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Detection of tomato brown rugose fruit virus through CRISPR-Cas12a and CRISPR-Cas9 systems

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Tomato brown rugose fruit virus (ToBRFV) is a single-stranded positive-sense RNA virus that targets tomato and pepper plants and is causing significant damage to crops in some regions of the world. ToBRFV is a highly contagious virus that is stable and rapidly spreads by mechanical methods and seeds. As a result, it may spread both locally and over long distances, and it is now recognized as a pandemic in plants. This study investigates the effectiveness of the systems CRISPR-Cas12a and CRISPR-Cas9, in conjugation with recombinase polymerase amplification (RPA), to detect ToBRFV in tomato plant samples collected from the field. The *trans*-cleavage activity of both nucleases, Cas12a and Cas9, was exploited to process a probe labelled with fluorescein and biotin to be resolved on a lateral flow device, thereby enabling a visual readout. We were able to detect the RNA genome of the virus in about 1 h at a low constant temperature. These results could pave way to offer a rapid, sensitive, and specific method for on-site detection of ToBRFV.

Keywords CRISPR diagnostics, Plant virus, Lateral flow assay

Plant viruses pose a significant challenge to agriculture, which are responsible for almost half of all new plant diseases, thereby leading to an estimated annual economic loss exceeding $30 billion[1]. The tomato brown rugose fruit virus (ToBRFV), a new member species of the genus *Tobamovirus*, causes significant damage to tomato crops in some regions of the world[2]. ToBRFV is related to several other viruses, including tobacco mosaic virus (TMV), tomato mosaic virus (ToMV), tomato mottle mosaic virus (ToMMV), cucumber green mottle mosaic virus (CGMMV), and odontoglossum ringspot virus (ORSV)[3–6]. ToBRFV is a single-stranded positive-sense RNA virus that targets tomato and pepper plants initially identified in 2014 in greenhouses in Israel and Jordan[7]. Despite the long-standing resistance of the Tm-2[2] gene against *Tobamoviruses*, ToBRFV has proven to overcome all known resistance genes in tomatoes[8,9]. This virus has been found to naturally infect tomato (*Solanum lycopersicum*) and pepper (*Capsicum annuum*)[7,8,10]. Tomato cultivars carrying the Tm-1, Tm-2, or Tm-2[2] *Tobamovirus* resistance (R) gene are susceptible to infection by ToBRFV[8,11,12]. Additionally, it can infect pepper cultivars that have the L alleles (L1 or L2); and in pepper plants that encode for the L3 and L4 alleles, it can cause a hypersensitivity reaction (HR)[13]. Tomatoes and peppers, similar to other crops, are consistently targeted by pests and pathogens. Primary pathogens are *Cucumoviruses*, *Begomoviruses*, *Potyviruses*, *Tospoviruses*, and *Tobamoviruses*[14,15]. Infection with these viruses diminishes crop yield, degrades the quality and marketability of the fruits, and results in significant economic losses[14–17]. The ToBRFV appears to be spreading rapidly, as the list of countries affected by ToBRFV has grown swiftly. To date, it has reached more than 45 countries worldwide[18–24].

The virus genome consists of a single-stranded positive-sense RNA molecule with specific open reading frames (ORFs) encoding essential proteins for viral replication. Notably, the production of a helicase and methyltransferase protein (p126) and an RNA-dependent RNA polymerase protein (p183) play crucial roles in the viral life cycle (Fig. 1)[7,25].

Traditional diagnostic methods for viral infections involve enzyme-linked immunosorbent assay (ELISA) tests for viral protein detection or polymerase chain reaction (PCR) techniques for genome amplification[26]. To detect ToBRFV, various serological and molecular techniques have been employed. Serological approaches, such as ELISA, are popular due to their ease of use and affordability[19,27]though they offer limited sensitivity

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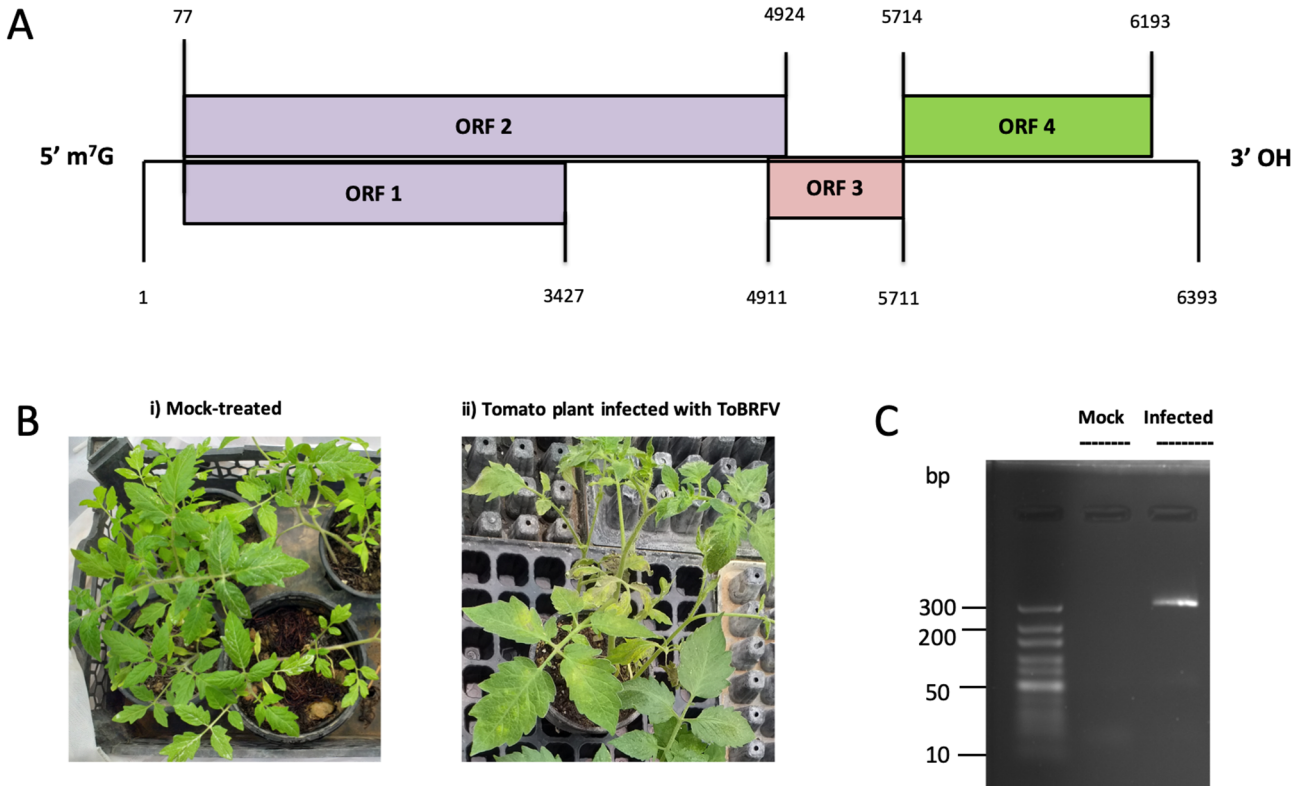

Fig. 1. Phenotypic effects of ToBRFV-infected plants and classical detection. (A) Genome architecture of ToBRFV. Each number indicates where an open reading frame (ORF) begins and ends in a representative Iranian ToBRFV isolate (OP557566, 6393 bases). (B) Images of *S. lycopersicum* L. plants infected by ToBRFV. (i) Mock-treated plants; and (ii) infected plants showing necrosis. C) Gel electrophoresis image showing the expected viral product upon RT-PCR (total RNA was used as starting material).

and specificity. Molecular techniques such as PCR[28,29] have been developed to address these limitations, and even isothermal methods such as loop-mediated isothermal amplification (LAMP)[29] have been put forward. These molecular methods provide high specificity and sensitivity for detecting ToBRFV and are helpful for field diagnostics, vital for early virus detection and management. However, reverse transcription PCR (RT-PCR) involves a lengthy assay time (approximately 2 h), requires skilled personnel, and relies on costly laboratory equipment. While LAMP-based techniques have shortened amplification times and have reduced instrumental dependence, they tend to be prone to false positives, thereby compromising their use for effective field applications. Notwithstanding, Cao et al. used reverse transcription recombinase-aided amplification (RT-RAA) to identify ToBRFV in a lateral flow assay (LFA)[30].

Some bacteria and archaea contain an innate immune system called clustered regularly interspaced short palindromic repeats (CRISPR)[31]which has resulted very versatile to be exploited in biotechnology. The use of CRISPR systems can add a higher level of specificity in the detection[32]. This kind of approaches have shown potential for identifying viral pathogens by targeting specific sequences within their genomes. The CRISPR-Cas12a and CRISPR-Cas13a/d systems have been exploited for the detection of RNA and DNA viral pathogens in plants[33–36]. The main difference between CRISPR type V (Cas12) and type VI (Cas13) systems is that DNA is targeted in former while RNA is targeted in the latter. Both nucleases have non-specific collateral cleavage upon target recognition. This collateral activity involves cleaving non-targeted single-stranded DNA (ssDNA) for Cas12 or single-stranded RNA (ssRNA) for Cas13. In particular, these systems have been already exploited to detect ToBRFV[27,37,38]. They have relied on producing a fluorescent signal upon specific recognition of the virus. For in situ applications, it would be convenient nonetheless to exploit LFA devices to monitor the output of the CRISPR reactions.

The *trans*-cleavage activity of the CRISPR-Cas9 system has been recently disclosed[39]adding this system to the toolbox of CRISPR diagnostics. Our work aims to investigate the use of the CRISPR-Cas12a and CRISPR-Cas9 systems to detect ToBRFV on an LFA device. By leveraging these innovative techniques, we present a rapid and accurate method for on-site identification of ToBRFV infections in tomato plants, offering a practical solution for monitoring and managing viral diseases in agricultural settings.

Results

Detection of ToBRFV with CRISPR-Cas12a

Custom RPA primers were designed to target a conserved sequence of ToBRFV. We considered the gene coding for the coat protein of the virus (Fig. 1). This region is convenient because it is included in all possible sub-genomic RNAs. Using samples collected from tomato (*S. lycopersicum* L.) plants infected with ToBRFV, we applied PCR with those RPA primers and subsequent gel electrophoresis to confirm the presence of the virus.

Then, a Cas12a-dependent CRISPR RNA (crRNA) was designed to target the resulting DNA amplicon. We ensured the presence of a suitable protospacer adjacent motif (PAM) for Cas12a recognition (TTTV). Cas12a was a commercial preparation from *Lachnospiraceae bacterium* and the crRNA was produced by in vitro transcription. Activation of Cas12a triggered collateral *trans*-cleavage of an ssDNA reporter molecule labelled with fluorescein and biotin, whose sequence reads TTATT. The cleavage of this molecule set apart fluorescein and biotin so that it could be measured in an LFA device. A reaction buffer previously used to detect plant viruses with this nuclease was considered[33]. Using the RPA product generated from the tomato plant samples, we demonstrated that the CRISPR-Cas12a assay coupled to LFA is suitable to detect ToBRFV with sufficient sensitivity and specificity with a visual readout (Fig. 2).

Detection reaction signals were robust to variations in Cas12a concentration, from 50 to 200 nM, and increased over time, from 30 to 120 min (Fig. S1). Signals at 50 nM and 2 h showed the highest visual signal-to-noise ratio, but with 1 h it was sufficient to obtain a significant signal. Moreover, we assessed the specificity of the test by using TMV-infected plant samples (Fig. S1).

Detection of ToBRFV with CRISPR-Cas9

Parallel experiments were conducted using the CRISPR-Cas9 system, knowing the ability of this other nuclease to process poly(T) strands. Within the previously used DNA amplicon, the PAM recognized by Cas9 (NGG) was also found. Thus, we designed a crRNA to target it. The main difference with respect to the Cas12a-dependent crRNA is that, in this case, the spacer is located in the 5' end. Moreover, Cas9 works with a bimolecule RNA, including a *trans*-activating crRNA (tracrRNA). Cas9 was a commercial preparation from *Streptococcus pyogenes* and, as before, the crRNA and tracrRNA were in vitro transcribed. In this case, we used a poly(T) probe of 18 nt (T_{18}) labelled with fluorescein and biotin to be processed upon activation of Cas9. Following the RPA step, we confirmed that the CRISPR-Cas9 assay coupled to LFA is also suitable to detect ToBRFV in tomato plant samples with a visual readout (Fig. 3). While the assay displayed sufficient sensitivity and specificity, in comparison to the CRISPR-Cas12a assay, the intensity of the test line here showed lower dynamic range.

Detection reaction signals with Cas9 could be increased by reducing the amount of probe in the reaction (from 100 to 50 nM; Fig. S2). Moreover, we analyzed the performance of the test upon variations in Cas9 concentration, from 50 to 200 nM, and incubation times, from 30 to 120 min. As before, signals at 50 nM and 2 h showed the highest visual signal-to-noise ratio, but this time with 30 min it was sufficient to obtain a significant signal. These results suggest roughly comparable, yet condition-dependent *trans*-cleavage activity of both Cas9 and Cas12a. Besides, we assessed a specific detection by challenging the system with TMV-infected plant samples (Fig. S2). Nonetheless, a faint band was noticed in the test line in the negative case, so further screening of primers and crRNAs might be performed to enhance the specific detection ability with Cas9.

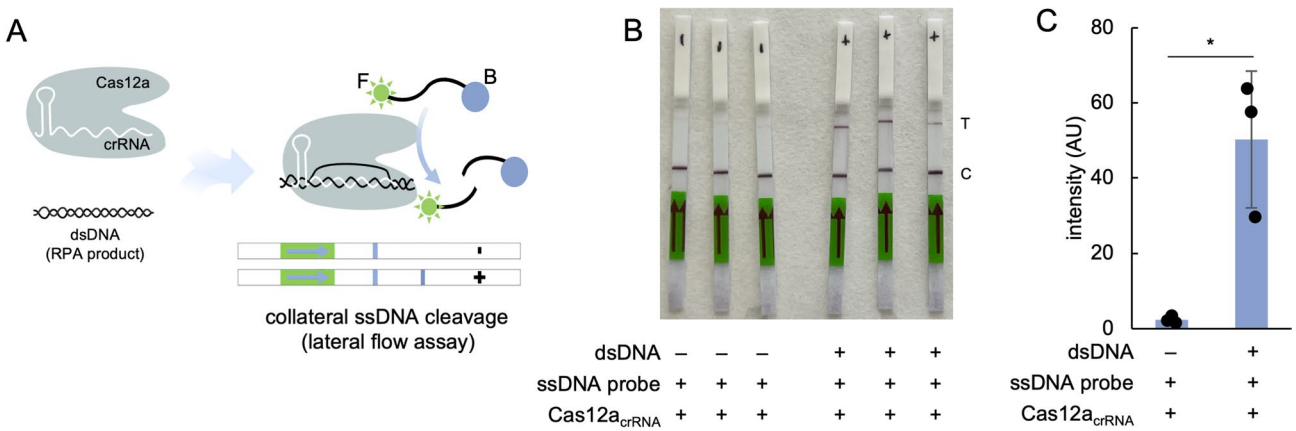

Fig. 2. Detection of ToBRFV with CRISPR-Cas12a. (A) Scheme of the detection in which the RPA product (dsDNA) generated from the viral genome is recognized by a programmed ribonucleoprotein (formed by *L. bacterium* Cas12a and ToBRFV-targeting crRNA) that in turn cleaves an ssDNA probe [labelled with fluorescein (F) and biotin (B)]. A commercial LFA strip is used to reveal the detection. (B) Image of the LFA strips when detecting ToBRFV in tomato plant samples (performed in triplicate). ToBRFV-derived dsDNA generated by RPA. (C) Quantification of test line intensity (bars represent means and standard deviations, $n = 3$). *Statistically significant change by a *t*-test, $P < 0.05$.

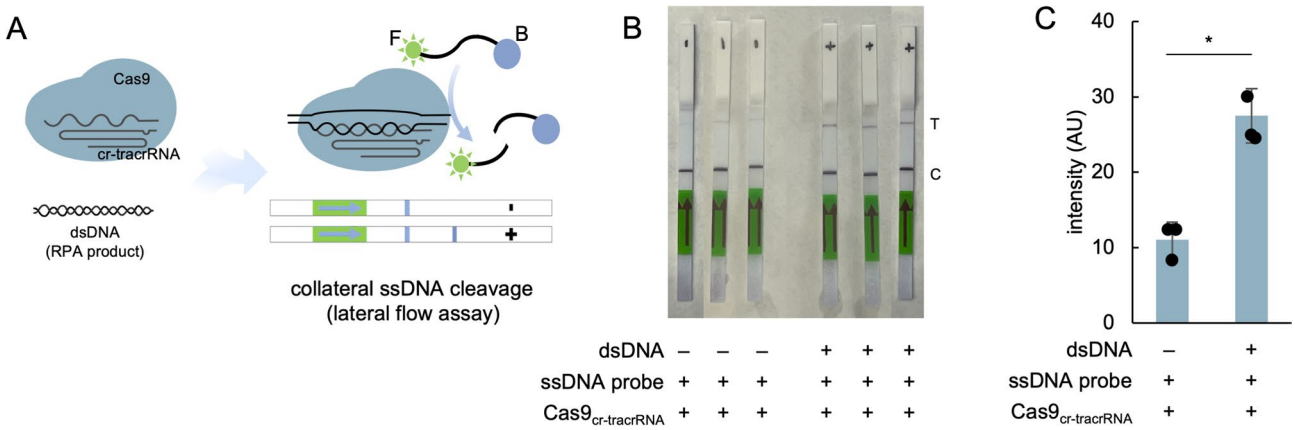

Fig. 3. Detection of ToBRFV with CRISPR-Cas9. (A) Scheme of the detection in which the RPA product (dsDNA) generated from the viral genome is recognized by a programmed ribonucleoprotein (formed by *S. pyogenes* Cas9 and ToBRFV-targeting crRNA) that in turn cleaves an ssDNA probe [labelled with fluorescein (F) and biotin (B)]. A commercial LFA strip is used to reveal the detection. (B) Image of the LFA strips when detecting ToBRFV in tomato plant samples (performed in triplicate). ToBRFV-derived dsDNA generated by RPA. (C) Quantification of test line intensity (bars represent means and standard deviations, $n = 3$). *Statistically significant change by a *t*-test, $P < 0.05$.

Discussion

The findings of this study suggest that both the CRISPR-Cas12a and CRISPR-Cas9 systems are instrumental to have a practical and rapid approach to detect ToBRFV in infected plant tissues using LFA devices. The *trans*-cleavage activity of both Cas12a and Cas9 was exploited to process appropriate ssDNA probes. The current study demonstrates that accurate detection can be achieved swiftly, within a 1.5-h timeframe, starting directly from harvested leaves and maintaining a constant low temperature of 37–39 °C. These methods are applicable to a diverse range of plant species and plant pathogens, thereby representing a promising and expedient approach for next-generation agricultural practices.

In the current study, the ToBRFV genome amplification was conducted using the RPA method, but nothing prevents using other isothermal approaches, such as LAMP. Indeed, Bernabé-Orts et al. utilized LAMP coupled to CRISPR-Cas12a for the detection of ToBRFV[27]. While RPA requires only two oligonucleotides and operates at 37–42 °C, LAMP uses six oligonucleotides and requires higher incubation temperatures of 60–65 °C, so its usage is more demanding in the field. The CRISPR step is important to add specificity to the detection. On the other hand, Nanopore sequencing represents an alternative approach[40]as the devices have increased portability. However, they still require a laptop and more sophisticated sample handling, so CRISPR-Cas detection systems are more suitable for rugged environments.

Yet, the use of CRISPR systems still faces some limitations for field deployment, such as the requirement for specialized reagents, including purified nucleases and RNAs. There is in addition the need for cold chain storage, which hinders their practical use in remote or resource-limited settings, unless working with samples that can be rehydrated[41]. Moreover, many CRISPR diagnostics rely on nucleic acid amplification steps, which complicate portability and increase the risk of contamination. Further work should focus on developing amplification-free strategies for plant virus detection[42].

In sum, we here successfully detected ToBRFV infections in tomato plants. As this study serves as a proof of concept, it is anticipated that these methods will be extended to other crops such as peppers, corn, and potatoes soon. Crucially, CRISPR-Cas systems are also being used as RNA interference tools to fight diseases and to develop more resilient and productive plant kinds[43,44]. In this sense, it is thought that integrated breeding, engineering, and diagnostic programs based on CRISPR-Cas would transform agriculture and contribute to addressing the issue of supplying enough food.

Materials and methods

Plant inoculation

The Iranian isolate of ToBRFV, Accession No. OP557566.1 (kindly provided by Dr. K. Bananej, IRIPP, Tehran), was used for mechanical inoculating four-week-old *S. lycopersicum* L. plants. An aliquot of 100 mg of frozen infected tissues was ground in a ball mill and homogenized in 20 volumes of ice-cold 50 mM phosphate buffer, pH 7.4 [3.1 g $NaH_2PO_4.H_2O$ and 10.9 g Na_2HPO_4 (anhydrous) in ultra-pure water] using an iced mortar and pestle. The homogenate was then centrifuged at 4 °C, and crude extract was obtained from the supernatant. Typically, up to 1 µg RNA per mg of tissue was recovered. A cotton swab soaked in the extract was used to spread the virus using carborundum onto the adaxial side of a plant leaf. A photoperiod of 12 h/day and 12 h/night was maintained in a growth chamber at 25 °C[45].

Preparation of tomato leaf samples

Systemic tissues (approximately 100 mg) were collected from non-inoculated upper leaves at 10 days post-inoculation (dpi). These aliquots were purified using 1 mL of ice-cold RNX-Plus (SinaClon, Karaj, Iran) according to the manufacturer's protocol. Also, different dilutions [by a factor of 10^{10} with ribonuclease-free (DEPC) water] of these original samples were prepared. Following the manufacturer's instructions, complementary DNA (cDNA) was synthesized from extracted RNA using the SinaClon cDNA Synthesis Kit. Furthermore, cDNAs were stored in a refrigerator at -80 °C until use.

ToBRFV detection by PCR

The presence of the virus within the inoculated plants was confirmed by RT-PCR (Eppendorf Mastercycler) followed by gel electrophoresis. 1 µL cDNA was mixed with 200 µM dNTPs (Thermo), 500 nM of forward and reverse primers, and 0.2 U/µL Phusion polymerase (Thermo) in a volume of 20 µL. Reactions were incubated at 98 °C for 30 s for denaturation, followed by 35 cycles of amplification at 98 °C for 10 s and 72 °C for 30 s (manufacturer's 2-step protocol). The forward primer reads AGTAGATGACGCAACGGTGGCTATAAGGAG and the reverse primer TACGTGCCTACGGATGTGTATGAACCATAC. These primers were either used for PCR or RPA.

Guide RNAs

The spacer of the Cas12a-dependent crRNA used in this work reads CAAUGGUCCUCUGCACCUGC, and the spacer of the Cas9-dependent crRNA AUCUCAAGAUGCAGGUGCAG. The crRNAs were synthesized through in vitro transcription using the TranscriptAid T7 High Yield Transcription kit (Thermo) with DNA templates obtained from IDT. Subsequently, the crRNAs were purified using RNA Clean and Concentrator spin columns (Zymo) and quantified using a NanoDrop spectrophotometer.

ToBRFV amplification using RPA

The TwistAmp Basic kit (TwistDX) was used for isothermal amplification of the ToBRFV genome. Forward and reverse primers (480 nM) were combined with 29.5 µL rehydration buffer to reach a total volume of 45.5 µL (adjusted with RNase-free water). The TwistAmp Basic reaction pellet was resuspended with this volume, and 22.8 µL was used for each reaction with 1 µL cDNA. The reaction was initiated by adding 14 mM magnesium acetate. Reactions were incubated at 39 °C for 30 min (Eppendorf Thermomixer) following manufacturer's instructions.

CRISPR-Cas12a reaction

A mix of Cas12a from *L. bacterium* (NEB) and crRNA was incubated for 30 min for ribonucleoprotein formation. 1 µL RPA product was mixed with the CRISPR-Cas12a ribonucleoprotein preparation (50 nM), ssDNA probe (200 nM) in NEBuffer 2.1 [10 mM tris-HCl, 50 mM NaCl, 10 mM $MgCl_2$, and 100 µg/mL BSA (pH 7.9); NEB] to make a total volume of 20 µL. Reactions were incubated for 1 h at 37 °C (Eppendorf Thermomixer). The ssDNA probe was FAM-TTATT-B7, where FAM is fluorescein and B7 biotin. To further assess the detection ability of the method, different nuclease concentrations (50, 100, and 200 nM) and reaction times (30, 60, and 120 min) were assayed with 1 µL RPA product. Besides, TMV-infected *Nicotiana tabacum* plant samples[45] were collected to perform a specificity assay. In particular, 1 µL RPA product from TMV-derived cDNA with the ToBRFV-targeting primers was used in a CRISPR-Cas12a reaction with the ToBRFV-targeting crRNA.

CRISPR-Cas9 reaction

The bimolecule crRNA-tracrRNA was constituted by hybridizing an equimolar amount of both RNAs, incubating them 3 min at 95 °C and then slowly reducing the temperature to 10 °C during 30 min (Eppendorf Mastercycler). A mix of Cas9 from *S. pyogenes* (IDT) and crRNA-tracrRNA was incubated for 30 min for ribonucleoprotein formation. 1 µL RPA product was mixed with the CRISPR-Cas9 ribonucleoprotein preparation (200 nM), ssDNA probe (50 or 100 nM) and COLUMBO[46] reaction buffer [1x Tris/Acetate/EDTA (TAE) buffer pH 8.5 (Invitrogen), 0.05% Tween 20, and 12.5 mM $MgCl_2$] to make a total volume of 20 µL. Reactions were incubated for 1 h at 37 °C (Eppendorf Thermomixer). The ssDNA probe was FAM-TTTTTTTTTTTTTTTTTT-B7. To further assess the detection ability of the method, different nuclease concentrations (50, 100, and 200 nM) and reaction times (30, 60, and 120 min) were assayed with 1 µL RPA product. Besides, TMV-infected *Nicotiana tabacum* plant samples[45] were collected to perform a specificity assay. In particular, 1 µL RPA product from TMV-derived cDNA with the ToBRFV-targeting primers was used in a CRISPR-Cas9 reaction with the ToBRFV-targeting crRNA.

Lateral flow assay

CRISPR-Cas reaction volumes were mixed with 80 µL GenLine Dipstick buffer (Milenia) for 1:5 dilution and the lateral flow strip (HybriDetect, Milenia) was dipped into the reaction tube. Images were captured with a smartphone after 2 min. Image quantification of the band intensity in the test line was done with Fiji software[47].

Data availability

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Author contributions
M.R. S. and H.R. designed research, M.B. and M.R.S. performed research, A.A. and M.F. helped to analyze data, and M.B. and M.R.S. wrote the paper. All authors read and approved the manuscript.

Declarations

Competing interests
The authors declare no competing interests.

Additional information
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