SEMINAR REPORT

CRISPR-Cas9 system for plant genome engineering

By

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DEPARTMENT OF PLANT BIOTECHNOLOGY

COLLEGE OF HORTICULTURE

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CERTIFICATE

This is to certify that the seminar report entitled **“CRISPR-Cas9 system for plant genome engineering”** has been solely prepared by **Ashwini M.N. (2018-11-006)**, under my guidance and has not been copied from seminar reports of any seniors, juniors or fellow students.

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This is to certify that the seminar report entitled **"CRISPR-Cas9 system for plant genome engineering"** is a record of seminar presented by **Ashwini M.N. (2018-11-006)** on 31st October, 2019 and is submitted for the partial requirement of the course MBB 591.

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INDEX

Sl. No.		Title	Page No.
1.	Introduction		8
	1.1	Phases of genome editing	8
2.	History of CRISPR		11
3.	Components of CRISPR		12
	3.1	Locus structure of CRISPR	12
	3.2	Cas genes and CRISPR subtypes	12
	3.3	CAS 9 enzyme	13
	3.4	Protospacer adjacent motifs	14
	3.5	crRNA , tracrRNA and gRNA	14
4.	Mechanism of CRISPR		15
5.	CRISPR-Cas Systems as a Gene-Editing Tool		15
6.	Steps for the improvement of plant properties through the CRISPR technique		16
	6.1	Pre-requisites	17
	6.2	How it works	17
7.	Applications of CRISPR CAS 9 in Agriculture		19
8.	Case study		21
9.	Limitations of CRISPR system		23
10.	Summary		23
11.	Conclusion		24
12.	References		25

13.	Abstract	26
14.	Discussion	29

LIST OF TABLES

Sl. No.	Title	Page No.
1.	Characteristics of different gene editing tools	10
2.	PAM sequences from different species and subtypes of Cas nucleases	14
3.	CRISPR techniques to overcome abiotic stress	20
4.	CRISPR techniques to overcome biotic stress	20
5.	CRISPR techniques to enhance nutritional and other traits	21
6.	Targeting mutagenesis efficiency in T_0 rice plants	22

LIST OF FIGURES

Sl. No.	Title	Page no.
1.	Phases of genome editing	8
2.	Zinc Finger Nucleases	9
3.	Transcription Activator-Like Effector Nucleases	9
4.	Simplified scheme of CRISPR array	12
5.	Classification of CRISPR system	13
6.	Mechanism of CRISPR system in Bacteria	15
7.	CRISPR-Cas workflow	17
8.	CRISPR system applications in agriculture	19
9.	pCAMBIA1300-OsU3-Cas9 binary vector	21
10.	Sequencing results of homozygous *Gn1a* and *DEP1* mutant rice plants	22
11.	Field yield of plants carrying novel alleles of *Gn1a* and *DEP1,* displayed in histograms	23

1. Introduction

With the increase in the global population, there is a demand for enhanced food production. Though several technologies has been developed in the past years, the production could be increased only up to certain extent. Breeders are in continuous efforts to develop crops with improved traits (Morrissey *et al.,* 2004). However, conventional method of crop improvement has several drawbacks as they are time consuming and require multiple generations of breeding. Soon after, the alternative approaches to knock down gene expression, such as antisense oligonucleotides and short interfering RNAs, have instead become standard. But these approaches only transiently reduce gene expression, and the effect is usually incomplete and can lead to off-target effects.

Recently mutation breeding and transgenic technologies are being used to understand the plant biology, but integration of the transgene into the host genome is nonspecific, sometimes unstable and is also a matter of public concern for the food crops (Stephens and Barakate, 2017). As a better alternative, genome editing technologies with site specific nucleases (SSNs) have gained popularity during the last decade.

Genome editing/ genome engineering is the process of addition, deletion, modification or replacement of DNA into genome of living organisms to produce desirable trait by generating double stranded breaks.

1.1.Phases of genome editing;

There are mainly two phases of genome editing as follows:

A) Creation of Double-Stranded Break (DSB)

B) Cell repair mechanisms

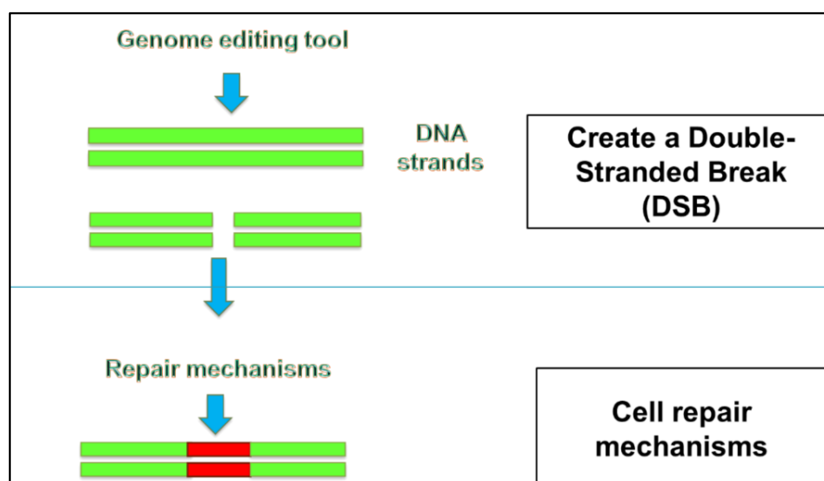

Fig. 1: Phases of genome editing

A) Creation of Double-Stranded Break (DSB)

Site-specific nuclease (SSNs) precisely generates DNA double strand breaks by breaking phosphodiester bond. ZFNs, TALENs and CRISPR are the three most popular genome editing tools.

a. ZFNs (Zinc Finger Nucleases)**:** It is composed of a three-finger binding domain and a *FokI* nuclease cleavage domain that are connected by a linker. Each finger binds to three base pairs. These arrays could be designed to bind a long stretch of DNA sequences when fused together (Kim *etal*., 1996). *FokI* domains dimerization generates DSBs.

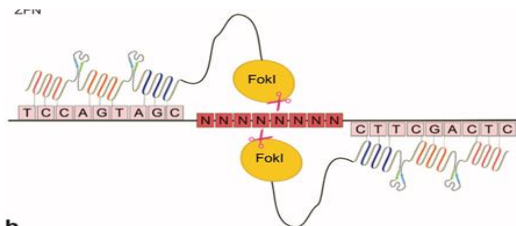

Fig. 2: Zinc finger nuclease

Despite the high specificity and precision of ZFNs they suffer from low reproducibility, cytotoxicity, sophisticated protein engineering for every single gene target (Ayman, 2019).

b. TALENs (Transcription Activator-Like Effector Nucleases)**:**These are natural proteins produced in the phytopathogen *Xanthomonas*. TALEN is a bi-modular protein which is made up of two domains namely a TALE DNA binding domain and a *FokI* endonuclease domain. The DNA-binding domain consists of monomers, each of them binds one nucleotide in the target nucleotide sequence. Monomers are tandem repeats of 33-39 amino acid residues, two of which are located at positions 12 and 13 and are highly variable and are referred to as Repeat Variable Diresidue (RVD) and are responsible for the recognition of a specific nucleotide (Gaz *etal*., 2013). Similar to ZFNs, dimerization of *FokI* domain generates staggered DNA cuts (sticky ends). However TALENs are more predictable, easy to design and faster than ZFNs. A clear disadvantage of TALENs over ZFNs is their larger size. This makes it harder to deliver and express a pair of TALENs into cells compared with ZFNs.

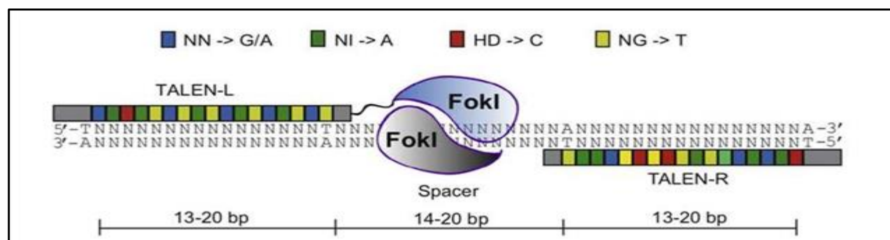

Fig.3:Transcription Activator-Like Effector Nucleases

However, the application ofZFNs and TALENs in genome engineering is limited due to delivery challenges, their large size, possible cytotoxic effects and the requirement of protein engineering for every DNA target of interest (Ayman , 2019).

c. CRISPR-Cas system (Clustered Regularly Interspaced Short Palindromic Repeats, 2012): Nearly about one to two years after the discovery of the TALEN proteins, another genome editing tool, CRISPR Cas system, which uses a combination of proteins and short RNAs to target specific DNA sequences for cleavage, was developed and started to be used extensively. Recognition of target DNA by the system is carried out by the complementary interaction between the target site DNA and a non-coding RNA. A complex of non-coding RNA and Cas proteins, which have nuclease activity, is formed and it will bind to target DNA to produce double stranded breaks.

B) Cell repair mechanisms

The DSBs are repaired through non-homologous end joining (NHEJ) or homology-directed repair (HDR) pathways.

a. HDR: Homology directed Repair, in which the cell uses a pre-existing DNA template to undergo the repair event (Ciccia and Elledge, 2010). It can be often used for gene correction, gene stacking and generation of protein variants of interest as it uses a repair template with pre-designed modifications (Krejci *et al*., 2012).

b. NHEJ: Non homologous end joining pathway, error prone which imprecisely re-ligate the DSBs and results in INDEL helps in functional studies of genes. As HDR is inefficient in most of the cell types and eukaryotic cells, NHEJ is harnessed for diverse applications in genome engineering (Mahfouz and Li, 2011).

Table 1:Characteristics of different gene editing tools

	ZFN	TALEN	CRISPR
Binding principle	Protein-DNA	Protein-DNA	RNA-DNA
Core components	ZFA-FokI fusion protein	TALE-FokI fusion protein	sgRNA and Cas9
Work mode (pair)	Pair	Pair	No
Design	Moderate	Easy	Very easy
Construction	Difficult	Easy	Very easy
Time for construction (days)	5–7	5–7	1–3

Cost	High	Moderate	Low
Efficiency	Variable	High	High
Off-target rate	High but variable	Low	High
Length of target sequence	~18- to 24-bp (including 5–7 bp spacer)	~50–60 bp (including 14–18 bp spacer)	~20 bp

(Source: Chen and Gao, 2012)

2. History of CRISPR :

- CRISPR was discovered by Yoshizumi Ishino and his colleagues (1987) in *Escherichia coli* in the course of the analysis of the gene responsible for isozyme conversion of alkaline phosphatase. They accidentally cloned a part of CRISPR sequence together with the "*iap*" gene.The organization of the repeats was unusual and at that time they did not know the function of the interrupted clustered repeats
- Later in 1993,Francisco Mojica discovered the existence of CRISPR sequence in bacteria and suggested that these sequences of DNA had repeats in them, with regularly-spaced intervals. His discovery of this concept was the basis for all future work done with CRISPR
- The term CRISPR was proposed by Jansen *et al*. (2002)
- In the early 2000s, the discovery of sequence similarity between the spacer regions of CRISPRs and sequences of bacteriophages, archaea, viruses, and plasmids finally shed light on the function of CRISPR as an immune system
- Mojica *et al*., 2003 proved that the CRISPR is an Adaptive Immune System of bacteria
- In 2005 the most substantial evidence was found, when three separate studies have revealed that the spacer short sequences in-between repeats are homologous to foreign genetic elements like plasmids and phage DNA (Bolotin *et al*., 2005; Pourcel (2005); Salvignol (2005); Vergnaud (2005).
- The function of the CRISPR-Cas system as a prokaryotic acquired immune system was finally experimentally proven in 2007, using the lactic acid bacterium *Streptococcus thermophilus* by Philippe Horvath and Rodolphe Barrangou
- Jennifer Doudna is considered as the Mother of CRISPR. She is among the first scientists to propose that the microbial immunity mechanism could be harnessed for programmable genome editing
- In 2011, Emmanuelle Charpentier the co-inventor of CRISPR together with Doudna, showed that the two RNAs (crRNA and tracrRNA) could function in vitro even when fused into a single-guide RNA (Concept of sgRNA)
- CRISPR systems are classified into two major classes; class 1 and class 2, where each is using a different mode of action (Makarova *et al*., 2015)

3. Components of CRISPR

- CRISPR locus
- Cas 9: CRISPR associated protein 9
- PAM: protospacer adjacent motif with sequence NGG (for Cas 9)
- gRNA: guide RNA – a construct/chimera of crRNA and tracrRNA

3.1.Locus structure of CRISPR

The CRISPR locus have several elements; Cas9 nuclease, tracrRNA, acquisition genes (Cas1, Cas2 & Csn2), CRISPR array leader sequence, spacers and repeats

The CRISPR array is made up of an AT-rich leader sequence followed by short repeats that are separated by unique spacers. CRISPR repeats typically range in size from 28 to 37 base pairs (bps). Each repeat is arranged in a palindromic fashion, sometimes leads to the formation of a secondary structure such as a stem-loop ('hairpin') in the RNA, The size of spacers in different CRISPR arrays is typically 32 to 38 bp (range 21 to 72 bp). These spacers function as an immunological memory bank, storing sequences from previous encounters with invading organisms. The number of spacers within a CRISPR array can range from as few as one to several hundred, depending on the species(Loureiro and da Silva, 2019).

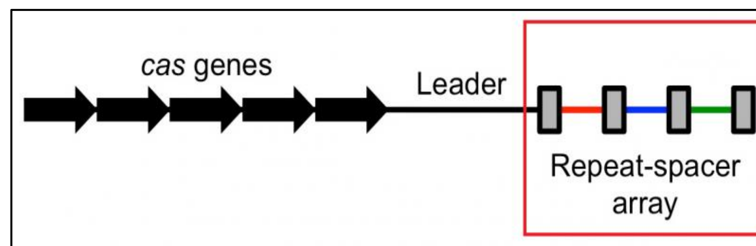

Fig. 4: Simplified scheme of CRISPR array

(Source: WikimediaCommons/AnnaJune)

3.2. Cas genes and CRISPR subtypes

Small clusters of cas genes are often located next to CRISPR repeat-spacer arrays. Variations in cas genes and different arrangements of CRISPR loci originate several types of CRISPR-Cas systems.

Recent studies suggest classifications of CRISPR/Cas system, with two classes and five - six types. Class 1 systems include types I, III, and IV, which all depend on multiprotein crRNA-complexes to execute their function; class 2 systems encompass types II, V, whose effector complex is composed of a single multi-domain protein. Further subgroups exist

within each type, with varied genetic composition and spacer structure. Cas proteins constitute the backbone of CRISPR systems. The distribution of Cas proteins among different types is highly variable. Some are present in most systems, such as Cas1 and Cas2, which participate in adaptation, while others are the signature proteins of specific types or subtypes.

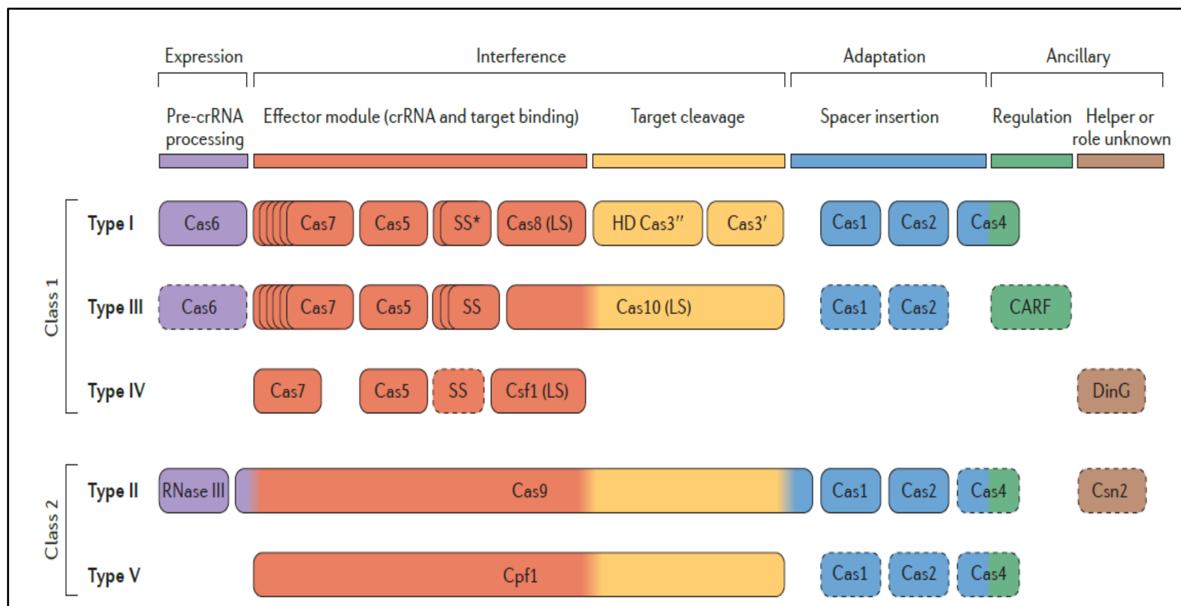

Fig. 5: Clasification of CRISPR system (Source: Mohanraju *et al.,* 2013)

3.3. CAS 9 enzyme

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 sequence (Belhaj *et al.*, 2013). It is a RNA-guided DNA endonuclease enzyme associated with the CRISPR adaptive immunity system in *Streptococcus pyogenes,* (hencealso called as SpyCas9).The organismutilizes Cas9 to memorize and later interrogate and cleave foreign DNA, such as invading bacteriophage DNA or plasmid DNA. Cas9 performs this interrogation by unwinding foreign DNA and checking for sites complementary to the 20 base pair spacer region of the guide RNA. If the DNA substrate is complementary to the guide RNA, the enzyme cleaves the invading DNA. In this sense, the CRISPR-Cas9 mechanism has a number of parallels with the RNA interference (RNAi) mechanism in eukaryotes.

Cas9 is a large (1,368-amino-acid) multidomain and multifunctional DNA endonuclease. It snips dsDNA 3 bp upstream of the PAM through its two distinct nuclease domains: an HNH-like nuclease domain that cleaves the DNA strand complementary to the guide RNA sequence (target strand), and an RuvC-like nuclease domain responsible for cleaving the DNA strand opposite the complementary strand (non-target strand). In addition

to its critical role in CRISPR interference, Cas9 also participates in crRNA maturation and spacer acquisition(Jaganathan *et al.,*2018)

3.4. Protospacer adjacent motifs

PAM is a 2-6 base pair DNA sequence immediately following the DNA sequence targeted by the Cas9nuclease in the CRISPR bacterial adaptive immune system. It is a component of the invading virus or plasmid, but is not a component of the bacterial CRISPR locus. Cas9 will not successfully bind to or cleave the target DNA sequence if it is not followed by the PAM sequence. PAM is an essential targeting component (not found in bacterial genome) which distinguishes bacterial self from non-self DNA, thereby preventing the CRISPR locus from being targeted and destroyed by nuclease.

Table 2: PAM sequences from different species and subtypes of cas nucleases

CRISPR Nucleases	Organism Isolated From	PAM Sequence (5' to 3')
SpCas9	*Streptococcus pyogenes*	NGG
SaCas9	*Staphylococcus aureus*	NGRRT or NGRRN
NmeCas9	*Neisseria meningitidis*	NNNNGATT
CjCas9	*Campylobacter jejuni*	NNNNRYAC
StCas9	*Streptococcus thermophilus*	NNAGAAW
LbCpf1	*Lachnospiraceae bacterium*	TTTV
AsCpf1	*Acidaminococcus* sp.	TTTV

3.5. crRNA, tracrRNA and gRNA

- **crRNA:** The integrated DNA is transcribed from the CRISPR locus to yield a mature transcript called the CRISPR RNA (crRNA) crRNA, specifically, is found in the CRISPR repeat/spacer library (loci) of prokaryotes as one long string of crRNAs. The cRNA is made of the spacer (complementary to the target) and a structural piece (the repeat) that complements with the tracrRNA. The cRNAs confer target specificity to Cas9, but they can't bind to Cas9 alone. The cRNAs have to complex with tracrRNAs, also known as the handle or scaffold region. Only then can the RNA:RNA /RNA:DNA duplex properly fit into Cas9. These tracrRNAs are not found inside the aforementioned library, but they're expressed separately by another promoter.
- **tracr RNA** : 25 bases - complementary to the CRISPR repeatstracrRNA and precursor of the crRNAs hybridized and processed into mature products by RNaseIII

- **gRNA/ sgRNA** : sgRNA is an abbreviation for "single guide RNA." As the name implies, a sgRNA is a single RNA molecule that contains both the custom-designed short crRNA sequence fused to the scaffold tracrRNA sequence. sgRNA can be synthetically generated or made *in vitro* or *in vivo* from a DNA template.

4. Mechanism of CRISPR

CRISPR-Cas9 was adapted from a naturally occurring genome editing system in bacteria. The bacteria capture snippets of DNA from invading viruses and use them to create DNA segments known as CRISPR arrays. The CRISPR arrays allow the bacteria to "remember" the viruses (or closely related ones). If the viruses attack again, the bacteria produce RNA segments from the CRISPR arrays to target the viruses' DNA. The bacteria then use Cas9 or a similar enzyme to cut the DNA apart, which disables the virus.

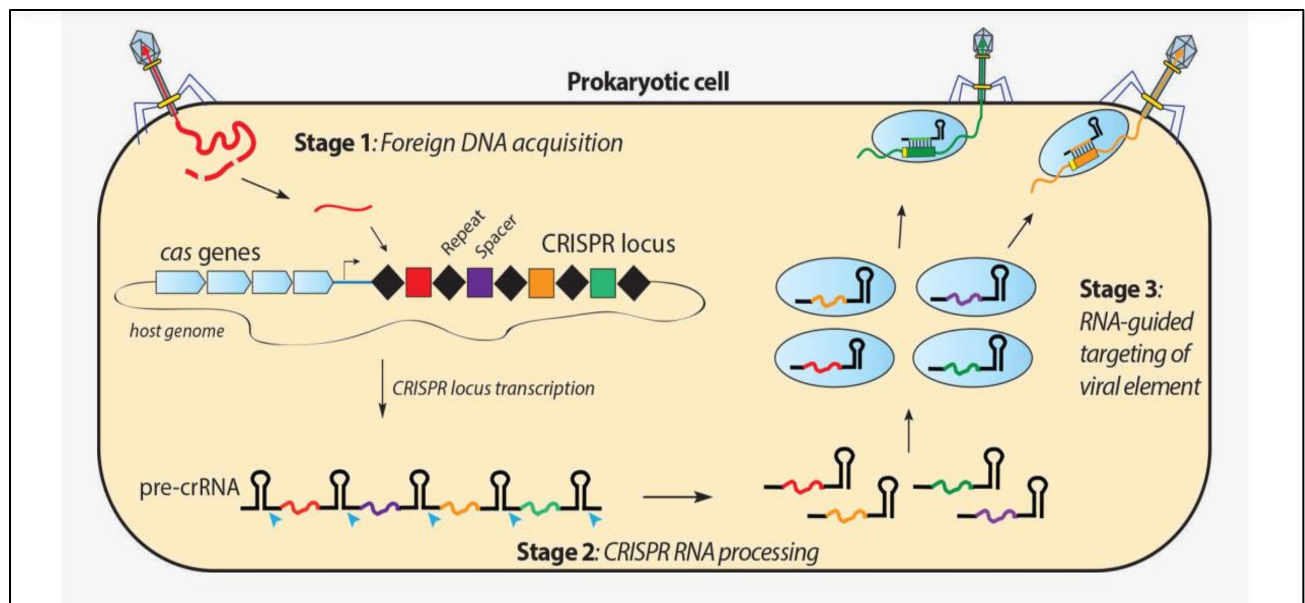

Fig. 6: Mechanism of CRISPR system in Bacteria

5. CRISPR-Cas Systems as a Gene-Editing Tool

The CRISPR-Cas9 system works similarly in the lab. Researchers create a small piece of RNA with a short "guide" sequence that attaches (binds) to a specific target sequence of DNA in a genome. The RNA also binds to the Cas9 enzyme. As in bacteria, the modified RNA is used to recognize the DNA sequence, and the Cas9 enzyme cuts the DNA at the targeted location. Although Cas9 is the enzyme that is used most often, other enzymes (for example Cpf1) can also be used. Once the DNA is cut, researchers use the cell's own DNA repair machinery to add or delete pieces of genetic material, or to make changes to the DNA by replacing an existing segment with a customized DNA sequence.

In a landmark paper released in June 2012 by Jinek and his peers laid the foundation to what would ultimately become a revolution in genome editing and transcriptional control. Jinek *et al*. (2012) hypothesised that in Type II systems, the dual guide RNA complex tracrRNA:crRNA of Cas9 could be fused into a single chimeric RNA by linking the 30 end of crRNA to the 50 end of tracrRNA. Such a technique would allow for programmed DNA cleavage through engineering of the chimeric RNA molecule, later designated as sgRNA or gRNA (guide RNA). This hypothesis was proven with the design five different gRNA molecules to target the green fluorescent protein (GFP) gene, which resulted in precise and efficient cleavage of a plasmid containing the GFP gene by the programmed Cas9 for all five gRNA molecules. Shortly thereafter, further discoveries unravelled the full potential of CRISPR as a tool for genetic editing. Jiang *et al*. (2013) used the CRISPR-Cas9 system to induce targeted mutations (insertions, deletions, and single-nucleotide substitutions) in the genome of *Streptococcus pneumoniae* and *E. coli* strains. Later they also demonstrated how CRISPR could be used as a new tool to regulate gene expression by either activating or repressing the transcription of bacterial genes. Soon, all sorts of cells and some multicellular organisms would be the object of CRISPR-mediated manipulation, such as human cell cultures, mice, plants, yeasts*etc*.The simplicity of Cas9 targeting has also inspired the generation of large gRNA libraries using array-based oligonucleotide synthesis. These libraries can be engineered to encompass multiple gRNAs for every target gene in a host organism, thereby greatly facilitating forward genetic screens and selections. In contrast to short hairpin RNA libraries(Sander and Joung, 2014).

The application of the bacterial CRISPR/Cas system to plants is very recent. In the August 2013 issue of Nature Biotechnology, three short reports described the first applications of the Cas9/sgRNA system to plant genome engineering. Shortly after, five more reports followed. The papers mainly focused on testing the CRISPR/Cas technology using transient expression assays, such as protoplast trans-formation and in-planta expression using *Agrobacterium tumefaciens* transient expression.

6. Steps for the improvement of plant properties through the CRISPR technique

1. Retrieve the target gene sequences
2. Analyze the gene structure
3. Select a suitable software
4. Design and select the gRNAs
5. Guide RNA synthesis and cloning
6. gRNA delivery
7. Screening of CRISPR edited plants

6.1. Pre-requisites

a. **The genomic target:** Itshould be
   - Unique compared to the rest of the genome
   - Present immediately adjacent to a PAM

b. **gRNA**
   - GC content
   - Length
   - Potential off-target effects

6.2. How it works?

- When present together, the Cas9 protein and the gRNA form a ribonucleoprotein complex.
- Cas9 undergoes a conformational change upon gRNA binding.
- Once the Cas9-gRNA complex binds a DNA target, the seed sequence will begin to anneal to the target DNA.
- The gRNA anneal to the target DNA in a 3′ to 5′ direction and Cas9 will then cut both strands of DNA at~3-4 nucleotides upstream of the PAM sequence.
- Mismatches between the target sequence in the 3′ seed sequence can abolish target cleavage, whereas mismatches toward the 5′ end is tolerated.

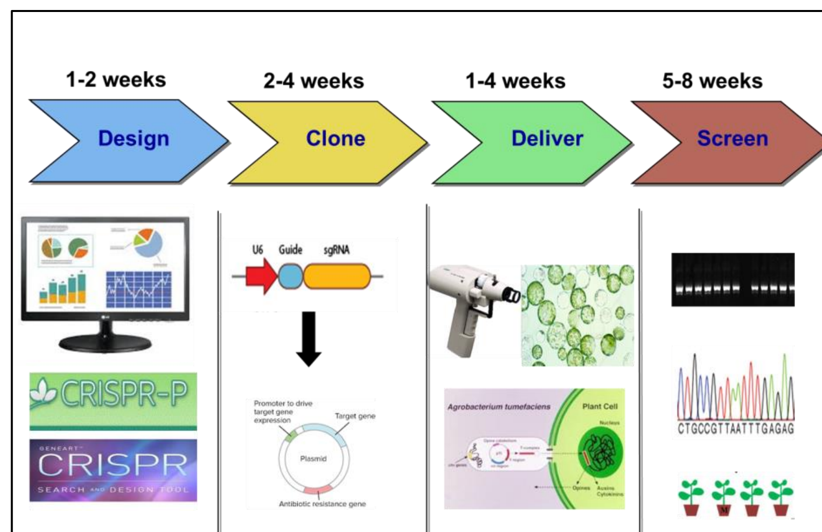

Fig. 7: CRISPR-Cas workflow

Step 1: Retrieve the target gene sequences

- Identify the gene of interest and obtain the desired gene sequence either from: Publicly available databases like NCBI, Phytozome.net, TAIR, MSU *etc.* or from a genome sequencing project

- It is recommended to sequence the target gene from the species you wish to work before you design gRNA

Step 2: Analyze the gene structure

- Find out exons and introns
- Genomic DNA sequences are used as input to design guides
- For activation and repression 5' UTR is used

Step 3: Selection of a suitable software

Most commonly used softwares are:

- CRISPR-P 2.0 (Plant specific)
- CRISPR PLANT (Plant specific)
- RGEN
- E-CRISP
- CRISPR DESIGN

Step 4. Designing and selection of gRNAs

Upload or paste the selected gene sequence to the specified window and set design parameters (length, GC content, organism, promoter *etc*.). The software will suggest a list of potential guide RNAs (20 nt and PAM) based on the on-score value.Higher the on-score value, better the efficiency.

Step 5: Guide RNA synthesis and cloning

- Get the oligos synthesized as top and bottom strands
- Anneal the top and bottom stands
- Restriction digestion of both gRNA and binary vector
- Ligation of gRNA to the vector and cloning to *E. coli*
- Mobilization to *Agrobacterium*
- Confirmation of clones by sequencing

Step 6: gRNA delivery: Common methods are *Agrobacterium* mediated transformation, Gene gun, Protoplast, Infiltration *etc*.

Step 7: Screening of CRISPR edited plants

For identification of putative transformants (Selection marker based), analysis of mutants can be done by:

- Sequencing by Sanger
- Restriction assays
- Real time PCR

7. Aplication of CRISPR-CAS 9 in Agriculture

CRISPR-Cas9 is currently used in genetic engineering, agriculture, and nutritional research.

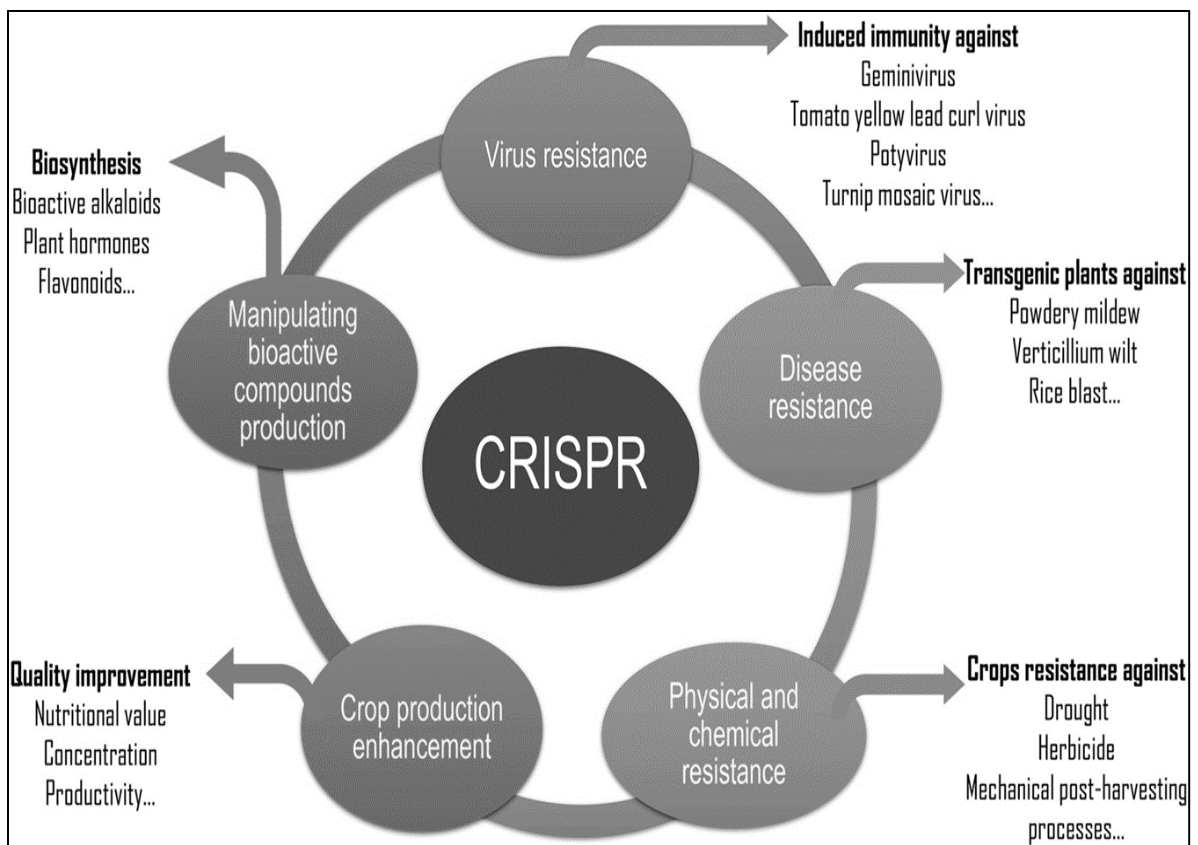

Fig. 8: CRISPR system applications in agriculture

(Source: Ismail *etal*., 2019)

The CRISPR-Cas gene-editing system is able to generate heritable, targeted mutations and also to address concerns over the presence of foreign DNA sequences as it can generate transgene-free plants.

Ricroch *et al*. (2017) studied about the agricultural applications related to the use of CRISPR systems in plants from 52 peer-reviewed articles published since 2014. They stated that, the main use of CRISPR systems is to achieve improved yield performance,

biofortification, biotic and abiotic stress tolerance in crop plants, with rice (*Oryza sativa*) being the most studied crop.

Table 3: CRISPR techniques to overcome abiotic stress

Crop	Target gene	Stress / trait	Reference
Rice	OsMPK2, OsDEP1	Yield under stress	Shan *et al*., 2014
Rice	OsDERF1, OsPMS3, OsEPSPS, OsMSH1	Drought tolerance	Zhang *et al.,* 2014
Arabidopsis thaliana	MIR169a	Drought tolerance	Zhao *et al*., 2016
Tomato	SIMAPK3	Drought tolerance	Wang *et al.,* 2017
Maize	ARGOS8	Increased grain yield under drought stress	Shi *et al.,* 2017
Rice	OsHAK-1	Low caesium accumulation	Cordones *et al*., 2017
Rice	OsPRX2	Potassium deficiency tolerance	Mao *et al*., 2018

Table 4: CRISPR techniques to overcome biotic stress

Crop	Target gene	Stress / trait	Reference
Bread wheat	TaMLO-A1, TaMLO-B1 & TaMLO-D1	Powdery mildew resistance	Wang *et al*., 2014
Arabidopsis thaliana	dsDNA of virus (A7, B7 & C3 regions)	Beet severe curly top virus resistance	Ji *et al*., 2015
Nicotiana benthamiana	BeYDV	Bean yellow dwarf virus resistance	Baltes *et al*., 2015
Rice (IR 24)	OsSWEET13	Bacterial blight resistance	Zhou *et al*., 2015
Nicotiana benthamiana	ORFs and IR sequence sDNA of virus	Tomato yellow leaf curl virus	Ali *et al*., 2015
Arabidopsis thaliana	eIF(iso)4E	Turnip mosaic virus resistance	Pyott *et al*., 2016
Rice	OsERF922 (ethylene responsive factor)	Blast resistance	Wang *et al*., 2016

Table 5: CRISPR techniques to enhance nutritional and other traits

Crop	Target gene	Stress/ trait	Reference
Maize	ZmIPK1A, ZmIPK & ZmMRP4	Phytic acid content	Liang *et al*., 2014
Tomato	Rin	Fruit ripening	Ito *et al*., 2015
Soybean	GmPDS11 & GmPDS18	Carotenoid biosynthesis	Du *et al*., 2016
Potato	ALS1	Herbicide resistance	Butler *et al*., 2016
Wheat	TaVIT2	Iron content	Connorton *et al*., 2017
Rice	25604 gRNA for 12802 genes	Creating genome wide mutant library	Meng *et al*., 2017
Cassava	MePDS	Carotenoid biosynthesis	Odipio *et al*., 2017

8. Case study

Developing superior alleles of yield genes in rice by artificial mutagenesis using the CRISPR/Cas9 system

Huang *et al*. (2018) showed the CRISPR mediated editing of alleles of two yield related genes in rice. The CRISPR-Cas9 system was used to edit two genes *viz*. Grain number 1a (*Gn1a*) and Dense and Erect Panicle1 (*DEP1*). Several mutants were identified by a target sequence analysis. Phenotypic analysis confirmed one mutant allele of *Gn1a* and three of *DEP1* conferring yield, superior to that conferred by other natural high-yielding alleles.

At first they constructed binary vectors (pCAMBIA1300-OsU3(Aar I)-Cas9) that contained the Cas9 cassette driven by the 35S promoter and simple guide RNA (sgRNA) cassette under the rice OsU3 promoter for efficient rice mutagenesis.

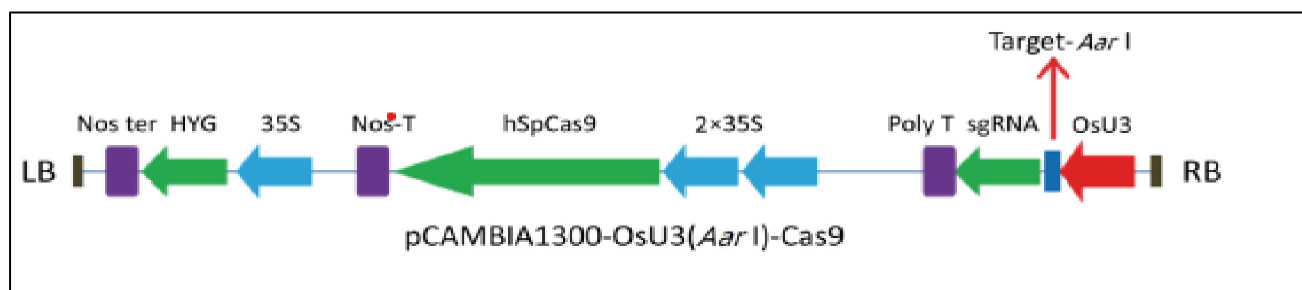

Fig.9: pCAMBIA1300-OsU3-Cas9 binary vector

Then the two binary vectors (p*Gn1a* and p*DEP1*) were transformed into the *Agrobacterium* strain EHA105 by electroporation and then further transformed into Nipponbare, a japonica rice cultivar**.**They generated the mutant rice plants and proceeded for genotyping of the mutations.

Table 6.Targeting mutagenesis efficiency in T_0 rice plants

Gene	Target sequence (5′–3′)	Position	T_0 Plants	Mutant Plants	Targeting efficiency (%)
1. *Gn1a*	AAGCAGTACCT GCCTTACTA	Exon 4	53	26	49
2. *DEP1*	AGCTGCGGTTG CAACGGCTG	Exon 5	48	19	39

All the artificial alleles were different from the natural ones, and most of them represented frameshift mutations.They selected 6 mutant alleles and performed sequencing followed by field evaluating the same along with the wild.

Fig.10:Sequencing results of homozygous *Gn1a* and *DEP1* mutant rice plants

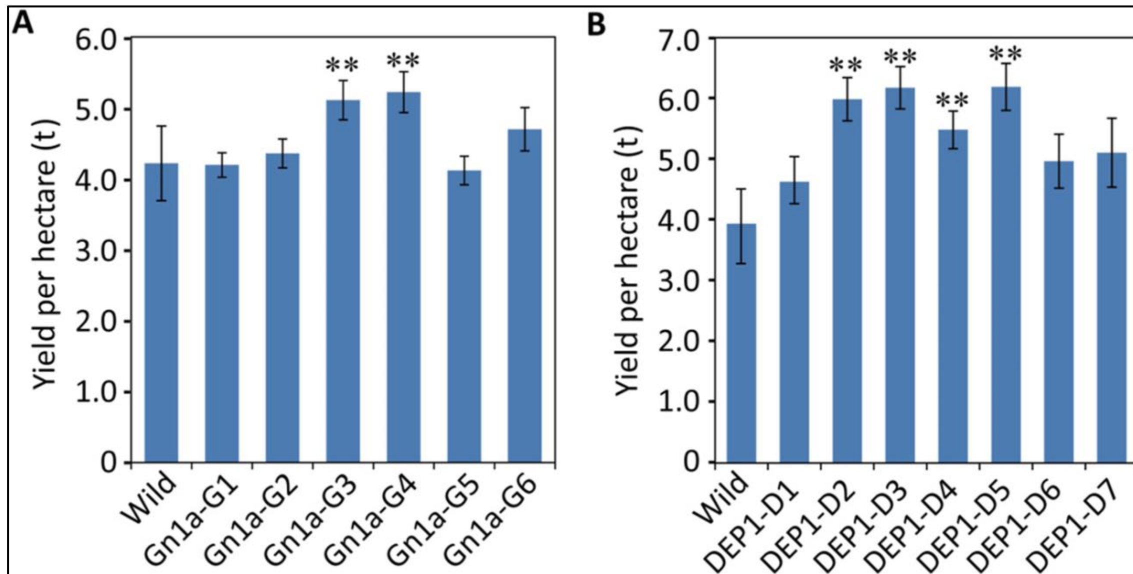

Fig.11: Field yield of plants carrying novel alleles of *Gn1a* (A) and *DEP1* (B), displayed in histograms

Result:

Phenotypic analysis by field yield evaluation confirmed that one mutant allele of *Gn1a* and three of *DEP1* conferring yield superior to that conferred by other natural high-yield alleles. The study reported that artificial mutagenesis by CRISPR could develop more superior alleles and provide ideal genetic materials for crop breeding.

9. Limitations OF CRISPR system

- This is not suitable for the introduction of more complex modifications such as targeted insertion of transgenes, recombinases or reporters, all of which still rely on homologous recombination in embryonic stems cells
- Off target effects

10. Summary

- Genome editing is the process of addition, deletion, modification or replacement of DNA into genome of living organisms to produce desirable trait by generating Double stranded breaks. There are mainly two phases in genome editing like creation of double stranded break and the cell repair mechanism
- Major tools for genome editing are Zinc Finger Nucleases (ZFN), Transcription Activator-Like Effector Nucleases (TALEN) andCRISPR-Cas9 system
- CRISPR-Cas9 was adapted from a naturally occurring genome editing system in bacteria

- The system has three important components viz. Cas9 (CRISPR associated protein 9), sgRNA (Single guide RNA)/ gRNA (which is a construct/chimera of crRNA and tracrRNA) and PAM (Protospacer Adjacent Motif)
- When present together, the Cas9 protein and the gRNA form a ribonucleoprotein complex. The Cas9-gRNA complex anneal to the target DNA in a 3′ to 5′ direction and Cas9 will then cut both strands of DNA at 3-4 nucleotides upstream of the PAM sequence
- Then the cell tries to repair the cut either through non homologous end joining or through homology directed repair, but the repair process is error prone which leads to mutation or changes in the target DNA sequence.
- The application of the bacterial CRISPR/Cas system in improving crop plants include crucial steps like retrieving the target gene sequence, analysing the gene structure, designing sgRNA using appropriate software), sgRNA synthesis and cloning, sgRNA delivery, screening for transformed plants and phenotypic analysis
- Potential applications of CRISPR system in Agriculture
- Case study on developing superior alleles of yield genes in rice by using CRISPR-Ca9 system
- Advantages and limitations of CRISPR-Cas9 system

11. Conclusion

CRISPR-Cas9 system has wide application in crop improvement such as enhancement of yield and nutritional value, pest and disease resistance, abiotic stress management etc. CRISPR-Cas9 based genome editing will gain popularity and be an essential technique to obtain 'suitably edited' plants that will help to achieve the zero hunger goal and maintain the growing human population.

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MBB 591: Masters Seminar

Name	:	Ashwini M. N.	**Venue**	:	Seminar hall
Admission No.	:	2018-11-006	**Date**	:	31-10-2019
Major Advisor	:	Dr. Haseena Bhaskar	**Time**	:	10.45 am

CRISPR-Cas9 system for plant genome engineering

Abstract

Genome engineering is the process of addition, deletion, modification or replacement of DNA into genome of living organisms to produce desirable trait by generating double stranded breaks.Genome editing technologies with site specific nucleases (SSNs) have gained popularity during the last decade. Zinc Finger Nucleases (ZFNs), Transcription Activator-Like Effector Nucleases (TALENs) and Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-Cas9 system are the most popular genome editing tools.

CRISPR is a family of DNA sequences found within the genomes of prokaryotic organisms such as bacteria and archaea. These sequences are derived from DNA fragments of bacteriophages that have previously infected the bacteria and are used to detect and destroy DNA from similar phages during subsequent infections. Hence, CRISPR provides acquired resistance against viruses in prokaryotes (Barrangou *et al*., 2007). There are two phases of genome editing *viz*. creating a double-stranded break (DSB) and cell repair mechanisms. The DSBs are repaired through non-homologous end joining (NHEJ) resulting in indels (deletions, insertions or substitutions) (Weterings and Chen, 2008) or homology directed repair (HDR) which introduces point mutations or total gene addition to the DNA sequence (Ciccia and Elledge, 2010).

The CRISPR-Cas9 system has three important components *viz*.Cas9 (CRISPR associated protein 9), sgRNA (Single guide RNA) - a construct/chimera of crRNA and tracrRNA and PAM (Protospacer Adjacent Motif) with sequence NGG (for Cas9). Cas9 is a large (1,368 amino acid) multidomain and multifunctional DNA endonuclease. An sgRNA is a single RNA molecule that contains the custom-designed short crRNA sequence fused to the scaffold tracrRNA sequence (Jinek *et al*., 2012). The sgRNA can be synthetically generated or made *in vitro* or *in vivo* from a DNA template.When present together, the Cas9 protein and the gRNA form a ribonucleoproteincomplex. The Cas9 undergoes a conformational change upon gRNA binding. Once the Cas9-gRNA complex binds to a DNA target,the seed sequence will begin to anneal to the target DNA. The gRNA anneal to the target DNA in a 3′ to 5′ direction and Cas9 will then cut both strands of DNA at 3-4 nucleotides upstream of the PAM sequence.

The application of the bacterial CRISPR-Cas9 system in improving crop plants include crucial steps like retrieving the target gene sequence, analysing the gene structure, designing sgRNA using appropriate software, avoiding off targets, sgRNA synthesis and cloning, sgRNA delivery, screening for mutants and phenotypic analysis. The CRISPR-Cas9 system has been efficiently utilized for improving the quality and yield traits and to improve the crop plants against various biotic and abiotic stresses (Ismail *et al*., 2019). Huang *et al*. (2018) showed the CRISPR mediated editing of alleles of two yield related genes in rice. The CRISPR-Cas9 system was used to edit two genes *viz*. *Grain number 1a* (*Gn1a*) and *Dense and Erect Panicle1* (*DEP1*). Several mutants were identified by a target sequence analysis. Phenotypic analysis confirmed one mutant allele of *Gn1a* and three of *DEP1* conferring yield, superior to that conferred by other natural high-yielding alleles. This study demonstrated that favourable alleles of the *Gnla* and *DEP1* genes, could be developed by artificial mutagenesis using genome editing technology.

CRISPR-Cas9 system has wide application in crop improvement such as enhancement of yield and nutritional value, pest and disease resistance, abiotic stress management *etc*. CRISPR-Cas9 based genome editing will gain popularity and be an essential technique to obtain 'suitably edited' plants that will help to achieve the zero hunger goal and maintain the growing human population.

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14.Discussion

1.How to overcome the limitations of CRISPR?

By Cas9 nuclease activity modifications, by using PAM variants, or by manipulating the sgRNA length we can overcome some of the limitations.Recently, new advancements over CRISPR technologies are developed like, Base editing or Prime editing.

2.What is the Indian scenario for CRISPR work?

Even though CRISPR workis started very recently, they are confined only to the laboratory research.Acceptance is less.

3.How CRISPR can be used for insect pest control?

If we know the molecular interaction between the plants and insect, then we can easily target the gene in the plant which is responsible for the interaction. By altering that gene the interaction will be lost, thus we can control the insect pests.

4.What is the basic difference between GMO and CRISPR Cas9 modified organisms?

GMO organisms are carrying foreign genetic materials, however, CRISPR alters the genetic material and the plants are free of foreign genetic materials.

5.How CRISPR edited plants will pass the regulatory procedures for transgenic ?

After editing the target sequence using CRISPR technology, the plants with the edited sequence are selected from the segregating population. In this way after two to three generation we will remove out the transgene from the plant. So there is no question of regulations as these plants are no more transgenic.

6.Is there any plant specific soft ware for CRISPR genome editing?

Yes,

1. CRISPR-P 2.0
2. CRISPR PLANT

7.Why the application of other genome editing technologies are limited?

The application of ZFNs and TALENs in genome engineering is limited due to delivery challenges, their large size, possible cytotoxic effects and the requirement of protein engineering for every DNA target of interest.