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CRISPR-CAS9 MEDIATED GENOME EDITING IN PLANTS AGAINST VIRUSES: AN UPDATED REVIEW

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Abstract

The novel clustered regulatory interspaced short palindromic repeats (CRISPR)/CRISPR associated nuclease 9 (Cas9) method is one of the key tool for the modification of plant genome against biotic stress. Viruses inflict a greater extent of losses to crop yields in the form of destructive diseases. Conventional approaches of augmenting disease resistance are rendered ineffective by ever evolving viruses. Moreover, the relatively advanced CRISPR/ Cas9 system is a quick and efficient method to confer resistance to plants against a broad range of viruses. CRISPR/Cas9 precisely alters the host genome against specific virus. However the mutation rate and tolerance response vary with type of plant species and with use of different methods as well as the targeting sites. Several researchers used this system against both RNA and DNA types of viruses. Here, we discussed the advantages of CRISPR-Cas9 technique in plants against different types of viruses. The present review will be useful to scientists to understand the range of options offered by this technology for enhancing resistance in economically important plant species against multiple viruses.

Key words: Biotic stress; CRISPR-Cas9; DNA/RNA viruses; Genome editing; Virus resistance.

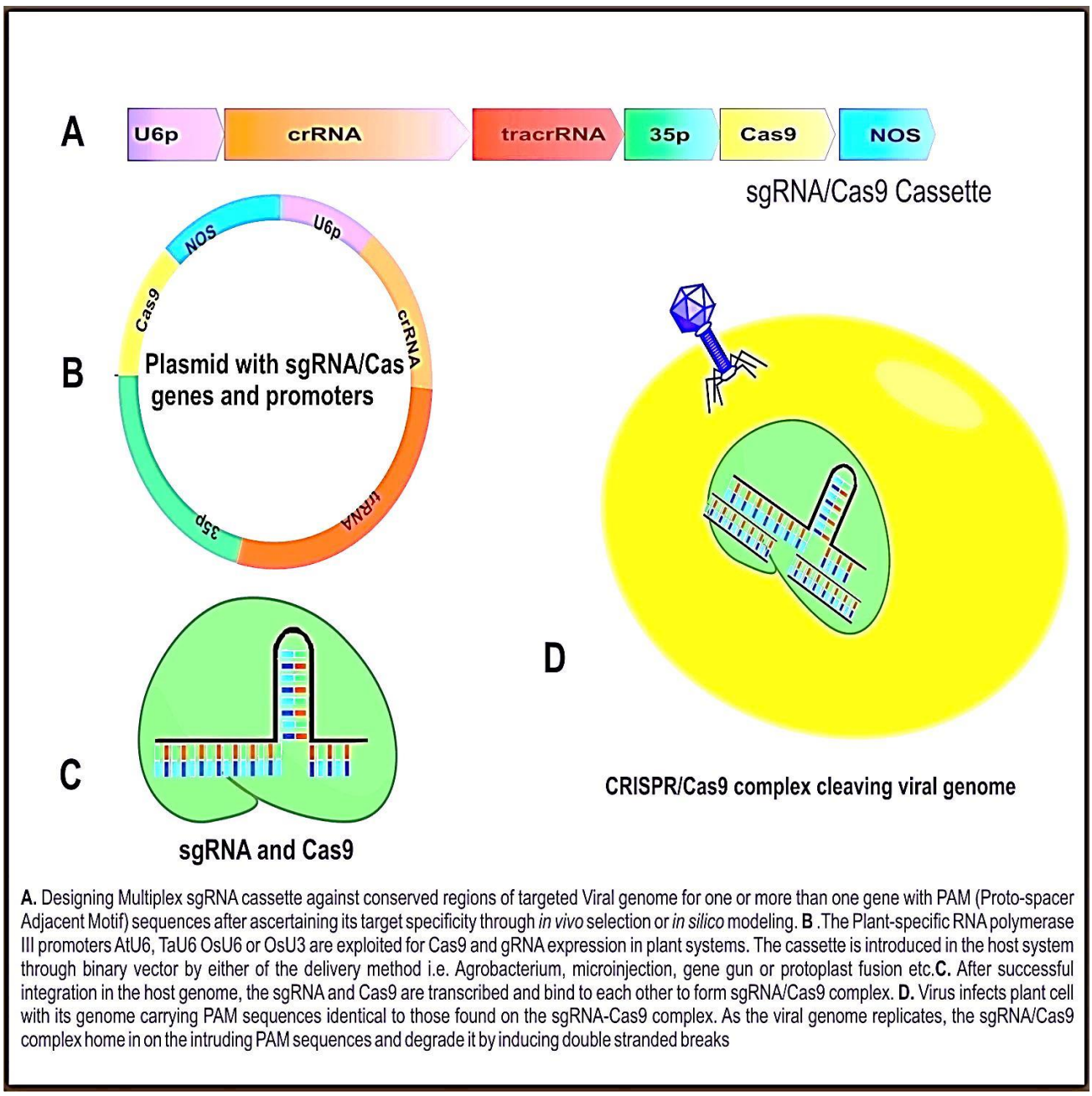
Introduction

Overview on CRISPR-Cas9 technology against plant viruses: The CRISPR/Cas technique is a novel and efficient method for plant genome editing (Khurshid *et al*., 2017; Shinwari *et al.*, 2017). Several researchers have used this system to boost up the immunity of several plant species against many lethal viruses. In early experiments, Ali *et al.*, (2015), Ji *et al.*, (2015), and Baltes *et al.*, (2015) used this technology to enhance tolerance in plants against geminviruses. In all three methods the Cas9-guided RNA complex targeted the double strand RNA (ds-RNA) and inhibited viral replication. The CRISPR-Cas system based engineered geminvirus resistant plants remain green and healthy compared to disease plants. Here, we summarized the latest literature on CRISPR/Cas9 approaches to develop resistant plants against a broad range of viruses. The methodology of CRISPR/Cas9 based genome editing against viruses is given in (Fig. 1). The detailed information on developing CRISPR/Cas9 based virus free genetic engineered plants is given in Table 1.

Applications of CRISPR/Cas9 system to develop genetic engineered plants against both RNA and DNA viruses: Biotic and abiotic factors affect the morpho-biochemical and molecular processes of many important plant species (Shah *et al*., 2016; Jan *et al.*, 2016; Hasanuzzaman *et al.*, 2017; Jan *et al.*, 2017; Nejat & Mantri, 2017; Shinwari *et al.*, 2020). Directed alteration of plant genomes is an amazing methodology for examining and building cell frameworks and bringing changes in economically important traits (Mahas *et al*., 2019). The simple, easy and efficient engineered CRISPR/Cas system is a useful genome modification method for many important eukaryotic species including plants against DNA viruses (Ali *et al*., 2015; Song *et al.*, 2018; Kennedy & Cullen, 2017). Tashkandi *et al.*, (2018) develop tomato yellow leaf curl virus (TYLCV) resistant transgenic *Nicotiana benthamiana* and tomato by using this system (Table 1). The *Agrobacterium* transformation method was used for the expression of sgRNA and Cas protein under the control of U6-26s and CaMV-35S promoters, respectively. The transgenic plants were selected by using kanamycin as a screening marker. The transgenic plants expressing Cas9 endonuclease were confirmed through anti- FLAG antibody western blotting techniques. Three transgenic *Nicotiana benthamiana* lines show positive results for Cas9 endonuclease. These lines show tolerance against TYLCV by cleaving at *CP, IR,* or *Rep* sequences. Similarly, six transgenic tomato plants were screened that showed positive PCR results for *CP* and *Rep* regions that lead a T7EI mutation. The rolling circle amplification assay (RCA) also showed a lower amount of viral DNA in the transgenic plants than non-transformed plants.

Liu *et al.*, (2018) used this novel system in model Wild-type *Arabidopsis thaliana* Col-0 plants against cauliflower mosaic virus (CuMV) (Table 1). They targeted the viral coat protein sequences that lead to short deletions or insertions and eventually caused early inhibition of the translation process. They also found short

small interfering RNAs (siRNA) at the 3- side of the sgRNA. However, they observed that resistance to CaMV was due to the presence of Cas9 protein not due to the siRNA. The resulting transgenic plants showed normal growth, remained fertile and no other adverse effects were observed (Liu *et al*., 2018). They also suggested that further modification would help to improve the high level of resistance against this virus. Zhang *et al.*, (2018) designed *Francisella novicida* based CRISPR-Cas9 system to produce transgenic *Nicotiana benthamiana* and *Arabidopsis* plants against two RNA viruses (cucumber mosaic virus (CMV) and tobacco mosaic virus (TMV). The plants having Fncas9 and specific sgRNA to both viruses showed lower infection rate and low level of RNA. Severe leaf shrinkage symptoms were observed in infected control. They also found similar results in the next progenies. Aman *et al.*, (2018) established a new CRISPR/Cas13a method against a RNA virus (TuMV) in model *N. benthamiana* plant. They found higher levels of interference of CRISPR RNA (crRNAs) in two important sequences like helper component proteinase silencing suppressor (*HC-Pro*) and green fluorescent protein (GFP). They recorded about 50% reduction in GFP signal in leaves at one week of days post infiltration (dpi) in transgenic plants. Ali *et al*., (2018) developed CRISPR-Cas-based engineered *Nicotiana benthamiana* and *Arabidopsis thaliana* plants against two types of viruses (Tobacco rattle virus (TRV) and Pea early browning virus (PEBV). They reported that these two viruses could efficiently deliver sgRNA to leaves and induce mutation. However, the mutation was higher in PEBV-based sgRNA compared to the TRV-based delivery. Ali *et al*., (2018) recommended this system for engineering of some other economically important plant species. .



A. Designing Multiplex sgRNA cassette against conserved regions of targeted Viral genome for one or more than one gene with PAM (Proto-spacer Adjacent Motif) sequences after ascertaining its target specificity through *in vivo* selection or *in silico* modeling. **B** .The Plant-specific RNA polymerase III promoters AtU6, TaU6 OsU6 or OsU3 are exploited for Cas9 and gRNA expression in plant systems. The cassette is introduced in the host system through binary vector by either of the delivery method i.e. Agrobacterium, microinjection, gene gun or protoplast fusion etc.**C.** After successful integration in the host genome, the sgRNA and Cas9 are transcribed and bind to each other to form sgRNA/Cas9 complex. **D.** Virus infects plant cell with its genome carrying PAM sequences identical to those found on the sgRNA-Cas9 complex. As the viral genome replicates, the sgRNA/Cas9 complex home in on the intruding PAM sequences and degrade it by inducing double stranded breaks

Fig. 1. CRISPR/Cas9 based genome editing in plants against viruses.

Table 1. Applications of CRISPR/Cas9 system in developing genetic engineered Virus free plants (Modified from Khatodia *et al.*, 2017).

Engineered plant	Targeting virus	Target sites	Reference
Hordeum vulgare	Wheat dwarf virus (WDV)	*CP, MP, LIR, Rep*	Kis *et al*., (2019)
A. thaliana	Banana streak virus (BSV)	*BSOLV, eBSOLV*	Tripathi *et al*., (2019)
N. benthamiana	TMV	*HC-Pro, GFP*	Aman *et al.,* (2018)
A. thaliana Col-0	CuMV	*CP*	Liu *et al.,* (2018)
N. benthamiana	GVBS, CTV	43 regions in the viral genome	Zhang *et al.,* (2018)
N. benthamiana	PVX, TCV and TMV	*AGO2*	Ludman *et al.,* (2017)
N. benthamiana, Solanum lycopersicum	TYLCV	*CP, IR, Rep*	Tashkandi *et al.,* (2018)
Cucumis sativus L.	CVYV, ZYMV and PRSMV-W	*eIF4E*	Chandrasekaran *et al.,* (2016)
A. thaliana	TMV	*eIF(iso)4E*	Pyott *et al.,* (2016)
Citrus paradisi	CK	*CsLOB1*	Jia *et al.,* (2017)
N. benthamiana	TYLCV, CLC KV, TYLCSV, MMV, BCTV	*IR, CP, Rep*	Ali *et al.,* (2016), Ali *et al.,* (2015)
N. benthamiana, A. thaliana	BSCTV	*IR, CP, Rep*	Ji *et al.,* (2015)
N. benthamiana	BYDV	*LIR, Rep/RepA*	Baltes *et al.,* (2015)

Chandrasekaran *et al*., (2016) developed virus resistant Cucumber plants for the first time by using Cas9 sub-genomic RNA (sgRNA) technique. The cas-sgRNA lead single nucleotide polymorphisms of transformed T_1 plants by targeting N^0 and C^0 of eukaryotic translation initiation factor 4E (*eIF4E*). The mutated non-transformed heterozygous plants were used for the formation of homozygous plants at T_3 stage by following Cas9/sgRNA based mutation at two sites of *eIF4E*. The resulted plants showed resistance to three different types of viruses (Cucumber vein yellowing virus (CVYV), Zucchini yellow mosaic virus (ZYMV) and Papaya ring spot mosaic virus-W (PRSV-W) as compared to the non-mutant and heterozygous plants. Ludman *et al.*, (2017) described the antiviral immunity role of Argonaute 2 (*AGO2*) gene in *N. benthamiana.* They used the CRISPR-Cas9 system for the production of broad range virus resistant *N. benthamiana* plants by inactivating the *AGO2* gene. The resulting *AGO2* mutant plants showed different sensitivity responses to three distinct viruses (potato virus X, turnip crinkle viruses and turnip mosaic virus (PVX, TCV and TuMV). A very efficient CRISPR genome editing system was used by Iqbal *et al.*, (2016) to bring leaf curl virus resistance in cotton. Jia *et al.*, (2017) used this tool for genome editing in Ducan grape fruit (*Citrus paradisi*), one of the key susceptible genotype against citrus canker disease. It was also used to bring modification in a type of canker susceptibility gene *CsLOB1* in six different susceptible grape fruit lines (DLOB2, DLOB3, DLOB9, DLOB10, DLOB11 and DLOB12). The genotype DLOB2 and DLOB3 showed low rate of mutation (31.58 and 23.80%). While other genotypes i.e. DLOB9, DLOB10, DLOB11 and DLOB12 showed a high rate of mutation (89.36, 88.79, 46.91 and 51.12%). All the genotypes were inoculated to *Xanthomonas citri* subsp. citri (Xcc) stress. The genotypes (DLOB2 and DLOB3) having a low rate of mutation showed susceptibility than other four highly mutated genotypes. However, the resistance plants showed small pustules caused by Xcc at a later stage. The pustule found in genotypes DLOB9 and DLOB10 did not produce any canker symptoms.

Conclusion

The engineered CRISPR/Cas9 technique has been successfully used in economically important crop species by different researchers against different types of viruses. The protocols developed by different researcher are efficient and provide durable resistance against a broad range of viruses at many generations. This method can suppress or enhance the expression of the target gene in a precise way. However, several modifications are needed to develop highly durable genetic engineered plants against many dangerous viruses. In the near future this technique can be used to produce new biotech crops against a wide range of viruses without any harmful effect to the environment or other living forms.

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