Engineering Plant Immunity via CRISPR/Cas13a System

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ABSTRACT

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Fatimah Rajeh Aljedaani

Viral diseases constitute a major threat to the agricultural production and food security throughout the world. Plants cope with the invading viruses by triggering immune responses and small RNA interference (RNAi) systems. In prokaryotes, CRISPR/Cas systems function as an adaptive immune system to provide bacteria with resistance against invading phages and conjugative plasmids. Interestingly, CRISPR/Cas9 system was shown to interfere with eukaryotic DNA viruses and confer resistance against plant DNA viruses. The majority of the plant viruses have RNA genomes. The aim of this study is to test the ability of the newly discovered CRISPR/Cas13a immune system, that targets and cleaves single stranded RNA (ssRNA) in prokaryotes, to provide resistance against RNA viruses in plants. Here, I employ the CRISPR/Cas13a system for molecular interference against Turnip Mosaic Virus (TuMV), a plant RNA virus. The results of this study established the CRISPR/Cas13a as a molecular interference machinery against RNA viruses in plants. Specifically, my data show that the CRISPR/Cas13a machinery is able to interfere with and degrade the TuMV (TuMV-GFP) RNA genome. In conclusion, these data indicate that the CRISPR/Cas13 systems can be employed for engineering interference and durable resistance against RNA viruses in diverse plant species.
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LIST OF ABBREVIATIONS

BCTV  Beet curly top virus
C2c2  Class II candidate 2
CaMV  Cauliflower mosaic virus
Cas13a CRISPR-associated endonuclease 13a
Cas9  Cas9 CRISPR-associated endonuclease 9
Cluc  Cyprindinia luciferase
CP    Coat protein
CRISPR Cluster Regulatory Interspersed Palindramic Repeat
crRNA CRISPR RNA
dCas13a Dead-Cas13a
dCas9  Dead-Cas9
dpi    days post infiltration
DR    Direct repeat
DSB   Double-stranded break
dsRNA Double stranded RNA
GES   Genome editing system
GFP   Green fluorescent protein
Gluc  Gaussia luciferase
gRNA  Guide RNA
HC-Pro Helper component proteinase silencing suppressor
HEPN  Higher eukaryotes and prokaryotes nucleotide binding domains
HR    Homologous recombination
LshCas13a Leptotrichia shahii Cas13a
LwaCas13a Leptotrichia wadei Cas13a
miRNA Micro RNA
N.benthamiana Nicotiana benthamiana
NHEJ  Non-homologous end joining
NLS   Nuclear localization sequence
PAM   Protospacer adjacent motif
pCas13a Plant codon-optimized Cas13a
PEBV  Pea early browning virus
PFS   Protospacer flanking site
RFP   Red fluorescent protein
RISC  RNA-induced silencing complex
siRNA Small interfering RNA molecules
ssRNA Single strand RNA
TALE  Transcription Activator-like Effector
tracrRNA Trans-activating crRNA
TRV   Tobacco rattle virus
TuMV  Turnip mosaic virus
TYLCV Tomato yellow leaf curl virus
ZFN   Zinc Finger Nuclease
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1. Introduction

Viruses are one of the major biotic factors that threaten the agricultural production and food security throughout the world [1]. It is estimated that half of plant emerging diseases is caused by viruses [2]. Plant viruses cause serious damaging effects in many crop species in the field and subsequently cause enormous losses, both in terms of quantity and quality of agricultural products as well as their economic value. However, it is estimated that the annual worldwide financial loss due to viruses in agriculture costs more than $30 billion [3]. Viruses infect plants in different ways including from contaminated agriculture tools, seeds, pollen infections but arthropod vectors serve as a major source of virus plant to plant transmission in the field [4]. The common methods of viral control strategies are focusing on limiting the viral spread among plants or preventing the viral introduction into the field and are either carried out by the use of insecticides to kill the transmission vectors, or applying clean culture practices by using certified seeds or plants, tested to be virus and pathogen free. But all such conventional viral control measures are difficult as they are costly, labor intensive, and have potential adverse environmental effects [1]. Moreover, plant viruses cannot be controlled chemically, therefore alternative and more effective and practical approaches are needed to control plant viral diseases [5]. One of the best, safe, and more practical strategies is to engineer molecular resistance against plant viruses.

Plant-virus interaction based studies helped to understand the molecular defense mechanisms and were used to develop tools to engineer resistance in plants against viruses. To cope with the ever challenging presence of viral load in open fields, plants developed very sophisticated innate immune systems like, RNAi, gene silencing, immune receptor signaling, hormone-mediated defense, protein
degradation and regulation of metabolism [3]. Viruses are mainly delivered into plants by insect vectors or through wounds. Inside the plant cell the virus particles are disassembled and release the viral genome and initiate the infectious cycle of virus genome replication, expression, cell to cell movement by virus-encoded movement protein, long-distance movement through vascular tissue, and finally transmission to new hosts by insect vectors [6]. In plants, the immune system recognize pathogen-associated molecular patterns (PAMPs or MAMPs), such as flagellin by transmembrane pattern recognition receptors (PRRs) or by the resistance (R) genes adaptive immune system, that recognize the virulence proteins of the viruses [7]. After recognition by R genes, the cellular hypersensitive response (HR) disrupts cell to cell viral movement by apoptosis-like cell death mechanism [5]. Different R genes have been identified and classified according to the N-terminal structure, that carries either a Toll–interleukin-1 receptor (TIR) domain or a coiled-coil (CC) domain, the most common members of these gene family members are the proteins containing a nucleotide-binding site (NBS) and leucine-reach repeats (LRRs) [5]. The *Nicotiana benthamiana* R gene is the best characterized example; TIR-NBS-LRR structure interacts directly with the helicase domain of the replicase of *Tobacco mosaic virus* (*TMV*), in the presence of ATP [5]. Another very important example of the R genes that are recessively inherited and are known as recessive resistance genes. Recessive resistance conferred by recessive gene mutation which correspond to the absence of appropriate host factors that are required for the virus replicative cycle. A large number of recessive resistance genes have been identified, one of the main such factors is the eukaryotic translation initiation factor (*eIF*)4E and *eIF*4G isoforms. They are mostly common in several crop plants, and are effective against a subset of viral strains [8]. These recessive
resistance \( (r) \) factors played an important role in plant breeding to control plant viral diseases caused by devastating pathogenic viruses in various crop species (Nicaise, 2014)(Sastry & Zitter, 2014).

Beside these dominant \( R \) and recessive \( r \) factors in plants, the post transcriptional RNA silencing, or the RNA interference (RNAi) is an important defense mechanism against viruses. Over the last two decades, RNAi has been widely used as an efficient molecular tool to induce antiviral defense systems against plant viruses [1].

1.1 RNA interference (RNAi)

RNA interference (RNAi) is a post-transcriptional gene silencing process in which small interfering RNA molecules (siRNA), or micro RNA (miRNA), guide cellular machinery for sequence specific degradation of a target RNA and thus inhibiting gene expression [1]. The critical breakthrough to regulate gene expression through RNAi pathway in the nematode worm Caenorhabditis elegans by Fire and Mello provided new tools to regulate gene expression in eukaryotes [9]. Subsequently, RNAi mechanism has been utilized to regulate gene expression in various eukaryotic species including plants [10, 11]. RNAi is initiated by endoribonucleases enzyme Dicer, which processes long double stranded RNAs (dsRNAs) to generate 21 to 25-nucleotides long small interfering RNAs (siRNA). Theses siRNAs bind to Argonaute protein in the RNA-induced silencing complex (RISC) and direct it to homologues RNAs to either block translation or to degrade the target RNA (Figure 1) [1].
RNAi has been harnessed to inhibit different plant viruses by engineering stable transgenic plants expressing a transgene hairpin loop derived from the pathogenic viruses. Over the past decades, these mechanisms have been widely employed to engineer resistance against plant viruses in many of the commercially cultivated crops [1]. But the drawbacks of this approach include variability of targeting efficiency, incomplete knockdown and off-targeting effects, which constitute major disadvantages of such antiviral defense mechanism [12] [13, 14]. Therefore, in the last decade new tools such as genome editing systems (GES) were adopted to confer targeted and stable resistance against plant viruses.

1.2 Genome engineering technologies
The significant progress in the development of genome engineering technologies based on programmable nucleases has expanded our capability to precisely manipulate eukaryotic genomes. The continuous advancements over the last few years in the efficiency, versatility, and simplicity of these technologies have paved the way to engineer genomes of any transformable species of choice with wide range of useful traits. Genome engineering relies on the introduction of double strand breaks (DSBs) at the desired genomic target sequence, and the subsequent repair of the break by one of the two major DNA repair pathways; the non-homologs end joining (NHEJ) or the homologous recombination (HR) repair pathways (Figure 2) [15]. NHEJ pathway re-ligates the two DNA ends at the break site without considering any sequence similarity between the two ends, generating small deletions or insertions of DNA nucleotides (indels) and therefore leading to the generation of DNA mutations [16]. In contrast, HR uses donor DNA template with homologous arms for the repair event, resulting in precise and accurate repair [16]. Various genome engineering tools have been developed to introduce DSBs in specific and precise manner including zinc finger nucleases (ZFNs) and transcription activator-like effector-based nucleases (TALEN) [17]. However, many hurdles hampered their practical adoption in many laboratories. For example, the fact that the specificity of these effectors depends on protein-DNA interactions, and protein engineering and construction for each new target is labor and resource intensive and expensive [17]. Recently, the clustered regularly interspaced short palindromic repeats (CRISPR)/and CRISPR associated genes have been developed as a highly efficient genome editing tool for genome editing [18].
1.3 The CRISPR/Cas adaptive immunity system

Clustered regularly interspaced short palindromic repeats (CRISPR)/ and CRISPR associated genes is an adaptive immunity systems of bacteria and archaea to defend against invading phages and foreign nucleic acids sequences [19]. The CRISPR/Cas adaptive immunity mechanism involves three main phases. The first phase is the acquisition of the molecular record or memory from the invading plasmid or virus. These sequences of molecular records are inserted in the CRISPR array ready for transcription (acquisition phase). The second phase is the transcription of the CRISPR array and the processing of the guide RNA molecules (transcription and maturation phase). In the final phase, the mature crRNAs guide an endonuclease or ribonuclease to bind the complementary sequence of the target nucleic acids, and subsequently cleave or degrade the DNA or RNA respectively (interference phase) (Figure 3) [19-21]. The CRISPR/Cas loci usually composed of a set of Cas genes and CRISPR arrays. The expression of CRISPR genes lead to the production of different CRISPR proteins including the Cas effector proteins.

DSBs can be repaired using one of the two predominant repair mechanisms. Non-homologs end joining (NHEJ) or homologous recombination (HR). NHEJ re-ligates the two DNA ends at the break site without considering any sequence similarity between the two ends, generating mutations, while HR uses a DNA repair template to provide a precisely homologues sequence that lead to accurate repair.

Figure 2: The repair of DNA double-strand breaks (DSBs)
endonucleases or ribonucleases) that mediate the interference; and other proteins involved in the adaptation phase of the CRISPR immunity such as Cas1 and Cas2 that mediate the acquisition and integration of spacers into the CRISPR array. On the other hand, the CRISPR arrays are non-coding genomic sequences that are comprised of a series of conserved direct repeats separated by spacer sequences, each spacer has been acquired from previous exposures to invading phages or plasmids [19]. All known CRISPR/Cas systems have been classified into two main classes which are further subdivided into different types and subtypes based on their loci organization and distinct signature proteins [22, 23]. Class I CRISPR/Cas systems (encompassing type I, III and IV) are the most abundant CRISPR/Cas systems found in bacteria and archaea, which are known to utilize multi-subunit effector complexes proteins. On the other hand, class II CRISPR/Cas systems (including type II, V and VI) is less common and found mostly in bacteria and recently reported to be found in archaea [24]. The CRISPR/Cas systems were originally discovered in 1980s as repetitive elements in E.coli genomes [25, 26]. Decades later, specifically in 2012, the comprehensive investigation of the type II Streptococcus pyogenes CRISPR/Cas9 components and its molecular mechanisms demonstrated the first insight for developing the system as a powerful genome engineering platform [27, 28]
1.3.1 The CRISPR/Cas9 system

Naturally, the type II CRISPR/Cas9 system from *Streptococcus pyogenes* is composed of three components, the Cas9 endonuclease, CRISPR RNA (crRNA), and trans-activating crRNA (tracRNA) (Figure 4) [29]. Cas9 endonuclease contains two nuclease domains, HNH and RuvC domains, which are able to cleave the complementary and the non-complementary strands of the target dsDNA, respectively (Jinek, Chylinski et al. 2012). The two small RNA molecules; CRISPR
RNA (crRNA) and the trans-activating crRNA (tracRNA) guide the Cas9 endonuclease to the target sequence and generate double-stranded break (DSB) at specific site in the target DNA. Fusion of crRNA to tracRNA has been successful to generate a functional single guide RNA (sgRNA) harboring at the 5’ end a 20-nucleotide targeting sequence (spacer), thus simplifying the system to a set of two components and facilitating its applicability for genome engineering purposes across diverse species and organisms [29]. The spacer sequence in the sgRNA is 20 nucleotides long and complementary to the targeted (protospacer) DNA sequence. Therefore, Cas9 can be reprogrammed to target and cleave any DNA sequence of interest by simple engineering of the 20-nucleotide spacer sequence that guides Cas9 via Watson-Crick base pairing to the targeted DNA locus, which must be immediately followed by 5’-NGG-3’ protospacer-adjacent motif (PAM) that is essential for SpCas9 activity [29] [30]. The absence of the PAM sequence within the direct repeat sequence prevents the targeting of the host genome by the CRISPR system [29, 30]

The natural CRISPR/Cas9 system is composed of three components including Cas9 endonuclease, crRNA and trans-activating crRNA. crRNA and trans-activating crRNA can be combined into a single guide RNA (sgRNA). Therefore, CRISPR/Cas9 is a two component system composed of Cas9 endonuclease and sgRNA. sgRNA can be engineered to target any sequence of interest for DSBs.

**Figure 4: CRISPR/ Cas9 components**
CRISPR/Cas9 has been the most widely used system and was harnessed for diverse genome engineering applications in prokaryotic and eukaryotic cells [16]. In addition, CRISPR/Cas9 has been employed as a molecular immunity tool to confer resistance against a variety of mammalian DNA viruses, and against plant DNA viruses as well. For example, CRISPR/Cas9 was used to confer resistance against HIV, hepatitis B, and herpes simplex virus, showing promising results against these and other viruses [31-33]. In plants, CRISPR/Cas9 system has proven efficient to confer resistance against plant DNA viruses. Different studies have shown that engineering plants to express CRISPR/Cas9 system targeting the coding and non-coding sequences in various DNA geminiviruses, including *Tomato yellow leaf curl* virus (TYLCV) [34] and *Beet curly top virus* (BCTV) [35] resulted in significant virus interference as demonstrated by the reduction of virus accumulation and delayed or eliminated symptoms, highlighting the enormous potential of CRISPR/Cas9 as a promising resistance strategy against plant DNA viruses [36, 37].

The fact that most of the plant viruses are RNA viruses [38] limited the applicability of Cas9 as an antiviral defense mechanism in plants. Therefore, other mechanisms to defend against RNA viruses are highly needed. The ability of CRISPR system to target nucleic acids has expanded beyond DNA targeting. Recent studies have found that other CRISPR/Cas variants are able to target RNA [39].

### 1.3.2 The CRISPR/Cas13a system

A study by Shmakov et al. has discovered a novel Class II CRISPR system, including Cas12b, Cas12c, and Cas13a (previously known as C2c2; Class II
candidate 2) [40]. Cas12b and Cas12c were classified into Class II type V-B
CRISPR/Cas systems due to the presence of RuvC-like domain, similar to Cpf1 [40,
41]. On the other hand, Cas13a was classified as a new Class II type VI-A due to its
unique features that have not been found in any CRISPR protein [41]. By analyzing
the Cas13a protein sequence, two higher eukaryotes and prokaryotes nucleotide
binding domains (HEPN) that are known to be associated with RNase activity [42]
were found, indicating that Cas13a might work as a single effector RNA-guided
RNA targeting protein [43].

The functional characterization of Cas13a by Abudayyeh et al. has shown an
RNA targeting and interference activity of Cas13a of *Leptotrichia shahii*
(LshCas13a) against the single strand RNA (ssRNA) of MS2 phage genome in
*E.coli*. The study has revealed the targeting requirements of Cas13a to recognize
and degrade ssRNAs. A 28-nt spacer sequence in the crRNA is needed to guide
Cas13a to a complementary sequence in the target RNA (Figure 5). The target
sequence “protospacer” must be flanked by a protospacer flanking site (PFS) that
immediately follows the targeted protospacer at its 3’ end. The PFS site was
characterized as H (A, U, or C but not G) (Figure 5). Indeed, this PFS site has been
hypothesized to be responsible for maintaining the interaction between the Cas13a
and its cognate crRNA [44]. Moreover, *in vitro* cleavage assays have shown that
Cas13a exhibit specific ssRNA cleavage activity, but not dsRNA. Furthermore,
Cas13a prefers to cleave uracil residues at multiple sites at the secondary structure
formed in the target RNAs. In addition, an important unique feature in Cas13a is the
presence of the two HEPN domains. However, mutations within HEPN domains,
specifically at the putative histidine and arginine catalytic residues, abolish the
cleavage activity of the Cas13a protein. Furthermore, HEPN domains are important
for the RNA cleavage activity, and by mutating these domains, inactive catalytic version of Cas13a, called dCas13a, can be generated. Similar to dead-Cas9 (dCas9), dead-Cas13a (dCas 13a) was able to bind specifically to the target RNA, but the cleaving activity will be absent, generating an RNA-guided RNA binding protein.

![Figure 5: CRISPR/ Cas13a system](image)

The Cas13a-crRNA complex recognizes target RNA with the cognate protospacer and cleaves its target RNA after binding. The target sequence must be flanked by a protospacer flanking site (PFS) that immediately follows the targeted protospacer at its 3’ end. However, binding of the Cas13a-crRNA activates the promiscuous RNase activity of Cas13a that lead to cleavage any RNA regardless of any base pair complementary.

In addition, the programmable RNA-guided ssRNA targeting activity of CRISPR/Cas13a system was demonstrated to specifically target non-phage RNA molecules in vivo. Cas13a was targeted against red fluorescent protein transcript in E.coli, resulting in specific knocking down the of the red fluorescent protein (RFP) protein expression [43]. However, the crystal structural of LshCas13a demonstrated
that the two enzymatic activities are independent and are accomplished by two different and separate catalytic sites. The RNA cleavage activity takes place in a catalytic site formed between the two HEPN domains. While the pre-crRNA processing takes place in the N-terminal helical-1 domain within the (REC) lobe [45].

However, Cas13a is distinguished with a unique property among other Cas proteins in which it has a collateral degradation of other nonspecific ssRNAs [39]. Once the Cas13a recognizes its target complementary sequence, the enzyme cleaves its target sequence as the presence of the PFS, however, the enzyme remains on active state in which it will bind and degrade any surrounded RNAs without considering any homology to the crRNA or the presence of the PFS [43, 46]. The promiscuous RNAse activity of Cas13a phenomenon prevents the spread of the viral infection as a natural mechanism to sense a host viral infection and as a result induce programed cell death (PCD). However, the collateral cleavage activity of Cas13a can be used to detect existing transcripts [47]. This was demonstrated by a study by Gootenberg and colleagues in which they used the promiscuous RNAse activity of Cas13a as a diagnostic tool for in vitro detection of DNA or RNA, opening the door for many research and diagnosis application [44].

1.3.3 RNA targeting with CRISPR-Cas13a system in mammalian cells

Cas13a of Leptotrichia wadei (LwaCas13a) was heterologously expressed in mammalian cell to knockdown RNA targets. LwaCas13a mammalian codon-optimized were cloned into an expression vector which fused with either C- or N-terminus or dual-flanking nuclear export sequence or nuclear localization sequence
NLS. In addition, msfGFP fused with LwaCas13a constructs were expressed and localized according to the localization sequence; in the cytoplasm if the vector fused with C terminus, and in the nucleus if it was fused with the N terminus. However, in order to evaluate the in vivo cleavage, two different luciferase reporters that express Gaussia luciferase (Gluc) and Cyprindinia luciferase (Cluc) under several promoters on the same vector were used as targets and as a dosing control. Then, several guide RNAs against Gluc designed and cloned into tRNA promoter. The LwaCas13a and the guide RNA vectors and a dual-luciferase construct were transfected into the HEK293FT cells and the luciferase activity measured. Amazingly, LwaCas13a-msfGFP-NLS showed a high level of knockdown compared to the control. Moreover, other endogenous genes were targeted by different guide RNA including KRAS, CXCR4, and PPIB, and also showed knockdown expression similar to RNAi results. Other cell line (A375 melanoma) tested also for the same targets, and similar results were obtained, confirming that LwaCas13a is able to knockdown targets and endogenous RNA levels in various mammalian cells [39].

1.3.4 RNA targeting with CRISPR-Cas13a system in plant cells

Three rice genes (Oryza sativa) were targeted to test Cas13a knockdown activity in plants. O. sativa protoplasts were co-transfected with LwaCas13a and guide vectors that targeted three rice genes. LwaCas13a cloned in expression vector under pOsActin promoter, and guide RNA designed against EPSPS, HCT, and PDS and cloned under pOsU6 promoter. Interestingly, after co-transfected the protoplasts with LwaCas13a and the guide vectors, the majority of O.stiva targets achieved
significantly high levels of knockdown in comparison with the controls, suggesting that Cas13a can be reprogramed with guide RNAs to effectively knockdown specific transcripts [39].

1.3.5 Establishing CRISPR-Cas13a system as a platform for studying RNA-virus interference in plants

Here, I attempted to evaluate and optimize CRISPR/Cas13a as a platform for targeting the genome of RNA viruses in plants. A plant codon-optimized Cas13a (pCas13a) of *Leptotrichia shahii* (LshCas13a) along with crRNA has been used against *Turnip Mosaic virus* (TuMV), a positive-strand RNA virus that belongs to *Potyviridae* family, to investigate the RNA virus interference in plants. To simplify the interference and virus detection a recombinant TuMV expressing GFP (TuMV-GFP) was used to infect plants in the presence CRISPR/Cas13a machinery (Figure 6). To test whether the plant-codon optimized Cas13a (pCas13a) could interfere with TuMV-GFP, transient assays in *N. benthamiana* leaves were performed (Figure 7). The successful interference with the TuMV-GFP genome would result in attenuated virus spread, which can be measured by monitoring the level and systematic spread of the virus-mediated GFP expression in the newly emerged leaves. To target TuMV-GFP, complementary crRNAs were designed to target four different regions of TuMV-GFP genome. Two crRNAs target in *GFP* regions (GFP target 1, and GFP target2), one target at the helper component proteinase silencing suppressor (*HC-Pro*), and one target at the coat protein (*CP*) sequence of the TuMV-GFP sequence. In addition, we engineered the RNA2 genome of *tobacco rattle virus* (TRV) to transiently and systemically express crRNAs under the *Pea early browning virus* (PEBV) promoter. To test the functionality of CRISPR/pCas13a in transient assays, mixed *Agrobacterium* cultures carrying the binary pK2GW7:pCas13a clones, TRV RNA1, and the engineered TRV-RNA2 genome expressing crRNAs along with TuMV-GFP
infectious clone were infiltrated into *N. benthamiana* leaves. A non-specific crRNA (ns-crRNA) with no sequence similarity to the TuMV-GFP genome was used as control. The interference activity of the CRISPR/pCas13a system against the TuMV-GFP will assessed 7 days post-infiltration (dpi) by visualizing the GFP signal in the plant systemic leaves under UV light.

![Figure 6. TuMV-GFP Genomic Structure](image)

Schematic representation of TuMV-GFP genomic structure. TuMV-GFP is a single strand RNA with 10000 nt long, the *GFP* gene expresses green fluorescent protein to facilitate the virus detection in plant leaves. Three sites were chosen to be targeted by crRNAs, GFP target 1, GFP target 2, HC-pro, and CP. Synthetic crRNAs complementary to 28 nt in each of the targets were designed and cloned with the respect of protospacer flanking site (PFS) in each target.

![Figure 7: Overview of transient assays in *N. benthamiana*](image)

*Agrobacterium tumefaciens* GV3101 strains harboring TRV RNA1, TRV RNA2 engineered with crRNA (*PEBV::gRNA*), and pK2GW7-pCas13a construct (*35S::pCas13a*) were mixed, in different combinations, and co-delivered into 3 to 4 weeks old *Nicotiana benthamiana* leaves. 7 days post infiltration, GFP expression was observed under UV light, and systemic leaves were collected and subjected to molecular analysis.
Next, we assessed the interference activity of CRISPR/pCas13a against TuMV-GFP in transgenic *N. benthamiana* and Arabidopsis thaliana constitutively expressing pCas13a protein (pCas13a-OE) under the control of *CaMV35S* promoter. These transgenic plants were generated via *Agrobacterium*-mediated transformation of *pK2GW7:pCas13a* clones into wild type *N. benthamiana* plants, and *pK2GW7:pCas13a.U6:crRNA* into wild type Arabidopsis thaliana. The crRNAs were delivered into pCas13a-OE *N. benthamiana* plants via RNA2 of the TRV system and expressed under *PEBV* promoter.

To test CPRISPR/pCas13a activity against TuMV-GFP in pCas13a-OE plants, we challenged the OE-plants with the TuMV-GFP. The second generation (T2) of the pCas13a-crRNA-OE Arabidopsis thaliana; crRNAs with specificity to the CP, Hc-Pro, GFP-T1, and GFP-T2 of the TuMV-GFP genome, were inoculated with 15ul sap isolated (13dpi) from TuMV-GFP infiltrated in wild *N. benthamiana*. A non-specific crRNA was used as negative control. Plants were assessed for the GFP signals under the UV-light at seven days post-infiltration (7dpi), and samples were collected and subjected to molecular analysis. Additionally, pCas13a-OE *N. benthamiana* plants were agro-infiltrated with TRV expressing crRNAs of the CP, Hc-Pro, GFP-T1, and GFP-T2 of the TuMV-GFP genome along with TuMV-GFP. Non-specific crRNA was used as a negative control. Similarly, GFP signals assessed under UV-light 7dpi, and samples were collected and subjected to molecular analysis (Figure 8).
Figure 8: Transient assays of molecular interference against TuMV-GFP in pCas13a OE N. benthamiana and Arabidopsis thaliana plants

*Agrobacterium tumefaciens* GV3101 strains harboring TRV RNA1, TRV RNA2 engineered with crRNA (*PEBV*:gRNA), and bacterial cells harboring TuMV-GFP, were mixed in different combinations, and co-delivered into 3 to 4 weeks old pCas13a-OE- *Nicotiana benthamiana* leaves. GFP expression was observed under UV light 7 days post infiltration, and systemic leaves were collected and subjected to molecular analysis.
2 Materials and methods

2.1 Plasmid construction

Plant codon optimized (pCas13a) was commercially synthesized using Blue heron (USA) gene synthesis facility in four overlapping fragments and cloned into pK2GW7 binary vector using LR Gateway recombination cloning to generate pK2GW7: pCas13a for expression in plants under the constitutive *cauliflower mosaic virus* promoter p35S. Here, the four overlapping fragments of pCas13a were assembled by unique restriction enzymes to generate a full-length clone flanked by *attL1* and *attL2* recombination sites, a nuclear localization signal fused to the C-terminus of the protein, and a 3x-HA tag fusion at the N-terminus to facilitate protein detection (Figure 9). On the other hand, the crRNAs were designed as primer dimers with overhangs (28nt guide with 28nt Cas13a binding scaffold were synthesized as a pair of custom synthesized oligos with extra nucleotides to make cohesive ends for respective final vector cloning sites) (Figure 10). The crRNA was cloned under the *pea early browning virus* (PEBV) promoter in TRV RNA2 by XbaI and XmaI. Clones were verified by Sanger sequencing using primers listed in Supplementary information. To express crRNA in permanent lines (Arabidopsis thaliana) the crRNA cloned under *U6* promoter into pK2GW7 by SacI restriction enzyme. pCas13a were cloned into pK2GW7-U6-crRNA by LR recombination to generate pK2GW7-pcas13a-crRNA final clone. Clones were confirmed by Sanger sequencing and transformed into GV3101 *Agrobacterium* strains.
A. Schematic assembly of the plant codon-optimized Cas13a (pCas13a). pCas13a was custom synthesized as four fragments. The F1 (with attL1 and 3x-HA), F2, F3, and F4 (with nls and attL2) fragments were assembled in the cloning vector using a restriction-ligation system.

B. Plant codon-optimized fused to HA tag (human influenza hemagglutinin epitope tag) and nls (nuclear localization signal) cloned in pK2GW7 under the expression of 35S promoter.

A. crRNAs were designed as primer dimers of 28nt guides with 28nt Cas13a binding scaffold with overhangs of extra nucleotides to make compatible cohesive ends for respective final vector

B. Cloning of crRNA into pK2GW7:pCas13a under U6 promoter as final destination vector into Arabidopsis thaliana plants
2.2 Agroinfiltration

Constructs, harboring the TuMV-GFP infectious clone, TRV RNA1, TRV RNA2 empty as control, TRV RNA2 containing crRNA under PEBV promoter, and pK2GW7-pCas13a under 35S promoter, were individually electroporated into Agrobacterium tumefaciens strain GV3101. Agrobacterium cultures were first grown overnight in 5 mL LB media containing suitable antibiotics, and subsequently cultured in 50 mL LB and grown to the OD600 between 1.0 and 1.5, followed by centrifugation at 3000 rpm for 15 minute at room temperature. The Agrobacteria were collected and resuspended in infiltration buffer (10 mM CaCl2, 10 mM MES, and 200 µM acetosyringone), and incubated at ambient temperature for 2 hours. Cultures were mixed at OD 600 ratio of 0.05: 1:1:1 for TuMV-GFP, TRV1, TRV2, pCas13a, respectively. The culture mixtures were infiltrated into 3 to 4-week-old leaves of N. benthamiana plants with 1-mL needleless syringe.

2.3 GFP imaging

GFP expression was observed 7 days post infiltration (dpi) by using a hand-held UV light. Images were taken by a Nikon camera under UV light as well as at normal light. Leaf samples were collected 7 dpi for molecular analysis.

2.4 Immunoblot Assay

Total proteins were extracted from 100 mg of leaf samples overexpressing pCas13a using IP protein extraction buffer composed of 100 mM Tris-Cl (pH8), 150 mM NaCl, 0.6 IGEPAL, 1 mM EDTA, 3 mM DTT with protease inhibitors, PMSF leupeptin, aprotinin, pepstatin, antipain, chymostatin, Na2VO3, NaF, MG132, and MG115. Proteins were separated on a 6% polyacrylamide gel at 100 V for 1 hour and 30 minutes. Immunoblot analysis was carried out using mouse α-GFP (1:2000;
Invitrogen) for TuMV GFP, and rat α-HA (1:500) antibody for pCas13a. The antigens were detected by chemi-luminescence using ECL-detection reagents (Thermo Scientific).
3 Results

3.1 pCas13a and TuMV-GFP targeting

CRISPR/Cas13a defends bacterial cells against RNA viruses, I attempted to test CRISPR/Cas13a against plant RNA viruses using in planta transient assays. TuMV was selected as RNA virus for targeting by the CRISPR/Cas13a system in plants. The modified TuMV (TuMV-GFP) expressing a mGFP cassette was used to facilitate the virus replication and systemic spread and detection, as well as to provide a visual estimation about CRISPR/Cas13a-mediated interference against the TuMV.

Subsequently, a plant codon-optimized pCas13a containing a C-terminus fused nuclear localization signal and N-terminus 3x-HA tag was cloned into pK2GW7 vector via Gateway recombination reactions to generate pK2GW7:pCas13a expression clones driven by 35S promoter. The pK2GW7:pCas13a clone was transformed into Agrobacterium tumefaciens Gv3101 strains via electroporation.

Next, to target the RNA genome of TuMV-GFP virus, three different regions in the virus genome were selected and four crRNAs with (28nt) complementarity to the TuMV-GFP RNA genome were designed. These cRNAs were designed to target two sequences within the GFP ORF (GFP target 1) and (GFP target 2), one target at the helper component proteinase silencing suppressor (HC-Pro), and one target at the coat protein (CP). RNA2 genome of the tobacco rattle virus (TRV) was engineered to transiently and systemically express these crRNAs under the Pea early browning virus (PEBV) in N. benthamiana plants (Figure 11).
3.2 Confirmation of pCas13a and GFP expression by immunoblot analysis

To confirm the transient expression of pCas13a protein in *N. benthamiana* plants, the pCas13a expressing binary vector was infiltrated via *Agrobacterium* into young leaves and samples were collected after 48 hours of infiltration. The samples were grinded and the extracted proteins were separated on poly-acrylamide gel and transferred to PVDF membrane and detected by Western blotting using α-HA antibody to detect HA tagged pCas13a protein. The Western blotting results clearly detected a 170 kDa protein corresponding to pCas13a-HA protein. These results confirmed the proper expression of the plant codon optimized clone and the correct size of pCas13a-HA protein in *N. benthamiana* leaves (Figure 12).
3.3 CRISPR/pCas13a interferes with TuMV-GFP in planta

In order to test the CRISPR/pCas13a interference against TuMV-GFP in plants, we performed transient assays in *N. benthamiana* leaves. The GFP expression can facilitate the detection of the interference activity under UV-light, as plants without viral interferences would give high GFP signal under UV-light, indicating systemic viral spread and movement in the whole plant, specifically in the young emerging leaves. On the other hand, plants with successful interference of TuMV-GFP RNA genome through CRISPR/Cas13a system would result in low or no GFP signal under UV-light. To confirm and validate this hypothesis, *N. benthamiana* plant leaves were co-infiltrated with mixed cultures of each TRV RNA2 expressing crRNA (GFP target1, GFP target2, HC-pro, or CP), TRV RNA1, *pK2GW7:pCas13a*, and TuMV-GFP infectious clone. A non-specific crRNA sequence (ns-crRNA) and empty *pK2GW7* vector were used as control. At 7 dpi GFP signals were estimated under UV light to determine the interference activity. We observed almost 50% reduction in GFP signal in the systematic leaves under UV light in our experiments using crRNAs targeting the virus and pCas13a compared to the controls (Figure 13). All of the crRNAs (*GFP1, GFP2, HC-pro*, and *CP*) showed lower GFP signals in the systemic leaves compared to nsgRNA control in which high GFP signals observed (Figure 14). Interestingly, *HC-pro* and
GFP-target2 exhibited a stronger reduction in the GFP signal detection compared to the GFP-target1 and CP. These initial results indicate the ability of CRISPR/pCas13a to interfere with TuMV-GFP in planta. We next assessed the interference activity of CRISPR/pCas13a against TuMV-GFP in stable transgenic *N. benthamiana* plants constitutively overexpressing pCas13a protein (pCas13a-OE) under the control of CaMV35S promoter, in which similar results were obtained confirming the ability of pCas13a to interfere with TuMV-GFP (Figure 15).

![Figure 13: Transient expression assays in *N. benthamiana* plants](image)

*N. benthamiana* leaves co-infiltrated with *Agrobacterium tumefaciens* GV3101 strains harboring TRV RNA1, TRV RNA2 engineered with crRNA (*PEBV*::gRNA), and *pK2GW7-pCas13a* construct (*35S::pCas13a*), in different combinations, and co-delivered into 3 to 4 weeks old *Nicotiana benthamiana* leaves. Photographs were taken 7dpi under UV light.
Similar but not exact results were obtained from pCas13-crRNA-OE.
Arabidopsis thaliana plants (Figure 15). Here, in Arabidopsis thaliana, the plants are stably expressing pCas13a as well as crRNAs of Hc-Pro, GFP, and CP. The second generation of pCas13-crRNA-OE Arabidopsis thaliana plants were challenged with TuMV-GFP (virus inoculation), and the interference activity was assessed at 7 to 10 dpi by GFP visualization. Imaging results of GFP under the UV lights showed strong interference with Hc-Pro crRNA, and GFP-T2 crRNA restricted the viral speared into systemic leaves (Figure 16).

<table>
<thead>
<tr>
<th>pCas13 OE</th>
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</thead>
<tbody>
<tr>
<td>Control</td>
</tr>
<tr>
<td>nsgRNA</td>
</tr>
<tr>
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<td>crRNA-HC-Pro</td>
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<td>crRNA-CP</td>
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**Figure 16. Suppression of Virus accumulation in pCas13-crRNA-OE Arabidopsis thaliana**

Arabidopsis thaliana plants engineered with Cas13aOE- and specific crRNAs show a notable reduction in GFP signals in systemic leaves compared to nsgRNA.

3.4 CRISPR/pCas13a interfered with TuMV-GFP and resulted in lower accumulation of the GFP protein and the RNA genome

The virus strain used (TuMV-GFP) is expressing a mGFP protein from the viral genome and any interference with the genome may results in lowering down the GFP expression. To confirm that the CRISPR/Cas13a based interference of the
TuMV-GFP would result in lower accumulation of the GFP protein, the samples were collected from the systemic leaves of the plants transiently expressing CRISPR/Cas13a machinery against TuMV-GFP.

The Western blotting results were consistent with the UV light based GFP images, i.e. in all four crRNA samples, a lower amount of the GFP protein was detected compared to vector controls. Similarly, the samples expressing the crRNA against GFP2 or HC-pro resulted in more severe reduction of GFP accumulation compared to crRNA against GFP1 and CP and the control samples. Overall, plants with specific crRNA targeting exhibited high GFP protein reduction compared to nsgRNA (Figure 17). These results demonstrated that CRISPR/pCas13 can interfere with TuMV-GFP virus at the RNA genome level.

![Figure 17: Virus interference assessed by GFP quantification from pCas13-OE N. benthamiana T1 plants](image)

Western blot of T1 nC2c2-OE *N. benthamiana* plant samples. GFP proteins were detected at 27 KD in all the samples by using mouse α-GFP (1:2000 invitrogen). Hc-Pro and GFP-T2 samples show less GFP accumulation compared to the other samples.
4 Discussion

Engineering and developing plant species resistant to viruses hold enormous promise to increase food production and enhance food security. Previously, RNAi was adopted by researchers to provide plants with a transgenic immune system to resist pathogens, including viruses [48]. However, few crops have been engineered against viruses via RNAi approach which has several disadvantages, i.e. they are labor intensive, expensive, unpredictable in reproducibility and have off-targeting activities. In recent years, CRISPR/Cas system emerged as one of the best and most effective strategies to engineer resistance to plant viruses. Several crops and model plants were engineered with CRISPR/Cas9 to develop resistance against invading DNA viruses. But very recently, CRISPR/Cas13a was shown to cleave RNA targets, in this work I attempted to test whether pCas13a could mediate RNA virus interference in plants. Our results of the transiently expressed CRISPR/Cas13a system showed that this system can be used as a platform for targeting RNA viruses in plants. Specifically, I employed pCas13a with crRNAs as RNA targeting module in the model plant system of N. benthamiana. I expressed and reconstituted the CRISPR/Cas13a machinery transiently in N. benthamaina leaves and targeted the TuMV genome. First set of experiments focused on establishing the assays system and ensuring the protein expression and reconstitution of the CRISPR/Cas13a machinery. In the second set of experiments, I conducted and studied the molecular interference against TuMV using the GFP signals as indication of the virus accumulation. All crRNA showed reduction in the levels of GFP expression in the systemic leaves under UV light compared to the controls (Figure 13, 14 and 15). Additionally, lower but detectable levels of reduction in the GFP signal were detected in GFP target 1 and CP crRNA, whereas higher reduction levels were
observed in GFP target 2 and HC-pro target (Figure 14). One possibility of the better targeting activity by the crRNA complementary to the HC-pro sequence of the TuMV genome may be because of function of the Hc-Pro within the viral genome as a helper factor to suppress the host defense mechanisms. Any change in Hc-Pro level can affect the viral replication within the plant cell. On the other hand, higher GFP signals were observed in the nsgRNA control and wild type N.benthamiana plants corroborating the usefulness of the CRISPR/Cas13a system in for virus interference. Overall, our date demonstrated the successful targeting and interference against TuMV-GFP. These findings indicate the effectiveness of CRISPR/pCas13a in targeting and interfering with RNA viruses for potential plant biotechnology applications.

Future work would focus on utilizing other Cas13 variants for developing more robust and specific targeting activities against devastating RNA viruses. The same approach described in this work can be used to determine the most effective Cas13 variant with robust ribonuclease activity, like the very recently identified Cas13a from Leptotrichia wadei (LwaCas13a) [39]. Our results show the power of this system for developing virus resistance in plants. This will provide a platform to focus on the most challenging problems associated with the virus rapid evolution and escapees from the CRISPR/Cas machinery. Furthermore, these promising results can be applied to produce different transgenic crops that are resistant to single and multiple viruses. We can engineer key plant species important for food security to express the CRISPR/Cas13 machinery against single and multiple viruses, and possibly other pathogens. These crops include rice, wheat, barley, tomato etc. Additionally, as Cas13 has been used as a diagnostic tool in detection of
different pathogen in mammalian cells, then, this system can be used in the plant system to identify and diagnose diverse array of pathogens in the field under natural conditions. In conclusion, CRISPR/Cas13a holds great promise as a precise RNA targeting tools in the plant system as well as in other eukaryotic species. The CRISPR/Cas13 system provides a revolutionizing technology to engineer plant immunity against single and multiple virus infections, a platform for genome wide interrogation of gene function, a diagnostic tool, and as an RNA knockdown mechanism against the plant transcriptome.
REFERENCES


**Supplementary Sequence 1:** pCas13a amino acid sequence (3xHA-pCas13a-nls) (3x-HA and NLS are highlighted)

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MYPYDVPDYA YPYDVPDYA YPYDVPDYA GNLFGHKRWYEVRDKKDFKIKRKVVKRN
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Supplementary Map 1: pCas13a sequence in pK2GW7-pCas13a (pK2GW7-3xHA-pCas13anls)
Supplementary Sequence 3: TuMV-GFP full length sequence with target sequences in different colors

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agggacaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaa
aaaa

Supplementary Sequence 4: Cas13a-repeat-cRNA-TuMV-GFP-GFP-target 1 sequence

CTAGACACCACCCAATATCGAAGGGGACTAAAACACACAGGTAGTTTTCCAGTAGTGCAAATATTTTTTTTG

Supplementary Sequence 5: Cas13a-repeat-cRNA-TuMV-GFP-GFP-T2 sequence

CTAGACACCACCCAATATCGAAGGGGACTAAAACACACAGGTAGTTTTCCAGTAGTGCAAATATTTTTTTTG

Supplementary Sequence 6: Cas13a-repeat-cRNA-TuMV-GFP-HC-Pro-T1 sequence

CTAGACACCACCCAATATCGAAGGGGACTAAAACACACAGGTAGTTTTCCAGTAGTGCAAATATTTTTTTTG

Supplementary Sequence 7: Cas13a-repeat-cRNA-TuMV-GFP-Cp-Pro-T1 sequence

CTAGACACCACCCAATATCGAAGGGGACTAAAACACACAGGTAGTTTTCCAGTAGTGCAAATATTTTTTTTG

List of the primers used in this study:

Forward primer for cloning of Cas13a-repeat-crRNA-GFP-T1 under PEBV promoter in TRV system

5’CTAGACACCACCCAATATCGAAGGGGACTAAAACACACAGGTAGTTTTCCAGTAGTGCAATATTTTTTTTG 3’
Reverse primer for cloning of Cas13a-repeat-crRNA-GFP-T1 under PEBV promoter in TRV system

5’GATCCAAAAAAAAATATTTCACACTGGAAATACCTGTGTTTTTAGTCCCC TTCGATATTGGGGTGTT 3’

Forward primer for cloning of Cas13a-repeat-crRNA-GFP-T2 under PEBV promoter in TRV system

5’CTAGACCACCCCAATATCGAAGGGGACTAAACCGTGCTCTTTGAATCGATT CCCTTAATTTTTTTTTT 3’

Reverse primer for cloning of Cas13a-repeat-crRNA-GFP-T1 under PEBV promoter in TRV system

5’GATCCAAAAAAAAATTAAGGGAATCGATTTCAAGGAGGACGGTTTTTAGTCCCC TTCGATATTGGGGTGTT 3’

Forward primer for cloning of Cas13a-repeat-crRNA-HC-Pro-T1 under PEBV promoter in TRV system

5’CTAGACCACCCCAATATCGAAGGGGACTAAACCGTGCTCTTTGAATCGATT AGCTCACTTTTTTTTTT 3’

Reverse primer for cloning of Cas13a-repeat-crRNA-HC-Pro-T1 under PEBV promoter in TRV system

5’GATCCAAAAAAAAAGTGAGCTATCCCAAGGACAAGCAAGCGTTTTTAGTCCCC TTCGATATTGGGGTGTT 3’

Forward primer for cloning of Cas13a-repeat-crRNA-Cp-Pro-T1 under PEBV promoter in TRV system

5’CTAGACCACCCCAATATCGAAGGGGACTAAACACACTGAAAGTTCCAGAGGTT CCAGCGTTTTTTTTTT 3’

Reverse primer for cloning of Cas13a-repeat-crRNA-Cp-Pro-T1 under PEBV promoter in TRV system

5’GATCCAAAAAAAAAACGCTGGAACCTCTGGAACTTTCAGTGTGTTTTTAGTCCCC TTCGATATTGGGGTGTT 3’

Forward primer for cloning of Cas13a-repeat-crRNA-Nonspecific-T1 under PEBV promoter in TRV system

5’CTAGACCACCCCAATATCGAAGGGGACTAAACACACTGAAAGTTCCAGAGGTT CCAGCGTTTTTTTTTT 3’
5’CTAGACCCAATATCGAAGGGGACTAAAAACTCCGGATCCAGAGAGATGATTCTCCGCTTTTTTTTTTG 3’

Forward primer for cloning of Cas13a-repeat-crRNA-Nonspecific-T1 under PEBV promoter in TRV system

5’GATCCAAAAAAAAGCGGGAGAATCATCTCTCTGATCCGGAGTTTTAGTCCCCTTCGGATATTGGGGTGGT 3’

Primer for making synthetic crRNA positive control

5’CCACCCCAATATCGAAGGGGACTAAAAACTTTTGCTCCCCCTCCACAAGAACATTGAGA 3’