Minireview Article

Enhancing Crop Resilience through CRISPR/Cas9-Mediated Development of Disease-Resistant Cultivars

Abstract

Agriculture faces a significant problem in protecting crops from diseases and pests. The development of cultivars that are resistant to disease is mainly achieved through plant breeding. Gene editing is an essential component of plant breeding to produce crops with desired traits. A recent development in gene editing technology is CRISPR (Clustered Regular Interspaced Palindromic Repeats)/Cas9 (CRISPR-associated protein). With the help of bacterial immune system recognition and degradation of the invading pathogenic genes, it can be used to take advantage of plant defense mechanisms against pathogen attack. The development of cultivars with heritable resistance to viral and bacterial diseases has been helped by advancements in plant breeding with the integration of CRISPR/Cas9. After the segregation of the Cas9/sgRNA transgene in the F1 generation, CRISPR/Cas9-mediated genetically engineered resistance can be passed down to future generations of crops. CRISPR/Cas9 is safe to use in plant breeding due to the segregation of the Cas9/sgRNA transgene. Although CRISPR/Cas9 has proved to be an amazing tool for revolutionizing plant breeding and developing a large range of disease-resistant cultivars, its effects on many physiological processes in plants still need to be thoroughly investigated.

Keywords: CRISPR/Cas9, Gene editing, Genome, Plant breeding, Resistance breeding.

Introduction

A major challenge is protecting crop cultivars against current pests and diseases as well as improving crop cultivars for greater yield. The lack of disease-resistant varieties of crops is the major reason farmers are facing loss in agriculture production. Plant breeding for pests, disease resistance, and higher productivity helps in the development of disease-resistant crop cultivars safeguarding food security [35]. Different genome editing and advanced molecular techniques with transgenic plants are integrated with plant breeding to achieve improved crop cultivars with

enhanced resistance to pests and diseases, termed as resistance breeding. Transgenic technology allows plant breeders to cross-crop species introducing genes from non-related plants and other organisms into the crop plants [35]. In order to meet the nutritional needs of the expanding population, which is predicted to reach 9.8 billion people by the year 2050 due to the world's population growth [4,30], an excessive amount of food must be produced. Plant pathogens, which include bacteria, viruses, fungi, and parasites, pose a threat to global food security [2,33]. In order to enhance crop yield and satisfy the world's food demand, it is vital to breed resistance in plants [13]. The ongoing struggle between plants and diseases to forge defences against one another is well known [19,46]. To protect themselves from infections, the plants have evolved "pattern triggered immunity (PTI)" and "effector triggered immunity (ETI)" [18]. The PTI is typically quickly activated by "pathogen associated molecular patterns (PAMPs)" via "pattern recognition receptors (PRRs)" [36,28].

Genetic variation is a major component of resistance breeding. To exploit the concept of resistance breeding, the creation of genetic variation with the enhancement of resistance against pathogenic genes is an indispensable part [48]. Considering these facts, different gene editing technologies have been used to develop genetic variations. One of the recent breakthroughs is CRISPR (Clustered Regular Interspaced Palindromic Repeats)/Cas9 (CRISPR- associated protein) bacterial immune system which is RNA-guided technology for efficient gene editing and gene regulation [32]. There are also other gene editing technologies like Zinc Finger Nucleases (ZFNs) and Transcription Activator Like Effector Nucleases (TALENS) however, they are less suitable as compared to CRISPR/Cas9 because of their large size and requirement of a pair of proteins for recognizing anti-parallel strands to induce Double Strands Break (DSB) [7]. CRISPR/Cas9 is regarded as a highly promising system for gene editing in crops because of its desirable features like precise specificity, multi-gene editing, minimal off-target effects, higher efficiency, and simplicity [32]. This unique system in bacteria acquires invading or foreign DNA fragments and utilizes them to recognize and degrade the further invading DNA or RNA sequences. CRISPR/Cas9 technology can be utilized to exploit defensive mechanisms in plants against disease attacks by recognizing and degrading the invading pathogenic genes.

CRISPR/Cas9 technology can be used for developing disease-resistant cultivars. With its ability of sequence-specific nuclease, CRISPR/Cas9 proves to revolutionize research in resistance breeding. This review paper will discuss on use of the CRISPR/Cas9 technique for the development of disease-resistant cultivars in plant breeding. In this review, research papers found in the online literature database at scopus.com and scholar.google.com with the use of keywords ‘CRISPR/Cas9’, ‘resistance breeding’, and ‘plant breeding’ are used. The first section of the body is a discussion on the use of the CRISPR/Cas9 system for gene editing and gene manipulation. The next section deals with the use of CRISPR/Cas9 on disease resistance. The third section analyzes the effectiveness of CRISPR/Cas9 in plant breeding for disease resistance. This review aims to facilitate researchers in further research providing information on current advances in CRISPR/Cas9 and its use in resistance breeding.

In CRISPR/Cas9 system, guide RNA binds to DNA, and the pre-designed sequences in RNA guide the Cas9 enzyme to cut the DNA strands at the right locations. Cutting of DNA proceeds with the removal and addition of required sequences into the target DNA. DNA recombination repairs the cut in the DNA and induces mutations. However, it also sometimes results in unwanted mutations as v to be complementary for binding with target DNA. This may result in the binding of guide RNA in the wrong places and cutting of non-target DNA strands which results in wrong mutations and part of essential DNA sequences with important information might be lost. Nonetheless, scientists are

working to make use of CRIPSR/Cas9 more accurate as the Cas9 enzyme can be regulated to target differentsites in DNA by changing sequences in guide RNA [49].

Plant diseases are foremost and imperative threat to agricultural development and global food security. According to the statistics published by FAO in 2015, 20–50% yield losses, worldwide, are due to different diseases [16,22]. Currently, the use of chemical pesticides is one of the key strategies for controlling crop diseases. However, the application of pesticides is directly or indirectly unsafe to other living organisms [11]. In addition, phyto-pathogens are also surging resistance against different pesticides and other chemicals due to their extensive and irregularuse [1]. Therefore, to minimize the negative impact of pesticides on environment and to protect fauna and flora, it is essential to avoid their application for food production. On the other hand, the development of disease-resistant crop varieties through plant breeding is an effective and eco-friendly strategy for sustainable agriculture. Conventional breeding of diseaseresistant varieties has been successful over the last few decades,but it has several limitations such as the introgression of resistance (R) genes and labor intensiveness. In addition, crossing/- selfing between two most compatible plants, unavailability of sufficient genetic variation in plant population and transfer of undesirable genes/traits along with desirable resistance gene/-traits are supreme limitations in conventional breeding for resistance [17]. Therefore, it is a big challenge for conventional breeding to keep pace with pathogen evolution and increasing food demand, particularly during an era of global climate change.

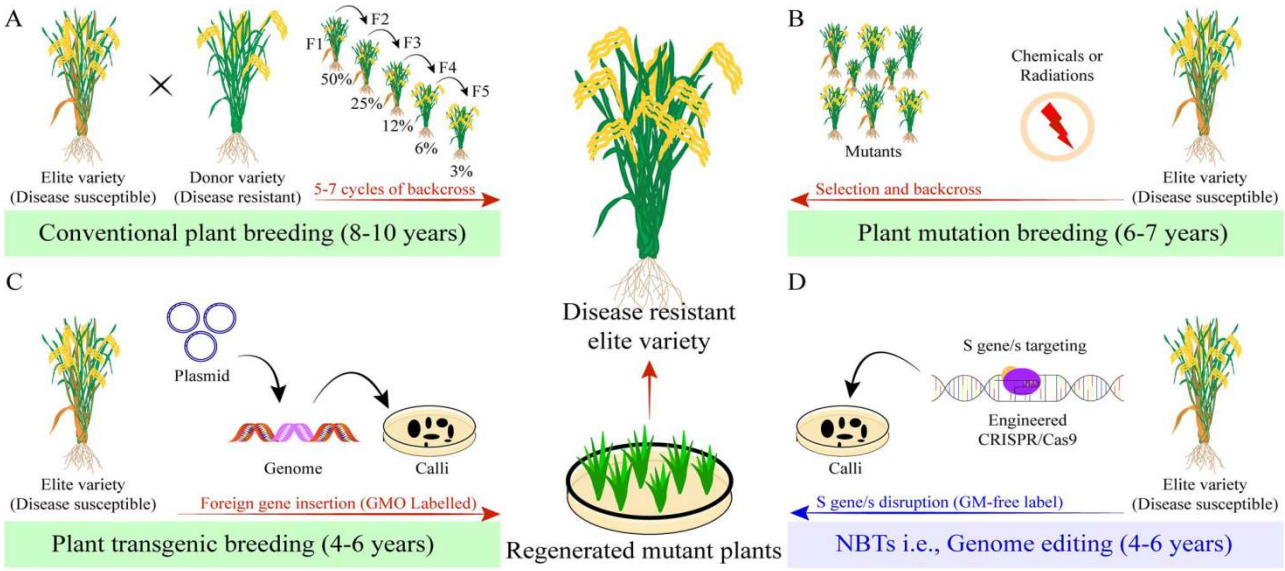

Figure-1: Illustration of comparison of plant breeding and mutagenesis methods used in crop (e.g. rice) improvement modified from Chen *et al*. [10]. {(A) The traditional method of plant breeding, known as cross breeding, has been used for a long time to enhance plant features, such as disease resistance, by crossing a donor parent line with a recipient parent line that possesses the desired trait. After numerous iterative backcrossing and severe selection cycles, an exceptional child with the desired trait or traits is chosen.This method has many drawbacks including being hard, time-consuming, and less effective. (B) Chemical or physical mutagenesis is what is meant by mutation breeding. Typically, different mutagens are applied to the seeds to produce mutations in the plant's genome. Mutants created using this method are subjected to stringent selection during the assessment of desired phenotype(s). This is another method for finding and mapping new genes in the genome. Additionally, the process is drawn out and takes about 6-7 years to produce desired outcomes. The random changes in the genome, which can occasionally be difficult to identify and anticipate, are one of the main drawbacks and limits of this technique. (C) Transgenic breeding, which involves moving a desired gene from one genome to another, has been used to successfully enhance a variety of agricultural attributes (e.g. Bt cotton, Bt maize, etc.).The targeted trait(s) are improved when the gene of interest is accurately and precisely introduced into the host elite variety's genome. However, because someone's genome has been altered by the introduction of alien DNA or elements, these plants are genetically altered. One of the major challenges with GMOs, among numerous other concerns, is that the public and a number of plant scientists around the world do not accept them as much.(D) For a sustainable future of crop development, new genetic modification techniques, commonly referred to as "new (breeding) techniques," like genome editing, constitute both promise and hype. By specifically focusing on and destroying any particular negative regulator(s) or gene(s), on a specific gRNA-guided target site, and rearranging chromosomes in the genome of elite kinds, plants are improved. When compared to other methods, improving traits via genome editing technologies like the CRISPR/Cas9 system is a reliable, effective, time- and labor-saving, and cost-effective method. Additionally, this method may allow the plants to escape GMO legislation and be labelled as non-GMOs because they contain no foreign DNA.}

Although mutation breeding and transgenic technology are also in practice, but they also have certain limitations due to which they are less demanding nowadays (Figure 1). These challenges to our current agricultural practices suggest the need for the introduction of newer and quicker means (i.e. genome editing technologies, referred as GETs) of creating materials for breeding new cultivars. Among the new GETs, clustered regularly interspaced short palindromic repeat (CRISPR) is a popular method currently employed to develop desirable plant material for sustainable food production [55].

CRISPR/Cas9 as a Tool for Gene Editing and Gene Manipulation in Plant Breeding

Gene editing and gene manipulation have always been interesting topics for research works in molecular biology and plant breeding. Gene regulation is an important aspect of regulating and controlling the expression of specific genes to obtain desirable traits and characteristics in an organism and has high use in the breeding of disease-resistant and higher-yielding cultivars. In plant breeding, gene editing is highly used to produce transgenic plants to introduce new resistant genes against crop pests and diseases. Production of transgenic plants for plant breeding with gene editing technology uses all the available genetic variation without any limitation of natural cross barriers resulting in plants that are not producible by conventional breeding methods. Various approaches for gene editing and gene manipulation serve as important tools for molecular biologists and plant breeders to integrate the essential genes in the genomes of important crops [32]. The principle behind gene editing consists of the binding domain and the effect or domain. The binding domain helps in the recognition and binding of sequence-specific DNA while the effector domain helps in the cleavage of DNA at the target site and regulates transcription [49]. In a CRISPR/Cas9 system, the CRISPR locus or array is located on the genome and consists of hypervariable spacers acquired from bacteriophage virus or plasmid DNA [8]. Cas genes are located upstream of CRISPR loci and encode for Cas protein for defense against invasive genetic materials [8].

CRISPR/Cas9 system as an adaptive immune system possessed by many bacteria works in a series of steps as shown in Figure 2. At first, CRISPR-containing organisms recognize foreign nucleic acid and acquire small fragments of DNA from invading bacteriophages and plasmids. Then the host incorporates the acquired fragments into its CRISPR locus as spacers between short DNA repeats. A short stretch of conserved nucleotides, Protospacer Adjacent Motifs (PAMS) act as recognition motifs for the acquisition of DNA fragments into the spacer [32]. The expression of Cas proteins then transcribes the spacers acquired CRISPR to form pre-CRISPR RNAs (pre-crRNAs) which after cleavage and maturation of pre-crRNAs results in CRISPR RNAs (crRNAs). These crRNAs contain a spacer sequence from a previous foreign nucleic acid that helps in the recognition and cleavage of invading genome, which matches with the spacer sequence and helps to protect the host cells [49]. This unique ability of bacteria to acquire invading or foreign DNA fragments and utilize them to degrade further invading DNA or RNA sequences confers CRISPR/Cas9 system as an acquired and heritable defense system [8].

Use of CRISPR/Cas9 on Disease Resistance CRISPR/Cas9 system can be used to create disease resistance in plants through guide RNA (sgRNA) technology. sgRNA is formed by the fusion of crRNA and trans-encoded CRISPR RNA (tracrRNA) [39]. Cas9 together with sgRNA forms an RNA-guided nuclease that regulates the sequence-specific cleavage and editing in the target genome [25]. The site-specific cleavage action of the sgRNA-Cas9 complex is defined by pre-designed sequences in guide RNA which has ~20 base pairs that are complementary to target DNA and help in the binding of guide RNA to strands of target DNA [32,49] (Figure 3).

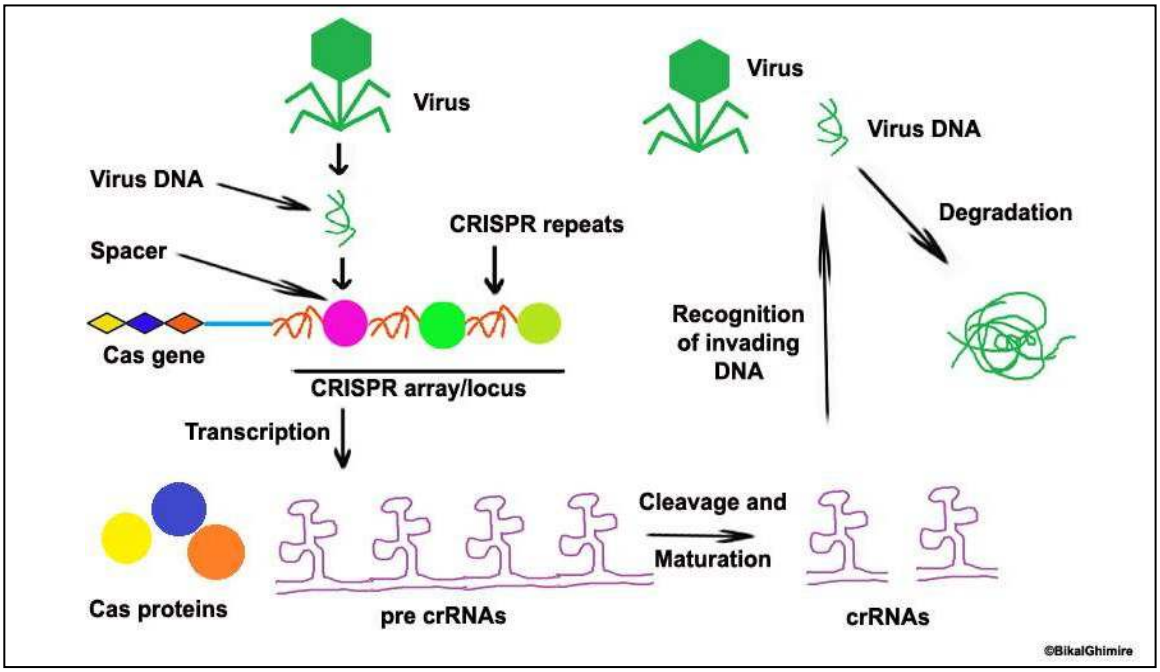

Figure 2: Working mechanism of CRISPR/Cas9 system [32]
[CRISPR-containing organism recognizes the invading DNA, takes a fragment of it, and incorporates that fragment in the spacer between CRISPR repeats. Transcription of the Cas gene produces crRNAs after maturation. These crRNAs can immediately recognize the invading DNA and cleave them to degrade which protects the host. The figure is based on a study of the CRISPR/Cas9 system in various kinds of literature and reviews].

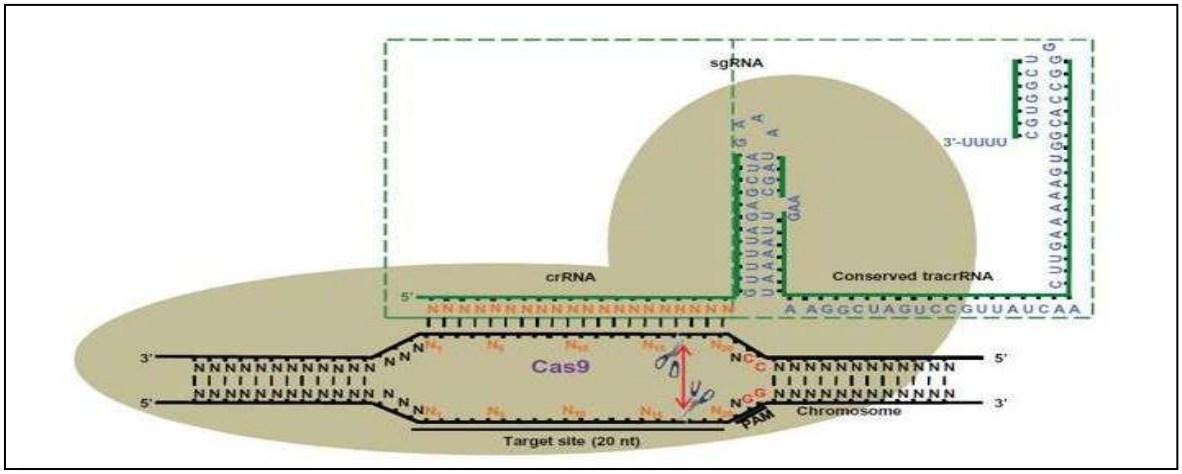

Figure- 3: Diagrammatic overview of CRISPR/Cas mediated cleavage mechanism. {Green and black lines represent the backbone of the RNA and genomic DNA molecule, respectively. The red arrow indicates the cleavage sites. Cas9, (CRISPR)-associated protein 9; crRNA, CRISPR RNA; PAM, protospacer adjacent motifs; sgRNA, Single guide RNA; tracrRNA, trans-encoded CRISPR RNA. This figure is extracted from [32]}

With the use of sgRNA as a binding domain, specific sequences of bacterial DNA can be edited, and invading foreign DNA such as phages can be cleaved by RNA-guided nuclease in a sequence-specific manner [9]. Guide RNA binds to DNA and the pre-designed sequences in RNA guide the Cas9 enzyme to cut the DNA strands at the right locations. Cutting of DNA proceeds with the removal and addition of required sequences into the target DNA [49]. This technique can be explored to create resistance to specific diseases in plants through the delivery of sgRNA and Cas9 into target cells for gene transformation.

1. Different methods are used for the delivery of sgRNA and Cas9 into plant cells like electroporation, via plasmids, agrobacterium-mediated transformation, shotgun methods,

particle bombardment, and polyethylene glycol-mediated transformation [24]. However, Agrobacterium-mediated gene transformation is easy and commonly used in many experiments. Baltes *et al*. [5]explained the use of the CRISPR/Cas9 technique for the protection of plants against the Gemini viruses. Ali *et al*. (2015)[3] experimented to demonstrate the efficacy of CRISPR/Cas9 against tomato yellow leaf curl virus (TYLCV) in *Nicotiana benthamiana* plants and their results exhibited profound evidence ofinterference against viral DNA by use of guide RNA mediated through *Agrobacterium tumefaciens* engineered tobacco rattle virus (TRV) with sgRNA specific for TYLCV into *Agrobacterium tumefaciens* and infiltrated it into the plant. Accumulation of TYLCV was foundless in plants infiltrated with sgRNA than in control plants [3]. Similar results were obtained when CRISPR/Cas9 technique integrated with *Agrobacterium-mediated* gene transformation was applied in *Nicotiana benthamiana* and *Arabidopsis thaliana* against beet severe curly top virus [23]. Interference of Cas9/sgRNA by binding to viral genetic element prevents replication of viral gene by blocking access of viral gene to replication protein or by cutting double-stranded (ds) DNA of virus to cease its replication or by causing error prone mutation of the viral genome [3]. The sgRNA also controls other viral DNA whose sequences match with it. Ali *et al*. (2015) were able to target three viruses TYLCV, beet curlytop virus (BCTV), and Merremia mosaic virus (MeMV) matching an invariant sequence of viruses within the intergenic region of CRISPR. The sequence of crRNAregulates the cleavage of specific DNA targets and by using variable crRNAs it is possible to design multi-target sgRNAs [32]. Table 1 shows the list of plant species in which gene transformation was integrated with CRISPR/Cas9 that induced resistance against differentdiseases in plant species.

Effectiveness of CRISPR/Cas9 in Resistance Breeding

CRISPR/Cas9 technique addresses the urgent need for efficient crop improvement schemes with advanced and reliable gene editing tools (Zhang & Zhou, 2014). This technique creates mutation in the target DNA with the removal of the target sequence and the addition of the gene of interest. It helps in the mutagenesis of inaccessible genes, multi-gene editing, and generates large gene deletion which ultimately helps in progressive plant breeding (Khatodia, Bhatotia, Passricha, Khurana & Tuteja, 2016). This precise modification into a plant genome can be inherited stably and the Cas9/sgRNA transgene can be removed to make the plant transgene-free for successive improvement of the crop variety in further generations (Xu *et al*., 2015; Fig. 4).

Table- 1: List of Plant Species with resistance to various diseases induced by gene transformation integrated with CRISPR/Cas9(Based on past research papers)

Plant Species	Medium for gene transformation	CRISPR/Cas9 integrated with gene transformation induced resistance against	References
Nicotiana benthamiana	*Agrobacterium tumefaciens*	Tomato Yellow Leaf Curl Virus (TYLCV), Beet Curly Top Virus (BCTV), Merremia Mosaic Virus (MEMV)	Ali *et al*.(2015)
Nicotiana benthamiana	*Agrobacterium tumefaciens*	Bean Yellow Dwarf Virus (BeYDV)	Baltes *et al*. (2015)
Nicotiana benthamiana, Arabidopsis thaliana	*Agrobacterium tumefaciens*	Beet Severe Curly Top Virus (BSCTV)	Ji *et al*. (2015)
Nicotiana benthamiana	*Agrobacterium tumefaciens*	Cabbage Leaf Curl Virus (CaLCuV)	Yin *et al*. (2015)

Oryza sativa	*Agrobacterium tumefaciens*	Bacterial Blight	Jiang *et al*. (2013)
Triticum aestivum	*Particle bombardment*	Powdery Mildew	Wang *et al*. (2014)
Oryza sativa	*Gibson Assembly and Protoplast transformation*	Rice disease	Xie & Yang (2013)

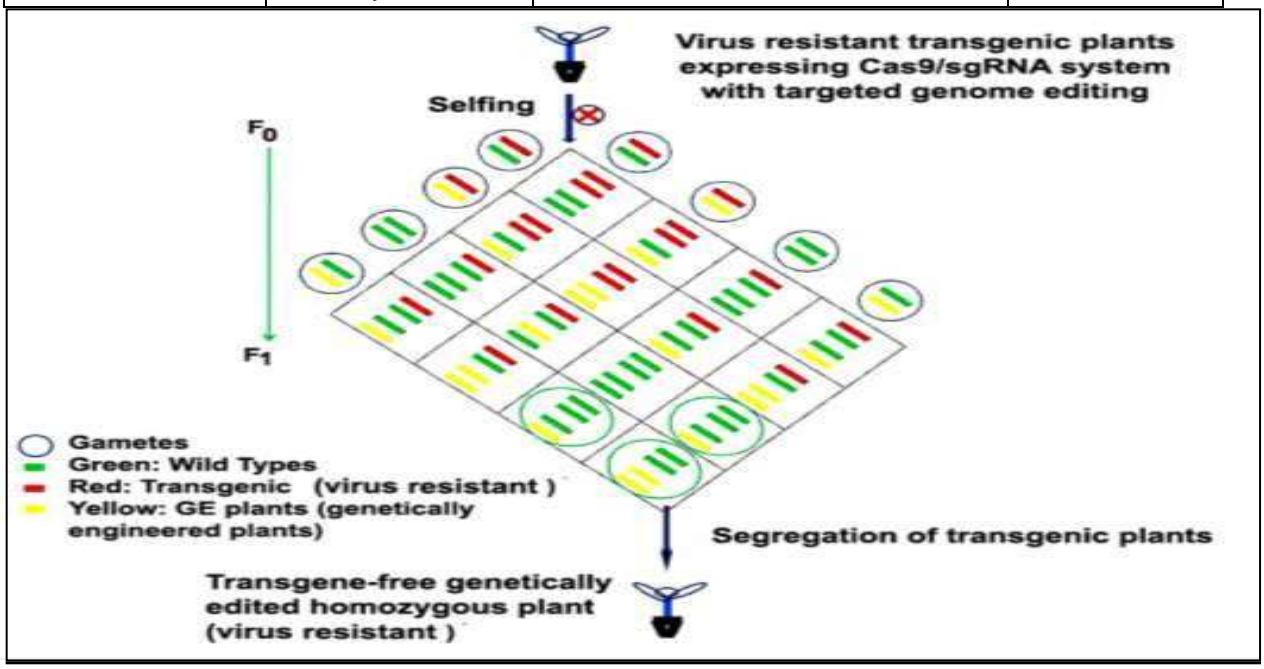

Figure- 4: Successive generation of transgene-free genetically edited (GE) viral-resistant crops. {Transgene-free viral resistant homozygous mutants having desirable modification at targeted gene site can be selected by selfing of F_0 generation plants. The segregation of transgene in the next F_1 generation will avoid possible modification in the genome by CRISPR/Cas9 [29]}.

Many studies have reported the heritability of CRISPR/Cas9-induced mutations in rice, tomato, and Arabidopsis with CRISPR/Cas9 transgene (Belhaj *et al*., 2015). With the use of this technique, transgene modification that confers the resistance against specific viral, bacterial, and pest-disease can be integrated into the plant genome and enables them to produce successive generations of plants resistant to specific viruses or diseases (Wang *et al*., 2014). The mutation generated from CRISPR/Cas9 system is stable and heritable. This mutation can be easily segregated from Cas9/sgRNA to avoid further modifications by Cas9/sgRNA and helps

in the development of transgene-free progeny by only one generation [57,56]. Xu *et al.* (2015)[53] reported the development of transgene-free rice with desired genes after mutation by CRISPR/Cas9 and segregating the transgene with self-fertilization in the F_1 generation.

The use of CRISPR/Cas9 to integrate Cas9/sgRNa for virus resistance helps in the development of virus-resistant transgenic plants. The selfing of plants having wild-type genes, virus-resistant transgene, and genetically engineered (GE) plants in the F_0 generation will produce different progenies in the F_1 generation. The segregation of the transgene in the F_1 generation prevents undesirable modification from CRISPR/Cas9 in further generations. The selection of transgene-free homozygous plants with resistant genes in F_1 generation helps in the further breeding of plants to developcultivars resistant to specific viral, bacterial, and pest-diseases [29] (Fig. 3). In autogamous plants it is possible to obtain 25% of F_2 plants without transgene by self-fertilization of F_1 plants [31]. However, in the case of self-incompatible or dioecious perennial woody plants, transgene-free biallelic mutants can be produced in 25% of the progeny by controlled crosses between male and female primary transformants with biallelic mutations [31].

CRISPR/Cas9 mediated resistance breeding limitations

CRISPR-based technology has revolutionised plant biology, and a number of creative solutions have been created to deal with a number of problems [45,38]. One of the most significant uses of CRISPR technology is the capacity to increase plant tolerance to diseases [27,12]. There are still a number of issues that need to be resolved before CRISPR technology can fully realise its potential in this field, notwithstanding recent developments in plant pathogen resistance [21,44]. Due to the present CRISPR-based techniques' specialised nature, plant pathogen resistance is constrained [42]. Because the existence of a protospacer adjacent motif (PAM) in the target DNA is necessary for CRISPR to be specific, creating CRISPR-based tools that target particular pathogens is difficult [37]. Off-target effects can also lead to undesired outcomes, including the disruption of crucial genes, the cleavage of non-target areas, or the beginning of epigenetic alterations [34,41]. This may have an impact on the plant's general wellbeing and rate of growth and lessen the power of the CRISPR-based technology [43]. Another restriction is the delivery of CRISPR-based plant pathogen resistance mechanisms to target cells [14]. One of the largest obstacles is the lack of effective delivery systems for CRISPR-based tools to target cells [40]. This could be a substantial obstacle, particularly in large-scale applications where delivery costs are a key concern. The effectiveness of CRISPR-based techniques is also restricted by the size of the target DNA [26,7]. When the target DNA is big, it is more challenging to design a CRISPR tool with effective targeting capability and achieve the desired result [51]. The number of targets that may be successfully addressed may be constrained, which would reduce the utility of CRISPR-based technologies [15]. Another disadvantage of CRISPR-based tools is their limited endurance. In contrast to conventional breeding methods, a CRISPR-based tool is not permanent and may need to be reapplied sometimes to preserve its efficacy. Reapplication may be expensive, particularly for extensive applications [20,47].

Conclusion

Increasing population, limited land resource, climate change, and the outbreak of new pests and disease has imposed a severe threat to crop production and global food demand challenging food security. Sustainable agriculture with gene editing technologies for the

breeding of disease-resistant varieties seems to be the key solution to these problems. CRISPR/Cas9 as an adaptive immune system in bacteria can be utilized as a powerful tool in plant breeding for editing genes to develop desirable traits in plants. Integration of this technology in plant breeding facilitates the production of cultivars with heritable resistance to viral and bacterial disease. The possibility of segregation of Cas9/sgRNA after the development of disease-resistant plants makes use of CRISPR/Cas9 safe in plant breeding. However, various studies on the effects of CRISPR/Cas9 on plant physiology are still lacking. Mutations might be induced by CRISPR/Cas9 in the virus and other pathogens which may undergo selection pressure to develop resistance against CRISPR/Cas9 system. Due to this, pathogens might change the invariant sequences in their genomes that match the intergenic sequences in the CRISPR array during evolution and may develop resistance against CRISPR/Cas9 technology. Also, CRISPR/Cas9 integration might affect the induced defence mechanism in plants to produce volatiles during a pest's attack. The study on the effects of CRISPR/Cas9 on the rate of photosynthesis is also lacking.

To conclude, CRISPR/Cas9 system renders itself a powerful tool in plant breeding for the development of various disease-resistant cultivars however, the effects of the CRISPR/Cas9 technique on plant physiology and possible mutations in viruses that may develop resistance against CRISPR/Cas9 system still needs to be studied.

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