JOURNAL OF WATER AND LAND DEVELOPMENT
e-ISSN 2083-4535
Polish Academy of Sciences (PAN) Institute of Technology and Life Sciences – National Research Institute (ITP – PIB)

JOURNAL OF WATER AND LAND DEVELOPMENT
DOI: 10.24425/jwld.2023.145345
2023, No. 57 (IV–VI): 147–158

Plant virus resistance biotechnological approaches: From genes to the CRISPR/Cas gene editing system

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RECEIVED 04.10.2022 ACCEPTED 13.03.2023 AVAILABLE ONLINE 02.06.2023

Abstract: Plant viruses cause crop losses in agronomically and economically important crops, making global food security a challenge. Although traditional plant breeding has been effective in controlling plant viral diseases, it is unlikely to solve the problems associated with the frequent emergence of new and more virulent virus species or strains. As a result, there is an urgent need to develop alternative virus control strategies that can be used to more easily contain viral diseases. A better understanding of plant defence mechanisms will open up new avenues for research into plant-pathogen interactions and the development of broad-spectrum virus resistance.

The scientific literature was evaluated and structured in this review, and the results of the reliability of the methods of analysis used were filtered. As a result, we described the molecular mechanisms by which viruses interact with host plant cells.

To develop an effective strategy for the control of plant pathogens with a significant intensity on the agricultural market, clear and standardised recommendations are required. The current review will provide key insights into the molecular underpinnings underlying the coordination of plant disease resistance, such as main classes of resistance genes, RNA interference, and the RNA-mediated adaptive immune system of bacteria and archaea – clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated Cas proteins – CRISPR/Cas.

Future issues related to resistance to plant viral diseases will largely depend on integrated research to transfer fundamental knowledge to applied problems, bridging the gap between laboratory and field work.

Keywords: CRISPR/Cas9, CRISPR/Cas13, plant disease resistance, plant immunity, plant viruses

INTRODUCTION

The production of crops is significantly harmed by phytopathogens, which also significantly lower yields and product quality globally. Losses from viruses are lower (3–7%) than those from bacterial and fungal pathogens (15%), but outbreaks of plant viral diseases can still cost 60–80 bln US dollars per year (Oerke and Dehne, 2004) in economic damage. Currently, physical barriers are built to defend against insect carriers of viruses or chemical pesticides (Ualiyeva *et al.*, 2022) and are applied widely throughout the world (Khan *et al.*, 2015). There are no effective or long-lasting ways to fight viral diseases in plants because of the epidemiological changes caused by viral disease outbreaks, the speed with which viruses change, and the fact that viral vectors are always moving (Zaidi *et al.*, 2016).

Introducing plant varieties that are resistant to viral infections into commercial production is the most cost-effective technique for reducing losses that are caused by viral infections. The threat to global food security necessitates the creation of crop types that are both highly productive and virus-resistant. Traditional antiviral techniques undoubtedly raise crop quality, but they are expensive and labour-intensive. Modern biotechnologies and in-depth studies of the molecular and biochemical processes that drive interactions between plants and viruses have opened up new ways to make plants' immune systems work better against viruses.

The urgency of this work was to get a complete picture of how the virus and host cell interact under biotic stress factors. This knowledge is required to develop security measures that prevent the virus from entering different regions, ensuring each country's phytosecurity. It is also critical to optimise the effectiveness of individual field control measures.

PLANT DISEASE RESISTANCE GENES

Pathogen-derived resistance (PDR) was proposed for the first time in 1985. According to the theory, a phytopathogen's genetic components expressed in plant cells give resistance to viral pathogenesis (Sanford and Johnston, 1985). Later, scientists began attempting to create plants resistant to viruses by injecting them with various viral genes. As a result, a market for crops resistant to viruses was successfully established (Wilson, 1993).

To successfully infect viruses, it is crucial to get through the system of complex plant defence mechanisms. Since several of these mechanisms are widespread and effective against the majority of viruses, the innate immune system is a representation of this response. Some viruses have other distinctive defence mechanisms in addition to the activation of resistance genes. The limited ability of phytoviruses to infect all plants is caused by the universal non-host resistance (NHR) mechanism (Ali *et al.*, 2022). There are two main forms of universal immune resistance in terms of mechanism and pathogen detection. The first kind of defence is the main line of defence against pathogen entrance into the body. This type of protection involves both thickening the cell wall and the synthesis of many secondary metabolites.

Once the pathogen overcomes the first kind of resistance, local necrosis causes the second type of universal resistance to become active (Baruah, 2020). Following that, the pathogen is recognised due to particular structures or proteins that are connected to it. These molecular patterns linked to microorganisms (microbial-associated molecular patterns, or MAMP) or pathogens (pathogen-associated molecular patterns, or PAMP) are recognised by pattern recognition receptors (PRR) on the plasma membrane of plants that identify conservative structures of phytopathogens (Jones and Dangl, 2006). Viruses can enter plant cells manually through inoculation or vectors such as nematodes, fungi, and insects since they are unable to overcome the first type of universal resistance on their own. They can penetrate the cell wall's physical defences because of this.

Although the plant's apoplast does not directly identify phytoviruses, Korner *et al.* (2013) showed that receptor-like kinases (RLKs) may be involved in this process. Phytoviruses may come across specific resistance genes as a form of defence. These genes offer defence against related viruses. There are two types of resistance genes: dominant genes, most of the NB-LRR type, and recessive partial resistance genes (Fig. 1). The Ty-1 from tomatoes is an illustration of a dominant resistance gene for the tomato yellow leaf curl virus (TYLCV). Even though the amount of virus in plant cells carrying Ty-1 is low, tomato plants do not show any symptoms after being infected with TYLCV (Verlaan *et al.*, 2013).

Dominant R genes cause a hypersensitive response or an extreme response. Plants' programmed cell death response, which targets infected cells and prevents the infection from spreading throughout the body, is involved in both processes. The expression of pathogenesis-PRP-genes (pathogenesis-related proteins), salicylic (SA) and jasmonic (JA) acids, nitride oxide (NO), ethylene, reactive oxygen species (ROS), Ca^{2+} ions, and other molecules are all activated during this process. Salicylic acid, ROS, and Ca^{2+} are the essential substances needed for the molecular mechanisms of viral infection resistance, as has been shown (Carr, 2010). Before now, it was thought that the resistance response's component, the hypersensitive response, was also present. However, recent studies on the signaling of the

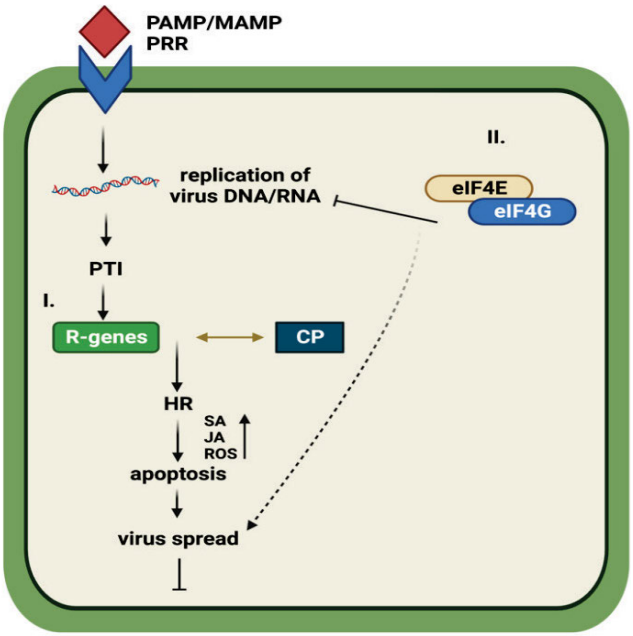

Fig. 1. Antiviral strategies based on susceptibility of dominant and recessive genes; I: dominant resistance is based on the interaction between the avirulence factor (capsid protein, CP) and the R gene product; II: recessive resistance, which corresponds to the absence of appropriate host factors (eIF4E/4G) required for the viral cycle, is a non-receptive resistance, passive and effective throughout plant colonisation; source: own elaboration

downstream protein MLA10 have shown that the physiological pathways for the hypersensitive response and resistance are different (Bai *et al.*, 2012). It should be highlighted that undesirable phenotypic traits are associated with the dominant genes for plant virus resistance that have been discovered. For instance, the *Arabidopsis thaliana* ssi2 mutant accumulates significant quantities of salicylic acid and displays aberrant dwarfism in addition to conferring *Cucumber mosaic virus* (CMV) resistance (Sekine *et al.*, 2004). Therefore, the evolution of plant viruses cannot benefit from these plant resistance genes. The consequences of recessive resistance are modulated by host plant susceptibility factors (Truniger and Aranda, 2009). Plant viruses recruit host cell translation factors not only to translate their viral RNAs but also to facilitate other infection processes, which led to the original identification of translation-related host factors as proviral factors. Because they encode translation initiation factors of the eIF4E/eIF4G family, recessive resistance genes are frequently used to halt the spread of the plant potyvirus family (Kang *et al.*, 2012). 4E/4G interacts with the viral transcript cap structure to translate. Potyviruses don't have cap structures, but they do have VPg proteins that make it possible to translate transcripts even in the absence of a cap. The requirement for the interaction of cap-like structures with eIF4E/eIF4G for translation implies the existence of strict selection in the host selection mechanism. Although recessive S-genes can have unwanted side effects such as spontaneous necrosis and dwarfism, dominant R-genes are more resistant to infections than recessive S-genes (Gawehns, Cornelissen and Takken, 2013).

Recessive genomic mutation methods and the introduction of resistance genes are employed to combat TYLCV and other viruses in crops (Kunik *et al.*, 1994; Verlaan *et al.*, 2013). However, antiviral strategies based on recessive resistance mostly

rely on the use of eIF4 and their homologs against potyviruses and other similar plant viruses. It is essential to discover and exploit more genes that increase host sensitivity to obtain efficient genetic resources against several different plant viruses with major economic impact.

THE EFFECTIVE APPROACH TO THE CREATION OF TRANSGENIC PLANTS RESISTANT TO VIRAL INFECTION – RNA INTERFERENCE

One of the early innate responses to viral infection is the reduction of intracellular viral RNA. The mechanism by which RNA silencing takes place in plants was first discovered in 1990 (Napoli, Lemieux and Jorgensen, 1990). When exogenous double-stranded (ds) RNA molecules are present, this mechanism – also known as RNA interference – inhibits transcript translation or their sequence-specific hydrolysis. RNA-dependent RNA polymerase (RDR), suppressor of gene silencing (SGS), dicer-like (DCL), argonautes (AGO), and other proteins are also involved in RNA interference. Through the cleavage and destruction of antisense RNA or the recruitment of DNA modifiers and histones, which in turn limit the transcription of the target gene, these proteins participate in the successive processes that result in the suppression of foreign gene expression (Fig. 2) dsRNA-mediated silencing can shield the plant host from viruses in addition to regulating how a plant grows and develops (Voinnet, 2005; Ding, 2010; Ipsaro and Joshua-Tor, 2015). To make virus-resistant transgenic plants, methods based on several precursor RNAs have been developed (Duan, Wang and Guo, 2012). RNA suppression technology has been used to successfully stop 60 types of economically important plant viruses, such as *the Papaya ringspot virus* (PRSV) (Bau *et al.*, 2003), *Plum pox virus* (PPV) (Hily *et al.*, 2007; Kundu *et al.*, 2008; Sidorova *et al.*, 2019), *Maize dwarf mosaic virus* (MDMV) (Zhang *et al.*, 2010; Zhang *et al.*, 2013), *Tomato yellow leaf curl virus* (TYLCV) (Fuentes *et al.*, 2016), and many more.

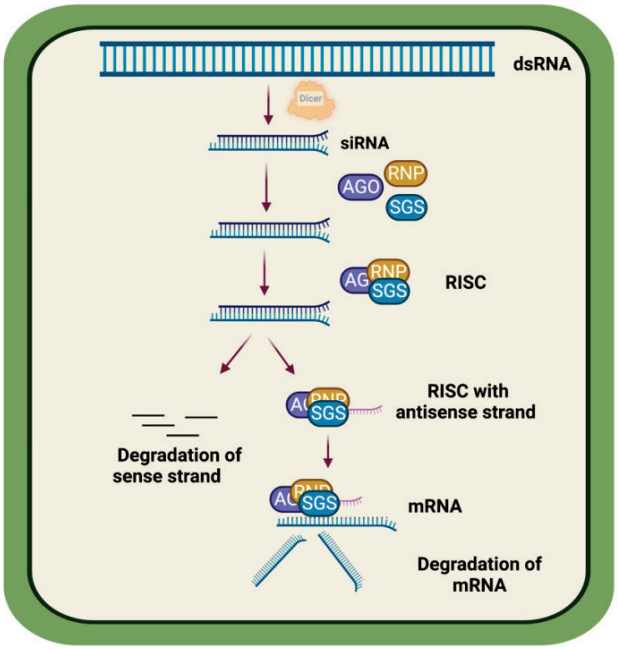

Fig. 2. RNA-induced gene silencing; source: own elaboration

These examples were all obtained using plant genetic modification techniques. Exogenous administration of naked dsRNA, which successfully initiates RNA silencing against plant viruses, has been used in approaches to allay public anxiety (Gogoi *et al.*, 2017; Worrall *et al.*, 2019; Das and Sherif, 2020). Using multilayer hydroxide nanosheets as carriers, Mitter *et al.* (2017) created a novel dsRNA delivery technique, developing *Cucumber mosaic virus* (CMV) resistance in tobacco plants.

The use of RNAi-based technology, therefore, has a great potential to overcome the shortcomings of conventional viral resistance breeding for several reasons, including the requirement for only viral sequence information (to crops with limited genome sequence information), the absence of the need for genetic crossing and the selection of segregating offspring (reducing the breeding period), and the induction of RNA silencing by exogenous application of dsRNA (in viral pandemics).

Due to co-evolution, the majority of plant viruses have developed defences against RNA interference. One of the most effective instances of this technique is the coding of RNA interference suppressor proteins by viruses (Omarov and Scholthof, 2012). In addition to controlling the process of plant RNA silencing, suppressor proteins can also be used to reduce DICER and RISC (RNA-induced silencing complex) activity, sequester dsRNA/siRNA and destabilise AGO proteins (Mann *et al.*, 2016; Iki, Tschopp and Voinnet, 2017; Kenesi *et al.*, 2017). The 19 kDa protein P19 of the *Tomato bushy stunt virus* (TBSV) is one of the best-researched suppressor proteins (Omarov *et al.*, 2006). By developing viral mutants inoculated with *Nicotiana benthamiana* and *Vigna unguiculate* plants, the dose-dependent effects of the P19 protein were investigated. Visualising GFP expression and identifying the siRNA/P19 complex allowed researchers to monitor viral RNA buildup and the RNA interference response (Qiu, Park and Scholthof, 2002; Shamekova *et al.*, 2014).

CRISPR/CAS AS NEW GENOME EDITING TOOLS

The science of genome editing is a brand-new, quickly evolving direction in the development of virus-resistant plants. Clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated Cas proteins offer acquired immunity against viruses and other mobile genetic elements in bacteria and archaea, analogous to RNAi-mediated plant defence against pathogens. About 40% of bacterial genomes and 70% of sequenced archaeal species have these CRISPR sequences, which are a component of the bacterial immune system and confer endogenous adaptive immunity (Mohanraju *et al.*, 2022; Dimitriu *et al.*, 2022). The CRISPR array stores a sort of "immune memory" of phages and plasmids in the form of brief spacer sequences that intercalate between repetitions and specify CRISPR/Cas immune targets. In addition to the spacer-repeat matrix, the locus contains operons containing CRISPR-associated Cas genes. The transcript is converted into individual spacer repeat units in the form of short guide crRNA molecules upon the introduction of a new phage, and the spacer repeat template is repeated as a lengthy pre-CRISPR RNA (pre-crRNA). These molecules instruct RNA-guided Cas nucleases to break foreign nucleic acids based on nucleotide complementarity. As a result,

the CRISPR system prevents infection by cleaving the viral or plasmid target DNA according to predefined sequences. The specificity of the CRISPR/Cas system depends on the specificity of the crRNA molecules that effectively direct the nuclease to its target, a complementary target DNA fragment, similar to the RNA interference process (Fig. 3).

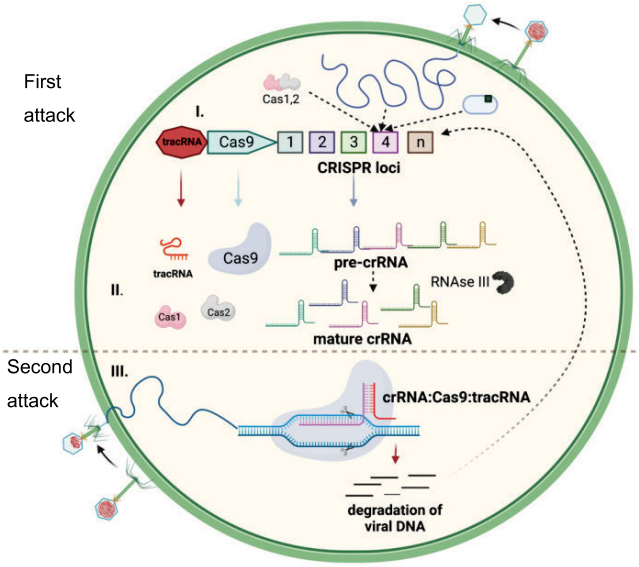

Fig. 3. Adaptive immunity through CRISPR/Cas systems in bacterial cells; source: own elaboration

CRISPR/CAS BASED DNA PLANT VIRUSES RESISTANCE

The CRISPR system is now divided into two classes and six subtypes according to a taxonomy (Shmakov *et al.*, 2017; Tal and Sorek, 2022). The nature of the effector complexes is the primary distinction between these groups. In bacteria and archaea, the first class (types I, III, and IV) produces multisubunit effector complexes by combining numerous Cas proteins and crRNA. The second class (types II, V, and VI) solely includes bacteria and uses one multidomain protein (Wang *et al.*, 2021). The immune system of *Streptococcus pyogenes* targeted against type II, second-class invading DNA molecules, yielded the CRISPR/Cas9 system that has been the subject of the most research (Gao, 2021). This complex, which consists of the Cas9 protein and the short RNAs tracrRNA and crRNA, can cleave viral or foreign plasmid DNA that enters a cell in vivo. A short nucleotide sequence known as a protospacer adjacent motif (PAM) motif is required for cleavage (Baltes *et al.*, 2017).

The CRISPR/Cas9 method has been employed in recent years to battle eukaryotic viruses, particularly in preventing the invasion of plant DNA viruses by altering the viral genome (Fig. 4) (Kis *et al.*, 2019; Robertson, Burger and Campa, 2022). There are primarily two methods for employing CRISPR/Cas technology to eradicate plant viruses. One method for preventing the reproduction and infection of invasive viruses is to specifically target the viral genome's breakdown. The other approach is to modify host susceptibility elements required for the viral life cycle or infection to boost plant immunity and prevent viral invasion.

Plant DNA viruses copy their genome in the nucleus after entering plant cells. The Cas9:sgRNA (subgenomic RNA)

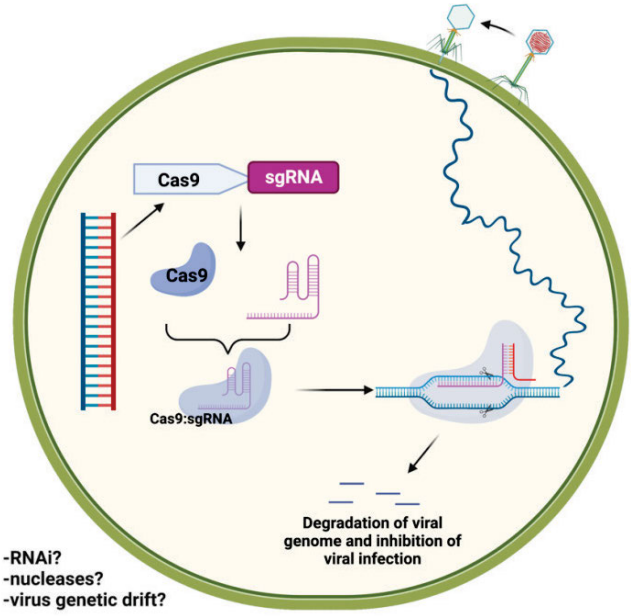

Fig. 4. CRISPR/Cas9-mediated viral interference on plant cells; source: own elaboration

complex is made up of the Cas9 protein and the gRNA (guide RNA), both of which are expressed by the plant genome. By cleaving the viral genome through the production of double-strand DNA breaks (DSB), which can be repaired by non-homologous end joining (NHEJ) repair, the Cas9:sgRNA complex targets viral dsDNA at complementary target sites. In contrast, the development of double-strand breaks might result in the viral genome.

This method has been applied to model plants as well as barley, conferring on plants a highly effective resistance to the *Wheat dwarf virus* (WDV) (Zhang *et al.*, 2022). Because eukaryotic viruses have not yet evolved the ability to withstand CRISPR immune systems, this method has an advantage. The CRISPR/Cas9 system's ability to target and cleave just dsDNA, however, means that it can only be used to combat DNA viruses (Singh *et al.*, 2022).

The families *Geminiviridae* and *Caulimoviridae*, which have 485 species with ssDNA and 85 species with dsDNA, respectively, are two of the most prominent DNA-genomic viral families. Studies aimed at creating technologies against the DNA of culimoviruses and geminiviruses are therefore quite interesting to reduce the threat to the agro-industry. The manipulation-friendly CRISPR/Cas technique soon gained popularity in plant antiviral engineering. Thus, resistance to the *Beet severe curly top virus* (BSCTV), *Bean yellow dwarf virus* (BeYDV), and *Cotton leaf curl Multan virus* was established on *N. benthamiana* and *A. thaliana* model plants (CLCuMuV). For the successful DNA interference of the aforementioned viruses (Yin *et al.*, 2019; Tripathi, Ntui and Tripathi, 2021; Li *et al.*, 2022), locations connected to viral replication and translation (RBS, three Rep motifs essential for rolling replication), and on fsGFP, respectively, were targeted by CRISPR/Cas constructs with single guide RNAs. However, Ali *et al.* (2016) discovered that secondary structure-targeted guide RNAs had more effective silencing of geminiviruses like *Cotton leaf curl Kokhran virus* (CLCuKoV) and *Merremia mosaic virus* (MeMV) (Kumar, Kumar and Sinha, 2022) compared to guide RNAs targeting the capsid protein and replicase of *Tomato yellow*

*leaf curl virus (*TYLCV) (Tashkandi *et al.*, 2018). This was done by comparing viral titer levels in tomato and *N. benthamiana*. These results show that geminivirus IRs, but not CRISPR/Cas9-induced ORF variations, can copy and spread throughout the body without being noticed by the CRISPR/Cas9 system.

The employment of several systems for delivering CRISPR/Cas9 constructs with guide RNAs, the most prominent of which is transitory expression of CRISPR genes, can result in the activation of protection mechanisms against transgenes in plant cells. The function of the RNAi defence system in plants can influence the method of genome editing. Mao *et al.* (2018) found that inhibiting RNA interference by co-expressing the TBSV P19 suppressor protein enhanced the efficacy of CRISPR/Cas9 in editing the AP1 and TT4 genes in *Arabidopsis* plants.

Because the evolution of eukaryotic viruses did not take place in the presence of prokaryotic Cas9, they are unlikely to have developed natural defensive mechanisms against Cas9 nucleases. However, Ali *et al.* (2016) discovered that some geminiviruses generate viral variants that are resistant to the CRISPR/Cas9 mechanism.

With the advancement of knowledge about the CRISPR/Cas system, several iterations of Cas proteins have been identified in vivo from diverse bacterial RNA strains. It was discovered in 2013 that a *Francisella novicida* Cas9 protein variation (FnCas9) can target RNA sequences (Schunder *et al.*, 2013). This gave a chance to use editing techniques to create targeted resistance to an RNA virus. In hepatoma cell lines, (Price *et al.*, 2015) employed CRISPR/FnCas9 to target the ssRNA-positive hepatitis C virus genome. When compared to the control, transient production of FnCas9, sgRNA complexes targeting the 5'- or 3'-untranslated regions of the hepatitis C virus genome resulted in a 50–60% reduction in viral protein expression. Plant virus RNA was also effectively modified and targeted by FnCas9. The system was modified to handle the CMV and TMV viruses. The CRISPR/FnCas9 system, which is regulated by a specific sgRNA sequence, has been found to target viral RNA and limit its infection in plants by 40–80% (T. Zhang *et al.*, 2018). It should be highlighted that viral suppression is accomplished by binding to the target RNA rather than through the ability to hydrolyse with FnCas9 (Tab. 1).

Viruses can resist the CRISPR/Cas9 system, notwithstanding its functional efficiency. To modify persistent viral resistance in plants, it is necessary to measure the degree and frequency of this natural resistance. Furthermore, employing the CRISPR/Cas9 system to target the viral genome resulted in the development of double-strand breaks. Error-prone non-homologous end joins are used to fix these damages. As a result of probable alterations in RNA sequences corresponding to critical regions necessary for Cas9 activity, such as spacer and PAM sequences, this repair mechanism may result in the production of viral variants capable of evading the CRISPR/Cas9 recognition mechanism. Because viruses evolve quickly and CRISPR/Cas9 does not accept mismatches in the original spacer sequences near the PAM, any change in this region reduces or destroys CRISPR/capacity Cas9's ability to target the virus, resulting in virus variants that can dodge the editing process.

When employing the CRISPR/Cas9 system in *A. thaliana*, sequence-nonspecific effects are well recognised, which can considerably limit the editing method's utility (Q. Zhang *et al.*, 2018). Furthermore, the presence of secondary structures of dsRNA domains in sgRNA can result in the creation of siRNAs, reducing the amount of sgRNA. This potential should be

Table 1. Application of the CRISPR/Cas9 system against viruses plants

Cas9	Plant	Virus	Target sequence	Reference
SpCas9	*Cassava* *Nicotiana benthamiana*	ACMV	ACMV	Mehta *et al.* (2019)
SpCas9	*Solanum lycopersicum* *N. benthamiana*	TYLCV	TYLCV	Tashkandi *et al.* (2018)
SpCas9	*Arabidopsis*	TBSV	AP1, TT4	Mao *et al.* (2018)
FnCas9	*N. benthamiana*	CMV	CMV	T. Zhang *et al.* (2018)
FnCas9	*Arabidopsis*	TMV	TMV	T. Zhang *et al.* (2018)
Cas9	*Oryza sativa*	RTSV	eIF4G	Macovei *et al.* (2018)
Cas9	*N. benthamiana*	MeMV	IR, CP, Rep	Kumar *et al.* (2022)
Cas9	*N. benthamiana*	BeYDV	LIR, Rep/RepA	Li *et al.* (2022)
Cas9	*N. benthamiana*	BSCTV	BSCTV	Tripathi *et al.* (2021)
Cas9	*Arabidopsis*	BSCTV	BSCTV	Tripathi *et al.* (2021)
Cas9	*Arabidopsis*	potyvirus	eIF9(iso)4E	Pyott, Sheehan and Molnar (2016)
Cas9	*Cucumis sativus*	CVYV	eIF4E	Chandrasekaran *et al.* (2016)
Cas9	*Cucumis sativus*	PRSMV-W	eIF4E	Chandrasekaran *et al.* (2016)

Explanations: ACMV = *African cassava mosaic virus*, TYLCV = *Tomato yellow leaf curl virus*, TBSV = *Tomato bushy stunt virus*, CMV = *Cucumber mosaic virus*, TMV = *Tobacco mosaic virus*, RTSV = *Rice tungro spherical virus*, MeMV = *Merremia mosaic virus*, BeYDV = *Bean yellow dwarf virus*, BSCTV = *Beet severe curly top virus*, CVYV = *Cucumber vein yellowing virus*, PRSMV-W = *Papaya ring spot mosaic virus*-W, AP1 = apetala1, TT4 = transparent testa glabra4, eIF4G, eIF9(iso)4E, eIF4E = translation initiation host factors, IR = intergenic region, CP = capsid protein, Rep = replication-associated protein, LIR = long intergenic region.
Source: own study.

considered while creating CRISPR/Cas9 target sequences. Thus, novel NGS-based systems, such as Perturb-Seq (CRISPR-Seq) (Dixit *et al.*, 2016), Guide-Seq (Tsai *et al.*, 2015), and immunoprecipitation with dCas9 (Kuscu *et al.*, 2014), can be used to forecast potential off-target effects. High temperature is another factor that influences the optimal effectiveness of Cas proteins. Repeated high-temperature treatments in *A. thaliana* result in a significant increase in Cas9 efficiency (LeBlanc *et al.*, 2018). Heat treatments also result in efficient Cas12a editing in *A. thaliana* (29°C) and maize (28°C) (Malzahn *et al.*, 2019).

Based on the above mentioned, active searches for novel Cas protein variations are now underway. This could serve as a platform for concentrating work on the most difficult issues connected with viruses' fast evolution and, as a result, their capacity to avoid the CRISPR/Cas editing process. As a result, the development of genomic editing technologies, particularly those focused on targeting RNA-containing viruses, is a pressing issue. It should be emphasised that this class contains around 70% of all viruses that infect plants.

To date, fully novel Cas systems of class 2 have been found; Cpf1, also known as Cas12 (Schunder *et al.*, 2013), is one of them. Following that, its two orthologs (Cas12b and Cas12c) were discovered to have considerable activity in eukaryotic cells (Zetsche *et al.*, 2017; Kurihara *et al.*, 2022). Cas12 proteins were classified into type VI-B of the CRISPR/Cas system's second class due to the presence of a RuvC-like domain in them. Cas12, on the other hand, does not require tracrRNA and is powered by a single RNA. This discovery is essential for the advancement of genetic engineering because the formation of stepwise breaks, as opposed to Cas9's blunt breaks, and the requirement for diverse PAMs considerably widens the collection of tools for various types of manipulations.

DIRECT CRISPR/CAS-MEDIATED INHIBITION OF RNA PLANT VIRUSES

Using bioinformatic analysis, Shmakov *et al.* (2017) predicted a new type of class 2 Cas protein named C2c2 (Cas13). This type has two nucleotide-binding domains of higher eukaryotes and prokaryotes (higher eukaryotes and prokaryotes nucleotide binding, HEPN) that are solely involved with RNase activity, while pre-crRNA processing takes place in the N-terminal domain of helical-1 (REC) (Knott *et al.*, 2017). All of this suggests that Cas13 can act as a single effector RNA-gated target protein that cleaves ssRNA (Fig. 5). Mutations in HEPN domains, particularly in the putative histidine and arginine catalytic residues, impede Cas13 protein cleavage, resulting in the formation of a catalytically inactive variant of the Cas13a enzyme (deadCas13a).

In the presence of PFS (protospacer flanking sequence), Cas13a cleaves its 22–28 nt crRNA (preferably A, Y, or C nucleotides). In contrast to Cas9, Cas13a displays in vitro collateral activity, which results in the hydrolysis of circulating RNAs in the cell regardless of crRNA homology or the presence of PFS.

Cas13a's nonspecific RNase activity restricts viral infection spread as a natural defence mechanism for identifying viral invasion. This defensive system causes programmed cell death. Nonspecific degradation of free-circulating RNAs is observed

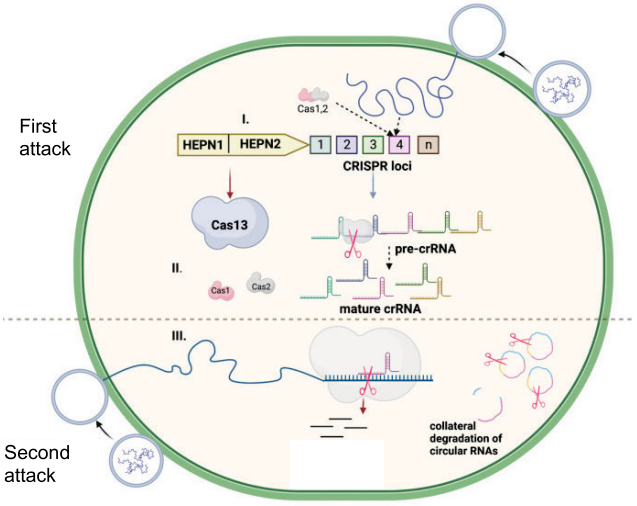

Fig. 5. CRISPR/Cas13-mediated viral interference in cells; I stage: adaptation, II stage: maturation, III stage: CRISPR/Cas13a-mediated interference. source: own study

exclusively in bacterial organisms in vitro but not in eukaryotic cells (Abudayyeh *et al.*, 2017). While this Cas13 collateral activity may appear to be a negative in terms of particular RNA editing, it has transformed the Cas13 family of enzymes into a potent tool for creating CRISPR/Cas-based diagnostics. As a result, the development of the diagnostic instrument SHERLOCK (Specific High-sensitive Enzymatic Reporter unLOCKing) proved the potential of employing Cas13 as a disease detection platform (Cox *et al.*, 2017; Gootenberg *et al.*, 2018). The modified SHERLOCK technology has also been used for nucleic acid detection in soybeans to research the glyphosate resistance gene. As a result, the flexibility and substantial potential of implementing this application were demonstrated (Gootenberg *et al.*, 2017). As a result, this Cas13 ortholog-based method may most likely be utilised to identify numerous viruses and viroids in plants in a short time. All of this will result in significant progress in efforts to establish innovative ways to control the quality of agricultural products.

In 2018 (Aman *et al.*, 2018; Abudayyeh *et al.*, 2019), additional research on planned ssRNA cleavage of plant viruses was conducted. *N. benthamiana* and *A. thaliana* Cas13a isolated from *Leptotrichia shahii* with crRNA targeting viral RNA sequences demonstrated quick and effective RNA interference with *Turnip mosaic virus* in transgenic plants, as predicted (TuMV). In a vector based on the *Tobacco rattle virus,* the *Pea early browning virus* (PEBV) promoter drove the crRNA (TRV). Due to the quick and effective expression of guide RNAs and/or Cas proteins during viral infection, using viral vectors to carry guide RNAs and/or Cas proteins dramatically improves the effectiveness of the CRISPR/Cas system. Transgenic tobacco and rice plants were made resistant to TMV, *Rice stripe mosaic virus* (RSMV), and *Southern rice black-streaked dwarf virus* (SRBSDV) using the CRISPR/LshCas13a system (Ashraf *et al.*, 2022). This method has also been demonstrated to be quite efficient in halting sickness (Zhang *et al.*, 2017). It does this by specifically targeting the reading frames of the P3, NIb, or CP *Potato virus Y* (PVY) viral proteins.

For targeted RNA knockdown in human cells, the Cas13a orthologue obtained from *Leptotrichia wadei* was employed

Table 2. Application of the CRISPR/Cas13 system against plant viruses

Cas13	Microorganism	Plant	Virus/ protein/ infection	Targeted sequence	Reference
Cas13d (CasRx)	*Ruminococcus flavefaciens*	*Nicotiana benthamiana*	TuMV-GFP	HC-Pro, CP, GFP	Ai, Liang and Wilusz (2022)
Cas13d (CasRx)	*Ruminococcus flavefaciens*	*N. benthamiana*	PVX-GFP	PVX	Ai, Liang and Wilusz (2022)
LshCas13a	*Leptotrichia shahii*	*N. benthamiana*	TuMV	HC-Pro, CP, GFP	Abudayyeh *et al.* (2019)
LwaCas13a	*Leptotrichia wadei*	plant	GFP	viral genome	Knott *et al.* (2017)
LshCas13a	*Leptotrichia shahii*	rice	SRBSDV	SRBSDV	Ashraf *et al.* (2022)
LshCas13a	*Leptotrichia shahii*	*Solanum tuberosum*	PVY	NIb, P3, CI, CP	Zhang *et al.* (2017)

Explanations: TuMV = *Turnip mosaic virus*, GFP = green fluorescent protein, PVX = *Potato virus X*, SRBSDV = *Southern rice black-streaked dwarf virus*, PVY = *Potato virus Y*, HC-Pro = helper component proteinase, CP = capsid protein, NIb = RNA-dependent RNA polymerase, P3 = potyviral membrane protein, CI = protein forms the laminate cytoplasmic inclusion bodies.
Source: own study.

(Abudayyeh *et al.*, 2017). The level of RNA knockdown was comparable to that of RNAi, while the CRISPR/Cas13 system outperformed RNAi in terms of selectivity. The effectiveness of RNA knockdown induced by CRISPR/LwaCas13 in plant cells was validated by the same group of researchers. Three distinct genes were targeted at rice protoplasts (*Oryza sativa*) in the experiment. The LwaCas13a and three additional vectors encoding crRNAs designed against the EPSPS, HCT, and PDS genes were transfected into plant protoplasts. A knockdown of around 50% was achieved 48 h after transformation. This highlights the applicability of the approach to a broad spectrum of modified organisms and shows that Cas13 can quickly diminish the pool of cytoplasmic RNA in plants.

Another HEPN-containing effector that targets RNA has been found using computational sequencing data mining. C2c6 (Cas13b) was the protein's given name (Zhan *et al.*, 2019). Prevotella sp. P5-Cas13b 125's were classified as class 2 subtype VIB. The great functionality of Cas13b for RNA degradation targeting in eukaryotic cells was shown through analysis to be independent of the stabilisation domain (msfGFP) as demonstrated in LwaCas13a. According to data on the viability of CRISPR/Cas13b-mediated manipulation in *N. benthamiana*, PspCas13b stably inhibits TMV-GFP accumulation with 50% and 52% effectiveness in agro infiltrated and systemic leaves, respectively (Yu *et al.*, 2022). However, nucleases of other Cas13 orthologues exhibited a higher index than Cas13b when compared to the average efficiency of viral titer interference, hence this nuclease was not used further in the tests. The authors of the study also co-inoculated plants with TuMV-GFP and CMV-DsRed viruses, and they used a lamp and filters to observe the growth of the two RNA viruses. The authors created pre-guide RNAs that were complementary to the sequences of TuMV-NIb, TuMV-GFP, CMV-1a, and CMV-2a. For TuMV, LwaCas13a and RfxCas13d had 95% and 91% suppression effectiveness, respectively, whereas for CMV, LwaCas13a and RfxCas13d had 68% and 66% suppression efficiency. This means that at least two Cas13 nucleases can make pre-guide RNAs, which can be used to make multifunctional gRNAs that can be used to target multiple related viruses or different RNA viruses in plants.

Small in size (930 amino acids), the newly discovered Cas13d protein from *Ruminococcus flavefaciens* can regulate the splicing of endogenous transcripts (Smargon *et al.*, 2017).

During research aiming at inhibiting TuMV infection by targeting the GFP, CP, or HC-Pro region in the TuMV-GFP genome, Cas13d was discovered to have significant advantages over other Cas13 variations (Konermann *et al.*, 2018). They also demonstrated that the Cas13d system may be used to simultaneously target two RNA viruses, increasing its utility. According to a recent study, using the CmYLCV promoter instead of the AtU6 promoter, which provided an efficiency of roughly 50%, enhances RNA interference efficiency for RxCas13d by 85% (Mahas, Aman and Mahfouz, 2019; Ai, Liang and Wilusz, 2022). Following authors demonstrated that guide crRNAs for Cas13 can downregulate viral and endogenous RNAs both in the presence and absence of Cas13 nuclease when exploring RNA silencing against TMV utilising the CRISPR/Cas system (Sharma *et al.*, 2022). Instead, a stable transgenic line expressing the guide crRNA is required in this instance.

To resist mixed viral incursions that happen in field, natural conditions, the system may have potential use in crops. Notably, the new coronavirus SARS-CoV-2 and influenza A virus have both been thwarted by the Cas13d-mediated PAC-MAN (Prophylactic Antiviral CRISPR in huMAN cells) RNA interference system in human lung cells (Tab. 2) (Abbott *et al.*, 2020).

All of these findings suggest that Cas13 can work as a component of a successful, adaptable CRISPR/Cas system with a lot of potential for precise, strong, and scalable RNA targeting applications (Tab. 3).

CRISPR/CAS-MEDIATED PLANT VIRUS RESISTANCE THROUGH PLANT SPECIFIC FACTORS

The CRISPR/Cas system can be used to identify specific plant components that interact with viral proteins for targeted editing. Combining these findings with computational biology's use of molecular dynamics research has a lot of potential. All of this will guarantee the continued development of numerous strategies to combat viruses that are evolving quickly. Research on multi-virus plant resistance has now shown that four specific groups of possible plant factors are interesting targets. These include: recessive genes involved in translation-eEF1A and eEF4; negative regulators of transcription-rgs-CaM; enzymes involved in post-translational modifications of proteins-HAT2 and HAT3, protein kinase SK4-1, NsAK, ubiquitin ligase; factors of phenylpropanoid

Table 3. General characteristics of CRISPR/Cas system variants

Specification	CRISPR/Cas variant					
	CRISPR/Cas9	**CRISPR/FnCas9**	**CRISPR/Cas12**	**CRISPR/Cas13a**	**CRISPR/Cas13b**	**CRISPR/Cas13d**
Class	class II, type II	class II, type II	class II, type II	class II, type VI-A	class II, type VI-B	class II, type VI-D
Microorganism	*Streptoc occus pyogenes*	*Francisell a novicida*	*Acidaminoco ccus* sp.	*Leptotrich ia* shahii, *Leptotrichia wadei*	*Prevotella sp.*	*Ruminococcus flavefaciens*
Effector protein	Cas9	Cas9	Cas12	Cas13a	Cas13b	Cas13d
Component	Cas9, sgRNA	Cas9, sgRNA	Cas12, gRNA	Cas13a, gRNA	Cas13b, gRNA	Cas13d, gRNA
Length targets	~18–22 nt	~18–22 nt	23–25 nt	~28–30 nt	~30 nt	~28–36 nt
PAM/PFS	G	G	T, C	A, U, C	A, U, C	A, U, C
Catalytic domains	RuvC, HNH	RuvC, HNH	NuC, RuvC	HEPN 1, 2, Helica I	HEPN 1, 2, Helica I	HEPN 1, 2, Helica I
Enzymatic activity	targeting of DNA	cytosolic RNA targeting	targeting of DNA	RNA knockdown	RNA knockdown	transcript splicing control with in vivo capability delivery, RNA knockdown
Multiplexing	+	+	+	+	+	+
Off-sites	different	different	different	collateral RNA degradation	collateral RNA degradation	–

Source: own study.

metabolism and secondary cell wall synthesis, the suppression of which delays infection with geminiviruses-4-coumarate, CoA ligase1, 4CL1, Bearskin2B, and BRN2 (Cao *et al.*, 2020).

PROSPECTS FOR EMPLOYING THE CRISPR/CAS SYSTEM

Genetic engineering and the creation of new virus-resistant plant species have enormous potential for increasing food production and food security. Through the NLS-cas13 fusion, Cas13 can target non-coding nuclear transcripts in contrast to RNAi, which can only target cytoplasmic transcripts. The CRISPR/Cas13 system is a ground-breaking method for developing plant immunity to both localised and broad viral infections. This platform can be utilised for genome-wide gene function research, as a diagnostic tool, and to understand how RNA knockdowns against the plant transcriptome work. Additional research is needed to establish whether RNA viruses may also live in the presence of Cas9 while avoiding the CRISPR/Cas13 system in plants. Recent studies have demonstrated that DNA viruses can survive in the presence of Cas9.

It is still mostly unknown how viral sequences are acquired for CRISPR arrays. It would be interesting to examine the viability of using the complete complement of CRISPR/Cas tools to acquire a spacer in eukaryotic cells. The prospective acquisition might make clear novel strategies for creating virus resistance, like the ability to repeatedly generate virus resistance. It will also provide data and the ability for site-specific DNA integration into eukaryotic genomes, opening up fresh possibilities for plant genomic engineering.

Additionally, the current method of genome editing will substantially and broadly boost viral resistance in crops, eventually permitting commercialisation. Because of this, the adoption of CRISPR technology may soon provide a useful and well-known means of enhancing crop resistance to viruses. The development of CRISPR/Cas-mediated virus resistance (see Fig. 6) is possible in any plant species with genomic sequences known to be resistant to any virus or set of viruses. This method has opened up new possibilities for studying the interactions between viruses and plants and for engineering broad-spectrum viral resistance.

The application of CRISPR/Cas gene editing technology may result in the following issues that must be addressed:

– essentially, CRISPR modulation results in the complete loss of target gene function, which can harm plants in a variety of ways; tissue-specific targeting as a new genetic tool based on

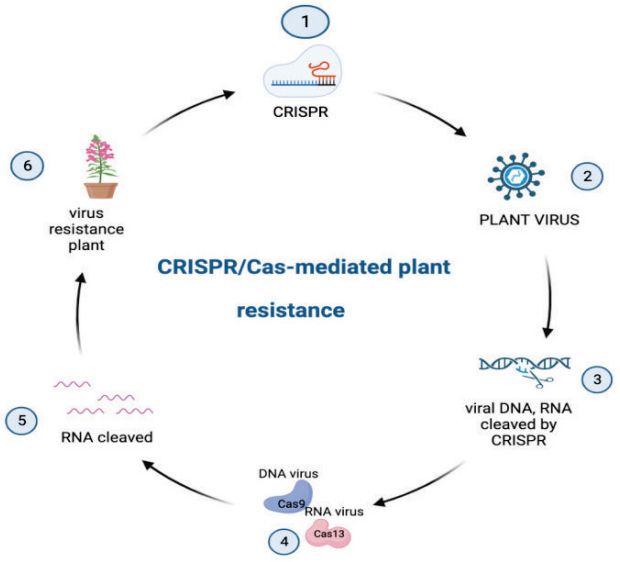

Fig. 6. CRISPR/Cas-mediated plant resistance; 1 = optimization of the CRISPR/Cas system for expression *in planta*, 2 = selection of target virus sequences, 3 = targeting the CRISPR/Cas system to the genetic material of viruses in plants, 4 = activation of Cas9 proteins on DNA, Cas13 on RNA virus genome, 5 = directed degradation of viruses in plants, 6 = obtaining plants resistant to viral diseases; source: own study

CRISPR should be developed to solve this problem; there is currently no information on the use of such a method in plants;
– in order to use CRISPR-based tools, more fundamental answers about plant-virus interactions, as well as the molecular mechanism of plant resistance, are required.

So far, reports indicate that knocking out specific plant genes can confer resistance to biotic stressors. The improved plant varieties that result will need to be approved by regulatory authorities for consumption and industrial use.

CONCLUSIONS

One of the highest priorities of studying viruses and their interactions with host cells is to combat the spread of viral infection in crop communities to reduce yield or quality losses. This work may lead to the development of more integrated methods for increasing plant resistance to viruses, as well as the stimulation of the interaction of genomic editing systems with plant internal defence mechanisms, which can not only protect crops but also increase their productivity. The current study also emphasises the importance of conducting systematic studies to not only document but also investigate the effects of using and optimising conditions for RNAi induction, CRISPR/Cas, and targeting gene silencing in viral infection.

Understanding the molecular mechanism of guide RNA selection in the CRISPR/Cas system in plants under various developmental and environmental conditions is one of the future directions of research in the development of pathogen-resistant plants.

These CRISPR-based tools will also help us understand the molecular mechanisms underlying viral infection, resistance, and host cell susceptibility. It is worth noting that these platforms are excellent for studying viral repair and recombination in plant cells' extrachromosomal DNA. Furthermore, one can try to build plants using the entire CRISPR/Cas system and investigate the presence of spacers in CRISPR arrays in the plant genome. These experiments will provide information on the integration of foreign DNA into the plant genome at a specific location, which will be useful in plant engineering. Genome-wide CRISPR/Cas systems may be used to identify host susceptibility and resistance factors to improve our understanding of the molecular basis of viral biology and to apply this knowledge through the development of plant immunity and virus-resistant crops. Furthermore, due to the ongoing arms race between viruses and host defence systems, studying and quantifying viruses evading CRISPR/Cas remains critical.

FUNDING

This research has been funded by the Science Committee of the Ministry of Education and Science of the Republic of Kazakhstan (Grant No. AP09258746).

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