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Mini Review

Use of Crispr/Cas9 for Development of Disease Resistant Cultivars in Plant Breeding

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Abstract

Crop protection against pests and diseases is a major challenge in agriculture. Plant breeding is a key solution for the development of disease resistant cultivars. Gene editing is an indispensable part of plant breeding to obtain desirable traits in crops. CRISPR (Clustered Regular Interspaced Palindromic Repeats)/Cas9 (CRISPR- associated protein) is a recent breakthrough in gene editing technology. It can be utilized to exploit defensive mechanism in plants against pathogen attack with recognition and degradation of the invading pathogenic genes by bacterial immune system. Advances in plant breeding with integration of CRISPR/Cas9 have facilitated the production of cultivars with heritable resistance to viral and bacterial disease. CRISPR/Cas9 mediated genetically engineered resistance can be inherited to further generation of crops after segregation of Cas9/sgRNA transgene in F1 generation. The segregation of Cas9/sgRNA transgene prevents undesirable genome modification in successive generation and makes use of CRISPR/Cas9 safe in plant breeding. CRISPR/Cas9 proves itself as a fascinating tool to revolutionize plant breeding for the development of various disease resistant cultivars however, effects of CRISPR/Cas9 system on different physiological process of plants still need to be studied.

Keywords: CRISPR/Cas9; plant breeding; resistance breeding

Introduction

Protection of crop cultivar against existing pest and disease as well as improvement of crop cultivar from higher productivity standpoint is a major challenge. 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 (Melchers & Stuiver, 2000). Different genome editing and advanced molecular techniques with transgenic plants are integrated with plant breeding to achieve improved crop cultivar with enhanced resistance to pest and diseases, termed as resistance breeding. Transgenic technology allows plant breeder to cross crop species introducing genes from non-related plants and other organisms into the crop plants (Melchers & Stuiver, 2000).

Genetic variation is an essential component of resistance breeding. To exploit the concept of resistance breeding, creation of genetic variation with the enhancement of resistance against pathogenic genes is an indispensable part (Van den Bulk, 1991). In light of these facts, different gene

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editing technologies have been used to develop genetic variations. One of the recent breakthrough 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 (Kumar & Jain, 2014). 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) (Belhaj *et al*., 2015). CRISPR/Cas9 is regarded as 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 (Kumar & Jain, 2014). This unique system in bacteria acquire invading or foreign DNA fragments and utilize them to recognize and degrade the further invading DNA or RNA sequences. CRISPR/Cas9 technology can be utilized to exploit defensive mechanism in plants against disease attack by recognizing and degrading the invading pathogenic genes.

CRISPR/Cas9 technology can be used for developing disease resistant cultivar. With its ability of sequence specific nuclease, CRISPR/Cas9 proves to revolutionize research in resistance breeding. This review paper will discuss on use of CRISPR/Cas9 technique for 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 the discussion on use of 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 researches providing information in current advances in CRISPR/Cas9 and its use in resistance breeding.

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

Gene editing and gene manipulation has always been an interesting topic for research works in molecular biology and plant breeding. Gene regulation is an important aspect for regulating and controlling the expression of specific genes to obtain desirable traits and characters in an organism and has high use in 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 genomes of important crops (Kumar & Jain, 2014). The principle behind gene editing consists of binding domain and effector domain. Binding domain helps in the recognition and binding of sequence specific DNA while effector domain helps in the cleavage of DNA at target site and regulates transcription (Wang *et al*., 2016). In a CRISPR/Cas9 system, CRISPR locus or array are located on the genome and consists of hypervariable spacers acquired from bacteriophage virus or plasmid DNA (Bhaya *et al*., 2011). Cas genes are located upstream of CRISPR loci and encodes for Cas protein for defense of invasive genetic materials (Bhaya *et al*., 2011).

CRIPSR/Cas9 system as an adaptive immune system possessed by many bacteria works in series of steps as shown in Fig. 1. 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 motif for the acquisition of DNA fragment into the spacer (Kumar & Jain, 2014). The expression of Cas proteins then transcribes the spacers acquired CRISPR to form pre CRIPSR RNAs (pre crRNAs) which after cleavage and maturation of pre crRNAs results into CRISPR RNAs (crRNAs). These crRNAs contains spacer sequence from 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 (Wang *et al*., 2016). 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 (Bhaya *et al*., 2011).

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) (Qi *et al*., 2013). Cas9 together with sgRNA forms RNA guided nuclease that regulates the sequence specific cleavage and editing in the target genome (Jinek *et al*., 2012). The site specific cleavage action of sgRNA-Cas9 complex is defined by pre designed sequences in guide RNA which has ~20 base pairs that are complementary to target DNA and helps in the binding of guide RNA to strands of target DNA (Kumar & Jain, 2014; Wang *et al*., 2016) (Fig. 2).

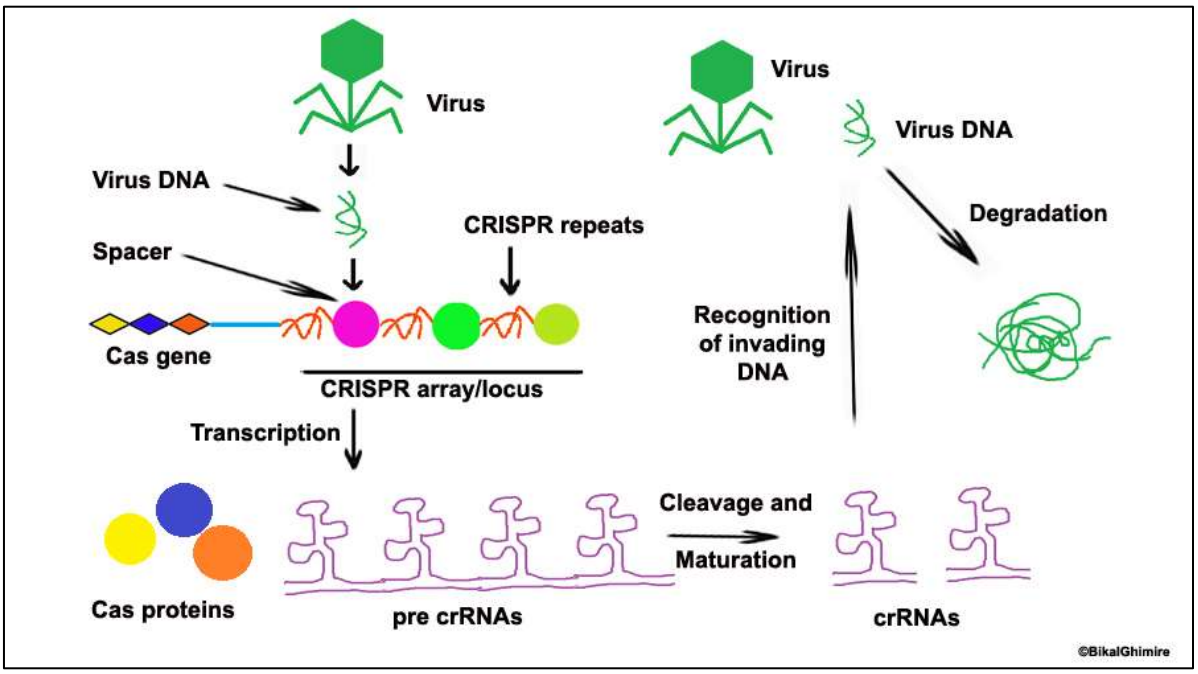

Fig. 1: Working mechanism of CRISPR/Cas9 system.

[CRISPR containing organism recognizes the invading DNA, takes a fragment of it and incorporates that fragment in the spacer between CRISPR repeats. Transcription of 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 study of CRISPR/Cas9 system in various literatures and reviews].

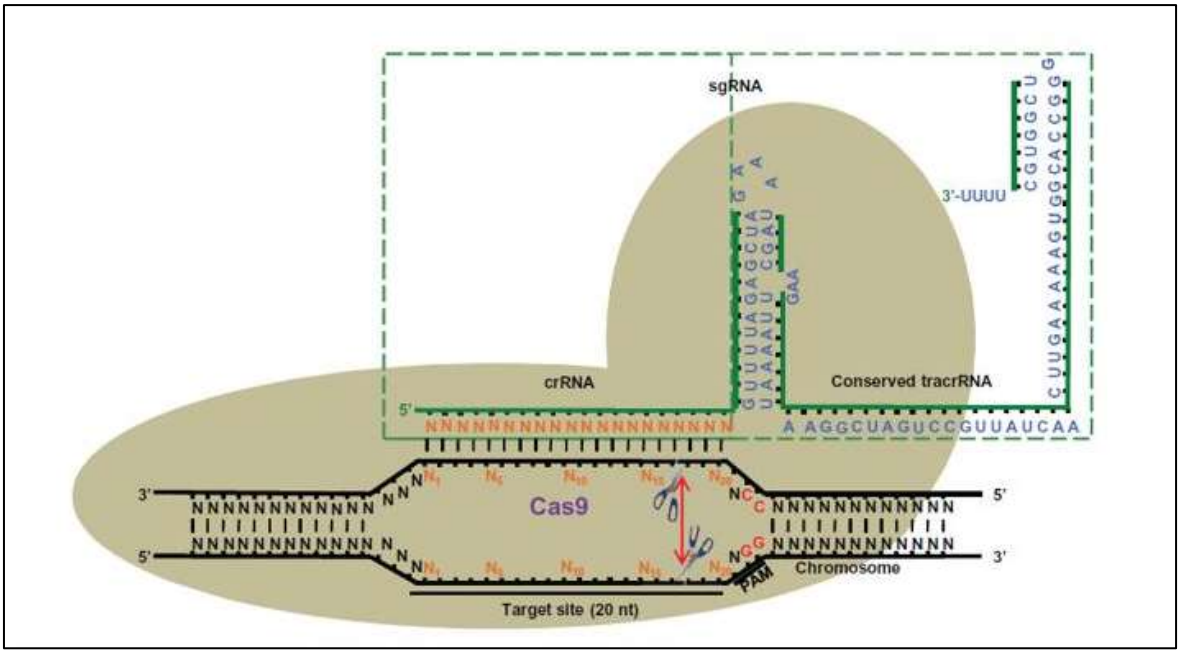

Fig. 2. Diagrammatic overview of CRISPR/Cas mediated cleavage mechanism.

[Green and black lines represent the backbone of the RNA and genomic DNA molecule, respectively. 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 Kumar & Jain (2014)].

With use of sgRNA as binding domain, specific sequence 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 (Chaparro-Garcia *et al*., 2015). Guide RNA binds to DNA and the pre-designed sequences in RNA guides Cas9 enzyme to cut the DNA strands at right locations. Cutting of DNA proceeds with removal and addition of required sequences into the target DNA (Wang *et al*., 2016). This technique can be explored to create resistance to specific disease in plants through delivery of sgRNA and Cas9 into target cells for gene transformation.

Different methods are used for delivery of sgRNA and Cas9 into plants cells like electroporation, via plasmids, agrobacterium mediated transformation, shotgun methods, particle bombardment and polyethylene glycol mediated

transformation (Jiang *et al*., 2013). However, Agrobacterium mediated gene transformation is easy and commonly used in many experiments. Ali *et al*. (2015), Baltes *et al*. (2015), Ji *et al*. (2015) explained the use of CRISPR/Cas9 technique for protection of plants against geminiviruses. Ali *et al*. (2015) performed experiment to demonstrate the efficacy of CRISPR/Cas9 against tomato yellow leaf curl virus (TYLCV) in *Nicotiana benthamiana* plants and their results exhibited profound evidence of interference against viral DNA by use of guide RNA mediated through *Agrobacterium tumefaciens*. Ali *et al*. (2015) engineered tobacco rattle virus (TRV) with sgRNA specific for TYLCV into *Agrobacterium tumefaciens* and infiltrated it into plant. Accumulation of TYLCV was found less in plants infiltrated with sgRNA then in control plant (Ali *et al*., 2015). 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 (Ji *et al*,, 2015). 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 viral genome (Ali *et al*., 2015). The sgRNA also controls other viral DNA whose sequences matches with it. Ali *et al*. (2015) were able to target three viruses TYLCV, beet curly top virus (BCTV) and Merremia mosaic virus (MeMV) matching an invariant sequence of viruses within the intergenic region of CRISPR. The sequence of crRNA regulates cleavage of specific DNA target and by using variable crRNAs it is possible to design multi target sgRNAs (Kumar & Jain, 2014). Table 1 shows the list of plant species in which gene transformation was integrated with CRISPR/Cas9 that induced resistance against different diseases in plant species.

Effectiveness of CRISPR/Cas9 in Resistance Breeding

CRISPR/Cas9 technique addresses the urgent need of efficient crop improvement schemes with advanced and reliable gene editing tools (Zhang & Zhou, 2014). This technique creates mutation in the target DNA with removal of target sequence and addition of gene of interest. It helps in mutagenesis of inaccessible genes, multi gene editing and generate large gene deletion which ultimately helps in progressive plant breeding (Khatodia, Bhatotia, Passricha, Khurana & Tuteja, 2016). This precise modification intro a plant genome can be inherited stably and the Cas9/sgRNA transgene can be removed to make the plants transgene free for successive improvement of the crop variety in further generations (Xu *et al*., 2015; Fig. 3).

Table 1: List of Plant Species with resistance to various disease 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)

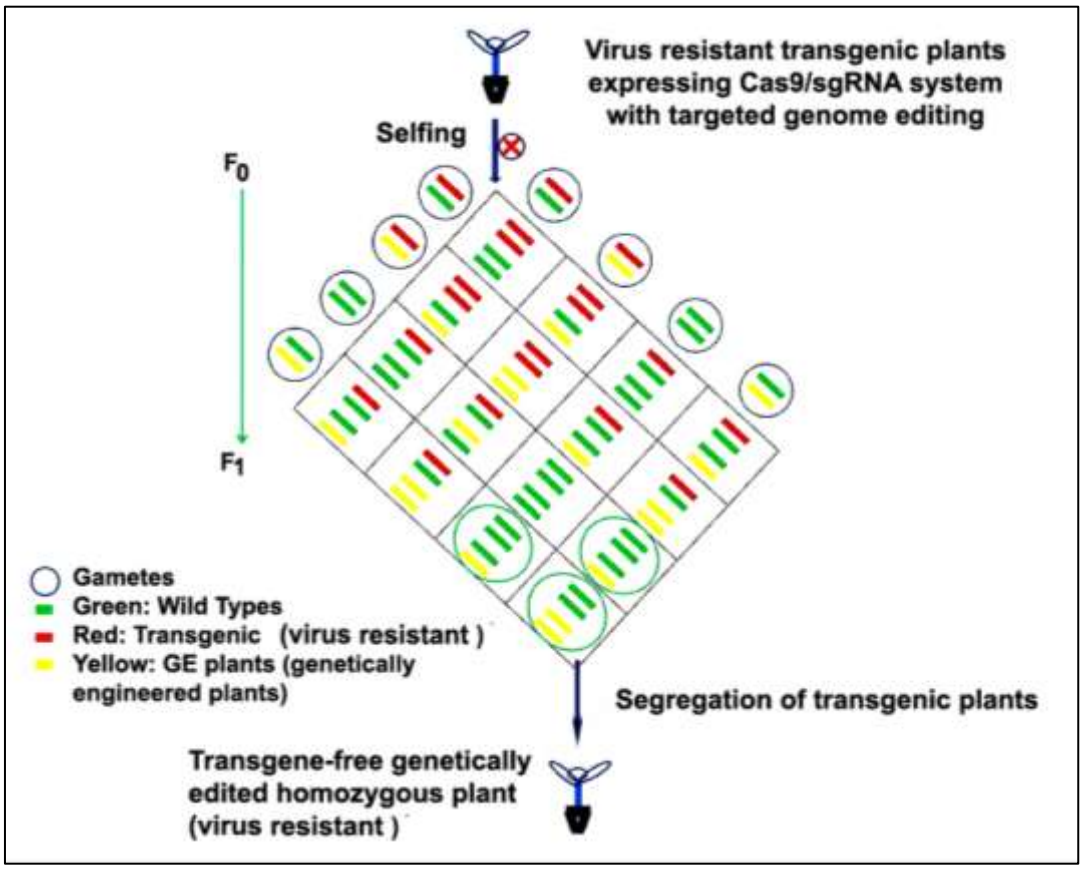

Fig. 3. Successive generation of transgene-free genetically edited (GE) viral resistant crops.
[Transgene-free viral resistant homozygous mutants having desirable modification at targeted gene site can be selected by selfing of F_0 generation plants. The segregation of transgene in the next F_1 generation will avoid possible modification in the genome by CRISPR/Cas9. This figure is extracted and modified from Khatodia *et al.* (2016)].

Many studies have reported 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 generation of plants resistant to specific virus or disease (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 development of transgene-free progeny by only one generation (Zhang & Zhou, 2014; Zhang *et al*., 2014). Xu *et al*. (2015) reported the development of transgene-free rice with desired genes after mutation by CRISPR/Cas9 and segregating the transgene with self-fertilization in F1 generation.

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 gene, virus resistant transgene and genetically engineered (GE) plant in F0 generation will produce different progenies in the F1 generation. The segregation of the transgene in F1 generation prevents undesirable modification from CRISPR/Cas9 in further generation. The selection of transgene-free homozygous plants with resistant genes in F1 generation helps in further breeding of plants to develop cultivars resistant to specific viral, bacterial and pest-disease (Khatodia *et al*., 2016) (Fig. 3). In autogamous plants it is possible to obtain 25% of F2 plants without transgene by self-fertilization of F1 plants (Kole *et al*., 2015). However, in 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 (Kole *et al*., 2015).

Conclusion

Increasing population, limited land resource, climate change and 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 breeding of disease resistant varieties seems to be 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 development of disease resistant plants makes use of CRISPR/Cas9 safe in plant breeding. However, various studies on effects of CRISPR/Cas9 on plant's 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, pathogen might change the invariant sequences in their genomes that

matches the intergenic sequences in CRISPR array in course of evolution and may develop resistance against CRISPR/Cas9 technology. Also, CRISPR/Cas9 integration might affect induced defence mechanism in plants for production of volatiles during pests attack. The study on effects of CRISPR/Cas9 on the rate of photosynthesis is also lacking.

In CRISPR/Cas9 system, guide RNA binds to DNA and the pre designed sequences in RNA guides Cas9 enzyme to cut the DNA strands at right locations. Cutting of DNA proceeds with removal and addition of required sequences in to the target DNA. DNA recombination repairs the cut in the DNA and induces mutations. However, it also sometimes results in unwanted mutations as it is not necessary that all of the 20 base pairs in guide RNA sequence needs to be complementary for binding with target DNA. This may result in binding of guide RNA in 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, scientist are working to make use of CRIPSR/Cas9 more accurate as Cas9 enzyme can be regulated to target different sites in DNA by changing sequences in guide RNA (Wang *et al*., 2016).

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

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