REVIEW ARTICLE

ADVANCES IN RESEARCH ON GENOME EDITING CRISPR-CAS9 TECHNOLOGY

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Background: The current era of genome engineering has been revolutionized by the evolution of a bacterial adaptive immune system, CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) into a radical technology that is making an expeditious progress in its mechanism, function and applicability. **Methods:** A systematic literature review study was carried out with the help of all available information and online resources. **Results:** In this review, we intend to elucidate different aspects of CRISPR in the light of current advancements. Utilizing a non-specific Cas9 nuclease and a sequence specific programmable CRISPR RNA (crRNA), this system cleaves the target DNA with high precision. With a vast potential for profound implications, CRISPR has emerged as a mainstream method for plausible genomic manipulations in a range of organisms owing to its simplicity, accuracy and speed. A modified form of CRISPR system, known as CRISPR/Cpf1 that employs a smaller and simpler endonuclease (Cpf1) than Cas9, can be used to overcome certain limitations of CRISPR/Cas9 system. Despite clear-cut innovative biological applications, this technology is challenged by off-target effects and associated risks, thus safe and controlled implementation is needed to enable this emerging technique assist both biological research and translational applications. **Conclusion:** CRISPR/Cas9 systems will undoubtedly revolutionize the study and treatment of both immunologic and allergic diseases. Concerned authorities should formulate and authorize such laws and regulations that permit the safe and ethical use of this emerging technology for basic research and clinical purposes.

Keywords: CRISPR-Cas9 technology; Off-target effects; Anti-CRISPR activity; Implications

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INTRODUCTION

A variety of bacteria are naturally equipped with a defence mechanism known as CRISPR or 'Clustered regularly interspaced short palindromic repeats.' It contains short repetitions of base sequences with intervening spacer DNA. Spacers are acquired from invading viruses and represent history of old infections so as to protect bacteria in case of their recurrence.[1] The CRISPR can be present on both chromosomal and plasmid DNA. CRISPRs account for about 90% and 40% of the sequenced archaea and bacterial genomes, respectively. Modification of this bacterial immune system has led to the development of a revolutionary genome editing tool, known as the CRISPR system. Its three distinctive types identified so far include type I, II and III, of which the type II CRISPR-Cas system is the foundation of present-day genome engineering applications. The type I and III systems exploit a large Cas proteins complex for crRNA-directed targeting.[2] Nonetheless, only a single protein is necessitated by the type II system for RNA-mediated DNA recognition and cleavage; a feature that showed immense advantageousness in genome engineering applications. This simple CRISPR system studied in *Streptococcus pyogenes* is orthogonal to the native *E.coli* system.[3] Genomic manipulation requires Cas9 protein and an engineered small guide RNA (sgRNA) with a PAM (Protospacer adjacent motif) sequence upstream of target complimentary sequence. PAM does not comprise a part of the bacterial CRISPR locus; instead, it is a constituent of the invader (virus or plasmid). The importance of PAM sequence, firstly as a crucial targeting component, is that it indirectly protects the bacterial CRISPR locus from nuclease attack by distinguishing between native and non-native bacterial DNA. Secondly, binding of the Cas9 protein to target sequence and subsequent cleavage of the latter cannot occur successfully in the absence of PAM sequence.[4] Cas9 endonuclease necessitates CRISPR RNA (crRNA) and a trans-activating CRISPR RNA (tracrRNA) that functions to hold the crRNA in place. Correct, site specific binding of crRNA with the targeted DNA sequence directs the endonuclease to its target site. This means that adaptive immunity in bacteria occurs in three stages;

a short spacer DNA sequence of the invader gets inserted into the CRISPR array followed by the transcription and maturation of precursor crRNA (pre-crRNA) into distinct crRNAs (each comprising of a repeat sequence and an invader-targeted spacer region) and finally the Cas proteins mediate crRNA-directed cleavage of the foreign genetic material at sites corresponding to the crRNA spacer sequence.[5] So, it was decided to modify the whole system and as a result, a tracrRNA-crRNA chimera (an RNA duplex) was designed, later called as a guide RNA (gRNA).[6] Cleavage by Cas9 nuclease does not require a unique DNA target; it can be present at multiple locations and still be targeted. If we want to add a new gene into the sequence, the system will now have three parts; Cas9, gRNA and host RNA that we ought to insert. As we break the DNA, host RNA is added and, in this way, a new part will be integrated into a sequence. CRISPR/Cas9 technology has enabled the scientists to engineer any human genomic segment with high fidelity. Acclaimed as a breakthrough in medical science, this development holds the promise of transforming the study and treatment of a wide range of diseases such as viral diseases (e.g. HIV) and heritable genetic maladies (e.g. haemophilia) and cancer. In the light of its prospects, this review provides an overview of the CRISPR-Cas system: its history, mechanism of action, specificity, off target effects and their minimization, and the anti-CRISPR activity of viruses. Moreover, the pertinent applications of CRISPR technology, in general and in plants, have been comprehensively reviewed. The paper also evaluates the pros and limitations of this emerging technology, the challenges faced and their practical solutions; and also, an assessment of associated risks and consequences.

HISTORY

Over the years, innovative genome engineering techniques such as Zinc Finger Nucleases (ZFNs), RNA Guided Endonucleases (RGENs), Transcriptional Activator like Effector Nucleases (TALENs) and the recently discovered CRISPR-Cas System have completely transformed genome editing in different organisms.[7] However, the effectiveness of ZFNs and TALENs was chiefly hurdled by the protein design, synthesis and validation for a particular DNA locus under study. In 2012, the CRISPR system was identified in the laboratories of Jennifer Doudna (UC Berkeley) and Emmanuelle Charpentier. A parallel revolutionary work was reported by Feng Zhang (Broad-MIT, Cambridge) in 2013. Many studies have described the characterization and engineering of CRISPR systems (Figure-1).[8] The CRISPR patents have been recently granted to Zhang in February 2017 by the US patent office.[9] In March 2017, the European patent office (EPO) has made a declaration of granting a broad patent to the University of California, the University of Vienna and Emmanuelle Charpentier.[10]

MECHANISM OF ACTION OF CRISPR-Cas SYSTEM

A cluster of CRISPR associated genes, or precisely Cas genes, adjoining the CRISPR locus are prerequisite for CRISPR activity as these encode (Cas) proteins vital to the immune response.[11] The two essential components of Cas9 protein, as revealed by crystal structure analysis, include a recognition lobe (REC) and a nuclease lobe (NUC). These play pivotal roles in CRISPR system. The REC lobe, comprising of a long α helix and two domains (namely, REC1 and REC2) is a Cas9-specified functional domain as it interacts with the repeat: anti-repeat duplex. The NUC lobe consists of the PAM-interacting (PI), RuvC and HNH domains; the last two domains named on the basis of their structural homology to the well-recognized nuclease domains: RuvC bears similarity to the E.coli RuvC domain that functions to resolve Holliday junctions while HNH is analogous to phage T4 endonuclease VII that carries out DNA binding and cleavage.[12] Another essential component of the CRISPR system is a 3 base pair PAM sequence which, adjoining the 3′ end of the DNA target site, enables its recognition. Even within the same species, the specificity of the PAM sequence to each Cas9 orthologue governs the targeted DNA locus of Cas9.[13] The versatility of the *S. pyogenes* Cas9 frequently employed in genome editing is attributed to its ability to identify 5′-NGG and 5′-NAG PAM sequences (the latter sequence with lower efficacy) present abundantly in the genome, i.e., every eight base pairs on an average.[14] The HNH and RuvC domains of Cas9 are employed in DNA cleavage; with the former cleaving the strand complementary to crRNA and the latter cutting the opposite DNA strand.[15] Basically, Cas9 utilizes a single guide RNA (sgRNA) that undergoes base pairing with the target DNA so that Cas9 can make a site-specific double-strand break (DSB) in the target sequence. Hence, the sgRNA is imperative for precise CRISPR activity as it furnishes genomic target recognition via the universal Watson–crick base pairing and also functions as a scaffold for endonuclease Cas9 binding. Thus, its unique structure links Cas9 and the target sequence. Also, since it is convenient for the researchers to use a single RNA, it has led to the extensive use of sgRNAs for genome editing. Particularly, the crRNA comprises of a 20-nt guide RNA (gRNA) and a 12-nt repeat region while the tracrRNA contains a 14-nt anti-repeat region and three stem loops, termed as loops 1, 2 and 3, respectively. Stem loop 1 is vital in

functional Cas9: sgRNA complex formation while the stem loops 2 and 3 influence the activity and stability of the CRISPR/Cas9 system. Of the Cas9 protein, both the REC and NUC lobes can recognize the repeat: anti-repeat duplex, stem loop 1 and the gRNA: target sequence; while the NUC lobe can specifically recognize the linker, and the stem loops 2 and 3.[16] All cells types and organism have two endogenous DNA repair mechanisms to repair Cas9-induced DSBs: Homology Directed Repair (HDR) and Non-Homologous End Joining (NHEJ).[17] Cells use these repair mechanisms to sustain cell viability and genomic integrity. If a homologous repair template is present, the CRISPR/Cas9 system can be utilized to bring about specific sequence modifications (for instance, mutation insertion/correction) via HDR.[18] HDR usually fixes double strand breaks when the cell is in the S and G2 phases of its division whereas the NHEJ does not depend on cell cycle and is the most prevalent pathway for repairing DSBs in mammalian cells. However, NHEJ can also generate indels which, if present in exons, can result in a frameshift and formation of a premature stop codon, thereby disrupting the normal open reading frame (ORF) and inactivating the corresponding protein.[19] Structural studies of Cas9 with X-ray crystallography and electron microscopy have revealed the large conformational change that the protein undergoes when it binds to the guide RNA followed by an additional change when it associates with the target double stranded DNA (dsDNA). It is, however, speculated but still non-validated that the conformational changes in Cas9 might be inclusive of the mechanism that entails unwinding of target dsDNA and gRNA strand invasion. The CRISPR-Cas facilitated defence process can be divided into three stages (Figure-2). The first or acquisition phase, involves integration of the invading plasmid or phage DNA fragments into the CRISPR loci as "spacers"; second, the CRISPR RNA (crRNA) biogenesis phase, in which the precursor crRNAs are transcribed and mature into crRNAs as a result of cleavage within the repeat sequences; third, the interference phase in which the Cas proteins enable the crRNA-guided cleavage of foreign nucleic acid.[20] An arginine-rich α helix in the Cas9 plays two important roles; it acts as a hinge between the protein's two structural lobes and also has a chief function in binding the hybrid (guide RNA–target DNA).[21] Also, the C-terminal domain of Cas9 has arginine motifs that interact with the PAM sequence within the major groove of the target DNA strand. The position +1 in the target DNA has a phosphodiester group that forms association with the duplexed PAM's minor groove. As a result, an R-loop formed directly upstream of the PAM possibly causes local strand separation.[22] Single-molecule experiments have suggested that R-loop association rates are influenced mainly by the PAM, while R-loop stability is chiefly affected by protospacer elements located distally to the PAM.[23] Therefore, the Cas9 endonuclease carries out target DNA cleavage by utilizing its two catalytic centres (also called as blades) for generating breaks in each target DNA strand at a site adjacent to the PAM sequence, in the presence of a complementary single guide RNA (sgRNA) sequence.

Comparable to the CRISPR/Cas9 system is the CRISPR/Cpf1 system with a similar mechanism of action comprising of three stages. It is native to Prevotella and Francisella bacteria and makes use of Cpf1, an RNA-guided endonuclease belonging to class II CRISPR/Cas system. This endonuclease, encoded by Cpf1 genes associated with the CRISPR locus, is smaller and simpler than Cas9. It is different from Cas9 in that it 1) requires one RNA instead of two, 2) produces staggered end cuts instead of blunt end cuts, 3) cleaves target DNA distal from recognition site as opposed to Cas9 that cuts proximal to PAM, 4) makes use of a T-rich PAM instead of a G-rich PAM and, 5) can recognize different PAM sequences. So, a few limitations of the CRISPR/Cas9 system can be possibly overcome with this tool, making it useful for prospective applications.[24]

OFF TARGET EFFECTS AND POSSIBLE STRATEGIES

Off-target effects, resulting from the interaction of Cas9 with an unintentional target, are of chief concern particularly for clinical and therapeutic applications of CRISPR.[25] These call for the need to develop strategies for prognosis and prevention. In this regard, one notable advancement is the engineering of chimeric single-guided RNAs (sgRNAs) that are based on CRISPR[3] and function in eukaryotic cells to guide the Cas9 nuclease to cut complementary genomic sequences with supervening 5′-NGG PAM sequence. However, all sgRNA cannot prompt precise and efficient editing. So, an optimized sgRNA design, functional Cas9 and a distinctive target sequence choice are imperative for refining potency and curtailing off-target mutations. It has been manifested through comprehensive experiments that mismatched bases between the sgRNA and target DNA are generally tolerated by the CRISPR system when they are less than three in number and are found on the 3′end of the sgRNA.[26] Nonetheless, off-target mutations occur at greater frequencies as compared to the intended mutation which may result in genomic instability and disruption of normal function of genes.[27] Keith Joung's group has recently established that slightly truncated gRNAs (having 18

nt rather than the usual 20 nt homology) maintain normal activity in mammalian cells with considerably reduced risk of off-target mutagenesis. Moreover, the authors showed that off-target mutagenesis caused by these truncated sgRNAs was reduced by up to 5,000-fold without compromising on-target efficiency of modifications.[28] The 20 base-pairing associations between target DNA site and gRNA furnishes much greater amount of energy than that mandatory for the binding and activation of Cas9 and so can tolerate a few mismatches. Truncated gRNAs necessitate perfect homology as these are believed to provide only the required energy upon binding of all nucleotides to the target. Enzyme concentration is also indispensable for Cas9 specificity; higher enzyme concentration has exhibited increased off-target sites[29] giving greater off-target activity whereas a lower Cas9 concentration augments enzyme specificity and reduces on-target cleavage activity. Paired Cas9 nickases (Cas9n), creating two nicks or single-strand breaks (SSBs) on different DNA strands, have been reported to show high specificity in human cells so as to avoid off-target mutations without compromising genome-editing efficacy.[27] The off-target sites of two separate gRNAs are improbably in close proximity which is an advantage, as it can only result in DNA nicks having low mutagenic potential. This concept was further refined by the development of Fok1-dCas9.[30] Here, dCas9 cannot cut the DNA itself, but is affixed to a Fok1 endonuclease domain. Fok1 cleaves DNA as a dimer that can again be successfully achieved with two gRNAs having closely adjoining target sites. This system exhibits more specificity than double-nicking as no DNA lesions are induced by a single Fok1 dCas9/gRNA complex.

Genome editing has been refined with the emergence of light activated CRISPR/Cas9 system that consists of two fusion proteins, i.e., C1b1 and CRY2, the former functioning as the genomic anchor probe by fusing with dCas9 for targeting the genome sequence with the help of sgRNAs, and the latter acting as an activator probe together with an activator domain. Cas9, modified by the heterodimerization of C1b1 and CRY2, can be directed to the target site for activating gene transcription when provided with a blue light stimulus (Figure-3).[31] Generally, the likely CRISPR/Cas off-targets can be managed similarly as RNAi off targets or second hits prompted by conventional mutagenesis methods. RNAi results are usually validated with another independent RNAi construct due to the RNAi associated chronic off-target problems. A comparable approach is suggested for CRISPR/Cas where a similar mutation can be practically generated with a second gRNA owing to the ease of gRNA cloning. It is quite rare for these distinct mutations to share similar off targets and thus, can be investigated individually or in a trans-heterozygous combination. Genetic rescue experiments can furnish additional assurance in the specificity of observed phenotypes. Mutations produced by classical unspecific mutagenesis methods, for instance X-rays or EMS are followed by thousands of undesirable mutations somewhere else in the genome. Hence, it is imperative to isolate desired mutations by numerous backcrosses into a wildtype background. Prospective CRISPR/Cas off-targets are assumed to be quite occasional and so the need for genetic clean-up operations is small. It might be promising to remove chromosomes devoid of the desired mutation during the establishment of stable fly lines.

ANTI CRISPR ACTIVITY OF VIRUSES

Bacteria have evolved various mechanisms to confer them protection against their widespread predation by bacteriophages (phages). Phages infecting Pseudomonas aeruginosa were found to have five specifics anti-CRISPR genes in their genomes. They seem to be lodged in a distinct operon intervening two highly conserved head morphogenetic genes. These phage-encoded anti-CRISPR genes might equip the phages with an extensive mechanism to overcome the highly prevailing CRISPR/Cas systems.[32] *P. aeruginosa* phages have been discovered to possess a more sophisticated countermeasure in the form of several encoded proteins that influence the activity of Type I-E and I-F systems. Despite their functions being unclear, these proteins do not seem to exert any influence on the expression of the crRNA or Cas proteins. They possibly obstruct the activity of CRISPR-Cas complexes.[33] Eventually, another study carried out the characterization of three of these anti-CRISPR proteins (AcrF).[34] Direct binding of AcrF1 and AcrF2 to the Csy complex (i.e., crRNA-guided surveillance complex) blocks target DNA binding. AcrF3, however, acts by directly interacting with and blocking the recruitment of Cas3 protein into Csy complex, consequently protecting phage DNA from the CRISPR/Cas system-mediated degradation.[35] The Csy complex carries out specific recognition of the complementary target DNA and then directs Cas3 for degrading the invading DNA.[12] Phages have evolved to avoid CRISPR interference by means of a single nucleotide substitution in the conserved PAM (for types I II CRISPR-Cas systems) or in the protospacer region in which the position of the mutation is particularly significant.[36] Phages having substitutions proximal to PAM (or seed sequence) escape CRISPR targeting[37,38] while those having several mismatches at PAM-distal protospacer positions do not. Such an evasion can also take place if PAM and/or

protospacer sequences are excised from the phage genome[36] with an assumption that this deletion does not compromise phage infectivity or fitness considerably. Nevertheless, in the case of unsuccessful interference, some infected host cells can persist by the direct attainment of new spacers from the attacking phage genome.[39] Moreover, the CRISPR-Cas subtype I-E system in *E.coli* is also equipped to procure multiple spacers rapidly upon the failure of the initial interference step, thus providing an increased range of phage resistance to this bacterium.[40]

GLOBAL ACQUISITION AND WIDESPREAD APPLICATIONS

Ever since CRISPR's identification as a gene editing tool about four years ago, researchers have explored its use in the genome alteration of almost any organism with an unparalleled ease and sophistication. Its range of applications, together with its speed and efficacy, has given it an edge over other gene editing tools and has revolutionized biological applications around the world.[41]

I. Genome Manipulation Through CRISPR-Cas9 System:

As a multiplex-able and dynamic genome editing tool, CRISPR/Cas9 enables accurate manipulation of specific genomic elements, thereby facilitating the researchers in elucidating the function of target genes in biology and ailments.[7]

a. Germline Research:

CRISPR has certainly the most debatable application in human germline research as any alteration made in the germ cells is heritable whereas gene editing in somatic cells will not be passed on to subsequent generations. Germline editing can be potentially useful and, in some cases, the only viable option in preventing the effects of a devastating genetic disease in a future child.[41]

b. Generation of disease models for human disease:

One of the most imperative achievements was the development of animal models using CRISPR to assist the researchers in studying diseases. Such models have been made for pigs, mice, monkeys, rats and even dogs.[41]

Using CRISPR/Cas9 system for the investigation of disease pathology and gene function in development, efficient and rapid generation of GM mice having either reporter or tagged alleles, null, conditional or accurately mutated genes can be done successfully utilizing a one-step approach. Mouse models with LKb1, KRAS, and p53 mutations were generated for lung adenocarcinoma with distinctive CRISPR/Cas9 systems that utilized various reagents such as chemicals, adeno-associated virus (AAV), and lentivirus to deliver gRNA into immune cells and neurons.[31]

c. Genome editing in plants:

CRISPR/Cas9 also finds remarkable applications in plants for producing valuable phenotypes or disease resistance. This was validated by Jiang et al. who inserted a gene for green fluorescence protein into tobacco and Arabidopsis genomes, and genes for bacterial blight susceptibility into rice genome. Thus, alteration of the crop genomes by CRISPR/Cas9 to improve crop quality will emerge as a novel breeding technique in the future.[7]

d. Genetic manipulation in specific tissues:

Using the CRISPR/Cas9 system, researchers can carry out direct and efficient genome editing of particular tissues such as the brain and liver tissues, using AAV vectors and/or hydrodynamic injection. Utilizing a direct hydrodynamic tail-vein injection for delivering a plasmid encapsulating Cas9 and sgRNAs to the liver, a cancer model has been generated with Pten and p53 mutations. Also, recently, an efficient and successful application of the CRISPR/Cas9 system has been described in mammalian nervous systems (or precisely, adult male mice) where in vivo injection of GFP tagged plasmids mix (AAV-spCas9 and AAV-spGuide) was made into the animal's hippocampal dentate gyrus.[31]

e. Investigation of Gene Function:

For a molecular biologist, an elucidation of the working of genomes and cells is a prerequisite for understanding complex genetic diseases. To quote, CRISPR has enabled 1) the study of epigenetic functions which effect gene expression, and 2) identification of the functions of non-coding DNA sequences.[41]

For functional genomics studies in cells, the CRISPR/Cas9 system provides a highly versatile and proficient approach for creating gene knockouts to determine gene function in both biological processes and diseases with high resolution. For example, the technology has been used to produce knockout of CCR5 and C4BPB genes in human myeloid leukemia K562 cells. Moreover, introduction of specific point mutations has also been done in zebrafish and mouse.[42]

f. To Generate Multiple Gene Mutations Simultaneously:

The ability of CRISPR/Cas9 system to produce various gene mutations in multiple organisms has been increasingly evidenced. One of the

major achievements, back in 2013, was the introduction of multiple gene mutations in one-cell stage rat embryos by a simultaneous delivery of six sgRNAs aiming at six genomic regions encoding Tet1 (sgTet1-1, sgTet1-2), Tet2 (sgTet2-1, sgTet2-2), Tet3 (sgTet3-1, sgTet3-2), along with Cas9 mRNA into the cell cytoplasm. For now, in Arabidopsis, numerous gene mutants using CRISPR/Cas9 system can be created for mosaics T1 generation while for non-mosaics T1 generation, specific expression of Cas9 and sgRNAs directing at ETC2, CPC and TRY in egg cells and one-cell stage embryos is required.[31]

g. For Correction of Genetic Mutations:
Although the most direct approach for treating a human genetic disease entails correcting the causal disease mutation(s) via gene therapy; but, recently it has been demonstrated that this can also be possibly done rapidly and proficiently by CRISPR/Cas9 system- mediated genome editing in the cultured human stem cells.[42]

h. Human Therapeutics:
Another impressive use of the CRISPR/Cas9 system is that it may be a unique therapeutic strategy against HIV because it can modify coding or non-coding sequences at pre-integration or provirus stages, thereby interrupting the viral expression and replication; and ultimately causing disruption of the infection. Moreover, the treatment of HBV (hepatitis B virus) infection has also been suggested using the CRISPR/Cas9 system that brings about inhibition of replication of HBV, resulting in the down regulation of HBV protein and covalently closed circular DNA (cccDNA). This can be done by injecting plasmids having Cas9 and sgRNA, directed at the HBV's conserved regions, into the tail vein.[31]

II. Epigenome Editing:
Besides the direct modification of target DNA sequences, the CRISPR system also encompasses alteration of the epigenome for the regulation of specific gene expression.

- **Suppresses DNA methylation**: By disruption of the catalytic domain of DNA methyltransferases (DNMTs), the CRISPR/Cas9 system has been employed to suppress DNA methylation in human cells that ultimately causes cell death.
- **Regulation of Gene Expression**: For the regulation of epigenetic processes and gene expression, the CRISPR/Cas9 system has been applied to long non-coding RNA (lncRNA) as well as enhancer RNA (eRNA). For example, in CH12F3 cell line, a subclone of the lgM+ CH12.LX lymphoma cell line, CRISPR/Cas9 system-induced deletions caused downregulation of both the eRNA-expressing element (lncRNA-CSR) and the lncRNA-expressing enhancers.[31]
- **Transcription Regulation:** CRISPRi or CRISPR interference, a modified CRISPR/Cas9 system has been recently developed for RNA-directed transcriptional regulation. Qi et al. created a Cas9 (dCas9) mutant, and a recognition complex made by co-expressing dCas9 with gRNA could interfere well with the transcriptional elongation and the binding of RNA polymerase and transcription factor.[7] Additionally, using dCas9 p300 core fusion protein (formed by the fusion of cCas9 to the catalytic histone acetyltransferase core domain of p300), gene expression can be feasibly regulated by altering acetylation at the promoters or proximal and terminal enhancers (target sites).[31]

III. Livestock /Large Animals Transgenesis:
The CRISPR–Cas technology has been harnessed in large animal/livestock transgenesis. Recent reports have described the first successful experiments using CRISPR–Cas systems for the genomic modifications of pig and cow. Allelic variants of agronomic interests can be directly introduced into the desired breed via oligonucleotide donors and targeted nucleases. For example, it is possible to genetically dehorn bulls by introduction of the Angus POLLED allelic variant.[43]

IV. RNA Manipulation:
Besides the manipulation of double-stranded DNA, editing of RNA sequences can also be done using the CRISPR/Cas9 system comprising of a Cas9 protein, PAM-presenting DNA oligonucleotide (PAMmer), gRNA and ssRNA (single strand RNA).[31]

V. Gene Therapy:
Gene therapy studies have been revolutionized using CRISPR/Cas9 system (with the latest engineered nucleases) that has emerged as an innovative and a very competent genome editing tool. Ebina *et al*. reported one notable advancement utilizing CRISPR/Cas9 that resulted in substantially reduced HIV-1 expression in infected human cells due to disruption of the long terminal repeat promoter in HIV-1 genome. The system can also be used to eliminate proviral genes that have integrated into the host cell genomes.[7] The induced pluripotent stem cells (iPSCs) are also of

considerable interest in gene therapy and disease modeling because of their indefinite self-renewing and multipotential differentiation property.[42] Using CRISPR/Cas9, Horri *et al*. generated an iPS cell model for facial anomalies syndrome (ICF) that is caused by DNMT3B gene mutation, immunodeficiency and centromeric region instability.[7] Current research is aimed at treating ocular genetic disorders, and editing the eye is a less technically complicated procedure than the intricate brain tissue, for instance.[41]

VI. Crispr-Cas9 in the Biology of Cancer:

Despite the extensive and vivid studies on the molecular landscape of human cancer over the last decade, the mutations causal to tumour initiation and progression are still not fully ascertained. Since CRISPR/ Cas9 system can virtually target several mutations at a time, it can be essentially employed to model complex genetic diseases like cancer. Sanchez-Rivera *et al*. created a unique CRISPR/Cas9-based method to rapidly interrogate the function of tumour suppressor genes (such as Pten and Apc) in mouse lung cancer models. Another breakthrough of the CRISPR/Cas system in cancer biology is its capacity to produce tumour-associated chromosomal translocations that normally occur during carcinogenesis via illicit non-homologous joining of two chromosomes; whereas CRISPR/Cas system does so by introducing DSBs at well-defined positions. This approach has been exploited for successful generation of primary cells and cancer cell lines having chromosomal translocations and replicating similarly to those found in cancers such as lung cancer, Ewing's sarcoma and AML.[42]

VII. Medicinal drugs:

Recently, many new companies e.g. Intellia Therapeutics and Editas Therapeutics have collaborated with drug companies with a hope to harness CRISPR/Cas9 technology for the development of new medicines for treating genetic diseases.[41]

IMPLICATIONS OF CRISPR IN PLANTS

CRISPR/Cas9 editing in plants was first reported in 2013, with successful application for transient expression and recovery of stable transgenic lines. Several studies have reported the applicability of the CRISPR/Cas9 system for genome editing in plants such as Nicotiana benthamiana, and Arabidopsis[44,45] and three crop species, i.e., rice[46], wheat[47] and sorghum[48]. In these studies, to achieve plants with heritable modifications, it was necessary to generate transgenic lines that stably expressed the Cas9 and gRNA; progeny with targeted modifications were then recovered in subsequent generations. However, the production of transgenics is time consuming and, therefore, efficient delivery methods are needed to expedite and maximize the usefulness of this technology for trait discovery and development.[49]

The CRISPR/Cas9 system can be potentially utilized for plant functional genomics and agricultural biotechnology applications by means of virus-mediated genome-editing. Tobacco rattle virus (TRV), delivered via Agrobacterium, is an efficient vector for virus-induced gene silencing in diverse plant species. Its small genome size facilitates cloning, multiplexing, library constructions, agroinfections and also the viral RNA does not integrate into the plant genome.[49]

The CRISPR/Cas9 system could be used in plants to provide molecular immunity against DNA viruses. In this regard, sgRNAs targeting the coding and non-coding sequences of tomato yellow leaf curl virus (TYLCV) were successfully delivered into *Nicotiana benthamiana* plants that showed stable overexpression of the Cas9 endonuclease. The virus was efficiently targeted by the CRISPR system for degradation, introducing mutations at the target sequences and subsequently challenging these plants with TYLCV.[50]

Of late, cucumbers resistant to potyviruses (papaya ring spots virus, yellow cucumber vein yellow virus and yellow mosaic virus, that cause a considerable 80% loss of the yield when transmitted by aphids) have been engineered using CRISPR by direct targeting of the eIF4E gene that plays a central role in the propagation of potyviruses.[51] Other notable examples include tomatoes resistant to numerous *Xanthomonas* and *Pseudomonas* bacteria; and powdery-mildew-resistant wheat. The technique is being explored by Monsanto to enhance yield, disease resistance and drought tolerance in some crops.[52] The CRISPR/Cas9 system is thus paving the path towards a new horizon for crop development and basic research.[53]

i. Rice:

Cas9/sgRNA-mediated small indels have been reported at single cleavage sites in transient and stable transformations; the genetic transmission of edits has been described in Arabidopsis and rice.[46,48,54,55] A highly adept Cas9/sgRNA platform has been employed for endogenous gene targeting in rice plants. Four distinct genomic loci were modified at a very high efficiency, together with an accomplishment of 100% di-allelic (heterogeneous or homogeneous) mutations that showed stable transmission into all the examined T2 generation plants.[56]

ii. Tomato:

The transient application of CRISPR/Cas9 has been recently reported in tomato roots[57] to introduce *Agrobacterium rhizogenes*-induced mutations at desired loci in hairy root structures; with the CRISPR/Cas9 transgene carried by the bacterium. But there was no regeneration of transgenic plants. In a study by Brooks *et al*., CRISPR/Cas9 construct was used to target adjoining sequences in the second exon of the tomato homolog of *Arabidopsis* ARGONAUTE7 (SlAGO7), owing to the fact that loss-of-function mutations are recessive and produce plants with needle like or wiry leaves instead of typical compound flat ones. Subsequently, stable transgenic tomato plants, with a set of desirable mutations efficiently introduced by the CRISPR/Cas9 system, were generated with a high rate of mutagenesis. However, homozygous deletion (facilitated by using two sgRNAs) of a desired size was observed in only in one of 29 T0 plants. This low efficiency could be probably explained by the fact that DNA cutting at an individual target must be simultaneous but the possibility of this occurring is considerably lower than asynchronous cuts and corrections at respective targets, resulting in mutations at each target.[58] These results, along with those of others[46,54,59], put forward the prospect of CRISPR/Cas9 system becoming the technology of choice to create gene knockouts for reverse genetics studies. For instance, forward genetics has recently been used to discover that the gene Solyc11g064850 controls various aspects of tomato reproductive development.[58] Also, CRISPR gene editing technology has recently been used by a group of researchers in Japan to create seedless tomatoes by virtue of a mutation that increased auxin levels-the hormone that stimulated the plant to grow irrespective of seed formation. No mutations were introduced into any other part of the tomato genome, hence accounting for the precision of this gene editing tool.[60]

iii. Soyabean:

The CRISPR technology has been applied for genetically modifying soybean genes. Introduction of CRISPR vectors targeting 11 loci into soybean through *Agrobacterium rhizogenes* has been reported for the generation of transgenic hairy roots. The genetic variations were made in 95% of the tested events as evidenced by the custom-amplicon sequencing of DNA derived from these roots. Even in somatic embryo cultures, alterations were detected that should produce soybean lines with germinal modifications.[61]

iv. *Nicotiana benthamiana*:

A rapid and powerful transient assay has been developed by Voytas to permit plant-specific optimization of the Cas9 system. Coexpression of GFP-Cas9 and sgRNA, delivered via *A. tumefaciens,* in *N. benthamiana* leaf tissue was done and sgRNA-directed, Cas9-induced mutations at the PDS locus were detected by digesting the genomic DNA with MlyI, followed by a PCR reaction. This strategy enriched DNA molecules having mutations that cause removal of the MlyI site.[44,62,63] Plants were regenerated from the modified *N. benthamiana* leaf sections that expressed Cas9 and the sgRNA. Increased MlyI-resistant PCR product was detected in 2 out of 30 regenerated plants. Such results were not observed in the negative control treatments, suggesting the non-toxic nature of sgRNA and the Cas9 and that the induced mutations can be transmitted to the whole plants.[44]

v. *Marchantia polymorpha*:

Sugano *et al*. reported genome targeted mutagenesis of the auxin responsive factor 1 (ARF1) gene in the gametophyte generation of *M.polymorpha*. The plant's U6 promoter was identified and cloned in a gRNA expression vector. Using an *Agrobacterium* mediated transformation, many mutant alleles of arf1 were attained using the 35Spro (Cauliflower mosaic virus 35S promoter) or the MpEFpro (*M. polymorpha* elongation factor 1α promoter) to express Cas9. Stable monoclonal clonal mutants with an auxin-resistant phenotype were isolated.[64]

vi. *Arabidopsis thaliana*:

Jiang *et al*. described a successful Cas9/sgRNA system mediated conversion of a non-functional, out-of-frame GFP gene to a functional GFP gene during the early development of transgenic Arabidopsis. The edited gene, confirmed in T1 plants, was stably inherited in the T2 and T3 generations that showed restored GFP function. Specificity of the CRISPR/Cas system was also verified as no off-targeting was observed in any of the tested targets, suggesting the prospects of this system for facile editing of plant genes.[65]

PROS OF CRISPR TECHNOLOGY

In a single step, numerous genetic variations can be triggered seamlessly with the help of CRISPR technology.[43] Small deletions or insertions are made through a repair pathway i.e. non-homologous end-joining (NHEJ) pathway. Using CRISPR technology, the NHEJ repair mechanism can be simultaneously activated at various endogenous loci via co-injection of multiple sgRNAs having the Cas9 mRNA.[43]

Crispri (Crispr interference) allows targeting of the promoter region to effectively stop the expression of the transcript.[66] In case of ZFN or TALENS, a new protein constituent is required for every single locus which, on a large scale, might not be cost-effective and feasible. In contrast, the CRISPR-Cas9 system requires a minute RNA load to achieve various gene targeting events and has a set up that is very simple, and cost and time effective. Secondly, engineering of the ~ 20 nucleotides sgRNAs is quite easy.[67] The CRISPR-Cas system is organized to have a gRNA, thereby eliminating the need for engineering an enzyme. In

order to target a particular sequence, only a single customized gRNA is needed, as the same Cas9 enzyme is suitable for all the sequence targets of the human genome.[68] Secondly, length of the gRNA sequences is very short; thus, avoiding problems related with the delivery of hugely repetitive and longer TALEN and ZFNs encoding vectors into the cells. Moreover, in order to acquire distinct target specificity, hardly 20 nucleotides are required to be changed in the gRNA. Conclusively, it can simply be said that the process of cloning is also not needed.[67] By in vitro transcription, a large number of gRNAs can be produced using two of the complementary oligonucleotides. Thus, a large library of gRNAs can be created, allowing this technology to be useful for high-throughput functional genomics.

In case of RNAi, the knockdown introduced is temporary, i.e., it lasts for 2–7 days, while genome editing through CRISPR-Cas9 gives rise to a permanent effect.[69] In contrast to the phage defence and other gene transfer processes, one of the main advantages of CRISPR interference is that this system can be easily reprogrammed to repudiate the invading DNA molecules that have not been confronted formerly.[1]

By means of this novel technology, the entire gene family or multiple genes can be disrupted which can be helpful in accelerating the generation of genetically modified animals having several mutated genes. Secondly, the function of several genes and their epistatic connection can be known and investigated.[7] In comparison to the TALENs and ZFNs, CRISPR technology can easily cut methylated DNA in human cells; thus, allowing genetic modifications that cannot be accessed by other nucleases. Even though in plants this attribute has not yet been studied but it is logical to presume that this property of the CRISPR technology is natural and is not reliant on the target genome. Generally, the CRISPR system is more adaptable for genetic modifications in plants as about 70% of the CpNpG/CpG sites in plants are methylated, essentially the ones found in the proximal exons and promoter regions. But this technology is chiefly suitable for the high GC content of monocots; for instance, rice.[67]

Lastly, the CRISPR research community has an open and free access strategy that has permitted the extensive use of this technology, unlike the ZFNs policy which has a proprietary nature. This community gives access to the web tools and plasmids (such as through the non-profit repository Addgene) in order to choose gRNA sequences and to predict specificity. Because of these amenities, new researchers in this field are encouraged to adopt this emerging technology.[68]

LIMITATIONS

On the basis of a recent study, it has been suggested that genome editing using CRISPR is more susceptible to off-target effects in comparison to other genome editing tools including TALENs or ZFNs.[66] Along with transfection or transduction, genomic manipulation using CRISPR-Cas9 is also dependent upon selection, confirmation of induced variation plus clonal expansion of engineered entities. In this context, it must be observed that, to date, CRISPR-Cas9 mediated engineering of primary cell types (significantly the post-mitotic cells) has been documented only with adenoviral vectors rather than the generally applied lentiviral vectors. Given these reasons, extra time and effort has to be expended on the CRISPRCas9 technology in contrast to RNAi.[69] However, the off-target mutation rates of varied CRISPR/Cas9 systems continue to be a major challenge.[31] Lastly, the direct and defined genome editing has given rise to ethical concerns such as the creation of engineered babies via gene alteration of human germline cells by means of the CRISPR/Cas9 system which initiated arguments and inquiries amongst both the scientists and public. Furthermore, grave concerns with regard to environmental balance as well as species safety have been raised due to the invention of MCR.[31] Off-target mutagenesis poses uncertainly regarding the use of CRISPR/Cas9 genome editing technology, particularly in the case of gene therapy.[42]

CHALLENGES FACED AND THEIR PRACTICABLE PANACEA

I. Delivery:

One of the major obstacles for clinical translation remains the delivery of editing systems to the targeted cell types [70]. RNA and DNA injection-based methods are employed for CRISPR/Cas9 delivery, for instance the injection of: CRISPR constituents (as RNA) and plasmids expressing gRNA and Cas9.[7] The delivery system, however, must be meticulously selected owing to the fact that nucleases may elicit immune responses or show off-target cleavage activity.

Viral vectors, especially AAV vectors, comprise the most promising delivery systems for in vivo applications and have recently been accepted for clinical applications. AAVs have different serotypes and high delivery efficiency for various tissue types such as the muscle, eye, liver and brain. But, the AAV vectors have relatively small packaging capacity that somehow challenges nuclease delivery. Delivering Cas9 in a single AAV entails packaging of short orthologs along with guide RNAs.[70] For CRISPR/Cas9 delivery, several researchers have studied other viral vectors that have greater capacities for accommodating larger payloads e.g. adenovirus and lentivirus, particularly for multiplex genome editing that requires numerous different guide RNAs.[71]

Despite the potentiality of AAV-facilitated in vivo nuclease expression, it still offers quite a few challenges that call for additional work. Firstly,

AAV-assisted nuclease expression is usually constitutive, while shutting down the nuclease expression following successful genome editing in the target cell would be desirable. Secondly, people previously exposed to AAV probably become immune to particular serotype**,** hence; AAV would not be a suitable delivery tool for such patients. These challenges can be overcome by using nanoparticle- and lipid-based in vivo mRNA or protein delivery systems that may be fascinating substitutes for viral vectors.[70]

II. Editing Efficiency:

For a long time, delivery and editing efficacy have been the essence of genetic alteration applications, chiefly for cancer. Cancer gene therapy, for example, requires high editing proficiency which is far from being achievable using existing CRISPR-Cas9 technologies. This problem can be solved by the future development of more effective delivery vectors, more robust sgRNA and more dynamic Cas9. Adeno-associated virus (AAV) vectors are generally utilized gene delivery tools for clinical trials and gene therapy research for their efficiency and safety. But, the size of the frequently employed Cas9 gene from Streptococcus pyogenes is much bigger as compared to the accommodating capacity of wildtype AAV.[72] Regardless of the limitation of using AAV vectors with respect to the size of Cas9 transgenes, scientists have developed engineered cytomegalovirus (CMV) AAV vector constructs for efficient incorporation of Cas9 and sgRNA. For reducing the overall size, evaluation of minimal promoter sequences and termination signals has been done and shown to have no effect on editing frequencies.[71]

III. Avoidance of Off Target Mutations:

Off-target mutations offer a challenge to the genome editing application of the CRIPSR/Cas9 system.[31] Genetic alterations are perpetual and detrimental off-target mutations can give rise to cancerous cells and cells with functional impairment or decreased fitness. Moreover, oncogenic mutations caused by off-target editing may result in the proliferation of edited cells, and so even low levels of off-target mutagenesis can have deleterious outcomes.[70] For the reduction of off-target effects, various methods have been developed. Firstly, the guide RNA's structure and composition can influence the rate of off-target effects. So, a practical approach for minimizing off-target effects demands selection of a target site having no homologous sequence in the genome.[72] Secondly, transforming Cas9 into a single-strand DNA nickase which predominantly produces DSBs by generating two distinct single-strand breaks on complementary DNA strands, through the expression of two discrete guide RNAs, decreases off-target mutagenesis at computationally estimated off target sites. In addition, targeting specificity can be improved by truncating the guide RNA or using an RNA-guided FokI nuclease created by fusing FokI nuclease domain and catalytically inactive Cas9.[70] For identification of potential off-target sites throughout the genome, Xiao *et al*. developed an adaptable searching tool called CasOT.[7] Hence, the advances in technology for therapeutic uses will require additional research into the specificity of CRISPR/Cas9 system along with probable optimization strategies, for example analyses using pairs of Cas9 nickases.[71]

IV. Reliance on PAM sequence:

In theory, application of CRISPR/Cas9 system can be done to any DNA sequence via engineered programmable gRNA. The CRISPR/Cas9 specificity entails gRNA/target sequence complementarity along with a PAM sequence (2~5 nt) located directly downstream of the target sequence. Different Cas9 orthologs have variable identified PAM sequences, such as NGG PAM from Streptococcus pyogenes, NNNNGATT PAM from Neisseria meningitidis and NGGNG and NNAGAAW PAM from Streptococcus thermophiles. Hsu *et al.* recently reported a NAG PAM that was shown to have only around 20% efficiency of NGG PAM for directing DNA cleavage.[7]

CONSEQUENCES AND RISKS DELIBERATION

Understanding the risks and consequences of a new technology is important for evaluating how it should be governed and what limitations might be appropriate. The risks raised by this technology can generally be divided into risks and consequences related to research (which includes both laboratory work and field trials) and those related to the deployment of the technology into wild populations.[41]

I. Off-and On-Target Effects:

One unsettled issue associated with this technology is the rate of off-target effects that can be potentially toxic to cells or lead to unwanted genomic rearrangements such as deletions and translocations.[41] Nevertheless, it is imperative to pay special attention to off-target mutations before the clinical applications due to the effect of these unwanted genetic changes on the health of individuals enrolled in a clinical trial; thus, causing potential depreciation of this biotechnology in the society.[73]

II. Misuse of Technology:

Given the far-reaching distribution, accelerated pace of development and low cost of this dual-use technology, its unintentional abuse may cause widespread national security and economic

implications. Although CRISPR is intended to improve the human gene pool, the misapplication of the technology has the potential to harm society not only through laboratory accidents, but also through biological weaponization.[74]

III. **Fortuitous Release:**

The affordability and effectiveness of CRISPR has also opened the door for use by non-institutionalized researchers, such as DIY biologists or citizen scientists. This raises questions about proper biosafety and containment measures for research not conducted in institutionalized laboratories. However, some have pointed out that the DIY community is very proactive in terms of codes of conduct and safety. A number of federal programs have worked with these communities to raise awareness about potential misuses of the technology as well as to educate users about broader risk assessments for their work.[41]

IV. **Effect on Ecosystem:**

Both accidental release and the purposeful deployment of the technology into wild populations could have unintended consequences for the environment. It will be important to understand the impacts on biodiversity and ecosystem balance that reducing, altering, or eliminating a plant or animal species may have. An additional consideration is whether the elimination of one species might lead to another (potentially more harmful) species taking its place. To date, most risk management, containment strategies, and ecological consequence studies have largely focused on mosquito populations. While these will provide a good starting point, considerable new research will be required to understand the full breadth of impacts for other organisms and their ecosystems.[41]

CONCLUSION

From simple Mendelian disorders to complex multifactorial diseases, CRISPR/Cas9 systems will undoubtedly revolutionize the study and treatment of both immunologic and allergic diseases. The current possibilities for building upon the indigenous design, gene modulation and multiplex epigenetic modifications have unravelled new research and therapeutic prospects to make far-reaching changes in the field. Nevertheless, the present knowledge of the CRISPR/Cas9 technology at crystal structural and biochemical levels is inadequate and necessities further investigation, including a deep probing into the Cas9 protein, one of the pivotal components of the CRISPR/Cas9 system. It is a need of the hour that government and associated social organizations should formulate and authorize such laws and regulations that permit the safe and ethical use of this emerging technology for basic research and clinical purposes

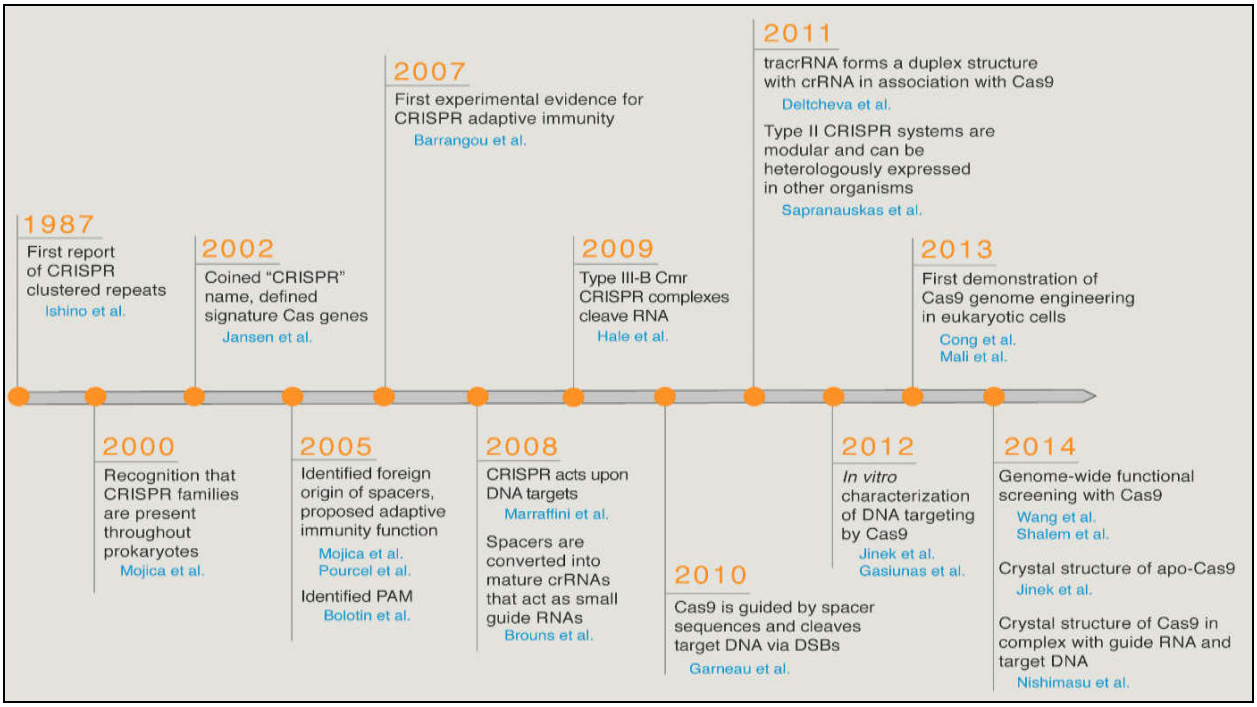

Figure-1: CRISPR Timeline: a concise history, from initial discovery to a radical genome editing tool. [Adapted from: Hsu, P., Lander, E., & Zhang, F. (2014). Development and Applications of CRISPR-Cas9 for Genome Engineering. Cell, 157(6), 1262–1278]

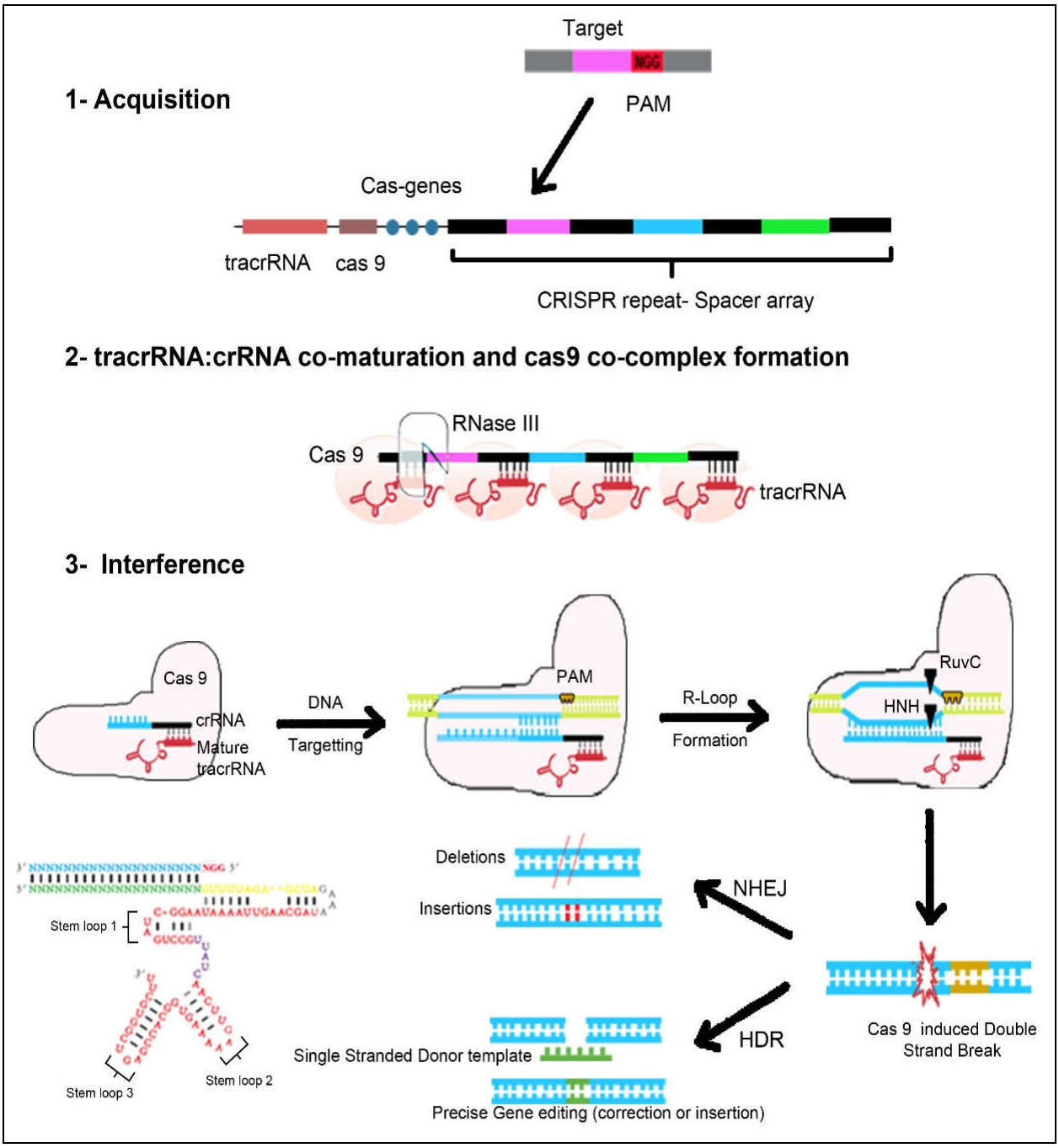

Figure-2: Mechanism of type II-A CRISPR-Cas System

The first step involves the recognition and integration of foreign DNA at leader side of the CRISPR locus as spacer DNA. Second step involves duplex formation (Cas9 with antirepeat-repeat RNA; tracrRNA) followed by RNA processing by RNase III enzyme resulting in further trimming, R-loop formation and cleavage of target DNA. In the presence of the sgRNA complimentary sequence and PAM sequence, a double stranded break (DSB) is generated which triggers the endogenous repair machinery of cellular DNA and results in the catalysis of Non-homologous end joining (NHEJ) and homology directed repair (HDR). **B)** Representation of the overall structure of sgRNA: target DNA complex.

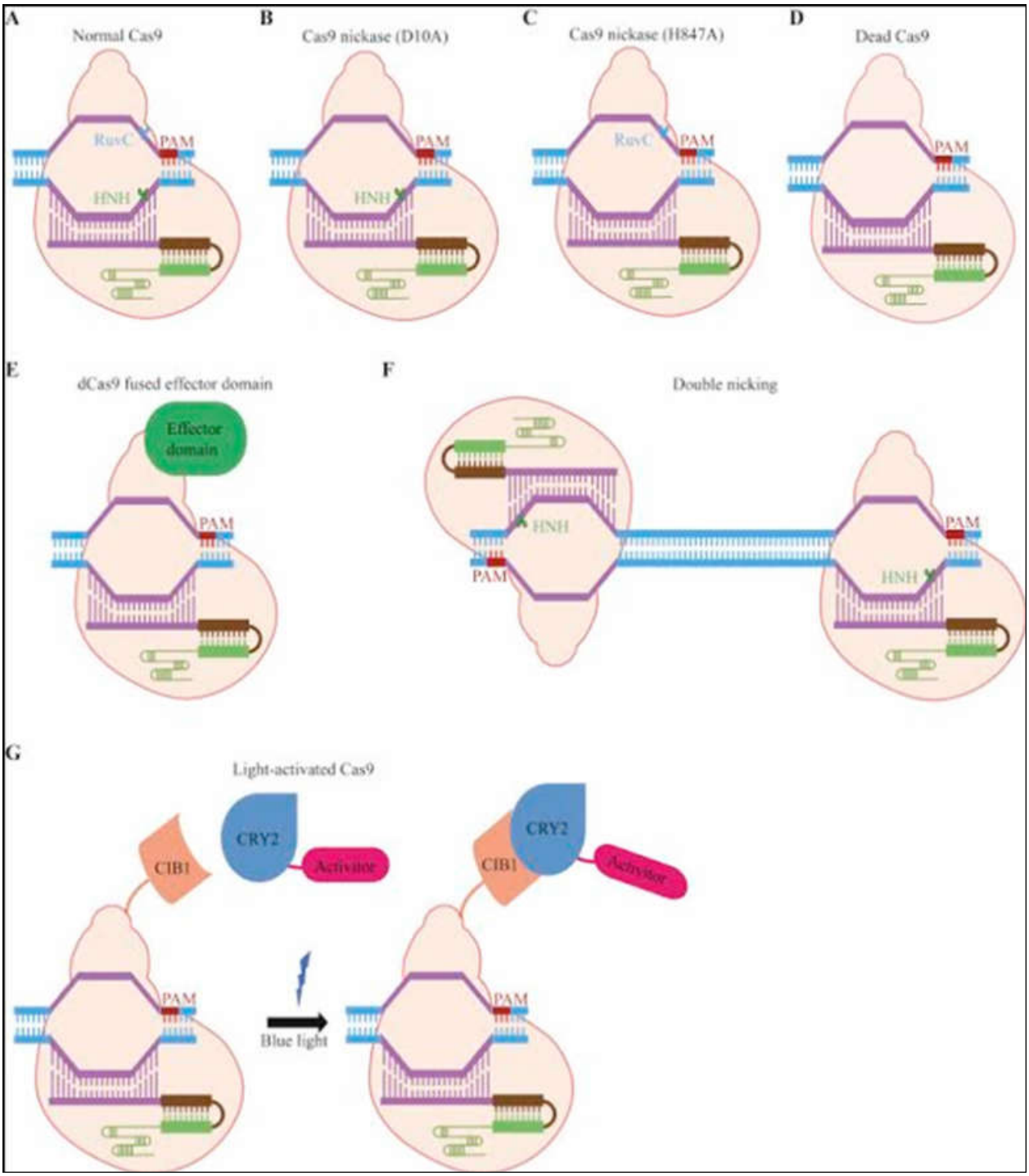

Figure 3- Modifications of Cas9 on the basis of its functional domains

For precise genome editing, several versions of Cas9 have been engineered. (A) Normal Cas9 with RuvC and NHN domains; (B-C) Cas9 with inactivating mutations in the RuvC and NHN domains, respectively; (D) Nuclease-null deactivated dCas9 created by inactivating nuclease activities of both the domains; (E) dCas9, with a fused effector domain, can stimulate or repress gene expression, respectively; (F) double-nicking with Cas9n and two distinct sgRNAs for a break in each DNA strand; and (F) Light activated Cas9. [Adapted from: Mei, Y., Wang, Y., Chen, H., Sun, Z. S., & amp; Ju, X. D. Recent progress in CRISPR/Cas9 technology. Journal of Genetics and Genomics, 43 (2), 63–75]

REFERENCES

1. Marraffini LA, Sontheimer EJ. CRISPR interference: RNA-directed adaptive immunity in bacteria and archaea. Nat Rev Genet 2010;11(3):181–90.
2. Brouns SJ, Jore MM, Lundgren M, Westra ER, Slijkhuis RJ, Snijders AP, *et al*. Small CRISPR RNAs Guide Antiviral Defense in Prokaryotes. Science 2008;321(5891):960–4.
3. Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science 2012;337(6096):816–21.
4. Mali P, Esvelt KM, Church GM. Cas9 as a versatile tool for engineering biology. Nat Methods 2013;10(10):957–63.
5. Makarova KS, Aravind L, Wolf YI, Koonin EV. Unification of Cas protein families and a simple scenario for the origin and evolution of CRISPR-Cas systems. Biol Direct 2011;6(1):38.
6. Jiang W, Bikard D, Cox D, Zhang F, Marraffini LA. RNA-guided editing of bacterial genomes using CRISPR-Cas systems. Nat Biotechnol 2013;31(3):233–9.
7. Zhang F, Wen Y, Guo X. CRISPR/Cas9 for genome editing: progress, implications and challenges. Hum Mol Genet 2014;23(R1):R40–6.
8. Hsu PD, Lander ES, Zhang F. Development and Applications of CRISPR-Cas9 for Genome Engineering. Cell 2014;157(6):1262–78.
9. Broad Institute prevails in heated dispute over CRISPR patents [Internet]. STAT. 2017 [cited 2017 Mar 2]. Available from: https://www.statnews.com/2017/02/15/crispr-patent-ruling/
10. European Patent Office to grant UC a broad patent on CRISPR-Cas9. Berkeley News. [Internet]. [cited 2017 Mar 30]. Available from: http://news.berkeley.edu/2017/03/28/european-patent-office-to-grant-uc-a-broad-patent-on-crispr-cas9/
11. Barrangou R, Fremaux C, Deveau H, Richards M, Boyaval P, Moineau S, *et al*. CRISPR Provides Acquired Resistance Against Viruses in Prokaryotes. Science 2007;315(5819):1709–12.
12. Van der Oost J, Westra E, Jackson RN, Wiedenheft B. Unravelling the structural and mechanistic basis of CRISPR–Cas systems. Nat Rev Microbiol 2014;12(7):479–92.
13. Hsu PD, Scott DA, Weinstein JA, Ran FA, Konermann S, Agarwala V, *et al*. DNA targeting specificity of RNA-guided Cas9 nucleases. Nat Biotechnol 2013;31(9):827–32.
14. Cong L, Ran FA, Cox D, Lin S, Barretto R, Habib N, *et al*. Multiplex genome engineering using CRISPR/Cas systems. Science 2013;339(6121):819–23.
15. Gasiunas G, Barrangou R, Horvath P, Siksnys V. Cas9-crRNA ribonucleoprotein complex mediates specific DNA cleavage for adaptive immunity in bacteria. Proc Natl Acad Sci U S A 2012;109(39):E2579–86.
16. Nishimasu H, Ran FA, Hsu PD, Konermann S, Shehata SI, Dohmae N, *et al*. Crystal structure of Cas9 in complex with guide RNA and target DNA. Cell 2014;156:935–49.
17. Davis L, Maizels N. Homology-directed repair of DNA nicks via pathways distinct from canonical double-strand break repair. Proc Natl Acad Sci 2014;111(10):E924–32.
18. Sander JD, Joung JK. CRISPR–Cas systems for editing, regulating and targeting genomes. Nat Biotechnol 2014;32(4):347–55.
19. Mladenov E, Iliakis G. Induction and repair of DNA double strand breaks: the increasing spectrum of non-homologous end joining pathways. Mutat Res 2011;711(1-2):61–72.
20. Doudna JA, Charpentier E. The new frontier of genome engineering with CRISPR–Cas9. Science 2014;346(6213):1258096.
21. Jinek M, Jiang F, Taylor D, Sternberg S, Kaya E, Ma E. *et al*. Structures of Cas9 Endonucleases Reveal RNA-Mediated Conformational Activation. Science 2014;343(6176):1247997.
22. Anders C, Niewoehner O, Duerst A, Jinek M. Structural basis of PAM-dependent target DNA recognition by the Cas9 endonuclease. Nature 2014;513(7519):569–73.
23. Szczelkun M, Tikhomirova M, Sinkunas T, Gasiunas G, Karvelis T, Pschera P, *et al*. Direct observation of R-loop formation by single RNA-guided Cas9 and Cascade effector complexes. Proc Natl Acad Sci U S A 2014;111(27):9798–803.
24. Ledford H. Alternative CRISPR system could improve genome editing. Nature 2015;526(7571):17.
25. Lin Y, Cradick T, Brown M, Deshmukh H, Ranjan P, Sarode N, *et al*. CRISPR/Cas9 systems have off-target activity with insertions or deletions between target DNA and guide RNA sequences. Nucleic Acids Res 2014;42(11):7473–85.
26. Ran FA, Cong L, Yan WX, Scott DA, Gootenberg JS, Kriz AJ, *et al*. In vivo genome editing using Staphylococcus aureus Cas9. Nature 2015;520(7546):186–91.
27. Mali P, Aach J, Stranges PB, Esvelt KM, Moosburner M, Kosuri S, *et al*. Cas9 transcriptional activators for target specificity screening and paired nickases for cooperative genome engineering. Nat Biotechnol 2013;31(9):833–8.
28. Fu Y, Sander JD, Reyon D, Cascio VM, Joung JK. Improving CRISPR-Cas nuclease specificity using truncated guide RNAs. Nat Biotechnol 2014;32(3):279–84.
29. Pattanayak V, Lin S, Guilinger JP, Ma E, Doudna JA, Liu DR. High-throughput profiling of off-target DNA cleavage reveals RNA-programmed Cas9 nuclease specificity. Nat Biotechnol 2013;31(9):839–43.
30. Tsai SQ, Wyvekens N, Khayter C, Foden JA, Thapar V, Reyon D, *et al*. Dimeric CRISPR RNA-guided FokI nucleases for highly specific genome editing. Nat Biotechnol 2014;32(6):569–76.
31. Mei Y, Wang Y, Chen H, Sun ZS, Ju XD. Recent progress in CRISPR/Cas9 technology. J Genet Genomics 2016;43(2):63–75.
32. Bondy-Denomy J, Pawluk A, Maxwell KL, Davidson AR. Bacteriophage genes that inactivate the CRISPR/Cas bacterial immune system. Nature 2013;493(7432):429–32.
33. Pawluk A, Bondy-Denomy J, Cheung VH, Maxwell KL, Davidson A. A New Group of Phage Anti-CRISPR Genes Inhibits the Type I-E CRISPR-Cas System of Pseudomonas aeruginosa. Mbio 2014;5(2):e00896.
34. Bondy-Denomy J, Garcia B, Strum S, Du M, Rollins MF, Hidalog-Reyes Y, *et al*. Multiple mechanisms for CRISPR–Cas inhibition by anti-CRISPR proteins. Nature 2015;526(7571):136–9.
35. Wang J, Ma J, Cheng Z, Meng X, You L, Wang M, Zhang X, *et al*. A CRISPR evolutionary arms race: structural insights into viral anti-CRISPR/Cas responses. Cell Res 2016;26(10):1165–8.
36. Deveau H, Barrangou R, Garneau JE, Labonte J, Fremaux C, Boyaval P, *et al*. Phage response to CRISPR-encoded resistance in Streptococcus thermophilus. J Bacteriol 2008;190(4):1390–1400.
37. Sapranauskas R, Gasiunas G, Fremaux C, Barrangou R, Horvath P, Siksnys V. The Streptococcus thermophilus CRISPR/Cas system provides immunity in Escherichia coli. Nucleic Acids Res 2011;39(21):9275–82.
38. Semenova E, Jore MM, Datsenko KA, Semenova A, Westra ER, Wanner B, *et al*. Interference by clustered regularly interspaced short palindromic repeat (CRISPR) RNA is governed by a seed sequence. Proc Natl Acad Sci U S A 2011;108(25):10098–103.
39. Datsenko KA, Pougach K, Tikhonov A, Wanner BL, Severinov K, Semenova E. Molecular memory of prior infections activates the CRISPR/Cas adaptive bacterial immunity system. Nat Commun 2012;3:945.
40. Richter C, Chang JT, Fineran PC. Function and regulation of clustered regularly interspaced short palindromic repeats

(CRISPR) / CRISPR Associated (Cas) systems. Viruses 2012;4(10):2291–311.
41. Beets R. Governing CRISPR: Evaluating Ethics, Risk, and Regulation in Gene Drive Research 2016. [Internet]. [cited 2017 Mar 17]. Available from: http://www.beccabeets.com/wp-content/uploads/Rebecca-Beets-Plan-B-Final.pdf
42. Pellagatti A, Dolatshad H, Valletta S, Boultwood J. Application of CRISPR/Cas9 genome editing to the study and treatment of disease. Arch Toxicol 2015;89(7):1023–34.
43. Seruggia D, Montoliu L. The new CRISPR–Cas system: RNA-guided genome engineering to efficiently produce any desired genetic alteration in animals. Transgenic Res 2014;23(5):707–16.
44. Nekrasov V, Staskawicz B, Weigel D, Jones JD, Kamoun S. Targeted mutagenesis in the model plant Nicotiana benthamiana using Cas9 RNA-guided endonuclease. Nat Biotechnol 2013;31(8):691–3.
45. Shan Q, Wang Y, Li J, Zhang Y, Chen K, Liang Z, *et al*. Targeted genome modification of crop plants using a CRISPR-Cas system. Nat Biotechnol 2013;31(8):686–8.
46. Zhang H, Zhang J, Wei P, Zhang B, Gou F, Feng Z, *et al*. The CRISPR/Cas9 system produces specific and homozygous targeted gene editing in rice in one generation. Plant Biotechnol J 2014;12(6):797–807.
47. Wang Y, Cheng X, Shan Q, Zhang Y, Liu J, Gao C, *et al*. Simultaneous editing of three homoeoalleles in hexaploid bread wheat confers heritable resistance to powdery mildew. Nat Biotechnol 2014;32(9):947–51.
48. Jiang W, Zhou H, Bi H, Fromm M, Yang B, Weeks DP. Demonstration of CRISPR/Cas9/sgRNA-mediated targeted gene modification in Arabidopsis, tobacco, sorghum and rice. Nucleic Acids Res 2013;41(20):e188.
49. Ali Z, Abul-faraj A, Li L, Ghosh N, Piatek M, Mahjoub A, *et al*. Efficient Virus-Mediated Genome Editing in Plants Using the CRISPR/Cas9 System. Mol Plant 2015;8(8):1288–91.
50. Ali Z, Abulfaraj A, Idris A, Ali S, Tashkandi M, Mahfouz MM. CRISPR/Cas9 mediated viral interference in plants. Genome Biol 2015;16(1):238.
51. Chandrasekaran J, Brumin M, Wolf D, Leibman D, Klap C, Pearlsman M. *et al*. Development of broad virus resistance in non-transgenic cucumber using CRISPR/Cas9 technology. Mol Plant Pathol 2016;17(7):1140–53.
52. Borel B. CRISPR, microbes and more are joining the war against crop killers. Nature 2017;543(7645):302–4.
53. Samanta MK, Dey A, Gayen S. CRISPR/Cas9: an advanced tool for editing plant genomes. Transgenic Res 2016;25(5):561–73.
54. Feng Z, Mao Y, Xu N, Zhang B, Wei P, Yang DL, *et al*. Multigeneration analysis reveals the inheritance, specificity, and patterns of CRISPR/Cas-induced gene modifications in Arabidopsis. Proc Natl Acad Sci U S A 2014;111(12):4632–7.
55. Xie K, Yang Y. RNA-guided genome editing in plants using a CRISPR–Cas system. Mol Plant 2013;6(6):1975–83.
56. Zhou H, Liu B, Weeks DP, Spalding MH, Yang B. Large chromosomal deletions and heritable small genetic changes induced by CRISPR/Cas9 in rice. Nucleic Acids Res 2014;42(17):10903–14.
57. Ron M, Kajala K, Pauluzzi G, Wang D, Reynoso MA, Zumstein K, *et al*. Hairy root transformation using Agrobacterium rhizogenes as a tool for exploring cell type-specific gene expression and function using tomato as a model. Plant Physiol 2014;166(2):455–69.
58. Brooks C, Nekrasov V, Lippman ZB, Van Eck J. Efficient gene editing in tomato in the first generation using the clustered regularly interspaced short palindromic repeats/CRISPR-associated9 system. Plant Physiol 2014;166(3):1292–7.
59. Gao Y, Zhao Y. Specific and heritable gene editing in Arabidopsis. Proc Natl Acad Sci U S A 2014;111(12):4357–8.
60. Ueta R, Abe C, Watanabe T, Sugano SS, Ishihara R, Ezura H, *et al*. Rapid breeding of parthenocarpic tomato plants using CRISPR/Cas9. Sci Rep 2017;7(1):507.
61. Jacobs TB, LaFayette RJ, Schmitz RJ, Parrott WA. Targeted genome modifications in soybean with CRISPR/Cas9. BMC Biotechnol 2015;15(1):16.
62. Goodin MM, Zaitlin D, Naidu RA, Lommel SA. Nicotiana benthamiana: its history and future as a model for plant–pathogen interactions. Mol Plant Microbe Interact 2008;21(8):1015–26.
63. Voytas DF. Plant genome engineering with sequence-specific nucleases. Annu Rev Plant Biol 2013;64:327–50.
64. Sugano SS, Shirakawa M, Takagi J, Matsuda T, Shimada T, Hara-Nishimura I, *et al*. CRISPR/Cas9 mediated targeted mutagenesis in the liverwort Marchantia polymorpha L. Plant Cell Physiol 2014;55(3):475–81.
65. Jiang W, Yang B, Weeks DP. Efficient CRISPR/Cas9-mediated gene editing in Arabidopsis thaliana and inheritance of modified genes in the T2 and T3 generations. PLoS One 2014;9(6):e99225.
66. Heintze J, Luft C, Ketteler R. A CRISPR CASe for high-throughput silencing. Front Genet 2013;4:193.
67. Bortesi L, Fischer R. The CRISPR/Cas9 system for plant genome editing and beyond. Biotechnol Adv 2015;33(1):41–52.
68. Ramalingam S, Annaluru N, Chandrasegaran S. A CRISPR way to engineer the human genome. Genome Biol 2013;14(2):107.
69. Barrangou R, Birmingham A, Wiemann S, Beijersbergen RL, Hornung V, Smith Av. Advances in CRISPR-Cas9 genome engineering: lessons learned from RNA interference. Nucleic Acids Res 2015;43(7):3407–19.
70. Cox DB, Platt RJ, Zhang F. Therapeutic genome editing: prospects and challenges. Nat Med 2015;21(2):121–31.
71. LaFountaine JS, Fathe K, Smyth HD. Delivery and therapeutic applications of gene editing technologies ZFNs, TALENs, and CRISPR/Cas9. Int J Pharm 2015;494(1):180–94.
72. Xiao-Jie L, Hui-Ying X, Zun-Ping K, Jin-Lian C, Li-Juan J. CRISPR-Cas9: a new and promising player in gene therapy. J Med Genet 2015;52(5):289–96.
73. Araki M, Ishii T. Providing appropriate risk information on genome editing for patients. Trends Biotechnol 2016;34(2):86–90.
74. Yoon J. The Imperative Need to Consider the Bioethics of CRISPR-Cas9 Technology. NHSJS. [Internet]. 2016. [cited 2017 Mar 30]. Available from: http://nhsjs.com/2016/the-imperative-need-to-consider-the-bioethics-of-crispr-cas9-technology/

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