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Joginder Pal
Ph.D. Scholar, Department of Plant Pathology, Dr. Y.S. Parmar University of Horticulture and Forestry Nauni, Solan, Himachal Pradesh, India

Sunita Chandel
Department of Plant Pathology, Dr. Y.S. Parmar University of Horticulture and Forestry Nauni, Solan, Himachal Pradesh, India

Ranjna Sharma
Department of Plant Pathology, Dr. Y.S. Parmar University of Horticulture and Forestry Nauni, Solan, Himachal Pradesh, India

Correspondence
Joginder Pal
Ph.D. Scholar, Department of Plant Pathology, Dr. Y.S. Parmar University of Horticulture and Forestry Nauni, Solan, Himachal Pradesh, India

CRISPR/Cas9 technology and its potential role in plant disease resistance: A review

Joginder Pal, Sunita Chandel and Ranjna Sharma

Abstract
The targeted genome editing that targets genomic sequences in a site specific manner has recently emerged as a most effective biotechnological weapon for boosting resistance in plants against widespread phytopathogens. Among various gene editing techniques, gene targeting notably by CRISPR-Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats) has abundantly aroused consequential excitement among agricultural scientists since its discovery in 2013. It is an inexpensive, easy, efficent and rapidly flourshing technique that occurs in nature as a prokaryotic immune system and confers resistance to foreign invading genetic elements such as plasmids and bacterial viruses by intruppting compatible host pathogen interaction. The most important advantage of this technique over other gene editing methods is that it enables precise genomic modifications with ease and more effective manner, reducing off targest effects and can also be capable of editing multiple genome site simultaneously. The CRISPR/Cas9 technique offers the opportunities to rewrite the effector-target sequence for avoiding effector-target molecular communication and also to modify effector-target promoters for increasing the expression of target genes and thereby engaged in the resistance process. Besides its widespread role in virus and bacterial disease resistance, recently its potentiality in fungal disease management has also been reported. The technique displayed plant resistance tremendously by acting upon the immunity components and proved as an excellent and indispensable approach for sustainable agriculture. The CRISPR/Cas9 is rapidly evolving technique and its application is constantly expanding year by year. In this review, we have summarized the CRISPR/Cas9 system and its role in developing plant resistance.

Keywords: CRISPR-Cas9, genome modifications, disease resistance, sustainable agriculture

Introduction
During the last few decades, the CRISPR-Cas based systems have opened the new era of molecular biology and become the most promising tool among agricultural scientists for editing and modifying genome in widespread organisms especially plants (Belhaj *et al*. 2015) [4]. In general, the CRISPR/Cas9 technology is evolved from prokaryotic organisms (a type II bacterial immune system) that acts on an adaptive immune system and thereby protect these organisms from invading DNA viruses and/or plasmids. Moreover, it represents a new array of targeted genome editing technology globally which can be applied extensively to nearly all organisms in a sustainable manner, including the potential for reduced pesticide usage. In CRISPR/Cas9 technique, site-specific modification is achieved by a single guide RNA (usually about 20 nucleotides) that is complementary to a target gene or locus and is anchored by a protospacer adjacent motif. Cas9 nuclease then cleaves the targeted DNA to generate double-strand breaks (DSBs), which are subsequently repaired by non-homologous end joining (NHEJ) or homology-directed repair (HDR) mechanisms. When combined with double or multiplex guide RNA design, NHEJ may also introduce targeted chromosome deletions, whereas HDR can be engineered for target gene correction, gene replacement, and gene knock-in (Song *et al*. 2016) [26].

Remarkably, CRISPR/Cas DNA editing system is able to achieve efficient gene editing in plants through either transient experiment or transgenic plants. In many cases, Cas9 and gRNAs are introduced inside plant cells by *Agrobacterium*-mediated T-DNA transformation or physical means, such as PEG-mediated transformation of protoplast or biolistic transformation of callus. In CRISPR technology, the Cas proteins, such as Cas9, are RNA-directed endonucleases which are able to recognize and cleave nucleic acids on the basis of sequence complementarities (Brouns *et al*. 2008) [5]. Cas9 can be targeted to specific DNA genomic sequences by engineering separately an encoded small guide RNA (sgRNA) with which it forms a complex. Thus, only a short RNA sequence must therefore be synthesized to confer recognition of a new target.

Historical background

S. No.	Year	Contribution
1	1987	First record of CRISPR cluster repeats was reported in *Escherichia coli* (Ishino *et al*. 1987) [12]
2	2000	Recognition and approval that CRISPR families are present throughout prokaryotic organisms (Mojica *et al*. 2000) [20]
3	2002	Description of term CRISPR along with defined signature cas genes was coined (Jansen *et al*. 2002) [13]
5	2007	The CRISPR-cas complex was successfully demonstrated and it was the first experimental evidence for CRISPR adaptive immunity (Barrangou *et al*. 2007) [3]
6	2008	CRISPR system can act upon specified DNA targets; Spacers are converted into mature crRNAs which acts as a small guide RNAs (Brouns *et al*. 2008) [5]
8	2010	Cas9 is directed by spacer sequences and cleaves target DNA via Double strands breaks (Garneau *et al*. 2010) [10]
9	2011	Tracer RNA designs a twofold structure with cr RNA in association with cas9:Type II CRISPR systems are transposable and can be heterologously expressed in different organisms (Deltcheva *et al*. 2011) [9]
10	2012	*In vitro* characterization of DNA targeting by cas9 (Jinek *et al*. 2012) [16]
11	2013	First demonstration of cas9 genome engineering in prokaryotic cells (Cong *et al*. 2013) [18]
12	2014	Description of genome -wide functional screening with cas9: crystal structure of cas9 in complex with guide RNA can rewrite DNA efficiently (Wang *et al*. 2014) [15]
13	2015	CRISPR/Cas systems were successfully refined against numerous geminiviruses (Ali *et al*. 2015) [1]
15	2016	Development of resistance to a ssRNA plant virus through CRISPR/Cas 9 deal mutagenesis of host gene elF(iso)4E that codes a protein which is necessary for virus replication and multiplication of same virus (Pyott *et al*. 2016)
16	2016	Evolution of resistance against three ssRNA plant viruses through CRISPR/Cas 9 mediated mutagenesis of host gene elF4F that codes for a protein required for virus replication (Chandrasekaran *et al*. 2016) [6]

Mechanism of CRISPR/Cas9 based genome editing

CRISPR-Cas9 is a new gene editing technology that offers the potential for substantial improvement over other gene editing technologies which offers the most reliable and versatile platform to engineer plant genome in a seguence specific manner. Irrespective of its large applications in animal sciences, it has now become the method of choice for genome engineering in plants for multipurpose (Puchta and Fauser, 2013) [23]. The CRISPR/Cas technology is initially regulated by the bacterial type-II CRISPR/Cas adaptive immune system that is displayed by the hosts to cleave invading phage or plasmid DNA (Doudna and Charpentier, 2014) [27]. In general, CRISPR- Cas9 system just requires three components, i.e., a protospacer-containing CRISPR RNA (crRNA), a trans-activating crRNA (tracrRNA) and a Cas9 endonuclease for function. The Cas9 nuclease is supervised by the crRNA tracrRNA duplex to cleave any trespassing DNA carrying the same protospacer sequence which is escorted by a protospacer adjacent motif (PAM). The PAM is absolutely crucial for target recognition as it carries the platform of the crRNA tracrRNA- Cas9 complex and consequitive and successive base pairing between the crRNA and the protospacer (Sternberg *et al*. 2014) [27].

In CRISPR/Cas9 technology, the Cas9 and sgRNA are expressed and design or construct a complex that fuses on targeted DNA near the NGG (PAM) site. A double stranded break (DSB) is induced at a targeted site that can be repaired either by non-homologous end joining (NHEJ) or homology directed repair (HDR). The repair by NHEJ usually results in the insertion or deletion (indel) or frame shift mutation resulting gene knockout by disruption. If a donor DNA is provided with end homology, then this can be inserted at the targeted site to modify a gene by adding changes in nucleotides or by gene insertion (Cong *et al*. 2013) [18]. In type II of CRISPR, attacking viral DNA or plasmids is divided into smaller pieces and integrated in CRISPR locus.The particular loci are reproduced or transcribed and these processed transcripts produce crRNA. These crRNAs regulate effect or endonuclease to target alien DNA depending upon complementarity or matching of sequence.

Spectacularly, the sgRNA programmed Cas9 appeared more effective in targeted gene modifications rather than individual trRNA and crRNA.Till date, genome-editing protocols have ratify three different types of Cas9 nuclease.The first Cas9 type can cut DNA site-specifically and results in the stimulation of DSB repair event to occur.Cellular NHEJ (Non-Homologous End Joining) mechanism is used to repair DSBs (Sternberg *et al*. 2014) [27]. As a culmination, insertions/deletions (indels) takes place that obstruct the targeted loci immediately. Apart from this, if any similarity between donor template and target locus is witnessed, the DSB may be repaired by HDR pathway (homology directed repair) allowing exact substitute mutations to be prepared (Sternberg *et al*. 2014) [27].

CRISPR/Cas9 technology mediated resistance in plants

The CRISPR/Cas9 system is a newly developed tool that can furnish important role in developing resistance in different agricultural crops. The CRISPR mediated genome editing has been used as a tool for imparting resistance to viruses in plants composed of Cas9 endonuclease of *Streptococcus pyogenes* and a synthetic guide RNA (gRNA), which combines functions of CRISPR RNA (cRNA) and trans-activating cRNA (tracrRNA) to direct the Cas9 protein to the DNA target sequence leading the protospacer-associated motif (PAM) (NGG in the case of *S. pyogenes*). Because the specificity of the system is determined by the 20-nucleotide sequence of the gRNA, it allows for peculiar and adequate genome.

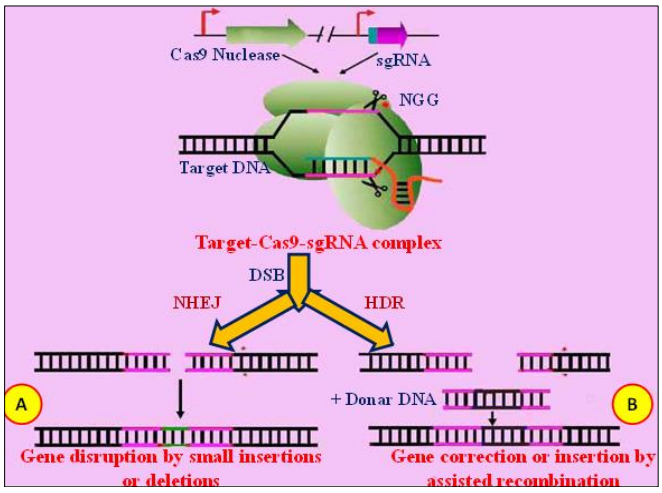

Fig 1: Schematic representation of Targeted genome editing via CRISPR-Cas9 system

The role of CRISPR/Cas9 system for developing resistance in plants can be described as:-

1. Antiviral Resistance in Plants Based on CRISPR-Cas Technology

The CRISPR/Cas system of genome editing has now been used efficiently as a tool for imparting resistance to viruses in several crop plants (Chaparro-Garcia *et al*. 2015) [7]. Very recently, three reports have been documented which described the CRISPR/Cas approach and its wide utility for protection to plants against geminiviruses (Ji *et al*. 2015) [14]. This technology admitted enhanced resistance to the plants against the geminiviruses species including BCTV (Beet curly top virus), TYLCV (Tomato yellow leaf curl virus), and MeMV (Merremia mosaic virus).

The CRISPR–Cas system initially recognize and targets the specific site of the genetic material of invading pathogens via; three stepwise processes, namely acquisition, expression, and interference. The acquisition step is the foremost or initial event which involves recognition and integration of foreign DNA as spacer at the leader side of the CRISPR locus, followed by duplication of the repeat. In general, in first step, short fragment of exogenous DNA are incorporated via CRISPR array in the bacterial genome, and acts as a new spacer sequence. In the expression step of the CRISPR–Cas system, the long pre-CRISPR RNA (pre-crRNA) first transcribed and is then actively processed into crRNAs with the help of specific Cas proteins as well as trans-activating crRNA (tracrRNA). During the third or final event as interference, a specific sequence of foreign genomic element is targeted and then cleaved into small fragments. The crRNA directs the Cas9 protein for cleavage to the complementary target region of the DNA of viruses and plasmids and therby leading induction of resistance in plants (Kumar and Jain, 2014) [17].

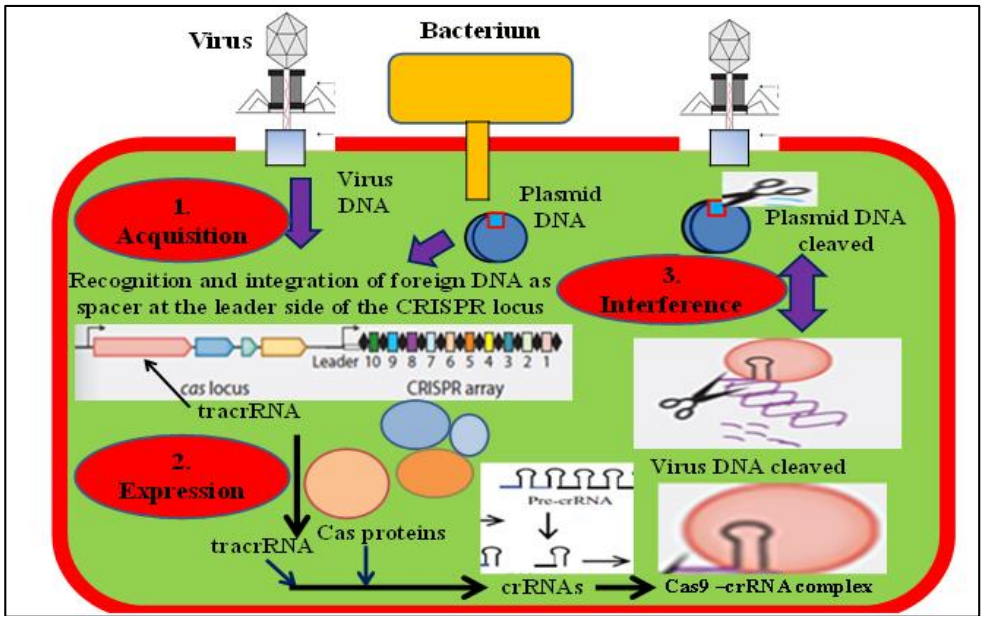

Fig 2: Diagrammatic representation of the bacterial CRISPR–Cas system, which provides protection against pathogen attack.

CRISPR/Cas9 based antiviral resistance in plants can be grouped into

a. Developing Plants Resistant to RNA Viruses

Earlier no single evidence of directly targeting and cleaving RNA viruses were found. But currantly CRISPR-Cas9 sgRNA system has now been used extensively to efficiently inactivate certain target genes and later these target regions of genome were mutated with the CRISPR/Cas9 system, in order to produce virus-resistant plants (Chandrasekaran *et al*. 2016) [6]. The only limitation of directly targeting RNA viral genomes is that the guide RNA-Cas9 system can only be used to target DNA viruses. But this could be change in the future, because Cas9 nuclease can be programmed and modify to cleave RNA and the type III-B CRISPR-Cas system mediates programmable cleavage of RNA sequences that are complementary to a guide RNA (Romay and Bragard, 2017. Currently, the usefulness of CRISPR/Cas9 technology for generating novel genetic resistance to the potyvirus TuMV was demonstrated greatly in *A. thaliana*, by deletion of a host factor, *eIF(iso)4E*, which is strictly required for virus growth and multiplication (Pyott *et al*. 2016). Development of virus resistance in cucumber (*Cucumis sativus* L.) was also explained by disrupting *eIF4E*, and developing non-transgenic heterozygous *eIF4E* mutant plants. These non-transgenic plants resulted partial resistance to an ipomovirus (*Cucumber vein yellowing virus*) and two important potyviruses (*Zucchini yellow mosaic virus* and *Papaya ring spot mosaic virus*- W) causing severe losses to cucrbits and papaya. Other examples of resistant to RNA viruses includes the targeting of two sites of eIF4 E gene in cucumber allowed developing plants resistant to infection by five positive strand RNA viruses (Chandrasekaran *et al*. 2016) [6]. The targeted genome sites were present with in the first and third exon sequences. Small indel and small nucleotide polymorphisms (SNPs) were also observed in the T1 generation. Homozygous T3 progeny with 20 and four deletions in the eIF4E gene were found immune to infection by cucumber vein yellowing virus (family *Potyviridae*, genus *Ipomovirus*), and resistant to papaya ring spot virus-W and zucchini yellow mosaic virus(family *Potyviridae*, genus *Potyvirus*). As expected, the plants remained susceptible to viruses that donot appear to highjack the host eIF4E to complete their cycle, such as cucumber mosaic virus (members of family *Bromoviridae*, genus *Cucumovirus*) or cucumber green mottle mosaic virus (family, *Virgaviridae*, genus *Tobamovirus*).

b. Developing Plants Resistant to DNA Geminiviruses

The geminiviruses are major group of single stranded DNA viruses and most of the previous research regarding CRISPR-mediated viral interference has been done on these ssDNA

geminiviruses. But very recently, the application of the CRISPR-Cas systems targeting geminiviruses has been reported to enhance resistance to tomato yellow leaf curlvirus (TYLCV, genus *Begomovirus*) and bean yellow dwarf virus (BeYDV, genus *Mastrevirus*) in *Nicotiana benthamiana* and to beet severe curly topvirus (BSCTV, genus *Curtovirus*) in *N. benthamiana* and in *Arabidopsis* (Ali *et al*. 2015) [1]. Ali *et al*. (2015) [1] has done excellent work in CRISPR/cas technology and engineered sgRNAs targeting coding and non-coding TYLCV sequences, including the conserved non-coding intergenic region (IR) of about 300 nt that can exhibit a stem- loop structure containing the origin of replication and promoter sequences for RNA polymerase II SgRNAs targeting the IR were demonstrated the most efficient inreducing TYLCV DNA titer that wholly or partially vanish the disease symptoms.The sgRNAs-Cas9 system was also found successful in simultaneously targeting of three geminiviruses TYLCV, beet curly top virus (genus *Curtovirus*) and merremia mosaic virus (genus *Begomovirus*) when sgRNAs specific for the IR sequence of each virus were used (Ali *et al*. 2015) [1]. SgRNA-Cas9 constructs intrrupted the virus DNA replication at different levels. Over-expressing sgRNA-Cas9 specifically targeting the viral DNA genome sequences resulted in virus-resistant plants. Disease symptoms were also attenuated to different levels, which ranged from severe to mild leaf curly symptoms). Ali *et al*. (2015) [1] demonstrateded that *N. benthamiana* plants expressing the CRISPR/Cas9 machinery mediated widespread resistance against TYLCV, *Beet curly top virus* (BCTV), and *Merremia mosaic virus* (MeMV).

Ali *et al*. (2015) [1] engineered sgRNAs targeting open reading frames encoding viral genes such as Rep, coat proteins and conserved noncoding intergenic region (IR) were targeted and it was reported that conserved intergenic region of gene was most effective target for minimizing the virus titre of tomato yellow leaf curl virus (TYLCV). Further, simultaneous resistance to tomato yellow leaf curl virus (TYLCV), beet curly top virus (BCTV) and merremia mosaic virus (MeMV) could be achieved with the use of sgRNA targeting conserved sequence from IR region. Cotton leaf curl disease caused by begomoviruses is one of the major and destructive diseases of cotton. For combating this, a multiplex CRISPR editing technique was developed as a broad spectrum method for control of leaf curl diseases of cotton (Iqbal *et al*. 2016). Baltes *et al*. (2015) showed that one sgRNA targeting the BeYDV genome could confer plant resistance without cleavage activity,which suggests that catalytically inactive Cas9 (dCas9) can be used to mediate virus interference, thereby eliminating concerns of off-target activities in the plant genome. Ali *et al*. (2016) [2] analysed that CRISPR/Cas system can be used to target and cleave *Cotton leaf curl Kokhran virus* (CLCuKoV) and they also recorded that this technology not targeting only the conserved nucleotide sequence but can can target multiple begomoviruses simultaneously and therefore conferring broad-spectrum gemini virus resistance activity. All of these studies showed that *N. benthamiana* plants expressing the CRISPR/Cas9 system displayed considerably reduced viral titers, which abolished or significantly reduced disease symptoms.

Ali *et al*. (2016) [2] systematically determined the ability of multiple geminiviruses geminiviruses to evade the CRISPR/Cas9 machinery by targeting coding and non-coding sequences.This study reveals that targeting coding sequences led to the generation of viral variants capable of evading the CRISPR/Cas9 machinery. Interestingly, targeting the non-coding intergenic sequences resulted high levels of virus interference, no detectable viral movements from the CRISPR/Cas9 machinery, and there by providing an effective strategy to confer potential durable resistance in plants against numerous DNA viruses.

Table 1: Examples of CRISPR/Cas technology developed to confers virus resistance in plants

CRISPR/Cas technology	Virus	Genus	Family	DNA targeted	References
	Bean yellow dwarf virus	*Mastrevirus*		Viral	Baltes *et al*. 2015
	Merremia mosaicvirus, Cotton leaf curl Kokhran virus, Tomato yellow leaf curl virus (TYLCV)	*Begomovirus*	*Geminiviridae*	Viral	Ali *et al*. 2015 [1], 2016
	Beet curly top virus (BCTV); *Beet severe curly topvirus* (BSCTV)	*Curtovirus*		Viral	Ali *et al*. 2015 [1]; Ji *et al*. 2015 [14]
	Cucumber vein yellowing virus	*Ipomovirus*	Potyviridie	Host	Chandrasekaran *et al*. 2016[6]
	Zucchini yellow mosaic virus, Papaya ring spot virus, Turnip mosaic virus	*Potyvirus*		Host	Chandrasekaran *et al*. 2016 [6]; Pyott *et al*. 2016

2. CRISPR/Cas confers resistance in plants against fungal and bacterial pathogens

Despite of large applications of CRISPR/Cas9 in managing plant virus disease, recently it has also proven to be extremely versatile tool for combating fungal and bacterial pathogens. Rice-pathogenic bacteria *Burkholderia glumae*, *Burkholderia gladioli*, and *Burkholderia plantarii*, which primarily cause grain rot, sheath rot, and seedling blight, respectively, can severely affects and reduce rice yield potential in all the major rice growing countries. For successful managaemnt of these diseases yet requires comprehensive studies and new tool to control these diseases only in the early stages. Although the genome of *B. plantarii* ATCC 43733T has many related features with those of *B. glumae* and *B. gladioli*, but this *B. plantarii* strain also has some distinctive features, including quorum sensing and CRISPR/CRISPR-associated protein (Cas) systems, signifying that *B. glumae* has evolved rapidly or has undergone rapid genome rearrangements or deletions in response to the host plants. Thus, this rice pathogenic Burkholderia species has unique features relative to other Burkholderia species of plants, animals and humans (Seo *et al*. 2015). The type II bacterial CRISPR/Cas9 system has been used to efficiently disrupt target genes in the smut causing maize pathogen *Ustilago maydis*. Two years ago TALEN and CRISPR/Cas9 technologies were used successfully to target the mildew-resistance locus O (*MLO*) in wheat, generating plants resistant to powdery mildew disease (Wang *et al*. 2014) [15]. GETs were used to generate plants resistant to bacterial leaf blight, caused by *Xanthomonas oryzae* pv. *oryzae*, impairing down the transcriptional regulation of S-genes by the effector and thereby leads to resistance process. Indeed, the plants firmly edited in the *OsSWEET14* promoter were resistant to bacterial strains since the effector was found incapable to activate the transcription of its target (Li *et al*.

2013). The metabolic pathways that regulate hormonal balance can be customized to enhance the IMC component of plant immunity.This goal was achieved by using GETs to cause the down-regulation of ethylene-responsive factors (*ERF*). In particular, the ethylene pathway in rice was successfully modified by targeting a mutation in OsERF922 using CRISPR/Cas9 technology to increase resistance to rice blast pathogen caused by *Magnaporthe oryzae* (Wang *et al*. 2016) [14].

The CRISPR/Cas9 also holds potential for generating mlo-type resistance in tomato against powdery mildew and other pathogens. *Pectobacterium atrosepticum*, a plant pathogen that causes soft-rot and blackleg disease in potato, has been used to explore protein-protein interactions and complex formation in the subtype I-F CRISPR/ Cas system including Cas1, Cas3, and the four subtype specific proteins Csy1, Csy2, Csy3 and Cas6f (Richter *et al*. 2012) [24]. The CRISPR/Cas9 system and a synthetic sgRNA targeting the CsPDS gene were delivered into sweet orange leaves via agroinfiltration facilitated by pretreatment with *Xanthomonas citri* subsp. *citri* (Xcc) DNA sequencing confirmed that the CsPDS gene was mutated at the target site with efficiency of approximately 3.2-3.9 % and without any off-target mutagenesis, suggesting targeted genome modification in citrus using the Cas9/sgRNA system-a system holds considerable promise for the study of citrus gene function and for targeted genetic modification (Jia and Wang, 2014) [15].

Advantages of CRISPR/Cas 9 technology

The ease, simplicity, a relatively high degree of precision and accessibility of the CRISPR/Cas9 as a new gene editing technology platform offers substantial improvement and advantages over other genome editing methods, on a genomic scale. The main advantages of genome engineering using the CRISPR-Cas9 system can be summarized as:

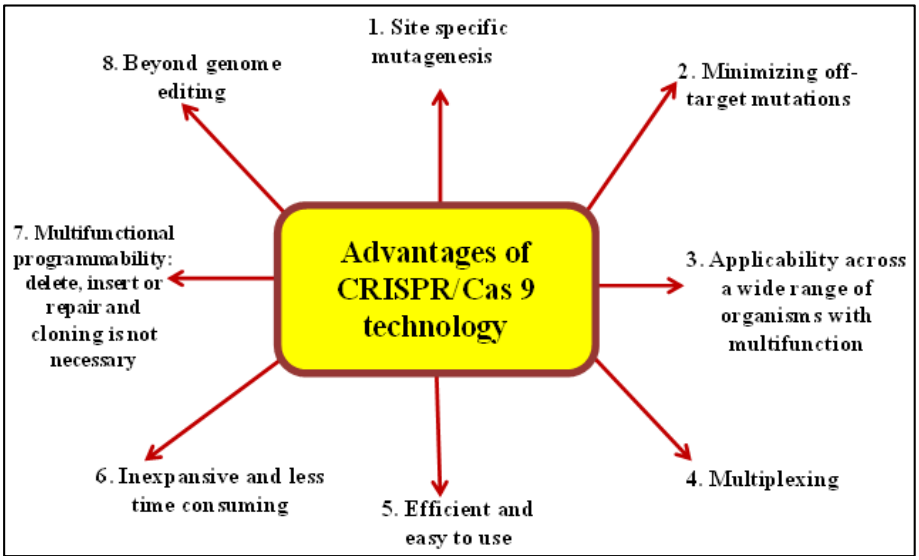

Fig 3: The main advantages of genome engineering using the CRISPR-Cas9 system can be summarized as

1. Site specific mutagenesis: The CRISPR/Cas 9 is a very versatile and comparatively precise approach to carry out strand-specific cleavage and modification of DNA at specific sites. In CRISPR, RNA-guided Cas9 nucleases use short RNAs to target and cleave DNA elements captured from foreign invaders (termed "spacers") in a sequence-specific manner (Horii and Hatada, 2014) [11]. To achieve site-specific DNA recognition and cleavage, Cas9 must be complexed with both a CRISPR RNA (crRNA) and a separate trans-acting crRNA (tracrRNA). The specificity is determined by a 20-nucleiotide (nt) sequence within the crRNA, which can be altered to match any desired sequence in the endogenous DNA. Site-specific Cas9 nuclease also allow targeted molecular trait stacking, i.e., the addition of several genes in close vicinity to an existing transgenic locus. Through CRISPR/Cas system it is now feasible to introduce multiple traits into crops with a low risk of segregation, which is difficult to achieve by classical breeding or even conventional genetic engineering.

2. Minimizing off-target mutations: To minimize off-target activity, a double nicking strategy can be employed to introduce DSBs at the target site of the genome. Cas9 nuclease is guided by a sgRNA to mediate a DSB at the target locus, e.g., the D10A mutant Cas9 nickase can be specified by a pair of appropriately spaced and oriented sgRNAs to simultaneously introduce single-stranded nicks on both strands of the target DNA. These DSBs from double nicking are then repaired via NHEJ and result in indel formation with similar levels of efficiency to that of WT Cas9. As single-stranded nicks are repaired without indel formation, DSBs would only occur if both sgRNAs are able to locate target sequences within a defined space. Thus, this strategy effectively doubles the number of bases that need to be specifically recognized at the target site and significantly increases the specificity of genome editing In general, low mutation rate in plants has been reported in CRISPR/Cas system as compared to previous gene editing technologies which simply indicated that this technology has less off-target effects (Li *et al*. 2013).

3. Applicability across a wide range of organisms with multifunction: CRISPR/Cas has already been discussed in many species with 33–92% success rate, nutritional values or stress and crop improvement in which genome engineering has been difficult. Irrespective of advantages of a highly efficient mutation rate, it requires only a simple system to design target specific sgRNA (Horii and Hatada, 2014) [11]. Precise genome modifications by CRISPR/Cas system excite the interest of scientists working in both basic science and applied fields, including gene therapy. Undoubtedly, targeted genome editing using artificial nucleases has the potential to

accelerate basic research as well as plant breeding by providing the means to modify genomes rapidly in a precise and predictable manner improving a wide variety of agronomic traits in economically important plants. Particularly, success in genome modification has been noticed among several animal and plant species that are difficult to be modified by other techniques Xing *et al*. 2014) [30]. Earlier, most of the studies have been conducted by using animal systems but during last few years, CRISPR-Cas9 mediated mutagenesis was performed successfully in arabidopsis, sorghum, tobacco, proving applicability of this technique to both dicot and monocot plants (Li *et al*. 2013).

4. Multiplexing: The main practical advantage of CRISPR/Cas is that it can simultaneously introduce multiple gene disruptions, which allows researchers to edit multiple genes in one plant line through a single transformation without time-consuming which is otherwise difficult to achieve through other gene editing technologies. Multiplex editing induced by CRISPR helps to create allelic variation at quantitative trait loci to modify multiple agronomic traits (quality, yield, disease resistance and abiotic stress tolerance). Through other gene editing technologies like ZFNs and TALENs it is very difficult to obtain multiplexed genes as they needs separate dimeric proteins specific for each target.The simultaneous introduction of DSBs at multiple sites can be used to edit several genes at the same time (Li *et al*. 2013) and can be particularly useful to knock out unnecessary genes effectively.

5. Efficient and easy to use: CRISPR/Cas9 technique is affordable, easy to use, and importantly it works for high throughput multi-gene experiments. CRISPR also give scientists a precise way to delete and edit specific bits of DNA-even by altering a single base pair. In CRISPR technology, Cas9 can be easily retargeted to new DNA sequences by simply purchasing a pair of oligos encoding the 20-nt guide sequence. Previous genome editing technologies ZFNs and TALEN requires separate dimeric proteins and construction of genes specific for each target which are on the other hand not essential in CRISPR technology. Moreover, Cas9 offers several potential advantages over conventional genome editing technologies, including the ease of customization, higher targeting efficiency and the ability to facilitate multiplex genome editing. Also in old gene editing techniques, cloning of gene is pre-requisitive step, which is not essentially required in CRISPR technology and may be therefore considered as best possible alternative to substitute conventional genome editing technology.

6. Inexpansive and less time consuming: Older genome editing tools, such as ZFNs and TALENs, are slow and expensive due to their use of proteins necessary for findng out the portion of DNA to be cut. Also these techniques are based on the protein-guided recognition mechanism, in which the targeting of a specific DNA sequence requires the modular assembly of pairs of recognition proteins units while the vector system is being constructed, which is time-consuming and tedious work. Due to expensive nature of ZFNs and TALENs, these have not been widely adopted by the plant research community until now. Any number of gRNAs can be produced by *in vitro* transcription using two complementary annealed oligonucleotides. Unlike its predecessors, the CRISPR/Cas9 system does not require any protein engineering steps, making it much more straight forward.This allows the inexpensive assembly of large gRNA libraries so that the CRISPR/Cas9 system can be used for high-throughput functional genomics applications, bringing genome editing within the budget of any molecular biology laboratory. These milestones confirmed that the CRISPR/Cas9 system was a simple, inexpensive and versatile tool for genome editing, which has rightly been become to known as the 'CRISPR craze' (Pennisi, 2013) [22].

7. Multifunctional programmability: delete, insert or repair and cloning is not necessary: The advent of CRISPR has now made it feasible to rewrite host DNA by introducing some major modifications. These modifications include gene replacement, deletions, inversion, knockouts, and translocations. Double-strand breaks induced by a nuclease at a specific site can be repaired either by non-homologous end joining (NHEJ) or homologous recombination (HR). Repair by NHEJ typically results in the insertion or deletion of accidental base pairs, causing gene knockout by disruption. If a donor DNA is available, which is simultaneously cut by the same nuclease leaving compatible overhangs, gene insertion by NHEJ can also be achieved. HR with a donor DNA template can be exploited to modify a gene by introducing precise nucleotide substitutions or to achieve gene insertion. The same strategy can also be used to engineer large genomic deletions or inversions by targeting two widely spaced cleavage sites on the same chromosome by targeting both endogenous genes and transgenes and by exploiting both NHEJ and HR to generate small deletions, targeted insertions and multiplex genome modifications (Li *et al.* 2013).

8. Beyond genome editing: Undoubtedly, the CRISPR/Cas 9 is currently emerging as the most ground-breaking technology for genome editing among scientific community worldwide. The application of CRISPR-Cas9 has now even made it possible to rewrite host DNA by introducing few major alterations in plant genome (Noman *et al*. 2016) [21]. Regardless of its prospective role in genome editing, the CRISPR/Cas has also found to contribute largely in various field e.g., the ectopic regulation of gene expression, which can provide useful information about gene functions and can also be used to engineer novel genetic regulatory circuits for synthetic biology applications. The accessibility of the CRISPR/Cas9 technology will assist both forward and reverse genetics and will boost basic research even in model species such as Arabidopsis, which already boast extensive (yet incomplete) mutant libraries. Most information concerning the properties of the CRISPR/Cas9 system is currently derived from studies in mammals, and although it assumess that many of the findings can be generalized it is still necessary to conduct similar studies in plants to ensure that system properties are translatable to different species

Box 1

Common terminologies used in CRISPR/Cas9 technology

CRISPR- Clustered Regularly Interspaced Short Palindromic Repeats
Cas9 -CRISPR associated protein 9
ZFN-Zinc Finger Nucleases
TALENs- transcription activator-like effector nucleases
PAM -Protospacer Adjacent Motif
crRNA- CRISPR RNA
tracrRNA- Trans acting crRNA
sgRNA- Single guide RNA
HR- Homologous recombination

HDR - Homology directed DNA repair
NHEJ - Non-homologous end-joining
DSBs- Double-strand DNA breaks
Indel - Insertion and/or deletion

Future aspects

The CRISPR/Cas9 technology presents innovative molecular scissors for engineering biology and has recently become one the most versatile and dominant platforms. One of the most important aspect of this technology is to allow precisely and predictable multiple genetic changes simultaneously. Although the CRISPR/Cas9 system is an excellent tool for genome editing, but the extent of off-target mutation needs to be explored with high cleavage efficiency among different but perfectly matched targets. However, improvement in this technique could be deployed in the upcoming future to combat deadly phytopathogenic diseases. In this direction research works by various agricultural scientists are in progress and hopefully, we will get the best possible results soon.

Concluding Remarks

The CRISPR/Cas system is an excellent promising tool for genome editing in plants due to its simplicity, efficiency, minimal off-target effects, and high specificity for targeted mutagenesis. It provides a novel platform for resistance activation through continous breeding in crops. Importantly this technique has now been evolved as principal technique that has made it possible to modify host genome by introducing some major modifications. These modifications include gene replacement, deletions, inversion, knockouts, and translocations. The most potential prospects of this technique for producing plants with mutations also contribute greatly to other disciplines of science, i.e., synthetic biology, biofuel production, disease resistance, abiotic stress tolerance, phytoremediation etc. Undoubtedly, CRISPR/Cas9 system can facilitate potent research on genome modifications in crop plants but improvements in sequence specificity and reduction of off-target effects must be corrected, aspects needed to be considered for the design of the gRNA-Cas9 complex. Through this technology recessive resistance could be exploited with the aid of the CRISPR/Cas9 system to create novel resistance alleles in crop plants to protect them against problematic viruses using host translation initiation factors. Another significant attribute of this CRISPR/cas9 system is that it is by far the most user friendly system among all the currently available genome editing techniques. With this powerful and innovative technique, resistance in plants could be advanced which thereby contribute for sustainable agriculture in the future for maximizing yield by combating abiotic and biotic stresses.

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