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CRISPER/CAS: A potential tool for genomes editing

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Abstract

The ability to engineer genomes presents a significant opportunity for applied biology research. In 2050, the population of this world is expected to reach 9.6 billion residents; rising food with better quality is the most promising approach to food security. Compared to earlier methodologies including Zinc Finger Nucleases (ZFNs) plus Transcription Activator-Like Effector Nucleases (TALENs), which were expensive as well as time-consuming, innovation in Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and related CRISPR (Cas) protein classifications allowed selective editing of genes for the enhancement of food. The basic mechanism of CRISPR Cas9 process and its applications on genome editing has been summarized in this manuscript. The method relies on Sequence Specific Nucleases (SSNs) to create Double Stranded Breaks (DSB) of DNA at locus of genome defined by user, mended by using one of two DNA mending ways: Non-Homologous End Joining (NHEJ) or Homology Directed Repair (HDR). Cas9, an RNA-guided endonuclease, was used to produce stable knock-in and knock-out mutants. The focus of this effort is to explore the CRISPR Cas9 genome editing to manage the gene expression and improve future editing success. This adaptable technique can be consumed for a wide range of applications of genome editing requiring high precision. Advances in this technology have sparked renewed interest in the possibilities for editing genome in plants.

Introduction

The field of genome editing is expanding quickly with the evolution of new methods and techniques. The present biotechnology methodologies discover the molecular mechanisms and biological functions of the genes in order to modify to rises better quality of food. In 2050, the population of the world is anticipated to reach 9.6 billion residents, higher quality plants are the most viable solution to food sustainability rather than clearing more land for production. Researchers have used traditional breeding methods or gene transfer methods to improve product, stress resistance and integration of further attractive traits. Non-specific and uncontrolled incorporation of transgenes into the genome of host by method of breeding and traditional transformation techniques for the development of genetically modified organisms causes public concern, specifically to eatable species [1].

Genome editing has been made easier due to programmable sequence specific nuclease (SSN) technologies that allow for the high-efficiency selective modification of endogenous genomic sequences [2]. The genome editing approaches provide sequence-specific breakage of DNA. Some SSNs develop double stranded breaks (DSBs) at the selected regions of the genome for genetic development in different species. These SSNs can precisely cut DNA at a specific place, and the breaks are repaired by Non-Homologous End Joining (NHEJ) or Homology Directed Repair (HDR), resulting in insertion/deletion or substitution changes in target regions, respectively [3, 4] (**Fig. 1**). The revolutionary developments in SSNs engineering have recently enabled progress in the particular manipulation of the model genome.

Until now, three SSNs have been involved in waves of innovation: Zinc-Finger Nucleases (ZFNs), Transcription Activator-Like Effector Nucleases (TALENs), and Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/Cas. The DNA of the desired trait was found in the organisms whose genomes were modified [5]. The targeted genome modifications (TGM) were traditionally carried out with ZFNs, artificial nucleases made up of synthetic ZFN domain attached to a FokI cleavage area. Endogenous genes have been modified using ZFNs in a diversity of cell types and organisms, as well as plant species including Arabidopsis, maize, tobacco, and soybean. TALENs recently emerged as targeted genome modifications substitute to ZFNs, also revealed to present a lot of promise for specific genome manipulation. TALENs, like ZFNs, have a FokI cleavage domain and an engineered special TALEDNA binding domain. Any sequence can be targeted by the TALE DNA binding domain, which is made up of nearly identical tandem repeat arrays. Plants have generally accepted TALEN-mediated genome alteration in the last years [6].

CRISPR/Cas is the third group of genomes editing technology, with easy-to-implement designs and methods that are both cost- and time-effective. The CRISPR/Cas method based on RNA has been efficaciously applied in a large number of species including plants, since the publication of first report in 2012 on a new genome engineering strategy focused on bacterial endonuclease Cas9 [3]. The Cas9 RNA-guided nucleases from microbial CRISPR/Cas systems are stable and adaptable tools for inducing error-prone double strand breaks in the cells, and has the ability to implement the cellular reparation mechanisms including Non-Homologous End Joining or Homology-Directed Repair pathways [2]. By complementary pairing of sgRNA having 20-base pair target sequence, the Cas 9 nuclease is controlled to the target cutting site, and the genomic DNA cut originates at the third base upstream of the protospacer adjacent motif (PAM). The domains including RuvC and HNH of the Cas9 nuclease plays the significant role to cleave non-complementary and complementary constituents of DNA to the guide RNA [1].

Development of Genome Editing Wonder

The CRISPR mechanism was reported in *Escherichia coli* genome in 1987led to an era of discoveries (**Fig. 2**).

Eventually, these repeated sequences were revealed in almost 40% of sequenced bacterial genomes and in archaea about 90%, while the function is still unclear [7]. With Ruud Jansen's support, in 2002, Haloferax mediterranei called them CRISPR sequences. In 2005, he revealed the resemblance between CRISPR regions and bacteriophage sequences and archaeal viruses. This discovery shed light on CRISPR as an adaptive immune system [8]. The immune response system of bacteria defends themselves by cleaving the foreign DNA of viruses. When paired with Cas genes, CRISPR assortments were found to offer defense against attacking viruses in 2007. Multiple guide RNAs with different

sequences might also be utilized at the same time to produce the high-efficient multiplex genome editing at various loci. Such interventions verified that the CRISPR/Cas 9 method was easy, budget-friendly and flexible technique for the editing of the genome.

Five papers were released in August 2013 describing the primary use of CRISPR technique based on editing of genome [9]. The initial collection of experiments established the tremendous applicability of CRISPR technique in the era of plant biology by taking up the model species "*Nicotiana benthamiana"* and "*Arabidopsis thaliana",* certain crops such as rice, and employing a range of transformation platforms including the creation of stable transgenic organisms. Wheat [9], Sorghum [10], and maize were among the crops studied in the succeeding years [11].

Earliest comparative or proportional data by these studies was provided on mutation performance, cleavage specificity, the resolution of locus structure to elucidate image, plus the ability to generate large deletion of chromosomes, as well as demonstrating that a number of promoters comprising those renowned by RNA polymerase II and III, can influence the expression of guide RNAs [9, 10, 12, 13].

CRISPR technique was recently demonstrated towards function in hairy roots of tomato after conversion with *Agrobacterium rhizogenes*, and also known as the first genome editing platform utilized in sweet orange [14]. Interestingly, separately 4 groups have presented that this system can directly present homozygous mutations in the first group of tomato generation as well as rice transformants, demonstrating the system's exceptional productivity [13, 15]. Cas9/gRNA-induced genetic changes were also found in the germ line of arabidopsis, rice, and tomato, and separated ordinarily in consequent generation groups without additional modifications [12-14, 16].

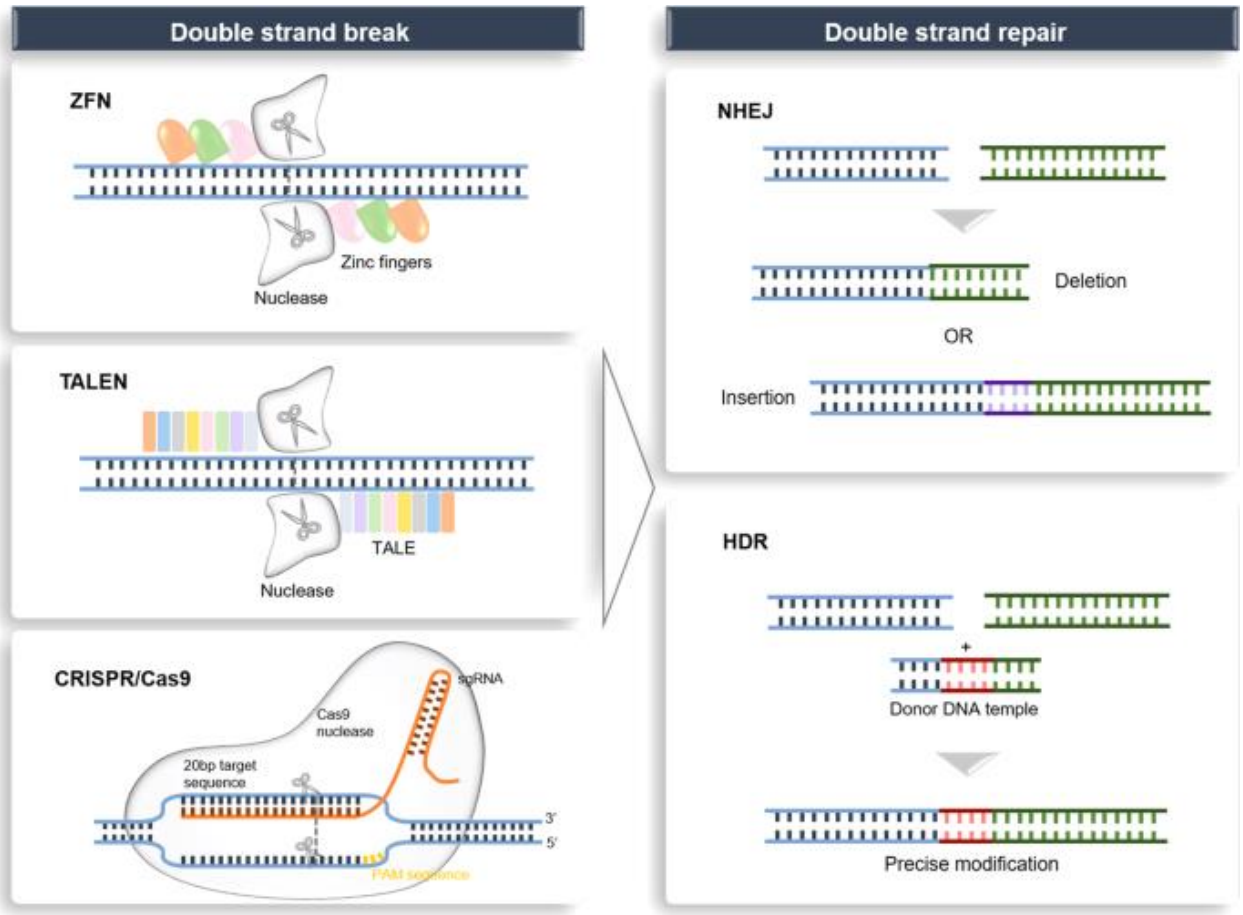

Fig. 1: Genome editing techniques. ZFNs proteins guide the FokI dimer to the particular DNA site to chop. The TALEN proteins guide the FokI dimer to the unique DNA site to be cut. CRISPR/Cas9 nicking led by the single guide RNA to repair double strand breaks by the non-homologous end joining (NHEJ) or homology directed repair (HDR).

Mechanism of CRISPR/Cas Mediated Defense System

In nature, the CRISPR/Cas facilitated defense mechanism works on by following three stages including adaptation primarily, expression, and interference (**Fig. 3**). Small DNA fragments are incorporated in the arrangement of CRISPR as new spacers in the first step as new spacers when a virus or bacterium infects the host cell. With the assistance of Cas proteins complex, CRISPR RNA precursor transcribed through a CRISPR locus, split down within repeats and mature crRNA molecules. A little guide RNA is a spacer flanked by tiny DNA repeats, is found in any mature crRNA and instructs Cas proteins to mount anti-viral response [17].

Finally, a 20-nucleotide crRNA binds to the target nucleic acids, instructing these proteins to destroy the virus targeted sequences or complementary plasmid of spacers [18]. After the PAM sequence, Cas nuclease chops three to four bases, which plays an important role in binding and nicking the target DNA [19]. In the genome editing CRISPR systems, two constituents are existing: a gRNA and a CRISPR-associated endonuclease/Cas protein [20]. The gRNA is categorized as small synthetic RNA with nearly 20-nucleotide and also Cas binding sequence that determines the DNA to be targeted for required changes [3]. The genomic goal of the Cas protein can be improved simply through changing target sequence in gRNA. The target DNA sequence would be distinct from the rest of the genome, and the target protein have to be located promptly corresponding to a PAM [21]. The PAM sequence is required for Cas nuclease to function, but particular sequence varies depending on utilized protein. Following the expression of the CRISPR locus, interactions between the scaffold of the gRNA and positively-charged grooves produced on the surface of Cas9 protein generate a ribonucleoprotein complex [22].

Cas9 undergoes a conformational transition after binding gRNA, which transforms the molecule from inactive to active state. The spacer of gRNA region is still able to bind to target DNA [23]. After connecting the target to the nuclease domains like RuvC and HNH, the complex undergoes a second conformational transition to cut the opposite strand of the target DNA [24]. Cas9-mediated DNA cleavage causes a DSBs in the target DNA [25].

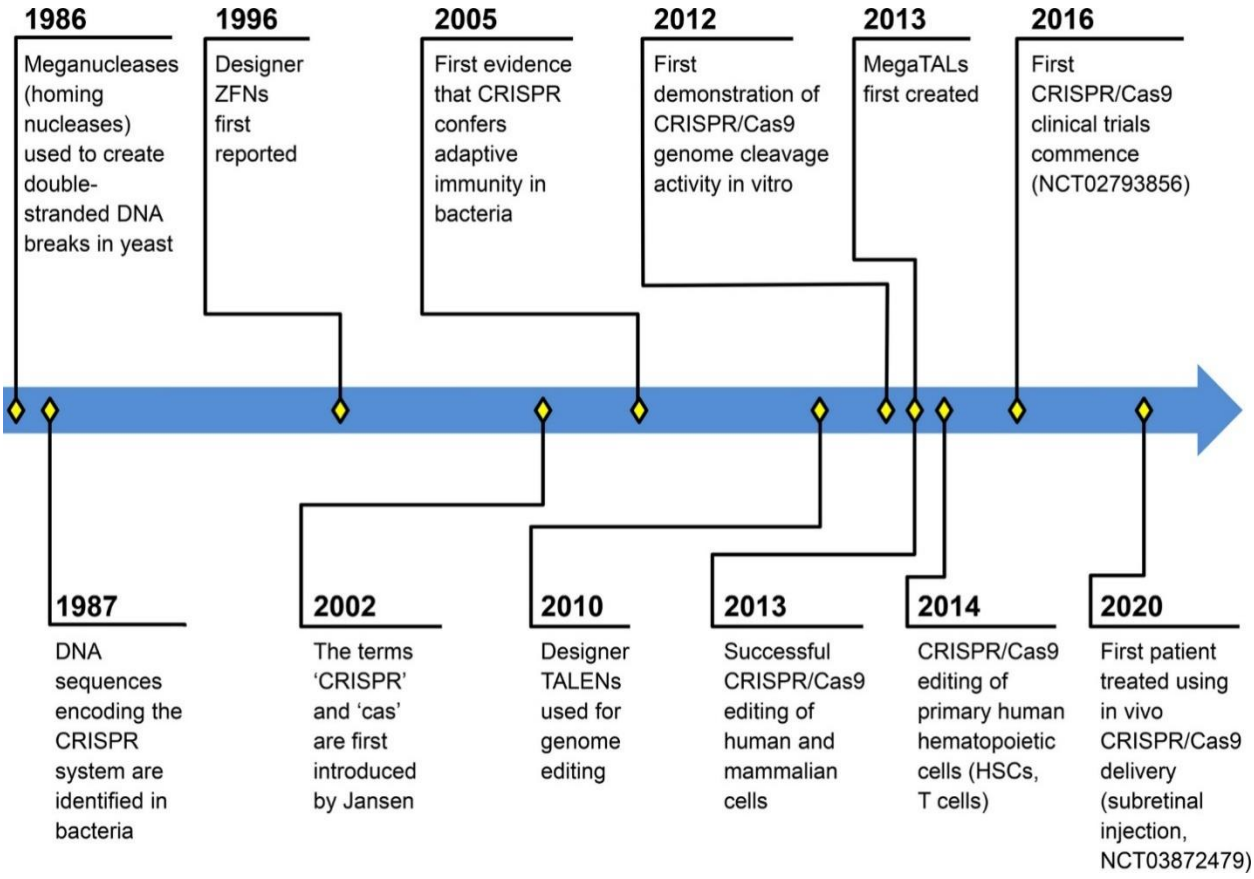

Fig. 2: Interventions in innovation of CRISPR/Cas9 technology.

Types of CRISPR-Cas System

These systems are divided into three groups based on the proteins required by host cells to induce an immune response. In systems of type I and type III, Cas protein complex contains several proteins, whereas in case of the type II system, Cas9 protein is known as a single multi-functional protein to process the crRNA as well as chopping down the target DNA. The type II structure is simpler to design than the type I system and type III system, and it can be speedily employed to act as genome editing mechanism [18]. The type-specific locus I have genes that code for a large Cas3 protein with helicase and DNase functions.

A significant number of proteins including Cas 5, 6, and 7 are included in the translation of lengthy spacer transcript into mature crRNA, according to repeat associated mysterious protein (RAMP) superfamily, which was recently discovered. However, there is an exception in the Type1C- scheme, where RNase activity was observed. Cas1 is often joined with RecB nuclease domain of Cas 4, where it played part in spacer acquisition [26]. The type II system has a "HNH" streptococcus like system. This method included a large Cas 9 protein that can cleave the target DNA as well as make crRNA [27]. RuvC has two nuclease domains at the amino terminal, and HNH domain in the mid of protein. The HNH domain controlled an endonuclease operation which frequently cleaved a target DNA [28]. Although, the exact function of Type II is unclear however it is assumed to be included in the formation of a duplex between tracer-RNA and the pre-crRNA repeat portion. For processing of precursor crRNA, first cleavage occurs at replicated area. dsRNA specific RNase III catalyzes this process in occurrence of Cas 9 protein [29].

The type III mechanism is involved in polymerase and RAMP action, which is accountable for the chopping of spacer-repeat transcripts. This system is distributed into two sub categories: III-a known as CasS6 plus III-B known as CasS7 [30]. The type III-A is accountable for cleavage of targeted plasmids, while type III-B is liable for RNA targeting. These two subtypes play a role in nucleic acid targeting, while the exact mechanisms are vague. Apart from Cas2 proteins, RAMP proteins are the ribonuclease in this system [31]. The type III has two more RAMPs for transcript processing in addition to Cas 6 [32].

Plant Genome Editing by CRISPR/Cas9

Since 2013, system of CRISPR/Cas has proved as a useful tool for editing of the plant genome. As compared to previous techniques like transcription activator-like effector nucleases and zinc finger nucleases, the technique of CRISPR/Cas allows for easy, low cost and the influential gene editing tool for the researchers [33].

The CRISPR/Cas9 has widespread usage in editing of plant genome for characterizing their gene function and improving qualities and yield. In plants, genome editing by using this system usually consists of four stages [1, 34].

The first step is to develop and construct a gene-specific sgRNA. For the computer-based design of sgRNAs, a number of online resources have been created. In the next step, before using sgRNA in genome editing, it is best to test its behavior in protoplasts. Cas 9 and sgRNA expression cassettes are usually stably introduced in the plant genome *via* agrobacterium-mediated transformation or particle bombardment. Finally, polymerase chain reaction (PCR) genotyping and sequencing are used to identify converted plants with the desired modifications [34].

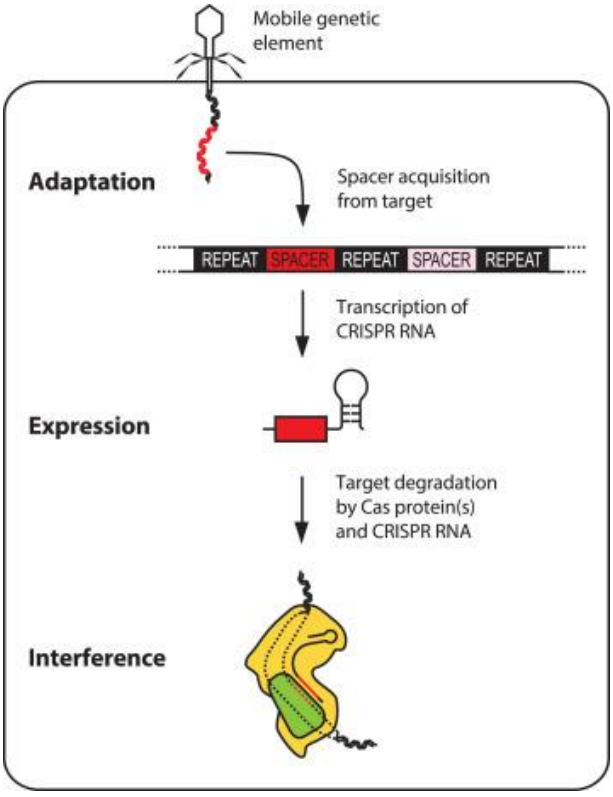

Fig. 3: CRISPR/Cas developed innate immunity.

Knock in by CRISPR/Cas9

In the genome editing, knock-in includes the gene insertion at specific sites is considered as a crucial genome editing strategy. After the DSBs caused by CRISPR/Cas9 occur, homology directed repair using donor template DNA results in the insertion of the target gene into the relevant region [15]. This system was applied to achieve glyphosate tolerance in *Oryza sativa* by knock-in [35] strategy. This system was made functional to achieve knock-in in *Arabidopsis thaliana* [36].

In most plants, generating stable knock-in plants remains a problem as it involves the co-delivery of donor template into the cells that complicates the process. The repair methods are also considered as the complication, NHEJ occurs at much faster rate than HDR by implementing DSB repair method [37].As a result, identifying the true knock-in lines takes more time and effort. To get around these problems, a DNA virus may be used as a donor template. The ability of Gemini viruses to attack genes has been recognized [38]. Owing to their DNA genome, the ability to act as repair models and their ability to replicate in large numbers enhances the 'knock in' efficacy in genome editing [39]. However, the CRISPR/Cas9 provides simple technique for generating DSBs for HDR; further research is needed to improve plant genome knock-in efficiency.

Knock Out by CRISPR/Cas9

In the plant genetic research, functional knockout of a target gene is a fundamental stage. In practice, it could be easily obtained by using Cas9/gRNA to incorporate insertion/deletion into the coding region, causing translation to be disrupted [39].

Gene knockout occurs through this system creates DSBs in the selected genes. The DNA repair mechanism, NHEJ, activates reparation of these breaks, but it causes deletions or insertions in the genome due to its error-prone nature. As a consequence, the target genes are silenced [40]. This system has been operated to obtain gene knockout producing tolerance to stress of cold, drought and salt [41, 42].

Target Base Editing by CRISPR/Cas9

A new technique known as ''target base editing" has recently been developed, allows for the transformation of one target base into other in a well-defined manner. This conversion is irreversible in nature with no use of donor template or DSB in the genome of mammals [43].

In this system, the enzyme APOBEC1 is merged with the N-terminus of a Cas9 nickase (Cas9n) which maintains the capacity to be line up with a gRNA and facilitate the direct exchange of cytidine to uridine, resulting in a high rate of C/T (or G/A) substitution. Two agriculturally significant genes of rice including NRT1.1B and SLR1 selected for editing to validate and determines the viability for using breeding of plant. A nitrogen transporter is encoded by NRT1.1B, a C/T substitution in this gene has been shown to increase nitrogen usage performance in rice [44] .

Applications of CRISPR/Cas9

The use of this system in editing of the plant genome has a wide range of applications, including tolerance from biotic to abiotic stress factors, as well as improved yield efficiency, bio fortification, and plant quality enhancement. The induced resistance to fungal, viral and bacterial diseases is included in biotic stress tolerance. In abiotic stress tolerance, herbicide and natural environmental stress resistance, including temperature, salt and drought are the major goals [33].

Plants were exposed to biotic stress *via* gene deletion utilizing the CRISPER/Cas9 system. By targeting the genes eIF (iso) 4E and eIF4E, tolerance to Potyvirus and Zucchini yellow mosaic virus was found in *Arabidopsis thaliana* and *Cucumis sativus*, respectively [45, 46]. Gene knockout of SlMlo in tomato plant resulted in confrontation to fungal disease [47]. The *Oryza sativa* plant was made resistant to bacterial blight disease by targeting the sucrose transporter gene OsSWEET13 by gene knockout through CRISPER/Cas9 [48]. Abiotic stress including herbicide, salt and drought resistance in plants was also facilitated by targeting the genes in '*Arabidopsis thaliana'*, '*Zea mays'* and '*Oryza sativa'* [41, 49, 50].

The applications of CRISPR/Cas9 in improving the yield and bio fortification of plants are of great interest. Increases in grain size, number, and weight, generation of parthenocarpic plants, and lingo cellulose biosynthesis have all been used to improve yield in rice, tomato, and orchid, respectively. Production of biotherapeutic proteins, rich amylase rice, and improved seed oil composition in plants including *Nicotiana tabacum*, *Oryza sativa* and *Camelina sativa* are the example of bio fortification

Table 1: Improvement in different plant species for the increase of the yield.

Plant species	Target gene	Application	Reference
Tolerance to virus stress			
Arabidopsis thaliana	*eIF(iso)4E*	Potyvirus resistant	[45]
Cucumis sativus	*eIF4E*	Zucchini yellow mosaic virus resistant	[46]
Tolerance to fungal stress			
Tomato	*SlMlo*	Powdery mildew resistant	[47]
Tolerance to bacterial stress			
Rice	Sucrose transporter gene *OsSWEET13*	Bacterial blight resistant	[48]
Tolerance to herbicide			
Arabidopsis thaliana	*BAR*	Glufosinate herbicide resistant	[51]
Rice	*Acetolactatesynthase* (*ALS*)	Herbicide resistant	[49]
Tolerance to salt stress			
Rice	*OsRAV2*	Salt stress resistant	[41]
Tolerance to drought stress			
Maize	*ARGOS8*	Drought stress resistant	[50]

mediated by CRISPR/Cas9 (**Table 1**) [33].

Conclusion

The CRISPR/Cas9 system represents a major development in the domain of molecular biology. With the recent advancements, it is gaining fame into the area of genome editing. Through this technology, genome editing has made significant progress in terms of developing genetically modified organisms of higher quality and yield. CRISPR/Cas9 editing may also be used to replace dysfunctional genes and to develop the organisms that are beneficial to humans. However, there is still some concern about the use of genome editing. This emerging approach is still in its early stages, and scientific activities must continue to achieve an established technique and understand its full prospects.

Future Prospects

Many potential applications of the CRISPR method were discovered after CRISPR mechanism completely revealed [3]. Thousands of genomes, ranging from viruses to plants, have been modified using this technique, which has been employed by scientists from several disciplines. Cas versions from many other species, including *S. aureus* and *S. thermophilus* have also been utilized for editing of the plant genome in addition to the classical CRISPR/Cas9 system of *S. pyogenes* [52].
Cas13a, a CRISPR variant that cuts RNA rather than DNA, was recently discovered and can thus be used for RNA editing in bacteria and plant. For commercial applications of Cas13a in medication and crop growing, further research is required. Cas13a has been shown to cause RNA degradation in bacteria, however these effects have not been identified in plant studies [53]. Aside from knock-ins and knock-outs, the CRISPR method can be used for regulating the expression of gene by fusing the DNA binding domains of genome-editing constructs (dCas) with activators or repressors, thus controlling the endogenous expression of the gene [54]. In addition, by fusing dCas9 (inactive) with sgRNA that targets a particular plant gene promoter, this system can be employed to activate or repress plant gene transcription [55]. The genome editing efficiency in rice was recently improved by using a bidirectional promoter to express Cas9 and gRNA in opposite directions [56]. As a result, these improvements may be used to enhance the editing efficiency.

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