Review

Engineering plant virus resistance: from RNA silencing to genome editing strategies

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Summary

Viral diseases severely affect crop yield and quality, thereby threatening global food security. Genetic improvement of plant virus resistance is essential for sustainable agriculture. In the last decades, several modern technologies were applied in plant antiviral engineering. Here we summarized breakthroughs of the two major antiviral strategies, RNA silencing and genome editing. RNA silencing strategy has been used in antiviral breeding for more than thirty years, and many crops engineered to stably express small RNAs targeting various viruses have been approved for commercial release. Genome editing technology has emerged in the past decade, especially CRISPR/Cas, which provides new methods for genetic improvement of plant virus resistance and accelerates resistance breeding. Finally, we discuss the potential of these technologies for breeding crops, and the challenges and solutions they may face in the future.

Introduction

The global population has increased by over 25% in the last 20 years and is projected to increase from 7.7 billion in 2019 to around 10 billion by 2050 (Tilman *et al*., 2011). As the global population continues to grow, the provision of adequate food has emerged as one of the major challenges at present (Cheeseman, 2016; Legg *et al*., 2014). However, the growth rate of grain production does not seem to have kept pace with population growth: the global *per capita* grain production has declined over the last 20 years (Legg *et al*., 2014; Suweis *et al*., 2015).

In addition to restricted arable land and water resources, the growth of global food production is further limited by pests and diseases. The yield of cultivated plants is threatened by pests which cause yield loss of 20–40%, while bacterial and fungal pathogens reduce crop yields by about 15% and viruses reduce yields by 3–7% (Oerke and Dehne, 2004). Compared with pests or other diseases, losses caused by viruses are not so great, but the outbreak of viral diseases can cause serious problems. Economic losses caused by viral diseases can reach USD 60–80 billion annually. This is due to the fact that chemical pesticides can be used for bacterial or fungal diseases and insect pests, but there is currently no traditional chemical pesticide that directly targets viral diseases. At present, the main strategy to control viral diseases in the field is to use pesticides or natural predators to control the vectors, or use physical barriers, such as reflective mulches and insect-proof nets (Legg *et al*., 2014). However, complex epidemiological factors associated with viral disease outbreaks, such as rapid evolution of viruses, vector migration dynamics and unpredictable expansion of viral host range, make it very difficult to develop effective long-term disease management strategies (Zaidi *et al*., 2016).

The use of virus-resistant varieties in agricultural production is the most economical and effective way to reduce losses caused by viral diseases, thus the current situation requires the development of highly effective and durable virus-resistant/immune crop varieties to combat increasingly serious viral diseases. Conventional antiviral breeding plays an essential role in crop improvement but usually requires large growing populations of crops over multiple generations, which is a rather time-consuming and laborious process. The emergence of genetic engineering, which directly alters the organism's genetic information using modern biotechnology, has significantly accelerated the process and efficiency of breeding (Christou, 2013).

Increasing knowledge about the molecular mechanism of plant–virus interactions and the advancement of biotechnology provides new opportunities for engineering plant resistance to viruses (Duan *et al*., 2012; Mahas and Mahfouz, 2018; Yin and Qiu, 2019). This review summarizes current antiviral biotechnology strategies, compares their advantages and disadvantages and discusses their application prospects and challenges.

Engineering RNA silencing-based resistance against viruses

As early as 1985, Sanford and Johnston put forward the elegant concept of pathogen-derived resistance (PDR; Figure 1), whose core theory was that expressing the pathogen genetic elements in plants will destroy the pathogenicity of the parasitic pathogens (Sanford and Johnston, 1985). The Beachy lab conducted pioneering work in 1986 which induced *Tobacco mosaic virus* (TMV) resistance in tobacco through the introduction of gene constructs expressing the viral coat protein (CP) (Figure 1; Abel *et al*., 1986). Subsequently, there have been numerous attempts to generate virus resistance by transforming plants with various viral genes or genome fragments, leading to successful development of virus-resistant crops for commercial application (Baulcombe, 1994; Beachy, 1993; Lomonossoff, 1995; Wilson, 1993), although the mechanisms of PDR were still unclear at that time (Baulcombe, 1996).

Meanwhile, the RNA silencing phenomenon in plants was first discovered in 1990 (Napoli *et al*., 1990), and has since been

widely characterized in many eukaryotic organisms such as fungi, animals and plants (Figure 1) (Baulcombe, 2004; Fire et al., 1998; Guo and Kemphues, 1995; Hannon, 2002; Romano and Macino, 1992). RNA silencing, also referred to as RNA interference (RNAi), is activated by the presence of double-stranded RNA molecules (dsRNAs) and induces gene expression inhibition or suppression in a nucleotide sequence-specific manner (Hannon, 2002; Voinnet, 2005). In plants, several key protein families are involved in RNA silencing, including Dicer-like (DCL), Argonautes (AGO), RNA-dependent RNA Polymerase (RDR) and Suppressor of Gene Silencing (SGS). As type III RNases, DCL proteins process dsRNA or miRNA precursors into siRNA or miRNA, respectively, of 20- to 24-nt long with a two-base overhang at the 3′ end. These siRNAs or miRNAs are incorporated into the endonuclease AGO proteins to form RNA-induced silencing complex (RISC). Directed by its containing siRNA/miRNA, RISC can bind to target mRNA or noncoding RNA and then silence the target gene expression by cleaving target RNA and rendering its degradation, or recruiting DNA and histone modifiers and inhibiting the transcription of the target gene. The cleaved target RNA may be recognized by RDR proteins which amplify the dsRNA to enhance the silence effect. SGS proteins stabilize the dsRNA substrate for DCLs to produce secondary siRNAs and reinforce the RNA silencing process (Figure 2; Ding, 2010; Ipsaro and Joshua-Tor, 2015; Voinnet, 2005).

Besides the regulatory roles in plant growth and development, the dsRNA-mediated RNA silencing also serves as a host antiviral defense mechanism (Ding, 2010). Since the progressive under-standing of the RNA silencing mechanism and its role in antiviral immunity, RNA silencing has been deployed in crop improvement for viral resistance (Ding and Voinnet, 2007). Generation of virus-derived dsRNA is a general feature when successful resistance is achieved in plants. Many approaches have been developed for engineering virus-resistant transgenic plants, mostly based on different precursor RNA for siRNA production, including sense/antisense RNA, hairpin RNA (hpRNA) and artificial miRNA precursors (Figure 2; Duan et al., 2012).

Thus far, RNA silencing technology has been successfully applied to target over 60 species of economically important plant viruses, including Papaya ringspot virus (PRSV) (Bau et al., 2003; Fitch et al., 1992; Ye and Li, 2010), Banana bunchy top virus (BBTV) (Elayabalan et al., 2013; Shekhawat et al., 2012), Citrus tristeza virus (CTV) (Soler et al., 2012), Plum pox virus (PPV) (Guo et al., 1998; Hily et al., 2007; Ravelonandro et al., 2014; Scorza et al., 2001; Wittner et al., 1998), Maize streak virus (MSV) (Shepherd et al., 2007), Maize dwarf mosaic virus (MDMV) (Zhang et al., 2010, 2013), Soybean mosaic virus (SMV) (Gao et al., 2015; Wang et al., 2001) and Tomato yellow leaf curl virus (TYLCV; Antignus et al., 2004; Fuentes et al., 2006). Nearly 30 crop species have been engineered to stably express small RNAs targeting various viruses, dozens of which have been approved for commercial release in several countries, such as papaya resistant to PRSV (Gonsalves, 2006; Ye and Li, 2010), potato resistant to Potato leafroll virus (PLRV) or Potato virus Y (PVY) and squash resistant to Cucumber mosaic virus (CMV) or Zucchini yellow mosaic virus (ZYMV) (Table 2).

These successful examples were all obtained by genetically modified methods; however, these transgenic approaches are not only time-consuming and expensive, but also suffer significant regulation and public acceptance issues. To address these limitations and public concerns, several approaches that involve exogenous application of naked dsRNA proved to successfully trigger the RNA silencing pathway against pathogenic viruses (Gan et al., 2010; Kaldis et al., 2018; Lau et al., 2014; Namgial et al., 2019; Robinson et al., 2014; Tenllado et al., 2003; Worrall et al., 2019). However, the obvious shortcoming of this strategy is that it has a very short virus protection window of 5–7 days post-application (Mitter et al., 2017b).Recently, a research group used a novel approach of delivering dsRNA using layered double hydroxide nanosheets as carriers and successfully established CMV resistance in tobacco plants (Mitter et al., 2017a). This approach not only increases the stability of dsRNA in plants, but also provides a sustained release of dsRNA to extend the virus protection period.

Engineering ZFN- or TALEN-based resistance against viruses

A decade ago, a new approach, referred to as genome editing, emerged that makes it possible to manipulate the genetic information in different cell types and organisms. Zinc finger nucleases (ZFNs) and transcription activator-like effector
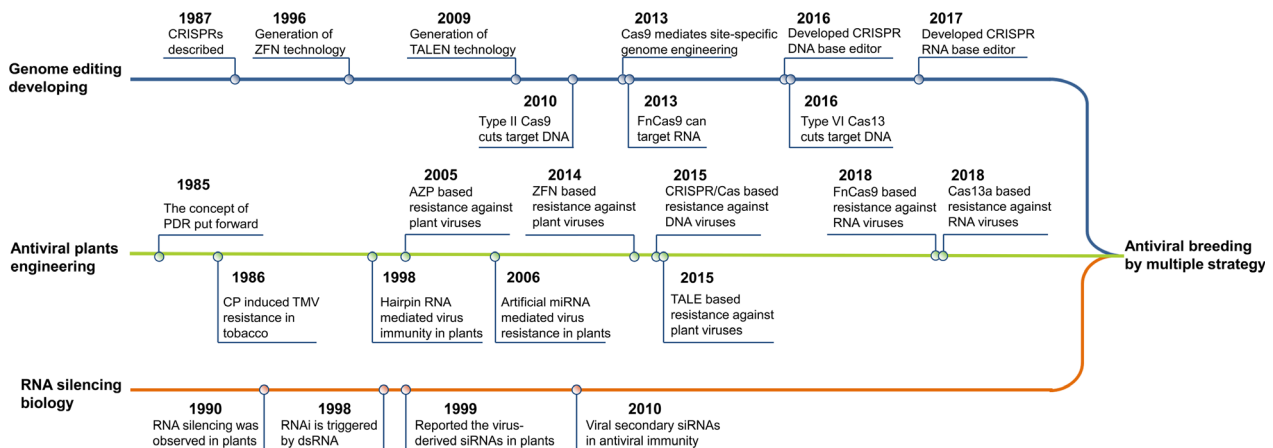

Figure 1 Timeline of antiviral plant engineering, genome editing and RNA silencing technology developing research fields. Key developments in all three fields are shown. In future, these fields will merged together, and multiple strategies will combined to server for antiviral breeding.
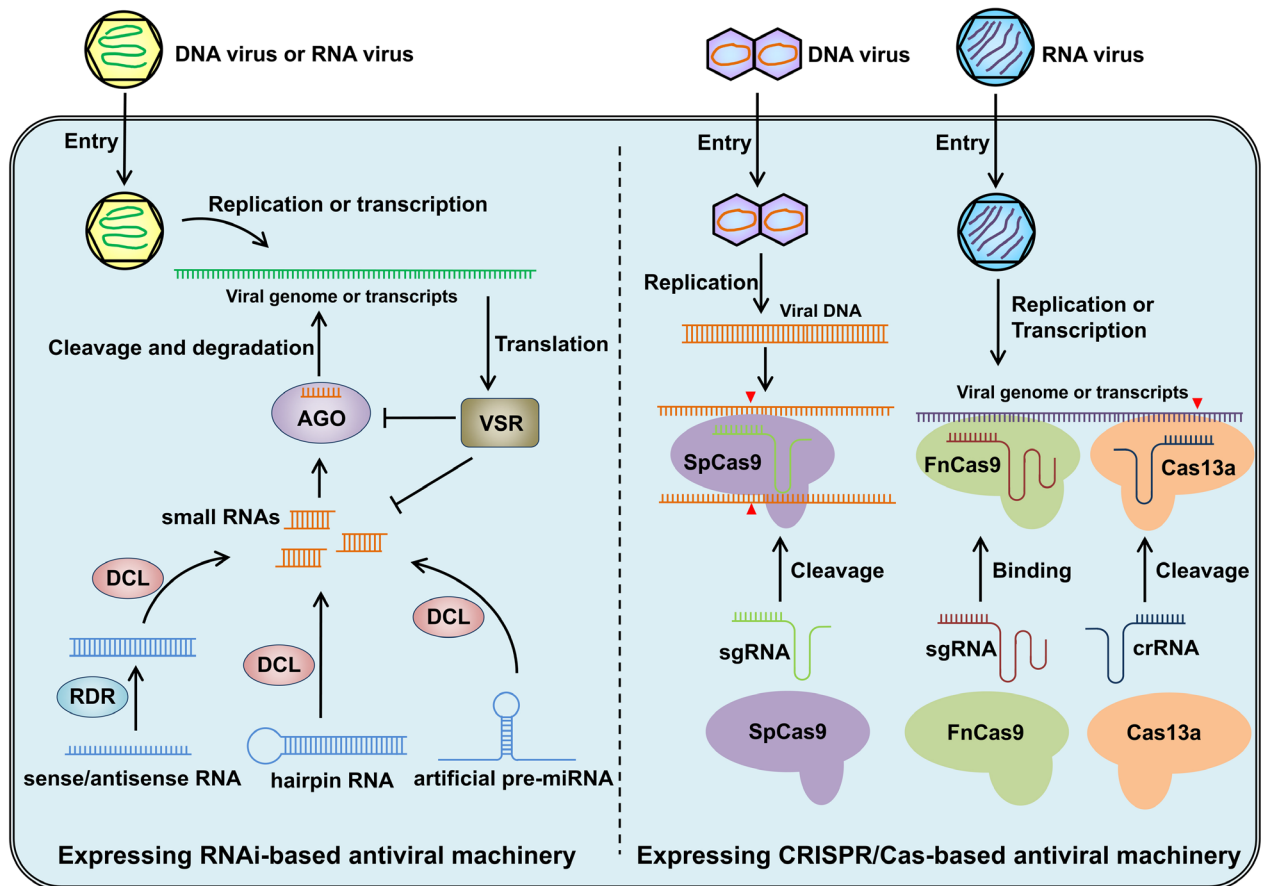
Figure 2 Schematic diagram depicting RNA silencing and CRISPR/Cas strategies to target plant viruses. The diagram on the left shows the mechanism of RNA silencing-based antiviral engineering. The plant cells are transgenic expressing or exogenous applied of virus-derived sense/antisense RNA, hairpin RNA, or artificial pre-miRNA to produce the small RNAs targeting viral genome or transcripts. The small RNAs are loaded into the AGO protein to guide the cleavage of the viral RNA, which induces the degradation of the viral genomic RNA or mRNA. Plant viruses can encode VSR to counter the RNA silencing based resistance by targeting the AGO protein or small RNAs. The diagram on the right shows the mechanism of CRISPR/Cas-based antiviral engineering. The CRISPR system consists of sgRNA and Cas protein. The transgenic or transit expression of the virus targeting sgRNA and its cognate Cas protein can effectively inhibit the virus infection. Upon DNA virus entry into the plant cell, for example the geminivirus, the viral genome is converted to a doublestranded DNA intermediate, which could be targeted and cleaved by the Cas9 protein from Streptococcus pyogenes (SpCas9). For RNA viruses, Cas9 from Francisella novicida (FnCas9) and Cas13a has been proved to confer virus resistance effectively. Guided by their cognate sgRNA or crRNA, the FnCas9 and Cas13a can bind or cleave the viral genome or transcripts, respectively. Arrowheads in red indicate cleave sites in the viral.

nucleases (TALENs) were the first-generation tools of genome editing technology (Figure 1) (Boch *et al.*, 2009; Kim *et al.*, 1996; Moscou and Bogdanove, 2009). Both ZFNs and TALENs are chimeric proteins created by fusing a DNA-binding domain (DBD) from a zinc finger protein or transcription activator-like effector, respectively, to the non-specific cleavage domain of the enzyme *Fok*I. The DBD determines a specific nucleotide recognition in the DNA target and the cleavage domain cleaves DNA to produce the double-strand breaks (DSB) in the targeted site (Boch *et al.*, 2009; Kim *et al.*, 1996; Moscou and Bogdanove, 2009; Urnov *et al.*, 2010). In eukaryotes, the DSBs are repaired by non-homologous end joining (NHEJ) or homologous recombination, and both repairing mechanisms may induce mutations in the particular genomic location (Wyman and Kanaar, 2006).

These genome editing techniques not only integrate, delete and/or mutate genes of interest, but also provide a new weapon in the arsenal against plant viruses. As early as in 2005, Sera developed an artificial zinc finger protein (AZP), which lacks the cleavage domain compared to ZFN, targeting the intergenic region (IR) of *Beet severe curly top virus* (BSCTV, family *Geminiviridae*) in *Arabidopsis* (Figure 1) (Sera, 2005). The IR of geminiviruses contains a stem-loop structure which is essential for virus replication by viral replication initiator protein (Rep) binding (Hanley-Bowdoin *et al.*, 2013). The transgenically expressed AZP efficiently binds the IR of BSCTV, thus blocking the Rep binding and subsequently suppressing the infection of the virus (Sera, 2005). Similar work has also been applied to reduce replication of *Rice tungro bacilliform virus* (RTBV) in *Arabidopsis* by expressing an AZP which was able to recognize and block the viral promoter sequences (Ordiz *et al.*, 2010). ZFN technology, unlike AZP, involves both DBD and DNA cleavage domains, and was also applied to target the Rep gene of two begomoviruses, *Tomato yellow leaf curl China virus* (TYLCCNV) and *Tobacco curly shoot virus* (TbCSV), in tobacco plants, and showed a significant inhibition of viral replication (Figure 1) (Chen *et al.*, 2014). TALEs, which lack the nucleases domain compared to TALEN, were developed to combat these begomoviruses using a similar approach. TALEs were engineered to target conserved motifs

among begomoviruses. Tobacco plants expressing the TALEs displayed resistance to TbCSV and TYLCCNV, while resistance to *Tomato leaf curl Yunnan virus* (TLCYnV) was partial (Figure 1) (Cheng *et al.*, 2015). TALEN technologies, a nuclease domain fused to TALE, has not been reported against plant viruses, although it has been explored for potential antiviral applications in some human viruses, such as h*epatitis B virus* (HBV), *hepatitis C virus* (HCV) and *human immunodeficiency virus* (HIV; Bloom *et al.*, 2015).

Although the use of these genome editing platforms has led to important advances, each has unique limitations, and their use in plants is far from routine. Thus, applications of ZFNs and TALENs have rapidly been surpassed by a new emerging genome editing system in various organisms, including plants.

Engineering CRISPR/Cas-based resistance against viruses

The clustered, regularly interspaced short palindromic repeats/ CRISPR-associated protein (CRISPR/Cas) system is based on an adaptive immune system that eliminates invasion of foreign plasmids or viral DNA via cleavage in bacteria and archaea (Bhaya *et al.*, 2011). CRISPR/Cas genome editing systems consist of an endonuclease Cas protein and a single-guide RNA (sgRNA) which directs the Cas protein to the DNA or RNA target. Moreover, sgRNA contains a scaffold for Cas protein binding and a user-defined approximately 20-nt long spacer sequence for genome targeting (Figure 1; Cong *et al.*, 2013; Mali *et al.*, 2013). Owing to its simplicity, high efficiency and affordability compared with precedent ZFN or TALEN, many labs working in different fields have turned to this technology. Many efforts are under way to reveal the potential application of CRISPR/Cas9 to control human viruses such as HIV, HBV, Epstein-Barr and plant viruses (Price *et al.*, 2016).

The original CRISPR/Cas system from *Streptococcus pyogenes*, used for genome editing, targets the DNA. Thus, the CRISPR/Cas9 machinery was first exploited to combat the geminivirus by targeting its viral genomic DNA during the replication stage (Mahas and Mahfouz, 2018; Yin and Qiu, 2019). Three groups reported the successful use of CRISPR/Cas9 to generate geminivirus resistance in tobacco and *Arabidopsis* (Figure 1, Table 1). They designed sgRNAs to target the IR, Rep or CP loci, and significantly reduced or abolished disease symptoms of several geminiviruses (Figure 2; Ali *et al.*, 2015; Baltes *et al.*, 2015; Ji *et al.*, 2015). In addition to its application to model plants, the same system was recently also used in barley and established highly efficient resistance against *Wheat dwarf virus* (WDV) (Kis *et al.*, 2019). For double-stranded DNA viruses, CRISPR/Cas9 has also been shown to be effective in inhibiting the virulence of *Cauliflower mosaic virus* (CaMV) in *Arabidopsis* (Table 1; Liu *et al.*, 2018).

Off-target effect is the major issue of genome editing, which occurs due to tolerance of sgRNA sequence mismatches and extended expression of Cas9 nuclease (Tsai and Joung, 2016). Ji *et al.* (2015, 2018) tested for the off-target effects in their *Arabidopsis* line expressing the virus-targeting CRISPR/Cas9 construct. In order to overcome the off-target effect, they used virus-induced promoters instead of constitutive promoter, to drive the Cas9 expression. Thus, the CRISPR/Cas9 antiviral system will be only expressed when the virus invades the plant cells. Ingeniously, no off-target effect was detected by deep sequencing in candidate sites of the virus-inducible genome editing *Arabidopsis* (Ji *et al.*, 2018). This kind of virus-inducible genome editing system could be widely applicable for generating virus-resistant plants without off-target costs (Table 2).

RNA viruses cause more serious losses in crops, as compared to DNA viruses, and enormous damage to agricultural production. With the development of more CRISPR/Cas systems from other bacterial strains, several Cas protein variants, such as the Cas9 from *Francisella novicida* (FnCas9) and the Cas13a from *Leptotrichia shahii* (LshCas13a) or *Leptotrichia wadei* (LwaCas13a), have been reported to target RNA *in vivo* (Abudayyeh *et al.*, 2016, 2017; Sampson *et al.*, 2013), which opens up new possibilities against RNA viruses. In the first case, FnCas9 and its sgRNA were engineered to target CMV and TMV, and reduced virus accumulation and attenuated disease symptoms were observed in tobacco and *Arabidopsis* expressing the antiviral system (Figure 1, Table 1) (Zhang *et al.*, 2018). Interestingly, RNA binding, but not cleavage capacity of FnCas9, is required for virus inhibition (Figure 2). Then, the LshCas13a system was reprogrammed and employed to antagonize RNA viruses in plants (Figure 2). Two groups successfully used the system to inhibit potyvirus infection in tobacco and potato (Aman *et al.*, 2018; Zhan *et al.*, 2019), while Zhang *et al.* (2019) established resistance to RNA viruses in both dicot and monocot plants. The LshCas13a system was designed to cleave genomic RNA of TMV in tobacco and to degrade the genomic RNA of *Southern rice black-streaked dwarf virus* (SRBSDV) and Rice stripe mosaic virus (RSMV) in rice plants (Figure 1; Zhang *et al.*, 2019). These cases demonstrated that the LshCas13a system can act against different types of RNA virus, including +ssRNA, -ssRNA and dsRNA genomes (Table 1). Targeting RNA genomes is superior as it would not lead to heritable off-target effect in the host genomic DNA, although it may lead to nonspecific RNA cleavage. Furthermore, the cleaved of viral genomic RNA will be further destroyed by RNAi system of plants. Therefore, RNA viruses have less chance to escape the CRISPR/Cas targeting antiviral system by mutating their genomes (Table 2).

In order for the infection to progress, virus needs to recruit many host factors to assist in replication, transcription, translation, etc. This feature provides us with a potential target of genome editing to limit virus infection. For example, viruses lack ribosomes in their virions, and the host translation machinery is essential for viral protein synthesis. In plants, the eukaryotic initiation factor 4E (eIF4E) and its isoform (eIFiso4E) are essential for some viruses to initiate viral protein translation (Sanfaçon, 2015). Previous work showed that the *Arabidopsis eIF(iso)4E* mutant has enhanced resistance to *Turnip mosaic virus* (TuMV) (Lellis *et al.*, 2002). By using CRISPR/Cas9, knockout of *Arabidopsis eIF(iso)4E* resulted in resistance to TuMV without affecting the plant growth (Pyott *et al.*, 2016), although these translation initiation factors are important for growth and reproduction (Table 2). Similar work was performed in cucumber, where the *eIF4E* gene destructed by CRISPR/Cas9 showed broad-spectrum resistance to the family *Potyviridae*, including *Cucumber vein yellowing virus* (CVYV), PRSV and ZYMV (Chandrasekaran *et al.*, 2016). Cassava encoded five proteins belonging to the *eIF4E* family, of which only nCBP-1 (novel cap-binding protein-1) and nCBP-2 interact with VPg (viral genome-linked protein) of *Cassava brown streak virus* (CBSV), which is a major constraint on cassava yields in East and Central Africa. The virus showed delayed and attenuated symptoms on the *ncbp-1/ncbp-2* double mutants generated by CRISPR/Cas9 (Table 1) (Gomez *et al.*, 2019).

Table 1 CRISPR/Cas technologies developed for viral pathogen resistance in plants. Cas9 was obtained from *Streptococcus pyogenes* (SpCas9), Cas9 from *Francisella novicida* (FnCas9) Cas13a from *Leptotrichia shahii* (LshCas13a)

Target	CRISPR system	Host plants	Virus	Genus	Family	Viral genome type	References
Viral genome	SpCas9	Tobacco	Tomato yellow leaf curl virus Cotton leaf curl Kokhran virus Merremia mosaic virus	Begomovirus	Geminiviridae	ssDNA	Ali *et al.* (2015, 2016)
		Tobacco and *Arabidopsis*	Beet severe curly top virus Beet curly top virus	Curtovirus			Ali *et al.* (2015), Ji *et al.* (2015)
		Tobacco	Bean yellow dwarf virus	Mastrevirus			Baltes *et al.* (2015)
		Barley	Wheat dwarf virus				Kis *et al.* (2019), Liu *et al.* (2018)
		Arabidopsis	Cauliflower mosaic virus	Caulimovirus	Caulimoviridae	dsDNA	
	FnCas9	Tobacco	Tobacco mosaic virus	Tobamovirus	Virgaviridae	+ssRNA	Zhang *et al.* (2018)
		Tobacco and *Arabidopsis*	Cucumber mosaic virus	Cucumovirus	Bromoviridae		Zhang *et al.* (2018)
	LshCas13a	Tobacco	Turnip mosaic virus	Potyvirus	Potyviridae	+ssRNA	Aman *et al.* (2018)
		Tobacco	Tobacco mosaic virus	Tobamovirus	Virgaviridae	+ssRNA	Zhang *et al.* (2019)
		Rice	Southern rice black-streaked dwarf virus	Fijivirus	Reoviridae	dsRNA	Zhang *et al.* (2019)
			Rice stripe mosaic virus	Cytorhabdovirus	Rhabdoviridae	-ssRNA	Zhang *et al.* (2019)
		Potato	Potato virus Y	Potyvirus	Potyviridae	+ssRNA	Zhan *et al.* (2019)
Host factor	SpCas9	Cucumber	Cucumber vein yellowing virus	Ipomovirus	Potyviridae	+ssRNA	Chandrasekaran *et al.* (2016)
		Cassava	Cassava brown streak virus				Gomez *et al.* (2019)
		Cucumber and *Arabidopsis*	Zucchini yellow mosaic virus Papaya ringspot virus Turnip mosaic virus	Potyvirus			Chandrasekaran *et al.* (2016), Pyott *et al.* (2016)

Table 2 Comparison of the strategies used in engineering antiviral plants

Strategies	Advantages	Disadvantages	Applications
RNA silencing	High efficiency and have several successful commercialized transgenic antiviral crops	Many viruses can encode VSRs to counter the defense of RNA silencing	Engineering resistance for the viruses without strong VSR, better effect on RNA viruses than DNA viruses
CRISPR/Cas targeting DNA	The eukaryotic plant viruses have not evolved to possess the ability to counter this immune defense coming from prokaryote	The targeted DNA viruses might be repaired and escape the engineered resistance. Off-target effect may produce some heritable mutations in the host genome	Suites for the viruses having dsDNA genome or dsDNA replication intermediate. A virus-induced promoter could reduce the off-target effect
CRISPR/Cas targeting RNA	The targeted RNA is further degraded and has less chance to produce mutant viruses	Off-target effect may affect the expression of some host genes, but will not change the plant genome	Engineering resistance to RNA viruses and DNA viruses with RNA intermediates
Host factors editing	The gene editing machinery can be removed by backcross, so it is able to engineer virus-resistant plant which is transgenic-free	Loss-of-function of some host factors may lead to lethality or impaired growth	Need to find a well-characterized host factor and mutation of the factor will not affect the growth and reproduction of the plants

Challenges and future aspects

Application of modern biotechnology has great potential to overcome the limitations of conventional viral resistance breeding. First, since in the case of both RNAi and genome editing technologies only viral sequence information is required, these approaches are particularly applicable to crops with limited genome sequence information. Second, resistance breeding using RNAi or genome editing does not require genetic crosses and selection of segregating progeny. Therefore, the breeding period can be greatly shortened. For some viral pandemics, rapid emergency response can be provided by exogenous application of dsRNA to induce RNA silencing against the virus. Third, destroying an essential host factor by CRISPR/Cas9 system is an effective way to generate virus-resistant crops, as in the case of eIF4E (Chandrasekaran *et al.*, 2016; Pyott *et al.*, 2016). Through several generations of backcross and screening, or even using DNA-free delivery of *in vitro* transcripts or ribonucleoprotein complexes of CRISPR/Cas9 by particle bombardment (Liang *et al.*, 2018), virus-resistant crops that are free of transgenic DNA can be generated, thus making it easier to release them for commercial production.

Nevertheless, these new technologies also have certain limitations. Through long-term evolution, plant viruses have developed

a range of counter-defensive measures against RNA silencing, one of which is the encoded viral suppressors of RNA silencing (VSR), which have become a major problem in RNA silencing approaches (Figure 2, Table 2) (Qu, 2010; Voinnet, 2005). Crop plants are often subjected to mixed viral infection. VSRs from untargeted viruses are able to disrupt the RNAi-mediated silencing process by targeting key components of RNAi pathways, and sometimes the targeted virus has a strong VSR that can break through the immunity conferred by RNA silencing (Kung *et al*., 2015). CRISPR/Cas is an immunity system derived naturally from prokaryotes, so eukaryotic viruses have not evolved in the presence of CRISPR/Cas, which implies they are unlikely to have CRISPR/Cas evasion strategies. Hence, using the genome editing strategy, or even combining RNA silencing and CRISPR/Cas system, can potentially and effectively solve the problem.

Zinc finger nuclease and TALEN approaches are not widely used due to their weaknesses related to affordability, simplicity and efficiency. In particular, with the rapid development of CRISPR technology, there are only a few examples of using these genome editing approaches to generate virus-resistant crops.

The establishment and development of CRISPR/Cas system is a definite milestone in genome editing technology, but it also has some problems in antiviral application. First, when CRISPR/Cas system is used to knockout an essential host factor to obtain viral resistance, such as eIF4E, the loss-of-function of these host factors often leads to lethality or impaired growth (Callot and Gallois, 2014; Gauffier *et al*., 2016). In some crops, the redundancy among *eIF4E* genes could reduce eIF4E-based resistance durability by making other members available to viruses (Bastet *et al*., 2017). The best strategy to develop eIF4E-based resistance would be to design functional alleles by introducing point mutations in the gene, which does not affect its function in plant growth but prevents interaction with the virus, instead of knocking it out (Bastet *et al*., 2017). This can be achieved *in situ* using recently developed CRISPR/Cas base editing technology (Figure 1; Kim, 2018; Rees and Liu, 2018), as well as by *de novo* design and construction using synthetic biology technologies (Bastet *et al*., 2018; Liu and Stewart, 2015). Second, as mentioned above, off-target effect can never be ignored when using genome editing strategies, irrespective of using ZFN, TALEN or CRISPR/Cas (Tsai and Joung, 2016). Using a virus-inducible genome editing system could effectively reduce off-target effect in antiviral breeding (Ji *et al*., 2018). In addition, CRISPR/Cas base editing technology, which chemically modifies the nucleotide instead of producing DSB (Kim, 2018; Rees and Liu, 2018), could be widely applied in crop antiviral breeding. Moreover, the RNA binding and cleaving of CRISPR/Cas13 system could target RNA viruses and the RNA intermediates of DNA viruses, and is ideal to avoid off-target genomic modifications in the host genome. Last but not least, when using CRISPR/Cas9 system in developing crops resistant to DNA viruses, the targeted viruses evolve mutations that escape from CRISPR/Cas9 cleavage (Ali *et al*., 2016; Mehta *et al*., 2019). Bacteria and archaea use the CRISPR system to defend against phages and plasmids, but most bacteria lack NHEJ as a DNA repair mechanism, and the cleaved invading DNA is usually degraded rather than repaired (Wigley, 2012). However, DNA repair mechanisms differ substantially in eukaryotes, and NHEJ enables efficient genome editing by effectively repairing cleaved DNA (Lieber, 2010). Thus, this efficient repair mechanism in eukaryotes makes CRISPR/Cas9-mediated DNA virus resistance more prone to evolving mutant viruses (Ali *et al*., 2016; Mehta *et al*., 2019). This mechanism will not only make the antiviral crops lose their effective virus defense ability, but it may even promote the evolution of viruses to produce super-viruses, which have greater pathogenicity and produce more severe symptoms (Table 2). Optimal selection of sgRNA target sites in the viral genome may help reduce the viral mutation rate, for example, targeting certain viral genomic regions, such as the noncoding intergenic sequences, within some plant virus genomes led to the formation of mutations that were deleterious to virus replication. Furthermore, multiplex targeting of different regions of the viral genome in one CRISPR/Cas system, which will cause deletion of large fragments that is difficult to repair, can minimize the generation of escapee mutants. Finally, as outlined above, CRISPR/Cas base editors can introduce a mis-sense mutation in a critical codon sequence outside of the protospacer seed sequence, thereby maintaining the ability of the sgRNA to recognize and bind to the targeted sequence, as well as preventing the formation of NHEJ-mediated escapee viruses.

Although various biotechnologies have their advantages and disadvantages (Table 2), the full potential of RNAi and CRISPR/Cas systems for engineering resistance against eukaryotic viruses has not yet been exploited. More research is needed to improve these systems for virus interference, including improving their accuracy, durability, convenience and safety of delivery. To overcome the shortcomings of each strategy, the combination of RNA silencing and CRISPR/Cas strategies has great potential in antiviral breeding.

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Conflict of interest

The authors declare that they have no conflict of interest.

Author contributions

Y.Z. and T.Z. jointly developed the conceptual structure of manuscript. Y.Z. and X.Y. were involved in the compilation of relevant literature and drafting the manuscript. T.Z. and G.Z. provided a critical feedback and edited the final manuscript.

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