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CRISPR-Cas9 Induced Genome Editing, New Hope for Plant Molecular Biology: A Review

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ABSTRACT

What makes CRISPR so revolutionary is that it's incredibly precise, the Cas9 enzyme is highly site specific and will act only on desired location. And also it's incredibly cheap and easy. In the past, it might have cost thousands of dollars and weeks or months of fiddling to alter a gene. The CRISPR/Cas (clustered regularly interspaced short palindromic repeats/CRISPR-associated proteins) system was first identified in bacteria and archaea and can degrade exogenous substrates. Gene editing itself isn't new. Various techniques to knock out genes have been around for years. It was developed as a gene editing technology in 2013. CRISPR/Cas9 technology is evolved from a type-II bacterial immune system and represents a new generation of targeted genome editing technology that can be applied to nearly all organisms. For the first time, researchers have been able to detect and characterize the mechanism of action by which the CRISPR complex binds and cleaves DNA using electron microscopy. We have unlimited possibilities. CRISPR helps us see that GMO/non-GMO binaries are overly simplistic. This one tool can perform many DNA nips and tucks and can up-regulate or down-regulate genes in ways that are not transgenic — yet are by no means inconsequential. Many CRISPR edits; won't involve any questions about foreign DNA, but will be equally dramatic in their effects. In crops and animals, "gene knockouts" can eliminate genes that affect food quality, divert energy away from valuable end products, and confer susceptibility to crop diseases.

Keywords: *CRISPR, DSB, NHEJ, SSNs, Gene Editing, Cas9*

INTRODUCTION

Early approaches to targeted DNA cleavage were through use of oligonucleotides, small molecules or self splicing introns for site-specific recognition of DNA sequences. Oligonucleotides coupled to chemical cleavage/ cross linking reagents such as bleomycin and psoralen **(Tabassum *et al.*, 2017)**. These methods were notrobust for site specific genome modification. Although Zinc Finger Nucleases (ZFN's) and TALENs are effective genome editing reagents they are not widely adapted because of the difficulty and validating such proteins for a specific DNA locus of interest (**Doudna and Charpentier, 2014)**. In 2010, **Fyodor Urnov** and his colleagues made explicit the reasons for adopting the expression genome editing to designate the use of the newly designed DNA scissors: the fact that they cut at precise positions in the genome with a limited number of off-targets, that their action does not lead to the insertion in the genome of additional sequences, and that they permit the efficient replacement of a mutated copy of a gene by a normal version of it were all good reasons to speak of genome editing **(Urnov *et al.* 2010)**.The editing or proofreading capacity of DNA polymerase, and in particular of the famous Klenow fragment of DNA polymerase-I extensively used in genetic engineering, wasscrutinized **(Morange, M. 2016).** The reliability of the information stored in DNA was not the consequence of the chemical stability of this macromolecule, but of these editing processes as well as of the repair mechanisms **(Loeb and Kunkel 1982).** The recent development of genome editing technology using programmable nucleases such as zinc finger nucleases (ZFNs); transcription activator-like effector nucleases (TALENs); clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated (Cas)

proteins (CRISPR/Cas) **(Kim and Kim 2014)** shed light on a new plant breeding approach; this technique can minimize the degree to which the target genome is genetically modified and can increase the specificity of the target locus **(Shan *et al*. 2013; Araki & Ishii 2015; Baltes and Voytas 2015; Kanchiswamy *et al*. 2015).**

WHAT IS CRISPR/CAS9?

The Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) Type II system is a form of prokaryotic immunity that has been adapted for genome engineering **(Bolotin et al., 2005)**. It consists of two components: a specific guide RNA (gRNA) and a non-specific CRISPR-associated endo nuclease protein from *Streptococcus pyogenes* (Cas9) **(Fig.1).** Identified in archaea and bacteria, short nucleic acid sequences are captured from invading pathogens and integrated in the CRISPR loci amidst the repeats. Small RNAs, produced by transcription of these loci, can then guide a set of endo nucleases to cleave the genomes of future invading pathogens, thereby disabling their attacks **(Gilbert et al, 2013).**

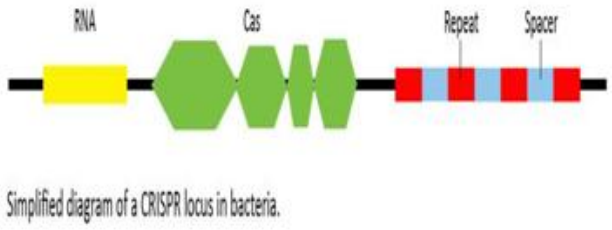

Fig1. *A simplified diagram of a CRISPR Locus in Bacteria (Courtsey: **Steph Yin**)*

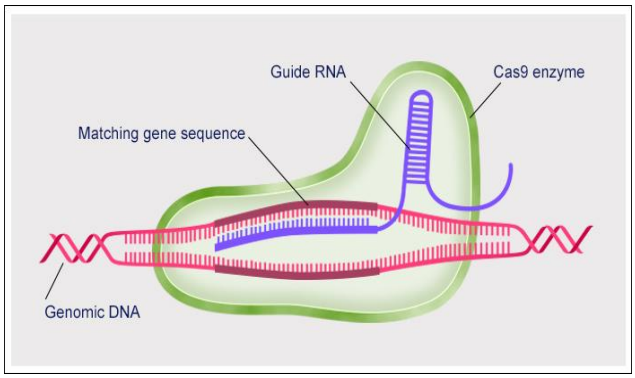

Fig2. *CRISPR comprises a single molecule of RNA (shown in purple) that performs two jobs; One end binds to the target gene (dark red), the other end delivers a DNA-cutting enzyme (in this case, Cas9) to the site. (Courtsey: **Bernie Hobbs**)*

In nature, Prokaryotes store small palindromic segments of DNA that are interspaced with other fragments of genetic material. These segments fall between CRISPR loci and correspond to fragments of viral DNA that the cell has previously encountered. After a prokaryotic cell successfully clears a viral infection or encounters a foreign plasmid, it stores fragments of foreign DNA as a way to retain a genetic memory in order to recognize and disable future infections. The main characteristic of CRISPR/Cas9 system is the Cas9 protein, **(Fig. 2)** which comprises two functional domains: the RuvC-like domain and the HNH nuclease domain **(Cong et al., 2013)**.The endo nuclease Cas9 can be guided by a synthetic single-guide RNA to recognize target sequences and produce double strand breaks (DSBs) at desired target sites **(Cong et al., 2013 and Jinek et al., 2012).**DSBs subsequently cause a series of complex DNA self-repair mechanisms in the cell and generate various site-specific genetic alterations through non-homologous end joining (NHEJ) or homology-directed repair (HDR). The NHEJ pathway is error-prone and typically generates insertions or deletions (indels) within the target sequence **(Zhou H *et al*., 2014)**. When these indels introduce a frame-shift mutation or disrupt important functional domains, the functions of the target genes will be damaged **(Shan Q *et al*, 2013; Zhou *et al*., 2014 and Gratz*et al*., 2013).** The possibility of homologous recombination will significantly increases in the present of homologous DNA fragments during the repair process (**Fig 3).** In

addition, the recombination efficiency caused by double strand breaks can be improved by one thousand-fold **(Rouet *et al.*, 1994).**

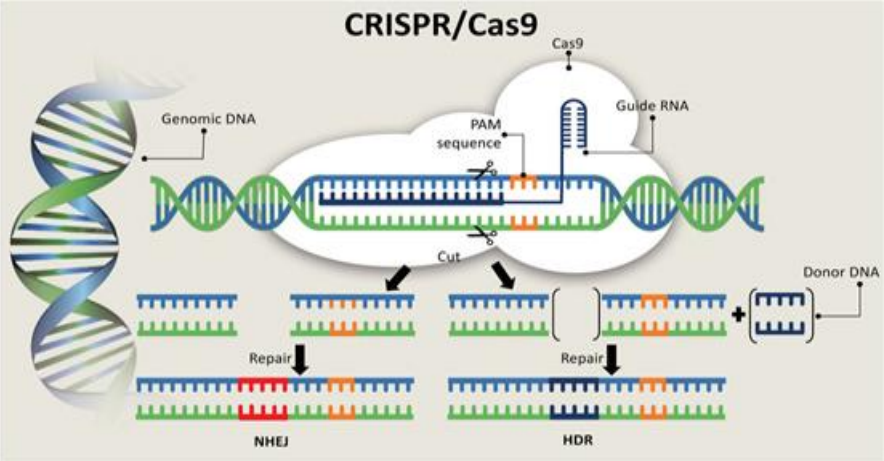

Fig3. *A diagram comparing how CRISPR works (Courtsey: CRISPR Bio-informatics R & D)*

Less than 5 years ago the CRISPR/Cas nuclease was first introduced into eukaryotes, shortly becoming the most efficient and widely used tool for genome engineering **(HolgerPuchta. 2017).** Editing a gene by CRISPR/Cas9 only has three requirements: (1) expression of the nuclear localized Cas9 protein; (2) production of a guide RNA (gRNA) molecule, whose first 20 nucleotides are complementary to the target gene; (3) the NGG PAM site that is located immediately adjacent to the 30 end of the target sequence **(Li et al., 2017).** Methods to specifically target and modify DNA sequences are indispensable for basic and applied research. Recently, the type II bacterial clustered, regularly interspaced, short palindromic repeats (CRISPR) system emerged as a simple and efficient tool to target and modify DNA sequences of interest in a variety of organisms **(Fig 4).** There are two components to the CRISPR system: a nuclear localized CRISP associated (Cas) 9 protein and a guide RNA (gRNA) as proposed by **Pattanayak *et al.*, 2013**. Cas9 is a large protein containing two nuclease domains, and the most commonly used one is derived from Streptococcus pyogenes. The gRNA is a synthetic 100 nucleotide (nt) RNA molecule, of which the first approximately 20 nt are the targeting site, and the 3′ end forms a hairpin structure that interacts with the Cas9 protein **(Jinek M et al., 2012)** Cas9 and the gRNA interact to identify DNA sequences complementary to the gRNA **(Basak and Nithin, 2015)** and generate a DNA double strand break (DSB) **(Fig 5).**

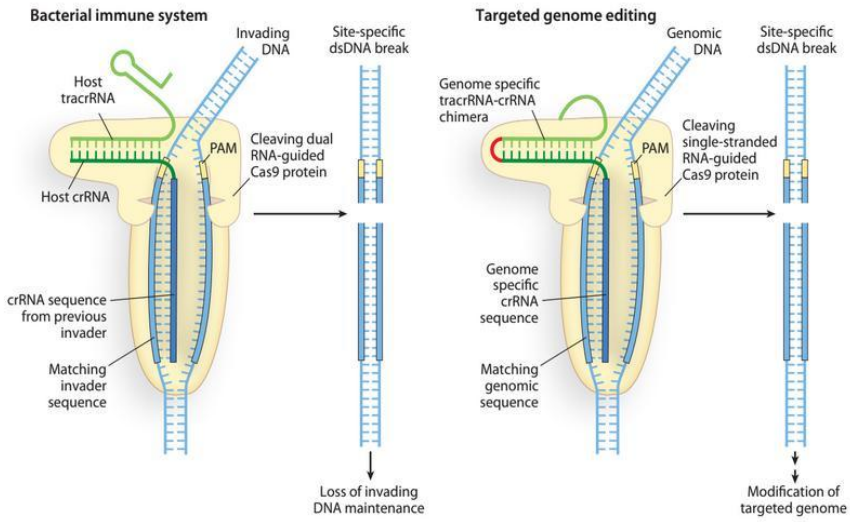

Fig4. *A diagram comparing how CRISPR works in the bacterial immune system and how it works in CRISPR/Cas9 genome editing. (Image by **H.Adam Steinberg).***

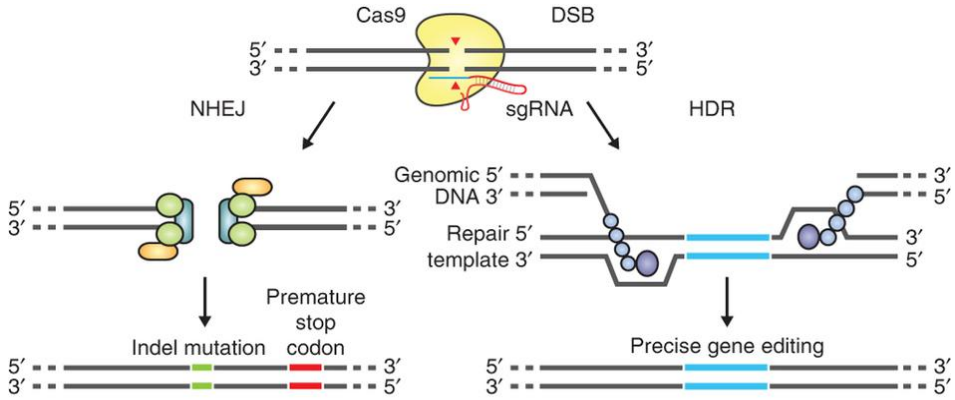

Fig5. *The guide RNA (sgRNA) directs Cas9 to a specific region of the genome, where it induces a double-strand break in the DNA. On the left, the break is repaired by non-homologous-end joining, which can result in insertion/deletion (indel) mutations. On the right, the homologous-directed recombination pathway creates precise changes using a supplied template DNA.* ***[Credit: Ran et al. (2013). Nature Protocols.]***

Invented in 2012 by scientists at the University of California, Berkeley, CRISPR/Cas9 has received a lot of attention these years. Scientists in Japan were the first to discover CRISPR in the DNA of bacteria in 1987. In their attempts to study a particular protein-encoding gene in E.Coli, the researchers noticed a pattern of short, repeating, palindromic DNA sequences separated by short, non-repeating, "spacer" DNA sequences. Francisco Mojica was the first researcher to characterize what is now called a CRISPR locus, reported in 1993. He worked on them throughout the 1990s, and in 2000, he recognized that what had been reported as disparate repeat sequences actually shared a common set of features, now known to be hallmarks of CRISPR sequences (he coined the term CRISPR through correspondence with Ruud Jansen. The Timeline of CRISPR Cas9 presented in **Fig. 6**, thoroughly described by Leaders in Pharmaceutical Business Intelligence (LPBI) group.

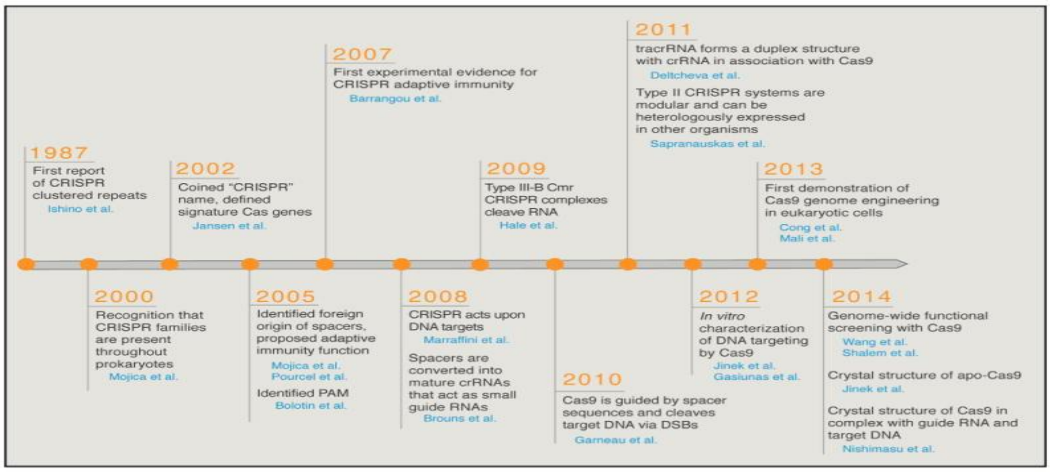

Fig6. *Timeline & History of CRISPR Cas 9 (Courtsey:* ***Leaders in Pharmaceutical Business Intelligence (LPBI) group)***

Genome Editing In Agricultural Sciences

Genome editing provides new approaches to reach objectives in food security, but uncertainty among regulators and segments of the public regarding the associated benefits and risks may impede implementation **(Bortesi L. & Fischer, 2015)**. This Review will meet a need for clarity by examining several aspects of genome editing of plants and animals for food, feed, fuel, and fibre including methods, applications and relation to other methods for improving plant and animal genetics, and issues impacting effective governance. An objective summary will help regulatory agencies and interested public stakeholders better understand the technology (**Zhang Y. et al 2016).** In recent years, sequence-specific nucleases (SSNs) have been demonstrated to be powerful tools for the improvement of crops **(Shan Q. et al. 2013)** via

gene-specific genome editing, and CRISPR/Cas9 is thought to be the most effective SSN **(Deltcheva et al., 2011).** Here, Wang reported the improvement of rice blast resistance by engineering a CRISPR/Cas9 SSN (C-ERF922) targeting the OsERF922 gene in rice **(Wang *et al.*,2016; Miah G *et al.*, 2013).** TALENs (transcription activator-like effectors nucleases) and CRISPR/Cas (clustered regularly interspaced short palindromic repeats and CRISPR-associated proteins) are potent biotechnological tools used for genome editing. In rice, species-tailored editing tools have proven to be efficient and easy to use **(Christian et al., 2010**). Both tools are capable of generating DNA double-strand breaks (DSBs) in vivo and such breaks can be repaired either by error-prone NHEJ (non-homologous end joining) that leads to nucleotide insertions or deletions or by HDR (homology-directed repair) if an appropriate exogenous DNA template is provided **(Bi and Yang, 2017).** As per the Joel McDade's research blog delivering of CRISPR Cas9 components to plant cell presented in **Fig. 7.** CRISPR components can be expressed stably or transiently depending on the delivery method and cell type in question. CRISPR components can be delivered and expressed transiently using a standard detergent, Polyethylene Glycol (PEG), although the application of this approach is limited to protoplast cells (plant cells whose cell wall has been removed). Another common delivery method is agro bacterium-mediated delivery, which uses the soil derived bacterium *Agrobacterium tumefaciens* as a vehicle to deliver your gene of interest into a target cell line or organism **(Fig. 7).**

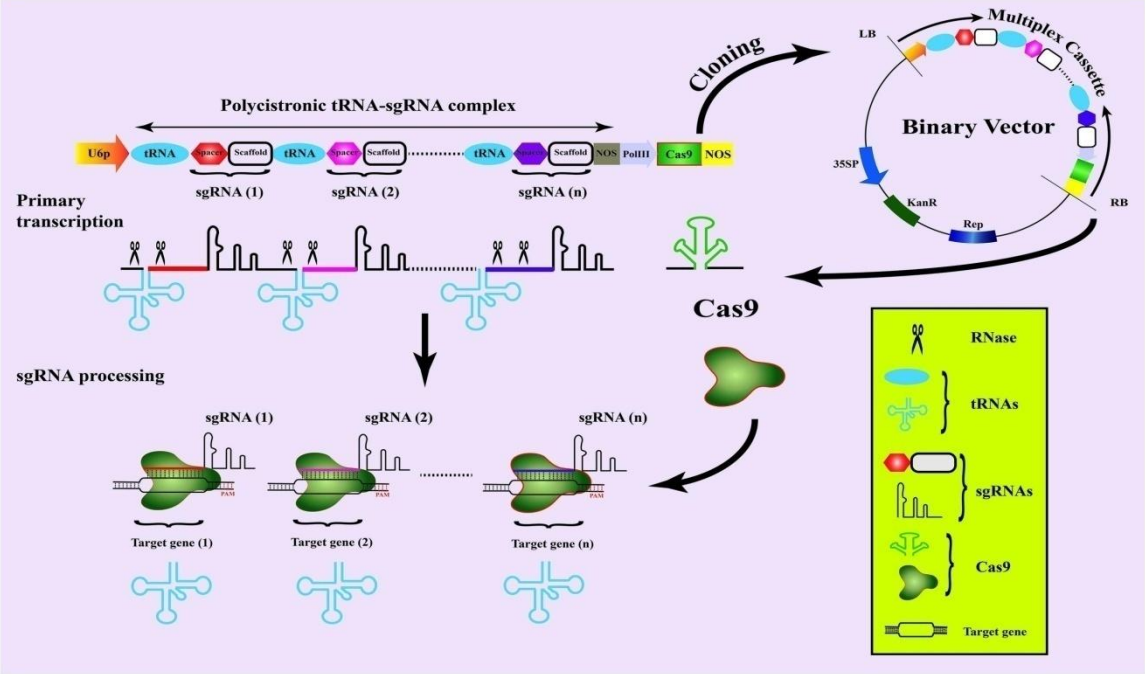

Fig7. *Delivering CRISPR Cas9 components to plant cell (Courtsey: Sattar MN)*

Sequence-specific nucleases have been applied to engineer targeted modifications in polyploidy genomes, but simultaneous modification of multiple homoeoalleles has not been reported **(Wang et al., 2014)**. They used transcription activator-like effectors nuclease (TALEN) and clustered, regularly interspaced, short palindromic repeats (CRISPR) - Cas9 technologies in hexaploid bread wheat to introduce targeted mutations in the three homoeoalleles that encode MILDEW-RESISTANCE LOCUS (MLO) proteins. Genetic redundancy has prevented evaluation of whether mutation of all three MLO alleles in bread wheat might confer resistance to powdery mildew, a trait not found in natural populations.

Similarly, knockdown of expression of the rice ERF gene **OsERF922** by RNA interference (RNAi) enhanced rice resistance to *M. oryzae*, indicating that **OsERF922** acts as a negative regulator of blast resistance in rice. A total of 22 dominant and 9 recessive BB resistant genes have been identified **(Nino et al., 2006 and Wang C. et al., 2009)**. Some of which have been widely used in rice production. Among these genes, Xa21 is the best studied for BB Resistance. Through phosphorylation **(Chen X et al., 2010 and Park et al., 2008)** and cleavage

of its intracellular kinase domain **(Park CJ et al., 2012)**, Xa21—a cell membrane receptor —perceives the presence of Xoo and relays the signal to the nucleus through multi-step signal cascades involving some key proteins such as XA21 Binding Protein 3 (XB3) **(Wang et al., 2006)** , mitogen-activated protein kinase 5 (MAPK5), MAPK12 **(Seo et al., 2011),** and transcription factors (TFs) including **OsWRKY62 and OsWRKY76 (Seo et al., 2011; Peng et al., 2008 and Peng et al., 2010)** in the nucleus. Some Xoo resistant genes, such as xa5, are transcription factors **(Jiang et al., 2006 and Iyer et al., 2004)**. Furthermore, many effectors from Xoo belong to the transcription activator-like (TAL) family, which facilitate injection into rice cells to activate susceptibility genes in the host to exert their functions15. The known Xoo effectors include avrxa516, avrXa717, avrXa1018 and avrXa2719, which trigger xa5-, Xa7-, Xa10- and Xa27-mediated resistance, respectively.

In fact, CRISPR has already been used to engineer the genome of many plant species, including commonly used model organisms like Arabidopsis and Medicago truncatula and several crop species including potato, corn, tomato, wheat, mushroom, and rice **(Khatodia et al., 2016; Yara A *et al.*, 2007; Miao J *et al.*, 2013; Wang C et al., 2015).** Alternatively, Cas9-mediated DSBs can be repaired by homology-directed repair (HDR) using an homologous DNA repair template, thus allowing precise gene editing by incorporating genetic changes into the repair template. HDR can introduce gene sequences for protein epitope tags, delete genes, make point mutations, or alter enhancer and promoter activities. In anticipation of adapting this technology for gene therapy in human somatic cells, much focus has been placed on increasing the fidelity of CRISPR-Cas9 and increasing HDR efficiency to improve precision genome editing **(Salsman and Dellaire 2017).** The type-II CRISPR/Cas RNA-guided nucleases are the most recent addition to the tool kit of sequence-specific nucleases. Intense interest has been focused on the CRISPR/Cas9 system from Streptococcus pyogenes following initial reports of its successful use for gene editing **(Jinek *et al.*, 2012).**

Cai *et al.*, 2015 successfully applied type II CRISPR/Cas9 system to generate and estimate genome editing in the desired target genes in soybean (Glycine max (L.) Merrill.). The single-guide RNA (sg RNA) and Cas9 cassettes were assembled on one vector to improve transformation efficiency, and they designed a sg RNA that targeted a transgene (bar) and six sg RNAs that targeted different sites of two endogenous soybean genes (GmFEI2 and GmSHR). The targeted DNA mutations were detected in soybean hairy roots. The results demonstrated that this customized CRISPR/Cas9 system shared the same efficiency for both endogenous and exogenous genes in soybean hairy roots. The team also performed experiments to detect the potential of CRISPR/Cas9 system to simultaneously edit two endogenous soybean genes using only one customized sg RNA.

Parthenocarpy in horticultural crop plants is an important trait with agricultural value for various industrial purposes as well as direct eating quality. **Risa Ueta *et al.*, 2017**optimized the CRISPR/Cas9 system to introduce somatic mutations effectively into SlIAA9—a key gene controlling parthenocarpy—with mutation rates of up to 100% in the T0 generation. Furthermore, they analysed off-target mutations using deep sequencing indicated that our customized gRNAs induced no additional mutations in the host genome. Regenerated mutants exhibited morphological changes in leaf shape and seedless fruit—a characteristic of parthenocarpic tomato. And the segregated next generation (T1) also showed a severe phenotype associated with the homozygous mutated genome.

As per **Damiano Martignago**, using CRISPR, scientists from the Chinese Academy of Sciences produced a wheat variety resistant to powdery mildew, one of the major diseases in wheat. Similarly, another Chinese research group exploited CRISPR to produce a rice line with enhanced rice blast resistance that will help to reduce the amount of fungicides used in rice farming. CRISPR/Cas9 has also been already applied to maize, tomato, potato, orange, lettuce, soybean and other legumes. Recently, scientists showed that is possible to edit the genome of plants without adding any foreign DNA and without the need for bacteria- or virus-mediated plant transformation. Instead, a pre-assembled Cas9-gRNA rib nucleoprotein (RNP) is delivered to plant cells in vitro, which can edit the desired region of the genome before being rapidly degraded by the plant endogenous proteases and nucleases. This non-GM approach can also reduce the potential of off-target editing, because of the minimal time that the

RNP is present inside the cell before being degraded. RNP-based genome editing has been already applied to tobacco plants, rice, and lettuce, as well as very recently to maize.

Editing plant genomes without introducing foreign DNA into cells may alleviate regulatory concerns related to genetically modified plants. **Je Wook Woo et al., 2015** transfected preassembled complexes of purified Cas9 protein and guide RNA into plant protoplasts of *Arabidopsis thaliana*, tobacco, lettuce and rice and achieved targeted mutagenesis in regenerated plants at frequencies of up to 46%. The targeted sites contained germline-transmissible small insertions or deletions that are indistinguishable from naturally occurring genetic variation. If anyone thinking about GMOs and the *Flavr Savr* Tomato, plants are somehow always in the first row when it comes to genetic modification. It is no different with CRISPR. Researchers are currently experimenting with ways to improve crop disease resistance and environmental stress tolerance using the gene-editing tool **(Boyd LA et al., 2013).** A research team from Rutgers is working on a long-term project to genetically modify wine grapes and turfgrass in such a way that the methods can be implemented in a variety of other crops. Imagine having jasmines blossoming the whole year in Scandinavian countries or harvest pumpkins in February.

Undoubtedly, crop biologists are striving hard to engineer resistance against diseases, enhancing tolerance to low precipitation or survival under degraded rhizosphere by introducing advantageous genes taken from other varieties of similar species **(Noman *et al.*, 2016).** In agriculture, CRISPR-Cas9 is presently being employed to knock-out unwanted genes from crops to promote preferable traits. For example, Chinese researchers developed wheat line resistant to powdery mildew. Genome editing may escort to a few surprising developments in agriculture. Different allergy causing proteins have been detected in peanuts **(Hourihane et al., 1997; Skolnick et al., 2001**). Getting rid of these proteins is not easy. But new technology may likely to offer allergy-free peanuts. CRISPR-Cas9 technique advocates important changes in plant genome within our access. Gene editing can help in overcoming a hurdle that is polyploid plants showing duplicate genome copies, i.e., Wheat. Successful editing of wheat genome in China demonstrates that CRISPR-Cas9 is definitely "multiplexed" with enormous ability to affect all gene copies or to target several genes at the same time. With simultaneous modification of multiple traits, the CRISPR-Cas9 system would provide highly competent method to pyramid breeding **(Bortesi and Fischer, 2015).** Negative regulators of plant disease resistance and grain development can be amended for increasing yield and granting resistance to the host plant against targeted pathogens **(Song et al., 2016)**. Researchers working in polyploid crops like sugarcane, wheat need information about variation of sequence among diverse allelic forms to design precise gRNAs **(Mohan, 2016).**

One drawback to the CRISPR/ Cas9 system in plants concerns off-target effects. To assess these effects in plants, whole genome sequencing is the current gold standard. We have unlimited possibilities. CRISPR helps us see that GMO/ non-GMO binaries are overly simplistic. This one tool can perform many DNA nips and tucks and can up-regulate or down-regulate genes in ways that are not transgenic — yet are by no means inconsequential. Many CRISPR edits; won't involve any questions about foreign DNA, but will be equally dramatic in their effects. In crops and animals, "gene knockouts" can eliminate genes that affect food quality, divert energy away from valuable end products, and confer susceptibility to crop diseases. Using the Cas9 enzyme's powerful ability to enhance or suppress gene activity could touch on many important processes of crop and livestock metabolism, resistance and yield.

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