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CRISPR/Cas in crop betterment: A review

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Abstract
Dynamic environment changes have been created a major impact on agriculture sector. To meet the nutritional demands of the future along with overcoming the barrier of climate change there is necessity of development of efficient technologies for crop betterment. CRISPR/Cas technology is one of them. Implementing the CRISPR/Cas 9 technology to understanding the plant growth and development will be helpful to design crop betterment strategies.

Keywords: Agriculture, CRISPR/Cas9, crop betterment, technologies

Introduction
The major thing behind the selection of any crop is that it should be first domesticated well and then breeders/scientist tends to go for improvement of that particular crop for economic purpose by the means of selection or any other breeding approach. According to FAO. 2017 [21] report, most of the crops are get damaged by various pathogens that leads to depletion in yield level. The purpose of improvement of crop is to make them resistance to various diseases, pest like biotic and abiotic stress and thereby improving yield factor. Initially most of the people were not aware about selection and improvement of crop. But after the Mendel's discovery on pea plant, we know that how the character of any plant are controlled by genes that contributed by both of its parent. By such evolutionary work there is forth coming base of modern breeding methods get fixed likewise selection, backcross, pure-line, transgenic breeding and rDNA technology.

Transgenic breeding includes incorporation or insertion of foreign desirable gene into an organism that leads to development of new character or traits to the host plant. But sometimes it leads to affect or stop the expression of other desirable gene which is present already in the genome (Gelvin *et al*., 2003) [26]. To avoid such barrier the newly emerged genome editing technology have greater influence on crop betterment without any side effect. The genome editing requires various types of nucleases more specifically site specific nuclease enzymes to cut the DNA at proper site to edit. Till now there are four nucleases family have been exploited in gene editing namely Meganucleases, Zing Finger Nucleases (ZFNs), Transcription Activator like Effector based Nucleases (TALENs) and Clustered Regularly Interspaced Short Palindromic Repeats Associated Nucleases (CRISPR/Cas) (Gaj *et al*., 2013) [22]. Out of these four families the first ever nuclease which were utilized in genome editing was the Meganucleases (Silva *et al*., 2011, Demirci *et al*., 2018) [76, 18]. Along with ZFNs and TALENs are also utilized in plant and animal genome editing to diagnose various genetic diseases particularly in humans (Zhang, 2020) [98-100].

Now, CRISPR/CAS system has grabbed the advertence of scientists and now it is used extensively for gene editing. CRISPR is Clustered Regularly Interspaced Short Palindromic Repeats, this system is usually present in prokaryotes mainly Bacteria and Archaea. Repeats of CRISPER are complementary to viral DNA that get enters into the bacterial cell after infection and then by combining with nuclease (*i.e*. Cas it invade that viral or foreign DNA make a cut on virus DNA particles that ultimately result in degradation of viral DNA particles (Zhang *et al*., 2020) [98-100]. In 1987, the first CRISPR gene was accidentally cloned from *E. coli* (Ishino, 1987) [35]. After that many trials has been carried out to clone the CRISPR sequence from most of the prokaryotes (Nakata *et al*., 1989; Hermans *et al*., 1991; Mojica *et al*., 1993; Jansen *et al*., 2002) [64, 62, 36]. More over researchers were non familiar about the function of these nucleases and their significance in prokaryotes.

Long stretches of tandem repeats have been discovered in the genomes of the halophilic Archaea Haloferax mediterranei and Haloferax volcanii by Mojica *et al*. (2016) [63]. These repeats interspersed with 33-39 bp unique sequences, a 30 bp sequence with dyad symmetry

(Containing 5 bp inverted repeats). This structure spans several kilobytes in the *H. mediterranei* chromosome and roughly 3 kilobytes in the *H. volcanii* chromosome. The tandem repeats (called TREPs) have a comparable distribution in both organisms, appearing once or twice in the chromosomes of *H. volcanii* and *H. mediterranei*. *H. volcanii* transformed with a recombinant plasmid containing a 1.1 kb segment of the TREPs showed considerable changes in the host cells, especially in terms of cell viability. DAPI staining shows that adding more copies of TREPs to the vector changes the distribution of the genome among the daughter cells dramatically. Although the precise biological purpose of tandem repeats in halobacteria cannot be determined, all evidence points to them being involved in replicon partitioning. So the appropriate work of CRISPR remained in dark until the researchers found the gene that code for Cas9 enzyme (Jansen *et al*., 2002) [36]. Since then, we've learned that the CRISPR/Cas system is a naturally occurring and acquired immune defense mechanism in bacteria and archaea that protects them against foreign DNA material, primarily viruses. This discovery explored a novel technique of gene editing to scientific community by utilizing the immune system's inherent defenses (Zhang *et al*., 2020) [98-100]. According to Gasiunas and co-workers (2012) Clustered, regularly interspaced, short palindromic repeats (CRISPR)/CRISPR-associated (Cas) systems provides adaptive immunity against viruses and plasmids in bacteria and archaea. Also in same year Jinek and co-workers (2012) [39] reported that the CRISPR/Cas systems provides adaptive immunity against virus and plasmid by using crRNA to guide the silencing of invading nucleic acids. Jinek and colleagues (2012) [39] demonstrate that CRISPR RNA (crRNA) and trans-activating crRNA (tracRNA) can be combined to generate a single chimeric synthetic RNA molecule known as single guide RNA (sgRNA), which is ideal for genome editing. This study is significant and it simplifies the DNA construction for creating the CRISPR/Cas system as a biotechnological tool for genome editing. Technique is relatively simple as compared to previously utilized genome editing tools like ZFNs and TALENs. As a result, these two investigations paved the way for future research into harnessing the CRISPR/Cas system to modify genome sequences for a number of applications (Zhang *et al*., 2020) [98-100].

The goal of this paper is to give brief idea about the key characteristics of the CRISPR/Cas9 genome editing technique and how it can be used to improve disease resistance in plants.

CRISPR/CAS Mechanism

Cas is the enzyme which is endonuclease, CRISPR/Cas9 *i.e*. SpCas9 which is derived from Streptococcus pyrogens having variety of applications in research field including crop research (Ding *et al*., 2018; Liu and Moschou, 2018) [18, 51]. The Cas9 have various domain namely RuvC domain, HNH domain and PAM- interacting domain that helps in binding and editing of the genome. (Anders *et al*., 2014, Nishimasu *et al*., 2014) [5].

Out of these two domain one get attached to one strand and another attached to second strand of targeted dsDNA molecule. So for proper functioning of these two domain *i.e*. for efficient cutting of strand Cas enzyme must require to join to dsDNA to form complex and initiate Double Strand Break (DSB) (Fig. 1). Joining of Cas enzyme to genome is depends on recognition of specific site within the genome, those site are recognized only when there is presence of PAM motif (Protospacer Adjacent Motif). It means the PAMs are the pre-requisite for the initiation of genome editing process. However, the generated DSB get repaired by host cell DNA repair machinery involves two types of mechanism 1) NHEJ (Non Homologous end Joining) 2) HDR (Homologous Directed Repeat).

In NHEJ mechanism has less precision but frequency of repair is more while HDR has high precision but frequency of repair is less (Zhang *et al*., 2020) [98-100].

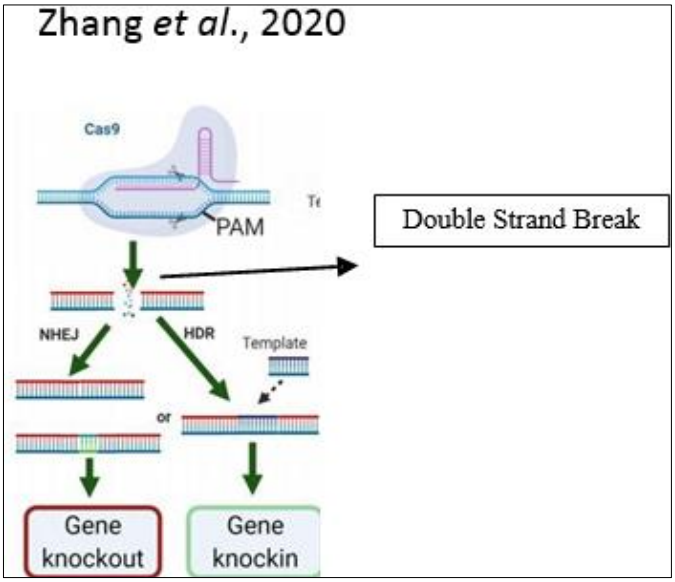

Fig 1: Double stand break

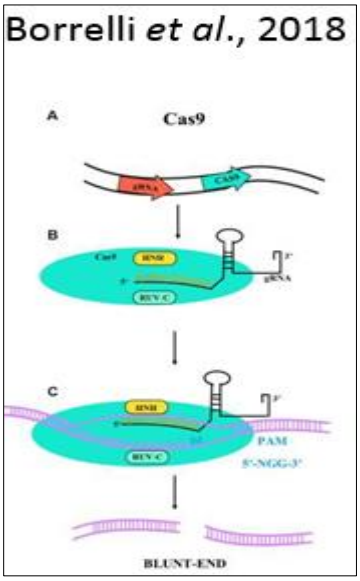

Fig 2: Blunt-End

NHEJ insert random nucleotides from the cut end so the chances of inserting wrong nucleotides may occur. On contarary, HDR requires template DNA which is homologous to targeted cut DNA. Template DNA may be sister chromatids or homologous or any other sequence which greatly related with targeted DNA. (Zhang *et al*., 2020) [98-100].

Why CRISPR/Cas system appropriately cut targeted sequences only? because system consist of SgRNA *i.e*. Single Guide RNA which can be made by combining CRISPR RNA (crRNA) with trans-activating crRNA (tracRNA) to generate

a single chimeric synthetic RNA molecule that works flawlessly for genome editing. (Jinek *et al*., 2012). CrRNA also called as Spacer and the function of that RNA is to join target sequence because it is complementary to target DNA (Borrelli *et al*., 2018). The tracRNA is responsible for identification/recognition of whole target DNA by identifying PAM present in that DNA and facilitating the recruitment of Cas enzyme at that site. So PAM serves as signal to bind the DNA sequence, hence without PAM no any recognition & cutting will take place. After that two domains of Cas *i.e.* RuvC & HNH make DSB at both strands with the help of Cas enzyme.

The specific requirement of PAM motif for recognition of cutting site limits the researcher to modify the genome by this CRISPR/Cas system. So, the main efforts could be spent on to modify the Cas enzyme to recognized other PAM sequences or by another way to construct/engineered naturally available Cas in such way that they should recognized different PAM sequences (Borrelli *et al*., 2018). Those Cas which are related to each other as well as those are different will be recognized by different PAM motif. So there were great scope to develop CRISPR/Cas system which is devoid of PAM will greatly enhance the research studies. (Zhang *et al*., 2020) [98-100]. Likewise xCas9 is evolved/derived from Spcas9 which have property to identify wide range of PAM sequence like NG, GAA & GAT in mammals, (Hu *et al*., 2018) where N from NG is any nucleotide (Gasiunas *et al*., 2012; Jinek *et al*., 2012). Another enzyme was Cpf1 is the Cas enzyme which is derived from the Prevotella & Francisella having PAM of TTTV in which V = A, C or G (Endo *et al*., 2016) Fig.3.

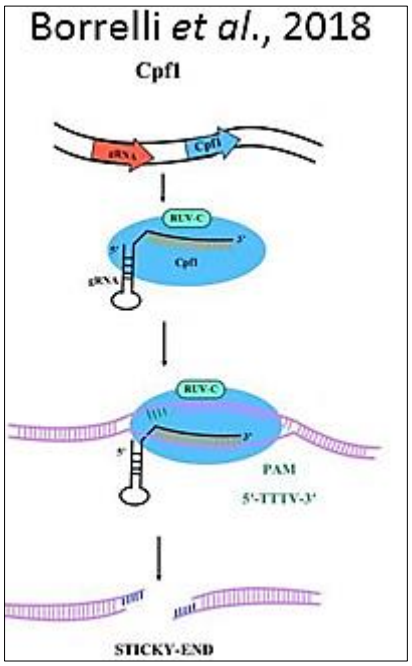

Fig 3: Sticky-End

Cpf1 is smaller than Cas9 and have ability to generate sticky/staggered ends while Cas9 produces blunt ends. So that Cpf1 can be a batter candidate to be used as an genetic engineer to get feasible gene combination by homologous directed repeat (HDR) mechanism.

For the successful and precise editing there is need to construct proper gRNA. Criteria should be made to design gRNA *i.e.* gRNA should be upto 20bp long, it should have intermediate GC content. If there is purine base in the most of PAM proximal positions then cutting efficiency of Cas will increase (Wang *et al*., 2014).

CRISPR sgRNA designing tools

To make out scientist and researchers easy for construction of sgRNA with proper peerless restriction sites for targeting insertions there are various tools are available. Already there are tremendous bioinformatics tools are available but these all are not equal some of them easy to use, some are difficult to handle, some are available online, some needs direct software, etc. In this review we provide various bioinformatics tools along with their availability for genome and web address. (Table: 1)

Table 1: CRISPR tools, availability and web address

Tool Name	Availability	Web Address
S	vertebrates, invertebrates, plants	http://cas9.cbi.pku.edu.cn/
CC Top	vertebrates, invertebrates, plants	http://crispr.cos.uni-heidelberg.de/
CGAT	Plants	http://cbc.gdcb.iastate.edu/cgat/
COSMID	vertebrates, invertebrates	https://crispr.bme.gatech.edu/
CRISPR design	vertebrates, invertebrates, arabidopsis	http://crispr.mit.edu/
CRISPRdirect	vertebrates, invertebrates, fungi	http://crispr.dbcls.jp/
Crispr Finder	Vertebrates invertebrates fungi	http://crispr.u-psud.fr/Server/
CrisprGE	various: plants, animals, fungi, prokaryotes, protists	http://crdd.osdd.net/servers/crisprge/
CRISPR Multitargeter	vertebrates, invertebrates, plants	http://www.multicrispr.net/
Crispr-P	Plants	http://cbi.hzau.edu.cn/crispr/

CRISPRseek	vertebrates, invertebrates, fungi, plants, protists	http://www.bioconductor.org/packages/release/bioc/html/CRISPRseek.html
CROP-IT	vertebrates: mouse and human	http://cheetah.bioch.virginia.edu/AdliLab/CROP-IT/homepage.html
E-crisp	vertebrates, invertebrates, plants, fungi, protists	http://www.e-crisp.org/E-CRISP/
flyCRISPR	invertebrates	http://flycrispr.molbio.wisc.edu/
GT-SCAN	vertebrates, invertebrates, plants, fungi	http://flycrispr.molbio.wisc.edu/
sgRNAcas9	vertebrates, invertebrates	http://www.biootools.com/col.jsp?id=140
SS Finder	N/A	https://code.google.com/p/ssfinder/

The ability to search for matching sgRNAs by gene name, offer options for using alternative PAM sequences, offer options to predict off-targets, separate and/or rank lists of identified targets, and aggregate all analyses into a single and all are aspects within single tool these all criteria for comparing sgRNA design tools (Brazelton *et al*., 2015). Here we providing some tools comparison regarding their service (Table: 2).

Table 2: Tools comparison on the basis of service

Tool Name	Web server	Search by gene name	Alternate PAM sequence	Predicts off-targets	Rank output	All in one tool
Cas9-Design	✓	×	×	✓	×	✓
CCTop	✓	×	✓	✓	×	✓
CGAT	✓	✓	×	✓	✓	✓
CHOPCHOP	✓	✓	✓	✓	✓	✓
COSMID	✓	×	✓	✓	✓	✓
CRISPR design	✓	×	✓	✓	✓	✓
CRISPRdirect	✓	✓	✓	✓	✓	✓
Crispr Finder	✓	×	×	✓	×	×
CRISPR Multitargeter	✓	×	✓	✓	×	×
Crispr-P	✓	✓	✓	✓	✓	✓
CRISPRseek	×	×	✓	✓	×	✓
CROP-IT	✓	✓	✓	✓	✓	×
E-crisp	✓	×	✓	✓	×	×
flyCRISPR	✓	×	✓	×	×	✓
GT-SCAN	✓	×	✓	✓	×	✓
sgRNAcas9	×	×	✓	✓	×	×
SS Finder	×	×	×	×	×	×

Transfer of CRISPR/Cas System into host genome

As we know that, to initiate the editing of any organism's genome the CRISPR/Cas system should be delivered into that organism. So for incorporation of this system to any organism following methods can be used (Zhang *et al*., 2020) [98-100].

1. Agrobacterium Mediated Gene Transfer.
2. RNA & Protein Level Delivery.
3. Biolistic/Particle Gun Transformation/Particle Bombardment.
4. Direct Transformation of Protoplast.

Char *et al*., 2017 carried out the transfer of nCRISPR/Cas system into maize via. Agrobacterium mediated gene transfer. Also in case of rice plants Agrobacterium and Biolistic methods are used for transformation. (Baysal *et al*., 2016) [8]. Particularly to increase copy number in host plants biolistic method is preferably used. Protoplast transient assay is becoming an efficient approach for assessing CRISPR/Cas in maize which is get revealed by lot of studies (Gao *et al*., 2013; Jiang *et al*., 2013; Xie and Yang, 2013; Zhou *et al*., 2014; Lowder *et al*., 2015; Li *et al*., 2016; Luo *et al*., 2016; Wang *et al*., 2016) [24, 38, 93, 105-106]. In case of wheat there is up to 90% efficiency of transformation was recorded by the Agrobacterium mediated transfer (Ishida *et al*., 2015 a, b) but bombardment method were most preferably adopted in wheat transformation and it became a standard method (Hakam *et al*., 2015; Wang *et al*., 2018). The large number of successful transformations were demonstrated with the help of Agrobacterium mediated transformation rather than biolistic method or any other method.

Besides of these above methods, the transfer of DNA or RNA which codes for Cas9 enzyme also preferred. (Svitashev *et al*., 2015; Zhang *et al*., 2016). Along with that *in vitro* assembled complex of Cas9 protein along with gRNA could be used for transformation. (Malnoy *et al*., 2016; Svitashev *et al*., 2016; Liang *et al*., 2017). It has been found that biolistic method and direct DNA transfer have increased the DNA repair mechanisms in host than other inclusions, so that it would prefer HDR than NHEJ (Borrelli *et al*., 2018).

Some researchers also used the promoters from model plant species like Arabidopsis and few research studies revealed that using the promoter from target species will increase the gRNA expression as well as editing work (Zhang *et al*., 2020) [98-100].

After transforming any gene the plant species there is need to set an appropriate protocol for transferring this gene to next generation through tissue culture. If such protocol is not available for regeneration for particular species then there is no means of transformation. To overcome such problems scientist carried out co-transformation of developmental regulators such as stm (SHOOT MERISTEMLESS) & wus (WUSCHEL) with CRISPR/Cas9 system in tobacco plant (Maher *et al*., 2020; Gallois *et al*., 2002), due to which transformed somatic cells of plant give rise to shoot directly, So this method introduces novel mechanism for obtaining genome editing events. However more research is needed to see if this strategy is utilized to developed other plant preferably important agricultural crops.

Applications in crop improvement

Crop yield ultimately improves when there is affinity/susceptibility towards pathogen infection is reduced. This target can be achieved by increasing the resistance in host plant to target the pest and stresses. Earlier scientist preferred traditional breeding methods to confer resistance into plants but now scientist got aware about mechanism of CRISPR/Cas system to increase the resistance against various viruses, fungi, bacteria & abiotic stress in various crops.

Biotic Stress

Resistance against fungal pathogens by CRISPER/Cas system:

It has been reported that about 30% plant diseases are caused by fungus (Giraud *et al*., 2010). According to Buschges *et al*., (1997), the MLO - Mildew Resistance Locus is accountable to support the infection caused by powdery mildew fungus in barley crop. MLO genes are responsible for encoding the one of the 7 trans-membrane protein which universally present in monocotyledon and dicotyledon plants (Acevedo-Garcia *et al*., 2014). The CRISPR/Cas9 system get utilized to deactivate MLO gene in bread wheat (Wang *et al*., 2014) by using TALEN the 3 MLO genes in the wheat get deactivated that enables in creating resistance against powdery mildew pathogen. This was the first ever attempt to develop disease resistance plant by using this system.

Thereafter a lot of research has been carried out to develop and edit the plant genome for economical purpose. In tomato 16 SlMlo genes were knockout by detection of 48bp region. The plants which were edited then selfed to obtain a new plant progeny designated as new strain "Tomelo" which is completely resistant to powdery mildew (Nekrasov *et al*., 2017). In grapes (Chardonnay variety) by targeting MLO-7 gene editing with the help of CRISPR/Cas9 system which is delivered by RNP protein system confers resistance against powdery mildew (Malony *et al*., 2016). In case of watermelon the Fusarium oxysporum f.sp.niveum (FON) causes vascular wilt disease and which is responsible for yield reduction upto 30-80% (Martyn & Netzer, 1991). In current there is no any cultivar or germplasm which having resistance against this fungal pathogen (Zhang *et al*., 2020) [98-100]. The knockdown lines confers resistance against wilt pathogen. In cotton Verticillium wilt caused by Verticillium dahilae pathogen and against this disease resistance is developed by knockout of the 14-3-3 genes by CRISPR/Cas9 system in cotton (Zhang *et al*., 2016) [102].

Resistance to bacteria by CRISPR/Cas

There are numerous bacterial species on earth but only few of them are responsible to cause damage to crops (Schloss & Handelsman, 2004).

Generally few research has been carried out to confer resistance against bacteria using CRISPR/Cas system. In rice, a disease bacterial blight which is caused by Xanthomonas oryzae, against which the resistance was developed by mutagenesis of promoter region of gene osSWEET 14 by using TALEN approach. It reduces the binding ability of effector to promoter and results in developing resistance to pathogen (Li *et al*., 2012). Similarly in same crop, and same disease the osSWEET 13 gene responsible for production of sucrose transporter which enables the more infection of X. Oryzae by more expression of osSWEET 13gene. So by editing the promoter sequence of this gene reduces disease severity by avoiding effector binding to the promoter (Zhou *et al*., 2015) & also Os8n3n gene *i.e*. SWEET 11 gene edited that confers resistance against blight disease (Kim *et al*., 2019).

In apple crop for fire blight disease, resistance developed by using 3 sg RNA namely RG2, RG4 and RG7 to make site specific DSB in the loci of DIPM-1, DIPM-2 & DIPM-4 respectively (Malony *et al*., 2016). In citrus plant disease resistance were developed for citrus canker caused by Xanthomonas citri pv citri (Peng *et al*., 2017) by knocking out the transcription factor CsWRKY22 (Wang *et al*., 2019) [69, 70].

The primary agent of tomato bacterial speck disease, Pseudomonas syringae pv. Tomato (Pto) DC3000, generates coronatine (COR), which increases stomata opening and enables bacterial leaf colonization. They discovered that tomato has a functional ortholog of AtJAZ2 that it accumulates preferentially in stomata and that SlJAZ2 was a significant co-receptor of COR in stomatal guard cells. The dominant JAZ2 repressors were created by editing SlJAZ2 with CRISPR/Cas9 to remove the C-terminal Jas domain (SlJAZ2Djas). SlJAZ2Djas conferred resistance to Pto DC3000 by avoiding stomatal reopening but not to the necrotrophic fungal pathogen Botrytis cinerea (Ortigosa *et al*., 2018).

Resistance to viruses by CRISPR/Cas

Virus are the host specific pathogenic organisms that causes destruction of whole crop. According to Roossinck and co-workers (2015) viruses are divided into following different groups by considering their genome organization –

1. dsDNA Virus
2. ssDNA Virus
3. Negative Sense Single Stranded-RNA (ssRNA-) Virus
4. Positive Sense Single Stranded-RNA (ssRNA+)

Geminiviridae is a major family of plant viruses that is responsible for widespread agricultural losses in Cucurbitaceae, Euphorbiaceae, Solanaceae, Malvaceae & Fabaceae family (Zaidi *et al*., 2016). Hence CRISPR/Cas editing of of ssDNA Gemini viruses has been carried out (Ali *et al*., 2015, 2016; Baltes *et al*., 2015; Ji *et al*., 2015). The first studies confirming resistance against virus was by using the CRISPR-Cas technology to target the bean yellow dwarf virus (BeYDV) genome and to prevent crop losses due to geminivirus infection. CRISPR- Cas reagents generated mutations within the viral genome and lowered virus copy quantity in transient tests utilizing BeYDV-based replicons. Transgenic plants that were challenged with BeYDV and expressed CRISPR-Cas reagents had lowered viral loads and symptoms, showing a unique technique for building geminivirus resistance (Baltes *et al*., 2015 and Ji *et al*., 2015).

Baltes and co-workers (2015) in plant N. benthamiana have employed nearly 11 sgRNA which target replication motifs as well as other binding sites and nucleotide sequence of BeYDV & demonstrated that 87% decrease in yield reduction. Pyott & co-workers (2016) used CRISPR/Cas9 technology to successfully create total resistance to Turnip mosaic virus (TuMV), a key disease in field-grown vegetable crops, by introducing sequence-specific detrimental point mutations at the eIF(iso)4Elocus in Arabidopsis thaliana.

Chandrasekaran and co-workers (2016) demonstrate that the development of virus resistance in cucumber (Cucumis sativus L.) by disrupting the function of the recessive eIF4E (eukaryotic translation initiation factor 4E) gene using Cas9/sub genomic RNA (sgRNA) technology. The N and C

termini of the eIF4E gene was targeted with Cas9/sgRNA constructs. In the eIF4E gene targeted locations of transformed T1 generation cucumber plants, small deletions and single nucleotide polymorphisms (SNPs) were found, but not in putative off-target regions. For the creation of non-transgenic T3 generation plants, non-transgenic heterozygous eIF4E mutant plants were chosen. Cucumber vein yellowing virus (Ipomovirus) immunity and resistance to the potyviruses like Zucchini yellow mosaic virus and Papaya ring spot mosaic virus-W were found in homozygous T3 progeny following Cas9/sgRNA targeting both eIF4E sites. Heterozygous mutant and non-mutant plants, on the other hand, were extremely vulnerable to these viruses. For the first time, virus resistance in cucumber has been created non-transgenically, with no visible effects on plant development and no long-term backcrossing, using a new approach that might be used for various crop plants. Rice Tungro Disease (RTD) is a major stumbling block to rice production in tropical Asia. The interaction of Rice Tungro Spherical Virus (RTSV) and Rice Tungro Bacilliform Virus causes RTD. The translation initiation factor 4 gamma gene is responsible for natural RTSV resistance (eIF4G). The Y1059, V1060, V1061 residues of eIF4G have previously been linked to RTSV reactions. Mutations in eIF4G were created using the CRISPR/Cas9 technology in the RTSV-susceptible variety IR64, which is widely farmed across tropical Asia, to establish new sources of RTD resistance (Macovei *et al*., 2018) [58].

Resistance against insect attack by CRISPR/Cas

Insect causes damage to the crop not only by feeding on different plant parts but also by serving as a vector for other parasitic viruses (Chang *et al*., 2021). Chemical pesticide may not be practicable due to their transient benefits which typically associated with negative environmental consequences and in some cases can exacerbate a farmers overall insect problem (Akhtar *et al*., 2009). Host plant resistance (HPR) is one alternative to chemical control of insect pests (HPR). Although HPR's full potential has yet to be realized, it is environmentally benign and compatible with other control methods. In a constantly changing environment, the key challenge for today is to create insect pest resistance types that can boost and sustain crop yield (Wani *et al*., 2022) [92].

Traditional breeding procedures can be speed up by using molecular markers to identify and deploy insect resistance quantitative trait loci (QTLs). Several QTLs for insect pest resistance have been cloned using positional cloning techniques. CRISPR/Cas9 genome editing technology are paving the way for customizing insect pest resistance loci for future crop design. Focused genome editing aided by CRISSPR associated nuclease protein (Cas) system has enabled accurate, efficient and targeted alteration of target genes linked with insect resistance and agronomically relevant trait as a complement to breeding efforts (Gui *et al*., 2020; Wang *et al*., 2018) [28].

Kandul *et al*.,(2019) in Drosophila, systematically developed numerous pgSIT systems that reliably produce 100% sterile males. It has further shown that pgSIT-generated sterile male were healthy and competitive, expecting that pgSIT will cause far more population suppression than currently existing self-limiting suppression methods, based on mathematical models. By the use of CRISPR/Cas9 technology the Gui *et al*., (2020) [28] carried out mutagenesis of vestigeal gene (vest) in Colorado potato beetle (CPB) and formed wingless adult having no elytron this will help to control the CPB in eco-friendly way. There are presence of olfactory receptors in insect for recognition of host for laying eggs, mating with female and also for some other interactions. So by mutating such loci which control olfactory action by CRISPR/cas9 helps to reduce affinity of insect towards host.

In 2016, carried out mutation of Or83b gene by CRISPER/Cas9 system that results in being defective olfactory receptors that abrupt the host selection to lay the eggs. In case of Spodoptera litura the gene Orco (Olfactory receptor coreceptor) was mutated by this system which cause disruption in insect ability to select the host and also in to the search the partner for mating.

Detoxicants are the chemicals especially enzyme released by the insects that destroy the host defense system and causes severe damage. By targeting and mutating the genes which produces such enzyme will help to reduce susceptibility of host. So in case of Helicoverpa armigera the gene CYP6AE get deactivated that ultimately results in detoxification of harmful chemicals (Wang *et al*., 2018a).

We can also able to edit genome of crop to make them insect resistant but this strategy has been less explored. In rice CYP71Al gene get mutated by CRISPR/Cas9 this gene responsible for coding the tryptamine 5-hydroxylase which leads to serotonin production and there by cause increase in resistance of plant. So such mutated plant confered resistance against striped stem borer (Chilo suppressalis) and brown plant hopper (Nilpavarata lugens) (Lu *et al*., 2018) [55].

B] Abiotic stress

There are several abiotic stresses like drought, salinity, temperature and different the environmental pollutions. Also there is several genes are responsible for expression of plant towards stress via regulatory gene, non-coding RNA (Zhang 2015) [97].

Shi *et al*., (2017) [77] edited AGROS8 gene from the maize crop with the help of CRISPR/Cas9 system that resulted in improving tolerance of maize to the drought. Tomato can able to grow normally even in heat stress due to knockout of SIAGL6 gene with the use of CRISPR technology (Kalp *et al*., 2017). Additionally in tomato there was also knockout of the AR4 transcription factor by CRISPR system to increase water use efficiency and osmotic pressure (Bouzroud *et al*., 2020). Recently in case of Arabidopsis the mutation of dpa4-sod7-aitr256 was done, that increased tolerance towards drought (Chen *et al*., 2019). The mutation of gs3 and dep1 gene in rice by CRISPR/Cas improve the rice tolerance to different abiotic factors but more specifically to the salinity (Cui *et al*., 2020). In rice again Wang and co-workers (2019), knocked the ppa6 gene offering tolerance to alkaline stress. A key positive regulator of the drought stress response is the abscisic acid (ABA)-responsive element binding protein 1/ABRE binding fact or (AREB1/ABF2). By converting chromatin to a relaxed state in Arabidopsis with histone acetyltransferase 1 (AtHAT1) enhances gene expression activation. The CRISPRa dCas9HAT system was used to activate the endogenous promoter of AREB1, and the resultant plants had a dwarf phenotype. Then, in comparison to the control plants, the gene positively controlled by AREB1 showed increased gene expression. Under water stress, the plants were showed increased chlorophyll content and faster stomatal aperture, as well as a higher survival rate in drought stress (Roca Paixao *et al*., 2019) [72].

C] Miscellaneous Application

The CRISPR system has been extensively utilized to investigate the effect of genes on plant growth and development as well as a reliable method for altering plant morphology to improve plant growth and development.

Arginase is an enzyme which responsible for regulation of process regarding development of root which is encoded by ARG gene. This ARG gene were knocked in cotton by CRISPR/Cas9 that resulted in increased lateral roots development in T_1 knockout generation of cotton by 25% (Wang *et al*., 2017). It also helps in absorption of more water and nutrients equally, facilitating plants growth and development (Peng *et al*., 2020) [69, 70]. In rice editing of transcription factor gene MADS78 &MADS79 belongs to MADS box to reduce the development time of seed in rice *i.e.* early development occurs (Paul *et al*., 2020) [68]. Double knockout mutant of either of these two gene prevent seed development in rice while single mutant increases earlier endosperm cellularization (Paul *et al*., 2020) [68]. Lee & co-workers (2020) [45] demonstrated that the hexokinase is important for pollen germination as well as for growth of pollen tube. So they created the rice mutant lines which shows male sterility *viz*, hxk5-1, hxk5-2, hxk5-3 & hxk5-4.

OP II complex (Coat Protein complex - II) responsible for sporophytic or gametophytic development of pollen in Arabidopsis (Liang *et al*., 2020). So by knocking out the Sar1b & sar1c gene by CRISPR/Cas 9 development of sterile mutant is achieved. In rapeseed Li *et al*., 2018 developed a mutant Bnspf3 which showed delay in phenotype development. In *Brassica napus* the mutant of BnaCO3 TFL1 gene resulted in early flowering & also mutant of BnaTFL1 gene caused the change in plant morphology (Sriboon *et al*., 2020) [78]. In case of tomato there was mutant generation by CRISPR/Cas 9 technology in promoter region of same gene resulted in improved inflorescence and also change in plant morphology (Rodriguez-Leal D *et al*., 2017) [73].

As we know the yield is most important factor in crop improvement. Yield traits are governed by various polygenes so far yield improvement is quite difficult because of its deleterious effect on environment & other inhibitory genes. According to Li and colleagues (2016), knocking down the gn1a, dep1, or gs3 gene dramatically increased the some rice production parameters such as grain size, grain weight, grain number, dense panicles, and erect panicles. Also in 2016 Xu & co-workers showed that by editing gw2, gw5 & tgw6 genes in rice by CRISPR/cas9 technology developed a mutant having increased weight of grain and size, before that mutant development of the same genes act as negative regulator of grain weight. In case of tomato, bigger fruits can be developed by editing the regulatory element CLV-WUS with this technology (Rodriguez-Leal D *et al*., 2017) [73]. By replacing negative promoter in maize by CRISPR/Cas system generate ARGOS 8 variant. The ARGOS8 variations boosted grain output by five bushels per acre under flowering stress circumstances and had no yield loss in well- watered conditions, according to field research. These findings shows that how the CRISPR/Cas9 system can be used to generate unique allelic variation for developing drought- tolerant crops (Shi *et al*., 2017) [77]. OsLOGL5, a rice cytokinin-activation enzyme-like gene, was over expressed in a constitutive ectopic manner, resulting in significant reductions in primary root growth, tiller number, and yield. Under well-watered, drought, normal nitrogen, and low nitrogen field circumstances in several geographical regions, mutations at the 3′-end of the OsLOGL5 CDS resulted in normal rice plant shape but enhanced grain yield (Wang *et al*., 2020). Rice with knocked out G protein genes namely gs3 and dep1 knocked out grew with better agronomic features, such as grain size and number per panicle (Cui Y *et al*., 2020). CRISPR/Cas technology has also greater impact on increasing quality level as well as production of several biomolecules. As there is presence of lycopene in tomato fruits which imparts pink colour to fruits. This lycopene have greater role in treating the diseases like cancer & cardiovascular disease in humans. CRISPR/Cas9 system used to edit several genes in tomato that leads to increase in lycopene production in tomato (Li x *et al*., 2018). The carbohydrates are the essential source for our body. CRISPR also utilized in enriching certain crop's starch & carbohydrate content. In rapeseed pat2/5 gene editing with CRISPR/cas9 causes the improvement of starch content in seeds (Zhang *et al*., 2019) [101]. Also in case of rice amylose content was increased about 25% by editing SBE III b gene (Starch Branching Enzyme gene) by CRISPR/Cas system (Sun Y *et al*., 2017). Moreover editing the same gene in sweet potato same result were obtained (Wang *et al*., 2019). The barley crop also shows reduction in prolamins, improvement in glutenins and protein matrix around the starch granules increased by knockout action of CRISPR on D-hordein gene (Yang *et al*., 2020) [95]. GBSS gene editing by this system changes the starch quality in potato (Anderson *et al*., 2017) [5]. There was also protein profile change of CRUCIFERIN C gene responsible for seed storage protein in Camelina sativa seeds by editing with this system (Lyzenga *et al*., 2019) [57] due to which editing valine, isoleucine & tyrosine content get depleted & proline, cysteine & alanine content get improved. In wheat by editing α-gliadin gene developed the wheat with low glutein content (Sanchez-Leon *et al*., 2018) [74].

As CRISPR system has been successfully utilized for protein, starch, carbohydrates, etc. biomolecules content improvement, in same manner it can be utilized to improve oil content in few crops. In rapeseed by editing BnSFAR4 & BnSFAR5 genes results in increased oil content of seed & this editing do not have any deleterious effect of agronomical character such as germination & vigour (Karunarathna *et al*., 2020) [41]. Editing of tt8 gene in *Brassica napus* resulted in increase in oil and protein concentration and simultaneous change in fatty acid content (Zhai *et al*., 2020) [104].

Conclusion

Vigorously developing world has created many hazardous effects on the environment, among which pesticides are most harmful for the agricultural crops as well as animals. Pesticides are widely used to control the diseases, insects & pests, but genetic modification approach by CRISPR/Cas technology have greater and best alternative to avoid any hazardous effect on environment along with crop improvement. A specific PAM motif is required for every CRISPR/Cas system now in use. PAM sequences give specificity by guiding the editing sites of an individual event. The PAM requirement, on the other hand, limits the genes that CRISPR/Cas can access for effective editing. The development of a CRISPR/Cas system independent of PAM motif would increase the use of CRISPR/Cas in both fundamental and applied research. It is also good idea to keep changing current Cas enzyme variants, especially the most widely used ones like Cas9, Cas12 & Cas13. As delivery of CRISPR system is prime action for editing genome the

CRISPR/Cas system's delivery strategy is equally crucial. It has an impact not only on the efficiency of transformation, but also the off-target effect and the purpose of later regulation. The present hurdle for genome editing in plants is the plant transformation and regeneration mechanism. As a result, developing an effective delivery and plant regeneration system is critically important. Structural genes are typically coding genes that regulate crop properties directly. They usually govern distinctive traits directly. Discovering a structural gene is therefore the greatest option for genome editing in order to improve the trait governed by those genes. However, while utilizing CRISPR approach, it is important to ensure that no large portions of the chromosome are deleted, as this could have a substantial impact on other features. Off-target effects are commonly documented and widely seen in genome editing events, however they occur less frequently in plants than in animals. Off-target effects might have unfavourable consequences, hence off-target effects must always be avoided while genome editing.

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