Review

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Review

When Intruder Comes Home: GM and GE Strategies to Counter Virus Infection in Plants

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Abstract: Viruses are silent enemies that intrude and take control of the plant cell's machinery for their own multiplication. Infection by viruses and the resulting damage is still a major challenge in the agriculture sector. Plants have the ability to fight back, but the ability of viruses to mutate at a fast rate helps them to hide from the host response. Therefore, classical approaches for introgressing resistance genes by breeding have obtained limited success in counteracting the virus menace. Genetic modification (GM) based strategies have been successful in engineering artificial resistance in plants. Several different approaches based on pathogen derived resistance, antisense constructs, hairpin RNA, double stranded RNA etc. have been used to enhance plant resistance to the virus. Recently, genome editing (GE) strategies mainly involving the CRISPR/Cas mediated modifications are being used for virus control. In this review, we discuss the developments and advancements in the GM and GE based methods in tackling virus infection in plants.

Keywords: pathogen derived resistance; RNA silencing; artificial miRNA; tasiRNA; epigenetic editing; prime editing; multiplexing CRISPR/Cas editing

1. Introduction

Viruses constitute nearly half of the microbial pathogens that are accountable for emerging and re-emerging the epidemics worldwide [1]. They also pose serious challenges to agriculture, by causing major plant diseases like bunchy top of banana, tobacco mosaic, wheat streak mosaic, potato tuber necrotic ring spot, cucumber green mottle mosaic, African cassava mosaic etc. So far, 131 families containing 803 genera and 4853 species of plant virus are known (ICTV, 2018). The viruses are responsible for a worldwide crop loss of $30 billion annually and it is predicted to reach up to $60-80 billion [2–4]. The plant viral diseases threaten global food security as they can cause severe food limitations for the world population, which is projected to elevate from 8 billion in 2022 to 10 billion by 2050 [5,6].

For resolution of any problem, it is important to implement appropriate prevention and control measures. However, the viruses cannot be controlled by chemicals like pesticides, although their carriers or vectors can be restrained to some extent [7] This simply leaves the possibility of finding or generating plant species with inherent resistance to the virus infections. The silver lining came from the McKinney's observation in 1929, that mild infection caused by Tobacco Mosaic Virus (TMV) green mosaic strain protected the tobacco plants from severe infection of TMV yellow mosaic strain, pointed towards the existence of mechanism(s) for cross protection [8]. This phenomenon involves the formation of a memory response in the host plant to combat the challenge posed by related virus [9] These early observations prompted search for host-based resistance genes and detailed understanding of plant-virus interactions [10,11].

The selection of dominant and recessive genes associated with virus resistance played an important role in breeding of naturally resistant plant varieties as a cheaper and better alternative for tackling viral diseases [4]. Classical approaches such as introgression of resistance genes by marker assisted selection (MAS), QTL mapping and gene pyramiding were extensively used to tackle the menance of viruses. However, there were several bottlenecks such as non-availability of markers near

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the resistance genes, transfer of undesired traits with resistance genes, and screening of plants for several generations, which made the process tedious and time consuming ands limited the success of these techniques [12].

The era of genetic engineering accelerated the process by allowing incorporation of genes in the plant genome as per the requirement. In the early 1980s, the concept of Pathogen Derived Resistance (PDR) laid the foundation of artificial resistance. PDR employed the use of virus proteins like coat protein (CP) to disrupt the virus replication by inactivating the viral components [10]. Subsequent studies unravelled the role of RNA silencing or RNA interference (RNAi) in symptom recovery and cross protection [13,14]. It also strengthened the possibilities for employing RNA silencing for generating virus resistance in plants. However, the silencing pathway has some limitations due to presence of silencing suppression activity in the viral encoded proteins. In recent times, CRISPR/Cas based tools are being used to edit the plant genome for generating virus resistant plants. In this review, we have discussed the improvements and advancements of gene modifications (GM) and genome editing (GE) approaches in managing plant virus infections.

2. GM approaches

These approaches involve the over-expression or knock-down of genes using a variety of genetic engineering tools. The methodology usually involves cloning the gene of interest (trans-gene) into the desired vector in either sense or antisense orientation followed by its transformation in plants [15]. Many old reports are available on engineering virus resistance in plants via this transgenic approach [16,17].

2.1. Pathogen Derived Resistance

In this approach the pathogen's own genetic material is used to generate resistance in the host plant. The concept of PDR was first presented by Sanford and Johnston (1985) and a year later it was proven to be successful in case of virus infections. The transgenic plants expressing coat protein (CP) gene of TMV were shown to exhibit resistance against TMV infection [18]. This ground-breaking discovery initiated the development of various virus-resistant transgenic plants [5]. PDR can be classified into different types depending on the strategy used to generate resistance towards the virus in the host plant.

2.1.1. Protein-Mediated Resistance

The process entails the expression of viral encoded proteins like CP, MP (movement protein) or Rep (replicase) in the plants to counteract the virus [5]. In 1986, Beachy's group first demonstrated that the expression of the CP gene of TMV in transgenic tobacco plants could provide considerable protection against virus infection [19]. It was observed that the expression of these viral proteins interfered with the disassembly of virus by formation of pseudo-capsids [5] Several transgenics were made using CP of *Cucumber mosaic virus* (CMV), *Papaya ringspot virus* (PRSV), *Potato Virus X* (PVX), *Plum pox virus* (PPV), *Zucchini yellow mosaic virus* (ZYMV), *Watermelon mosaic virus* (WMV), *Tomato yellow leaf curl virus* (TYLCV), *Tomato mosaic virus* (ToMV) and so on [20]

PDR also provided cross-protection against infection by related viruses. This technique was more effective in case of closely related viruses than those which are distantly related [21]. When TMV CP-transgenic plants, were infected by different viruses of the Tobamovirus family their inhibition was differentially compromised. Some viruses like Tomato mosaic virus (ToMV), *Pepper mild mosaic virus* (PMMV) and *Tomato mild green mosaic virus* (TMGMV) were inhibited by ~98% while no resistance was observed against others like *Cucumber mosaic virus* (CMV), *African mosaic virus* (AMV), *Potato virus X* (PVX) and *Potato virus Y* (PVY), though there was decrease in symptoms and spread [22]. In field trials, CP-TMV expressing tomato and CP-PVX/PVY expressing potato transgenics performed better than the wild-type [23].

In case of PVX infection, it was shown that the interaction of the 5' end of viral RNA with CP expressed in the host plant suppressed the expression of RNA dependent RNA polymerase (RdRP)

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encoded by the virus [24,25] The region and quantity of protein expression were important determiners for the generation of resistance. For instance, if CP was expressed in mesophyll but the viral RNA was introduced in epidermal cells then resistance did not develop [26]. Expression of mutant CP in the transgenics also resulted in resistance against wild-type CP, as the mutant CP could interact with the wild-type CP subunits or the host factors, which were involved in virion disassembly [27,28]. The extent of aggregation of CP correlated with level of Coat protein mediated resistance (CPMR) [29]. This observation suggested that homology between the amino acid sequences of the challenging viruses provided cross-protection in the transgenic [5].

Likewise, the MPs, which have role in cell to cell spread of the virus, were also utilized for triggering PDR. This technique worked better for large genera of viruses irrespective of the viral MP expressed in plants. The expression of defective or mutant MPs could also block virus transmission, since the mutant MPs competed with wild-type MPs of the virus for the plasmodesmatal sites [30]. Mutant TMV-MP provided resistance against Potexviruses, Tobamoviruses and Cucumoviruses [31]. Several groups tried to multiplex this approach by generating transgenics with more than one type of MP, by using overlapping regions of the ORFs which are also known as Triple gene block (TGB). However this approach did not provide wider resistance, possibly due to insufficient interaction of TGB with the plasmodesmata [32].

In subsequent studies, it was shown that use of modified non-structural proteins like Rep/RdRP were able to provide better results even at low levels of expression [33]. Mutations in the ori or NTP-binding sites of Rep gene (AC1) provided resistance against Geminiviruses [34,35]. The Rep/RdRP proteins could directly interfere with virus replication or bind with host factors involved in the viral replication process [36]. Detailed molecular mechanisms behind the responses were established for few viruses like TMV. Although later reports demonstrated that these proteins mediated resistance mainly through the RNA silencing machinery instead of direct protein interactions [37,38].

Despite several examples of the successful implementation of this technology, it faced some challenges due to evolutionary pressure provided by the expression of transgene. The genomic content of the virus interacting with the transgenic got altered due to recombination between transgenic and viral CP transcripts [39]. This trans-capsidation of the CP in transgenics imparted a new potential to the infecting virus isolate. Mixed infection by two unrelated viruses was another issue as it led to antagonism and synergism of defence responses. For instance, different outcomes were produced during mixed infection with *Papaya ringspot virus* (PRSV) and *Papaya mosaic virus* (PapMV). In the case when plants were first infected with PRSV followed by PapMV or simultaneously inoculated with both a synergistic response was observed. However, when first inoculation of PapMV was followed by PRSV an antagonistic response was observed, which caused reduction in PRSV titres due to activation of defence responses [40].

2.1.2. Defective interfering virus mediated resistance

Plant viruses are often associated with subgenomic/subviral RNAs known variably as satellite (sat) or defective interfering (DI) virus [41]. The DI viruses lack a critical portion of the virus genome, which makes them defective for replication. They require the presence of the complete functional virus, known as the helper virus, for their replication and encapsidation [42]. DI viruses are basically the defective versions of helper viruses formed by mainly deletions, duplication and inversion processes [43]. There is often a synergistic interaction between the DI virus and its helper as they can produce mild symptoms on one host plant but elicit severe symptoms on another host or in combination with a different strain of helper virus [44,45]. The observations that DI virus can attenuate symptoms of unrelated helper virus in co-infected cells by inhibiting the production and long-distance movement of helper virus [46,47]. It was used in transgenic approaches to decrease virus symptoms and/or titers [42]. These sat RNAs compete with the viral genome for binding to replicase for replication which indirectly suppresses the virus replication [47]. In 2015, it was shown that CMV sat-Y derived siRNAs directed against the VSR, 2b of CMV and P10 of Tombusvirus, caused displacement of the VSR-bound siRNAs resulting in down regulation of the viral target genes

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[48]. The major limitation of this strategy was a probable risk that the DI virus could mutate to a virulent form in conjunction with the infecting virus in the transgenic plants.

2.2. RNA silencing as a major antiviral weapon

The discovery of non-coding small RNAs and their role in gene silencing provided a paradigm shift in the understanding of host defense to virus and generation of virus resistance. RNA silencing is an evolutionarily conserved, fundamental cellular mechanism for regulating gene expression [49]. The process involves small RNA mediated degradation or inactivation of specific mRNA in sequence dependent manner [50]. In plant immunity, it contributes as first line defences against virus infection as is often referred to as virus induced gene silencing (VIGS). Earlier it was understood that the genome of RNA viruses and viroids were subject to RNA silencing [51,52] but later it was shown to be effective against the DNA viruses such as Geminiviruses (having ssDNA) and Caulimoviruses, Tungroviruses (having dsDNA with RNA intermediate) [53,54].

The RNA silencing machinery is triggered by the production of double stranded (ds) RNA either as replicative intermediate or self-folded complementary region of single strand (ss) viral transcript (Figure 1). The dsRNA is diced by the microprocessor protein complex containing Dicer Like Proteins (DCLs) to generate 20-24 nt small RNAs [55]. The small RNAs derived from the virus are normally categorised as viral small interfering RNA (vsiRNA). They are loaded onto RNA induced silencing complex (RISC) to direct Transcriptional Gene Silencing (TGS) or Post- Transcriptional Gene Silencing (PTGS) [55,56]. In TGS, the target gene gets methylated to block transcription [57,58], whereas in PTGS, the target mRNA is cleaved to block translation [59]. The visiRNAs also act as mobile signals as they can spread from cell to cell [60] [61]. These primary siRNAs also get amplified with the help of SGS3 and RDR6 to produce secondary siRNAs to provide protection throughout the plant [55,62].

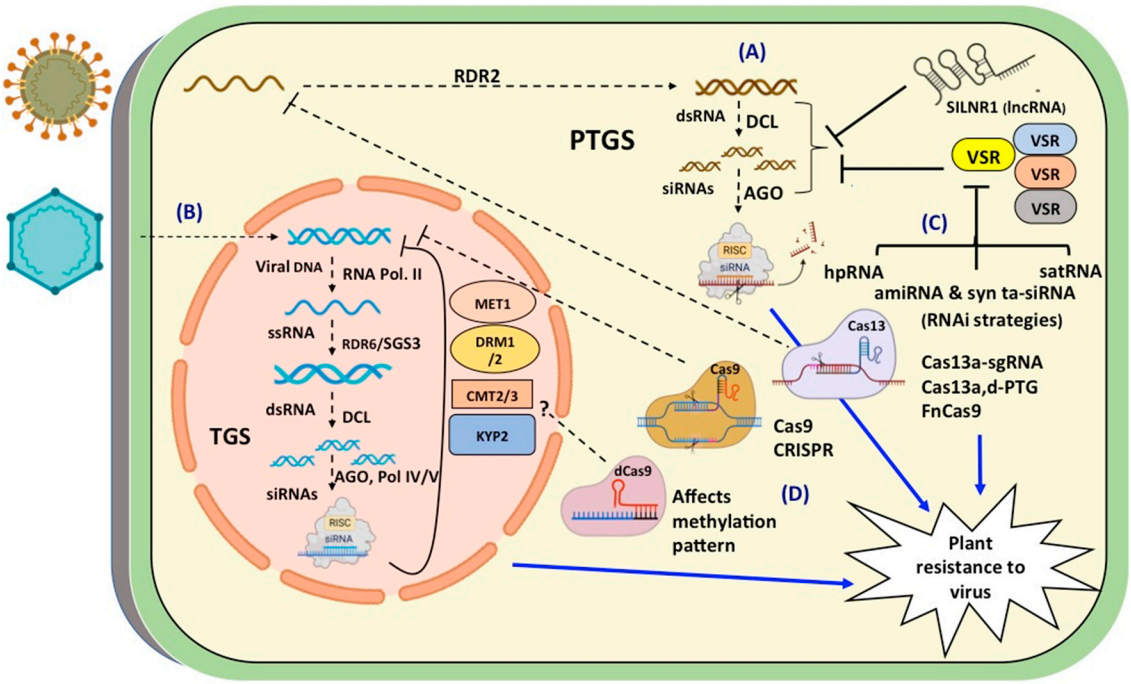

Figure 1. Schematic diagram to represent the RNAi pathway and the targets in genome editing strategies that ehance plant resistance to virus infection. (A) Infection of virus triggers Post-transcriptional Gene Silencing (PTGS) in plant cell. Long non-coding RNAs (incRNAs) like SILNR1 and viral encoded suppressor of RNA silencing (VSR) interfere with multiple steps in the silencing pathways. (B) Infection of DNA virus can also triggers Transcriptional Gene Silencing (TGS) in plant cells. Components of methylation machinery like Methyltransferase 1 (MET1), Domain rearranged methyltransferase 1/2 (DRM1/2), Chromomethylase 2/3 (CMT2/3) and Kryptonite 2 (KYP2) are

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involved in small RNA directed methylation. (C) The RNAi pathway can br artificially triggered by expression of hairpin RNA (hpRNA), artificial miRNA (amiRNA), synthetic trans acting siRNA (syntasiRNAs) and satellite or DI virus RNAs directed against the viral genome. (D) The GE strategies use nucleases such as FnCas9 and Cas13a,d to target the viral RNAs mainly the VSRs.

2.2.1. Role of TGS in virus infection

RNA silencing has a great impact on regulating viral genes as well as host genome via epigenetic modifications. The small RNAs initiate RNA directed DNA methylation (RdDM) of complementary DNA sequences [63,64], which reduces or blocks the transcription of mRNA, so the gene undergoes silencing [65,66]. Some proteins like AGO4, DCL3, RNA polymerase IVa, RDR2 which are involved in TGS would also lead to initiates systemic silencing. Many small RNAs as well as dsRNA were reported to have a role in targeted methylation by triggering RdDM.

Studies have shown that a typical small RNA mediated methylation pathway involves transcription from the target locus by RNA polymerase IVa [67]. The ssRNA is converted into dsRNA by action of RdRP/RDR2, which is then diced by DCL3. The siRNAs thus generated, associate with AGO4 containing RISC and polymerase IVb complexes to search for the target DNA sequences. At the target site RNA polymerase V is recruited to form AGO4:siRNA:PolV complex which attracts de novo methyltransferases [68]. Normally cytosine methyltransferases like DOMAIN REARRANGED METHYLTRANSFERASE 1/2 (DRM1/2), CHROMOMETHYLASE 2/3 (CMT2/3) (for CHG/CHH methylation), METHYLTRANSFERASE 1 (MET1) (for CG methylation) and KRYPTONITE 2 (KYP2) get recruited at the target site alongwith DEFECTIVE IN RNA-DIRECTED DNA METHYLATION 1 (DRD1), DEFECTIVE IN MERISTEM SILENCING 3 (DMS3) and DEFICIENT IN DNA METHYLATION 1 (DDM1) [69]. Cytosine methylation engages the histone deacetylases and other methyltransferases to initiate histone modifications [70]. It has been shown that methylation in promoter region, rather than coding region, is more effective for silencing [71]. Moreover, CG and CHG methylation can be maintained after cell division and without RNA triggers but CHH methylation cannot. [63,72,73].

DNA viruses can be easily targeted through TGS, because of their ssDNA and dsDNA replication process (Figure 1). The replication intermediates of Pararetroviruses and Geminiviruses form mini chromosomes, which are common targets of TGS [74–76]. VIGS was shown to initiate RdDM and methylation deficient mutants of *Arabidopsis* were hypersusceptible to infection [69]. The accumulation of visiRNAs to threshold levels at the target DNA recruits DRM2 for initiating de novo methylation [77]. Methionine cycle (MTC) plays a crucial role in small RNA mediated methylation during virus infection by maintaining continuous supply of S-adenosyl methionine (SAM) and removal of methyl transferase inhibitor, S-adenosyl-homocysteine (SAH). S-adenosyl-L-homocysteine hydrolase (SAHH) converts SAH to L-homocysteine (HCY) and then HCY converted into methionine by Methionine synthase (MS). SAH has been shown to inhibit Hua enhancer 1 (HEN1), which is responsible for siRNA stability through 2′-O-methylation of their 3′ pentose sugar (Mäkinen & De, 2019). It was hypothesized that viral encoded RNA silencing suppressor proteins like HC-Pro interact with SAM synthease and reduce its activity which results in decreased levels of SAM [78]. Mixed virus infections of potyvirus and potexvirus create imbalances in MTC and GSH biosynthesis which induce oxidative burst and lead to more symptoms in infected plants [79].

2.2.2. Role of PTGS in virus infection

The replicative intermediates of RNA viruses and the transcripts of DNA viruses are targets of PTGS in the cytoplasm [76]. Plants show symptoms of recovery when the vsiRNAs are generated and spread through the vascular system. Arabidopsis mutants of *sgs3, ago1, sgs2* showed greater susceptibility to virus infection [80]. Studies with Tomato bushy stunt virus (TBSV) infection in *Nicotiana benthamiana* and PVX and Turnip crinkle virus (TCV) in Arabidopsis have shown that the antiviral defense involves the AGO2 pathway [81–83].

To counteract RNA silencing, viral proteins contain suppressor of RNA silencing (VSR) activity (Figure 1). The VSRs interact with the components of PTGS at multiple points. They can interrupt

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silencing process by making dsRNAs unavailable for DCL action, inhibiting the siRNAs transport, quenching the siRNAs to prevent their loading in RISC and inactivation of RISC [84]. For instance, 2b protein, the VSR of Tomato aspermy virus (TAV), shows non-specific binding with siRNA and dsRNA [85]. Whereas, V2, the VSR is of Tomato yellow leaf curl virus binds with SGS3 which has an important role in dsRNA amplification [86,87].

2.2.3. small RNA shields in virus resistance

RNA silencing has been effectively used for generating virus resistant plants [49,88]. A variety of small RNAs have been used through various strategies to trigger the RNA silencing mediated defense responses against viruses (Table 1). The regions of the viral genome that encode VSRs eg. P69, HC-Pro and AC2 are usually selected as the targets of the small RNAs. Expression of small RNAs can be from various precursors, which may be in cis or trans and single or polycistronic [89,90]. Initial GM approaches involved the use of antisense constructs or hp-RNAs as precursors to generate a pool of siRNAs, which could target the viral transcripts. Subsequently, these were replaced by more specific tools through the use of constructs expressing antiviral artificial microRNAs (amiRNAs) and synthetic trans-acting small interfering RNAs (syn-tasiRNAs).

Table 1. List of small RNA based GM approaches used against plant viruses.

Strategy	Virus	Target	Plant	References
hpRNA (IR)	TMV	Movement protein	Tobacco	[91]
	CMV	Replicase		
	PPV	P1 and HC-Pro		[92]
	CGMMV	Coat protein		[93]
hpRNA	PSTVd	Viroid sequence	Tomato	[94]
	TYLCV	Replicase (C1)		[95]
		Coat protein		[96]
	PVX	ORF2	Potato	[97]
	PVY	HC-Pro		
	PLRV	CP		
	PRSV	CP	Melon	[98]
	SbDV	CP	Soyabean	[99]
	SMV	HC-Pro (VSR)		[100]
	WCMV	Replicase	White clover	[101]
	ACMV	Rep (AC1)	Cassava	[102]
	BBTV	Replicase	Banana	[103]
	CTV	P20, p23, p25 (VSRs)	Mexican lime	[104]
DI Virus-derived RNA	PVY	Coat protein (DI virus)	Tobacco	[105]
	TBSV	Movement protein (DI		
	GLFV	virus)		
dsRNA	ACMV	DNA-A and DNA-B	Tobacco	[106]
	RGSV	pC5, pC6	Rice	[107]
	RTBV	ORF IV		[53]
amiRNA (precursor miR395)	WSMV	Virus genome (conserved region)	Wheat	[89]
amiRNA (precursor miR319)	GFLV	CP	Grape	[103]
amiRNA/single monocistronic				
AthMIR156	CGMMV	CP	Tobacco	[108]
AthMIR159a				
AthMIR167b				
AthMIR169a				

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amiRNA/single polycistronic OsaMIR395	WSMV	5′UTR+P1+HC-Pro+ P3	Wheat	[89]
amiRNA/single monocistronic in tandem repeats AthMIR159a	PVX, PVY TuMV, TYMV	P25 (PVX) + HC-Pro (PVY) HC- Pro (TuMV) + P69 (TYMV)	N. tabacum	[109]
amiRNA/multiple monocistronic in trans AthMIR159a AthMIR390a	RBSDV, RSV TuMV TBSV	CP + HC-Pro 5′ terminal TBSV (+) RNA	Arabidopsis Tobacco	[110] [111]
ta-siRNAs AthTAS3a	TuMV, CMV	Multiple genomic positions	Arabidopsis	[112]
syn-tasiRNA/ single polycistronic AthTAS1c	TSWV	RdRP	Tobacco	[113]

1) microRNA shield

MicroRNAs (miRNAs) are genome encoded small RNAs that are processed by DCL1 from imperfectly paired, hairpin precursor RNAs [114,115]. The miRNAs belong to large gene families and each miRNA can modulate plethora of targets involved in plant immunity and phytohormonal pathways [116]. High throughput sequencing tools have provided useful insights for identifying the miRNAs involved in virus resistance. miR156, miR395, miR159, miR166, miR168, miR160 and miR444 have important roles in virus attack [117–120]. Recent report has shown that a novel miRNA, Seq 119 is down-regulated during Rice stripe virus (RSV) infection. Overexpression of this miRNA reverses the symptoms caused by RSV [121].

In many cases, amiRNA have been used to over-express a synthetic sequence designed to target a key viral gene. In Arabidopsis plants, amiRNAs were used first to target TYMV and TuMV [122]. This technique has the advantage of enhanced specificity and reduce off target effects. The amiRNA is composed of two components namely, a miRNA precursor (pre-miR) scaffold and a synthetic RNA insert. Special consideration is placed on selection of the pre-miR backbone to ensure that the correct amiRNA is processed by the cellular machinery [123]. Studies with TuMV population dynamics showed that inadequate levels of amiRNA build up more mutations in the target site and reduce the efficacy of virus resistance [124].

To synthesize the amiRNA, the miRNA/miRNA* sequence of the pre-miR is replaced by a synthetic sequence complementary to the viral target by performing overlapping PCR [125]. The sequence of the amiRNA is designed to have maximum complementarity with the target and minimum hybridization energy while ensuring sufficient mismatches to retain the secondary structures or bulges [126,127]. The other parameters include avoidance of mismatches at 10 or 11th nucleotides and instability at the 5′ end. The design of an effective amiRNA also necessitates incorporation of Uracil (U) at first base and Adenine (A) or U at 10th nucleotide position for making amiRNA biologically active [126,128]. Targeting an exposed region in a viral genome can increase the chances of DCL accessibility and efficiency of the amiRNA [129]. Tomato transgenics overproducing the amiRNAs to silence the conserved regions of ToLCV- AV2/AC2 genes could tolerate various leaf curl viruses of tomato [130]. The amiRNA technique faced the limitations of stringent design principles and the level of amiRNA expression.

2) siRNA shield

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The siRNAs are 21-24 nt in length and are derived from perfectly paired dsRNA molecules [131,132]. The siRNA-mediated gene silencing serves as a primary defense mechanism against plant viruses [76,133–136]. Transitivity of siRNAs is useful in propagating the silencing signal through the production of secondary siRNAs [55,137].

It was observed that better virus resistance was achieved when a dsRNA of the viral sequence was expressed in plant cell compared to the case of expressing either sense or antisense viral RNA [138]. This resulted in construction of intron splicable hairpin RNA (hpRNA) constructs with viral gene or gene-fragments. The hpRNA constructs offered the strongest resistance to RNA and DNA viruses [139–141]. The GM approaches involve expression of short-hairpin RNA (shRNA) constructs as precursors of the siRNAs in the plants to tackle the virus. The precursors are mobilized into the Agrobacterium and stably integrated into plants [142]. In majority of the cases, the VSR gene (s) have been used as a silencing target to generate virus resistance [140]. In Brazil, transgenic bean resistant to a Bean golden mosaic virus (BGMV) was created by expressing a hpRNA of Rep gene and it is being commercially cultivated [139].

Tomato plants expressing hpRNA corresponding to the TYLCV- Rep coding sequence produce 21- and 22-nt siRNAs and are resistant to TYLCV [143]. Similarly, Cassava transgenics containing hpRNA homologous to the overlapping region of South African cassava mosaic virus (SACMV)-Rep and the AC1/AC4, were tolerant to cassava mosaic disease [144]. In another study, three distinct intron hpRNA constructs comprising sequences of AC2, AC4, and fusion of AC2 and AC4 (AC2+AC4) of seven begomoviruses were used. All transgenic lines showed resistance against MYMIV infection when compared with untransformed controls [145].

3) Synthetic tasiRNA shield

The tasiRNAs are derived from non-protein-coding TAS transcripts that are capped as well as poly-adenylated and contain a binding site mostly for 22-nt miRNA [146]. The miRNA cleavage products are stabilized by Suppressor of Gene Silencing 3 (SGS3) and converted into a dsRNA form by RDR6 [147,148]. The dsRNA intermediate is then processed by DCL4 and dedicated dsRNA-Binding Protein 4 (DRB4) to phased 21-nt siRNAs in a 'head-to-tail' phased pattern. The transitive siRNAs are incorporated into AGO-RISC for targeting complementary sequences [147–150]. In Arabidopsis thaliana eight tasiRNA producing loci have been identified that fall into four TAS groups (TAS1-TAS4).

Since TAS precursors are able to generate multiple secondary siRNAs once they are cleaved by the miRNA, they work more efficiently against the rapidly evolving viruses that can evade the single amiRNA approach. The Arabidopsis TAS DNA sequences can be engineered to silence targeted viral sequences, by replacing some of the phased tasiRNA producing sequences by single or multiple siRNAs of different sequences but of equivalent length of base-pairs. When the replacing siRNAs are processed in the tasiRNA pathway from the engineered vectors as desired, these are called as the synthetic-tasiRNAs (syn-tasiRNAs), which consequently silence their targets in a usual manner using the RNA silencing machinery of the host plants. It was observed that most plants expressing higher levels of syn-tasiRNA were resistant to TSWV (Tomato Spotted Wilt Virus), while plants with particularly low levels of syn-tasiRNAs were infected. Several automated design tools have been used for designing syn-tasiRNA constructs, which are able to target distinct locations within a single or multiple viral RNAs. More efficient, long-lasting, and widespread resistance could be produced by simultaneous co-expression of multiple syn-tasiRNAs [151,152].

4) long non-coding RNA shield

Long non coding RNAs (lncRNAs) are 200 nt in length and are mainly produced by the action of polymerase II [153]. The lncRNAs resemble the mRNAs in having a 5'm7G cap and 3'poly(A) tail [154], [155], though their sequences show less conservation [156]. On the basis of origin they can be categorised as intergenic, intronic or exonic. They can act either in cis or trans and may have sense or antisense orientations [157,158]. NGS analysis has played an important role in identification of differentially expressed lncRNAs and their roles in host interaction studies [159]. RNA-seq of TYLCV-resistant tomato cultivar showed that some lncRNAs such as slylnc0048, slylnc00449, slylnc0483,

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slylnc0531 and slylnc0934 were up regulated while slylnc0475, slylnc0476, slylnc0673 and slylnc1052 were down regulated after virus infection [160].

LncRNAs can act as sources for the generation of small RNAs and regulate DNA or histone methylation by guiding the DNA methyltransferase. LMT1 encoded by *Citrus tristeza virus* (CTV) plays an important role in susceptibility of host plants. Mutation in LMT1 caused increase in the SA levels and enhanced virus resistance [161]. Recent reports have shown a relation between RNA silencing and viroid replication. Viroids are free RNA molecules which do not code for any protein and these are like circular lncRNAs [162]. Several horticulture crops such as apple, avocado, grapevine, peach, tomato etc are facing viroid infections. *Potato spindle tuber viroid* (PSTVd) is a good model for studying viroid-host interactions. PSTVd induced small RNAs cause the cleavage of several host transcripts coding for pyrophosphatase, callose etc [163,164].

The lncRNAs can also quench the function of small RNAs and act as target mimics of the miRNAs [165], [157]. SlLNR1 (lncRNA of tomato) involved in regulating leaf growth and development, contained sequences complementary to the vsiRNA derived from intergenic region (IR) of the virus. In susceptible lines, viral intergenic siRNAs targeted the SlLNR1 resulting in stunted growth and curling of leaves [166].

3. GE approaches

Genome editing (GE) offers the most precise tool to modify a target gene by using the endonucleases to create breaks in the DNA strands that lead to both target-specific mutagenesis (addition, removal or alteration) and gene replacement [167,168]. It offers the advantage of being less expensive, simple to design and execute and better acceptable. Several GE techniques have been implemented against different viruses and have produced better outcomes (Table 2). By complementing it with NGS, it will become easier to identify the fast mutating viral sequences and redesign the targets which influence the susceptibility response in host-virus interactions. [169].

Genome editing is now emerging and expanding as a modern tool in biotechnology. From basic techniques such as Meganucleases (MNs), ZFNs (zinc finger nucleases), TALENS (transcription activator-like effector nucleases to advanced techniques like CRISPR/Cas system. GE methods rely on the introduction of specific mutations to inactivate certain target genes. Modifications like CRISPR ON and OFF, use of Base Editors and Prime editors restricts the possibilities of accidental host targets.

3.1. Different GE tools

The onset of GE lies in the use of specialized restriction endonucleases known as meganucleases (MNs), which could target broad recognition sites of around 12-40 bp [170,171]. These nucleases create the double-stranded breaks (DSBs) that were repaired by non-homologous end joining (NHEJ), resulting in gene mutations [172]. MNs have been studied in model plant systems such as *Arabidopsis, Gossypium* and maize. They exhibited high efficiency of target recognition and moderate mutation rate. The length of target sequence recognised by the MN was around 68-88 bp, which was quite long as compared to others and it did not require another endonuclease (Ahmar S et al 2020). However, the major limitation was that production of these enzymes was costlier and time consuming. It was not easy to manipulate the MNs as their catalytic and DNA binding domains were intermingled and become difficult to detach [173].

Zinc-finger nucleases (ZFN) emerged as specialized cutter that could cleave dsDNA sequences [174], [175,176]. ZFN-induced double-strand breaks were subject to cellular DNA repair processes. ZFNs were engineered by fusing the non-specific DNA cleavage domain of Fok1 (*Flavobacterium okeanokoites* 1) with the Cys2-His2 zinc finger (ZF) DNA Binding domain [177,178]. Fok1 is a type II endonuclease that can bind to DNA regions in a non-specific manner, but it can cleave the double strand only when two monomers bind to the target sequence (overall 9-18 bp). Each monomer binds to 5-6 bp long sequences within the target region, so 4 monomers were required for generating 2 cleavage sites [179]. The breaks are repaired by homology directed repair (HDR) or NHEJ (when homologous sequences are not found) resulting in errors [180]. NHEJ creates deletions or insertions

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at target sites that further leads to frame shift and results in gene knockout while HDJ can insert new sequences at the break site that result in knock-in [181].

ZFNs were used to mutate the AC1 gene, which encodes Rep protein, in Tomato yellow leaf curl China virus [182]. Artificial ZFN proteins (AZPs) have been designed using conserved sequences for blocking the DNA binding sites, which are used by viruses [183,184]. For instance, AZPs bind to the IR region of Beet severe curly top virus (BSCTV) and interfere with binding of Rep thus suppressing replication of virus in the host [185]. The ZFN based technique allowed easy manipulation and could target any site but the efficiency was limited by chances of off-target cleavage. ZFN mediated gene editing has been used in many crops such as Arabidopsis, tobacco, maize, and soybean etc. [172,177,186,187].

TALENS (Transcription Activator-like effectors Nucleases) were produced by fusing DNA binding domains of transcription activator-like (TAL) effectors to DNA cleavage domains of Fok1 to induced dsDNA breaks that could be repaired by NHEJ or HDR repair mechanisms [188]. TAL effectors are produced by bacteria *Xanthomonas* in plants through the type III secretion system. [189]. These effectors alter the transcripts of the host by binding with the promoter region via repetitive amino acid residues in the central domain and activating gene expression [190,191]. TALENs were used to generate disease resistant plants by mutagenizing the susceptible genes in host plants [192]. TALENs were used to develop resistance of tobacco plants against *Tobacco curly shoot virus* (TbCSV) and *Tomato leaf curl China virus* (TYLCCNV) [193].

Recently, CRISPR-Cas has become popular in the scientific community as it has tremendous potential to improve crop traits (section 3.2). The attractive features of this technology include ease, efficiency, and rapidity of genetic manipulations. CRISPR system is rapidly becoming diverse and now includes different types of base editors, prime editors and endogenous tRNA systems. These modifications have given greater specificity and multiplex targeting abilities, which ultimately makes GE more precise (Table 2).

3.2. Manipulation of genome through CRISPR/Cas system

The CRISPR-Cas system was first identified as unique strategy used by bacteria and archaea, for adaptive immune response against invading phages or plasmids [194–196]. In this system, the prokaryotes were able to acquire small pieces of the infecting virus and then use it as memory for protection against further attacks. CRISPR stands for Cluster Regularly Interspaced Short Palindromic Repeats and the Cas protein is the RNA dependent DNA endonuclease. The Cas protein interacts with two special non-coding RNAs namely CRISPR RNA (crRNA) and transactivating crRNA (tracrRNA). The crRNA function as a short guide as it consists of a unique 20-base sequence that enables target recognition. The tracrRNA is a trans-encoded small RNA, which provides the stem loop structure [197]. The fusion of crRNA and tracrRNA generates a synthetic RNA of around 100 nucleotides (nt), called the single guide RNA (sgRNA).

In the initial step, Cas9 protein binds to sgRNA to form a catalytically active Cas9-sgRNA duplex [198,199]. The Cas 9 protein contains two REC domains, an arginine-rich Bridge Helix, a proto-spacer adjacent motif (PAM) interacting domain, and two DNA nuclease domains (HNH and Ruv C). The REC I domain is responsible for binding to the sgRNA, while the bridge helix is crucial for initiating cleavage activity upon binding of target DNA [200]. The PAM domain helps in recognizing the consensus NGG (N, any nucleotide; G, guanine) at 3′ end of the target DNA strand for initiating binding of Cas9 to target DNA [197,201–203]. After binding the Cas9 unzips the DNA to allow crRNA (within sgRNA) to anneal to the target DNA. After annealing, both the strands are cleaved 3 bases upstream to the PAM thereby creating blunt ends at the target site. The DSB can be repaired by the error prone NHEJ or HDJ to generate the mutations.

3.2.1. Cas9 editing

This technique has been beneficial to target DNA and RNA viruses with precision and high efficiency (Table 2) [204,205]. Studies using *Arabidopsis thaliana* or *Nicotiana benthamiana* showed that sgRNAs targeting the stem-loop sequence within the geminiviral genome or the non-coding

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intergenic region (IR) showed a stronger interference as compared those targeting the viral genes like CP or Rep. When gRNAs were used to target non-coding IRs in CLCuKoV and TYLCSV their systemic movement in host was blocked [206]. The sgRNAs also rendered a broad-spectrum resistance to different mono- & bipartite geminiviruses [207]. Combination of two sgRNAs against IR and Rep of CLCuMuV in a single plasmid enhanced the overall resistance of tobacco plants [208].

Several extensions or modifications of the CRISPR/Cas system have been developed either by mutating the Cas 9 enzyme or by using other variants of Cas. One of the class 2 effectors, Cas12a (also called Cpf1) has only RuvC domain and no HNH domain, hence it generates staggered DSBs instead of blunt [209,210]. Staggered DSB is near to the 3′ end of target sequence, which creates a 5′ overhang. This could be beneficial for knock-in experiments and also helpful in insertion of DNA fragments precisely and with perfect orientation through complementary ends by HDR [210]. Also it requires shorter crRNA than Cas9 and there is no requirement for tracrRNA as per the current reports [211].

3.2.2. Epigentic editors

An engineered Cas variant known as dead Cas9 (dCas9) contains inactivated HNH and RuvC nuclease domains [180]. It targets specific genomic region by recruiting chromatin modifiers and effector proteins, which leads to programmable epigenetic editing [212,213]. There is enormous potential for dCas9 to gain insights on plant-virus interactions and for performing transcriptional engineering and chromatin alterations [214].

3.2.3. RNA editors

The FnCas9 (class 2, Type II) derived from *Francisella novicida*, has RNA targeting and editing properties [215,216]. The FnCas9 with RNA-targeting gRNA has been used to cleave the ssRNA viruses for the resistance against CMV and TMV in *N. benthamiana* and *A. thaliana* [217]. CRISPR techniques especially CRISPR-Cas12a and CRISPR-Cas13a/d are also useful in detection of DNA and RNA virus amplicons generated through isothermal amplification [218].

Cas13 is a new type of protein isolated from *Ruminococcus flavefaciens* that comes in class 2 type VI effectors [219]. It has programmable RNA-targeting properties, which broaden the application of CRISPR/Cas system [204]. Recent studies have demonstrated the effectiveness of using various Cas13 systems successfully for targeting multiple RNA viruses [220]. Transgenic rice and tobacco harbouring LshCas13a (*Leptotrichia shahii* Cas13) with specific protospacer showed resistance to *Rice stripe mosaic virus* (RSMV), *Tobacco mosaic virus* (TMV), and *Southern rice black-streaked dwarf virus* (SRBSDV) [221]. The effectiveness of using LshCas13a to target the helper component proteinase (HC-Pro) and coat protein (CP) segments of the Turnip Mosaic Virus (TuMV) RNA genome in *Nicotiana benthamiana* and *Arabidopsis thaliana*, respectively has been demonstrated. Cas13 has many variants of which Cas13d is more advantageous than Cas13a, Cas13b, or others [222]. CRISPR techniques especially CRISPR-Cas12a and CRISPR-Cas13a/d are also useful in detection of DNA and RNA virus amplicons generated through isothermal amplification [218].

3.2.4. Base editors

These are formed by the fusion of enzyme deaminase with Cas nuclease domain. Base editors are used to create point mutations without the requirement of donor template and DSBs [223,224]. This approach is useful for targeting genes responsible for host susceptibility [225]. Cytosine base editors (CBE) and Adenosine base editors (ABE) are two commonly used editors in base editing. The former converts C:G to T:A and later converts A:T to G:C [226]. In this process, the availability of PAM is important to edit the target DNA. Advancements in the base editing technology such as variants of CBEs and ABEs are being used to create randomized mutations in targets [227,228]. In Arabidopsis resistance to *Clover yellow vein virus* (CIYVV) was obtained by using a novel CBE to convert a susceptible allele of eIF4E1 into a resistance allele by a point mutation of N176K [225].

3.2.5. Prime editors

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Prime editing was achieved by using an engineered nCas9 fused with a reverse transcriptase and prime-editing guide RNA (pegRNA). The pegRNA has protospacer sequence that directs Cas nuclease to cleave target region and add extra sequences, which are responsible for specific changes [229,230]. Like base editing, this technique does not require double stranded breaks (DSBs).

3.2.6. Multiplexing CRISPR/Cas editing

Targeting multiple sites on a virus in a single go is being developed as an effective strategy to minimize the chances of virus escape. In cotton, three sgRNAs were designed to target three viral regions for modifying six overlapping genes (AV2/AV1, AC2/AC3, AC1/AC4). The analysis showed relatively low virus titres in the plants transformed with all three gRNAs as compared to plants expressing single gRNA and the wild-type plants [231].

This includes simultaneous delivery of Cas9 mRNA with multiple gRNAs expression cassettes in one plasmid, artificial gRNAs with Cas9 flanked by Csy4/HH-HDV ribozymes for cleavage and release of gRNAs, multiple gRNA precursors with Cas9 flanked by tRNAs etc and so on [232]. Coupling of CRISPR/Cas13d with an endogenous tRNA system for expression of four sgRNA was found to be effective against PVY, PVX, PVS or PLRV viruses in potato [233]. Combination of dCas9 with cytosine/adenosine deaminase and gRNA complex was used for generating point mutations in targets [223,234].

Multiple gRNA strategy is also helpful in case of mixed viral infections [235]. In Potato, CRISPR/Cas13a was coupled with an endogenous tRNA system and four different gRNAs to produce a polycistronic tRNA-gRNA (PTG) against PVY. However the transgenic lines exhibited similar levels of tolerance as observed in case of single gRNA lines, indicating that multiple target sites did not affect viral interference [233].

Major hurdles in the GE approaches are the generation of off-targets due to the constitutive expression of Cas nuclease or because of sequence mismatches in sgRNA (Mushtaq, M et al., 2020). Error prone repairing process creates selection pressure on viruses which results in generation of viral variants having resistance towards editing. Replacement of constitutive promoter by a virus inducible promoter for expression of Cas nuclease is quite useful to bring down the off-targets [236].

Table 2. List of GE approaches available against Plant DNA and RNA viruses.

Strategy	Virus	Plant	References
For DNA Viruses			
ZFN	TYLCCNV, TbCSV		[182]
	BeYDV	Tobacco	[237]
TALENS	TYLCCNV, TbCSV, TLCYV		[193]
AZP	BSCTV, TYLCV		[183], [238], [239], [240],
	RTBV		[241], [242]
CRISPR/Cas9	TYLCV, CLcKV, BSCTV, BCTV,	Tobacco	[243], [206]
	BeYDV, CLcV		[244]
	CaMV, BSCTV	Arabidopsis	[245]
	WDV	Barley	[246]
	BSV	Banana	[247]
	ACMV	Cassava	[248], [249]
For RNA Viruses			
MNs	TRV	Tobacco	[250]
SpCas9	BYSMV	Tobacco	[251]
	SYNV		[252]
FnCas9	TMV, CMV	Tobacco	[217]
	CMV	Arabidopsis	
LshCas13a	TuMV	Tobacco	[205]

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	TMV		[221]
	SRBSDV, RSMV	Rice	
	PVY	Potato	
Cas13d/PTG	PVX or PLRV, PVY, PVS	Potato	[233]
Cas13a/PTG	PVY	Potato	[233]
Cas13a with multiplex gRNAs	PVY	Potato	[253]
LbCas12a	CLcMV	Cotton	[254]
	TEV	Tobacco	[255]
Cas12f	CLCuV	Cotton	[256]
Multiplex Cas9-gRNAs (9 duplex and 2 triplex)	ChiLCV	Tobacco	[245]

Summary and Future perspectives

Viruses invade plant cells and hijack the host cellular machineries for their own multiplication. The plants fight back by activating the cellular defense responses resulting in genetic and epigenetic events to block viral gene expression. Several attempts were made to introgress resistance to virus in cultivated plants by breeding with wild relatives. After decades of failure, strategies for targeting the virus to confer resistance offered great promise in saving the crops from virus infection. Therefore, GM and GE have emerged as effective strategies to engineer resistance to the viruses (Figure 1).

The early GM approaches were based on engineering PDR by over-expressing CP or other viral proteins and it proved to be effective against many viruses. However, with the discovery and understanding of the underlying principles of RNA silencing and its role in providing resistance to plant viruses, there was a paradigm shift in the strategies. The first generation silencing tools employed the dsRNA or hpRNA precursors directed against specific viral genes. The transgenic papayas expressing siRNAs against *Papaya ringspot virus* (PRSV) proved to be a boon for the Hawaian papaya industry. However, the siRNAs had the limitations of being less specific and generating undesirable off targets [260]. This resulted in development of amiRNA tools by engineering the natural pre-miRNA backbones. These second generation tools were more specific but had limited targeting capacity, so syn-tasiRNA vectors were favoured and developed as the third generation tools. The GM technology has helped to save the papaya grown in Hawaii and reduce potato crop losses in USA and Canada. It holds the potential to prevent losses in other crop to virus and other diseases.

However, the scepticism regarding acceptance of GM crops by the public has hindered the active implementation of the technology. This branched off the search for exogenous application of dsRNA precursors or small RNAs to activate the host silencing pathways (Table 3). Several reports have highlighted the use of high pressure (4-6 bar) for efficient transfer of dsRNA in the nucleus for spray induced epigenetic modifications (SPIEM). When high pressure spraying was used to transfer 333 bp dsRNA in *N. benthamiana*, there was no induction of methylation at the target region at 3 days post spraying (dps), while 28% samples were methylated at 6 dps and 40% of samples were methylated at 10 dps [262]. However, spraying with low pressure did not result in any methylation. The RNA based bioformulations are in various stages of development and are anticipated to prove useful in tackling the virus infections.

Table 3. List of exogenously delivered dsRNAs for induction of RNA silencing against plant viruses.

dsRNA coating and inoculation	Plant	Virus	References
LDH nanosheets	Cowpea	CMV	[250]
	Tomato	TYLCV	[260]
Agroinfiltration and Direct spray	Cucumber	CGMMV	[261]
		ToLCNDV	
High pressure spraying of free siRNAs	Tobacco	CaMV 35S promoter	[258]

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Mechanical inoculation of free dsRNAs in vivo and in vitro synthesized dsRNAs	Tomato	ToMV	[262]
	Orchid	CymMV	[263]
	Tobacco Chenopodium Quinoa	CMV	[264]
CQAS-dsRNA nanoparticles and Carbon Quantum Dots (CQDs) through Root soaking, infiltration	Tobacco Tomato Pepper	PVY	[265]

The emergence of GE tools has added another arsenal in the fight against viruses infecting the crop plants. CRISPR/Cas9 provides a robust system to generate more effective and prolonged resistance against viruses. The recent additions of Cas13 and Cas12 variants have proved much effective in targeting the RNA viruses. The efficacy of CRISPR-based approaches primarily depends on the method of delivery and design of the sgRNA sequence. A number of delivery methods including Agrobacterium-mediated T-DNA transformation, protoplast transfection, microprojectile bombardment and virus-based methods have been deployed to introduce CRISPR/Cas components in plants. As viruses have the tendency to mutate with the faster rate, the multiplexing technologies can contribute for precise editing and broadening the range of the targeted viruses. Although the use of CRISPR/Cas for development of viral-resistant plants has been demonstrated to be effective, further improvements in the technology are awaited to attain efficient, durable and broad-spectrum resistance.

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