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REVIEW ARTICLE

A Systematic Review to mitigate biotic constrains in Agricultural crops using CRISPR/Cas9 system: Endless Advancement in Plant Molecular Biology

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ABSTRACT

The sessile nature of plants subjects them to a wide range of biotic as well as abiotic stresses which reduces and limits agriculture productivity. Due to climate change and increase in environmental pollution, they are often imposed on the number of abiotic stresses such as radiation, salinity, floods, drought, extremes in temperature, heavy metals, etc. Drought stress primarily imposes osmotic stress on plants, altering metabolic processes of the cell, leading to the less nutritional quality of grain and ultimately affecting crop yield. For ensuring food security in the future, development of crop varieties resilient to abiotic stresses is urgently required. The conventional plant breeding method has contributed enormously to the development of elite cultivars for feeding the increasing population of the world. However, conventional plant breeding methods used tools such as natural selection, use physical or chemical mutagens. But it has the demerit of taking more time in developing a new variety and transfer of extra genome fragments along with desirable genes. This method cannot edit any particular gene in the genome, so combining it with new technology can accelerate the knockout of any unwanted gene and addition of desirable one. The recent gene editing techniques such as Zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), RNA interference (RNAi), anti-sense RNA (asRNA), and clustered regularly interspaced short palindromic repeats (CRISPR) has shown enormous potential for crop improvement. Above all gene editing tools, CRISPR- Cas9 is preferable among plant biotechnologist due to its specificity and simplicity. Moreover, in combination with nano-particles, gene delivery can be efficiently achieved. In this review, the present status of applicability of CRISPR/Cas9 in crop improvement towards various biotic and abiotic stresses is discussed and merits of CRISPR among all gene editing such as RNAi, Zinc finger Nuclease and TALENs are discussed.

Keywords*: CRISPR/Cas9, genome editing, RNAi, nano-particles, and crop improvement.*

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INTRODUCTION

A gradual increase in global population is a major concern, affecting the food security and continuously increasing demand for food. In addition to this, due to sessile nature plants continuously faces several biotic and abiotic stresses that limit the growth and yield in crop productivity [1]. The biotic stress includes microbial pathogens invasion, pest, nematodes, and virus attack, etc. [2]. Besides, abiotic stress consists of water-logging, salinity, drought, and heat stress [3]. Even though following the strict control safety measurements, many of the crops associated with economically importance are subjected to a significant range of yield loss. Genetic editing has, in some form or the other, existed in the arena of plant biology for several years. Modern genome editing technologies, however, are a less cumbersome and highly efficient successor to methods like selective breeding. Moreover, breeding strategies take much time to gene

transfer, and crop improvement, which is much laborious and costly [4]. To accelerate food production, the incorporation of gene editing tool such as RNAi, TALENS, CRISPR/Cas can serve an opportunistic model in improve the crop quality in less time.

RNA interference (RNAi)-based gene silencing has provided potential biotechnological tools for crop improvement. Several studies have reported successful application of RNAi to make plants resistance to disease or to increase the shelf life of vegetable crops. Recently, host delivered double stranded RNA for Chymotrypsin-like serine protease (CTLP), Alpha-amylase (α-amylase), and Tropomyosin (TPM) has shown tolerance against pod borer, *Maruca vitrata*, an important pest of legumes [5]. Similarly, another study has shown improved tolerance in *Nicotiana tabacum* against plant parasitic nematodes (PPNs) *M. incognita* with combinatorial gene silencing of four esophageal genes using host-delivered gene silencing (HD-RNAi) strategy [6]. In addition, *in-vitro* silencing of FLP genes demonstrate the broad-spectrum resistance against nematode in rice and wheat cultivars [7].

With advancement of plant biotechnology and genetic engineering, crop protection from the pest and climatic factors has marked the neoteric horizon. Incorporation and utilization of the wide range of insect resistance genes, including *Bacillus thuringiensis* - insecticidal proteins (*Bt*-ICPs); a number of crops have been modified using genetic engineering tools [8, 9]. Hence, genetic engineering exhibits a robust tool to enhance the sustainability of plants in the harsh environment or pathogenic attack. Moreover, mutation in a target gene leads to loss or gain of functionality [10]. Therefore, mutation in toxin genes does not allow interacting with the target site and barricade to form complex with their receptor. This in turn, resists the interaction of insect-toxin with the host plant. Hence, a combination of ICPs can be advantageously used to generate a broad range of insect-resistance crops. On the other hand, anti-sesne RNA approaches (asRNA) were also employed to improve the crop production. asRNA technology, involves engineering a antisense sequence which pairs to the target RNA sequence, thereby limiting the gene expression. One major study demonstrates the increased shelf life of tomato by regulating the expression of polygalactouronase (PG) enzyme [11]. A similar study was conducted in potato for enhancement the shelf life and storage by silencing *StAs1/2* gene [12].

Beside the above discussed technologies, CRISPR/Cas9 is a clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9 system is well-known biotechnology tool which is widely being used for crop improvement. CRISPR-Cas9 component consist of 'genetic pair of scissors' consists of a gRNA (guide RNA) and the Cas nuclease protein and it is essentially a bacterial adaptive immune response. When bacterial cell is invaded by bacteriophage/virus invades a, a small fragment of the viral DNA is cut off and retained along with bacterial own genome and form CRISPR array, and this array get transcribed to form RNA [13]. In the case of re-infection with same virus, RNA transcribed from CRISPR array region binds to incorporated viral segment and it, direct the Cas9 nuclease and induces a double stranded break, incapacitating the virus. This results in the formation of an immune memory which can be used in re-infection [14]. Further, scientists utilized gRNA composed of the CRISPR RNA and the trans-activating CRISPR RNA to identify the target region in genome for editing [15]. With the gRNA acting as a reliable guide, Cas9 can easily induce genetic changes with immense precision and efficiency. Working in tandem, Cas9 recognizes the protospacer adjacent motif (originally saved in the first infection). Site-specific genome editing is possible with the aid of the CRISPR-Cas9 complex [16]. An engineered complex features a synthetically created piece of RNA acting as the gRNA that binds to a specific region of the genome. The gRNA curates the Cas9 nuclease to act on the DNA according to the presence of the intended site. Genetic insertions and deletions can be induced via the cell's own repair machinery. CRISPR gene knock-out refers to the methodology in which the double stranded break created by Cas9 is indelicately repaired by the non-homologous repair pathway, leading to insertions and deletions [17]. The resulting frame-shift mutation renders the gene non-functional and is termed as gene knock-out. In CRISPR gene knock-in, the HDR pathway repairs the double stranded break, which can induce a gene of interest via a donor template [18, 19]. The donor template carries the sequence or gene of interest along with complementary regions which match the sequences of the cut. The resulting integration is termed a gene knock-in. In this article, authors are emphasizing to cover the recent advancement of gene editing tool accelerating the crop improvement. This review also enhances the deep knowledge of students and researchers to understand the utility of CRISPR/Cas 9 in crop improvement.

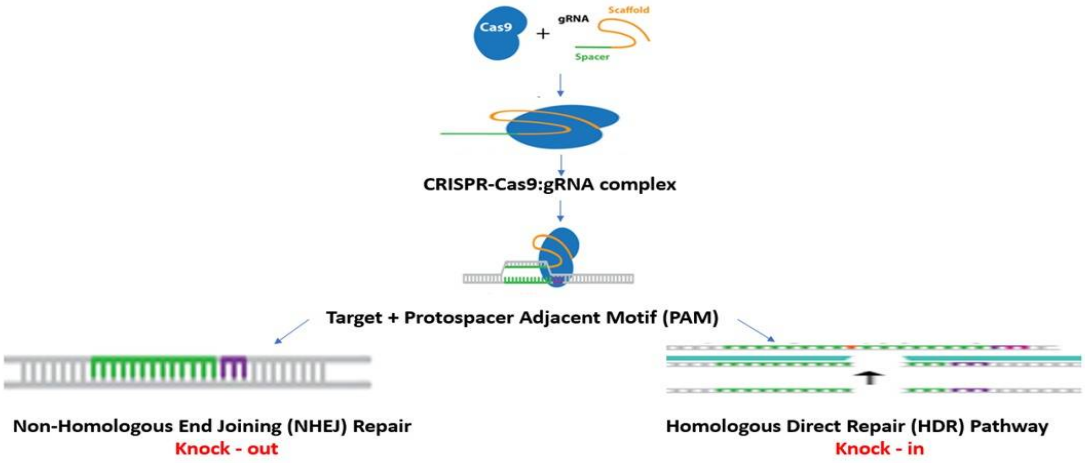

Figure 1: A schematic representation of the routes taken for the insertion/deletion processes.
CRISPR-Cas9 forms a complex along with gRNA, which further binds to the target sequence and act by two different way- a) Gene knock-out; it involved non-homologous end joining (NHEJ) repair and b) Knock-in; it is the gene insertion or repair process, which is followed by homologous direct repair (HDR) pathway.

Mitigation of Food Security Using CRISPR/Cas9 System

Due to climatic alteration a significant amount of yield loss is globally increased. For instance, biotic stresses, including insects, pathogens and viruses are the major challenges affecting grain quality, and reducing yield productivity [20]. It has been noticed that genetic stability and virus free crop propagation is time taking and several hormonal manipulations is prerequisite [21]. Therefore, CRISPR/Cas9 technology; a genome editing tool, directed by RNA-guided nucleases is widely used to secure the crop improvement and food quality. CRISPR/Cas9 technology is highly acceptable because of versatile, specificity and efficiency of Cas9 protein as compare to other genetic engineering tools. The CRISPR/Cas9 system is an evolution reference of adaptive immune response mediated by bacteria and classified as type I and type IICRISPR system, respectively. A detail mechanism of CRISPR/Cas9 system is well explained by Shahriar et al. 2021 [22].

An increasing threat to global food security due to loss of arable land, unpredictable weather changes and depletion of water tables in the big three producers, including India, China and the USA has led to a bleak outlook on the future of global hunger [23]. In modern plant biotechnology, tools of genetic editing are already in play to bring sustainable changes in the agriculture sector [24]. Crop improvement strategies such as genetically modified crops are subject to strict regulation [25]. Furthermore, there are concerns about agrochemical usage and the presence of antibiotic resistance genes, resulting in an expensive pipeline process. This has the two-pronged disadvantage of making crops inaccessible to farmers and extremely expensive to develop. In this regard, new plant breeding technologies (NPBT) offer an interesting and more accessible avenue [26]. Precise genetic editing offerings include CRISPR and TALEN. Such options are gaining momentum as they do not carry the disadvantages of multi-step processes like mutagenesis and GM crops. Concerns like presence of foreign DNA in consumer products can be eliminated as the genome editing facilitated by CRISPR and TALEN is extremely precise [27, 28].

Conventional Methods	New Plant Breeding Technologies
Final product may contain undesirable substances. (Eg: antibiotic resistance genes)	High specificity ensures minute Genetic changes.
Expensive pipeline process.	Cost of designing new sequences.
Inaccessible to local farmers.	Less hazardous with fewer maintenance costs.

Table 1: Comparative summary of conventional methods and new plant breeding technologies

Since the transgenic *Bt* technology has been well developed and adapted by many researchers to develop transgenic crops but the presence of insecticidal proteins in *Bt* mediated crops is the major concern of health as well as food related security. Therefore, efforts are being forward to silence the receptors in

order to increase the efficacy of resistance management. Keeping these beneficial points, cadherin receptor was successfully knockdown as it was genetically linked to Cry1 Ac. This strategy can be successfully utilized to generate resistance pigeon pea crop against the *H. armigera* attack [29]. In addition, defense related genes play crucial role to enhance the tolerant threshold during stress conditions [30, 31]. Therefore, alteration in defense related genes act as barricade for the pathogen invasion. Similarly, recent study indicates the susceptible genes in rice linked to pathogen resistance such as *enhanced disease resistance 1* (*EDR1*) and ethylene signaling including *Ethylene Response Factor 922* (*ERF922*) was also altered by genome editing tool in plants in order to modify their trait [32]. Moreover, genetically altered rice crop was also developed by modifying the genes *OsERF922* and *OsSEC3A*. Modification in such genes using CRISPR/Cas9 technology confirmed the resistance against the *M. oryzae* [33, 34]. In this way, transgenic crops can be generated that reflect the promising source of food security and crop improvement.

Apart from these strategies, CRISPR/Cas9 system has advantage to manage the food security. Unlike RNAi technology, which is dependent of mRNA sequence, CRISPR required DNA sequence to execute the editing activity. On the other hand, RNAi is not that much promising tool due to its poor reproducibility. While such complications are not the part of CRISPR system. Taking this advantage, CRISPR is used in many crop improvement programs. It has been reported that target genes sequence can halt the chemo-communication and generate the hindrance the identification of mating partners. These two factors-chemical communication and mating partner identification is the versatile nature of insect to get the entry into host plant. Therefore, researchers have blocked such a way of communication. Host plants are well recognized by the insects with the help of their olfactory receptors. Such receptors also help in identification of odorant of mating partner. In this way, an insect pathogen is sufficient to destruct the potential crop yield. Similarly, mutation in Drosophila gene sequence - *Or83b*; halted the identification of the host site for egg-laying and depraved the detection capacity of olfactory receptor [35].

Overview of New Plant Breeding Technologies

Traditional plant breeding approaches are time dependent and it takes several years to generate a crop resistance against stress environment [36, 37]. CRISPR, along with ZFNs and TALENs comprise the umbrella of NPBTs. ZFNs are usually difficult to customize according to editing needs, so a fair comparison would be between TALEN (Transcription activator-like effector nucleases) and CRISPR [38]. Comparatively, while both technologies are advantageous, CRISPR is impressive in ease of use. TALEN requires constant re-construction for every new sequence and DNA binding domain is specific for the particular target region. Whereas, CRISPR gRNA needs to be designed for the specific target sequence (only 20 nucleotides long). This also decreases the general time taken in the procedure to conduct CRISPR based research. CRISPR can induce multiple changes (multiplexing) whereas TALEN is limited by simple mutations [39, 40, 41, and 42]. TALEN editing can lead to mosaicism, compared to the higher efficiency of CRISPR. Although each method has its own advantages, they remain preferable over traditional methods that rely upon viral vector delivery.

Functionality	CRISPR	TALEN	RNAi
Specificity	Potential to target any site preceding PAM.	Roughly 18,742 human genes.	Off site target and homology to 19 basepair
Ease of Use	Easy, only gRNA requires modification	Difficult due to repeat sequences.	Interfere with target RNA synthesis
Multiplexing	Yes	No	No

Table 2: Summary of differences in CRISPR, TALEN and RNAi.

The CRISPR solution

In 1983, the first genetically modified crop, a tobacco resistant to antibiotics was produced [43]. Today, genetically modified crops occupy an area greater than 179.7 million hectares [44]. Genome editing in crop plants can improve their tolerance towards biotic and abiotic stress and increase their yield [45]. CRISPR can be used as a DNA-free gene editing system. In fact, it is already in active usage worldwide to develop plant lines resistant to stress and disease. In 2020, in a study, the gene knock-out method was employed to increase cold tolerance in rice [46]. In a similar vein, in 2021, canker resistant orange was developed by scientists [47]. Disease resistance can be enhanced via the targeting the genes as demonstrated in rice, which led to resistance to blast fungus, which wreaks havoc on the global food chain. Viral pathogens can

also be protected against, by employing CRISPR to target plant genes. Rice tungro resistant lines were developed in this fashion, by using the gene knock-out method to disrupt elF4G [48, 49, 50]. Besides disease, stress plays a huge role in crop disruption. Genome editing technologies, particularly CRISPR can be used to improve stress tolerance. An increase in industrial activities has contributed heavily towards soil pollution, particularly soil contaminated by heavy metals. These pose a threat to both plant and human health. CRISPR-Cas engineered systems can be used to silence metal transporter genes [45, 51, 52, 53]. A detailed table on diverse crop using CRISPR technique has been mentioned in table 4.

S.No.	Gene	Crop improved	Phenotype	References
1	Homoserine kinase (*ObHSK*)	Sweet basil	Significantly reduced susceptibility to Downy Mildew Disease caused by *Peronospora belbahrii*	54
2	*OsPIN5b* (a panicle length gene), *GS3* (a grain size gene) and *OsMYB30* (a cold tolerance gene)	Rice	Increased panicle length, enlarged grain size and increased cold tolerance	55
3	*drought and salt tolerance* (*DST*)	Rice	Improved *drought and salt tolerance* and grain yield	56
4	*MLO-7*	Grape	Resistance to powdery mildew in grape cultivar	57
5	***DIPM-1, DIPM-2***, and ***DIPM-4***	Apple	Increased resistance to fire blight disease.	57
6	*ZmCLE7*	Maize	Increased multiple maize grain-yield-related traits	58
7	***SiALS***	Foxtail millet	Homozygous herbicide-tolerant mutant plan	59

Table 4. CRISPR-dependent crop improvement by altering diverse genes.

Beside these complications, weeds are the major concern globally. Weeds significantly reduce the crop yield and multiplication at the exponential rate. Rice crop is highly susceptible to weeds and when encounter to weeds, resulting in a huge loss of farmers and Asian countries. Therefore, using CRISPR/Cas9 system, generating an herbicide-resistance/tolerance rice crop is an efficient way to mask the yield loss. In order to keep this crucial concern, recently a novel allele *G628W* was developed in rice by engineering the *acetolactate synthase* (*OsALS*) gene. This novel allele was the result of G-to-T transversion in *OsALS*gene (60) Thereby, representing a highly tolerance mechanism against the herbicide.

CRISPR in Yield and Nutrition Improvement

Besides building additional stress tolerance and ensuring crop longevity, another use of CRISPR in crop improvement is increasing the yield. In theory, high yielding plants could deliver better and more quantity, in shorter space, thereby being of great use in areas lacking arable space. In a similar manner, nutritional value could be manipulated, in order to produce crops that can fulfil global nutritional deficits. Greater yield efficiency would also lead to greater food production. In this respect, CRISPR has made great progress [61, 20]. The biosafety threat and regulatory measures that guard GM crops, are also not of much concern in CRISPR modified crops. This was recently observed in the tomato plant - two research groups recently demonstrated that silencing of *pectate lyase* and *alcobaca* led to increased shelf life without affecting nutritional value [62]. Another fascinating discovery was the identification of the aluminium-activated malate transporter 9, which determines malate content in tomatoes, was achieved via CRISPR-cas9 [63]. However, although progress has been made on this front, quality of nutrition within the plant source has notably been affected via CRISPR intervention to secure longevity. An example of achieving both increased nutritional value and longevity would be the genetic modifications achieved in the rice plant. Rice is a popular choice of genetic engineering due to a small genome size, high transformation efficiency and widespread usage across the globe. While rice production is essential to many cultures, rice agriculture is slowing down due to the issues of global warming and loss of arable land. Both CRISPR-Cas9 and CRISPR-Cpf1 have been successfully employed in genetic modification of rice and stable and heritable mutations were successfully generated in two endogenous rice genes *OsPDS* and *OsBEL.* The high mutation efficiency established the use of CRISPR systems in generating targeted mutants in rice. Furthermore, a study demonstrated the multiplexing ability of CRISPR - 46 target sites were edited with a mutation frequency of 85.4% [64, 65, 66, 67]. Similar multiplexing studies have repeatedly generated results with long term potential.

Value Added	CRISPR Functionality
Crop Longevity	Successful, with limited effect on nutritional content.
Crop Nutrition	Possible in species with small genomic sizes and high transformation efficiency.
Yield Efficiency	Successful.
Stress Tolerance	Highly successful, with multiple species responding to CRISPR based engineering. Disease, stress and pollution resistance can be induced.

Table 3: Summary of CRISPR in Crop Improvement.

Pitfalls and Long-term Strategies

In theory, CRISPR-Cas engineered systems tend to be very precise, however, in practice errors may arise. Such unexpected errors that may lead to unpredictable changes across the genome are called off target effects. Off target effects are random, imprecise and cause unnecessary changes which can lead to unwanted side effects. Especially, in the context of crop improvement, unwanted side effects could lead to potential health hazards. Off target specificity impedes optimal CRISPR utilization in crop improvement as well as therapeutic and clinical applications. However, an important concern is to maintain the off-target mutation frequency which is identified in CRISPR engineered plants compared to conventionally bred plants. Most off-target edits in CRISPR engineered lines were in protein-coding regions, with a few mismatches in the target sequence. There are also ethical concerns with genetic engineering that have followed CRISPR as well [68]. Optimization at the genetic level has caused a similar controversy in GM crops, including *Bt* Brinjal.

CONCLUSION AND FUTURE PERSPECTIVE

In the past few decades, consumption of genetically modified (GM) food has been increased significantly. Several government and private organization support the food biotechnology to minimize the food shortage and fulfill the nutritional demands [69]. However, a large population has fear to consume the GM food due to the concern related to health impact. This concern has pushed the evolution of several biotechnology approaches. Genome editing is an emerging tool, hence is the choice of many scientists and researcher to elucidate or uncover the gene function and translational utility in breeding programs. RNAi, is important tool to edit plant and recently it has been observed that exogenous RNAs can be taken up from environment either by spraying on leaf or by adding into medium and gene silencing has been achieved for various plant pathogen. Moreover, gene silencing can avoid cumbersome process of transgenics development for host induced gene silencing approach for gene silencing via RNAi effect. CRISPR remains a favored technology compared to other new plant breeding technologies due to its high efficiency, lower cost and high biosafety. It has been used in a variety of plant species to create stress-tolerant, disease resistant and high yield producing crops. CRISPR is also a popular choice for scientists due to its highly malleable properties such as lack of transgene application. In general, such gene editing technologies are gaining momentum due to wider applicability and higher efficiency compared to traditional vector delivery-based technologies.

Although, number of other molecular approaches can be used to improve the nutrition quality or grain yield but genome editing is less time consuming and does not require large number of screenings in field conditions. Despite successful incorporation of CRISPR/Cas9 system in plants to provide economic benefits, very less information is available to design new strategies in order to make plants exhibiting resistance behavior against the insect pathogens as well as harsh climatic conditions. Genome editing should be economical friendly and do not affect the food chain. This is because editing is not a single step procedure and it required a hierarchy of alteration in insect or host plant genome. In spite of having a huge advantage over the crop improvement, due to some governmental policy CRISPR/Cas system could be prohibited in some countries as a part of gene-editing concern including GM foods. However, it is not that much logical because CRISPR system is very precise and accurate and it is not based on the random target like mutations, application of chemical and physical mutagenesis etc. Genome editing technologies including CRISPR has delivered a number of opportunities to improve the crop and breeding programs but only tip of iceberg uncovering the genome complexity. Moreover, nanoparticles (NPs) can play advantageous role on combining with CRISPR/Cas technology. NPs along with CRISPR/Cas may enhance the gene transfer efficiency [70]. Due to surface charge and quick delivery system, NPs has showed boon in sustainable agriculture [70, 71]. In addition, excellent transformation capacity has been reported using NPs [72]. For example, such efficient transformation using silicon carbide has been demonstrated in maize, tobacco, cotton, and rice crop [73]. Therefore, we are expecting endless possibilities in the future and the same information can be utilized to maintain the crop production and yield improvement.

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CONFLICT OF INTEREST
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