CRISPR Applications in Plant Virology: Virus Resistance and Beyond

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ABSTRACT

Clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated genes (Cas) is a prokaryotic adaptive immune system which has been reprogrammed into a precise, simple, and efficient gene targeting technology. This emerging technology is revolutionizing various areas of life sciences, medicine, and biotechnology and has raised significant interest among plant biologists, both in basic science and in plant protection and breeding. In this review, we describe the basic principles of CRISPR/Cas systems, and how they can be deployed to model plants and crops for the control, monitoring, and study of the mechanistic aspects of plant virus infections. We discuss how Cas endonucleases can be used to engineer plant virus resistance by directly targeting viral DNA or RNA, as well as how they can inactivate host susceptibility genes. Additionally, other applications of CRISPR/Cas in plant virology such as virus diagnostics and imaging are reviewed. The review also provides a systemic comparison between CRISPR/Cas technology and RNA interference approaches, the latter of which has also been used for development of virus-resistant plants. Finally, we outline challenges to be solved before CRISPR/Cas can produce virus-resistant crop plants which can be marketed.

Keywords: CRISPR/Cas, genetics and resistance, plant viruses, RNA interference, virology, virus detection, virus resistance

Technology for exquisitely targeting the modification of specific genes in plants has great potential for elucidating gene functions and also for improving crop traits. The latest and most advanced gene-editing technology which can be exploited to achieve this is based on prokaryotic clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated genes (Cas) systems, which are apparently simpler, more efficacious, and more versatile than other editing techniques that use zinc finger nucleases and transcription activator-like effector nucleases. CRISPR/Cas technology has already revolutionized various areas of life sciences, in particular medicine and biotechnology, and offers a novel, powerful alternative (or complementary) approach to classical plant breeding for trait improvement. Given that plant viruses can induce devastating losses to crop productivity, it is not surprising that, in recent years, there has been an interest in utilizing diverse facets of CRISPR/Cas technologies to develop plants that are resistant to

viral pathogens (Borrelli et al. 2018; Green and Hu 2017; Makarova et al. 2018; Wolter and Puchta 2018; Zhang et al. 2018).

CRISPR/Cas systems could be utilized in two main ways to improve crop plant resistance to plant viruses. First, CRISPR/Cas systems can be used to introduce targeted mutations into specific host plant genes to interfere with their capacity to act as part of the machinery that permits successful virus replication and spread in the plant (a compatible infection). Second, CRISPR/Cas systems may be engineered to effectively function in plants to target viral genomes. For example, DNA-targeting CRISPR/Cas9 systems could target directly the DNA of DNA-containing viruses, whereas other CRISPR/Cas systems which can specifically cleave RNA, such as reprogrammed Cas9 from *Francisella novicida* (FnCas9) (Price et al. 2015) or Cas13a (previously known as C2c2) (Abudayyeh et al. 2016), could attack viruses that have RNA genomes (Green and Hu 2017; Wolter and Puchta 2018).

Although CRISPR/Cas systems have great potential in the control of plant virus infections in crops and crop improvement in general, there is a significant bottleneck in their deployment, which is the poor efficacy with which the CRISPR/Cas reagents can be delivered into plant cells (Baltes et al. 2014). To overcome such limitations, it is essential to consider how and in which form these CRISPR/Cas reagents can be delivered to plant tissues so that suitable protocols can be established to ensure optimal and environmentally friendly utility. It is also critical that the correct CRISPR/Cas editing system is selected for a given purpose.

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It is also worth noting that RNA silencing (also known as RNA interference [RNAi]), a conservative eukaryotic mechanism which refers to small interfering RNA-mediated sequence-specific gene silencing, has also been successfully deployed in crop improvement for viral resistance (Dubrovina and Kiselev 2019). Recent important achievements include the exogenous application of virus-specific double-stranded RNA (dsRNA) to confer viral resistance. Thus, despite the excitement surrounding CRISPR/Cas technologies, RNAi still retains a strong position in the array of useful tools for engineering plant viral resistance.

CRISPR: A PROKARYOTIC IMMUNE SYSTEM ENGINEERED INTO A EUKARYOTIC GENE EDITING TOOL

The CRISPR/Cas system is an RNA-based prokaryotic (archaea or bacteria) defense system that operates by integrating foreign DNA fragments from invading plasmids or viruses into the CRISPR/Cas loci as spacer sequences which are intercalated between identical repeat regions. Subsequent to integration, precursor CRISPR RNAs (precrRNAs) are transcribed from these loci and, after maturation into crRNAs, they then associate with and direct Cas endonuclease proteins to recognize and cleave newly invading plasmid or viral DNA, which have sequence complementarity to the crRNA. These acquired fragments allow prokaryotic organisms to generate a molecular memory of past viral and plasmid intrusions and provide immunity against repeated incursions.

The chief components of the natural CRISPR/Cas machinery are remarkably diverse in their protein and crRNA composition, architecture, genome locus structure, and mechanisms for crRNA biogenesis and activity (Koonin et al. 2017). This is reflected in their complex classification into two major classes and six types, which are further divided into several subtypes (Koonin et al. 2017). For example, class 1 systems (composed of types I, III, and IV) employ multisubunit effector complexes, whereas class 2 systems (including types II, V, and VI) utilize a single, multidomain protein. In this review, we will focus on the class 2 systems which have been most commonly adopted and exploited for genome editing because of their simplicity.

Notably, the class 2 type II CRISPR/Cas9 system of *Streptococcus pyogenes* was the first and most widely to be used as a gene-editing tool and, as such, its machinery is quite well elucidated (Koonin et al. 2017). Activation of this system requires an additional small RNA, a transactivating crRNA (tracrRNA), which is involved in the maturation of crRNA and is transcribed separately from a genomic locus that exists upstream of the CRISPR locus. The tracrRNA base pairs with precrRNA repeat sequences, generating dsRNA regions, which are then cleaved by RNase III to produce mature crRNA that are further trimmed at their 5' end by an unknown enzymes (Jiang and Doudna 2017). The resultant mature crRNA:tracrRNA can associate with the Cas9 endonuclease via its tracrRNA portion, whereas a single spacer sequence complementary to the foreign DNA present in the crRNA portion drives the targeting of the complex to alien DNA. The complex will not attack the foreign nucleic acid unless it contains immediately downstream protospacer adjacent motifs (PAMs) (Fig. 1) (Sternberg et al. 2014), with the PAM effectively behaving as a nonselfrecognition motif. Cas9 proteins originating from different prokaryotic species may recognize different PAM sites. For example, the most widely used *S. pyogenes* Cas9 (SpCas9) interacts with a 5'-NGG-3' PAM (Anders et al. 2014). This interaction facilitates target DNA strand unwinding, formation of an RNA spacer-DNA hybrid, and DNA cleavage. Cas9 contains two nuclease domains, HNH and RuvC, both of which introduce nicks 3 nucleotides upstream of the PAM but in different strands (Jinek et al. 2012). HNH nicks the DNA strand complementary to the crRNA, whereas RuvC cleaves the other strand, leading to a double-strand break in the invading DNA.

These studies were followed by a real revolution in gene editing: it was demonstrated that class 2 CRISPR/Cas systems could be reprogrammed to become a next-generation tool for genome editing in eukaryotic organisms (Cong et al. 2013; Jinek et al. 2012). To simplify the technology, it was shown that the CRISPR/Cas9 nucleic acid targeting system could also be guided by a single recombinant (synthetic) RNA formed by the fusion of crRNA and tracrRNA, termed a "single guide RNA" (sgRNA) (Jinek et al. 2012). The sgRNA binds Cas via its scaffolding tracrRNA sequence and the specific approximately 20-nucleotide spacer (designed using bioinformatic web tools) in the crRNA which defines the genomic target (immediately adjacent to PAM) to be modified. After deployment in eukaryotes, sgRNA/Cas9 creates a double-strand break (DSB) at the target DNA site (approximately 3 to 4 nucleotides upstream of the PAM motif), after which cells engage in DNA repair through either homology-directed repair (HDR) or nonhomologous end joining (NHEJ) (Cong et al. 2013).

When homologous sequences are delivered to the cell with Cas9 and sgRNA, HDR can occur, and this can be exploited for target-site correction (such as point mutation) or insertion of foreign genes of interest, via the homologous recombination between the donor DNA template provided by the system and host genomic DNA. In contrast, NHEJ does not require a homologous template but is, however, error prone and typically results in small nucleotide insertions or deletions (indels). NHEJ is the most efficient repair mechanism and often destroys the open reading frame (ORF) in the cleaved region and, hence, causes a target gene knock out (and loss of gene function) (Cong et al. 2013).

In addition to intended mutations introduced via CRISPR/Cas technology, unintended off-target mutations can occur, which may cause genomic instability and disrupt the functionality of otherwise normal genes (Tang and Fu 2018). To reduce the frequency of potential off-target mutations by Cas9, a "nicking" D10A Cas9 variant (Cas9n, nickase) has been generated (Tang and Fu 2018). For example, in the presence of a pair of offset sgRNAs complementary to opposite strands of the target sequence, Cas9n is capable of introducing single-strand nicks rather than a DSB and, because nicks are usually repaired with high fidelity, Cas9n significantly enhances gene-editing specificity (Ran et al. 2013). In addition, the use of another class 2 Cas protein, type V Cas12a (formerly Cpf1), can also reduce off-target effects due to its low mismatch tolerance (Kim et al. 2016). Cas12 has some similarities with Cas9; however, in contrast to Cas9, Cas12 does not require tracrRNA and depends only on crRNA for its activity to induce DSBs in target DNA sequences (Fig. 1) (Zetsche et al. 2015).

CRISPR-BASED RNA EDITING

A new, striking turn in the development of CRISPR/Cas technologies is related to discovery of Cas nucleases targeting RNA molecules. One of the most well-known is the class 2, type VI Cas13 protein (including Cas13a and Cas13b). Unlike most CRISPR/Cas systems, Cas13 lacks a DNase motif but, instead, contains two higher eukaryotes and prokaryotes nucleotide-binding domain (HEPN) RNase domains. Cas13 exclusively cleaves target RNA molecules (Abudayyeh et al. 2016) and is able to cleave single-stranded RNA (ssRNA) molecules containing sites homologous to crRNA. Interestingly, like Cas12a, Cas13 does not need tracrRNA and depends only on crRNA (Fig. 1) (Abudayyeh et al. 2017; Cox et al. 2017). Given that most plant viruses contain RNA genomes, these findings open up new technological opportunities to combat plant viruses, which will be discussed below.

Intriguingly, whereas the Cas13-crRNA complex mediates highly specific RNA targeting and cleavage in eukaryotes, it behaves

differently in prokaryotes and in vitro, in that it has the capacity to nonspecifically attack other ssRNA once activated by initial specific cleavage (East-Seletsky et al. 2016). This collateral effect can be exploited for the development of highly sensitive virus diagnostic methods that utilize the so-called specific with a high sensitivity enzymatic reporter unlocking (SHERLOCK) approach developed by Gootenberg et al. (2017) for precise detection of nucleic acids (see below).

Another widely used RNA editing nuclease is FnCas9 (reprogrammed from *F. novicida*) (Price et al. 2015). This enzyme also operates in combination with an RNA-targeting guide RNA to target endogenous RNA in vivo. However, in contrast to Cas13, this protein does not contain HEPN domains. It has been suggested that FnCas9 may either recruit endogenous RNases to cleave target RNA or possesses alternative domains with endonucleolytic activity (Ma et al. 2016). Like Cas13, FnCas9 has been reprogrammed to target and inhibit both human and plant RNA viruses by blocking

translation and replication of viral RNA (Price et al. 2015; Zhang et al. 2018) (see below).

DELIVERY SYSTEMS FOR CRISPR/CAS REAGENTS

CRISPR/Cas cargo can be “packaged” into different forms for delivery into plant cells: (i) DNA plasmids encoding both Cas protein and sgRNA, (ii) a Cas mRNA (which will be translated into Cas in planta) provided with a separate sgRNA, and (iii) ribonucleoprotein (RNP) complexes composed of the Cas protein and sgRNA (Ran et al. 2017). Current methods for introducing CRISPR/Cas reagents into plant cells include *Agrobacterium*-mediated gene transfer, protoplast transfection, plant virus vector-mediated delivery, biolistic delivery, and use of nanoparticle platforms (Ran et al. 2017).

***Agrobacterium*-mediated gene transfer.** *Agrobacterium*-mediated gene transfer can be used for transient or transgenic expression of both components of the CRISPR/Cas system: sgRNA

FIGURE 1

Schematic representation of the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated genes (Cas) complexes. **A**, The Cas9 endonuclease in a complex with two small RNAs, the CRISPR RNA (crRNA) and transactivating crRNA (tracrRNA), binds to the DNA target site and induces blunt double-strand break (DBS) 3 nucleotides (nt) immediately upstream of the protospacer adjacent motif (PAM). DNA recognition is mediated by the 20-nt guide sequence of crRNA (shown in red). REC = recognition lobe; PI = PAM-interacting domain; and HNH and RuvC = nuclease domains cleaving the target and nontarget strands, respectively, as shown by arrows. **B**, Cas12a operates in conjunction with a single small RNA, crRNA, and binds to the target DNA site downstream of PAM. DNA recognition is mediated by a 23- to 25-nt guide sequence of crRNA (shown in red). Cas12a induces a PAM-distal double-strand break with staggered 5' and 3' ends. Nuc and RuvC = nuclease domains cleaving the target and nontarget strands, respectively, as shown by arrows. **C**, Cas13a operates in conjunction with single small RNA, crRNA, and binds to the target RNA site. RNA recognition is mediated by guide sequence of crRNA (shown in red). HEPN = nuclease domain cleaving the RNA target site remote from the recognition site (shown by arrow). Adapted from Schindele et al. (2018).

and Cas protein genes. This is the most widely used delivery method for editing genomes of a broad spectrum of model plants and crops (Ran et al. 2017). However, it has some disadvantages, including (i) dependence on plant species and cultivar, because some plants, monocots in particular, are recalcitrant to transformation or whole plant regeneration; (ii) introduction of foreign DNA into plant cells which may be incorporated into the plant genome and, as such, impede the commercialization of transgenic (genetically modified) plants; and (iii) prolonged CRISPR/Cas expression, which may increase frequency of off-target events.

Virus vector-mediated delivery. For more than 20 years, plant viruses have been developed and exploited as autonomously replicating vectors for expressing foreign proteins and RNAs (Cody and Scholthof 2019; Zaidi and Mansoor 2017). Recently, virus vectors have also been reported as vehicles for efficient delivery of gene-editing components in plant cells. Notably, both DNA (*Bean yellow dwarf virus* [BeYDV], *Cabbage leaf curl virus* [CaLCuV], and *Wheat dwarf virus* [WDV]) and RNA (*Tobacco rattle virus* [TRV]) virus vectors have been shown to be efficient systems for CRISPR/Cas reagent delivery in model plants and crops (Ali et al. 2015; Baltes et al. 2014; Čermák et al. 2015; Ran et al. 2017; Zaidi and Mansoor 2017).

BeYDV, CaLCuV, and WDV belong to the family *Geminiviridae* and contain circular ssDNA (Zaidi and Mansoor 2017). To increase the limited cargo capacity of virus vectors derived from these geminiviruses, they were converted into replicating but non-infectious systems (replicons) by replacing genes encoding the movement protein (MP) and capsid protein (CP), which are not essential for replication, with foreign sequences such as cassettes for expression of Cas protein and sgRNA (Čermák et al. 2015). Such replicons are very efficient and exhibit high rates of accumulation of CRISPR/Cas reagents, which concomitantly results in a high frequency of gene-editing events. However, due to the absence of an MP, such high frequency occurs only locally because geminivirus vectors are limited to inoculated leaves and do not spread systemically. Moreover, use of such a vector system can make suitably transformed cells hard to regenerate into whole plants (Zaidi and Mansoor 2017).

TRV is a positive-strand ssRNA virus and has been converted into an efficient virus vector mainly for analysis of gene function by silencing (Ratcliff et al. 2001). It has also been used for delivery of the sgRNA component of the CRISPR/Cas system (Ali et al. 2015). However, due to the inability of the TRV vector to express sequences encoding large proteins such as Cas, it should be inoculated onto transgenic plants that overexpress the Cas protein.

To overcome technical hurdles precluding successful expression of the large *Cas9* gene from viral vectors, Kaya et al. (2017) used a split-protein approach. It was shown that split-*Cas9*, with two fragments transiently expressed from *Tomato mosaic virus* vector and *Agrobacterium*, respectively, was able to reassemble to form the active *Cas9* protein and induce targeted mutagenesis in plant cells. Thus, the split-*Cas9* approach may provide a new, powerful tool for DNA integration-free plant genome editing, avoiding the necessity for the use of transgenic plants. Another important advance was made when Cody et al. (2017) adapted a *Tobacco mosaic virus* (TMV)-derived vector (a CP deletion mutant of TMV) for rapid and highly efficient transient delivery of sgRNAs. These virus-based approaches, which were developed for transient delivery of *Cas9* and sgRNAs, would be advantageous tools for integration-free genome editing and could be of great utility for functional genomics studies (Cody and Scholthof 2019; Kaya et al. 2017). However, getting rid of the virus afterward may be a significant problem, making difficult the practical applications of plant virus-based editing systems. This hurdle may possibly be overcome during the meristematic tissue culturing, because this is well known to lead to loss of virus in infected material.

Protoplast transfection. Protoplast transfection has been demonstrated as an efficient method for delivery of CRISPR/Cas reagents in some plant systems (Ran et al. 2017). Moreover, protoplasts may be transformed not only with DNA which expresses sgRNA and Cas endonuclease but also with RNAs (sgRNA and Cas mRNA) or even with preformed sgRNA:Cas RNP complexes, which excludes the possibility of integrating foreign DNA into the plant genome (thus, regenerated plants will be completely free of transgenes). This technology has been successfully exploited for gene-editing in tobacco, potato, and lettuce (Ran et al. 2017). It is worth noting, however, that a potential bottleneck of this approach may be regeneration of protoplasts into viable plants, because this is a long, laborious process that often requires optimization, depending on the plant species and crop cultivar.

Biolistic delivery. Biological ballistics (or biolistics) is a method that allows transfection of cells by bombarding them with microcarriers (such as gold or tungsten microprojectiles) decorated with DNA (Ran et al. 2017). DNA is delivered into the cell using a gene gun machine which facilitates penetration through the plant cell wall. This method has allowed the delivery of DNA plasmids encoding sgRNA as well as Cas proteins into *Brachypodium* spp., rice, soybean, and wheat, leading to gene modification. After integration of the DNA into the plant genome, viable plants could be regenerated from the treated tissues.

Recently, for DNA-free editing, in vitro transcripts or preformed sgRNA:Cas9 RNP complexes have been biolistically bombarded into maize and wheat cells that were consequently successfully regenerated into whole plants, producing transgene-free gene-edited plants (Ran et al. 2017; Svitashv et al. 2016).

Chitosan platform for delivery of CRISPR/Cas by vacuum infiltration. Nanobiotechnology offers various platforms for delivery of macromolecules or their complexes into cells, including plant cells. Recently, we have developed a new technique using chitosan nano- and microparticles as a platform for delivery of various macromolecules, including preformed sgRNA:Cas9 RNP complexes, into plant cells by means of vacuum infiltration (Khromov et al. 2018; Makhotenko et al. 2019). Due to its polycationic nature, chitosan can form complexes with negatively charged polymers such as nucleic acids, proteins, and nucleoproteins and can promote plant cell permeability, which leads to greater uptake of these assemblies (Il'ina and Varlamov 2005). Chitosan particles functionalized by CRISPR/Cas reagents designed to target phytoene desaturase (*PDS*) or coilin genes were introduced into apical meristems of potato plants, which were then regenerated into viable plants. Genetic and phenotyping analyses revealed successful gene-editing events. The advantage of the use of apical meristem tissue is the simplicity of regeneration. However, in these experiments, we were not able to modify all alleles of a coilin or *PDS* gene in tetraploid potato. Another curious feature of this approach is that large deletions (up to 600 nucleotides) have been produced by CRISPR/Cas in the region flanking the sgRNA binding site of the target gene in the potato genome but classical short indels have not been observed (Khromov et al. 2018; Makhotenko et al. 2019). Thus, the position of the target site should be considered so that any large deletion completely lies within the gene to be knocked out. Large deletions induced by CRISPR/Cas have previously been detected in animal cells exhibiting high mitotic activity (Kosicki et al. 2018). Thus, our data are in good agreement with this observation, because meristematic cells are also rapidly dividing cells.

To summarize this section, we can note that at least some of the approaches for delivery listed above are probably suitable for functional genomics but they must be further optimized for practical applications. With regards to the form in which CRISPR/Cas reagents should be delivered, the use of sgRNA:Cas RNP complexes is probably the preferable option over DNA plasmids, because it can reduce the regulatory limitations incurred by the possible insertion of transgenic DNA into the plant genome.

Another advantage of using sgRNA:Cas RNP is the lower frequency of off-target events, which is likely related to the limited time of RNP action.

DIRECT CRISPR-MEDIATED INHIBITION OF DNA AND RNA VIRUSES

As mentioned above, CRISPR technology provides new, expanding tools to generate plant virus resistance. These include approaches to inhibit viruses directly or to confer virus resistance in host plants by mutating their “susceptibility” genes (Table 1).

DNA viruses. Most of the studies on direct targeting of plant viruses with CRISPR/Cas have been carried out on viruses belonging to the family *Geminiviridae* (Table 1), which consists of nine genera. Epidemics of geminiviral diseases cause significant yield losses in some economically important crops worldwide, increasing global demand for virus resistance strategies. Geminiviruses are geminate icosahedral viruses with monopartite or bipartite circular ssDNA genomes which produce dsDNA during replication (Hanley-Bowdoin et al. 2013) and, as such, could be a direct target for CRISPR during replication.

The first experiments to engineer CRISPR-mediated resistance to geminiviruses were conducted on monopartite *Beet severe curly top virus* (genus *Curtovirus*) and *BeYDV* (genus *Mastervirus*) in *Nicotiana benthamiana* and *Arabidopsis thaliana* plants (Table 1) (Baltes et al. 2015; Ji et al. 2015). CRISPR/Cas9 (including sgRNA and Cas9) constructs were engineered to target and cleave coding (such as the *replication-associated protein* [Rep] gene) and noncoding regions in viral genomes (such as Rep-binding sites and the invariant noncoding nanonucleotide [TAATATTAC] sequence common to all geminiviruses contained in the intergenic region [IR]), and were transformed into plants for transgenic expression. Resultant transgenic plants were shown to exhibit high levels of resistance to the targeted virus, which was manifested in significant (up to 87%) reductions in virus accumulation and symptom expression (Baltes et al. 2015; Ji et al. 2015).

In another approach, Ali et al. (2015) engineered sgRNA molecules specifically targeting coding and noncoding sequences of another bipartite geminivirus, *Tomato yellow leaf curl virus* (TYLCV) (genus *Begomovirus*), including the CP gene, the RCR II (a metal-binding site involved in protein conformation and DNA cleavage) motif of the Rep gene, and the conserved noncoding stem-loop sequence contained in the IR, which were delivered into *N. benthamiana* plants transgenically expressing Cas9. The sgRNAs, expressed from a TRV plant virus vector, all mediated anti-TYLCV resistance, with the strongest virus inhibitory effect achieved by the sgRNA targeting the stem-loop sequence in the IR. Thus, it could be suggested that all other geminiviruses containing the same stem-loop region could also be targeted by this CRISPR/Cas9 system. In good agreement with this suggestion, it has been shown that the same sgRNA (when delivered into Cas9-overexpressing *N. benthamiana* transgenic plants) appeared to confer resistance against the mixed infection with monopartite *Beet curly top virus* (genus *Curtovirus*) and the bipartite *Merremia mosaic virus* (MeMV) (genus *Begomovirus*), geminiviruses that share this invariable stem-loop sequence (Ali et al. 2015).

Another interesting effect of CRISPR/Cas9 on geminiviral infection has been described by Ali et al. (2016). When the CRISPR/Cas9 system was engineered to interfere with coding sequences of TYLCV, MeMV, and *Cotton leaf curl Kokhran virus* (a monopartite virus belonging to the genus *Begomovirus*), new mutated virus variants eventually arose which could evade the CRISPR/Cas9 activity and continue to replicate and spread systemically. Thus, this genome-editing system may represent some risk, because it may potentially rapidly lead to and release newly emerging virus variants into the environment. It is worth

noting, however, that such new virus forms, able to replicate and spread, were not detected when sgRNA was designed to target IR sequences. Although the reason for these differences remains unclear, noncoding viral sequences presumably may be a better target to achieve CRISPR/Cas-mediated resistance.

In another approach to increase efficiency and durability of CRISPR-mediated resistance, Yin et al. (2019) used transgenic *N. benthamiana* plants expressing Cas9 and dual sgRNAs which simultaneously targeted two different sequences (Rep-coding sequence and IR) in the genome of another monopartite geminivirus, *Cotton leaf curl Multan virus* (CLCuMuV) (genus *Begomovirus*). This led to these plants being completely resistant to CLCuMuV.

Interestingly, mutational deactivation of catalytic endonuclease Cas9 domains did not completely prevent its ability to suppress geminiviral infections (Khan et al. 2019). It was shown that catalytically inactive Cas9 (dCas9) is still able to partially inhibit replication and accumulation of the monopartite geminivirus *Cotton leaf curl virus* (genus *Begomovirus*), when supplied with sgRNA targeting the conserved nanonucleotide sequence involved in initiation of virus replication (Khan et al. 2019). This suggests that binding DNA by Cas9 on its own (without further cleavage) may lead to inhibition of virus replication, although further cleavage may significantly increase efficiency of the virus suppression.

Although most studies on the CRISPR-mediated resistance against geminiviruses have been carried out on model plants, translation of these studies into crop plants has been recently initiated. In particular, Kis et al. (2019) produced barley plants with resistance to WDV (monopartite virus belonging to the genus *Begomovirus*) while Tashkandi et al. (2018) generated tomato plants with resistance against TYLCV; although the targeted sites in these studies lay in the coding regions, induction of new virus forms were not reported. In contrast, other attempts to engineer resistance to geminiviruses using CRISPR systems targeting the coding regions were not so successful (Mehta et al. 2019). These authors failed to induce resistance against *African cassava mosaic virus* (ACMV) (a bipartite member of the genus *Begomovirus*) in transgenic cassava plants that overexpresses Cas9 and sgRNAs that target the AC2 gene encoding the transcription activator protein and the AC3 gene encoding the replication enhancer protein. Instead, they found that CRISPR editing led to formation and escape of new (CRISPR-resistant) ACMV variants which were probably generated as a result of postcleavage repair (NHEJ). These events may trigger rapid evolution of the virus, potentially leading to the development and release of new pathogenic virus forms (Mehta et al. 2019), as discussed above.

In addition to geminiviruses, the CRISPR/Cas tool has also been applied to *Cauliflower mosaic virus* (CaMV) (family *Caulimoviridae*, genus *Caulimovirus*), which is a dsDNA-containing pararetrovirus. Liu et al. (2018) reported that transgenic expression of Cas 9 together with multiple sgRNAs targeting the CaMV CP gene exhibited significant resistance against CaMV. However, in addition, they also found some virus mutated forms which could escape and spread in systemically infected leaves.

Some pararetroviruses such as *Banana streak virus* (BSV) (family *Caulimoviridae*, genus *Badnavirus*) may integrate its DNA into the plant host genome, forming an endogenous virus (eBSV) which can be activated under stress conditions (Harper et al. 1999). Thus, to eliminate the pathogenic virus from the host genome, Tripathi et al. (2019) generated transgenic banana plants expressing Cas9 and sgRNA, which target coding areas in the eBSV genome. The obtained transgenic plants contained mutations in the targeted sites which disrupted the potential coding capacity of eBSV, and were resistant to reactivation of the latent virus under water stress compared with control nontransgenic plants.

RNA viruses. RNA viruses are the most common plant viruses that lead to devastating yield losses in a broad range of important crop plants. Discovery of Cas endonucleases (FnCas9 and Cas13)

that target RNA molecules could lead to new ways to control RNA virus infections in plants. In the first study on CRISPR-mediated resistance to plant RNA viruses, Zhang et al. (2018) transgenically expressed FnCas9 and sgRNAs that were targeted to attack various regions in the RNA genome of TMV (family *Virgaviridae*, genus *Tobamovirus*) and *Cucumber mosaic virus* (family *Bromoviridae*, genus *Cucumovirus*) in *N. benthamiana* and *A. thaliana* plants. The transgenic plants were found to have significantly less accumulation (40 to 80%) of both these viruses relative to the wild-type controls. Interestingly, mutational inactivation of the known nuclease domains in FnCas9 did not affect its virus inhibitory activity (Table 1) (Zhang et al. 2018). This may suggest that FnCas9 either possesses another unrecognized yet (hidden) RNase domain or its endonuclease function is not required for inhibition of virus accumulation; it is possible that the mere binding of viral RNA by FnCas9 may lead to inhibition of replication or translation of viral RNA.

Another RNA-targeting endonuclease, Cas13, has also been deployed to engineer resistance against plant virus infection (Aman et al. 2018). For example, *N. benthamiana* plants transiently or transgenically overexpressing Cas13a and sgRNA (Table 1) (Aman et al. 2018), which target *Turnip mosaic virus* (TuMV, genus *Potyvirus*) or TMV (Zhang et al. 2019) RNA, had potent resistance to the corresponding virus. Similar approaches were successfully used for generation of the resistance in crops against positive-strand ssRNA *Potato virus Y* (PVY) (family *Potyviridae*, genus *Potyvirus*) in potato (Zhan et al. 2019) and negative strand ssRNA rhabdoviruses (family *Rhabdoviridae*), *Southern rice black-streaked draft virus* (genus *Fijivirus*) and *Rice stripe mosaic virus* (genus *Cytorhabdovirus*) in rice (Zhang et al. 2019).

Collectively, these data show that Cas endonucleases that directly target viral RNA (such as FnCas9 or Cas13) are a potentially powerful tool in engineering plant RNA virus resistance. However, such antiviral approaches require permanent expression of the Cas component, which is currently achieved only in transgenic plants. Thus, practical applications of direct-acting CRISPR/Cas antivirals may be limited, because the plants in question are transgenic and may be subjected to tight genetically modified organism (GMO) regulations, and overcoming these regulatory constraints will be a challenge for future research.

CRISPR-MEDIATED HOST GENOME EDITING THAT GENERATES VIRUS RESISTANCE

To mitigate barriers associated with GMO regulations, another strategy for CRISPR-mediated engineering of virus resistance in plants has been developed. This includes transgenic expression of the CRISPR tool, consequent modification of a host gene leading to virus resistance, and then segregating out the CRISPR transgene sequences to produce nontransgenic plants that have the virus resistance trait (Borrelli et al. 2018; Chandrasekaran et al. 2016; Macovei et al. 2018; Pyott et al. 2016) (Table 1).

Among the known susceptibility factors required to establish successful infection with some plant RNA viruses are the translation initiation factors eIF4E, eIF(iso)4E, and eIF4G (Sanfaçon 2015). In these cases, the viruses require a specific translation initiation factor isoform to replicate and become established in the plant. Inactivation of such a single isoform can induce resistance to a virus without effect on general health and fitness of the plant because there is functional redundancy between the different isoforms in the host (Sanfaçon 2015).

Using technology described above, Chandrasekaran et al. (2016) prepared nontransgenic knockout *eif4e* cucumber plants and demonstrated their resistance to several members of the *Potyviridae* family: *Cucumber vein yellowing virus* (genus *Ipomovirus*), *Zucchini yellow mosaic virus* (genus *Potyvirus*), and *Papaya ring spot mosaic virus-W*

(genus *Potyvirus*). Similar approaches were successfully exploited to engineer resistance to TuMV in *A. thaliana* plants (Pyott et al. 2016) and to *Rice tungro spherical virus* (RTSV) (family *Sequiviridae*, genus *Waikavirus*) in rice (Macovei et al. 2018) by knocking out *eIF(iso)4E* and *eIF4G*, respectively.

In addition to knocking out translation initiation factors, CRISPR/Cas9 has been utilized to introduce mutations into these factors. This was achieved using a Cas9 variant which was inactivated at one catalytic site (the nickase D10A mutant; see above) and fused to a cytidine deaminase to convert cytosine to thymidine at the targeted site without DSBs in rice and tomato (Shimatani et al. 2017). Using this editing technique, Bastet et al. (2019) introduced a single substitution mutation (N176K) into *eIF4E* in the plant host genome and demonstrated that this mutation is sufficient to generate resistance in *A. thaliana* plants against the potyviruses *Clover yellow vein virus*.

A promising alternative approach to achieve transgene-free genome edits, and to even avoid the use of DNA at all, is the delivery of CRISPR/Cas9 as an RNP (Ran et al. 2017) (see above). RNPs act rapidly without the need of transcription and translation and, as such, result in a significant reduction in unintended off-target events. This approach has recently been applied to engineer virus resistance by targeted mutagenesis of coilin genes in potato plants (Makhotenko et al. 2019). Coilin is a signature protein of Cajal body subnuclear structures and has been shown to modulate different plant–virus interactions and, in particular, increase susceptibility of plants to PVY (Shaw et al. 2014). Therefore, to generate resistance against PVY, we deployed CRISPR/Cas9 systems to target the coilin gene (Makhotenko et al. 2019). CRISPR/Cas9 RNPs preassembled from Cas9 and sgRNAs that target the coilin gene were delivered into apical meristematic tissues of potato plants using gold and chitosan microparticles via biolistic bombardment or vacuum infiltration, respectively. Genetic and phenotyping analyses of the obtained plants revealed large deletions in the targeted sites (as discussed above) and indicated that editing at least one allele of coilin gene in potato tetraploid plants caused strong resistance to PVY (Makhotenko et al. 2019).

Due to significant instrumental advances in the transgene-free CRISPR-mediated technology for host genome editing and its opportunity to ameliorate GMO-related public concerns, it is likely the most readily applicable approach to produce virus resistant crops. However, at present, it is strongly limited by the lack of knowledge of appropriate target genes; thus far, only two groups of plant genes (encoding eIF4s and coilin) have been used for CRISPR modification, which affects viruses within a narrow range (potyviruses and RTSV) (Table 1). To make a real breakthrough in practical applications of CRISPR technology for production of virus-resistant plants, new scientific advances in understanding plant–virus interactions are needed to identify novel virus susceptibility (target) genes that can influence a broad spectrum of plant viruses.

CRISPR technology, which is increasingly used as a revolutionary functional genomics tool in plants (Bortesi and Fischer 2015), could be used to uncover and identify new virus resistance strategies. Cas9 endonuclease could be exploited to further elucidate plant and virus interactions, particularly from the gene function perspective, whereas the RNA-targeting capacity of Cas13 opens up new prospects for research relating to the functions of long noncoding RNAs, which are implicated in many processes, including resistance to pathogens (Wolter and Puchta 2018), and, as such, may represent a new source of virus resistance.

CRISPR APPLICATIONS BEYOND VIRUS RESISTANCE: DIAGNOSTICS AND IMAGING

As mentioned above, the effect of the “collateral” (nonspecific) RNA cleavage caused by Cas13 upon activation by a target RNA in

vitro has been exploited to develop new diagnostic tools (called SHERLOCK) applicable for detection of viral RNA and DNA (Gootenberg et al. 2017). In this approach, diagnostic samples undergo amplification to enrich target DNA (if present) using recombinase polymerase reactions (RPA) or, where the target is RNA, it undergoes reverse transcription prior to RPA amplification. The DNA- or RNA-derived RPA products are then transcribed into

RNA using T7 RNA polymerase. These transcripts are subjected to Cas13 targeting and cleavage in the presence of a quenched fluorescent ssRNA reporter that releases a quantifiable signal when collaterally cleaved by Cas13, indicating the presence of the target virus DNA or RNA. Recently, this technique has been significantly simplified and adapted for practical diagnostic management as a fast, inexpensive, highly sensitive lateral-flow “paper strip” test

TABLE 1
Virus-resistant plants generated using clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated genes (Cas) technology

Virus ^a	Plant species	Effector protein	Target sequence ^b	Delivery/ expression ^c	Reference
Direct effect on DNA viruses					
BSCTV	<i>Nicotiana benthamiana</i> , <i>Arabidopsis thaliana</i>	SpCas9	<i>CP</i> , <i>Rep</i> genes; IR	<i>Agrobacterium</i> ; Cas9/sgRNA-transgenic plants	Ji et al. 2015
BeYDV	<i>N. benthamiana</i>	SpCas9	<i>Rep/RepA</i> genes; LIR	<i>Agrobacterium</i> ; Cas9/sgRNA-transgenic plants	Baltes et al. 2015
TYLCV	<i>N. benthamiana</i>	SpCas9	<i>CP</i> , <i>Rep</i> genes; IR	<i>Agrobacterium</i> ; Cas9-transgenic plants; TRV vector-mediated sgRNAs delivery	Ali et al. 2015, 2016
BCTV	...	...	...	...	Ali et al. 2016
MeMV	...	...	...	...	Ali et al. 2016
CLCuKoV	...	...	...	...	Ali et al. 2016
CLCuMuV	<i>N. benthamiana</i>	SpCas9	<i>C1 (Rep)</i> gene and IR	<i>Agrobacterium</i> ; Cas9/sgRNA-transgenic plants	Yin et al. 2019
CLCuV	<i>N. benthamiana</i>	SpdCas9	IR	<i>Agrobacterium</i> ; dCas9/sgRNA-transient expression	Khan et al. 2019
WDV	<i>Hordeum vulgare</i> (barley)	SpCas9	<i>MP/CP</i> , <i>Rep/RepA</i> genes and LIR	<i>Agrobacterium</i> ; Cas9/sgRNA-transgenic plants	Kis et al. 2019
TYLCV	<i>Solanum lycopersicum</i> (tomato), <i>N. benthamiana</i>	SpCas9	<i>CP</i> , <i>Rep</i> genes	<i>Agrobacterium</i> ; Cas9/sgRNA-transgenic plants	Tashkandi et al. 2018
ACMV	<i>Manihot esculenta</i> (cassava)	SpCas9	AC2 and AC3 genes encoding viral proteins TrAP and Ren, respectively	<i>Agrobacterium</i> ; Cas9/sgRNA-transgenic plants	Mehta et al. 2019
CaMV	<i>A. thaliana</i>	SpCas9	<i>CP</i> gene	<i>Agrobacterium</i> ; Cas9/sgRNA-transgenic plants	Liu et al. 2018
BSV (eBSV)	<i>Musa</i> spp. (banana)	SpCas9	Viral ORF1, ORF2, and ORF3 integrated into plant genome	<i>Agrobacterium</i> ; Cas9/sgRNA-transgenic plants	Tripathi et al. 2019

(Continued on next page)

^a BSCTV = Beet severe curly top virus, BeYDV = Bean yellow dwarf virus, TYLCV = Tomato yellow leaf curl virus, BCTV = Beet curly top virus, MeMV = Merremia mosaic virus, CLCuKoV = Cotton leaf curl Kokhran virus, CLCuMuV = Cotton leaf curl Multan virus, CLCuV = Cotton leaf curl virus, WDV = Wheat draft virus, TYLCV = Tomato yellow leaf curl virus, ACMV = African cassava mosaic virus, BSV = Banana streak virus, eBSV = endogenous BSV, CaMV = Cauliflower mosaic virus, TMV = Tobacco mosaic virus, CMV = Cucumber mosaic virus, TuMV = Turnip mosaic virus, SRBSDV = Southern rice black-streaked draft virus, RSMV = Rice stripe mosaic virus, CIYV = Clover yellow vein virus, PVY = Potato virus Y, CVYV = Cucumber vein yellowing virus, ZYMV = Zucchini yellow mosaic virus, and PRSMV = Papaya ring spot mosaic virus.

^b CP = coat protein, Rep = replication-associated protein, IR = intergenic region, LIR = long IR, MP = movement protein, TrAP = transcription activator protein, Ren = replication enhancer protein, ORF = open reading frame, UTR = untranslated region, and GFP = green fluorescent protein.

^c sgRNA = single guide RNA, TRV = Tobacco rattle virus, and RNP = ribonucleoprotein.

(Gootenberg et al. 2018) applicable for rapid and reliable in-field detection of plant viruses.

CRISPR tools can also be used as novel live imaging techniques. The need for live imaging has emerged in conjunction with the understanding that many plant molecular processes are determined by localization and trafficking of macromolecules, including nucleic acids. Ma et al. (2016) developed an imaging technique using catalytically inactive Cas9 (dCas9) fused to a fluorescent

marker in complex with an sgRNA which recognizes target DNA sites. This tool can be used for live visualization of specific regions within the plant genome. Similarly, catalytically inactive Cas13 (dCas13) has been used to image RNA transcripts in live cells (Abudayyeh et al. 2017). With regard to applications in plant virology, this approach may be particularly important for direct visualization of localization and intra- and intercellular trafficking of viral RNA in virus-susceptible and virus-resistant plants.

TABLE 1
(Continued from previous page)

Virus ^a	Plant species	Effector protein	Target sequence ^b	Delivery/ expression ^c	Reference
Direct effect on RNA viruses					
TMV	<i>N. benthamiana</i> , <i>A. thaliana</i>	FnCas9	ORF1, ORF2, ORF3, CP gene and 3'UTR	<i>Agrobacterium</i> ; FnCas9 or FndCas9 /sgRNA-transgenic plants	Zhang et al. 2018
CMV	...	...	...	...	...
TuMV	<i>N. benthamiana</i>	LshCas13a	HC-Pro, CP genes; GFP gene (integrated into viral genome)	<i>Agrobacterium</i> ; Cas13a transgenic plants; TRV vector-mediated sgRNAs delivery	Aman et al. 2018
TMV	<i>N. benthamiana</i>	LshCas13a	Various sequences in TMV genome	<i>Agrobacterium</i> ; Cas13a/sgRNA-transient expression	Zhang et al. 2019
SRBSDV	<i>Oryza sativa</i> (rice)	LshCas13a	Various sequences in SRBSDV and RSMV genomes	<i>Agrobacterium</i> ; Cas13a/sgRNA-transgenic plants	Zhang et al. 2019
RSMV	...	...	...	...	...
PVY	<i>S. tuberosum</i> (potato)	LshCas13a	P3, CI, NIb, CP genes	<i>Agrobacterium</i> ; Cas13a/sgRNA-transgenic plants	Zhan et al. 2019
Virus resistance mediated by host modification					
CVYV	<i>Cucumis sativus</i> (cucumber)	SpCas9	elF4E gene	<i>Agrobacterium</i> ; Cas9/sgRNA-transgenic plants with further transgene segregation	Chandrasekaran et al. 2016
ZYMV	...	...	...	...	...
PRSMV	...	...	...	...	...
TuMV	<i>A. thaliana</i>	SpCas9	elF(iso)4E gene	<i>Agrobacterium</i> ; Cas9/sgRNA-transgenic plants with further transgene segregation	Pyott et al. 2016
RTSV	<i>O. sativa</i> (rice)	SpCas9	elF4G gene	<i>Agrobacterium</i> ; Cas9/sgRNA-transgenic plants	Macovei et al. 2018
CIYV	<i>A. thaliana</i>	Sp-nCas9-cytidine deaminase	elF4E gene	<i>Agrobacterium</i> ; nCas9/sgRNA-transgenic plants with further transgene segregation	Bastet et al. 2019
PVY	<i>S. tuberosum</i> (potato)	SpCas9	Coilin gene	RNP (Cas9/sgRNA); vacuum infiltration or bombardment	Makhotenko et al. 2019

COMPARISON OF THE CRISPR AND RNAI APPLICATIONS IN GENERATION OF PLANT VIRUS RESISTANCE

RNAi is another genome technology which has been very dominant in fundamental and applied plant sciences in recent decades. RNAi is a dsRNA-mediated mechanism which leads to transient sequence-specific gene silencing at the posttranscriptional level, and is a key aspect that targets viral and other foreign genetic material for destruction and also may regulate endogenous plant gene expression (Guo et al. 2019). Like CRISPR, this natural mechanism has been successfully deployed in functional genomics and crop improvement (Dubrovina and Kiselev 2019). This approach has attracted particular attention due to recent important achievements in the application of exogenous virus-specific dsRNA to confer virus resistance. Here, we analyze these two technologies in the context of conferring plant virus resistance.

Direct effect on plant viruses. dsRNA technologies have been successfully exploited to induce resistance to many viruses in a broad range of crop plants (Dubrovina and Kiselev 2019). In contrast to currently existing CRISPR technology, which may use transgenic expression of Cas nucleases, dsRNA can induce virus resistance in plants without the use of transgenes, thus overcoming the regulatory GMO hurdles. dsRNA-mediated protection can be induced by spraying plants with exogenous dsRNA complementary to virus sequences; however, the duration of protection is low (plants can be protected from infection up to 7 days after dsRNA application). However, the duration of protection can be extended significantly by loading the dsRNA onto nanoplateforms (Dubrovina and Kiselev 2019).

Effect on host plants to render them virus resistant. As discussed above, one of the approaches to engineer plant resistance is transgenic expression of the CRISPR tool to mediate knockout or modification of a host susceptibility gene, and then subsequent segregation of the CRISPR transgene sequences to produce resistant nontransgenic plants. This is a laborious and time-consuming procedure which still carries the risk of yielding plants with genomes that contain random insertions of introduced DNA. In addition, this gene-editing approach, like those where CRISPR DNA is deployed through transient expression or via RNP/Cas endonuclease/sgRNA complexes, still requires a plant regeneration stage, which may be problematic for many agronomically important crops or cultivars. The genome editing performed using CRISPR tools induces permanent and irreversible genome modifications (which may not be suitable for essential genes whose knockout could be lethal). In contrast, dsRNA applications (for RNAi) induce knockdown phenotypes and may control gene expression in a temporal manner, only when it is required (e.g., when epidemiological risk is increased). dsRNA designed to silence a given virus can be applied to intact plants without the need for plant regeneration; in addition, modification of individual cultivars or crops is not required because the same dsRNA is applicable practically to all plants infected by this virus.

Thus, despite the excitement surrounding CRISPR/Cas technologies, RNAi still retains a strong position in the array of useful tools for engineering plant virus resistance, at least for the time being, before CRISPR offers new technological solutions addressing the challenges listed above.

CONCLUSIONS AND FUTURE PROSPECTS

CRISPR/Cas technology has proved to be an invaluable research tool for basic plant biology. Unprecedented advances have been achieved in plant functional genomics, including generation of CRISPR/Cas9 mutant libraries for developing genome-wide mutation screens (Bortesi and Fischer 2015) which are well

complemented by new CRISPR imaging techniques allowing DNA/RNA localization studies in live plants.

Viral vector-based platforms for rapid and efficient transient expression of the CRISPR tools (split-Cas9 and sgRNAs) (Cody and Scholthof 2019; Kaya et al. 2017) provide a further step toward the development of a screening tool for accelerated plant functional genomics. This should facilitate the necessary technological progress for future applications in crop improvement. However, the rapid progress in CRISPR-based research also puts forward some societal concerns and technical challenges.

All current approaches to engineer CRISPR virus resistance by direct targeting of viral DNA or RNA require permanent maintenance of CRISPR reagents in plants, which has been achieved thus far by expression of at least one of them (Cas endonuclease) from a DNA transgene (Table 1). This may preclude or limit practical application of these approaches due to the presence of stringent GMO regulations. On a technical level, generation of transgenic plants is labor intensive, time consuming, and may also be problematic for agronomically important crops. Therefore, before new techniques alternative to plant transformation have been developed, the investigator may consider RNAi-based applications.

Another problem of direct targeting of viral genomes is that CRISPR editing could lead to formation and escape of new (CRISPR-resistant) geminivirus variants which were presumably generated as a result of postcleavage reparation (NHEJ). These events may potentially trigger rapid virus evolution and lead to the development and release of new pathogenic forms. To avoid emergence of new viruses, multiple viral genome regions should be targeted or the use of CRISPR-based nickases along with paired sgRNAs to introduce larger deletions should be employed to decrease recombination frequency.

Most approaches to modify plant host susceptibility genes to render the plants virus resistant are also currently based on the transgenic expression of CRISPR reagents (Table 1). Independently of further steps to segregate out CRISPR transgenes, introduction of foreign DNA into plants may represent the same concerns as listed above. Development of viral vector-based platforms for rapid and efficient delivery of the CRISPR tools (split-Cas9 and sgRNAs) (Cody and Scholthof 2019; Kaya et al. 2017) for transient genome editing with subsequent virus eradication through meristematic culturing may overcome these regulatory and technical constraints and will be a challenge for future research.

The use of preassembled RNP consisting of CRISPR reagents (sgRNA and Cas proteins) may be another option that prevails over transgenic approaches for engineering virus resistance in crop plants. However, one of the technical challenges to achieving the full potential of this approach is the delivery of the CRISPR tools into living cells of the plant. It is highly desirable that such techniques could avoid steps of plant regeneration from tissue culture because this step may not be achievable for many crops. One possibility is to use pollen grains for editing to allow production of edited seed without regeneration, a technique which has recently been used for plant transformation (Zhao et al. 2017) and which is applicable to a broad range of crop plants. To intensify penetration rates to ensure best delivery, various nanoplateforms can be tested to identify the most efficient delivery means for a given application.

Finally, identification of novel host susceptibility genes that should be inactivated or modified by CRISPR to induce plant virus resistance is desperately required. To the best of our knowledge, this has been done for only two groups of plant genes (encoding eIF4s and coilin). The future progress of this technology in plant protection against viruses is absolutely dependent on the identification of host genes involved in controlling interactions with a broad spectrum of plant viruses.

Although addressing these challenges and concerns takes some time, another application of the CRISPR tool such as diagnostics is

readily applicable in applied plant virology. A SHERLOCK (Cas13)-based paper strip test strip is now widely used to detect pathogens, tumor DNA, or any genetic signature of interest in human and animal systems, and the time has now come for broad applications of such tests in plant pathology and virology, in particular.

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