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Review

CRISPR/Cas9; A robust technology for producing genetically engineered plants

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Abstract: CRISPR/Cas9 is a technology evolved from modified type II immune system of bacteria and archaea. Exploitation of this bacterial immune system in all eukaryotes including plants may lead to site-specific targeted genome engineering. Genome engineering is objectively utilized to express/silence a trait harbouring gene in the plant genome. In this review, different genetic engineering techniques including classical breeding, RNAi and genetic transformation and synthetic sequence-specific nucleases (zinc finger nucleases; ZFNs and transcription activator-like effector nuclease; TALENs) techniques have been described and compared with advanced genome editing technique CRISPR/Cas9, on the basis of their merits and drawbacks. This revolutionary genome engineering technology has edge over all other approaches because of its simplicity, stability, specificity of the target and multiple genes can be engineered at a time. CRISPR/Cas9 requires only Cas9 endonuclease and single guide RNA, which are directly delivered into plant cells via either vector-mediated stable transformation or transient delivery of ribonucleoproteins (RNPs) and generate double-strand breaks (DSBs) at target site. These DSBs are further repaired by cell endogenous repairing pathways via HDR or NHEJ. The major advantage of CRISPR/Cas9 system is that engineered plants are considered Non-GM; can be achieved using *in vitro* expressed RNPs transient delivery. Different variants of Cas9 genes cloned in different plasmid vectors can be used to achieve different objectives of genome editing including double-stranded DNA break, single-stranded break, activate/repress the gene expression. Fusion of Cas9 with fluorescent protein can lead to visualize the expression of the CRISPR/Cas9 system. The applications of this technology in plant genome editing to improve different plant traits are comprehensively described.

Key words: Plant genome editing; CRISPR/Cas9; ZFNs; TALENs: RNPs.

Introduction

Genome editing technology has been evolved gradually from the use of physical or chemical mutagenic agents towards designed sequence-specific nucleases (SSN) that can leads to the gene deletion via non-homologous end joining and gene modification via homologous end joining (1). A large number of plants, filamentous fungi and yeast strains are beneficial for biotechnological applications, but they are non-domesticated and have complex genomes which are difficult to manipulate. But precise genome editing which is a substitute of natural mutation has been successfully used to conquer such challenges. Implementation of genome editing can be done in very diverse and countless areas because it can target any part of genome including the subject of gene deletion, gene addition (reporter gene), promoter study, gene activation/repression, in hereditary diseases, biomedicine, gene drive, gene tagging (2).

Targeted genome engineering is a substitute of traditional plant breeding and genetic transformation methods because classical breeding is laborious and time taking process and there are some concerns about transgenic food that these may have undesirable effects on the environment, biodiversity and human health, that are traditionally used to make better the crop plants. So far SSNs including mega-nucleases, zinc finger nucleases (ZFN) and transcriptional activator-like effector nucleases (TALEN) were used to design DNA-binding proteins which can create target specific double-strand DNA breaks (DSBs) but they are very difficult to design and have an extensive assembly of specific DNA binding proteins for each targeted gene. The newly developed technique is named as CRISPR (clustered regularly interspaced short palindromic repeats)/Cas9(CRISPR-associated 9-nuclease) system based on bacterial type II immune system that can target foreign nucleic acids (3). Among all biotechnological tools for genome editing, CRISPR/Cas9 is the most favourable due to the specificity, effectiveness, easy to construct, have very low cost and can edit multiple genes at a time. Cas9 nuclease generates DSBs which stimulates DNA repair mechanisms: non-homologous end joining (NHEJ) and homology-directed repair (HDR) to produce genomic alterations, gene indels and replacement of defective genes and insert the exogenous DNA at the aimed place. NHEJ has frequently used DNA repairing mechanism in somatic plants (4). The emergence of CRISPR/Cas9 has made genome editing to restructure the basic and applied biology and is extensively used in plants for distinguishing gene function and upgrade the traits (5). CRISPR/Cas9 is the latest technique in genome engineering requiring only two basic components; a single guide RNA and Cas9 endonuclease protein making a complex together. Such a complex identifies and cuts the specific target sequence of the 20bp present in a genome. Target recognition is determined by complementary base pairing between the target sequence and single guide RNA (sgRNA) which makes the target extremely specific and precise for editing. Upon cutting the target

site, error-prone endogenous DNA repair mechanism can use two types of repairing methods named NHEJ and HDR, makes small deletion or insertion at target site typically consequences failure of gene functioning. Implementations of CRISPR are not only the mutagenesis but also the target site cleavage can be used to promote sequence insertion or replacement by recombination (6). NHEJ mechanism results in the addition or deletion of nucleotides while HDR repairing is the full DNA re-establishment procedure and can only be used when the DNA template is present and it is more efficient and precise process (7). Whilst DSBs considerably induces to increase the efficacy of HDR but this is two times less efficient than NHEJ (8).

Comparison of CRISPR/CAS9 with other techniques of genome engineering

The CRISPR/Cas9 used as a novel technique from "methods of the year" in 2011 to "breakthrough of the year" in 2015 for its fascinating genome editing and this prokaryotic system was immediately accepted for eukaryotic genome editing (9, 10). Before CRISPR/Cas9 discovery, different other techniques were in use for manipulation of the genome to activate/repress gene expression. Those techniques include RNA interference, genetic transformation and classical plant breeding and crossing. Comparison of these techniques with CRISPR/Cas9 has been described in Table 1.

CRISPR/CAS9: Bacterial type II immune system

CRISPR/Cas9 is a prokaryotic adaptive immune system that provides sequence-specific adaptive immunity by integrating short virus sequences into cell CRISPR locus, recognizes infection and protects bacterial cell from viruses or other mobile genetic elements by degrading their genomes (21, 22). CRISPR locus was firstly found in *Escherichia coli* (23) and is present in about 84% archaea and 45% bacteria for their defence against viral attack (24, 25). The locus of CRISPR contains CRISPR as well as Cas genes (26). CRISPR/Cas9 system is able to integrate DNA/RNA sequences acquired from viruses (24). Cas9 is an important protein that helps to incorporate the phage sequence into spacer regions found in CRISPR array, for memorizing the bacterial cells (27). Transcription of CRISPR repeat arrays leads to the formation of CRISPR RNAs (crRNAs), each is

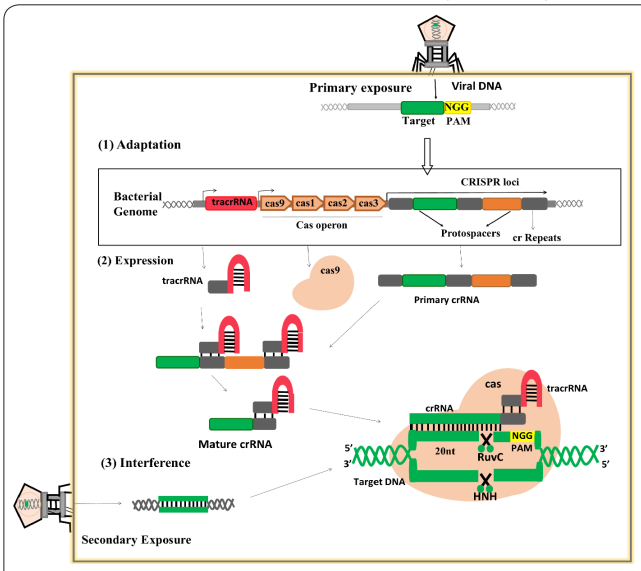

Figure 1. Representation of naturally occurring bacterial CRISPR/Cas9 system, contains tracrRNA, Cas9 genes and cr repeats/protospacers and based on 3 main steps 1) Adaptation: in which spacer acquisition occurs from target DNA into the bacterial genome. 2) Expression: involves transcription of CRISPR locus which is expressed into tracrRNA, Cas9 protein and crRNA and further processing of crRNA. 3) Interference: involves target selection via PAM, identification and breakdown of foreign nucleic acid through crispr complex (cas9 protein+gRNA). DSBs are generated by Cas9 nuclease via utilizing two cleaving domains HNH and RuvC.

Table 1. Comparison of CRISPR/Cas9 with other techniques of genome manipulation.

Characters	CRISPR/CAS9	RNAi Method	Stable transformation Methods	Conventional breeding Methods
Methodolgy	Introduction of in-vitro expressed gRNA/Cas9, RNPs into the cell can produce non-transgenic plants. (11)	Gene knockdown is induced by expressing antisense RNA. This technique can be exploited in both ways as a transgene or by transient expression (12)	Genetically modified plants possess transgenes.	Involves the Cross-pollination and selection of superior genes to get a hybrid (13)
Working principles	Induces DSBs at target sequence which are repaired by host endogenous repair mechanisms (8)	It targets mRNA and can silent the gene expression.	Different transformation techniques can be used to introduce transgenes and endogenous genes for genome modification (14)	Superior genes pool in a single plant species is achieved by selection. It is very laborious and time-consuming method.
Multiple genes editing	Allows multiplex genome editing (15)	It lowers the expression of the transcript by transcriptional and post-transcriptional gene silencing. Multiple gene silencing can be done through RNAi technology (16)	Stacking of different transgenes is possible in the single transgenic plant	It is very complex to pool multiple genes through the crossing and it requires continuous crossing and selection (17)
Off-target activity	Off-target effects are minimized due to stringent sequence specificity of gRNA (18)	Off-target effects of RNAi are produced due to transitivity of small inferring RNAs which guide the RISC complex for targeting transcripts	Imprecise and entirely random integration of transgenes can cause off-target mutations (19)	Genes are transferred in an ordered way within species that cause no mutation (20)

formed with a variable sequence transcribed from attacking DNA, is called protospacer sequence and a part of CRISPR repeats. Each crRNA binds to transactivating CRISPR RNA (trace RNA) (28). These two RNAs: crRNA and tracrRNA are fused to form a chimeric single guide RNA (sgRNA) that binds with Cas9 protein to make a complex (9). Protospacer sequences that are present into CRISPR locus, guide Cas9 protein to cleave viral DNA having an adjacent short sequence called protospacer adjacent motifs (PAMs) (9). When the bacterial cell is reinfected, it uses the stored viral sequence to degrade the attacking pathogenic nucleic acid (29).

Bacterial CRISPR/Cas immune system based on three main steps**.** Adaptation, which is the acquisition of new spacers into CRISPR locus (Figure 1). Adaptation is of two types; naïve and primed. Both are dependent upon protospacer adjacent motif (PAM) and cas1-cas2 complex. Naive spacer integration occurs when no previous information is present about the target in CRISPR locus, while primed spacer acquisition requires already present spacer in CRISPR locus that matches with the target DNA in the presence of cas3 along cascade complex (24, 30, 31). Expression, system activation occurs by the expression of cas9 genes and transcription of CRISPR that leads to the formation of precursor RNA (pre-crRNA) which is further processed into mature crRNA via Cas protein and accessory factors. Interference, foreign nucleic acid is recognized by crRNA which destroys invading sequence accompanied by Cas protein that works in a complex with crRNA (32-38).

Comparison of CRISPR/Cas9 with other synthetic genome editing techniques

ZINC finger nucleases

ZFNs are the fusions of cleavage domain of FokI restriction enzyme with ZF-proteins. The cleavage domain dimerization in ZFNs would create DSBs at a specific genomic site that are further repaired. A single ZF-protein comprises approximately 30 amino acids which recognize a triplet DNA sequence by making protein-DNA interaction (39). It requires specific expertise in designing the ZF-libraries and uses effortful selection methods such as phage display and B_2H system. A ZFN usually binds to 9 to 18 bp sequences (40). Methods of engineering ZF domains are (i) modular assembly method (ii) context-sensitive selection method. In the former method includes usage from pre-selected libraries of ZF modules which are developed by selection of combinatorial libraries or rational design. While latter method includes a selection of new ZF arrays from randomized libraries by using oligomerized pool engineering which is based on context depended interaction amongst adjacent fingers. Another approach is developed which uses both of the above methods in which ZF modules are pre-selected based on context-dependent interaction to generate longer tandem by modular assembly (41). Target recognition by ZFN is often faulty which leads to off-side cleavage and results in apoptosis (42) (Figure 2A).

Transcription activator-like effector nucleases

TALENS are the fusions of FokI cleavage domain with DNA binding domains derived from TALE proteins. FokI dimerization at cleavage site would induce cut at target site that activates damage response pathway (43). TALE proteins are the naturally derived from pathogenic bacteria Xanthomonas. A single TALE protein comprises of multiple 33 to 35 amino acid repeat domains, each recognizes a DNA sequence of 1bp only. It utilizes protein-DNA interaction for targeting (44). TALE repetitive array can be engineered no matter what length is required.TALE specificity is determined by 2 hypervariable amino acids that are called repeat variable di residues (RVDs). RVD code is utilized to make new TALE arrays that bind with a target at a greater affinity of 96%. A drawback of this technique is that the TALE binding site always starts with T base (45, 46). TALE domains are engineered by Modular assembly method and wide-range assembly of TALES can also be done by FLASH system (47, 48). Fewer off-side mutations are achieved by TALENs and cause less cell toxicity as compared to ZFNs (49) (Figure 2B).

CRISPR/Cas9 system in plants

CRISPR system Type II is characterized from Streptococcus pyogenes which can be used to introduce site-specific double-stranded breaks (DSBs) and edit the genome of other organisms. In this technique, we mainly use two main components, that are entered or expressed into cells/organisms: Cas9 endonuclease and guide RNA. At 5' end of guide RNA, almost twenty nucleotides of crRNA guide the Cas9 protein to target DNA site by RNA-DNA complementary base pairing that are consequently cleaved by endonuclease activity (Figure 2C). The target sequence is present upstream of protospacer adjacent motif (PAM) sequence which is a trinucleotide (NGG) sequence. The recognition of alternative sites of PAM sequence (i.e NAG) is also found but these are less efficient (9). The Cas9 protein having

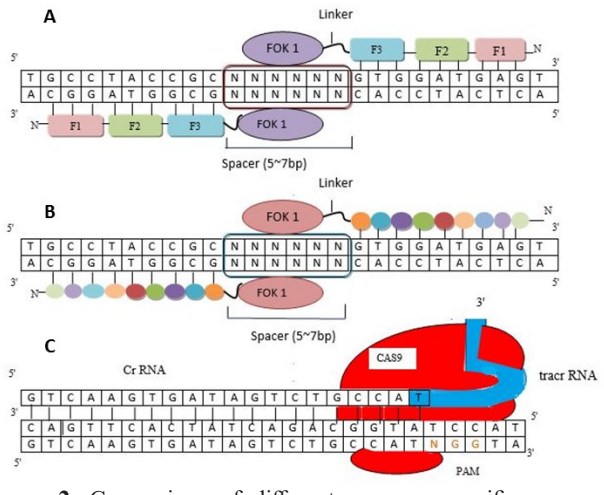

Figure 2. Comparison of different sequence-specific genome-editing techniques. Panel A. showing Zinc finger nuclease dimer bound to target DNA. A pair of zinc finger nuclease consists of 3 zinc finger proteins which cause DSB in the target DNA. Panel B. is representing transcription activator-like effector nucleases bound to its target DNA. Each TALEN consists of N-terminal transcription activator-like effectors (TALEs) and the cleavage domain of FokI endonuclease at C-terminal. Each TALE is composed of 9 repeat variable di-residues (RVDs). Panel C. showing CRISPR/Cas9 complex (cas protein+crRNA+tracrRNA) bound with target DNA which is 20 nucleotides long adjacent to the PAM (protospacer adjacent motif; NGG).

Table 2. Comparison of CRISPR/Cas9 with other synthetic endonucleases.

Characters	CRISPR/Cas9	ZFNs	TALENs
Off-target activity	CRISPR/Cas9 limits off-target activity because target recognition entirely depends upon gRNA sequence. Many of the gRNAs are not involved in creating off-side mutations(50).	Target recognition by ZFN is faulty and leads to off-side cleavage results apoptosis or cell death (42).	Fewer off-side mutations are achieved by TALENs and cause less cell toxicity as compared to ZFNs (49).
Vector delivery	cDNA that makes a Cas9 protein is about 4.2kb in size and gRNA (is of 100nt) should be delivered at the same time. Their larger size makes them difficult to deliver via a viral vector or as an RNA (50).	ZFN genes are easily lodged by size restricted vectors (i.e AAV) and delivered. cDNA that makes ZFN is of the only 1kb in size (51).	TALENs bigger size and recurring character limit their delivery by size-restricted vectors i.e AAV.cDNA that makes TALEN monomers of about 3kb in size (52).
Multiplexing	Multiplex genome editing is a unique characteristic of the CRISPR/Cas system (53).	Multiplexing is not done because of its laborious design of ZNF (54).	TALENs assisted Multiplex editing (TAME)is difficult to design and is not successfully done (55).
Gene regulation	dCAS9 can repress or activate transcriptionof a gene (56).	Gene regulation cannot be achieved by ZNF nucleases (56).	Gene regulation is not the characteristic feature of TALENs(56).

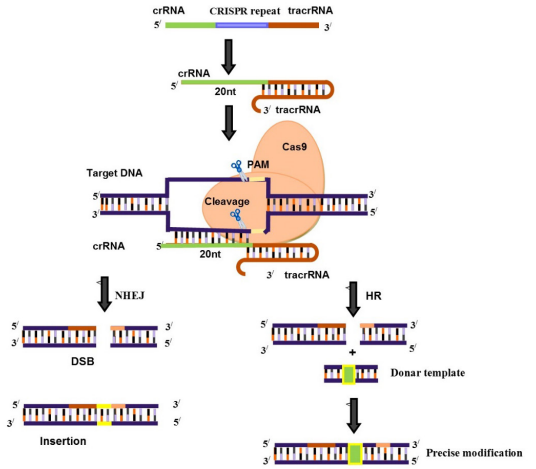

Figure 3. Graphical representation of plant genome editing via artificially induced CRISPR/Cas9 system by engineering a crRNA/tracrRNA chimera via creating a linker loop. This chimera has the same functionality as the crRNA/tracrRNA duplex in the endogenous Cas9 system. DNA target recognition requires both base pairing with the crRNA and the presence of a short sequence, the protospacer adjacent motif (PAM), adjacent to the targeted sequence in the DNA. Double-strand break (DSB) done by Cas9 is repaired by the host through non-homologous end joining (NHEJ) or homology-directed repair (HDR) mechanism.

two nuclease domains similar to HNH and RuvC. The HNH- like domain cuts complementary DNA strand while Ruv nuclease domain cuts the non-complementary strand so the blunt cleavage is produced at target site (9).

So basically in CRISPR/Cas9 activity is used to target DNA sequence of the form N_{20}-NGG by changing the sequence of first twenty nucleotides of guide RNA. Other species of bacteria recognize other substitute PAM sequences and uses distinct crRNA and trcrRNA sequences, their CRISPR TYPE II system is also modified for genome editing (57).

After cutting the target DNA sequence of 20 bp by Cas9 protein, the target site may be restored by two repair mechanisms either NHEJ or HDR. The target sequence is mostly repaired by NHEJ which is an error-prone mechanism that results in minute sequence insertion or deletion (indel) (58). Indel within gene coding sequences ultimately consequences the insertion or deletion of triplet codon, or produces frameshift mutation that leads to failure of function of a gene. Although CRISPR/Cas9 is a precise mutation tool, recognition of 20 bp target is very accurately occur as compared to previous random mutation techniques but end result alteration by error-prone NHEJ still a random event. Off-target breakage with similar but non-identical sequences may occur with CRISPR/Cas9. Mismatches are better endured at 5' of guide RNA as compared to 3' end. Off-target activity can be reduced either by choosing sequence having the lowest similarity with other sequences or by using mutated Cas9 nuclease (nickases) that cuts only one strand of DNA (59). Cas9-induced DSBs are repaired by using single-stranded oligonucleotide donor template or HDR by using double-stranded plasmid DNA.CRISPR/Cas9 can be utilized to edit multiple sites by expressing a Cas9 and numerous guide RNAs (53) (Figure 3). Different crop plants including potato, corn, tomato, wheat, mushroom, and rice are successfully been edited using CRISPR/Cas9 (60). The three synthetic nuclease based technologies have been summarized in Table 2.

CRISPR/Cas9 delivery methods

Transient gene expression can be achieved when CRISPR/Cas9 components are transiently expressed into plant cells. After their expression, some DNA portion is incorporated into the plant nuclear genome while some unincorporated portion is still expressed and work for a little time. This strategy is beneficial to use on wheat callus cells where transgenes are difficult to insert (61). Stable gene expression can also be achieved by CRISPR/Cas9 when delivered into plants by plasmids (62). These plants are considered as GMOs which complicates the path of commercialization of improved varieties. Albeit transgenes can be detached by genetic segregation but it is not possible in plants which propagate asexually. Even if transgenes are removed, plants are still not acceptable by some countries because plants were produced by recombinant DNA technology (63). There are two ways of CRISPR/Cas9 delivery system (a) transgene containing plasmid delivery of CRISPR/Cas9 components (64). (b) transgene-free genome editing by direct delivery of purified CRISPR/cas

ribonucleoprotein(RNP) complex or a mixture of cas9 encoding mRNA and gRNA (65). One drawback of transgene-free genome editing is the working of Crispr/Cas9 RNA is short-term that is why more optimization is required to shield the stability of RNA and it produces transient expression (66).

A transgene containing plasmid delivery

Vector delivery method would result in the stable expression of CRISPR/Cas9 plasmid. Delivery methods utilize either Binary vectors or all-in-one vector system to deliver CRISPR/Cas9 into cells. Binary vectors involve two separate vectors containing genes for a gRNA and a Cas9, both are compatible with each other and are co-trasfected into cells (67). while the all-in-one vector system expresses both gRNA and a Cas9 nuclease within a single vector. This type of vector is particularly constructed for multiplex genome editing where Golden gate cloning method is used to ligate expression cassettes of multiple gRNAs and a Cas9 nuclease into a single vector (53).

Due to large sized (>10Kb) CRISPR/Cas9, transformation methods include electroporation, microinjection and heat shock methods. Viral vectors such as *Bean Yellow dwarf virus*, Wheat dwarf virus and Cabbage leaf curl virus based vectors can also be used for transformation. Antibiotic resistance genes are used to select the cells that are gene-edited using vector delivery method of CRISPR/Cas9 (68).

Transgene-free technology

Plasmid-mediated delivery of CRISPR/Cas9 has some drawbacks such as undesired insertion of the transgene and constant expression. Additionally, off-target mutations may lead to cytotoxicity, apoptosis and gross chromosomal rearrangements (62). Plants developed from this technique are labelled as GMOs due to the occurrence of transgene (72, 73)-92).

Whereas, RNPs provide instant genome editing at the aimed place after which quick degradation occurs, thereby limiting the off-target activity and without the need of transgenes (74). USDA has categorized such plants as non-GMOs since these are edited via targeted mutagenesis inducing self-repair mechanisms, which is identical to naturally occurring genetic mutations. So they may fall outside the true definition of GMOs and these may be considered as NON-GMOs (75). USDA has excluded the Maize and Canola from the GMO regulations because they are free from transgenes (54).

These RNPs are intact complexes of purified Cas9 protein and in vitro transcribed gRNA which are preassembled and are directly delivered as functional complexes. The vector-free standard delivery techniques include microinjection, electroporation, liposome- and polyethylene glycol-mediated transformation, which are used to transport CRISPR/Cas9 RNPs into protoplasts {Martin-Ortigosa, 2014 #2231. While Mesoporous Silica Nanoparticles (MSN) and cell-penetrating peptides both methodologies are used to transform RNPs directly into plant cells by crossing their cell walls and overcome the limitation of regeneration of various recalcitrant protoplasts (76). MSN-mediated delivery of RNPs are successfully done on maize plants because this method ensures the stability of RNPs {Martin-Ortigosa, 2104 #1976}. After target mutagenesis, these complexes are instantly cleared by degradation pathways hence it reduces the time for which Cas9 is available for off-target activity and also no clue of transgene remain behind (62). Rice, lettuce, sorghum, Arabidopsis and tobacco have also been successfully edited by transiently transported RNPs (65). Selection of cells edited via RNPs can be done on the basis of fluorescent reporters used for the visualization of the genome (5).

Applications

miRNA editing

CRISPR/Cas9 can be used to edit miRNAs (77). Transient delivery of crispr components vectors into soybeans via particle bombardments can lead to NHED gene knockouts in miR1514 and miR1509 (78). Hexaploid plants can be easily edited by CRISPR/Cas9. Transiently delivered CRISPR/Cas9 vectors can edit TaMLO in bread wheat protoplasts which induces mildew resistance (79).

Gene expression control

CRISPR/Cas9 is a versatile tool to control the gene expression level either by activate or repress protein-coding and non-coding genes. The inactivated dCas9 fused with activator peptide EDLL, directed by gRNA can stimulate transcription of PDS while dCas9 fused with repressor peptide SRDX, directed by gRNA can repress gene expression in plants (80). dCas9 fused with VP64 directed by gRNA can inverse gene silencing occurred via methylation of AtFIS2 in Arabidopsis (70) (Table 3).

Table 3. Different variables of CRISPR/Cas9 fused with different proteins to achieve different functions.

Plasmid Functions	Achievements	Plasmid required	References
Cut	The entirely active Cas9 enzyme is made to produce DSB (cut) at a site defined by coexpressed gRNA-target sequence	pRGEB32	(69)
Nick	Nickase is the mutated cas9 enzyme used to create single-strand DNA break (nick) at a location defined by the co-expressed gRNA target sequence	pHSN501	(59)
Activate	dcas9(inactive cas9 enzyme) fused with activator peptide leads to transcription activation of a gene	pYPQ152	(70)
Interfere	dcas9 inactive enzyme fused with repressor peptide leads to knock down the transcription expression of a gene	pYPQ153	(70)
dcas/fok1	Catalytically inactive enzyme dcas9 is joined with half fok1-nuclease which dimerize to produce DSB.	FokI-dCas9	(46)
Visualize	d cas9-fluorescent protein complex is used to visualize or labels the specific genomic loci.	pSLQ1658-dCas9-EGFP	(71)

Bi-allelic mutation

Bi-allelic rice mutants can be achieved by transferring Cas9/sgRNA vectors having HDR sequence into Oryza sativa calli can edit ALS gene (1). CRISPR/Cas9 inducing HDR repairing is done to create transmissible alterations in various plants i.e tomato (81), maize (82) and soybean (3).

Fluorescent imaging

Fluorescently tagged Cas9 labelling of particular DNA sequences has been successfully done by coupling Cas9 with fluorescent reporters enables live imaging, visualizing the 3D structure of genome and painting the entire chromosome to follow its position in the nucleus. Modification in Cas9 structure and sgRNAs would create different colours and multi-loci capabilities that increase the utilization of CRISPR/Cas9 instead of FISH Technique which was previously used for visualizing chromosomes (5).

Epigenetic control

For epigenetic control dCas9 is converted into DNA binding domain and joined with epigenetic enzymes such as methyltransferase, demethylase etc. hence, these Epigenetic Cas9 effectors are used to fix or eliminate the epigenetic marks (DNA methylation or histone acetylation) at a particular site in the genome. Both of these events help in modelling the regulatory networks of the genome and reducing crosstalk with other endogenous proteins (83).

Multiplex genome editing

Multiplex genome targeting via CRISPR/Cas9 is used to control the gene expression, genetic improvement and methylation by designing multiple gRNAs coupled with functional domains such as repressors, activators (53). Multiplex genome targeting is successfully done in Bombyx mori by constructing 3 gRNAs and a Cas9 expression vectors to target exon 2 in BmKu70 gene. By co-transfection into Bombyx mori cell line, precise deletion is done at 3 different ku70-1, ku70-2 and ku70-3 sites respectively in the same genome (67).

Future prospective and conclusion

The biology of plant genome editing is passing through a revolution by the advent of the CRISPR/Cas9 system. Great progress has been made in this technology only a single decade of its age but still, there are more questions to be answered about its off-target effects, nearby genes effects, chromatin structure effects. Besides all these questions the CRISPR/Cas system has some advantages over TALENs and ZFNs because of its simplicity and robustness. In SSNs target is recognized by proteins, therefore, designing of these SSNs is complicated which is overcome by CRISPR/Cas9 technology which relies on sgRNA for target genome specificity. RNA based guidance of CRISPR/Cas system makes it easier with the potential to target multiple sites by using multiple sgRNAs in a single CRISPR array. Its robustness and simpler use will lead to more crops plants in the market which will have genome edited with this technology and these will not be considered as GMOs. Keeping its pitfalls in mind researchers are taking this challenge to improve the technology and to minimize its off-target effects.

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