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REVIEW

Biotechnological Approaches to Plant Antiviral Resistance: CRISPR-Cas or RNA Interference?

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Abstract—Established genome editing technologies, such as CRISPR-Cas and RNA interference (RNAi), have significantly advanced research studies in nearly all fields of life sciences, including biotechnology and medicine, and have become increasingly in demand in plant biology. In the review, we present the main principles of the CRISPR-Cas and RNAi technologies and their application in model plants and crops for the control of viral diseases. The review explores the antiviral effects they provide, including direct suppression of genomes of DNA- and RNA-containing viruses and inhibition of activity of host genes that increase plant susceptibility to viruses. We also provide a detailed comparison of the effectiveness of CRISPR-Cas and RNAi methods in plant protection, as well as discuss their advantages and disadvantages, factors limiting their application in practice, and possible approaches to overcome such limitations.

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INTRODUCTION

Viral diseases of plants present a serious danger to global agriculture by adversely affecting yield, food safety, and economic stability [1]. There is an urgent need to control epidemics caused by the spread of plant viruses and their new variants appearing due to the genetic evolution, transmission from natural plant reservoirs, changes in agriculture, mixed infections, and impact of global warming [1]. Almost half (47%) of all repeatedly emerging outbreaks of plant diseases are caused by viruses (i.e., more than by any other plant disease agent) [1, 2].

Currently, the major approach to combating viral diseases is the use of agrochemicals and resistant crop varieties. Although application of pesticides to eliminate natural vectors of viral infections (mites, nematodes, aphids, thrips, cicadas, and whiteflies) is quite effective, it is nonselective and can be harmful to other (beneficial) organisms, leading to the disruption in the ecological balance [3].

The use of genetically resistant plant varieties is commonly believed to be the most efficient, cost-effective, and consumer-friendly approach to controlling viral diseases. However, many crops lack genetic resistance to viruses due to a deficiency of resistance genes in genetically compatible relatives. Selection of resistance genes even by modern molecular methods, such as quantitative trait locus (QTL) mapping, marker-assisted selection, and whole-genome sequencing, is often time-consuming, labor intensive (i.e., due to the difficulties in crossing elite lines with wild plant species), and requires monitoring of large plant populations over a number of generations, which may take several years in crops with long-term breeding cycles [4].

Other plant protection technics include preventive measures, such as quarantine, certification, cross-protection, removal of infected plants, and micropropagation to obtain virus-free planting material [5];

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however, they are often insufficient to reduce the harmful effects of viral diseases on agricultural plants.

Achievements in genetic engineering and plant transformation methods in the early 1980s have made it possible to obtain virus-resistant lines of transgenic plants [6] by producing plants expressing viral proteins (envelope proteins, replicases, transport proteins, etc.) or their fragments, as well as non-coding viral nucleotide sequences [7-10]. These techniques also have significant limitations due, in particular, to the fact that the acquired resistance is usually highly specific to a particular virus strain and is eventually overcome because of a uniquely great capacity of viruses for mutagenesis and their ability (especially of RNA-containing viruses) to rapidly evolve [11]. Another approach was to induce plant resistance by transgenic expression of cellular genes, including natural resistance genes, as has been done by many research groups [6]. However, legal restrictions on the use of genetically modified organisms (GMOs) significantly reduce or even prohibit cultivating transgenic plants in many countries.

An expanding knowledge on the molecular mechanisms of plant interaction with viruses along with the rapid progress of genetic technologies in recent decades, have opened up new and completely different prospects for the development and implementation of effective and environmentally friendly approaches to plant defense against viral infections. Currently, the most promising genetic tool is genome editing mediated by the CRISPR-Cas system [12-16]. CRISPR-Cas enables introduction of directed changes into the target genes. It is based on the RNAi mechanism [17, 18] that allows to specifically inhibit viral replication by cleaving the viral RNA. The CRISPR-Cas technology has already led to significant advancements in nearly all fields of life sciences, including biotechnology and medicine, and has become increasingly common in plant biology. RNAi has long been used as a transgenic tool (host-induced gene silencing, HIGS) for the degradation of viral RNAs or inactivation of genes responsible for viral susceptibility in agricultural plants [6, 19-21]. Recently developed RNAi-based methods (spray-induced gene silencing, SIGS) that use exogenous double-stranded RNAs (dsRNAs), small interfering RNAs (siRNAs), and short hairpin RNAs not only cause the degradation of viral RNA, but also appear to be more stable, safe, and socially acceptable alternative to transgenic methods [17, 18, 22].

The use of CRISPR-Cas and RNAi technologies to provide plants with virus resistance, as well as the underlying mechanisms, have been described in detail in many reviews [12, 14, 16, 17, 22-25], however, comparative analysis of these technologies has received very little attention in the published literature. In this article, we aimed to fill the gap in the information on the applicability of CRISPR-Cas and RNAi tools with special emphasis on the outcome of their use for the generation of virus-resistant plants. We also discussed advantages and disadvantages of the CRISPR-Cas and RNAi methods, as well as their prospects in crop production and plant protection.

CRISPR-Cas: ENGINEERING TOOLKIT FOR ANTIVIRAL PROTECTION

The structure, properties, and principles of the CRISPR-Cas genome-editing system have been described in many publications [12-14, 25, 26]. The CRISPR-Cas system includes clustered, regularly interspaced short palindromic repeats (CRISPRs) and CRISPR-associated proteins (Cas) and has originated from the adaptive immune system of bacteria and archaea that prevents intrusion of foreign plasmids and bacteriophages by cleaving their DNA [27]. The active CRISPR-Cas complex consists of the Cas endonuclease and guide RNA (gRNA) which directs the Cas protein to the target DNA or RNA. gRNA contains a scaffold for the Cas protein binding and a spacer sequence of approximately 20 nucleotides (nt) for recognizing the target sequence in the phage or plasmid DNA [28]. Researchers have adapted this bacterial immune system for DNA editing in eukaryotes. When introduced into cells, gRNA recognizes the target DNA sequence and the Cas9 enzyme cuts DNA at this site, similar to the natural process taking place in bacteria. After DNA cleavage, cell apparatus repairs the breaks using either homologous recombination or non-homologous end joining mechanisms, resulting in the insertion or deletion of genetic material or DNA modification accompanied by the replacement of the native DNA segment with a new sequence, which eventually leads to the loss of function of the targeted gene.

The CRISPR-Cass-based methods of antiviral defense are classified into two categories: (1) direct cleavage or degradation of the viral genome and (2) modification (mutation) of the host plant genes required for the virus life cycle.

DIRECT EFFECT OF THE CRISPR-Cas SYSTEM ON THE VIRAL GENOME

DNA-containing viruses. The original CRISPR-Cas genome-editing system was derived from *Streptococcus pyogenes* and included Cas9 endonuclease responsible for the DNA cleavage. Because of this fact, the CRISPR-Cas9 technique was first tested against a variety of DNA-containing geminiviruses (Geminiviridae family). Thus, transgenic tobacco

and *Arabidopsis thaliana* plants expressing CRISPR-Cas components (Cas9 endonuclease and gRNAs against several coding and non-coding regions of the geminivirus genome) were more resistant to the viral infection due to the direct action of CRISPR-Cas on the geminivirus genome [29, 30]. Successful inhibition of geminivirus propagation in model plants has been replicated in agricultural crops, such as cotton, tomatoes, potatoes, peppers, watermelon, soybeans, beans, barley, etc. [16]. The CRISPR-Cas9 system has been also used to induce immunity to other DNA-containing plant viruses. For example, transgenic *A. thaliana* plants expressing Cas9 and gRNAs targeting the genome of cauliflower mosaic virus (CaMV; Caulimoviridae family) were highly resistant to this virus [31]. However, in some cases, DNA-containing viruses generated mutants that overcame resistance mediated by CRISPR-Cas9 and were capable of rapidly spreading in the environment. Hence, the CRISPR-Cas system poses some potential risk by stimulating generation and spread of new pathogenic virus variants [32].

RNA-containing viruses represent the most serious threat to agricultural plants by causing significant agronomic losses, including reduced crop yields, lower product quality, and shorter shelf life. The discovery of RNA-specific endonucleases associated with the CRISPR system, such as Cas9 from *Francisella novicida* (FnCas9), Cas13 from *Leptotrichia shahii* (LshCas13a), and Cas13 from *Ruminococcus flavefaciens* (RfCas13d), has allowed to develop systems that directly cleave viral RNA genomes [12, 14, 16]. Transgenic plants expressing RNA-specific Cas endonucleases together with gRNAs directed at viral RNA targets exhibited a significant resistance to RNA-containing viruses. Interestingly, deactivated forms of Cas proteins lacking the RNA endonuclease activity retained the antiviral effect. It was hypothesized that in this case the infection was inhibited at the levels of viral RNA translation and/or replication [33]. The high efficiency of this approach has been confirmed by successful suppression of more than 15 RNA-containing viruses (both with positive and negative genomes) in a wide range of model and agricultural plants [12, 14, 16].

Although Cas endonucleases are important technological tools capable of providing plant resistance to viruses by directly affecting the viral genome, this approach requires constant presence of these enzymes and associated gRNAs in plant cells, which can be achieved only through their transgenic expression. In this regard, the practical application of the CRISPR-Cas system may be strongly limited by the regulations on the use of GMOs. Another factor restricting implementation of this method is accelerated generation of new mutant viral variants capable of overcoming the CRISPR-Cas-based resistance and release of these viruses into the environment.

EDITING OF HOST PLANT SUSCEPTIBILITY FACTORS

Since viruses have to use and modify host plant systems for successful infection (replication and propagation through the plant) and depend on many host cellular mechanisms (for example, interaction between the virus and plant proteins is necessary to perform certain viral functions). Neutralization of these protein partners should inevitably lead to the inhibition of viral infection and can be achieved by introducing mutations into the corresponding plant genes using the CRISPR-Cas system, as it was demonstrated for the recessive genes encoding eukaryotic translation initiation factors eIF4E and eIF4G and their isoforms eIF(iso)4E and eIF(iso)4G [16, 34]. These factors, also known as cap-binding proteins, are key elements of protein synthesis in eukaryotes. As parts of the complex also including eIF4G and eIF4A proteins, they bind methylated guanine residue added post-transcriptionally to the 5′-end of eukaryotic mRNAs, which triggers assembly of the translation initiation complex, ribosome binding, and initiation of protein synthesis [34]. It was shown that many plant viruses also require interaction with eIF4E/eIF4G to ensure a successful infection.

Mutagenesis of the eIF4E gene using the CRISPR-Cas system induced resistance of cucumber plants against zucchini yellow mosaic virus (ZYMV), papaya ring spot mosaic virus W (PRSV-W), and cucumber vein yellowing virus (CVYV) [35]. Rice plants with the modified eIF4G gene allele introduced using CRISPR-Cas9, were resistant to Rice tungro spherical virus (RTSV) [36]. A mutation in the eIF(iso)4E gene induced by CRISPR-Cas9 in cassava and *A. thaliana* plants, provided full resistance to cassava brown stripe virus (CBSV) [37] and turnip mosaic virus (TuMV) [38], respectively. It should be noted that the genome of cassava encodes additional eIF4E-like proteins (nCBP-1 and nCBP-2). Mutations introduced with CRISPR-Cas9 into the *nCBP-1* and *nCBP-2* genes established plant resistance to cassava brown steak virus [37]. The use of Cas9 fused with cytidine deaminase enabled a highly efficient editing of the target codons and introduction of the N176K mutation into the eIF4E1 gene allele in *A. thaliana*, resulting in the generation of plants resistant to the clover yellow vein virus (ClYVV) [39].

Despite obvious achievements in the induction of antiviral resistance by introducing mutation in the alleles of translation initiation factor genes, the following aspects should be taken into account: (1) significant redundancy of such factors, which allows viruses to use unmodified factors for their replication; (2) a possible effect of mutations on the translational apparatus of the host plant and, as a result, on plant physiology; (3) a high frequency of overcoming induced

resistance by RNA-containing viruses capable of rapid evolutionary changes [34].

Resistance to viral infections can be enhanced by knocking out other susceptibility genes (S-genes) in addition to mutations in the eIF4E/eIF4G genes. For example, the knockout of the *SlPelo* gene led to the increased plant resistance to the tomato yellow curl virus [40]. Similarly, disruption of the chloride channel (*CLC-Nt1*) gene in *Nicotiana benthamiana* plants suppressed potato virus Y (PVY) replication [41]. Editing at least one allele of the coilin (structural protein of Cajal subnuclear bodies) gene dramatically improved resistance of potato plants to the PVY infection, as well as their tolerance to salt and osmotic stresses [42]. Other S-genes whose editing enhanced the antiviral resistance include soybean *GmF3H1*, *GmF3H2*, and *GmFNSII-1*, wheat *TaPDIL5-1*, *Arabidopsis Tom1*, and *N. benthamiana NbUbEF1B* and *NbCCR4/NOT3* [25]. These data reveal the potential of employing S-genes to create new plant varieties with a wide range of tolerance to viruses using the CRISPR-Cas9 system. However, it should be remembered that beside controlling viral infections, many S-genes are involved in important endogenous processes, such as plant growth and development, so that their editing can have an unexpected negative effect on the plants. For example, the *ssi2* mutation in *A. thaliana* significantly promoted accumulation of salicylic acid (phytohormone involved in the antiviral defense), but also induced phenotypic abnormalities in the growing plants [43].

Hence, the search of promising targets for the CRISPR-Cas-mediated editing is one of the most principal tasks in the creation of virus-resistant plants. Recent achievements in the functional genomics, including the CRISPR-Cas system itself, has brought this search to a new level. The programmability of the CRISPR system has proven to be very useful for the high-throughput identification of genes with specific functions [44]. Development of simple methods for the synthesis of gRNA libraries has made it possible to obtain large populations of designed plants and to screen them for genes with specific function, which in turn can be effective in developing new strategies for the regulation of antiviral resistance. Cas9 can be used for the analysis of plant–virus interactions, particularly, in terms of the host gene functions. Moreover, the ability of Cas13 to cleave RNA opens up new prospects for studying the functions of long non-coding RNAs, which are involved in many processes, including resistance to pathogens [45] and, therefore, may provide a new basis for the antiviral resistance.

Another problem in genome editing of host plant genes using the CRISPR-Cas system is that many editing techniques are based on transgenic CRISPR-Cas components. In recent years, alternative plasmid-free methods have been developed for the delivery of gRNAs and Cas9 protein to plant cells, that have eliminated the need for the transgenic system application. For example, Cas9 and gRNA can be directly delivered to the cells as a preassembled ribonucleoprotein (RNP) complex. RNP complexes can be introduced into plant protoplasts by transfection or can be transferred into immature plant embryonic cells, callus cells, and epidermis cells by particle bombardment using gold and tungsten microparticles or mesopore silicon nanoparticles [12]. The main advantage of DNA-free technology is the lack of need for the DNA introduction, since in conventional DNA-based methods, such DNA integrates into the genome at random sites and constitutive expression of the *Cas9* gene from this DNA likely leads to the off-target editing. Another advantage of the Cas9–gRNA complex delivery is that it ensures fast genome editing followed by rapid degradation of the editing complex in the cell, which also reduces the likelihood of adverse off-target effects [12]. Plasmid-free methods for the transfer of genome-editing complexes are both promising and preferable, since they facilitate the generation of plants exhibiting specified properties without being the subjects of anti-GMO regulations.

Another approach is removal (deletion) of foreign sequences encoding Cas and gRNAs from initially edited (transgenic) plants by crossing them with their wild-type counterparts [37, 38]. Plants obtained by this method are resistant to the selected viruses without being transgenic.

The above approaches enable to obtain nontransgenic plants with the required characteristics. However, it remains unclear whether plants produced using the CRISPR-Cas technology should be classified as GMOs. Moreover, crossing with the wild-type plants to obtain plants with certain properties but lacking transgenic sequences is impossible for some species, in particular, plants reproducing by vegetative propagation (e.g., potatoes), since production of seeds can alter the characteristic cultivar properties.

We discovered another interesting feature of the CRISPR-Cas-mediated genome editing when chitosan nanoparticles were used to deliver preformed gRNA–Cas9 complexes targeting genes for phytoene desaturase (PDS) and coilin to the apical meristem cells that were then regenerated into viable potato plants [42, 46, 47]. Typically, editing plant genome with the CRISPR-Cas system leads to the appearance of insertions or deletions (indels) 10-20 base pairs (bp) in length. However, we observed large deletions (up to 600 bp) in the region flanking the gRNA-binding site in the target gene, but no short indels [42, 46, 47]. Therefore, when using this approach, it is important to choose the targeted site so that any large deletion would be located entirely within the knocked-out gene. Large deletions caused by CRISPR-Cas have also

been previously detected in animal cells with a high mitotic activity [48], which correlates with our data, since meristem cells are rapidly dividing cells.

CRISPR DIAGNOSTICS

Unlike in eukaryotic cells, where Cas13 mediates exclusively highly specific RNA cleavage directed by gRNA, in prokaryotes and *in vitro* reaction mixtures, specific cleavage of a target RNA enables Cas13 to engage in the collateral (nonspecific) degradation of other single-stranded RNAs [49]. This side effect has been used in the development of highly sensitive virus diagnostic methods that employ the SHERLOCK (specific high-sensitivity enzymatic reporter unlocking) approach for high-accuracy detection of nucleic acids, in particular, viral RNA and DNA [50]. In SHERLOCK, analyzed samples are amplified to enrich the target DNA (if present) using recombinase polymerase reaction (RPA). If the identified molecule is RNA, it is reverse transcribed before the RPA. The products of RPA are transcribed into RNA-by-RNA polymerase and the obtained transcripts are cleaved by Cas13 in the presence of a quenched single-stranded RNA fluorescent reporter. The concomitant cleavage by Cas13 generates fluorescence signal, thus indicating the presence of the target viral DNA or RNA. Recently, this method has been significantly simplified and adapted for practical diagnostics in a form of fast, inexpensive, and highly sensitive paper-strip test [50] applicable for rapid and reliable detection of plant viruses in the field.

RNAi: RNA-DIRECTED TECHNOLOGY FOR PLANT ANTIVIRAL RESISTANCE

RNAi and plant protection. The concept of pathogen-derived resistance postulated in the 1980s [51] has initiated a breakthrough in the field of plant protection biotechnology by proposing the use of viral genes or their fragments to suppress viral infections. First, transgenic plants with an increased resistance to viruses were created via expression of viral structural and replicative proteins (replicases) [6, 22, 52]. The following discovery of RNAi has marked a new phase in plant biotechnology, as it enabled more specific and efficient plant antiviral defense [6, 22, 52].

RNAi is an evolutionarily conserved, sequence-specific mechanism for suppressing gene expression in most eukaryotes, including plants. It controls expression of endogenous genes and leads to the degradation of foreign nucleic acids. RNAi is triggered in the presence of dsRNA precursors originating from the host plant hairpin RNA structures or from foreign dsRNA intermediates (e.g., replicative forms of viral RNA). dsRNA molecules are cleaved by Dicer-like endonucleases (DCLs) into siRNAs 21-24 bp in length or microRNAs. This process is further amplified with the involvement of host RNA-dependent RNA polymerases (RDR1 and RDR6), leading to the formation of secondary siRNAs. These siRNAs are loaded into a complex formed by ARGONAUTE (AGO) family proteins with the generation of activated RISC (AGO-containing RNA-induced silencing complex) and direct the degradation or translational repression of complementary (specific) target RNA molecules [53, 54]. RNAi has become one of the most efficient approaches to obtain virus-resistant plants by expressing virus-specific dsRNA.

Specific antiviral effect of exogenous dsRNAs. The commercial use of transgenic plants with artificially induced disease resistance is limited by the regulations targeting GMOs, as well as negative public perception, which creates the need for more sustainable, efficient, environmentally friendly, and socially accepted alternative approaches to plant protection. The SIGS technology based on spraying plants with exogenous dsRNA meets such requirements, and, moreover, has already been successfully applied to induce antiviral resistance in a wide range of crops [12, 13, 17, 18, 55-57]. Mechanical inoculation with dsRNAs and high-pressure spraying have also been proven to protect plants from viruses. Exogenous technologies have been used in laboratories to suppress the replication of more than 20 economically important DNA- and RNA-containing plant viruses from various taxonomic groups in more than 10 plant species [17, 18, 58].

It is generally believed that the mechanism of the antiviral action of exogenous dsRNA is similar to the classical RNAi mechanism, including involvement of DCL, RDR, AGO, and other RNAi system components, since the effect of exogenous dsRNA in plants is specific to the targeted DNA/RNA sequence [17, 59-66]. However, there is no direct evidence supporting this concept. Our recent detailed comparative analysis of short RNAs formed in potato plants infected with PVY revealed that exogenous treatment of plants with the PVY-specific dsRNA was accompanied by the formation of non-canonical RNA fragments 18-30 nt in length vs. classical 21- and 22-nt siRNAs induced by the PVY infection in control plants [67]. Formation of the 21- and 22-nt siRNAs was consistent with the data on the size of siRNAs formed during infection with other RNA-containing viruses [67]. Such siRNAs are produced with the involvement of CL4 and DCL2, respectively, and then interact with AGO1 and AGO2 to form the RISC, which ensures hydrolysis of viral RNA (RNA silencing). Interestingly, similar size distribution of short RNAs was described by Tabein et al. [63] and Rego-Machado et al. [64] for the external plant

treatment with dsRNAs against tomato spotted wilt virus (TSWV) and tomato mosaic virus, respectively. Therefore, despite the fact that the anti-PVY dsRNA specifically inhibited the replication of PVY (and not of unrelated viruses) [67], these results may indicate that exogenous dsRNA can be processed through a previously unknown DCL-independent mechanism. It is also possible that the antiviral effect of exogenous dsRNA is mediated by some mechanisms other than the classical RNAi, but this suggestion requires further investigation.

Nonspecific antiviral effect of exogenous dsRNAs. Beside the ability to induce RNAi, dsRNAs can serve as effectors of pattern-triggered immunity (PTI) [68, 69]. RNA-based pathogen-associated molecular patterns (PAMPs) are well-known inducers of immunity in animals [70, 71] and plants [68, 72]. Defensive responses caused by viruses and dsRNAs are canonical for PTI and include generation of reactive oxygen species (ROS), induction of phytohormonal signaling, activation of mitogen-activated protein kinases, and triggering of expression of protective genes [68, 73, 74]. Unlike RNAi, dsRNA-induced PTI does not depend on the RNA sequence and can also be activated by non-viral dsRNAs, such as synthetic polyinosinic-polycytidylic acid [poly(I:C)] or GFP-specific dsRNA [68]. For example, poly(I:C), which induced the expression of PTI marker genes, also caused strong antiviral protection against oilseed rape mosaic virus (ORMV) infection [68].

Considering that dsRNA can function as a signal inducing antiviral plant defense by triggering both specific RNAi and non-specific PTI-type defense responses, we hypothesized that these two pathways could also be induced by the application of exogenous dsRNAs, resulting in changes in the susceptibility of the dsRNA-treated potato plants to PVY. We analyzed the impact of exogenously applied PVY-specific dsRNA on both defense mechanisms (RNAi and PTI) and studied its effect on the accumulation of homologous (PVY) and unrelated (potato virus X. PVX) viruses [66]. The use of dsRNA against PVY in potato plants induced accumulation of siRNAs (RNAi markers) and transcripts of genes coding for PTI-associated proteins, such as WRKY29 (transcription factor, molecular marker of PTI), RbohD (respiratory burst oxidase homolog D), EDS5 (increased susceptibility to diseases 5), SERK3 (somatic embryogenesis receptor kinase), and PR-1b (pathogenesis-related protein 1b) [66]. At the same time, the treatment suppressed only the PVY replication, but produced no effect on the PVX infection, despite the PTI induction in the presence of PVX [66]. Since the RNAi-mediated antiviral immunity is the main resistance mechanism, it can be assumed that the dsRNA-induced PTI alone was not sufficient to suppress viral infection under these conditions. However, it should be mentioned that Necro et al. [75] demonstrated the ability of a non-specific (anti-PVY) dsRNA to suppress replication of the non-homologous PVX, although with a lower efficiency than the specific anti-PVX dsRNA.

In addition to the induction of RNAi and PTI, anti-PVY dsRNA was able to regulate poly(ADP-ribosyl)ation (PARylation), which is a protein post-translational modification during which ADP-ribose chains are added to the target protein by poly(ADP-ribose) polymerases (PARPs) [66]. PARylation plays an important role in plant resistance to the genotoxic stress, DNA repair and transcription, cell cycle control, and response to biotic and abiotic stresses, including processes associated with programmed cell death and regulation of plant immunity [76, 77]. Poly(ADP-ribose) (PAR) residues can be removed by poly(ADP-ribose) glycohydrolase (PARG). Interestingly, we found that anti-PVY dsRNA increased the content of PARG in potato plants, which correlated with a decrease in the PAR accumulation and ultimately contributed to the suppression of PVY infection [66].

Therefore, exogenous dsRNAs can be used as “multitools” that mainly trigger RNAi as the key mechanism of antiviral resistance, as well as induce other mechanisms involved in PTI/PARylation, which produce a cumulative antiviral effect and represent an extra protective strategy when RNAi alone proves ineffective against viral infection.

Taken together, these data suggest the further development of technologies combining dsRNA-induced protective responses [RNAi, PTI, and modulation of poly(ADP-ribose) metabolism] in a coordinated manner will be able to ensure a high level of crop protection against viruses.

TECHNICAL FACTORS LIMITING THE USE OF dsRNAs

Viral suppressors of RNAi. Despite the above examples of successful use of RNAi-based technology, this approach has certain limitations because of the existence of viral RNA silencing suppressors [12, 78]. Many plant viruses encode silencing suppressors that interfere with RNAi through various mechanisms and at different stages. For example, viral suppressors can bind tRNAs or siRNAs, thus preventing the functioning of DCL or AGO, respectively [12, 17] and inevitably reducing the antiviral effect.

Delivery of dsRNAs to plant cells and tissues is another factor that limits the use of exogenous dsRNAs. For exogenous nucleic acid to enter plant cells, it should maintain its integrity on the leaf surface against the effects of environmental factors, such as ultraviolet radiation, wind, rain, pH, and attack

of bacterial nucleases. Next, dsRNA should penetrate numerous natural barriers, such as leaf cuticle, cell wall, and plasma membrane, as well as compensate for a low efficiency of absorption by the cell [79, 80]. Moreover, for producing a reliable and stable RNA phenotype, dsRNAs (or siRNAs formed from them) should spread throughout the plant via the intercellular transport system (plasmodesmata) and the long-distance transport system (phloem) [81]. Finally, inside the cell, dsRNA should be integrated into the RNAi mechanism occurring as a series of events, e.g., dsRNA processing by DCL with the generation of primary siRNA duplexes, amplification of primary miRNAs by RDR, loading of siRNAs into the RISC, and recognition of the target RNA site and its cleavage. These stages are strictly compartmentalized and determine the biological function of RNAi. An intuitive assumption would be that the sites of dsRNA generation (e.g., virus replication sites) can be mechanically linked to the compartments associated with the RNAi machinery, and therefore, endogenously produced dsRNAs would be able to naturally integrate into the RNAi mechanism, which can then operate as a conveyor system, in which the products of the previous stage are moved for the use at the subsequent stage (for example, the primary siRNAs obtained by the cleavage of dsRNA are transferred to the stage of siRNA amplification, etc.). Hence, to ensure an efficient RNAi operation, exogenous dsRNA should be incorporated into the RNAi "conveyor".

Solving the problem of dsDNA delivery has currently become a priority. At least in part, this problem can be managed by using additives and carriers that can improve the stability of dsRNA molecules and/or facilitate their adhesion and penetration into plants. Such function can be performed by cationic oligopeptides, various nanoparticles, surfactants, liposomes, artificial extracellular vesicles, and chromosome-free minicells (developed by AgroSpheres; https://www.agrospheres.com/) [82]. Thus, it has been found that nanocarriers can function as extremely efficient delivery systems that are currently commonly used for dsRNA delivery [24, 79, 80, 83]. A combination of RNAi technology and nanotechnology opens very encouraging prospects in plant protection. One example is the use of degradable dsRNA-containing clay particles (BioClay) that not only significantly facilitate the delivery of dsRNAs to plants, but also considerably prolong the time of their antiviral action (from 7 to 24 days) [62]. Further application of laboratory developments in crop production will depend on the cost and effectiveness of developed nanocarriers.

Methods for dsRNA synthesis and their cost-efficiency. Exogenous DNAs are currently produced by *in vitro* transcription, microbial expression in bacteria and fungi, and cell-free synthesis [24, 83].

In vitro transcription involves the use of a target sequence flanked by two convergent (i.e., oriented toward each other) 5′-RNA polymerase promoters (for example, T7 bacteriophage RNA polymerase promoter). Such DNA template enables transcription of both sense and antisense RNA strands, which rapidly anneal in the same reaction mixture with the formation of dsRNA [83].

In vivo dsRNA synthesis in microbial systems is mainly carried out in *Escherichia coli* HT115 (DE3) strain defective by RNase III (enzyme that specifically degrades dsRNA). The methods used for the dsRNA production in *E. coli* are similar to those employed for the synthesis of recombinant proteins. After transformation with a plasmid containing a fragment coding for the dsRNA sequence against a specific RNA target and placed under the control of the T7 RNA polymerase promoters, the cells are grown to the exponential phase and induced with IPTG. After incubation for ~4-6 h, the cells are collected and lysed, and dsRNA is purified [83].

Cell-free transcription/translation systems use cell lysates for the mRNA transcription coupled with the *in vitro* protein translation. Cell-free extracts are optimized to contain most of the cellular cytoplasmic components necessary for transcription and translation and have several advantages over *in vivo* bacterial systems. For example, elimination of secondary processes necessary to maintain cell viability and growth makes it possible to fully utilize the activity of RNA polymerase and the pool of ribonucleotides in the reaction mixture. The absence of cell walls makes it easier the control the synthesis and facilitates the process of sample preparation. Cell-free systems are currently successfully used for the production of RNA vaccines. Since for the application in the field, dsRNAs have to be produced in large amounts at a relatively low cost, the purity of such dsRNAs might be less than the purity of nucleic acid preparations intended for the medical use. GreenLight Biosciences Company (https://www.greenlightbiosciences.com/) [84] has developed a unique cell-free biotechnological platform that provides large-scale dsRNA production at a low cost ($0.5/g) compared to fermentation ($1/g), *in vitro* transcription ($1000/g), and chemical synthesis ($100,000/g) [24, 83].

CRISPR-Cas OR RNAi: HOW TO MAKE THE RIGHT CHOICE?

As discussed above, both dsRNA-based RNAi and CRISPR-Cas technologies are powerful tools for the production of virus-resistant plants. However, which of these seemingly competing approaches is most appropriate for a specific application? Before making

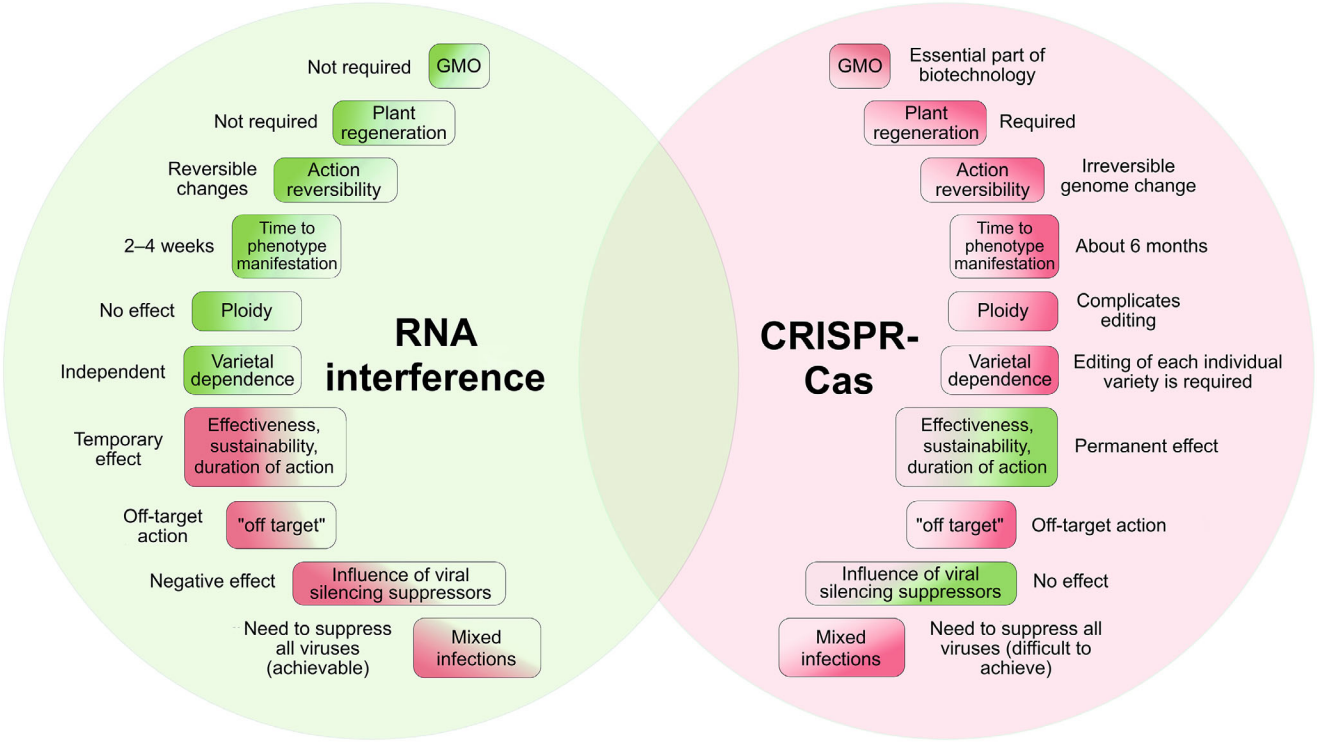

Fig. 1. Advantages and disadvantages of CRISPR-Cas and RNAi in protecting plants from viral infections.

the choice, it is necessary to consider several technical and methodological aspects (Fig. 1).

Direct impact on viruses. GMOs. As noted above, some aspects of the CRISPR-Cas functioning, in particular, direct action on RNA- and DNA-containing plant viruses, require a constant presence of the CRISPR-Cas system components in plants, which can only be achieved by transgenic methods, while the RNAi-based SIGS technology does not require generation of transgenic organisms (GMOs). Exogenously applied dsRNAs ("biopesticides") offer a clear advantage due to less strict GMO regulations and less public concern. In this regard, it is still necessary to establish an evidence base to support the approval of the application of biopesticides (dsRNAs) in the field. At the same time, it is essential to monitor the fate of dsRNAs in the environment, their impact on non-targeted organisms, and overall safety.

Plant regeneration. Inactivation of genetic susceptibility to viruses in host plant does not require a constant presence of Cas and gRNA, therefore, removal of transgenes encoding Cas9 and gRNA or delivery of editing reagents in a form of mRNAs or RNP complexes can help to avoid the use of transgenic plants. However, regeneration of whole plants from the edited cells and further identification of edited plant lines are typically time-consuming, technically complex, and expensive. Moreover, many agricultural plants and varieties cannot be regenerated from cells. So far, transgenic-independent CRISPR methods have been implemented only in a limited range of plant species and varieties. In contrast, the SIGS approach does not include the regeneration stage.

Reversibility. Classical DNA-editing techniques usually result in irreversible genome modification and cause complete loss of gene function. This significantly restricts the editing of essential (e.g., housekeeping) genes, because the knockout of such genes will lead to plant death. The dsRNA-based SIGS method induces reversible changes in the gene expression over a given period of time, which is important because some plant genes can be deactivated only at certain stages of plant development, as they play an important functional role at other stages. Moreover, the same genes can simultaneously determine resistance to some adverse factors and susceptibility to others.

Time to phenotype manifestation. Production of genetically modified plants takes at least six months, while a new dsRNA preparation can be obtained in less than 2-4 weeks. The time aspect is especially important in unforeseen circumstances, such as an emergence of a new virus type or a new strain. Sequencing of its genome followed by the fragment cloning to obtain dsRNA, fits well within the allotted time frame.

Ploidy. The efficiency of genome editing depends on the plant ploidy (genetic heterogeneity). Polyploidy, or the presence of more than two sets of chromo-

somes in the cell nucleus, is common in plants (for example, all cultivated potato varieties are tetraploid and wheat plants are hexaploid). The presence of several homologous genes as editing targets requires more meticulous work and specialized techniques to assess the success of genome editing procedure [85]. The use of exogenous dsRNAs does not depend on the plant ploidy, since in this case, RNAi is induced simultaneously for all alleles.

Plant varieties. When using the CRISPR technology, each variety should be edited and tested independently, whereas the SIGS-RNAi tools designed to target conserved gene sequences can be applied in multiple varieties.

Efficiency, sustainability, and duration of action. In ~75% cases, SIGS causes a gene knockdown and produces the phenotypic effect which sometimes can be observed only in the treated leaves. The efficiency and duration of action vary and depend on the environmental factors and/or the presence of viral RNAi suppressors. These parameters can be improved by using stabilizing nanoplatforms that facilitate the delivery of dsRNAs into cells, as well as by repeated treatment. The efficiency of the CRISPR-Cas editing is 10-40% per allele, but the effect is stable, permanent, and inherited.

Non-specific (off-target) action is common to both approaches. The off-target effect can be eliminated (or at least minimized) by selection of specific dsRNAs (RNAi) or gRNAs (CRISPR-Cas) that would not interfere with the off-target genome regions, which can be achieved by using advanced bioinformatic programs. At the same time, the RNAi technology has a greater potential, since dsRNAs could be directed (at least, theoretically) at any region of the targeted transcript, while gRNAs can be directed only to a specific target site located next to the protospacer adjacent motif (PAM) situated right after the DNA sequence targeted by the Cas9 endonuclease.

CONCLUSION AND PROSPECTS

The CRISPR technology has already led to a considerable progress in almost all fields of life sciences, including biotechnology and medicine, and is now becoming increasingly popular in plant biology. However, despite its high popularity and great technical capabilities, the application of the CRISPR technology in agriculture may be somewhat limited. In medicine, the CRISPR method is used to correct pathological changes in the genomes of individual patients, while plant biotechnology involves genome alteration in all plants of the modified cultivar. In the context of antiviral protection, the CRISPR system has a number of disadvantages (see Fig. 1).

Direct editing (or degradation) of viral genomes by CRISPR-Cas is achievable only with the use of transgenic plants expressing components of the CRISPR-Cas system, which conflicts with current legislation and public opinion in many countries. In addition, editing virus susceptibility genes can result in the formation of new, more pathogenic viral variants (superviruses) capable of overcoming plant resistance and causing more pronounced infection symptoms [86, 87]. Since genome editing is carried out in isolated cells (or tissues), the regeneration stage is required to obtain intact plants, which may be technically difficult for some crops (or their varieties). In polyploid cultures, genome modification can also be hindered due to the presence of multiple alleles of the same gene. As a result, plant genome editing is a time-consuming process, which is a disadvantage when new infections emerge and urgent measures have to be implemented to protect the plants. In this case, the use of exogenous dsRNAs for the antiviral defense induction seems to be a preferred technology. However, to fully realize its potential, the following challenges must be addressed:

- improvements in the delivery of exogenous dsRNAs compatible with the RNAi machinery (e.g., by using various polymers and nanoplatforms that facilitate the penetration and controlled release of dsRNAs);
- optimization and scaling of dsRNA production;
- continuous monitoring of viral populations by deep sequencing methods to ensure the rational design of dsRNAs;
- identification of new target genes responsible for the plant susceptibility to viruses, whose suppression by RNAi (and/or CRISPR-Cas) would enhance plant antiviral resistance without producing side effects;
- development of RNAi strategies that would minimize the effect of viral RNAi suppressors in the dsRNA-directed antiviral defense.

Another important aspect that should be considered when developing RNAi and CRISPR-Cas approaches for plant protection is the existence of mixed infections. In this case, it is necessary to suppress the replication of all viruses simultaneously to avoid the situation when a vacated niche can be filled by the viruses whose replication has not been suppressed by specific dsRNAs, which might facilitate the spread of new infections.

Abbreviations. AGO, ARGONAUTE family proteins; DCL, Dicer-like endonuclease; dsRNA, double-stranded RNA; gRNA, guide RNA; PAR, poly(ADP-ribose); PTI, pattern-triggered immunity; PVY, potato virus Y; RNP complex, ribonucleoprotein complex; RNAi, RNA interference; SIGS, spray-induced gene silencing; siRNA, small interfering RNAs.

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