



# CRISPR/Cas systems versus plant viruses: engineering plant immunity and beyond

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Update

## Abstract

Molecular engineering of plant immunity to confer resistance against plant viruses holds great promise for mitigating crop losses and improving plant productivity and yields, thereby enhancing food security. Several approaches have been employed to boost immunity in plants by interfering with the transmission or lifecycles of viruses. In this review, we discuss the successful application of clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein (Cas) (CRISPR/Cas) systems to engineer plant immunity, increase plant resistance to viruses, and develop viral diagnostic tools. Furthermore, we examine the use of plant viruses as delivery systems to engineer virus resistance in plants and provide insight into the limitations of current CRISPR/Cas approaches and the potential of newly discovered CRISPR/Cas systems to engineer better immunity and develop better diagnostics tools for plant viruses. Finally, we outline potential solutions to key challenges in the field to enable the practical use of these systems for crop protection and viral diagnostics.

## Introduction

The exceptional simplicity, efficacy, versatility, accuracy, and multifunctionality of the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR-associated genes (Cas) (CRISPR/Cas) system has made it an unprecedented tool for genome editing, gene regulation, engineering virus resistance, nucleic acid-targeting, and diagnostics for eukaryotic systems (Jinek et al., 2012; Cong et al., 2013; Malina et al., 2013; Doudna and Charpentier, 2014; Ali et al., 2015a, 2020a; Piatek et al., 2015; Aman et al., 2020a). CRISPR/Cas has been successfully employed to boost immunity and to produce diagnostics for eukaryotic viruses, including plant viruses (Ali et al., 2015b; Ali et al., 2016; Pyott et al., 2016; Aman et al., 2018a; Ali et al., 2020a). Here, we discuss the unrivaled application of CRISPR/Cas systems

(Box 1) against phytopathogenic viruses for viral interference and diagnostics. We also describe the development of plant viruses as tools to deliver the CRISPR/Cas machinery into plant cells (Box 2), for targeted genome editing, and thus enhance virus resistance.

Phytopathogenic viruses and viroids cause severe diseases in plants (Kovalskaya and Hammond, 2014; Sastry and Zitter, 2014). Plant viruses have small, highly compact genomes (2–20 kb genome) that encode only a few proteins (replication initiation factors, host immune system silencers, and coat proteins); these proteins allow the virus to use cellular resources to complete its lifecycle (Heinlein, 2015). Viroids, an even simpler type of small infectious phytopathogen, are composed of a 250–430 bases single-stranded

### ADVANCES

- CRISPR/Cas systems can boost plant immunity by directly targeting the viral genome (DNA or RNA) and have the potential to provide resistance against multiple pathogenetic viruses (mixed population in the field).
- CRISPR/Cas systems can be used to help fully grown plants recover from virus symptoms and pathogenicity.
- CRISPR/Cas systems can modify host genes to increase recessive resistance against pathogenic viruses.
- A CRISPR/Cas-based field-deployable plant virus diagnostic system is now feasible.
- Plant virus-based vectors can deliver genome modification reagents for efficient mutagenesis and gene targeting and delivery through RNA virus vectors can generate nontransgenic genetically modified elite crops.

circular RNA that encodes no protein but can cause plant diseases (Tsagris et al., 2008). Based on the genome organization, plant viruses are divided into six major types: double-stranded DNA (dsDNA) viruses, single-stranded DNA (ssDNA) viruses, double-stranded RNA (dsRNA) viruses, sense (positive) strand ssRNA (ssRNA+) viruses, and antisense (negative) strand ssRNA (ssRNA–) viruses (Roossinck, 2011). Similarly, plant viruses are named based on their visible disease symptoms and the host from which

the virus was first isolated, e.g., *Tobacco mosaic virus* (TMV) and *Tomato yellow leaf curl virus* (TYLCV). The current list of disease-causing phytopathogenic viruses includes almost 1,000 viruses (Lefkowitz et al., 2018).

Despite tremendous efforts aimed at prevention, plant viruses cause 10–15% yield losses (60 billion USD) annually by infecting cucurbits like cucumbers (*Cucumis sativus*), melons (*C. melo*), cotton (*Gossypium hirsutum*), beet (*Beta vulgaris*), beans (*Phaseolus vulgaris*, *P. coccineus*, *Vicia faba*), tomato (*Solanum lycopersicum*), tobacco, cassava (*Nicotiana tabacum*), alfalfa (*Medicago sativa*), sugarcane (*Saccharum officinarum*), eggplants (*S. melongena*), flax (*Linum usitatissimum*) spinach (*Spinacia oleracea*), squash (*Cucurbita maxima*, *C. pepo*), barley (*Hordeum vulgare*), wheat (*Triticum aestivum*), maize (*Zea mays*), petunia (*Petunia axillaris*), pansy (*Viola tricolor*), geranium (*Geranium aculeolatum*), delphinium (*Delphinium elatum*), oranges (*Citrus limon*, *C. reticulata*, *C. x paradise*), peaches (*Prunus persica*), apple (*Malus domestica*), banana (*Musa x paradisiaca*), tulip (*Tulipa x gesneriana*), and lily (*Lilium candidum*; Loebenstein, 2008; Nicaise, 2014). Moreover, severe, uncontrolled viral attacks can cause 100% crop losses in tomato, cotton, beans, and cassava, with disastrous effects on agriculture and food security (Briddon and Markham, 2000; Dovas et al., 2002; Hanssen et al., 2010; Chikoti et al., 2019). Multiple factors including the movement of plants and plant products in the food trade, the use of unhealthy seeds, mixed crop culture practices, vector migration, unexpected vector outbreaks, and vector resistance to pesticides, viral diversity, host range break, the presence of a mixed population of different plant viruses in the field, and the rapid evolution of the viral genome increase the virus/viroid transmission and thus pose great risks to sustainable food production (Loebenstein and Katis, 2014;

### Box 1. CRISPR/CAS SYSTEM AND PLANT VIROLOGY

The CRISPR/Cas system originated as a bacterial defense against viruses and therefore has the innate ability to facilitate virus resistance and interference, and it can be harnessed for diagnostics in plant virology. Moreover, the simplicity and diversity of the CRISPR/Cas systems make it an unrivaled toolbox to cope with all aspects of phytopathogenic viruses, such as the direct targeting of viral genomes. Even though they have not been studied thoroughly, both ZFNs and TALENs were shown to partially inhibit the replication of plant viruses, but in vivo viral genome cutting using these nucleases has never been reported (Sera, 2005; Cheng et al., 2015). Similarly, multiplexing for multisite targeting is almost impossible to perform with ZFNs and TALENs. Moreover, both ZFNs and TALENs can only be designed to target DNA sequences and are unable to target the genomes of plant RNA viruses. By comparison, multiple CRISPR/Cas modules can be used to target any genome (DNA or RNA), and it is very simple to perform multiplexing with these systems to target any plant virus or mixed virus infections (Ali et al., 2016; Aman et al., 2018a; Mahas et al., 2019).

Similarly, plant virus management requires an inexpensive, simple, specific, field-deployable virus diagnostic system. However, all currently used diagnostic tools are expensive, complicated, and cannot be deployed in the field by farmers. Even though CRISPR/Cas-based diagnostic tools are still in the development phase, they will soon fulfill all of the requirements of an agriculturally feasible diagnostic system (Aman et al., 2020a).

Virus-based delivery of genome editing machinery and the use of gene targeting reagents have severe consequences in animal systems (Chira et al., 2015). However, the short life spans of plants and the easy removal of viral vector remnants from plants in subsequent generations make plant viral vectors the best choice for delivering genome engineering reagents into plant cells.

## Box 2. LIMITATIONS OF THE CURRENT SYSTEM AND NEW PERSPECTIVES

CRISPR/Cas9 has been successfully employed for genome editing and to enhance virus resistance. However, this system has some limitations, including its inherent inability to perform some of the required steps in virus resistance in plants and the additional steps or helper systems needed for particular tasks. For example, multisite targeting of the virus genome is required to avoid virus escape, but Cas9 cannot process its sgRNA. Therefore, several promoters or tRNA–sgRNA structures have been employed to target plant virus genomes at multiple sites. Similarly, the PAM requirement for Cas9 is very strict, and Cas9 cannot target ssDNA.

The newly discovered CRISPR/Cas systems offer precise and simple solutions to these problems (preventing viral escape, multiplexing DNA targeting, and even easy viral diagnostics). For example, CRISPR/Cas12 is widely used and much more versatile than CRISPR/Cas9. Specifically, the compact toolkit of Cas12 is well suited for plant virology. Cas12 requires only a short crRNA (making engineering easy), can process polycistronic crRNAs (making multiplexing possible, with no chance of virus escape, allowing multiple genomic loci to be edited at once), targets ssDNAs, and dsDNAs, and degrades ssDNAs via trans activity, Cas12 is comparatively small, and can easily be delivered via deconstructed viral vectors. Similarly, the Type I system can be used to cut the target DNA and to chop the entire viral genome into pieces or completely remove any susceptibility genes from the host genome with Cas3 enzyme.

Similar to viruses, viroids are causing severe diseases in plants. Based on the crRNA processing capability of the CRISPR/Cas13, a well-designed multiplex system to target ssRNA of viroid genome would greatly help to mitigate viroid-based diseases in plants.

Furthermore, the nonspecific degradation of ssDNAs or ssRNAs (reporters) upon recognition of a specific target by Cas12, Cas13, and Cas14 variants provides the opportunity to develop an efficient diagnostic system for deployment in the field. Coupling of the target specificity and nuclease activity of Cas variants with target enrichment (via isothermal amplification, LAMP, RPA) and signal amplification (CONAN or SENSR) has the potential to change the entire scope of plant virus detection and control measures.

[Moreno and López-Moya, 2020](#)). Conventional methods, i.e., breeding resistant varieties and propagation of virus-free material in nurseries played a major role in food security, but these methods are time-consuming and require tremendous economic investment.

Antibiotics and other chemicals can limit the spread of some phytopathogens (such as bacteria and fungi), but no reagents are currently available to recover virus-infected plants or eliminate viral infection ([Cao et al., 2020](#)). Most of the conventional control strategies for plant viruses are based on controlling viral vectors (nematodes, mites, and insects) using pesticides, natural predators, physical barriers, replicative mulches, sticky pads, or UV-absorbing sheets ([Bragard et al., 2013](#)). Good agricultural practices, such as selecting virus-free stock/seeds, early sowing, and employing weed and crop management techniques are used to limit viral pathogenicity ([Goldbach, 1998](#); [Hilje et al., 2001](#); [Singh and Srivastava, 2020](#)). However, designing long-term strategies to manage plant viruses using conventional approaches remains challenging.

Fighting an invisible foe outside of the cell is challenging. Therefore, strategies to restrict pathogenic attack or target the pathogen inside the cell are good options to avoid diseases in plants. Different molecular approaches have been employed to boost plant immunity against viruses, including molecular breeding of resistance-related quantitative trait loci (QTLs), mutagenesis ([Lellis et al., 2002](#)), and the introduction of resistance genes (*R* genes; [Seo et al., 2006](#)) or RNA interference (RNAi) constructs via transgenics ([Pooggin](#)

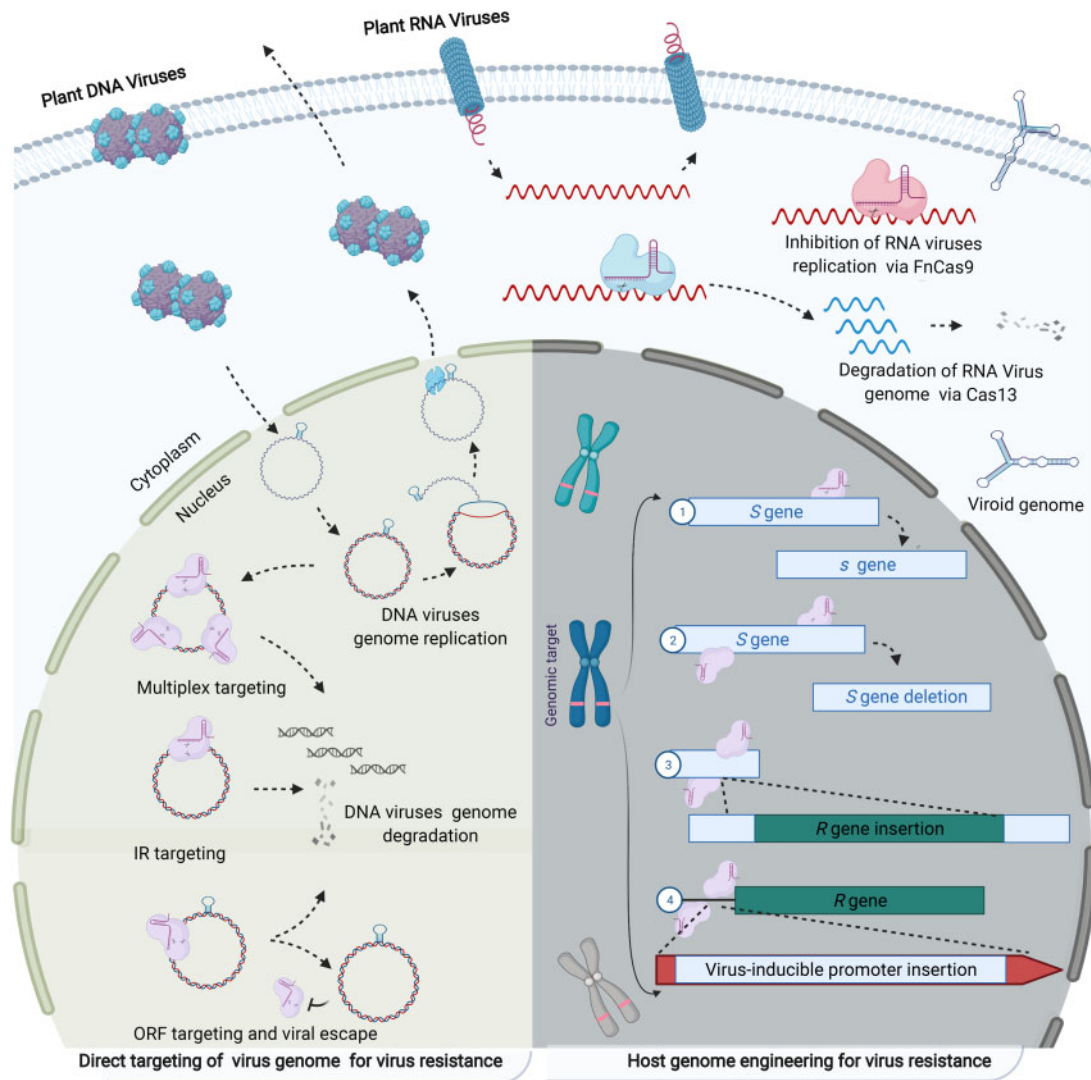
[et al., 2003](#); [Zhang et al., 2011](#)). However, the lack of information about such QTLs in most crops, the rapid evolution, and diversity of viruses, and the labor-intensive, time-consuming methods needed to introduce plant resistance using these strategies limit their broad-scale application in modern agriculture ([Dong and Ronald, 2019](#)). Similarly, the introduction of dominant/recessive genes for viral resistance and RNAi is still in use in some crops (for detailed reviews, see [Wang et al., 2012](#); [Hashimoto et al., 2016](#)). However, the inability to transform and regenerate many crop varieties, the emergence of new viral strains, resistance breaks, the lack of specificity, growth penalty issues for RNAi systems, and regulatory constraints limit the widespread use of these techniques in the field.

Even though they have not been fully exploited to target plant viruses, the development of the sequence-specific nucleases zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) have attracted the attention of plant virologists for their ability to confer direct immunity against phytopathogenic viruses ([Sera, 2005](#); [Mahfouz et al., 2011](#); [Chen et al., 2014](#); [Cheng et al., 2015](#)). The use of ZFNs and TALENs provides only partial interference but opens the possibility for the use of any nucleic acid-targeting system against viruses ([Sera, 2005](#); [Cheng et al., 2015](#)). The major problem with these systems is that both ZFNs and TALENs are protein-based, and must be assembled for each target independently. For an ever-changing foe such as viruses, designing ZFNs, and TALENs remains challenging.

CRISPR/Cas employs Cas9 as a guided endonuclease to cleave the target DNA in a site-specific manner. Cas9 is guided to the target DNA sequence by a short single guide RNA (sgRNA) containing a 20-nucleotide sequence complementary to the target site. A short NGG sequence (the adjacent protospacer motif, PAM) following the 20-nucleotide target DNA sequence is the only other requirement for the targeting of DNA by CRISPR/Cas9 (Jinek et al., 2012; Cong et al., 2013; Doudna and Charpentier, 2014). Plant scientists have taken advantage of the simplicity and robustness of the CRISPR/Cas9 system to study basic biological questions and have applied this revolutionary technology for crop improvement, including plant protection against pathogenic

viruses (Ali et al., 2015a, 2015b, 2016, 2020b; Baltés et al., 2015; Beying et al., 2020).

CRISPR/Cas reagents expressed inside the cell can target the viral genome and thus provide resistance against phytopathogenic viruses (Figure 1, Table 1). Even though viral infection is highly efficient and quick, the virus lifecycle provides several opportunities (Dangl et al., 2013) for the use of a CRISPR/Cas-based system to attack the viral genome or interrupt the virus's lifecycle (Chaparro-García et al., 2015; Chandrasekaran et al., 2016). For example, viruses replicate in the host cell, and their genomes are exposed to the cellular machinery. Moreover, the versatility of the CRISPR/Cas systems and recent discoveries have provided



**Figure 1** Schematic of CRISPR/Cas-mediated resistance to plant viruses. Left, CRISPR/Cas-based direct targeting and interference with the genome of phytopathogenic viruses. Multisite targeting or targeting the conserved inverted repeat (IR) region provides resistance by cutting the genome of DNA viruses into multiple pieces inside the plant nucleus. Targeting plant virus genomes at the open reading frame (ORF) sequence can lead to virus escape via evolution of new ORF sequence variants that can complete the virus life cycle but are not targeted by the existing CRISPR/Cas system. Similarly, inhibition of RNA virus genome replication or degradation of the RNA genome was achieved via FnCas9 and Cas13 variants in the cytoplasm. CRISPR/Cas systems can also be designed to target the RNA genomes of viroids. Right, genetic engineering of susceptibility (S) and resistance (R) genes in the host genome can provide durable resistance to multiple viruses. CRISPR/Cas editing of S genes mimics natural resistance (point repair). Removal of S genes and insertion of R genes into neutral, high-expression loci or under the control of strong promoters, including virus-inducible promoters, via homology-directed repair can result in over-expression of R genes for virus resistance.

Table 1 Summary of CRISPR/Cas-mediated virus resistance

CRISPR/Cas system used	Targeted plant virus	Plant used	Main observation of the study	References
Sp-CRISPR/Cas9	TYLCV	<i>Nicotiana benthamiana</i> , Tomato ( <i>Lycopersicon esculentum</i> )	CRISPR/Cas9 validation for plant virus resistance / Virus genome cutting	(Ali et al., 2015b; Tashkandi et al., 2018)
Sp-CRISPR/Cas9	BSCTV	<i>Nicotiana benthamiana</i>	CRISPR/Cas9 validation for plant virus resistance / Virus genome cutting	(Ji et al., 2015)
Sp-CRISPR/Cas9	BeYDV	<i>Nicotiana benthamiana</i>	CRISPR/Cas9 validation for plant virus resistance / Virus genome cutting	(Baltes et al., 2015)
Sp-CRISPR/Cas9	MeMV	<i>Nicotiana benthamiana</i>	Multiple virus targeting with single sgRNA	(Ali et al., 2016)
Sp-CRISPR/Cas9	BCTV	<i>Nicotiana benthamiana</i>	Multiple virus targeting with single sgRNA	(Ali et al., 2016)
Sp-CRISPR/Cas9	CLCuMV	<i>Nicotiana benthamiana</i>	Multiplex targeting and virus escape, Replication inhibition	(Ali et al., 2016; Khan et al., 2019; Yin et al., 2019)
Sp-CRISPR/dCas9				(Roy et al., 2019)
Sp-CRISPR/Cas9	ChLCV	<i>Nicotiana benthamiana</i>	Multiplex targeting and virus escape	(Liu et al., 2018)
Sp-CRISPR/Cas9	CaMV	<i>Arabidopsis thaliana</i>	Multiplex targeting and virus escape	(Tripathi et al., 2019)
Sp-CRISPR/Cas9	endogenous eBSV	banana ( <i>Musa</i> spp.)	Multiplex targeting and virus escape	(Kis et al., 2019b)
Sp-CRISPR/Cas9	WDV	barley ( <i>H. vulgare</i> L. cv Golden promise)	Multiplex targeting of overlapping sequences of different genes in the virus genome	
Sp-CRISPR/Cas9	ACMV	<i>Nicotiana benthamiana</i>	virus escape	(Mehta et al., 2019)
Fn-CRISPR/Cas9, Fn-CRISPR/dCas9	TMV	<i>Nicotiana benthamiana</i>	Replication inhabitation of RNA virus	(Zhang et al., 2019)
Fn-CRISPR/Cas9, Fn-CRISPR/dCas9	CMV	<i>Nicotiana benthamiana</i>	Replication inhabitation of RNA virus	(Zhang et al., 2019)
Lsh-CRISPR/Cas13a	TuMV	<i>Nicotiana benthamiana</i> , <i>Arabidopsis thaliana</i>	RNA virus genome targeting	(Aman et al., 2018a, 2018b, Zhan et al., 2019)
CRISPR/Cas13	PVY	Potato ( <i>S. tuberosum</i> )	RNA virus genome targeting, Multiple strains targeting, broad-spectrum resistance	(Zhan et al., 2019)
Lsh-CRISPR/Cas13a	Southern rice black-streaked draft virus (SRBsDV)	Rice ( <i>Oryza sativa</i> )	RNA virus genome targeting	(Zhan et al., 2019)
Lsh-CRISPR/Cas13a	TuMV	<i>Nicotiana benthamiana</i>	Characterization for robust CRISPR/Cas system for RNA virus genome targeting	(Mahas et al., 2019)
Lwa-CRISPR/Cas13a				
Psp-CRISPR/Cas13b				
Rf-CRISPR/Cas13d				
Sp-CRISPR/Cas9	Cucumber vein yellow virus	Cucumber ( <i>C. sativus</i> L.)	Host genome (eIF4E) modification for broad-spectrum resistance to potyviruses	(Chandrasekaran et al., 2016)
Sp-CRISPR/Cas9	CuVYV), Zucchini yellow mosaic virus (ZYMV)	Cucumber ( <i>C. sativus</i> L.)	Host genome (eIF4E) modification for broad-spectrum resistance to potyviruses	(Chandrasekaran et al., 2016)
Sp-CRISPR/Cas9	Papaya ring spot virus-W (PRSV-W)	Cucumber ( <i>C. sativus</i> L.)	Host genome (eIF4E) modification for broad-spectrum resistance to potyviruses	(Chandrasekaran et al., 2016)
Sp-CRISPR/Cas9	TuMV	<i>Arabidopsis thaliana</i>	Host genome (eIF4E) modification for resistance to RNA viruses	(Pyott et al., 2016)
Sp-CRISPR/Cas9	Rice tungro spherical virus (RTSV)	Rice ( <i>O. sativa</i> )	Host genome (eFiso4E) modification for broad-spectrum resistance to RNA viruses	(Macovei et al., 2018)
Sp-CRISPR/nCas9-base editor	Clover yellow vein virus (CIYVV)	<i>Arabidopsis thaliana</i>	Mimicking natural resistance alleles eIF4E (N176K; amino acid substitutions)	(Bastet et al., 2019)
Sp-CRISPR/Cas9	Cassava brown streak virus (CBSV)	Cassava plant ( <i>Manihot esculenta</i> Crantz)	Host genome (eIF4E isoforms nCBP-1 and nCBP-2) modification for broad-spectrum resistance to RNA viruses	(Gomez et al., 2019)
Sp-CRISPR/Cas9	PVY	Potato ( <i>S. tuberosum</i> )	Host genome (Collin) modification for broad-spectrum resistance to RNA viruses	(Makhotenko et al., 2019)
Sp-CRISPR/Cas9	Soya bean mosaic virus (SMV)	soya bean [ <i>Glycine max</i> (L.) Merr.]	Host genome (GmF3H1, GmF3H2, and GmFNSII-1, members of the metabolites Isoflavonoids pathway) modification for broad-spectrum resistance to RNA viruses	(Zhang et al., 2020)

the CRISPR/Cas-based antiviral toolbox with all the gadgets needed to foster robust immunity against pathogenic viruses regardless of their genome composition.

## Direct inhibition of plant DNA viruses

DNA viruses belonging to the Geminiviridae family infect many crops, including cotton, tomato, cassava, tobacco, potato, pepper, watermelon, melon, cowpea, soybean, common bean, mung bean, barley, and banana (Seal et al., 2006). The genomes of geminiviruses are very compact, contain genes encoding only six to seven proteins on both the sense and antisense strands, and are expressed from a bidirectional intergenic region (IR) promoter. Both the IR and open reading frames of protein-coding genes contain important conserved regions essential for the replication/lifecycle of the virus (Chatterji et al., 2000). Any alterations in these conserved regions can interfere with virus replication (Figure 1).

Based on their economic importance, the pioneering work of engineering CRISPR/Cas-mediated virus interference in plants began with virus inhibition strategies against geminiviruses (genus *Begomoviruses*; Ali et al., 2015b, 2016; Baltes et al., 2015; Ji et al., 2015). The CRISPR/Cas9 system was programmed to boost the molecular immunity of *Arabidopsis thaliana* and *N. benthamiana* against TYLCV, *Beet severe top curly virus* (BSCTV), and *Bean yellow dwarf virus* (BeYDV), belonging to three different genera: Begomovirus, Curtovirus, and Mastervirus. All three studies focused on the same important regions of the virus: Rep, coat proteins, and the IR harboring the conserved nine-nucleotide (TAATATTAC) replication initiation sequence. Baltes et al. (2015) and Ji et al. (2015) both used T-DNA plasmids to express Cas9 and sgRNA to target BSCTV and BeYDV. Virus targeting was first confirmed transiently by infiltrating the plants with *Agrobacterium* containing the CRISPR/Cas9 expression cassette. Next, CRISPR/Cas9-expressing transgenic plants were regenerated and progeny plants were challenged with BSCTV and BeYDV (Baltes et al., 2015; Ji et al., 2015, 2018). Plants expressing CRISPR/Cas9 against BSCTV and BeYDV developed no viral symptoms, and the accumulation of the viral genome decreased by 80%. A modified version of a virus-inducible virus targeting system was subsequently developed using a viral promoter to express the CRISPR/Cas9 machinery against BSCTV. This system provides highly efficient virus resistance, as any replication of BSCTV inside the cell simultaneously induces the CRISPR/Cas9 machinery against BSCTV (Ji et al., 2018).

Ali et al. (2015b) employed another interesting combinatorial approach to boost plant immunity against plant viruses via CRISPR/Cas9. In this approach, the sgRNA targeting TYLCV was systemically delivered via the well-established *Tobacco rattle virus* (TRV) into Cas9-expressing plants. After establishing CRISPR/Cas9-mediated immunity (7 d), plants were challenged with TYLCV. Sequencing of the targeted TYLCV genomic fragments (IR, RCR II, and CP [encoding coat protein]) revealed typical Cas9 cutting signatures. However, the robust inhibition of TYLCV genome

accumulation and reduced TYLCV symptoms were observed only by targeting the conserved regions of the IR and Rep (RCR II, metal ion binding motif) compared to CP-targeted plants. In plants, the cutting of target DNA by CRISPR/Cas9 is generally followed by nonhomologous end joining-based DNA repair, leading to random insertions and deletions (indels) at the DNA target site (Ali et al., 2015b). Ali et al. (2015b) reasoned that indel formation in the essential conserved regions, IR, RCR II, and RCR III (Rep), renders the TYLCV genome unable to replicate. However, indels in the CP sequence lead to variants that replicate properly.

Indels in a nonconserved sequence generated via CRISPR/Cas9 may lead to the escape of mutated virus from the existing CRISPR/Cas9, as the newly formed sequence (with indels) is no longer complementary to the sgRNA and cannot be digested by Cas9. In a follow-up study, the viral escape mechanism was evaluated in detail and demonstrated that CRISPR/Cas9-mediated targeting of the CP induces indels in the CP sequence that can create variants of intact CP still capable of packing viral DNA (Ali et al., 2016). Also, it was observed that an sgRNA targeting the conserved stem-loop nine-nucleotide (TAATATTAC) sequence of TYLCV was capable of targeting multiple geminiviruses, including *Merremia mosaic virus* (MeMV) and *Beet curly top virus* (BCTV; Ali et al., 2016).

Targeting the viral genome at multiple loci by CRISPR/Cas9 can eliminate the possibility of virus replication (Figure 1). Ali et al. (2016) coupled tRNA-sgRNA (Xie et al., 2015) with the TRV system to express three independent sgRNAs targeting the virus genome at three different loci. This multiplex approach led to enhanced resistance in the targeted plants, which were free of any TYLCV symptoms (Ali et al., 2015b, 2016). Later effectiveness of the multiplex targeting (IR, Rep, and CP) approach was confirmed by increase resistance against *Cotton leaf curl Multan virus* (CLCuMV; Yin et al., 2019), *Chilli leaf curl virus* (ChLCV; Roy et al., 2019), and against dsDNA of the pararetrovirus *Cauliflower mosaic virus* (CaMV; Liu et al., 2018).

ZFNs and TALENs inhibit viral replication without cutting the viral genome (Sera, 2005; Cheng et al., 2015). Inhibition of plant viruses without cutting the genome is also quite feasible using the CRISPR/Cas system. A mutation in the nuclease domain renders Cas9 unable to cut DNA, but dCas9-sgRNA still possesses a high binding affinity to the target site. Khan et al. (2019) produced transgenic plants expressing dCas9-sgRNA to demonstrate the inhibition of CLCuMV replication without genome cutting. However, compared to the efficient inhibition by wild-type Cas9, targeting of the dCas9-sgRNA to the IR provides only partial protection against CLCuMV (Khan et al., 2019).

After successfully producing CRISPR/Cas9-based immunity against phytopathogenic viruses in model plants, various groups used CRISPR/Cas9 to generate viral immunity in crop plants, including tomato, cassava, barley, and banana. Tashkandi et al. (2018) produced transgenic tomato plants expressing CRISPR/Cas9 machinery against TYLCV (Tashkandi et al., 2018). Similarly, multiplex CRISPR/Cas9-

based immunity was engineered in barley against *Wheat dwarf virus* (WDV; Kis et al., 2019a). CRISPR/Cas9 efficiently targeted the IR sequences of TYLCV and WDV in tomato and barley, and the transgenic plants were free of viral symptoms. Mehta et al. (2019) generated transgenic cassava plants expressing the CRISPR/Cas9 machinery to target the coding regions of transcription activator protein (AC2) and replication enhancer protein (AC3) in *African cassava mosaic virus* (ACMV). Similar to observations by Ali et al. (2016) for CLCuMV and Liu et al. (2018) for CaMV, targeting of the coding sequences of ACMV by CRISPR/Cas9 resulted in the generation of ACMV variants that were capable of replication and infection (Mehta et al., 2019). These findings (Table 1) are critical for designing virus resistance strategies and applying the CRISPR/Cas system to plant virology.

Plant viruses genome rarely integrate into the host plant genome. However, the genomes of pararetroviruses such as *Banana streak virus* (BSV) integrate into the genome of banana, generating an endogenous BSV called eBSV. These integrated viral genomes remain a continuous threat, as eBSV can be activated at any stage in the plant lifecycle. Tripathi et al. (2019) designed a CRISPR/Cas9 system to inactivate the eBSV genome present in the banana genome. Regenerated banana plants expressing CRISPR/Cas9 against the eBSV genome were produced, demonstrating the efficient targeting of the eBSV genome and mitigating the potential risks of eBSV activation (Tripathi et al., 2019).

## Direct inhibition of plant RNA viruses

RNA viruses are the most abundant phytopathogens worldwide, causing devastating diseases in cultivated plants. The genomes of plant RNA viruses are composed of positive-strand ssRNA, negative-strand ssRNA, or dsRNA. Multiple CRISPR/Cas system variants (Fn-CRISPR/Cas9 and CRISPR/Cas13) have the potential to increase plant immunity against RNA viruses (Figure 1, Table 1). Zhang et al. (2018) produced *N. benthamiana* and Arabidopsis plants expressing FnCas9, FndCas9 (nuclease inactive), and multiple sgRNAs to target the RNA genomes of TMV and *Cucumber mosaic virus* (CMV). Interestingly, both FnCas9 and its modified dead version FndCas9 provided efficient resistance against both TMV and CuMV, suggesting that the binding of FnCas9 or FndCas9 to the RNA genomes of TMV and CuMV is sufficient to block viral replication in plant cells (Zhang et al., 2018). Aman et al. (2018a, 2018b) engineered a multiplexed CRISPR/Cas13 system to cut viral genome and provide resistance against *Turnip mosaic virus* (TuMV) and TMV in *N. benthamiana* and Arabidopsis plants. The successful targeting of the RNA genomes of viruses in model plants (Aman et al., 2018a, 2018b) paved the way for applying this powerful technology to crop plants to increase resistance to viruses. Zhan et al. (2019) successfully used the CRISPR/Cas13 system to efficiently inhibit the replication of *Potato virus Y* (PVY) in potato (Zhan et al., 2019). Similarly, CRISPR/Cas13 was used to generate transgenic rice

resistance to *Southern rice black-streaked draft virus* and *Rice stripe mosaic virus* (Zhang et al., 2019).

Plant RNA viruses are highly diverse, and their genomes evolve rapidly. Thus, it is crucial to develop a CRISPR/Cas system capable of providing efficient, broad-spectrum resistance to RNA viruses. The Type VI CRISPR/Cas13 system is divided into four subtypes (A–D) based on the phylogeny of Cas13 variants (O'connell, 2019). Some Cas13 variants have more robust catalytic activity and specificity than the well-known LshCas13a (Abudayyeh et al., 2017; Cox et al., 2017; Gootenberg et al., 2017; Konermann et al., 2018; Yan et al., 2018). Mahas et al. (2019) characterized a set of Cas13 family members (LwaCas13, LwaCas13a, PspCas13b, and Cas13d) *in planta* to identify a robust system for RNA virus interference in plants. Most of the Cas13 variants provided resistance against plant RNA viruses, but CasRx (CRISPR/Cas13d) conferred robust resistance against the RNA virus TuMV (Mahas et al., 2019). Interestingly, nuclease-inactive (dCasRx)-based targeting did not mediate virus interference, indicating that in contrast to dCas9-sgRNA and FnCas9-sgRNA (Sera, 2005; Zhang et al., 2018), binding of only dCasRx-crRNA complex to viral genomic RNA is not sufficient to interfere with virus replication. In the same study, multiplexing of CasRx-crRNAs to target two different RNA viruses was successfully used to restrict the accumulation of the genomes of both viruses in plant cells (Mahas et al., 2019).

All Cas13 variants upon digestion of the specific viral RNA target, the activated Cas13 nonspecifically degrades cellular RNA transcripts, representing an essential component of bacterial immunity to restrict further viral spread via programmed cell death (Meeske et al., 2019; O'connell, 2019). Interestingly, such nonspecific promiscuous degradation of cellular RNA transcripts in trans has not been observed in plants. Still, the underlying molecular mechanism must be experimentally confirmed. Perhaps accessory/helper factors or structures with a specific confirmation enable Cas13 variants to promiscuously digest their nonspecific targets in bacterial cells in trans, but such factors are absent in plants, and Cas13 variants only target viral genomes for degradation (Mahas et al., 2019). The ability of CRISPR/Cas13 to precisely cut the target viral RNA while not disturbing cellular transcripts are of great importance for providing crops with an efficient immune system against RNA viruses.

## CRISPR/Cas-mediated host genome editing for virus resistance

The genomes of plant viruses encode only a few proteins, including proteins important for suppressing the host immune system and initiating viral replication, coat proteins for genome encapsidation, and movement protein for systemic infection of the plant body. To replicate and complete their lifecycles, viruses hijack the host's cellular machinery to facilitate the infection process (Dong and Ronald, 2019). Most of these host factors function as susceptibility factors (S factors) for plant viruses (Van Schie and Takken, 2014).

Modifying these S factors (Figure 1) to limit their availability or to prevent them from interacting with the viral lifecycle can mitigate the pathogenicity of viruses in plants (Dong and Ronald, 2019). The first recessive virus-resistant mutants were discovered in *Arabidopsis* and demonstrated that the potyvirus *Tobacco etch virus* could not infect plants containing the *eIFiso4E* (*Eukaryotic initiation factor*) variant (Lellis et al., 2002). Such recessive resistance to different plant RNA viruses was subsequently observed in many resistant cultivars of pepper, melon, lettuce, the wild tomato *S. habrochaites*, rice, and barley. For a detailed review, see Hashimoto et al. (2016). These observations prompted all of the initial efforts to modify the host genome for virus resistance to focus on *eIF4E*, *eIFiso4E*, and *eIF4G*. Chandrasekaran et al. (2016) used a transgene-free approach to deliver CRISPR/Cas9 to cucumber targeting *eIF4E*. The *eIF4E*-mutated (*elf4e*) cucumber plants showed efficient resistance to *Cucumber vein yellow virus*, *Zucchini yellow mosaic virus*, and *Papaya ring spot virus-W* (Chandrasekaran et al., 2016). Using the same approach, *eIF4E* in *Arabidopsis* and *eIFiso4E* in rice were mutated for resistance against TuMV and *Rice tungro spherical virus* respectively (Pyott et al., 2016; Macovei et al., 2018). Similarly, the inactivation of the *eIF4E* isoforms *nCBP-1* and *nCBP-2* by CRISPR/Cas9 reduced susceptibility to *Cassava brown streak virus* in cassava plants (Gomez et al., 2019).

Mimicking natural resistance alleles (amino acid substitutions) where the original physiological functionality of *eIF4E* is not affected but the engineered *eIF4E* (N176K) allele (Gao et al., 2004) can provide broad-spectrum virus resistance, are preferred in plant biotechnology (Zaidi et al., 2020). Bastet et al. (2019) applied the CRISPR/Cas9-coupled base editing approach to convert the wild-type susceptible *eIF4E* allele to the virus-resistant *eIF4E* (N176K) allele. The specific conversion of N176K conferred resistance against *Clover yellow vein virus* in *Arabidopsis* (Bastet et al., 2019).

In addition to *eIF4E*, several other host factors were identified as recessive susceptibility factors for plant viruses. *Coilin*, encoding a signature protein of subnuclear structures Cajal bodies mediate susceptibility to PVY (Shaw et al., 2014; Love et al., 2017). Makhotenko et al. (2019) delivered ribonucleo-protein complex (Cas9-sgRNA) to the apical meristem of potato via biolistics, to mutate the *Coilin* locus in the genome. Interestingly, editing of at least one allele of *Coilin* in the tetraploid genome of potato was sufficient to provide resistance against PVY (Makhotenko et al., 2019).

Secondary metabolites play a critical role in plant-microbe interactions, including virus resistance (Mishra et al., 2020). Particularly, accumulation of isoflavones contents resulting from the *Glycine max isoflavone synthase* (*GmIFS's*) polymorphisms is considered to be associated with *Soybean mosaic virus* (SMV) resistance in soya bean (Cheng et al., 2010). To demonstrate a direct connection of isoflavones content and SMV resistance in soybean, Zhang et al. (2020) employed CRISPR/Cas9-mediated multiplex gene-editing. Simultaneous targeting of *GmF3H1*, *GmF3H2*, and *GmFNSII-1* doubled the leaf isoflavone content, and reduced the SMV by one-third,

confirming the direct relationship of the increased isoflavone content to SMV resistance in soybean (Zhang et al., 2020).

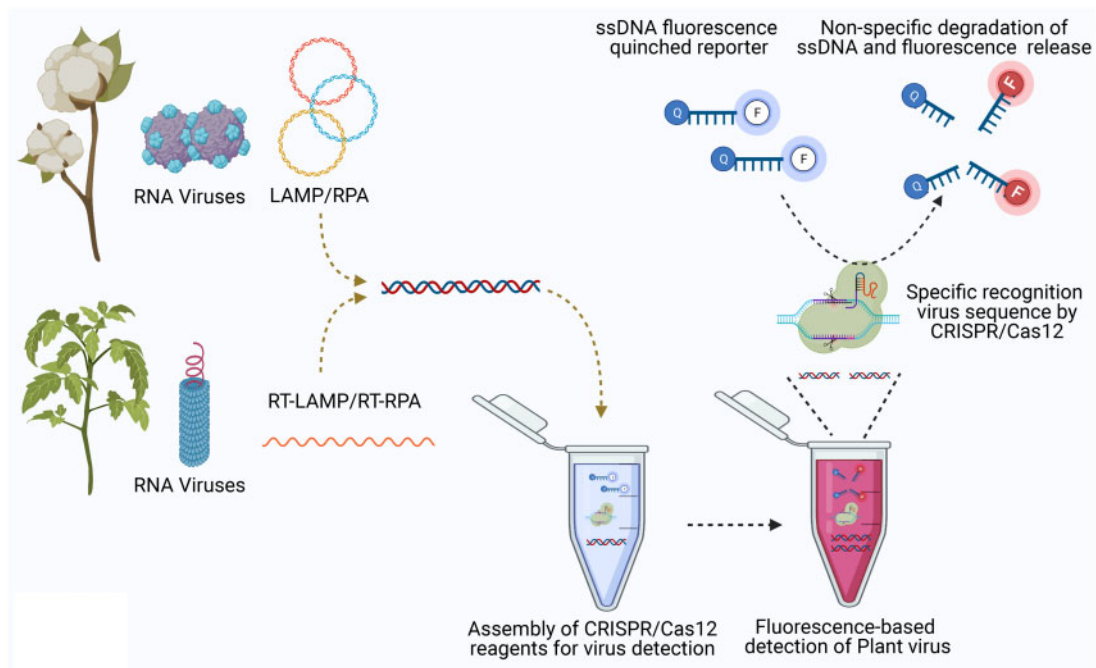
## CRISPR/Cas and viral diagnostics in plants

Early detection of a pathogen can give growers enough time to devise a disease prevention strategy. Molecular techniques such as PCR and RT-PCR and field-based isothermal techniques such as LAMP, RT-LAMP, RPA, and NASBA are commonly used to detect phytopathogens (Szemes et al., 2002; Lu et al., 2018; Wilisiani et al., 2019; Edgü et al., 2020; Wang et al., 2020b). However, these techniques are time-consuming, expensive, have a high ratio of nonspecificity or low sensitivity, and cannot be applied to bulk plant samples in the field. Simple, precise, inexpensive, rapid, large-scale, field-deployable diagnostic tools are still needed to detect phytopathogens in crops in the field.

The use of CRISPR/Cas systems as sequence-specific nucleases revolutionized the field of genome editing (Doudna and Charpentier, 2014), but also, techniques based on the ability of CRISPR/Cas variants to promiscuously degrade any ssDNA or ssRNA in the vicinity following its specific target recognition and digestion (Chertow, 2018) brought the field of molecular diagnostics to the next level (Wang et al., 2020a). In the past 5 years, more than 100 different CRISPR/Cas-based molecular diagnostic strategies were reported to detect nucleic acids, including plant viruses. After cutting the target DNA, Cas12a nonspecifically degrades the reporter ssDNA (Chen et al., 2018). In most cases, ssDNA (fluorescence quenched) reporter upon nonspecific degradation by Cas12, releases the fluorescent signal for visual detection (Kellner et al., 2019; Ali et al., 2020a). Like Cas12, most Cas13, and Cas14 variants possess collateral activity against ssDNA or ssRNA in trans. Variants of Cas12, Cas13, and Cas14 have been harnessed to develop efficient nucleic acid diagnostic platforms such as DETECTR, SHERLOCK, HOLMES, HOLMESv2, CaT-SMelor, iSCAN, and SHINE for specific and highly sensitive (up to attomole) detection of nucleic acids and small molecules (Gootenberg et al., 2017; Li et al., 2018; Kellner et al., 2019; Li et al., 2019; Liang et al., 2019; Ali et al., 2020a; Arizti-Sanz et al., 2020; Broughton et al., 2020). The impressive advancements in molecular diagnostics via CRISPR/Cas prompted plant scientists to apply these techniques as specific and sensitive diagnostic platforms for plant virus detection. Specifically, the coupling of the isothermal amplification assays LAMP and RPA and reverse transcription techniques to enrich target and visual detection systems (lateral flow strips, fluorescence detection systems) with CRISPR/Cas can provide an inexpensive, field-deployable diagnostic system (Figures 2 and 3) for plant viruses (Mahas et al., 2021).

Early detection of RNA viruses and viroids in apple can reduce financial losses when combined with viral spread prevention strategies. Jiao et al. (2021) developed the first inexpensive, field-deployable CRISPR/Cas12a based diagnostic system to detect the prevalent RNA viruses/viroids of





**Figure 2** Field-deployable CRISPR/Cas-based detection of plant viruses. Nucleic acid (DNA, or RNA) isolated from plants is subjected to isothermal amplification (loop-mediated isothermal amplification, LAMP or recombinase polymerase amplification, RPA for DNA viruses or reverse transcriptase coupled loop-mediated isothermal amplification, RT-LAMP, reverse transcriptase coupled recombinase polymerase amplification, RT-RPA for RNA viruses). Upon specific recognition and digestion of virus nucleic acid, Cas12a nonspecifically degrades an ssDNA reporter and releases the quenched fluorescence. End-point fluorescence (color change) is visualized with the naked eye using a P51 fluorescence lightbox.

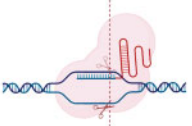
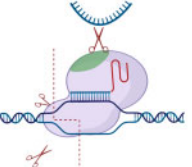
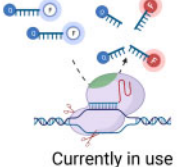
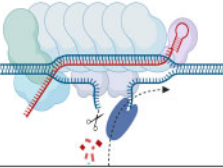
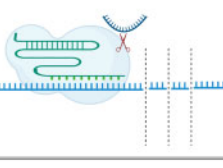
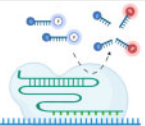
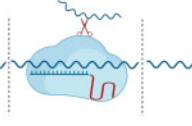
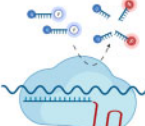
apple. A CRISPR/Cas12a-based platform was designed to detect multiple apple viruses, including *Apple necrotic mosaic virus*, *Apple stem pitting virus*, *Apple stem grooving virus*, *Apple chlorotic leaf spot virus*, and *Apple scar skin viroid* (Cho et al., 2016). Using reverse transcription-recombinase polymerase amplification (RT-RPA), the target viral RNA is first converted to DNA, and the amplified DNA is then detected using the CRISPR/Cas12a detection system. This visual detection system is based on a linear linker ssDNA and oligonucleotide-conjugated gold nanoparticles. In a positive sample, upon target recognition, Cas12a nonspecifically degrades the linker-ssDNA to prevent aggregation of DNA/2-AuNPs (there is no possibility of cross-linked hybridization), and the solution remains red (positive result). However, in the absence of the viral target (no activity of Cas12a and no degradation of linker-ssDNA), the intact linker-ssDNA crosslinks with the complementary ssDNA conjugated to gold nanoparticles. This results in the aggregation of DNA and gold nanoparticles, and solution becomes colorless (negative result; Jiao et al., 2021). The only drawback of this system is its relatively low sensitivity (250 viral copies per reaction), as it would not be possible to detect viral attack before a certain incubation period in the field.

To make the plant virus detection easier, recently Aman et al. (2020b) developed a much simpler but sensitive CRISPR/Cas12a-based assay to detect plant RNA viruses. The original in vitro Specific CRISPR-based Assay for Nucleic

acids detection (iSCAN; Ali et al., 2020a) protocol was modified by replacing RT-LAMP with a sensitive RT-RPA technique to develop a one-pot assay named iSCAN one-pot (iSCAN-OP) for the detection of *Potato virus X* (PVX), PVY, and TMV. With iSCAN-OP a very low titer of the virus (up to 10 pM) can be easily detected within 15–20 min in a single tube. Moreover, the presence of the virus (positive readout) is visible to the naked eye (Aman et al., 2020b). The iSCAN-OP detection system can be used for large-scale, reliable, affordable, and rapid screening of plant viruses in the field.

Selection of disease-free seeds and early detection of virus at nursery level is important for sustainable agriculture. Very recently, Mahas et al. (2021) developed an inexpensive LAMP-coupled CRISPR–Cas12a module for rapid and sensitive detection of plant DNA viruses. This in field, easy-to-interpret visual readout CRISPR–Cas12a based detection module was shown to detect TYLCV and *Tomato leaf curl New Delhi virus* (ToLCNDV) in less than 1 h (Mahas et al., 2021). These molecular diagnostics systems are low-cost, field suitable, and can be used in any point-of-use applications.

The development of such systems is just the beginning (Figure 3). Fozouni et al. (2021) recently developed a new CRISPR/Cas13a diagnostic method that does not require pre-amplification of the target sequence, and the results can be evaluated using a smartphone (Fozouni et al.,

CRISPR/Cas system for plant virus resistance	Possibilities and advantages for virus targeting	CRISPR/Cas system for plant virus diagnostics	Possibilities and advantages for virus resistance
 <p>CRISPR/Cas9</p>	<p>Can target dsDNA viruses. Multiplexing needs extra step. Virus escape is possible. Delivery via virus vectors is difficult. Good for targeted mutagenesis in host genome for virus resistance.</p>	<p>Not used yet for plant virus detection.</p>	<p>Can be used but needs extra steps.</p>
 <p>CRISPR/Cas12</p>	<p>Can target dsDNA and ssDNA viruses. Multiplexing very easy. Virus escape is not possible. Delivery via virus vectors is easy. Good for targeted mutagenesis and repair in host genome for virus resistance.</p>	 <p>Currently in use</p>	<p>Easy to develop for ssDNA, dsDNA viruses, and possible for RNA virus detection. Field deployable.</p>
 <p>CRISPR/Cas3</p>	<p>Can target dsDNA viruses. Multiplexing very easy. Virus escape is not possible. Delivery via virus vectors is difficult. Requires multiple factors for targeted mutagenesis in host genome for virus resistance.</p>	<p>Not feasible for plant virus detection.</p>	<p>Not applicable.</p>
 <p>CRISPR/Cas14</p>	<p>Can target ssDNA viruses. Multiplexing very easy. Virus escape is not possible. Delivery via virus vectors is very easy. Not used yet in plants.</p>	 <p>Not used yet for plant virus detection.</p>	<p>Easy to develop for ssDNA, dsDNA viruses, and possible for RNA virus detection. Field deployable.</p>
 <p>CRISPR/Cas13</p>	<p>Can target RNA viruses. Multiplexing very easy. Virus escape is not possible. Delivery via virus vectors is possible.</p>	 <p>Not used yet for plant virus detection</p>	<p>Easy to develop for RNA viruses, and possible for DNA virus detection. Field deployable.</p>

**Figure 3** Applications of CRISPR/Cas systems comparison of CRISPR/Cas systems for virus resistance and diagnostics. Targeting and digestion site pattern in the dsDNA or ssDNA and RNA are shown by small dashed lines. For each system, the possibilities and advantages are described. For plant virus diagnostics, the fluorescence detection method is presented.

2021). Adopting, the amplification-free method could make a truly field-deployable plant virus detection system.

### Plant viruses as CRISPR/Cas delivery tools for virus targeting and resistance

Apart from their devastating effects on plant health, the programmability of plant virus nucleoprotein nanostructures allows these natural foes to be converted into efficient tools to deliver DNA, RNA, proteins, medicines, and reagents of agricultural importance into living cells (Chira et al., 2015; Abrahamian et al., 2020; Kalinina et al., 2020; Varanda et al., 2021; DeHart and Kamrud, 2018). In a scenario where antibiotics and chemicals cannot be used against plant viruses, but CRISPR/Cas machinery can confer virus resistance in plants, the delivery of the CRISPR/Cas system via viruses has several advantages. Beginning with the work of Baltes et al. (2014; DNA replicons) and Ali et al. (2015a), (TRV, RNA virus) now more than 30 different viral vectors (Table 2 and

Box 3) have been designed for the delivery of CRISPR/Cas machinery into plant cell for multiple purposes (for detailed reviews, see Puchta, 2002; Wigge, 2011; Fauser et al., 2012; Puchta and Fauser, 2013; Gover et al., 2014; Lozano-Durán, 2016; Zhang et al., 2016; Schmidt et al., 2019; Van Vu et al., 2019; Liu and Zhang, 2020; Ma et al., 2020; Varanda et al., 2021). Here, we discuss only the pioneering studies, major milestones, and potential applications of some of these vector systems (for viral vectors expressing the whole CRISPR/Cas machinery see Box 3 and Table 2) to deliver the CRISPR/Cas machinery into plant cells for virus resistance in plants.

The RNA virus, TRV is widely used to deliver (200–500) stretches nucleic acid into plant cells (for detailed reviews, see Creager et al., 1999; Ratcliff et al., 2001; Senthil-Kumar and Mysore, 2011; Ali et al., 2015a). The delivery of short RNA, such as sgRNAs systemically to the entire plant provides multiple opportunities. In their pioneering study, Ali et al. (2015a) successfully engineered TRV2 using a short RNA-dependent RNA polymerase

### Box 3. VIRUS VECTORS FOR CRISPR/CAS SYSTEM DELIVERY INTO PLANTS

Plant virus-based vectors known as replicons (Gover et al., 2014; Lozano-Durán, 2016; Liu and Zhang, 2020; Ma et al., 2020) were developed to deliver whole CRISPR/Cas9 machinery or only sgRNA (*Cabbage leaf curl virus*) machinery into tobacco and potato, leading to high rates (75%–85%) of targeted mutagenesis and herbicide resistance (Baltes et al., 2014; Yin et al., 2015; Butler et al., 2016).

Mimicking natural resistance (s alleles, loss-of-function mutations in susceptibility factors) and to avoid silencing, insertion of R factors or virus resistance QTL into safe harbors via gene targeting (homology-directed repair) is important to create elite crop varieties (Puchta, 2002; Puchta and Fauser, 2013; Van Vu et al., 2019). Geminivirus replicons were developed to enhance the frequency of repair 1.5- to 9-fold in tomato, tobacco, *M. truncatula*, wheat, and barley (Fauser et al., 2012; Čermák et al., 2017; Schmidt et al., 2019). Similarly, the BeYDV replicon system (Baltes et al., 2014) was modified to make freely available repair template (Fauser et al., 2012), this CRISPR/Cas9-based recovered gene targeting events at the *CRTISO* and *PSY1* loci in 25% of T<sub>0</sub> tomato plants (Dahan-Meir et al., 2018).

Germline cells genome engineering can increase the recovery of modified plants in the next generation. Ellison et al. (2020) modified the TRV-based sgRNA expression system (Ali et al., 2015a; Ellison et al., 2020. Lei et al. (2021) modified *Cotton leaf crumple virus* replicons by fusing an mRNA FT-translocation signaling motif (Wigge, 2011) or tRNA (Zhang et al., 2016) to the 3' end of sgRNA to enhance its translocation into germline cells (Lei et al., 2021).

Very recently, simple mechanical inoculation of PYX and negative-strand RNA viruses vectors were used to deliver sgRNAs for simultaneous multisite genome editing or the whole CRISPR/Cas machinery into plant tissue to increase (up to 90%) the recovery of foreign DNA free genome-edited plants (Ariga et al., 2020; Ma et al., 2020; Uranga et al., 2021). Mechanical inoculation could be used to directly introduce constructs harboring new S and R genes for virus resistance into mature plants such as fruit trees to regenerate foreign DNA-free plants. The direct recovery of genome-edited seeds will eliminate the need for time-consuming tissue culture and can enhance the speed of crop breeding for virus resistance and could potentially reshape agriculture.

promoter from the related RNA virus *Pea early browning virus* (PEBV) to express sgRNA with a proper 5' end to target the TYLCV genome. The systemic targeting of TYLCV by Cas9-sgRNA resulted in a high rate of TYLCV genome editing, blocked the spread and accumulation of virus genome, and led to the recovery of *N. benthamiana* plants devoid of any TYLCV, CLCuMV, MeMV, and BCTV symptoms (Ali et al., 2015b, 2016).

Next, TRV-based sgRNA expression for CRISPR/Cas13-based was engineered to restrict the replication of RNA viruses like TuMV in *N. benthamiana* (Aman et al., 2018a). Similarly, TRV-based sgRNA system was adopted to identify a robust Cas13 ortholog for RNA virus targeting. The use of TRV vectors readily identified Cas13d from *Ruminococcus flavefaciens* XPD3002 (CasRx) as an efficient RNA virus targeting protein (Mahas et al., 2019) compared to Cas13a from *Leptotrichia wadei* (LwaCas13a), Cas13b from *Bergeyella zoohelcum* (BzCas13b), and Cas13b from *Prevotella* sp. P5-125 (PspCas13b).

### Conclusion

The CRISPR/Cas system is currently the only molecular tool used to cut the viral genome inside plant cells without

perturbing the biochemical functionality of the cell (Ali et al., 2015a; Baltes et al., 2015; Ji et al., 2015). Plant replicons have the potential to deliver CRISPR/Cas-based machinery into mature plants to fight against pathogenic viruses. As an alternative to the direct targeting of viral genomes, the CRISPR/Cas system can be used to modify host factors to provide cellular immunity against plant viruses. The recently discovered CRISPR/Cas variants, i.e., CRISPR (Cas3, 12, 13, 14) are offering the accurate tools that can cover all aspects including, virus resistance and field-deployable diagnostics. Similarly, modifications (base editing, EvolvR, prime editing) to the existing CRISPR/Cas9 system, and Replicon-based delivery of CRISPR/Cas to cut and repair a host factor to mimic the natural resistance allele or to insert an *R factor* into a highly expressing locus in the genome, can provide efficient resistance against plant viruses.

As a whole, CRISPR/Cas system-based nucleic acid-targeting and detection represents the best toolbox in the field of plant virology. In the Outstanding Questions Box, we describe some challenges faced when using the CRISPR/Cas system in the field. Successfully overcoming these challenges could lead to the development of efficient resistance against plant viruses in the field.

**Table 2** Summary of virus vectors for CRISPR/Cas machinery delivery into plant

Plant virus vector	Genome	Target plant	Main goal of virus-based delivery	Reference/s
BeYDV replicons	DNA	<i>Nicotiana benthamiana</i> , <i>potato (S. tuberosum)</i> , Tomato ( <i>L. esculentum</i> ), <i>Medicago truncatula</i>	Virus replicons development for CRISPR/Cas system delivery, for host genome editing and HDR-based repair, gene targeting	(Baltes et al., 2014; Butler et al., 2016; Čermák et al., 2017; Dahan-Meir et al., 2018)
Cabbage leaf curl virus (CaLCuV)	DNA	<i>Nicotiana benthamiana</i>	VIGE (Virus induce genome editing), systemic delivery of sgRNA to increase the recovery of edited regenerated plants	(Yin et al., 2015)
WDV	DNA	wheat ( <i>T. aestivum</i> ), Rice ( <i>O. sativa</i> ), Barley ( <i>H. vulgare</i> )	Virus replicons development for CRISPR/Cas system delivery and HDR based repair	(Čermák et al., 2017; Wang et al., 2017)
Cotton leaf crumple virus (CLCrV)	DNA	<i>Arabidopsis thaliana</i>	sgRNAs delivery stem apical meristem and germline cells	(Lei et al., 2021)
TRV	RNA	<i>Nicotiana benthamiana</i>	Systemic delivery of sgRNA, sgRNA-FT, sgRNA-tRNA to invade germline cells to increase the recovery of edited plants, to obviate tissue culture	(Ali et al., 2015a, 2015b, 2016; Aman et al., 2018a, 2018b; Mahas et al., 2019; Ellison et al., 2020)
PEBV	RNA	<i>Nicotiana benthamiana</i> , <i>Arabidopsis thaliana</i>	Systemic delivery of sgRNA to increase the recovery of edited plants	(Ali et al., 2018)
TMV	RNA	<i>Nicotiana benthamiana</i>	To enhance systemic delivery of sgRNA to increase the recovery of edited plants, sgRNAs processing	(Cody and Scholthof, 2020)
Beet necrotic yellow vein virus (BNYVV)	RNA	<i>Nicotiana benthamiana</i>	Multiple sgRNAs delivery	(Jiang et al., 2019)
Foxtail mosaic virus	RNA	Foxtail ( <i>Setaria viridis</i> ), Maize ( <i>Z. mays</i> )	sgRNAs delivery to monocots	(Mei et al., 2019)
Barley yellow striate mosaic virus (BYSMV)	RNA	<i>Nicotiana benthamiana</i>	Delivery of the whole CRISPR/Cas system to plant tissue to increase the recovery of foreign DNA free genome-edited plants	(Ma et al., 2020)
Sonchus yellow net rhabdovirus (SYNV)	RNA	<i>Nicotiana benthamiana</i>	Delivery of the whole CRISPR/Cas system to plant tissue to increase the recovery of foreign DNA free genome-edited plants	(Ma et al., 2020)
PVX	RNA	<i>Nicotiana benthamiana</i>	Delivery of tandemly arranged sgRNAs for simultaneous multisite genome editing and the whole CRISPR/Cas system to plant tissue to increase the recovery of foreign DNA free genome-edited plants	(Ariga et al., 2020; Uranga et al., 2021)

### OUTSTANDING QUESTIONS

- How can CRISPR/Cas systems be delivered into mature plants (vaccination) such as field crops or fruit trees to mitigate virus attack?
- What is the best CRISPR/Cas system for providing efficient, broad-spectrum resistance to viruses in the field?
- How can viral escape be controlled?
- How can CRISPR/Cas systems be used to identify additional susceptibility (S) factors to be modified for broad-spectrum viral resistance?
- How can resistance genes (R) be created or mimicked to produce virus-resistant elite crop varieties?
- How can genome-modified, virus-resistant elite crop varieties be created without the need for tissue culture?
- How should genome editing, field trials, and innovations be regulated?

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