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Targeting Plant ssDNA Viruses with Engineered Miniature CRISPR-Cas14a

Muhammad Zuhaib Khan,[1] Sibtain Haider,[1] Shahid Mansoor,[1] and Imran Amin[1,*]

CRISPR-Cas14a is a highly compact protein with great potential to be used as a guided genome editing tool for single-stranded (ss) DNA cleavage. Recently isolated from noncultured archaea, its unrestricted and sequence-independent cleavage makes it an ideal tool for engineering resistance against economically important plant ssDNA viruses.

Resistance against Plant Viruses

Plant single-stranded (ss) DNA viruses are an important group of plant pathogens that pose a great threat to global agriculture and food security [1]. Various strategies have been implemented to control epidemics and restrict crop losses caused by ssDNA viruses, including protecting plants from insect vectors and the introduction of virus-tolerant/resistant crop varieties by conventional breeding and transgenic approaches [2]. Despite the introduction of virus-resistant crop varieties, resistance is often overcome due to the rapid evolution of new viral strains. There is a need to develop new strategies to combat these viruses in the field. Multiple defense mechanisms have evolved in bacteria to resist phage infection. The CRISPR-Cas restriction modification system that exploits the targeted nuclease activity of Cas proteins could be engineered into plants to resist virus infection. This adaptive defense mechanism identifies and cleaves the DNA motifs of invading pathogens to ensure host protection [3].

Genome Editing and Programmable Nucleases

The class II type II CRISPR-Cas9 system, with its multidomain protein guided by an RNA molecule to perform binding and editing, can efficiently target double-stranded (ds) DNA with great precision. However, targeted cleavage is often restricted by the absence of a protospacer adjacent motif (PAM) sequence. This system can also target dsDNA efficiently [3]. A class II type VI system, CRISPR-Cas13a (previously known as C2c2), is able to efficiently target and cleave ssRNA [4]. These two systems are limited by the large size of their Cas proteins (Cas9, ~100–200 kDa; Cas13a, ~168 kDa) and their requirements for restrictive sequences [a PAM by Cas9 and a protospacer flanking sequence (PFS) by Cas13a] [5]. The availability of a PAM sequence is required to perform cleavage at the target site. Using computational biology and data-mining approaches, the search for smaller Cas proteins has continued; this search led to the identification of the Cas14 locus, which encodes a 40–70-kDa protein. CRISPR-Cas14a, a variant of this system, can target any ssDNA sequence with great precision without requiring restrictive signal sequences [6] (Figure 1).

Characteristic Features and Mode of Action of CRISPR-Cas14a

Using comparative sequence analysis, different variants of Cas14 proteins have been clustered into three subgroups (Cas14a–c). CRISPR-Cas14a is a compact (400–700 amino acids) RNA-guided targeting system that can cleave ssDNA. It is almost half the size of the previously characterized RNA-guided DNA-targeting Cas9 protein. A RuvC nuclease domain is present in all Cas14 variants, which is a characteristic of type V RNA-guided DNA-targeting systems. Phylogenetic analysis showed similarities between Cas14a and the C2c10 and C2c9 systems, which also contain an RuvC nuclease domain. Metagenomic analysis of the assembled DNA sequences of CRISPR-Cas14 loci demonstrated the diverse nature of the CRISPR array, with active acquisition of DNA spacers on new infection. Compared with other CRISPR systems (Cas9 and Cas12e) where mature CRISPR RNAs (crRNAs) and trans-activating crRNAs (tracrRNAs) are produced by the dsRNA-cleaving RNase III enzyme, no such system exists in the genomes of hosts containing the Cas14a system. Biochemical analysis also suggests that Cas14a is not able to process its own pre-crRNAs [6].

Unlike other known class II systems, Cas14a does not require a flanking sequence (PAM) near the target site [6,7]. *In vitro* validation of the PAM requirement showed that Cas14a could cleave the target site irrespective of the different sequences adjacent to the targets of these different guides. Interestingly, cleavage was performed beyond the guide-complementary region of the ssDNA. Moreover, when purified Cas14a–crRNA–tracrRNA was incubated with radiolabeled targets (dsDNA, ssDNA, and ssRNA), only ssDNA targets were selectively cleaved. This datum suggests either Cas14a is unable to target dsDNA templates or it might do so without the requirement for a PAM sequence. Mutations in the RuvC domain of Cas14a diminish its nuclease activity, suggesting that RuvC is the functional domain for DNA cleavage [6].

Harrington and colleagues [6] also observed target-activated nonspecific ssDNA interference for Cas14a. The system needs the guide to be 100% complementary to the target for guided cleavage. This suggests the presence of an internal seed sequence distinct from the PAM proximal region. Consequently, any mismatch in guide–substrate, truncation, or secondary structures in ssDNA substrate severely reduces the cleavage activity of Cas14a.

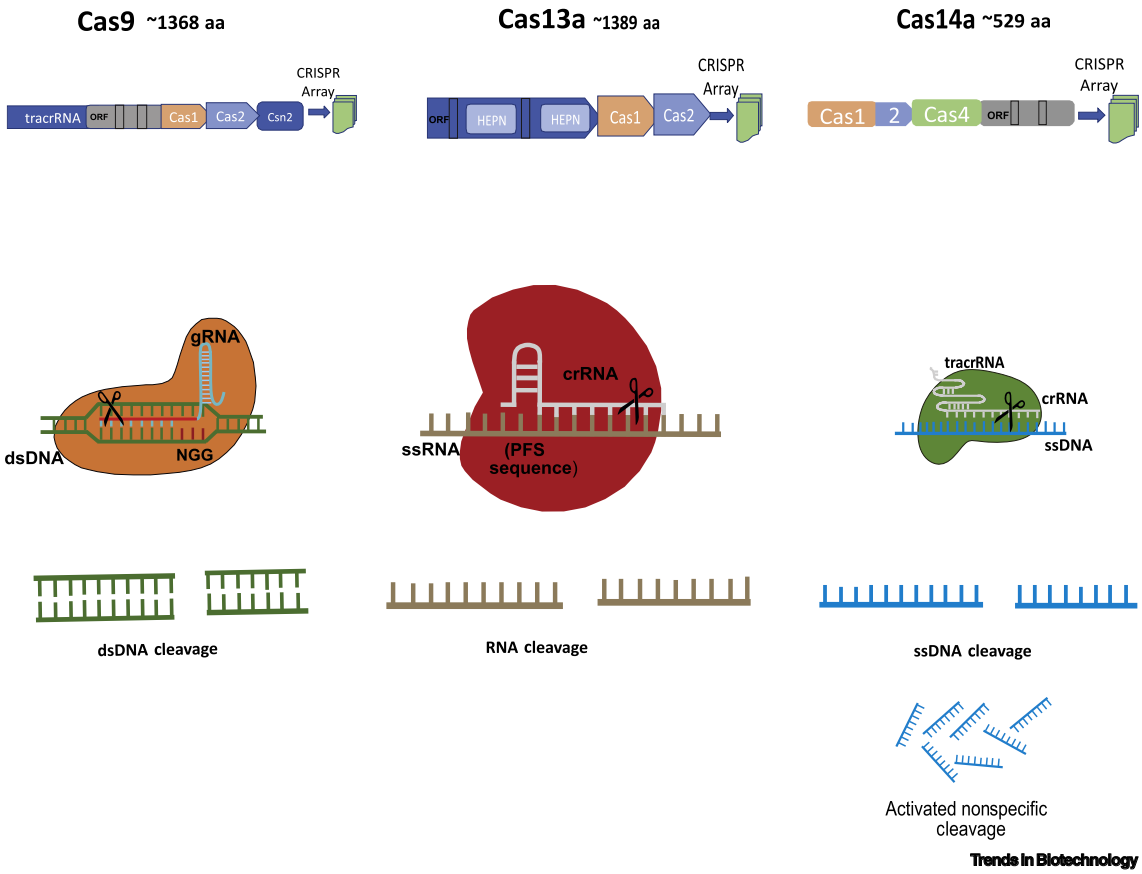


Figure 1. Mechanism of Action of Different CRISPR-Cas Systems. In class II type II CRISPR-Cas systems, Cas9 nuclease [~1368 amino acids (aa)] guided by a 20-bp guide (g)RNA reaches its target and cleaves the double stranded (ds)DNA. The class II type VI Cas13a (~1389 aa) is guided by a 28-bp gRNA and cleaves single stranded (ss)RNA. Once activated, the class II type V Cas14a (~529 aa), half the size of Cas9 and Cas13a, is guided by CRISPR RNA (crRNA) and recruited by trans-activating crRNA (tracrRNA) and cleaves ssDNA specifically and other ssDNA targets nonspecifically. Abbreviation: PFS, protospacer flanking sequence.

CRISPR-Cas14a for ssDNA Virus Interference

CRISPR-Cas14a is a potential system for engineering resistance against plant ssDNA viruses such as those belonging to the families *Geminiviridae* and *Nanoviridae* [8]. Geminiviruses possess mono- or bipartite circular ssDNA genomes, whereas nanovirus genomes comprise multiple circular ssDNA components. These viruses replicate via rolling-circle replication through a double-stranded replicative form [9]. Cas14a can be harnessed to target highly conserved (possibly replication associated protein, pre-coat protein, and intergenic region) sequences in the viral genome. The unique and major advantage of Cas14a is that it does not require any restrictive sequence to target and cleave the viral genome. Thus, virtually any sequence in ssDNA virus genomes can be targeted with high fidelity. The major limitation posed by other systems is the PAM requirement for the Cas protein to cleave a target site. This PAM sequence restricts options in designing guides to target viral genomes. However, using Cas14a, genes that play vital roles in virus replication and survival could be targeted by choosing any sequence as a guide.

Another exciting feature of CRISPR-Cas14a is the collateral activity on target-mediated activation, as shown in Figure 1 [6]. The occurrence of multiple viruses has been observed during geminivirus infection [10]. On activation of its collateral activity, Cas14a will also target other ssDNA viruses in its vicinity. Thus, this system can be leveraged to target different ssDNA viruses in a multiplex approach. This property furthermore suggests the possibility of using Cas14a in high-throughput diagnosis of ssDNA viruses. Paper-based lateral-flow assays (LFAs), which are instant and low-cost diagnostic tools that can detect virus particles in tissue samples, can be developed to detect viral infections. CRISPR-Cas14a based LFAs could be developed for real-time

qualitative and quantitative detection of viral infection. Finally, by incorporating a small number of point mutations in the RuvC domain this system may become capable of targeting dsDNA as well.

Dual Immunity against ssDNA Viruses

A major advantage of CRISPR-mediated viral interference is the availability of protocols [11] that help to develop highly efficient systems that shut down escape routes for ssDNA viruses. This system can possibly be used alongside the CRISPR-Cas9 system, shown in Figure 2, which targets dsDNA. Consequently, both the ssDNA genome and the dsDNA replicative form can be targeted [12]. This will act like a powerful 'two-hit' system to provide resistance against these viruses. Additionally, PAM-independent targeting and cleavage make this proposed system more versatile than other available mini-orthologs of Cas9 that can also target ssDNA. This property also gives Cas14a a unique advantage over Cas13a, which targets only ssRNA and not DNA, with a strictly required PAM sequence.

Apart from many advantages, the targeting of ssDNA viruses will limit use of Cas14a to an ecosystem abundant in ssDNA viruses. Furthermore, on activation this system may indiscriminately target transposable elements that will be important for plant development. Despite

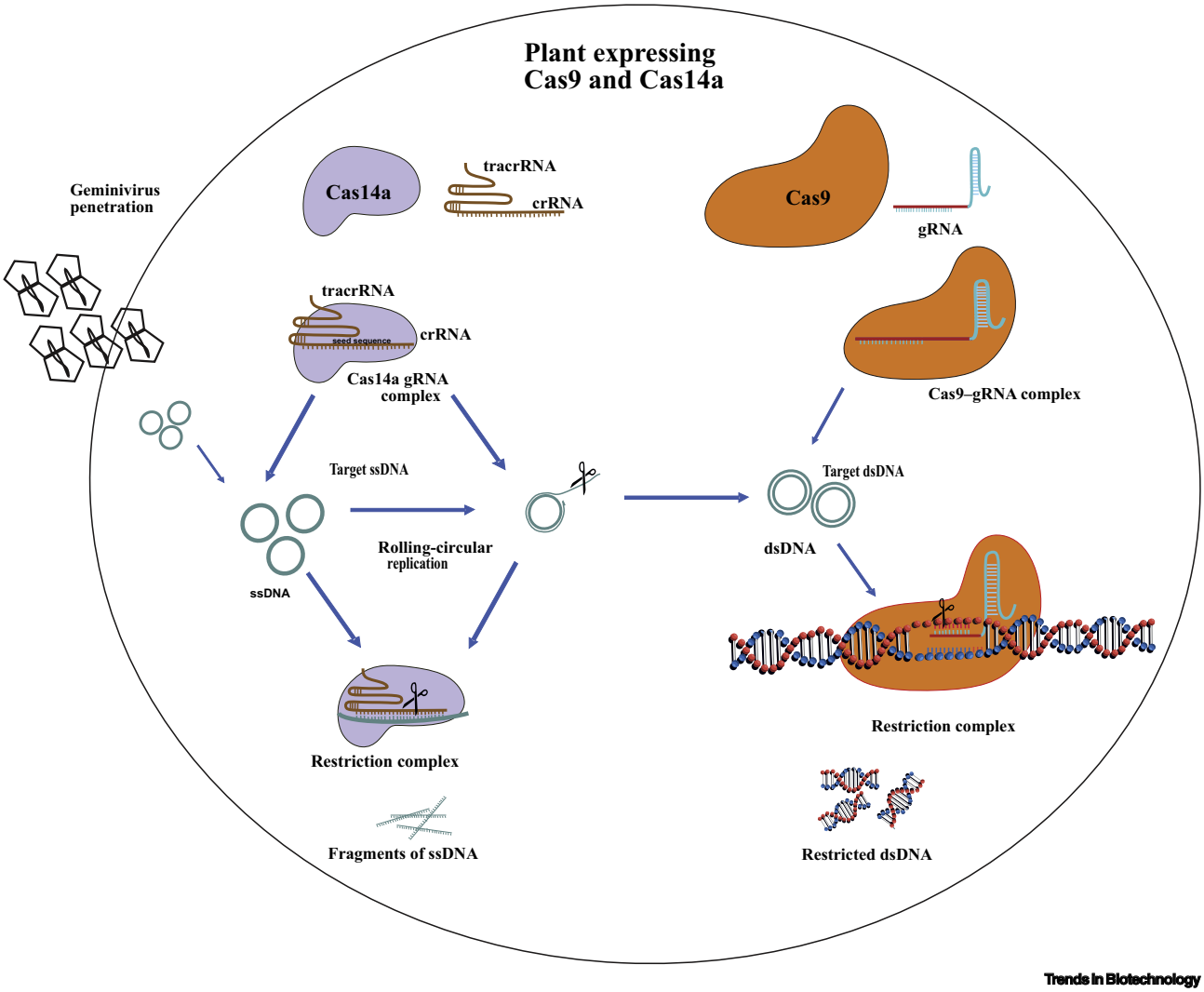


Figure 2. CRISPR-Cas14a and Cas9 to Provide Dual Immunity. After single stranded (ss)DNA virus entry into the cell, CRISPR-Cas14a will target its genome. If the virus escapes this immune system and performs rolling-circle replication, Cas9 will target the double stranded (ds)DNA replicative form of the virus. Abbreviations: crRNA, CRISPR RNA; gRNA, guide RNA, tracrRNA, trans-activating crRNA.

these challenges, this system is still a good candidate to target and restrain highly prevalent viruses belonging to *Geminiviridae* and *Nanoviridae*, which have ssDNA genomes. Further research is needed to explore this system and forge it into a powerful targeting tool for engineering resistance in plants against ssDNA viruses.

Author Contributions

I.A. provided the idea and outlines. M.Z.K. and S.H. wrote the manuscript and prepared the figures. I.A., S.M., M.Z.K., and S.H. worked on and improved the original draft and figures. The first draft of the manuscript was approved by all coauthors.

[1]National Institute for Biotechnology and Genetic Engineering (NIBGE), Faisalabad, Pakistan

*Correspondence:
imranamin1@yahoo.com (I. Amin).

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