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CRISPR: Boon for agriculture

Suman Rawte and Ritu R Saxena

Abstract
Humans have been improving the yield and disease resistance of crop for hundreds of years through traditional agricultural methods. Targeted genome engineering also known as genome editing has emerged as an alternative to classical plant breeding and transgenic (GMO) methods to improve crop plants and ensure sustainable food production. The CRISPR/Cas system has emerged as a powerful tool to create targeted mutations in plants. CRISPR/Cas is a microbial adaptive immune system that uses RNA-guided nucleases to cleave foreign genetic elements. This technology can be used to investigate the function of a gene of interest or to correct gene mutations in cells via genome editing. The technique is extremely simple, economical and versatile in many applications with minor modifications. This simple, affordable, and elegant genetic scalpel is expected to be widely applied to enhance the agricultural performance of most crops in the near future.

Keywords: CRISPR, genome editing, plants

Introduction
Humans have been improving the yield and disease resistance of crop for hundreds of years through traditional agricultural methods. Gene editing, a type of genetic engineering in which DNA is added, deleted or replaced in a target genome, is revolutionizing plant breeding across the world. Many gene families that regulate key processes are highly redundant and spread across diverse chromosomal locations in plants. To understand gene function this necessitates the ability to simultaneously target and mutate distinct loci in a highly specific manner without affecting other genes (Peterson *et al*., 2016). Targeted genome engineering also known as genome editing has emerged as an alternative to classical plant breeding and transgenic (GMO) methods to improve crop plants and ensure sustainable food production (Belhaj *et al*., 2013) [2].

Traditionally the crop were being improved by conventional breeding method or mutational breeding method but both of these are tedious and lengthy, which are now getting constrain by declining the existing genetic variation of plants. Than in agricultural world the application of genetic engineering have involved, but the disadvantage of this technology is that it cannot be controlled where exactly the gene is to be inserted in the genome.

Targeted genome engineering has emerged as an alternative to classical plant breeding and transgenic (GMO) methods to improve crop plants and ensure sustainable food production (Belhaj *et al*., 2013) [2]. Genome editing with site-specific nucleases allows reverse genetics, genome engineering and targeted transgene integration experiments to be carried out in an efficient and precise manner. It involves the introduction of targeted DNA double-strand breaks (DSBs) using an engineered nuclease, stimulating cellular DNA repair mechanisms (Bortesi *et al*., 2015) [4].

The CRISPR/Cas system has emerged as a powerful tool to create targeted mutations in plants (Lozano and Cutler, 2014) [30]. CRISPR/Cas is a microbial adaptive immune system that uses RNA-guided nucleases to cleave foreign genetic elements. This technology can be used to investigate the function of a gene of interest or to correct gene mutations in cells via genome editing. The technique is extremely simple, economical and versatile in many applications with minor modifications (Song *et al*., 2016). CRISPR is an acronym for clustered regularly interspaced short palindromic repeats and Cas9 is a nuclease associated with CRISPRs.

Important Terms

Term	Defenition
Cas	CRISPR associated protein
crRNA	CRISPR RNA, used to guide effector endonucleases that target invading DNA bases on sequence complementarity
tracrRNA	trans-activating crRNA, required for crRNA maturation.
HNH	An endonuclease domain named for characteristic histidine and asparagine residues
RuvC	An endonuclease domain named for an *E. coli* protein involved in DNA repair
PAM	Protospacer Adjacent Motif; Necessary for Cas9 to bind target DNA; Must immediately follow the target sequence
gRNA	Guide RNA, a synthetic fusion of the crRNA and tracrRNA; Provides both targeting specificity and binding ability for cas9 nuclease; does not exist in nature; also referred as "single guide RNA"

(Reis *et al*., 2014)

History

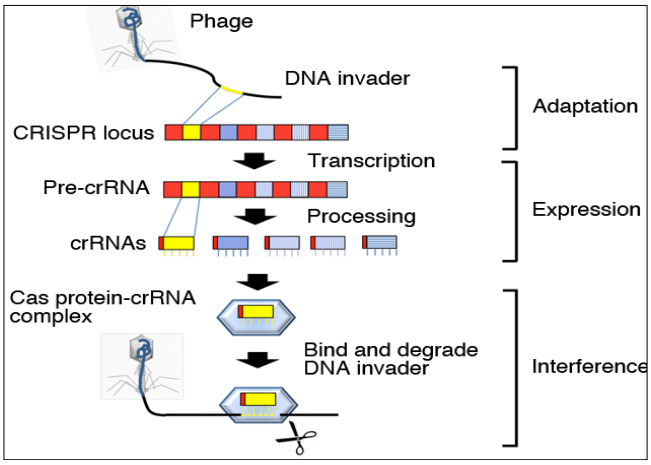
CRISPR system was first time reported in bacteria in 1987 by Ishino and his colleague in *E-coli*. Later it was found in 40% of sequenced bacterial genomes and 90% of archaea (Horvath and Barrangou, 2010) [32]. Meanwhile, several types of Cas genes were found to be well conserved and adjacent to repeat elements (Jansen *et al.*, 2002) [23]. These CRISPR/Cas systems can be classified into types I, II, and III, with the type II system requiring only the Cas9 nuclease to degrade DNA that matches a single guide RNA (sgRNA) (Horvath and Barrangou, 2010) [32]. The year 2005 was remarkable in the CRISPR/Cas9 epoch; in that year the spacer sequences were found to be originated from phage genomes(Bolotin *et al*., 2005 [3], Mojica *et al*., 2005 and Pourcel *et al*., 2005). Based on this discovery and the findings that viruses are unable to infect archaeal cells carrying sequences matching their own genomes, CRISPR/Cas systems were hypothesized to serve as a critical immune system to protect owners from pathogen invasion (Mojica *et al*., 2005) [32]. By 2011, the mechanism by which Cas9 works with CRISPR RNA (crRNA) and transactivator crRNA (tracrRNA) to attack foreign DNA that matches the crRNA was decoded (Deltcheva *et al*., 2011) [10]. Soon, the tracrRNA and crRNA were combined into a single guide RNA molecule, an advance that has since rapidly accelerated the application of the CRISPR/Cas9 system in practice. In 2013 first time it was used in plant system by many scientists. Recently in 2016 this system was first time used to treat lung cancer directly in human by Lu and his colleague in china.

A comparison of CRISPR/Cas9, ZFNs and TALENs

ZFNs and TALENs function as dimers and only protein components are required. Sequence specificity is conferred by the DNA-binding domain of each polypeptide and cleavage is carried out by the *FokI* nuclease domain. In contrast, the CRISPR/Cas9 system consists of a single monomeric protein and a chimeric RNA. Sequence specificity is conferred by a 20-nt sequence in the gRNA and cleavage is mediated by the Cas9 protein. The design of ZFNs is considered difficult due to the complex nature of the interaction between zinc fingers and DNA and further limitations imposed by context-dependent specificity. Commercially available ZFNs generally perform better than those designed using publicly available resources but they are much more expensive. TALENs are easier to design because there are one-to-one recognition rules between protein repeats and nucleotide sequences, and their construction has been simplified by efficient DNA assembly techniques such as Golden Gate cloning (Engler *et al*., 2008). However, TALENs are based on highly repetitive sequences which can promote homologous recombination in vivo (Holkers *et al*., 2013) [19]. In comparison, gRNA-based cleavage relies on a simple Watson–Crick base pairing with the target DNA sequence, so sophisticated protein engineering for each target is unnecessary and only 20 nt in the gRNA need to be modified to recognize a different target.

CRISPR System

It was originally discovered in the 1980s as a distinctive genomic locus in *E. coli* (Stern *et al*., 1984 [58] and Ishino *et al*., 1987) [2] and was later characterized to serve as an adaptive immune system in many bacteria and archaea (Wiedenheft *et al*., 2012) [20]. However, the molecular mechanism of the CRISPR/Cas system from *Streptococcus pyogenes* was not deciphered until 2012(Jinek *et al*., 2012) [27]. Foreign plasmid or viral DNA entering the bacterial cells are degraded by a single protein, the nuclease Cas9. The target specificity is governed by a short socalled CRISPRRNA (crRNA), which is encoded in the CRISPR locus and which is complementary to the invading DNA so that it can bind directly to the foreign DNA using a stretch of 20 nts. An additional short sequence motif next to the target sequence, termed protospacer adjacent motif (PAM), is needed for the correct recognition of the target site. A second short RNA, the transactivating CRISPRRNA (tracrRNA), binds to the crRNA, and a stable complex is formed with Cas9. The foreign DNA is then cleaved by two nuclease domains of Cas9. Furthermore, it was shown that the two RNAs can also be fused together to form a socalled singleguide RNA.



Basic logic behind this technology is to induce cells own DNA repair mechanism at precise locus in its genome by inducing the double strand break in the DNA.

Types of CRISPR System

CRISPR–Cas systems are diverse in terms of the content and organization of *cas* genes and have been classified into three main types and at least 11 subtypes (Makarova *et al*., 2011) [3]. There are major mechanistic differences between the

variants of the system; nevertheless, the general mode of action of all three types of CRISPR–Cas systems involves three distinct stages: adaptation, expression and interference (see the figure) (Westra *et al*., 2014) [64].

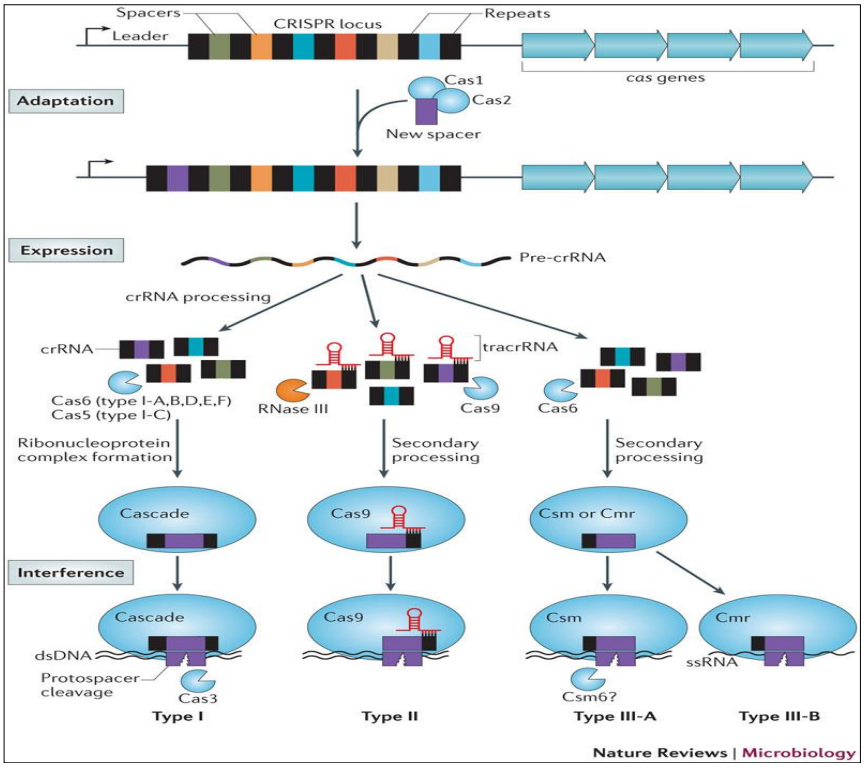
During adaptation, expansion of the CRISPR array occurs by the addition of an MGE-derived spacer sequence, which involves the duplication of a repeat sequence (Yosef *et al*., 2012) [70]. Spacer acquisition occurs in a polarized manner: new spacers are typically integrated at the leader-proximal end of the array, which involves the duplication of the first repeat of the array (Yosef *et al*., 2012) [70]. This process requires Cas1 and Cas2 (Yosef *et al*., 2012) [70], which are encoded by all CRISPR–Cas systems, but it might also involve additional Cas proteins in some systems (Makarova *et al*., 2011) [32].

During the expression stage, CRISPR loci are transcribed from an upstream promoter in the AT-rich leader sequence and the resulting pre-CRISPR RNA (pre-crRNA) is processed into short crRNAs by cleavage in the repeat sequences. In type I and type III systems, pre-crRNA cleavage is carried out by Cas endoribonucleases (Brouns *et al*., 2008 [32] and Carte *et al*., 2008) [7]. In type II systems, this process involves the expression of a transactivating crRNA (tracrRNA), which base-pairs with the repeats in the pre-crRNA transcript. The resulting duplexes are cleaved in the repeat sequences by RNase III in a Cas9-dependent reaction (Deltcheva *et al*., 2011) [10] (see the figure). Thus, in all CRISPR–Cas systems, cleavage of the pre-crRNA occurs in the repeat sequences, hence, mature crRNA consists of a spacer flanked by partial repeats.

In some systems, further processing of the crRNA takes place (Hale *et al*., 2008 [16] and Hale *et al*., 2009) [17]. In type II systems, the tracrRNA remains bound to the crRNA and the mature crRNA–tracrRNA duplexes are complexed with Cas9. In type I and III systems, mature crRNA is bound by a Cas protein complex. Type I ribonucleoprotein complexes are known as Cascade, whereas type III-A and type III-B complexes are known as Csm and Cmr complexes, respectively. During the interference stage (see the figure) crRNAs function as guides for the Cas proteins, as they recognize and bind to complementary nucleic acids in invading MGEs. The target nucleic acid (known as a protospacer; purple) is usually double-stranded DNA (dsDNA) (in type I, type II and type III-A systems) (Marraffini *et al*., 2008 and Westra *et al*., 2012) [65], but the type III-B system targets complementary single-stranded RNA (ssRNA) (Hale *et al*., 2008 and Zhang *et al*., 2012).

Target cleavage is carried out either by the Cas–crRNA ribonucleoprotein complex itself (in type II and type III-B systems) or by recruiting a Cas nuclease (in type I and type III-A systems) (Reeks *et al*., 2013). In type I systems, the surveillance complex (which is composed of Cascade and the crRNA) binds to dsDNA(Jore *et al*., 2011) and then recruits the Cas3 nuclease to degrade the target(Westra *et al*., 2012). In type II systems, Cas9 (which is loaded with crRNA and tracrRNA) binds to and cleaves target dsDNA (Jinek *et al*., 2012). In type III-A systems, a Csm–crRNA complex (Rouillon *et al*., 2013) binds to, and presumably degrades, invader dsDNA (Marraffini *et al*., 2008), possibly by recruiting Csm6 (Hatoum-Aslan *et al*., 2013). Type III-B Cmr–crRNA complexes cleave complementary RNA (Hale *et al*., 2009) [17].

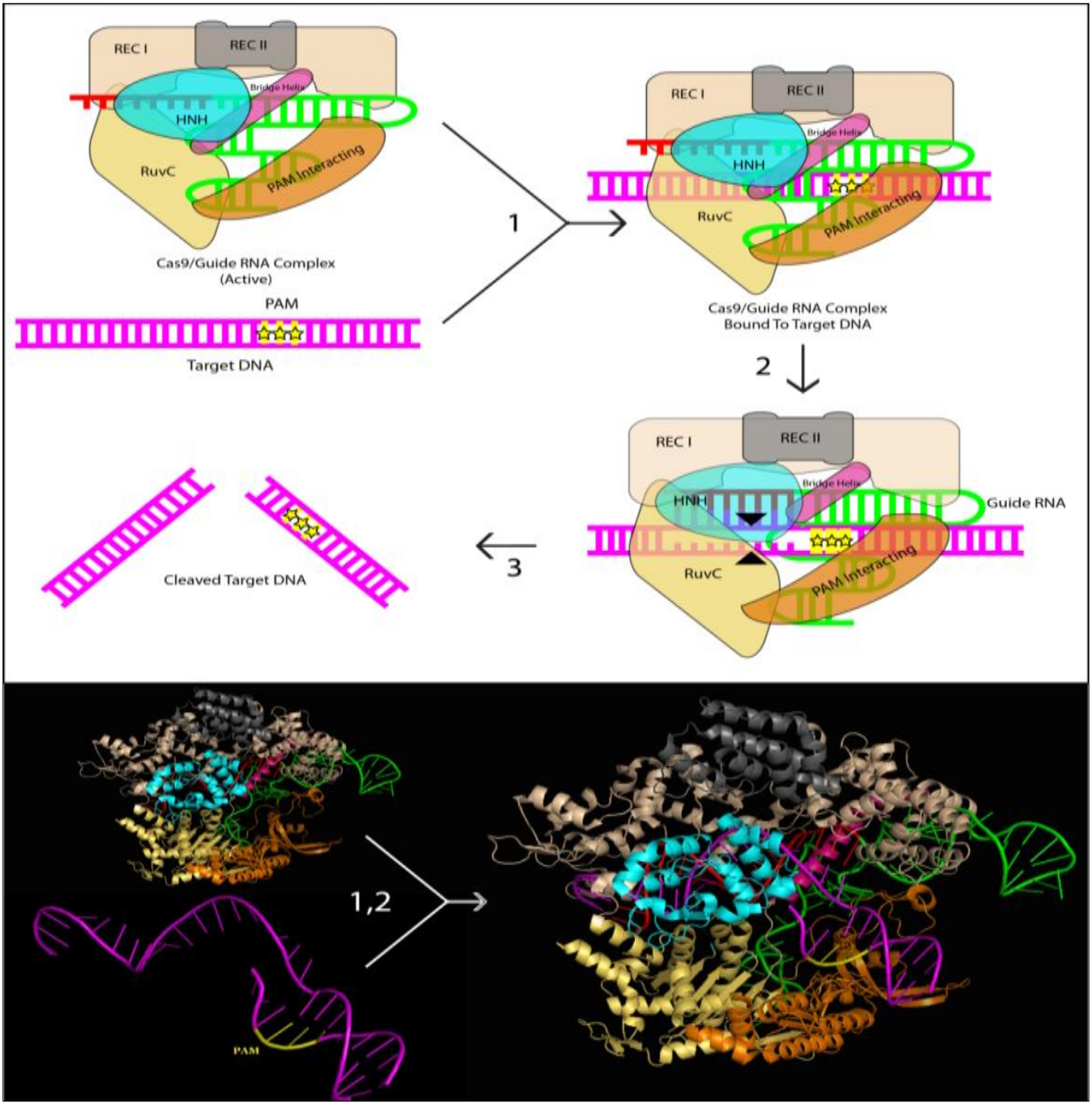
Currently used CRISPR technology is based on the type II adaptive immune system of *Streptococcus pyogenes.* The simplicity of the type II CRISPR nuclease, with only three required components (Cas9 along with the crRNA and trRNA) makes this system amenable to adaptation for genome editing. This potential was realized in 2012 by the Doudna and Charpentier labs (Jinek *et al*., 2012). Based on the type II CRISPR system described previously, the authors developed a simplified two-component system by combining trRNA and crRNA into a single synthetic single guide RNA (sgRNA). sgRNA programmed Cas9 was shown to be as effective as Cas9 programmed with separate trRNA and crRNA in guiding targeted gene alterations.



Different type of CRISPR system

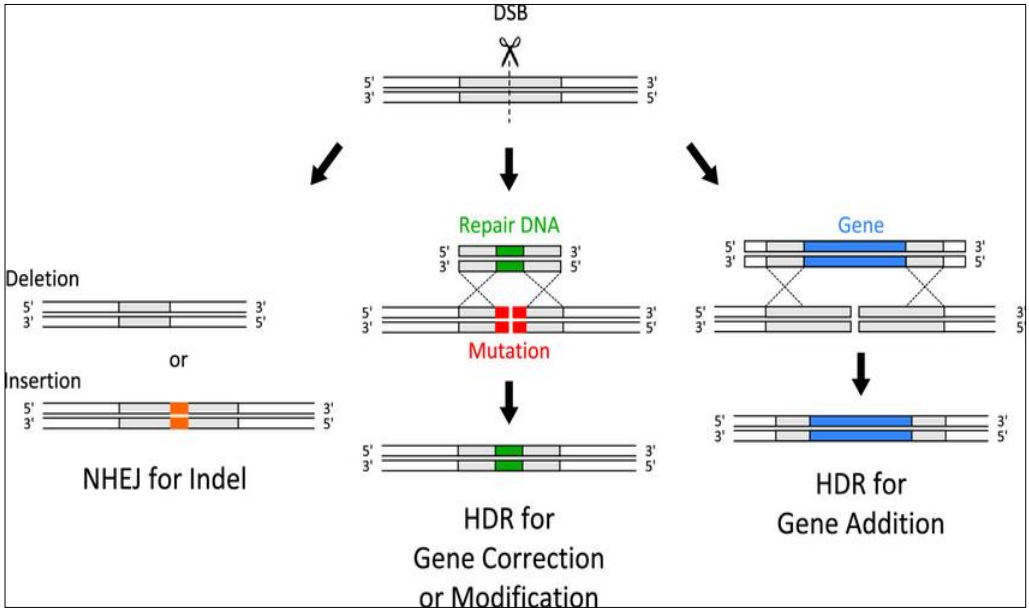
CRISPR Machanism

Cas9, a hallmark protein of the type II CRISPR-Cas system, is a large monomeric DNA nuclease guided to a DNA target sequence adjacent to the PAM (protospacer adjacent motif) sequence motif by a complex of two noncoding RNAs: CRIPSR RNA (crRNA) and trans-activating crRNA (Deltcheva *et al*., 2011 [10], Jinek *et al*., 2012 [27] and Sorek *et al*., 2013) [57]. The Cas9 protein contains a conserved core and a bilobed architecture including adjacent active sites and two nucleic acid binding grooves: a large recognition (REC) lobe and a small nuclease (NUC) lobe that are connected by a helix bridge (Nishimasu *et al*., 2014, Jinek *et al*., 2014 [16] and Anders *et al*., 2014) [27]. REC determines the Cas9specific function, whereas the NUC incorporates two nuclease domains, RuvC and HNH, and a protospaceradjacent motif (PAM) interacting domain (PI). Under natural conditions, Cas9 is inactive. It is activated when combined with the sgRNA at its REC lobe. The Cas9sgRNA complex scans a DNA double strand for rigorous PAMs (the trinucleotide NGG) using Watson–Crick pairing between sgRNA and targeted DNA. Once anchored at the proper PAMs, the HNH nuclease domain cleaves the RNA–DNA hybrid, while RuvC cleaves the other strand to form a doublestrand break (DSB).



Mechanism of CRISPR Cas9 system

DSBs can be repaired by nonhomologous end joining (NHEJ) and homologydirected repair (HDR) mechanisms that are endogenous to both prokaryotes and eukaryotes (Puchta, H. 2005). NHEJ employs DNA ligase IV to rejoin the broken ends, an operation that can introduce insertion or deletion mutations (indels), whereas HDR repairs the DSBs based on a homologous complementary template and often results in a perfect repair. The error prone NHEJ has advantages for gene knockout. HDR is used for gene replacement and gene knockin in plants.



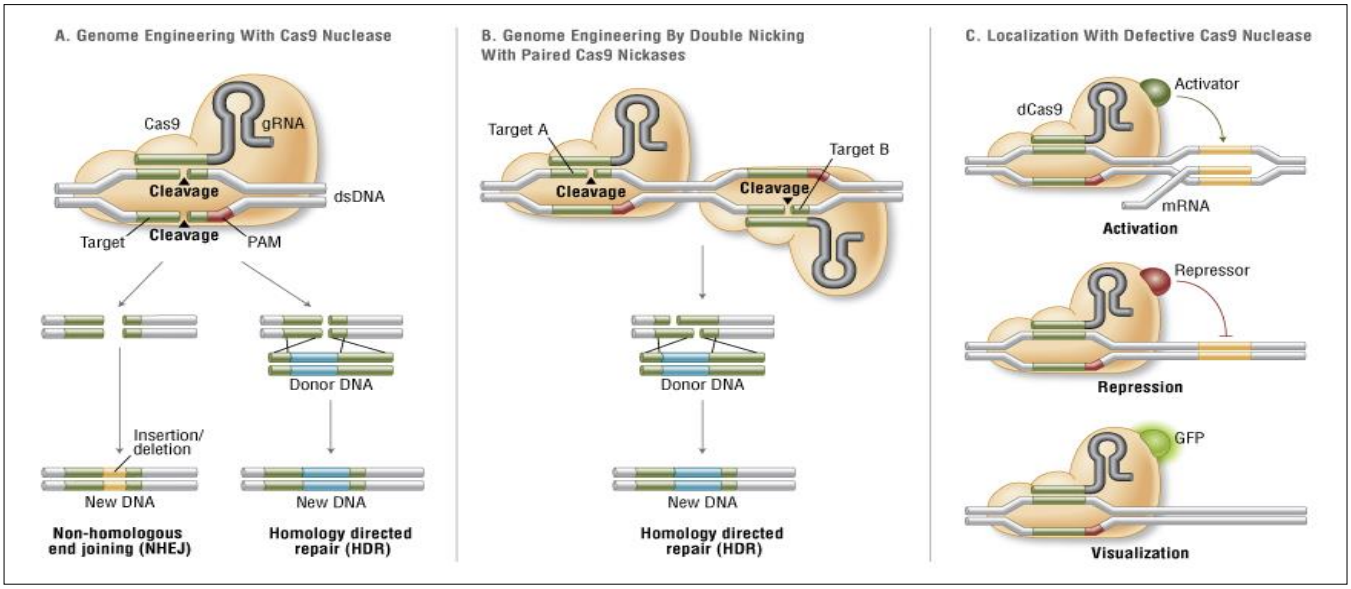
dsDNA repair mechanism

Variants of Cas9

To date, three different variants of the Cas9 nuclease have been adopted in genome-editing protocols. The first is wild-type Cas9, which can site-specifically cleave double-stranded DNA, resulting in the activation of the doublestrand break (DSB) repair machinery. DSBs can be repaired by the cellular Non-Homologous End Joining (NHEJ) pathway (Overballe-Petersen *et al*., 2013) [39], resulting in insertions and/or deletions (indels) which disrupt the targeted locus. Alternatively, if a donor template with homology to the targeted locus is supplied, the DSB may be repaired by the homology directed repair (HDR) pathway allowing for precise replacement mutations to be made(Gong *et al*., 2005 and Overballe-Petersen *et al*., 2013).

Cong and colleagues (cong *et al*., 2013) took the Cas9 system a step further towards increased precision by developing a mutant form, known as Cas9D10A, with only nickase activity. This means it cleaves only one DNA strand, and does not activate NHEJ. Instead, when provided with a homologous repair template, DNA repairs are conducted via the high-fidelity HDR pathway only, resulting in reduced indel mutations (Jinek *et al*., 2012, Cong *et al*., 2013 and Davis *et al*., 2014). Cas9D10A is even more appealing in terms of target specificity when loci are targeted by paired Cas9 complexes designed to generate adjacent DNA nicks (Ran *et al*., 2013) [64].

The third variant is a nuclease-deficient Cas9 (Qi *et al*., 2013) [14]. Mutations H840A in the HNH domain and D10A in the RuvC domain inactivate cleavage activity, but do not prevent DNA binding (Gasiunas *et al*., 2012) [13]. Therefore, this variant can be used to sequence-specifically target any region of the genome without cleavage. Instead, by fusing with various effector domains, dCas9 can be used either as a gene silencing or activation tool (Maeder *et al*., 2013 [31], Gilbert *et al*., 2013 [14], Perez-Pinera *et al*., 2013 [40] and Hu *et al*., 2014) [33]. Furthermore, it can be used as a visualization tool. For instance, Chen and colleagues used dCas9 fused to Enhanced Green Fluorescent Protein (EGFP) to visualize repetitive DNA sequences with a single sgRNA or nonrepetitive loci using multiple sgRNAs.

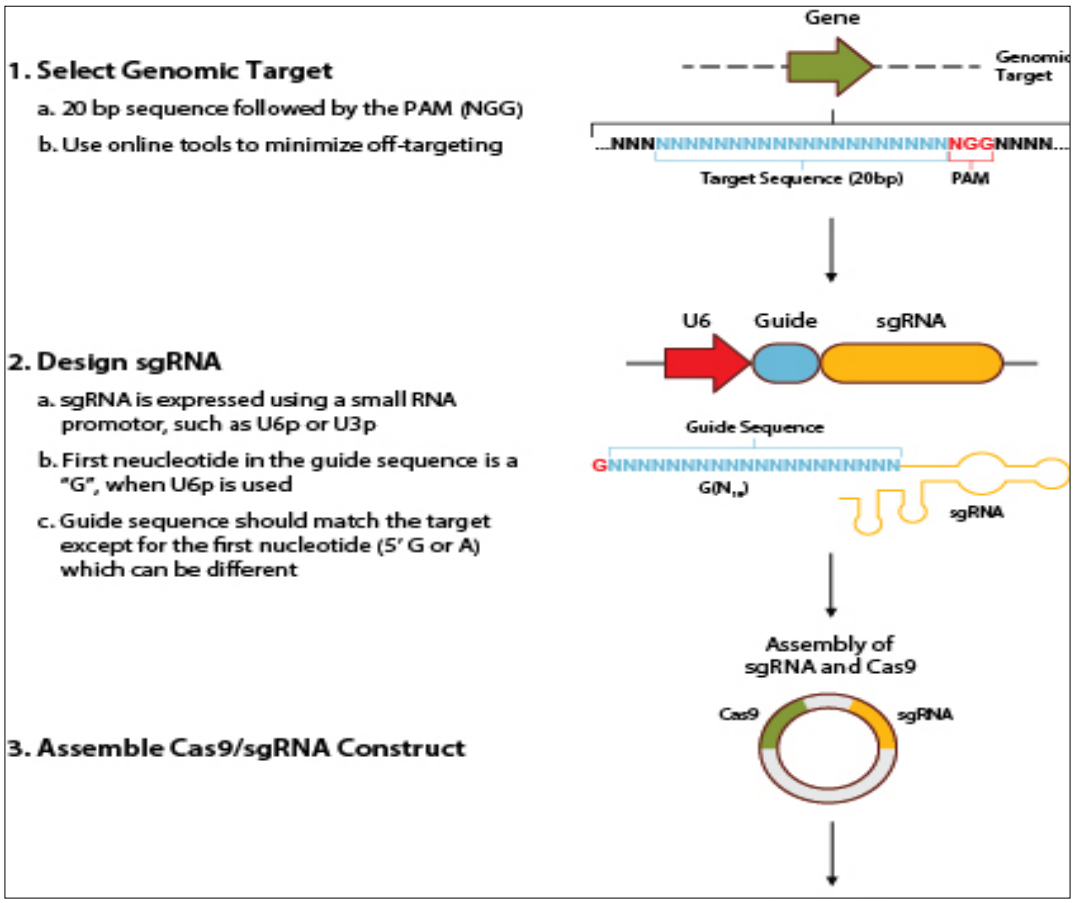


Variants of Cas9 protein

Steps of CRISPR system in plants

The single guide RNA (sgRNA) is the second component of the CRISPR/Cas system that forms a complex with the Cas9 nuclease. As mentioned above, the sgRNA is a synthetic RNA chimera created by fusing crRNA with tracrRNA (Jinek *et al*., 201) [16]. The sgRNA guide sequence located at its 5 end confers DNA target specificity. Therefore, by modifying the guide sequence, it is possible to create sgRNAs with different target specificities. The canonical length of the guide sequence is 20 bp. Consequently, a DNA target is also 20 bp followed by a PAM sequence that follows the consensus NGG. Interestingly, DNA targets and sgRNA guide sequences that differ from the canonical 20 bp length have been reported in some plant studies (Shan *et al*., 2013 [63], Feng *et al*, Mao *et al*., Xie *et al*. and Miao *et al*., 2013) [35, 67], while in the mammalian field targets of the consensus (N) NGG are normally used. Therefore, DNA targets validated in plants deviate from the strict (N) NGG and to date follow the consensus (N) 19-22NGG. The extent to which target sequences that deviate further from this consensus can affect the recognition by the Cas9/sgRNA system remains to be determined.

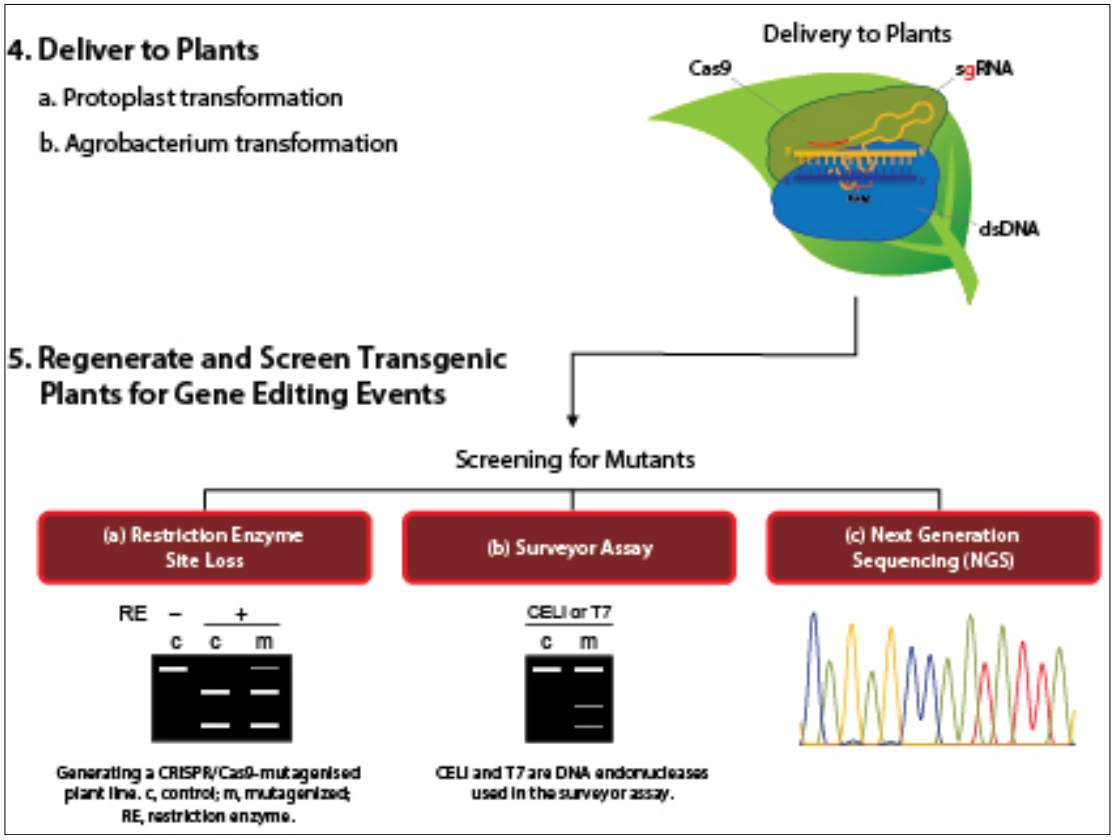
In plants, sg RNAs have been expressed using plant RNA polymerase III promoters, such as U6 and U3. These promoters have a defined transcription start nucleotide, which is "G" or "A", in the case of U6 or U3 promoters, respectively. Therefore, the guide sequences in the sgRNAs, used to target plant genomic loci, follow the consensus G(N)19–22 for the U6 promoter and A(N)19–22 for the U3 promoter, where the first G or A may or may not pair up with the target DNA sequence (Nekrasov *et al*., 2013 [5] and Jiang *et al*., 2013) [25]. On the other hand, in mammalian systems, sg RNA guide sequences normally follow the consensus G(N)19–20 where the first G may or may not pair up with the target (Yang *et al*., 2013 [67] and Wang *et al*., 2013) [62].



CRISPR/Cas genome editing assays in plants

In plants the CRISPR/Cas9 system has been implemented using transient expression systems, therefore enabling rapid execution and optimization of the method. Widely used transient assays in plant research are (i) protoplast transformation and (ii) leaf tissue transformation using the agroinfiltration method. Both methods have been used for Cas9 and sgRNA. The advantage of the protoplast strategy is the possibility to achieve high levels of gene co-expression even from separate plasmids. However, isolation of protoplasts from plant tissue requires enzymatic digestion and removal of the cell wall. The procedure can be time consuming, and protoplast cultures are fragile and prone to contamination (Belhaj *et al*., 2013) [2].

An alternative is the agroinfiltration assay, which is performed on intact plants, and relatively less time consuming compared to protoplasts. This system is based on infiltration of *A. tumefaciens* strains carrying a binary plasmid that contains the candidate genes to be expressed (Van der Hoorn *et al*., 2000) [60]. Efficiency of gene co-expression by agroinfiltration appears to be lower than in protoplasts, and combining multiple genes of interest in one vector is preferable. However, not all plant species are amenable to transformation by these methods and options can be limited depending on the plant species of interest.



To readily detect induced mutations generated by the CRISPR/Cas method, one approach is to target a restriction enzyme site and use the restriction enzyme site loss assay. Since the Cas9 nuclease introduces a blunt cut in the DNA predominantly 3 bp away from the PAM, it is advantageous to identify a DNA target with an overlapping restriction site proximal to the PAM motif. In this case, the repair of a DSB via the error-prone NHEJ pathway will result in mutations that will disrupt the restriction site. Therefore, mutations can be detected by amplifying the genomic DNA across the target and digesting resulting amplicons with the restriction enzyme. This assay can be more sensitive when the PCR-amplification is performed on genomic DNA template pre-digested with the restriction enzyme (Nekrasov *et al*., 2013 [2] and Jiang *et al*., 2013) [25].

An alternative assay is the Surveyor assay (Voytas *et al*., 2013) [61]. PCR amplified DNA from the Cas9/sgRNA treated sample is first denatured and then allowed to anneal before being subject to *CELI* or T7 endonuclease I that cleave heteroduplexes formed by the WT and the mutated DNA (Mao *et al*., 2013 and Xie *et al*., 2013) [67]. It is worth considering that the Surveyor assay is less sensitive than the restriction enzyme site loss assay and requires a higher rate of mutagenesis to be successfully applied. However, it can in principle be applied to any target sequence (Belhaj *et al*., 2013) [2].

Application in Agriculture

- Can be used to create high degree of genetic variability at precise locus in the genome of the crop plants.
- Potential tool for multiplexed reverse and forward genetic study.
- Precise transgene integration at specific loci.
- Developing biotic and abiotic resistant traits in crop plants.
- Potential tool for developing virus resistant crop varieties.
- Can be used to eradicate unwanted species like herbicide resistant weeds, insect pest.
- Potential tool for improving polyploid crops like potato and wheat.

Case study

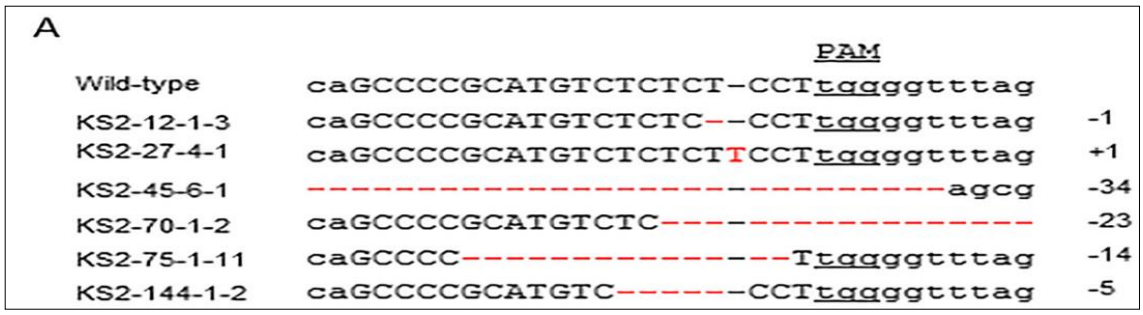
Enhanced Rice Blast Resistance by CRISPR/ Cas9-Targeted Mutagenesis of the ERF Transcription Factor Gene OsERF922 by Wang *et al*., 2016.

The adoption of host resistance has proven to be the most economical and effective approach to control rice blast. In recent years, sequence-specific nucleases (SSNs) have been demonstrated to be powerful tools for the improvement of crops via gene-specific genome editing, and CRISPR/Cas9 is thought to be the most effective SSN.

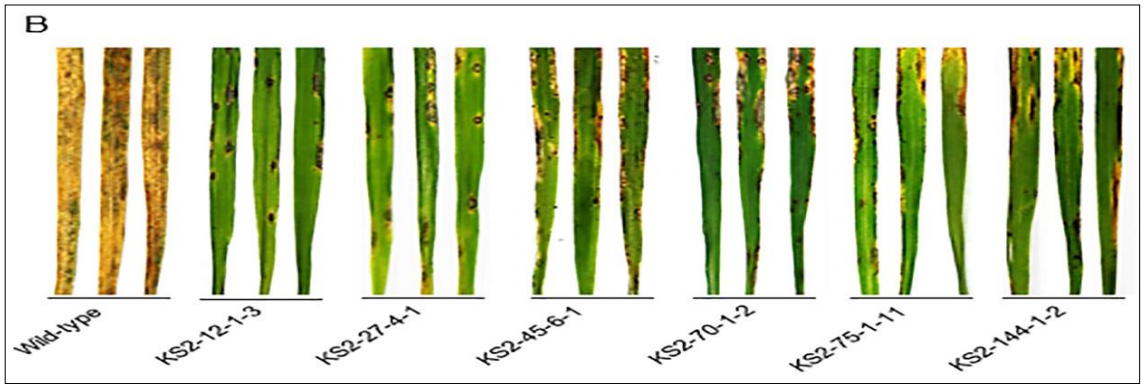
Here, they report the improvement of rice blast resistance by engineering a CRISPR/Cas9 SSN (C-ERF922) targeting the *OsERF922* gene in rice. Twenty-one C-ERF922-induced mutant plants (42.0%) were identified from 50 T_0 transgenic plants. Sanger sequencing revealed that these plants harbored various insertion or deletion (InDel) mutations at the target site. They showed that all of the C-ERF922-induced allele mutations were transmitted to subsequent generations. Mutant plants harboring the desired gene modification but not containing the transferred DNA were obtained by segregation in the T_1 and T_2 generations. Six T_2 homozygous mutant lines were further examined for a blast resistance phenotype and agronomic traits, such as plant height, flag leaf length and width, number of productive panicles, panicle length, number of grains per panicle, seed setting percentage and thousand seed weight. The results revealed that the number of blast lesions formed following pathogen infection was significantly decreased in all 6 mutant lines compared with wild-type plants at both the seedling and tillering stages.

Furthermore, there were no significant differences between any of the 6 T_2 mutant lines and the wild-type plants with regard to the agronomic traits tested. They also

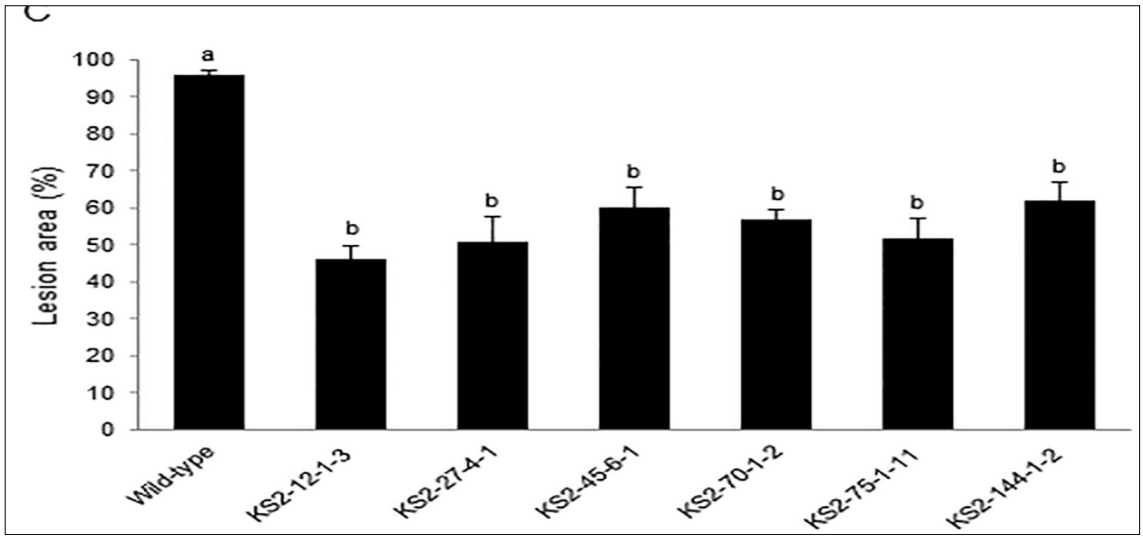
simultaneously targeted multiple sites within *OsERF922* by using Cas9/Multi-target-sgRNAs (C-ERF922S1S2 and C-ERF922S1S2S3) to obtain plants harboring mutations at two or three sites. Their results indicate that gene modification via CRISPR/Cas9 is a useful approach for enhancing blast resistance in rice.



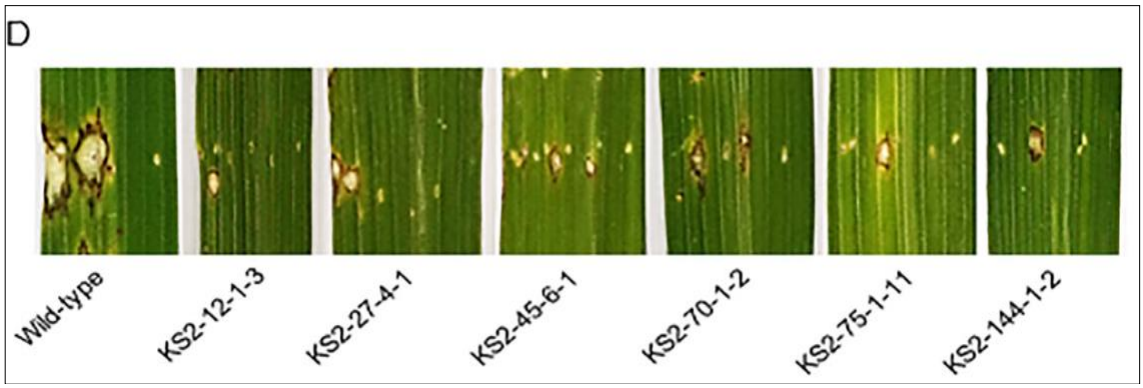
Nucleotide sequences of the target site



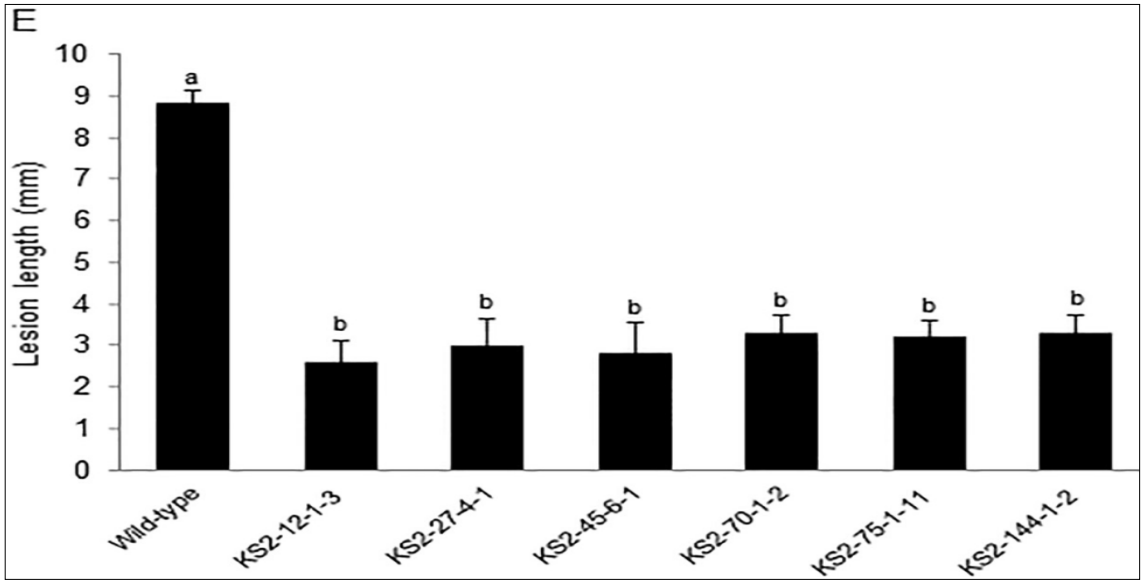
The seedling stage



Histogram showing Average area of lesions formed



The tillering stage



Histogram showing Average length of lesions formed

Some CRISPR/Cas approaches

Crops	Target Gene	References
Rice	PDS, BADH2, MPK2, Os02g23823	Shan *et al*. (2013) [63]
	MPK5	Xie and Yang (2013) [67]
	SWEET14	Jiang *et al*. (2013b) [25]
	KO1, KOL5, CPS4,CYP99A2, CYP76M5, CYP76M6	Zhou *et al*. (2014)
	OsPDS, OsBADH2	Shan *et al*. (2013) [63]
	OsMYB1	Mao *et al*. (2013) [12]
	CAO1, LAZY1	Miao *et al*. (2013) [35]
	ROC5, SPP, YSA	Feag *et al*. (2013) [12]
	PDS, PMS3, EPSPS, DERF1, MSH1, MYB5, MYB1, ROC5, SPP	Zhang *et al*. (2014) [29]
	BAL	Xu *et al*. (2014) [68]
	SWEET1a-1b-11-13	Zhou *et al*. (2014)
	OsPDS, OsBADH2	Shan *et al*. (2013) [63]
	GUUS	Miao *et al*. (2013) [35]
Wheat	MLO	Shan *et al*. (2013) [63]
	MLO-A1	Wang *et al*. (2014) [24]
	PDS, INOX	Upadhyay *et al*. (2013) [59]
	INOX	Upadhyay *et al*. (2013) [59]
	MLO-A1	Brooks *et al*. (2014) [5]
Maize	IPK	Liang *et al*. (2014) [29]
Orange	PDS	Jia *et al*. (2014) [24]
Tomato	GFP, SHR	Ron *et al*. (2014) [49]
	SIAGO7, Solyc08g041770, Solyc07g021170, Solyc12g044760	Brooks *et al*. (2014) [5]

Advantages of CRISPR Cas system

Everything that can be achieved with the CRISPR/Cas9 systemcan in principle also be achieved using either ZFNs or TALENs. Nevertheless, the appearance of such a large number of publications based on the CRISPR/Cas9 technology in such a short time, including virgin reports of genome editing in species such as sweet orange, highlights the clear advantages of CRISPR/Cas9 in terms of simplicity, accessibility, cost and versatility.

The immediate benefit for plant scientists is the possibility to rapidly create mutations in genes where no known T-DNA insertion or EMS mutant is available. Use of this method will therefore lead to a more complete understanding of gene function in plants.

This approach can not only be applied to genes with unknown functions but also to genes for which we must revise our current knowledge due to the option to produce true knock-out mutants.

Specific changes of single amino acids or integration of a larger piece of DNA in the plant genome can be achieved by using Cas9-based GT systems, while efficient multiplex systems will allow the complex rearrangement of chromosomes.

Thus, as well as coming closer to developing synthetic plant genomes, we will be also able to obtain plants with a single engineered point mutation that cannot be discriminated from natural varieties.

Disadvantages of CRISPR Cas system

Naturally, CRISPR/Cas9 system also has certain disadvantages that have to be taken into account. One of them is a reported high incidence of nonspecific DNA cleavage; while this has cooled some of the initial enthusiasm about this method, a potential remedy is the expression of two CRISPR modules with nickase activity against two genomic sites that are closely adjacent to one another.

Then there is a problem of mosaicism, where mutant allele is produced in only some of the cells, as nucleases may not inevitably cut the DNA during one stage of embryonic development. The production of multiple mutations in one

organism is also possible, which can create phenotyping bottlenecks in mouse models.

Regardless of those burning problems, CRISPR/Cas9 genome-editing technique presents staggering opportunities for addressing a number of illnesses beyond the reach of previous treatment modalities. Taking into account the accelerating rate of technological progress, as well as a wide range of research and clinical applications, the road ahead of us will certainly be a thrilling one.

Conclusion

Although much progress has been made in CRISPR/Cas9based genome editing technology in the last few years, some problems remain to be solved: offtarget effects, influence of chromatin structure, side effects on nearby genes, mechanisms underlying the different effects of different sgRNAs on mutation efficiency, and methods for efficient delivery in polyploid plants. Despite these challenges, with the tremendous enthusiasm of the research community, gene editing technologies as represented by the CRISPR/Cas9 system will improve rapidly. This simple, affordable, and elegant genetic scalpel is expected to be widely applied to enhance the agricultural performance of most crops in the near future.

Every evolutionary process involving host–pathogen interactions is an arms race featuring adaptations and counter-adaptations to overcome the opponent. Therefore, some viruses may well have evolved anti-CRISPR strategies to evade this bacterial immune system, and these as yet undiscovered regulators may provide additional tools to modify and control the activity of the CRISPR/Cas9 system. Given the large number of researchers working with CRISPR/Cas9 technology and the speed at which it has developed since the first reports of genome editing only 2 years ago, further advances in our understanding and control of the system are likely to come rapidly, potentially leading to the design of a new generation of genome editing tools.

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