biotechnology, it is widely used in crop improvement.

Keywords

Genome editing, SSNs, CRISPR, Cas9, Crop improvement

Introduction

Crop plants, possess a complex genome organization and gene expression hence it is difficult or impossible to perform site-specific mutagenesis for the development of desirable agronomic trait - more indirect methods must be used, such as silencing the gene of interest by RNA interference (RNAi). But sometime gene disruption by siRNA can be variable or incomplete. The advent of genome editing, or genome editing with engineered nucleases (GEEN) or targeted genome editing (TGE) (type of genetic engineering in which DNA is inserted, replaced, or removed from a genome using artificially engineered nucleases, or "molecular scissors") through which targeted genome editing is accomplished in wide variety of agronomically important crop species using sequence specific nucleases (SSNs) (Kamburova et al., 2017). The current applications of genome editing in plants, focuses on its potential for crop improvement in terms of adaptation, resilience, and end-
use. Novel breakthroughs are extending the potential of genome-edited crops and the possibilities of their commercialization (Wang et al., 2015). Basically the success of genome editing relies on two natural DNA repair mechanisms they are 1) Non Homologous End Joining & 2) Homology Directed Repair. Nuclease such as ZFNs, TALENs, Mega-nuclease and CRISPR/Cas can cut any targeted position in the genome and introduce modifications which are impossible using conventional RNAi. Unlike ZFNs, TALENs and mega-nuclease chimeric proteins target site recognition by CRISPR/Cas9 system is accomplished by the complementary sequence based interaction between the guide (noncoding) RNA and DNA of the target site and the guide RNA and Cas protein complex has the nuclease activity for exact cleavage of double-stranded DNA using Cas9 endonuclease (Kamburova et al., 2017). In addition ZFNs, TALENs as a tools of genome editing they are very costlier as they require a protein engineering prior to use them as genome editing tool than the ideal genome editing tool called CRISPR/Cas9, which is very much cheaper and has very high efficiency in target genome editing and it is widely used in genome editing of plants in order to develop novel genotypes with desirable agronomic traits to strengthen the global food security (Fig. 1).

**CRISPR/Cas9**

Clustered regularly interspaced short palindromic repeats are the segments of prokaryotic DNA containing, repetitive base sequences. CRISPR plays a key role in a bacterial defense system, form the basis of a genome editing technology known as CRISPR/Cas9 that allows permanent modification of genes within organisms. CRISPRs are found in approximately 40% of sequenced bacterial genomes and 90% of sequenced archaea (Mojica et al., 2005)

**Major breakthroughs in CRISPR timeline**

In the mid-2000s few microbiology and bioinformatics laboratories began investigating CRISPRs (clustered regularly interspaced palindromic repeats), which had been described in 1987 by Japanese researchers as a series of short direct repeats interspaced with short sequences in the genome of Escherichia coli (Ishino et al., 1987). Predictions were made about CRISPR as their possible roles in DNA repair or gene regulation (Makarova et al., 2002; Guy et al., 2004). A major breakthrough came in 2005 with the observation that many spacer sequences within CRISPRs derive from plasmid and viral origins (Bolotin et al., 2005; Mojica et al., 2005; Pourcel et al., 2005). Together with the finding that CRISPR loci are transcribed (Tang et al., 2002) and the observation that Cas (CRISPR-associated) genes encode proteins with putative nuclease and helicase domains (Bolotin et al., 2005; Pourcel et al., 2005; Jansen et al., 2002; Haft et al., 2005), it was proposed that CRISPR-Cas is an adaptive defense system that might use antisense RNAs as memory signatures of past invasions (Makarova et al., 2006). In 2007, infection experiments of the lactic acid bacterium *Streptococcus thermophilus* with lytic phages provided the first experimental evidence of CRISPR Cas-mediated adaptive immunity (Barrangou et al., 2007).

This finding led to the idea that natural CRISPR-Cas systems existing in cultured bacteria used in the dairy industry could be harnessed for immunization against phages a first successful application of CRISPRC as for biotechnological purposes (Barrangou et al., 2012). In 2008, mature CRISPR RNAs (crRNAs) were shown to serve as guides in a complex with Cas proteins to interfere with virus proliferation in E. coli (9). The same year, the DNA targeting activity of the
CRISPR-Cas system was reported in the pathogen *Staphylococcus epidermidis* (Marraffini et al., 2008).

**Natural CRISPR system**

CRISPR-Cas loci comprise a CRISPR array of identical repeats intercalated with invader DNA-targeting spacers that encode the crRNA components and an operon of Cas genes encoding the Cas protein components. In natural environments, viruses can be matched to their bacterial or archaeal hosts by examining CRISPR spacers (Andersson et al., 2008; Sun et al., 2013). Adaptive immunity occurs in three stages i) insertion of a short sequence of the invading DNA as a spacer sequence into the CRISPR array; (ii) transcription of precursor crRNA (pre-crRNA) that undergoes maturation to generate individual crRNAs, each composed of a repeat portion and an invader targeting spacer portion; and (iii) crRNA-directed cleavage of foreign nucleic acid by Cas proteins at sites complementary to the crRNA spacer sequence.

**Types of CRISPR/Cas system**

Three CRISPR/Cas system types (I, II, and III) use distinct molecular mechanisms to achieve nucleic acid recognition and cleavage (Makarova et al., 2011; Makarova et al., 2011). The protospacer adjacent motif (PAM), a short sequence motif adjacent to the crRNA-targeted sequence on the invading DNA, plays an essential role in the stages of adaptation and interference in type I and type II systems (Deveau et al., 2008; Horvath et al., 2008; Mojica et al., 2005; Shah et al., 2013).

The type I and type III systems use a large complex of Cas proteins for crRNA-guided targeting (Brouns et al., 2008; Nam et al., 2012; Haurwitz et al., 2010; Hatoum-Aslan et al., 2011; Rouillon et al., 2013; Hale et al., 2009). However, the type II system requires only a single protein for RNA-guided DNA recognition and cleavage (Jinek et al., 2012; Gasiunas et al., 2012) a property that proved to be extremely useful for genome engineering applications (Fig. 2 and 3).

There are three types of CRISPR/Cas systems, which vary in their specific target and mechanism of action (Makarova et al., 2011).

Type I systems cleave and degrade DNA,
Type II systems cleave DNA,
Type III systems cleave DNA or RNA.

**CRISPR/Cas9 is a type II CRISPR/Cas system**

**Key components of CRISPR/Cas9**

**crRNA**

Contains the guide RNA that locates the correction section of host DNA along with a region that binds to tracrRNA (generally in hairpin loop form) forming an active complex (Fig. 4) (Jinek et al., 2014).

**TracrRNA**

Binds to crRNA and forms an active complex (Jinek et al., 2014)

**sgRNA**

Single guide RNAs are a combined RNA consisting of a tracrRNA and at least one crRNA (Jinek et al., 2014).

**Cas9**

Protein whose active form is able to modify DNA. It as different subunits like HNH domain, RuvC domain, PAM interacting domain etc. (Fig. 5) (Jinek et al., 2014).
CRISPR-Cas9 as a genome editing tool

Different strategies for introducing blunt double-stranded DNA breaks into genomic loci, which become substrates for endogenous cellular DNA repair machinery that catalyze nonhomologous end joining (NHEJ) or homology-directed repair (HDR). Cas9 can function as a nickase (nCas9) when engineered to contain an inactivating mutation in either the HNH domain or RuvC domain active sites. When nCas9 is used with two sgRNAs that recognize offset target sites in DNA, a staggered double-strand break is created. Cas9 functions as an RNA-guided DNA binding protein when engineered to contain inactivating mutations in both of its active sites. This catalytically inactive or dead Cas9 (dCas9) can mediate transcriptional down-regulation or activation, particularly when fused to activator or repressor domains. In addition, dCas9 can be fused to fluorescent domains, such as green fluorescent protein (GFP), for live-cell imaging of chromosomal loci. Other dCas9 fusions, such as those including chromatin or DNA modification domains, may enable targeted epigenetic changes to genomic DNA (Fig. 6) (Doudna et al., 2014).

General protocol for employing CRISPR/cas9 as a genome editing tool

Select target Genomic region

- 20 bp sequence followed by the PAM (NGG)
- Use online tools to minimize off-targeting
- CHAPCHOP

Few tools which can help in designing the sgRNA complementary to the expected target site within target genomic location are

Select which Cas protein is to be used

*Streptococcus pyogenes* Cas9 (SpCas9) is the most common Cas9 for genome engineering

Different Cas proteins are used depending upon PAM sequence availability near the target genome

Design sgRNA

Assembling Cas9-sgRNA construct and transferring to the desirable vector

Mobilization into the host

Lipofection
Electroporation
Agrobacterium transformation
Particle bombardment

Evaluation/Screening of target gene in the host

Sequencing
Gene Specific markers
Southern hybridization
RT PCR
Western hybridization

Potential application of CRISPR/Cas9 in agriculture

Development of viral resistant crop plant

One of the most common viral infections that notably reduces plant harvest worldwide is
caused by Geminiviruses (from the Geminiviridae family) CRISPR-Cas9 system with modified sgRNA was used to target six different regions of the bean yellow dwarf virus (BeYDV) genome in order to reduce geminivirus replication in a transgenic plant model (Baltes et al., 2015). Significant reduction in copy number of BeYDVs was observed in plants that were treated with CRISPR-Cas9 utilizing four engineered sgRNAs (gBRBS +, gBM3+, gBM1−, and gB9nt+). Tashkandi et al., (2018) developed a CRISPR-Cas9 system to engineer Nicotiana benthamiana and Solanum lycopersicum plants to induce immunity against tomato yellow leaf curl virus (TYLCV).

Development of disease resistant crop plants

CRISPR-Cas9 system was also evaluated for the delivery of mutations in the TaMLO-A1 and TaMLO-B1 gene of bread wheat to generate transgenic plant resistant to powdery mildew, which is a common fungal disease caused by fungi. Study was undertaken in the allotetraploid cotton genome using two sgRNAs (GhMYB25-like-sgRNA1 and sgRNA2) with high mutation frequency (Li et al., 2017). The study was successful in providing plants with resistance against Verticillium wilt.

Development of abiotic stress resistant crop plants

Maize is majorly cultivated using dry farming techniques (Tykot et al., 2006), and drought-tolerance in maize is a major issue. Precise CRISPR-Cas9 genome editing was carried out at the ARGOS8 locus, which is a negative regulator of ethylene response, in order to generate drought tolerant breeding (Shi et al., 2017). Comparing to the wild-type, ARGOS8 variants exhibited improved grain yield under flowering and grain-filling stresses. CRISPR-Cas9-mediated mutation targeting ALS1 and ALS2 increased herbicide-resistance in maize (Svitashev et al., 2015). ALS2 gene editing using single-stranded oligonucleotides as repair templates could successfully provide chlorsulfuron resistance to maize.

Enhancing the level of crop production

By Improving the nutritional quality of crop

Application of CRISPR-Cas9 genome editing machinery to increase amyllose and resistant starch content in cereals such as rice. (Sun et al., 2017) employed the CRISPR-Cas9 system to produce targeted mutagenesis in SBEI and SBEIib genes in rice. Generated rice mutant presented amyllose and resistant starch content increased by 25 and 9.8%, respectively, which, consequently, improved nutritional properties of starch in rice grain.

The seed company Corteva Agriscience (a merger of the companies Dow, Dupont and Pioneer) has taken the lead in using CRISPR-Cas technology for crop improvement. In the spring of 2016, the company’s scientists developed the first commercial crop with this technology: a new generation of waxy maize. While the starch from ordinary maize kernels consists of 25% amyllose and 75% amylopectin, the grains of waxy maize contain almost exclusively amylopectin (97%). Amylopectin starch is relatively easy to process and is widely used in the food processing industry and in the production of adhesives. For example, the glue on cardboard boxes and on the adhesive strips of envelopes is often derived from amylopectin starch. The problem was that the first generation of waxy maize - developed through traditional breeding - had a lower yield than traditional varieties. This has now been remedied thanks to CRISPR-Cas. The researchers at Corteva Agriscience not only
succeeded in deleting the waxy gene, they did this in most of the current elite varieties. This makes it possible to create waxy maize varieties much faster and in a way that avoids the loss of yield. These maize varieties are expected to appear on the American market in a few years, pending field trials and regulatory testing (Table 1 and 2).

**Table.1** Examples of some of the crops modified through CRISPR/Cas9 (Harrison et al., 2014)

| CROPS     | DESCRIPTION                       | REFERENCES                |
|-----------|-----------------------------------|---------------------------|
| Corn      | Targeted mutagenesis              | Liang et al., 2014        |
| Rice      | Targeted mutagenesis              | Belhaj et al., 2013       |
| Sorghum   | Targeted gene modification         | Jiang et al., 2013        |
| Sweet orange | Targeted genome editing           | Jia and Wang, 2014       |
| Tobacco   | Targeted mutagenesis              | Belhaj et al., 2013       |
| Wheat     | Targeted mutagenesis              | Upadhyay et al., 2013; Yanpeng et al., 2014 |
| Potato    | Targeted mutagenesis              | Shaohui et al., 2015      |
| Soybean   | Gene editing                      | Yupeng et al., 2015       |

**Table.2** Advancements made in the field of agriculture and food sector through CRISPR/Cas9 (Harrison et al., 2014)

| GENOME EDITING TOOL | TRANSFORMATION METHOD | CROPS MODIFIED                          | TARGETED GENES                                                                 |
|---------------------|-----------------------|-----------------------------------------|-------------------------------------------------------------------------------|
| CRISPR/Cas9         | Stable integration    | *Arabidopsis thaliana*                  | TT4, GAI, BRI1, JAZ1, CHLI, AP1                                              |
| CRISPR/Cas9         | Protoplasts; *Agrobacterium* T-DNA (Transient) | *Arabidopsis thaliana*; Nicotianabenthamiana | AtPDS3, AtRACK1C, NbPDS3                                                     |
| CRISPR/Cas9         | Protoplasts; *Agrobacterium* T-DNA (Transient) | *Arabidopsis thaliana*; Tobacco; Sorghum; *Oryza sativa*               | OsSWEET14                                                                     |
| CRISPR/Cas9         | Protoplasts;Bombardment | *Oryza sativa*; *Triticum aestivum*     | OsPDS, OsBADH2, Os02g23823, OsMPK2, TaMLO                                     |
| CRISPR/Cas9         | Stable integration    | *Arabidopsis thaliana*                  | Transgene                                                                     |
| CRISPR/Cas9         | Stable integration    | *Arabidopsis thaliana*                  | AtBRI1, AtJAZ1, AtGA1, OsROC5, OsSPP, OsYSA                                   |
| CRISPR/Cas9         | *Agrobacterium* T-DNA (Transient) | *Sweet orange*                        | PDS                                                                          |
| CRISPR/Cas9         | Protoplasts           | *Zea mays*                              | IPK                                                                           |
| CRISPR/Cas9         | Stable integration    | *Oryza sativa*                          | SWEET11/13/1a/1b                                                             |
| CRISPR/Cas9         | Stable integration    | *Oryza sativa*                          | PDS, PMS3, EPSPS, DERF1, MSH1, MYB5, MYB1, ROC5, SPP, YSA                    |
| CRISPR/Cas9         | Stable integration    | *Arabidopsis thaliana*                  | ADH1, TT4                                                                     |
| CRISPR/Cas9         | Protoplasts           | *Arabidopsis thaliana*                  | PDS3                                                                          |
| CRISPR/Cas9         | Protoplasts; Stable integration | *Oryza sativa*               | Labdane-related diterpenoid gene clusters on chr 2, 4 and 6                |
**Fig.1** Types of molecular scissors

![Diagram of molecular scissors](image)

**Fig.2** CRISPR timeline (Wang et al., 2018)

1987
- The 29 nt tandem interval repeat sequences was found in E. coli in 1987

2000
- A genome editing technique CRISPR/Cas9 was developed in vitro in 2012

2005
- CRISPR/Cas9 system was engineered and used to edit mammalian cells in 2013

2010
- CRISPR/Cas9 was used to inactivate oncogenic mutations in 2016

2015
- In 2016 CRISPR/Cas9 nuclear dynamics and target recognition were revealed in living cells

2020
- In 2017 CRISPR/Cas13 can target RNA in mammalian cells

**Fig.3** CRISPR/Cas9 Cascade (Zhu et al., 2019)

![Diagram of CRISPR/Cas9 Cascade](image)
Fig. 4 Genomic CRISPR Locus (Jinek et al., 2014)

Fig. 5 Subunits of Cas9 protein (Jinek et al., 2014)

Fig. 6 Working mechanism of CRISPR/Cas9 as a genome editing tool (Khatodia et al., 2016)
**Fig. 7** General protocol of CRISPR/Cas9 mediated genome editing in plants (Eş et al., 2019)

![Diagram of CRISPR/Cas9 protocol](image1)

**Fig. 8** Chronological timeline of CRISPR/Cas9 achievements (Kamburova et al., 2017)

![Timeline of CRISPR/Cas9 achievements](image2)

CRISPR-Cas9 technology is very important to produce potato with higher yields in a shorter time. Gene knockout of tetraploid potato (*Solanum tuberosum*) was performed by transient expression of CRISPR-Cas9 (Andersson et al., 2017). RNP-delivery of CRISPR-Cas9 machinery resulted in commercial lines with higher yields without the integration of DNA.
Manipulating plant genome in order to produce bioactive compounds

Butt et al., (2018) used CRISPR-Cas9 system in rice (Oryza sativa) in order to disrupt the carotenoid cleavage dioxygenase 7 (CCD7) gene, which modulates plant growth, reproduction, senescence, and controls an essential step in SL production. Two sgRNAs (gRNA1 and gRNA2) were engineered to target the 1st and the 7th exon and subsequently produce knockout phenotypes. Some mutants could present a significant increase in tillering. Kim et al., (2016) also achieved improved lycopene and isoprene production in E. coli by manipulating the MVA pathway (mvaK1, mvaE) and ispA. Xylose production in E. coli was also significantly improved by after manipulation of the xylose pathway (xylA, xylB, tktA, talB) using the CRISPR-Cas technology (Zhu et al., 2017) (Fig. 7 and 8).

Advantages

Simple design and preparation
Multiplexing genes- editing more than one gene at a time
Cheaper compare to other genome editing techniques
Possible to alter the gene expression even without altering genome-cas13 mediated gene editing

Some pitfalls/Limitations of this technology

Off target indels (insertions and deletions)
Limited choice of PAM sequences

Solutions to overcome limitations

Proper selection of gRNA
Make sure that there is no mismatch within the seed sequences (first 12 nucleotides adjacent to PAM)
Use smaller gRNA of 17 nucleotides instead of 20 nucleotides

Sequencing the crop plant before working with it
Use NHEJ inhibitor in order to boost up HDR (Homology Directed Repair)

In conclusion the genome editing is a revolutionary technology for making rapid and precise changes in the genetic material of living organisms. This can be done in the DNA of plants, microbes, animals and humans. Using this technology, scientists can change a specific DNA letter, replace a piece of DNA or switch a selected gene on or off. Over the last years, genome editing has transformed life sciences research (Genome editing was selected by Nature Methods as the 2011 Method of the Year (Baker et al., 2011). This is mainly due to one very successful form of the technology: CRISPR-Cas9.

According to the journal Science, CRISPR/Cas was the scientific breakthrough of the year in 2015. User friendly and easiness in sgRNA design makes CRISPR/Cas9 system superior over others. CRISPR/Cas9 systems use RNA for target recognition which helps this system to recognize DNA sites that cannot be recognized by ZFNs and TALENs. Genome editing with the help of CRISPR/Cas9 is highly sophisticated and reliable genome editing tools for both applied and basic plant research and breeding. Engineered nucleases can help us to modify genetics of any plant species by gene insertions or deletions or through regulation of gene expression. It is now possible to regulate metabolic pathways to get desired products with ultimate enhanced plant yield. A better understanding of mechanisms involved in response to abiotic and biotic stress along with processes involved in nutrient and water absorption will also be investigated in near future. Hence replacing of age old, time consuming traditional breeding techniques by genome editing tool like CRISPR/Cas9 direct the evolution of crops as required by mankind, and it ensures sustainability in food and agriculture sector.
References

Andersson, A.F. and Banfield, J.F., 2008. Virus population dynamics and acquired virus resistance in natural microbial communities. *Science*, 320(5879), pp.1047-1050.

Andersson, M., Turesson, H., Nicolia, A., Fält, A.S., Samuelsson, M. and Hofvander, P., 2017. Efficient targeted multiallelic mutagenesis in tetraploid potato (*Solanum tuberosum*) by transient CRISPR-Cas9 expression in protoplasts. *Plant cell reports*, 36(1), pp.117-128.

Baker, M., 2011. Gene-editing nucleases.

Baltes, N.J., Hummel, A.W., Konecna, E., Cegan, R., Bruns, A.N., Bisaro, D.M. and Voytas, D.F., 2015. Confering resistance to geminiviruses with the CRISPR–Cas prokaryotic immune system. *Nature Plants*, 1(10), p.15145.

Barrangou, R. and Horvath, P., 2012. CRISPR: new horizons in phage resistance and strain identification. *Annual review of food science and technology*, 3, pp.143-162.

Barrangou, R., Fremaux, C., Deveau, H., Richards, M., Boyaval, P., Moineau, S., Romero, D.A. and Horvath, P., 2007. CRISPR provides acquired resistance against viruses in prokaryotes. *Science*, 315(5819), pp.1709-1712.

Belhaj, K., Chaparro-Garcia, A., Kamoun, S. and Nekrasov, V., 2013. Plant genome editing made easy: targeted mutagenesis in model and crop plants using the CRISPR/Cas system. *Plant methods*, 9(1), p.39.

Bolotin, A., Quinquis, B., Sorokin, A. and Ehrlich, S.D., 2005. Clustered regularly interspaced short palindrome repeats (CRISPRs) have spacers of extrachromosomal origin. *Microbiology*, 151(8), pp.2551-2561.

Brouns, S.J., Jore, M.M., Lundgren, M., Westra, E.R., Slijkhuis, R.J., Snijders, A.P., Dickman, M.J., Makarova, K.S., Koonin, E.V. and Van Der Oost, J., 2008. Small CRISPR RNAs guide antiviral defense in prokaryotes. *Science*, 321(5891), pp.960-964.

Butt, H., Jamil, M., Wang, J.Y., Al-Babili, S. and Mahfouz, M., 2018. Engineering plant architecture via CRISPR/Cas9-mediated alteration of strigolactone biosynthesis. *BMC plant biology*, 18(1), p.174.

Cai, Y., Chen, L., Liu, X., Sun, S., Wu, C., Jiang, B., Han, T. and Hou, W., 2015. CRISPR/Cas9-mediated genome editing in soybean hairy roots. *PLoS One*, 10(8), e0136064.

Deveau, H., Barrangou, R., Garneau, J.E., Labonté, J., Fremaux, C., Boyaval, P., Romero, D.A., Horvath, P. and Moineau, S., 2008. Phage response to CRISPR-encoded resistance in Streptococcus thermophilus. *Journal of bacteriology*, 190(4), pp.1390-1400.

Doudna, J.A. and Charpentier, E., 2014. The new frontier of genome engineering with CRISPR-Cas9. *Science*, 346(6213), 1258096.

Eş, I., Gavahian, M., Marti-Quijal, F.J., Lorenzo, J.M., Khaneghah, A.M., Tsatsanis, C., Kampranis, S.C. and Barba, F.J., 2019. The application of the CRISPR-Cas9 genome editing machinery in food and agricultural science: Current status, future perspectives, and associated challenges. *Biotechnology advances*.

Gaj, T., Gersbach, C.A. and Barbas III, C.F., 2013. ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering. *Trends in biotechnology*, 31(7), pp.397-405.

Gasiunas, G., Barrangou, R., Horvath, P. and Siksnys, V., 2012. Cas9–crRNAribonucleoprotein complex
mediates specific DNA cleavage for adaptive immunity in bacteria. *Proceedings of the National Academy of Sciences, 109*(39), pp.E2579-E2586.

Guy, C.P., Majerník, A.I., Chong, J.P. and Bolt, E.L., 2004. A novel nuclease-ATPase (Nar71) from archaea is part of a proposed thermophilic DNA repair system. *Nucleic acids research, 32*(21), pp.6176-6186.

Haft, D.H., Selengut, J., Mongodin, E.F. and Nelson, K.E., 2005. A guild of 45 CRISPR-associated (Cas) protein families and multiple CRISPR/Cas subtypes exist in prokaryotic genomes. *PLoS computational biology, 1*(6), p.e60.

Hale, C.R., Zhao, P., Olson, S., Duff, M.O., Graveley, B.R., Wells, L., Terns, R.M. and Terns, M.P., 2009. RNA-guided RNA cleavage by a CRISPR RNA-Cas protein complex. *Cell, 139*(5), pp.945-956.

Harrison, M.M., Jenkins, B.V., O’Connor-Giles, K.M. and Wildonger, J., 2014. A CRISPR view of development. *Genes & development, 28*(17), pp.1859-1872.

Hatoum-Aslan, A., Maniv, I. and Marraffini, L.A., 2011. Mature clustered, regularly interspaced, short palindromic repeats RNA (crRNA) length is measured by a ruler mechanism anchored at the precursor processing site. *Proceedings of the National Academy of Sciences, 108*(52), pp.21218-21222.

Haurwitz, R.E., Jinek, M., Wiedenheft, B., Zhou, K. and Doudna, J.A., 2010. Sequence-and structure-specific RNA processing by a CRISPR endonuclease. *Science, 329*(5997), pp.1355-1358.

Horvath, P., Romero, D.A., Coûté-Monvoisin, A.C., Richards, M., Deveau, H., Moineau, S., Boyaval, P., Fremaux, C. and Barrangou, R., 2008. Diversity, activity, and evolution of CRISPR loci in Streptococcus thermophilus. *Journal of bacteriology, 190*(4), pp.1401-1412.

Huang, L., Zhang, R., Huang, G., Li, Y., Melaku, G., Zhang, S., Chen, H., Zhao, Y., Zhang, J., Zhang, Y. and Hu, F., 2018. Developing superior alleles of yield genes in rice by artificial mutagenesis using the CRISPR/Cas9 system. *The Crop Journal, 6*(5), pp.475-481.

Ishino, Y., Shinagawa, H., Makino, K., Amemura, M. and Nakata, A., 1987. Nucleotide sequence of the iap gene, responsible for alkaline phosphatase isozyme conversion in Escherichia coli, and identification of the gene product. *Journal of bacteriology, 169*(12), pp.5429-5433.

Jansen, R., Embden, J.D.V., Gaastra, W. and Schouls, L.M., 2002. Identification of genes that are associated with DNA repeats in prokaryotes. *Molecular microbiology, 43*(6), pp.1565-1575.

Jia, H. and Wang, N., 2014. Targeted genome editing of sweet orange using Cas9/sgRNA. *PloS one, 9*(4), p.e93806.

Jiang, W., Bikard, D., Cox, D., Zhang, F. and Marraffini, L.A., 2013. RNA-guided editing of bacterial genomes using CRISPR-Cas systems. *Nature biotechnology, 31*(3), p.233.

Jiang, W., Zhou, H., Bi, H., Fromm, M., Yang, B. and Weeks, D.P., 2013. Demonstration of CRISPR/Cas9/sgRNA-mediated targeted gene modification in Arabidopsis, tobacco, sorghum and rice. *Nucleic acids research, 41*(20), pp.e188-e188.

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

Jinek, M., Jiang, F., Taylor, D.W., Sternberg, S.H., Kaya, E., Ma, E., Anders, C., Hauer, M., Zhou, K., Lin, S. and Kaplan, M., 2014. Structures of Cas9 endonucleases reveal RNA-mediated conformational activation. Science, 343(6176), p.1247997.

Kamburova, V.S., Nikitina, E.V., Shermatov, S.E., Buriev, Z.T., Kumpatla, S.P., Emani, C. and Abdurakhmonov, I.Y., 2017. Genome editing in plants: an overview of tools and applications. International Journal of Agronomy, 2017.

Khatodia, S., Bhatotia, K., Pasricha, N., Khurana, S.M.P. and Tuteja, N., 2016. The CRISPR/Cas genome-editing tool: application in improvement of crops. Frontiers in plant science, 7, p.506.

Kim, S., Kim, D., Cho, S.W., Kim, J. and Kim, J.S., 2014. Highly efficient RNA-guided genome editing in human cells via delivery of purified Cas9 ribonucleoproteins. Genome research, 24(6), pp.1012-1019.

Kim, S.K., Han, G.H., Seong, W., Kim, H., Kim, S.W., Lee, D.H. and Lee, S.G., 2016. CRISPR interference-guided balancing of a biosynthetic mevalonate pathway increases terpenoid production. Metabolic engineering, 38, pp.228-240.

Larson, M.H., Gilbert, L.A., Wang, X., Lim, W.A., Weissman, J.S. and Qi, L.S., 2013. CRISPR interference (CRISPRi) for sequence-specific control of gene expression. Nature protocols, 8(11), p.2180.

Li, C., Unver, T. and Zhang, B., 2017. A high-efficiency CRISPR/Cas9 system for targeted mutagenesis in cotton (Gossypium hirsutum L.). Scientific reports, 7, p.43902.

Liang, Z., Zhang, K., Chen, K. and Gao, C., 2014. Targeted mutagenesis in Zea mays using TALENs and the CRISPR/Cas system. Journal of Genetics and Genomics, 41(2), pp.63-68.

Lusser, M., Parisi, C., Plan, D. and Rodríguez-Cerezo, E., 2012. Deployment of new biotechnologies in plant breeding. Nature biotechnology, 30(3), p.231.

Makarova, K.S., Aravind, L., Grishin, N.V., Rogozin, I.B. and Koonin, E.V., 2002. A DNA repair system specific for thermophilic Archaea and bacteria predicted by genomic context analysis. Nucleic acids research, 30(2), pp.482-496.

Makarova, K.S., Aravind, L., Wolf, Y.I. and Koonin, E.V., 2011. Unification of Cas protein families and a simple scenario for the origin and evolution of CRISPR-Cas systems. Biology direct, 6(1), p.38.

Makarova, K.S., Grishin, N.V., Shabalina, S.A., Wolf, Y.I. and Koonin, E.V., 2006. A putative RNA-interference-based immune system in prokaryotes: computational analysis of the predicted enzymatic machinery, functional analogies with eukaryotic RNAi, and hypothetical mechanisms of action. Biology Direct, 1(1), p.7.

Makarova, K.S., Haft, D.H., Barrangou, R., Brouns, S.J., Charpentier, E., Horvath, P., Moineau, S., Mojica, F.J., Wolf, Y.I., Yakunin, A.F. and Van Der Oost, J., 2011. Evolution and classification of the CRISPR–Cas systems. Nature Reviews Microbiology, 9(6), p.467.

Marraffini, L.A. and Sontheimer, E.J., 2008. CRISPR interference limits horizontal gene transfer in staphylococci by targeting DNA. Science, 322(5909), pp.1843-1845.
Mojica, F.J., Diez-Villasen or, C., Garcia-Martinez, J. and Soria, E., 2005. Intervening sequences of regularly spaced prokaryotic repeats derive from foreign genetic elements. J. Mol. evol, 60(2), pp.174-82.

Nam, K.H., Haitjema, C., Liu, X., Ding, F., Wang, H., DeLisa, M.P. and Ke, A., 2012. Cas5d protein processes pre-crRNA and assembles into a cascade-like interference complex in subtype IC/Dvulg CRISPR-Cas system. Structure, 20(9), pp.1574-1584.

Pourcel, C., Salvignol, G. and Vergnaud, G., 2005. CRISPR elements in Yersinia pestis acquire new repeats by preferential uptake of bacteriophage DNA, and provide additional tools for evolutionary studies. Microbiology, 151(3), pp.653-663.

Rouillon, C., Zhou, M., Zhang, J., Politis, A., Beilsten-Edmands, V., Cannone, G., Graham, S., Robinson, C.V., Spagnolo, L. and White, M.F., 2013. Structure of the CRISPR interference complex CSM reveals key similarities with cascade. Molecular cell, 52(1), pp.124-134.

Shah, S.A., Erdmann, S., Mojica, F.J. and Garrett, R.A., 2013. Protoscaler recognition motifs: mixed identities and functional diversity. RNA biology, 10(5), pp.891-899.

Shi, J., Gao, H., Wang, H., Lafitte, H.R., Archibald, R.L., Yang, M., Hakimi, S.M., Mo, H. and Habben, J.E., 2017. ARGOS 8 variants generated by CRISPR-Cas9 improve maize grain yield under field drought stress conditions. Plant biotechnology journal, 15(2), pp.207-216.

Sun, C.L., Barrangou, R., Thomas, B.C., Horvath, P., Fremaux, C. and Banfield, J.F., 2013. Phage mutations in response to CRISPR diversification in a bacterial population.

Environmental microbiology, 15(2), pp.463-470.

Svitashev, S., Young, J.K., Schwartz, C., Gao, H., Falco, S.C. and Cigan, A.M., 2015. Targeted mutagenesis, precise gene editing, and site-specific gene insertion in maize using Cas9 and guide RNA. Plant physiology, 169(2), pp.931-945.

Tang, T.H., Bachellerie, J.P., Rozhdestvensky, T., Bortolin, M.L., Huber, H., Drungowski, M., Elge, T., Brosius, J. and Hüttenhofer, A., 2002. Identification of 86 candidates for small non-messenger RNAs from the archaeon Archaeoglobus fulgidus. Proceedings of the National Academy of Sciences, 99(11), pp.7536-7541.

Tashkandi, M., Ali, Z., Aljedaani, F., Shami, A. and Mahfouz, M.M., 2018. Engineering resistance against Tomato yellow leaf curl virus via the CRISPR/Cas9 system in tomato. Plant signaling & behavior, 13(10), p.e1525996.

Tykot, R.H., Burger, R.L. and Van Der Merwe, N.J., 2006. The importance of maize in initial period and early horizon Peru. Histories of maize (eds. JE Staller, RH Tykot, BF Benz), pp.187-197.

Upadhyay, S.K., Kumar, J., Alok, A. and Tuli, R., 2013. RNA-guided genome editing for target gene mutations in wheat. G3: Genes, Genomes, Genetics, 3(12), pp.2233-2238.

Wang, D., Wang, X.W., Peng, X.C., Xiang, Y., Song, S.B., Wang, Y.Y., Chen, L., Xin, V.W., Lyu, Y.N., Ji, J. and Ma, Z.W., 2018. CRISPR/Cas9 genome editing technology significantly accelerated herpes simplex virus research. Cancer gene therapy, 25(5), pp.93-105.

Wang, P., Zhang, J., Sun, L., Ma, Y., Xu, J., Liang, S., Deng, J., Tan, J., Zhang, Q., 2013.
Tu, L. and Daniell, H., 2018. High efficient multisites genome editing in allotetraploid cotton (*Gossypium hirsutum*) using CRISPR/Cas9 system. *Plant biotechnology journal*, 16(1), pp.137-150.

Wang, S., Zhang, S., Wang, W., Xiong, X., Meng, F. and Cui, X., 2015. Efficient targeted mutagenesis in potat by the CRISPR/Cas9 system. *Plant Cell Reports*, 34(9), pp.1473-1476.

Wang, Y., Cheng, X., Shan, Q., Zhang, Y., Liu, J., Gao, C. and Qiu, J.L., 2014. Simultaneous editing of three homoeoalleles in hexaploid bread wheat confers heritable resistance to powdery mildew. *Nature biotechnology*, 32(9), p.947.

Wiedenheft, B., Sternberg, S.H. and Doudna, J.A., 2012. RNA-guided genetic silencing systems in bacteria and archaea. *Nature*, 482(7385), p.331.

Zhu, X., Clarke, R., Puppala, A.K., Chittori, S., Merk, A., Merrill, B.J., Simonović, M. and Subramaniam, S., 2019. Cryo-EM structures reveal coordinated domain motions that govern DNA cleavage by Cas9. *Nature structural & molecular biology*, 26(8), pp.679-685.

Zhu, X., Zhao, D., Qiu, H., Fan, F., Man, S., Bi, C. and Zhang, X., 2017. The CRISPR/Cas9-facilitated multiplex pathway optimization (CFPO) technique and its application to improve the Escherichia coli xylose utilization pathway. *Metabolic engineering*, 43, pp.37-45.

Qi, Y. ed., 2019. *Plant Genome Editing with CRISPR Systems: Methods and Protocols*. Humana Press.

How to cite this article:

Abhishek K. Gowda, S. B. Mishra, Mainak Barman and Saikumar, M. 2020. CRISPR/Cas9: A Revolutionary Tool for Recent Advances in Crop Improvement: A Review. *Int.J.Curr.Microbiol.App.Sci*. 9(11): 200-214. doi: [https://doi.org/10.20546/ijcmas.2020.911.024](https://doi.org/10.20546/ijcmas.2020.911.024)