Technical Advance

Toward a CRISPR-Based Point-of-Care Test for Tomato Brown Rugose Fruit Virus Detection

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Abstract

Implementing effective monitoring strategies is fundamental to protecting crops from pathogens and ensuring sufficient food supply as the global population continues to grow. This is especially important for emergent plant pathogens such as tomato brown rugose fruit virus (ToBRFV), which overcomes the genetic resistance resources used in tomato breeding against tobamoviruses and has become a pandemic in less than a decade. Here, we report the development of a CRISPR/Cas12a-based test to detect ToBRFV in the laboratory and potentially in a field setting. Using different tobamoviruses to assess specificity, our test showed a clear positive signal for ToBRFV-infected samples, whereas no cross-reactivity was observed for closely related viruses. Next, we compared the limit of detection of our CRISPR-based test with a widely used reference real-time quantitative PCR test, revealing similar sensitivities for both tests. Finally, to reduce complexity and achieve field applicability, we used a fast nucleic acid purification step and compared its results side by side with those of a commonly used column-mediated protocol. The new protocol saved time and resources but at the expense of sensitivity. However, it could still be useful to confirm ToBRFV detection in samples with incipient symptoms of infection. Although there is room for improvement, to our knowledge, this is the first field-compatible CRISPR-based test to detect ToBRFV that combines isothermal amplification with a simplified nucleic acid extraction protocol.

Keywords: Cas12a, diagnostic, resistance breaking, tobamovirus, tomato

Emergent plant viruses are responsible for disease outbreaks that reduce the yield and quality of many crops (Jones 2021). The occurrence and reach of these outbreaks are accelerating along with globalization and climate change (Trebicki 2020), thus making it necessary to implement effective monitoring and eradication strategies.

Tomato brown rugose fruit virus (ToBRFV, genus *Tobamovirus*, family *Virgaviridae*) is an example of an emergent virus that is presently having an important impact on solanaceous crops such as tomato and pepper. ToBRFV has an ssRNA (+) genome

encompassing four open reading frames (ORFs) that encode the small subunit of the replicase (RdRp, ORF1), the large subunit of the RdRp (ORF2), the movement protein (MP, ORF3), and the coat protein (CP, ORF4). The genus *Tobamovirus* contains examples of notorious plant viruses including tobacco mosaic virus (TMV) and tomato mosaic virus (ToMV). To date, tomato breeding has taken advantage of the *Tm-1*, *Tm-2/Tm-2²* resistance genes to protect new varieties against TMV and ToMV (Hall 1980; Pelham 1966). Nevertheless, ToBRFV overcomes tomato resistance genes (Luria et al. 2017). Furthermore, tobamoviruses are very stable and highly infectious through mechanical transmission (Smith and Dombrovsky 2020; Tomlinson 1987). As a result, ToBRFV has rapidly spread worldwide. Hence, since its first description in the Middle East (Luria et al. 2017; Salem et al. 2016), ToBRFV has expanded to Europe, Asia, and America (Alfaro-Fernández et al. 2020; Fidan et al. 2019; Ling et al. 2019; Menzel et al. 2019; Panno et al. 2020; Skelton et al. 2019; Yan et al. 2019), evidencing the necessity of implementing new measures to prevent its further spread.

Specific testing methods that can be used to detect ToBRFV can critically contribute to the success of intervention and eradication strategies. The most commonly used method for RNA detection is the reverse transcription real-time quantitative polymerase chain reaction (RT-qPCR). Several protocols have been developed using the RT-qPCR technique to detect ToBRFV, including the International Seed Federation (ISF)-ISHI-Veg protocol (ISF 2020) and others, such as the set of primers and probes described by Panno et al. (2019). However, RT-qPCR needs to be performed by qualified operators in a laboratory environment, employing equipment that is not always available, thus limiting its availability, especially in low-resource settings. In contrast, isothermal nucleic acid amplification methods such as loop-mediated amplification (LAMP) (Notomi et al. 2000) can be performed over crude plant extracts at a constant temperature by using a water bath or thermoblock. Some colorimetric LAMP methods have also been developed to easily detect ToBRFV by the naked eye (Rizzo et al. 2021; Sarkes et al. 2020), but these rely on a type of amplification that can be nonspecific. On the other hand, the clustered regularly interspaced short palindromic repeats and its associated proteins (CRISPR/Cas) systems are trespassing the genome editing frontiers and revolutionizing diagnostics. Certain RNA-guided endonucleases such as LbCas12a (former Cpf1)

(Zetsche et al. 2015), after recognizing the target DNA, exhibit an unspecific DNase collateral activity in vitro that can be exploited to degrade a reporter molecule, thereby adding an extra layer of specificity to the LAMP reaction (Chen et al. 2018) (Fig. 1).

Typically, a nucleic acid extract is used as a template for the amplification of the targeted sequence by using LAMP (DNA template) or reverse transcription LAMP (RT-LAMP; RNA template). Next, the LAMP product is recognized by the RNA-guided endonuclease LbCas12a through the specific base pairing of the 20-bp target sequence (purple in Fig. 1) and the guide RNA (gRNA; black in Fig. 1). Target recognition triggers the collateral activity of LbCas12a, producing the unspecific trans-cleavage of an ssDNA reporter oligonucleotide. The molecule conjugated to the 3' end of the ssDNA marks the readout of the assay. When using a fluorophore, the endonuclease activity dissociates the fluorochrome from the quencher at the 5' end of the probe, allowing it to be detected. Conversely, the use of a biotin ligand enables the detection of the LbCas12a activity by the naked eye with the aid of a lateral flow assay (LFA) strip (Fig. 1). This system has already been employed in the diagnosis of human viruses such severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) (Broughton et al. 2020; Curti et al. 2021), influenza A and B (Park et al. 2021), and human papillomavirus (HPV) (Tsou et al. 2019), among others. In plants, isothermal amplification coupled with LbCas12a-mediated detection has also been employed in a handful of studies, mainly for pathogen diagnosis (Aman et al. 2020; Jiao et al. 2021; Mahas et al. 2021) but also for transgenic trait detection (Zhang et al. 2020). Although these works have been carried out to develop field-operative testing methods for pathogen diagnosis (Aman et al. 2020; Jiao et al. 2021; Mahas et al. 2021), only Jiao et al. (2021) actually used nucleic acid isolation protocols that were compatible with field settings. However, this work was carried out using symptomatic samples with a likely elevated titer of the target pathogen, which was perhaps well above the limit of the analytical sensitivity of the detection method. Therefore, the effect of the rapid nucleic acid extraction method on the detection limit of the CRISPR-based tests has not been accounted for and still needs to be evaluated.

Here, we report the design and validation of a CRISPR-based test for the detection of ToBRFV in the laboratory and potentially in a field setting. This test is based on coupling the isothermal amplification of two viral sequences from the ToBRFV MP

FIGURE 1

Pipeline for CRISPR/Cas12a-mediated detection processes. A nucleic acid extract is used as a template for the loop-mediated amplification (LAMP) or reverse transcription LAMP of the target sequences. The amplification product is recognized by the RNA-guided endonuclease LbCas12a through base-pair complementarity of the guide RNA and the target sequence, thus triggering the collateral activity of the LbCas12a, which, in a nonspecific manner, degrades the ssDNA probe, revealing the presence of the target sequence. The reporter molecule attached to the probe defines the readout of the assay, either by fluorescence or using a lateral flow strip.

ORF, with subsequent detection by using the RNA-guided endonuclease LbCas12a and a specific gRNA. The performance of the CRISPR-based test was compared with that of a standard RT-qPCR test recommended by the European Plant Protection Organization (EPPO), after which the protocol was adapted for field application.

MATERIALS AND METHODS

Virus isolates and plant inoculation

The Spanish ToBRFV isolate from Vicar (Almería, Spain) (Alfaro-Fernández et al. 2021) was provided by the Laboratorio de Producción y Sanidad Vegetal (La Mojonera, Almería, Spain). Isolates of the other tobamoviruses used in this study were acquired from the DSMZ collection: TMV (PV-1252), ToMV (PV-0141), pepper mottle mosaic virus (PMMoV, PV-0093), and tobacco mild green mosaic virus (TMGMV, PV-0124). Approximately 50 mg of dried plant tissue was homogenized from each isolate in 2 ml of 30-mM phosphate buffer (pH = 8) using a mortar and pestle. Homogenates were used to mechanically inoculate 25- to 26-day-old leaves (4 to 5 true leaves) of *Nicotiana benthamiana* plants and the first pair of true leaves of 7- to 10-day-old tomato plants (cultivar M82). For this, the leaves to be inoculated were first dusted with carborundum powder (600 mesh) and then rubbed with the homogenate manually. The inoculated plants were kept separately in a confined greenhouse under controlled conditions set at a 16/8-h photoperiod and 26/22°C in a day/night cycle. After 10 to 15 days, the leaves that showed obvious symptoms of infection were collected, cut, mixed, and divided into samples of approximately 100 mg. The samples were frozen in liquid nitrogen, ground with a Retsch Mixer Mill MM400 for 1 min at 30 Hz, and stored at -80°C for later analyses.

Column-mediated RNA extraction

Column RNA extraction was performed using a NucleoSpin RNA Plant kit (MACHEREY-NAGEL, Germany) following the manufacturer's instructions. The RNA was checked by running a 1% agarose gel, its concentration measured with a NanoDrop One (Thermo Scientific, U.S.A.), and adjusted to a working concentration of 10 ng/μl to be used as a template for the RT-LAMP, RT-PCR, and RT-qPCR.

RT-LAMP

Loop-mediated isothermal amplification primers (Supplementary Table S1) were designed using PrimerExplorer v.5 (<https://primerexplorer.jp/e/>). RT-LAMP reactions were performed using the WarmStart LAMP Kit (NEB, U.S.A.) at a final volume of 10 μl. Two sets of primers were designed for amplification of the ToBRFV MP ORF (MP1 and MP2) and an additional set as a positive detection control (PDC) to amplify the 25S ribosomal RNA from solanaceous species. Primers were added at a final concentration of 0.2 μM for F3 and B3, 1.6 μM for FIP and BIP primers, and 0.8 μM for LF and LB primers. The reactions were performed independently for each set of primers (MP1, MP2, and 25S) using 2 μl of input RNA. The amplification was performed at a constant temperature of 62°C for 25 to 45 min (50 to 90 cycles of 30 s each) in an Applied Biosystems StepOnePlus Real-Time PCR System (U.S.A.) and tracked with a DNA-intercalant green fluorophore provided with the WarmStart LAMP Kit (NEB). The RT-LAMP amplification was set to 45 min for experiments illustrated in Figures 2, 3A, and 3B. This incubation was reduced to 25 min for experiments in Figures 3C, 3D, and 4.

LbCas12a trans-cleavage assay

LbCas12a trans-cleavage assays were performed using fluorescent and biotinylated reporters, as described by Broughton et al. (2020) and Tsou et al. (2019). When using a fluorescent reporter, 40 nM of LbCas12a (EnGen Lba Cas12a, NEB) was pre-incubated with 40 nM of chemically synthesized gRNA (Synthego, U.S.A., Supplementary Table S1), and 100 nM of an ssDNA fluorescent (Supplementary Table S1) reporter for 10 min at 37°C. Then, 2 μl of LAMP product was combined with 18 μl of LbCas12a-gRNA complex and incubated for 10 min at 37°C (20 cycles of 30 s each) in an Applied Biosystems StepOnePlus Real-Time PCR System to detect the resulting fluorescence. When using a biotinylated reporter, the amount of LbCas12a was increased to 100 nM and the gRNA to 125 nM, maintaining the pre-incubation time and the 100 nM of the reporter molecule (Supplementary Table S1). Again, 2 μl of LAMP product was mixed with 18 μl of LbCas12a-gRNA-reporter mixture and incubated for 10 min at 37°C. Finally, 80 μl of 1× HybriDetect assay buffer was added to the reaction mixture, and the lateral flow strip (Milenia HybriDetect - Universal Lateral Flow Assay Kit, Germany) was immersed for 2 min. Images of the LFA strips were collected using an office scanner and quantified with the ImageJ software.

RT-PCR

The cDNA was prepared using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Switzerland) following the manufacturer's instructions, and AB-783 oligonucleotide priming the 3'-UTR of ToBRFV. The resulting cDNA was diluted 1/10 with water and used as a template for PCR amplification of a fragment of the MP ORF by using Phusion Hot Start II High-Fidelity DNA Polymerase (Thermo Fisher, U.S.A.) and a final concentration of 0.5 μM of AB-782 and AB-783 primers (Supplementary Table S1). The PCR product was assessed by 1% agarose gel electrophoresis and cleaned using GeneClean Turbo Kit (MP Biomedicals, U.S.A.) silica gel columns.

LbCas12a-mediated restriction reaction and efficiency estimation

A restriction reaction was set containing 250 ng of the same PCR product obtained in the previous section, "RT-PCR", 1 μl, 1 μM of LbCas12a; 1 μl, 1 μM of gRNA of each target (MP1, MP2, or 25S); 3 μl of 10× NEB2.1; and RNase-free water up to 30 μl. The reaction mixture was incubated for 1 h at 37°C, after which the restriction product was resolved in a 2% agarose gel for 45 min. The efficiency of each target was estimated using ImageJ for the quantification of the digested and nondigested bands. The value of the digested band was divided by the sum of the nondigested plus the digested bands, and the resultant value was multiplied by 100 to obtain the %Efficiency shown in Supplementary Figure S3.

In vitro transcription

RNA fragments of the ToBRFV MP ORF were synthesized from a PCR product (see the "RT-PCR" section) that included the T7pol promoter. One microliter of PCR product was employed to produce the transcripts with the T7 RNA polymerase (Promega, U.S.A.), setting a 20-μl reaction for 2 h at 37°C. Then, 3 μl of DNase I (NEB) was added, and the mixture was incubated for 15 min at 37°C. The RNA was precipitated by adding water to 100 μl, 10 μl, 3M of sodium acetate and 250 μl of ethanol and incubated

for 1 h at -80°C . The RNA was sedimented by centrifugation at 13,000 rpm for 30 min at 4°C , the supernatant was discarded, and finally, the pellet was air-dried and resuspended with 20 μl of RNase-free water. The resulting RNA was checked as described in the “Column-mediated RNA extraction” section.

RT-qPCR

Real-time quantitative PCR was performed using KAPA PROBE FAST Universal One-Step qRT-PCR (Roche, Switzerland), a specific pair of primers, and a probe for amplification and detection of ToBRFV (Supplementary Table S1). Reactions of 10 μl were carried out using a final concentration of 100 mM of oligonucleotides and up to 100 ng of RNA template. A StepOne-Plus Real-Time PCR System (Applied Biosystems) thermal cycler was employed following this program: reverse transcription at 42°C for 5 min; denaturation at 95°C for 3 min; 40 cycles of amplification with a denaturation step at 95°C for 3 s; and annealing and elongation steps at 60°C for 30 s.

ToBRFV time-course experiment

In total, twelve 5-week-old tomato plants of cultivar M82 (3 to 4 pairs of leaves approximately) were mechanically inoculated (see above). Three plants per data point (1, 2, 3, and 4 days post-inoculation) were sampled, collecting the first pair of new leaves observed to accumulate more virus (van de Vossen et al. 2020). Then, the leaves were finely sliced and mixed, after which 100 mg of tissue was introduced into a 2-ml tube with a pair of metal beads for subsequent RNA extraction following the column- or the paper strip-mediated protocols. These samples were frozen in liquid nitrogen and stored at -80°C for later analysis.

Paper strip-mediated RNA isolation

A rapid RNA extraction protocol was performed following the protocol reported by Zou et al. (2017). Briefly, samples were lysed by shaking them manually in 500 μl of lysis buffer (20 mM of Tris,

FIGURE 2

Design and assessment of a CRISPR-based testing method for the detection of tomato brown rugose fruit virus (ToBRFV) movement protein (MP) open reading frame (ORF). **A**, Representation of the ToBRFV genome and the oligonucleotides used for the detection of the MP ORF sequence (MP1 and MP2). The position of the reverse transcription loop-mediated amplification primers is represented by the black rectangles (F3, B3, FIP, BIP, LF, and LB). The white rectangles represent the binding position of the F1c and the B1c halves of the FIP and BIP primers. The blue rectangles represent the location of the guide RNAs (gRNAs MP1 and MP2). An additional set of primers was used to amplify the rRNA 25S as a positive detection control (25S, not shown). **B**, Evaluation of the specificity of the CRISPR-based test MP1, MP2, and 25S targets using a no-template control, a healthy-plant RNA extract (wild-type), and samples infected with different tobamoviruses (pepper mottle mosaic virus [PMMoV], tobacco mild green mosaic virus [TMGMV], tomato mosaic virus [ToMV], and tobacco mosaic virus [TMV]) related to ToBRFV, using a fluorescent reporter. Bars represent the average of three technical replicates (black dots). **C**, Evaluation of the specificity using a biotinylated reporter with lateral flow strips and TMV-, ToMV-, TMGMV-, PMMoV-, and ToBRFV-infected tomato plants (T is the test line and C the control line).

pH = 8.0, 25 mM of NaCl, 2.5 mM of EDTA, 0.05% SDS). Next, a homemade cellulose dipstick was introduced three times in the crude extract to retain the nucleic acids and then washed three times in 1.75 ml of wash buffer (10 mM of Tris, pH = 8.0, 0.1% Tween-20). After this, the nucleic acids retained to the dipstick were directly eluted into the LAMP mixture.

RESULTS

Design and specificity of a CRISPR-based test for ToBRFV detection

The MP ORF (ORF3 in Fig. 2A) is the genetic determinant for overcoming the genetic resistance conferred by the *Tm-2²* allele to other tobamoviruses such as TMV and ToMV (Hak and Spiegelman 2021); based on this, we reasoned that the MP might be an appropriate target for our CRISPR-based testing method.

We first screened the ToBRFV MP ORF sequence to identify LbCas12a gRNA candidates (Kim et al. 2017). After filtering candidates considering specificity and structural criteria (Bernabé-Orts et al. 2019), gRNA MP1 and gRNA MP2 were

chosen (Fig. 2A, Supplementary Fig. S1, and Supplementary Table S1). Next, we devised two sets of RT-LAMP primers flanking each gRNA target (Fig. 2A, Supplementary Fig. S1, and Supplementary Table S1). From this point on, we will refer to each set of gRNA and primers as MP1 and MP2, respectively. Importantly, MP1 and MP2 overlap the ISF-ISHI-veg RT-qPCR CaTa28 test (ISF 2020), facilitating comparisons. We also included a complementary PDC called 25S, which targets the 25S subunit of the ribosomal RNA from solanaceous species. Using RNA extracts obtained from plants infected with different tobamoviruses (PMMoV, TMGMV, ToMV, TMV, and ToBRFV), we demonstrated that our CRISPR-based test can detect ToBRFV without cross-reactivity with closely related viruses (Fig. 2B). Although all of the samples were positive for the 25S PDC, only the ToBRFV-infected sample gave rise to fluorescent signals above the background levels for all three targets, indicating that ToBRFV-negative results were due to the absence of this virus rather than a testing failure of MP1 or MP2. This result was corroborated when using LFA strips with the TMV-, ToMV-, TMGMV-, PMMoV-, and ToBRFV-infected samples (Fig. 2C). The ToBRFV extract showed intense test lines for MP1, MP2,

FIGURE 3 Comparison of the detection limits of the CaTa28 RT-qPCR and the CRISPR-based tests for the detection of tomato brown rugose fruit virus (ToBRFV). Limit of detection of RT-qPCR and CRISPR estimated with serial dilutions of a synthetic RNA transcript fragment of the movement protein open reading frame (A, B) or an RNA extract from ToBRFV-infected tomato leaves (C, D). Each RNA dilution was assessed in triplicate. As negative controls, no-template control and healthy tomato plant (wild-type) RNA extracts were used. A healthy tomato plant RNA extract was used as diluent in all cases. The dotted lines show the cutoff value.

and 25S, whereas the rest of the samples were positive only for the 25S target, aligning with the previous results obtained with the fluorescent reporter. Interestingly, the test lines of the MP2 and the 25S targets were more intense than that for the MP1. An evaluation of the efficiency of the three gRNAs showed that MP2 gRNA was the most efficient (62%), followed by the 25S (48%) and the MP1 (39%) (Supplementary Fig. S2), revealing a correlation between the gRNA efficiency and the LFA output, as Zhang et al. (2020) previously showed. Altogether, these results confirmed that our CRISPR-based testing method specifically detected ToBRFV while discarding false negatives at the same time, thanks to the testing process with the 25S PDC. Importantly, the test also allowed for the adaptation of the visualization of the results by fluorescence and/or by the naked eye.

Performance of the CRISPR-based test for ToBRFV detection

Next, we sought to assess the performance of our CRISPR-based detection platform and compare it with the CaTa28 RT-qPCR test described in the ISF-ISHI-Veg protocol (ISF 2020), supported by the EPPO (Fig. 3). This RT-qPCR test is directed to the MP ORF of ToBRFV, near our MP1 and MP2 target sites,

thus ruling out a possible bias in the efficiency due to positional effects. First, the limit of detection of both methods was assessed using serial dilutions of a synthetic transcript encoding a fragment of the MP to accurately evaluate the number of viral copies per reaction (rxn) that each method was able to detect (Fig. 3A and B). These experiments revealed that under our particular conditions of equipment, material, and reagents, the CaTa28 RT-qPCR test reliably detected up to 100 copies of transcript/rxn, although one technical replicate corresponding to the 10 copies/rxn dilution was also positive (Fig. 3A). Similarly, our CRISPR-based method detected up to 100 copies/rxn dilution, although with some difficulties for the MP2 target, which failed to detect the synthetic transcript in one technical replicate (Fig. 3B). The sensitivity of both techniques was also compared using serial dilutions of an RNA extract obtained from a tomato plant infected with ToBRFV (Fig. 3C and D). In this case, the CaTa28 RT-qPCR test (Fig. 3C) outperformed the CRISPR-based tests (Fig. 3D) by detecting the viral RNA in a dilution one order of magnitude higher (10^{-5} ng/rxn) than the CRISPR method (10^{-4} ng/rxn). Although some technical replicates were positive beyond this 10^{-4} ng/rxn dilution, this was the last point consistently detected, suggesting that this is the limit of detection of our CRISPR-based method.

FIGURE 4

Influence of the RNA extraction protocol on the CRISPR-based detection. Twelve tomato brown rugose fruit virus-inoculated tomato plants (P1 to P12) were sampled at 1, 2, 3, and 4 days post-inoculation, three plants each time. The pair of nascent leaves was collected, and the sampled tissue was finely sliced, mixed, and split into two subsamples used for the conventional column-mediated RNA extraction protocol or a rapid paper strip-mediated nucleic acid extraction protocol to capture the nucleic acids (Zou et al. 2017). Fluorescent readout from the column-extracted samples (A) and the rapid-extracted samples (B). Bars represent the average of three replicates (black dots). Signal quantification from the lateral flow assay strips of the column-extracted samples (C) and the rapid-extracted samples (D). Each value was normalized to the highest value. As negative controls, no-template control and healthy tomato plant (wild-type) RNA extracts were used. Quantification of the test line was performed using ImageJ. The dotted lines in A and B show the cutoff value.

In summary, these results showed that our CRISPR detection method possesses a similar sensitivity to the CaTa28 RT-qPCR, at least when a synthetic transcript is used as a template. When using dilutions of an RNA extract, the CRISPR test showed one order of magnitude less sensitivity than the RT-qPCR test.

Development of a rapid protocol for CRISPR testing

RNA isolation is a critical step that consumes most of the time and resources of the testing process, influencing at the same time the output of the subsequent detection method. For this reason, we sought to couple a rapid RNA extraction protocol, which requires a paper strip to retain the nucleic acids (Zou et al. 2017), with our CRISPR-mediated testing protocol and compare it with the standard column-mediated RNA extraction protocol that we had been using in our previous experiments (Figs. 2 and 3). To this end, we generated a set of samples by mechanically inoculating 12 tomato plants (P1 to P12) and sampling them at different days post-inoculation (dpi) in subsets of three biological replicates. Our purpose was to obtain a collection of samples with a variable range of viral load, use them to extract the genetic material of the virus with both protocols, and compare the performance of our test after that.

Figure 4A shows the data obtained when using the column-mediated RNA extraction protocol. These results revealed that ToBRFV could be detected in systemically infected tissue as soon as 1 dpi, at least for plant 1 (P1), which was positive for both MP1 and MP2 targets. P2 and P3 were positive only for some replicates and only for one target. At the following time points, only P6 was negative, detecting one replicate of the MP2 target. ToBRFV-negative plants were positive for the 25S PDC, indicating that the absence of a signal for MP1 and/or MP2 targets reflected a low titer or absence of the virus in these samples rather than test failure. Overall, the signal intensity of the MP1 and MP2 targets increased as the infection progressed, indicating an increase in the viral load. For example, at 1 dpi, the signal of P1 for the MP1 and MP2 targets was 3 to 4 times higher, respectively, as compared with the wild-type sample, whereas this difference was 10 to 14 times higher at 4 dpi. In contrast, the signal intensity of the 25S target remained steady through all the data points, indicating the stability of the RNA extraction for all the time points. When using the paper strip-mediated RNA extraction protocol (Fig. 4B), clear positives were only detected at 4 dpi (P10 to P12). Before this time point, some biological replicates were inconsistently positive (e.g., P1 or P8), not allowing for a clear diagnosis of these samples with this protocol. Furthermore, in general, the signal intensities were lower than for column-mediated RNA extractions (compare Fig. 4A and B). Finally, to check the visual output using the LFA strips, we selected the replicates that were positive in the previous experiments (Fig. 4A and B) and used them to perform the LbCas12a-mediated detection, this time with a biotinylated reporter. The signal intensities of the LFA strip test lines shown in Supplementary Figure S3 were quantified and the resulting values plotted in the heat maps shown in Figures 4C (column-mediated extraction) and 4D (paper strip extraction). As expected, the results aligned perfectly well with those obtained with the fluorescent reporter. Even the replicates that displayed low fluorescent signals close to the detection limit (e.g., MP2 target on P1 Fig. 4A or MP1 target on P1 in Fig. 4B) were easily detected with the LFA approach, confirming that fluorescent results can be extrapolated to naked-eye visualization. In sum, despite the considerable reduction in time, labor, reagents, and equipment associated with the paper strip-extraction procedure, the column-mediated protocol was the most

efficient, allowing for detection of ToBRFV systemic infection 3 days earlier than the paper strip protocol.

DISCUSSION

One important step in disease control is the unambiguous identification of the pathogen to limit or prevent its spreading. RT-qPCR tests have become the gold standard due to their versatility and ability to detect a few molecules of nucleic acid from the disease agent. However, the technical complexity of RT-qPCR restricts its application to laboratory settings.

CRISPR-based diagnostics promises to transfer the versatility, sensibility, and specificity of genetic testing methods from the laboratory to field settings, accelerating the decision-making process while reducing costs. In the past few years, the natural diversity of CRISPR/Cas systems has been harnessed to develop many testing methods with different chemistry and analyte specificity. For a sensitive detection, all of these methods require a pre-amplification step of the targeted sequence from the pathogen genome. The difference among these methods stems from the molecule that the Cas enzyme can recognize. Thereby, the endoribonuclease Cas13a recognizes an ssRNA analyte (Gootenberg et al. 2017), the endonuclease Cas12a a dsDNA (Chen et al. 2018), and the Cas14 an ssDNA (Harrington et al. 2018). This directly influences the chemistry of the detecting process, imposing some limitations. For example, when using the Cas13a system, an RNA polymerase is needed to transcribe the amplified dsDNA target sequence to an ssRNA moiety that can be recognized by the endoribonuclease. Similarly, Cas14a requires a T7 exonuclease to obtain an ssDNA sequence from the amplified target. Conversely, the LbCas12a can detect the dsDNA amplification product directly, saving steps that require additional enzymatic activities. Therefore, to our understanding, we selected the most straightforward approach, using the LbCas12a endonuclease to develop our CRISPR-based testing method for ToBRFV.

A pre-amplification step must be carried out to improve the sensitivity of the CRISPR-mediated detection method. Usually, to circumvent the utilization of sophisticated thermal cyclers, isothermal amplification methods such as recombinase polymerase amplification (RPA) (Piepenburg et al. 2006) or LAMP (Notomi et al. 2000) are the best choice for point-of-care applications. Both isothermal amplification systems are comparable in terms of sensitivity, rate of amplification, and tolerance to inhibitors (Zou et al. 2020). The main differences are found in the enzymatic activities involved in the process, which determine the molecular mechanism underlying the amplification and the temperature of incubation. Thus, similar to PCR, RPA only requires two oligonucleotides and can work at 37 to 42°C. In contrast, the LAMP reaction involves six oligonucleotides and higher incubation temperatures of 60 to 65°C. Apparently, as RPA and LbCas12a share the same incubation temperature of 37°C, this could be the best match for a single-tube amplification plus detection reaction. However, the different chemistry of both processes complicates their combination into a single-tube reaction, or at least reduces the sensitivity of the detection, as described by Kellner et al. (2020) when using Cas13a. Aman et al. (2020) used this approximation for plant virus detection, although no direct comparison with other testing methods was carried out. In addition, the simplicity of the RPA is a double-edged sword, as this technique is more tolerant to mismatches and thus to spurious amplifications (Li et al. 2020). Altogether, we decided to use LAMP and LbCas12a in a two-tube reaction and eventually upgrade our protocol to a single-tube reaction. To this end, the amplification and detection reactions can be physically separated with an inorganic phase, as previously done by Wang et al.

(2021), or combined by using a thermostable Cas enzyme such as AapCas12b endonuclease (Joung et al. 2020). In this latter work, an extensive screening of chemical adjuvants was conducted to enhance the simultaneous amplification and detection reactions, evidencing the incompatibility of both processes and reinforcing our strategy.

Hence, we targeted two different positions of the MP ORF (MP1 and MP2) and added a PDC (25S), thus facilitating the interpretation of the results. Our ToBRFV CRISPR-based assay was considered positive if there was detection in MP1 and MP2 or presumptive positive if there was detection in either MP1 or MP2. A negative test would be when only 25S was detected. Examples of these outputs can be found in Figure 4. First, we confirmed the specificity of our test using plants infected with different tobamoviruses, showing that only ToBRFV-infected samples gave rise to a strong signal for both MP1 and MP2, whereas the 25S PDC was detected in all the samples except the no-template control. Next, we assessed the detection limit of our CRISPR-based test by comparing it with the CaTa28 RT-qPCR test standard. Note that to capture all the amplification events, including samples with a low number of copies, the amplification time was set to 45 min in the first instance (Fig. 3A and B). However, being aware that a long amplification time might reduce the field applicability of our CRISPR-based method, this incubation was reduced to 25 min in the following experiments, illustrated in Figure 3C and D and Figure 4. Using a synthetic RNA extract as a template, the CaTa28 test and our CRISPR-based test showed equivalent detection limits. Conversely, when using dilutions of an RNA extract obtained from a ToBRFV-infected tomato plant, the CaTa28 RT-qPCR test showed a detection limit one order of magnitude higher than our CRISPR-based test. Furthermore, the signal from the CRISPR test decreased with the magnitude of the RNA dilutions, which could reflect a decrease in the LAMP product due to the reduction in the amplification time. Altogether, these results showed that our CRISPR detecting method possessed a similar sensitivity to the CaTa28 RT-qPCR test, at least when the LAMP amplification time was extended and a synthetic transcript was used as a template. Decreasing to half the amplification time reduced the limit of detection by one order of magnitude but shortened the detection process considerably. Finally, to adapt our test to a field setting, we shifted from a conventional column-mediated RNA extraction protocol to a rapid RNA extraction protocol that only involves a strip of paper to adsorb the nucleic acids present in a crude plant extract (Zou et al. 2017), as previously used by Zhang et al. (2020) to detect the *Bt* transgene in rice. The results revealed that the RNA extraction protocol critically influenced the output of the test. The column-mediated protocol outperformed the rapid one, which was only able to detect ToBRFV at 4 dpi, when the plants presented slight symptoms of infection such as subtle blistering and/or mottling. Therefore, in practice, this rapid protocol could be reserved for plants that present clear symptoms of virus infection. In the future, more rapid extraction procedures such as that described by Silva et al. (2018) need to be tested to assess their compatibility and influence on the performance of our assay.

While writing this manuscript, Alon et al. (2021) reported on the use of CRISPR/Cas12a to detect ToBRFV. Notably, the authors designed and screened efficient gRNAs for the differential detection of ToMV and ToBRFV and evaluated their ability to detect serial dilutions of an end-point PCR product using a fluorescent reporter. In spite of the significance of the work, important aspects for virus detection such as an extensive validation of the assay specificity by using several tobamoviruses, or the estimation of the detection limit, were not addressed in this study. Our work was aimed at developing a CRISPR-based test to de-

tect ToBRFV, which could potentially be deployed in the field. To this end, we contemplated all the steps involved in the testing process, including the amplification of the RNA template, and not only the CRISPR/Cas12a-mediated detection. We chose an isothermal amplification method such as RT-LAMP, which is compatible with a field setting, in comparison with the RT-PCR used by Alon et al. (2021). Importantly, the isothermal amplification imposes some design constraints (e.g., primers, amplicon size) that drastically reduce the target selection, so a good gRNA might not necessarily match with a good primer set for amplification. Therefore, the selection of a proper primer set in conjunction with the gRNA design is crucial for achieving good sensitivity levels, as Joung et al. (2020) demonstrated. In addition to a field-compatible amplification method, we also checked the sensitivity of our CRISPR-based test coupled with a paper strip-mediated RNA isolation protocol, which is also applicable outside of the laboratory. Finally, aside from the fluorescent reporter, we also used LFA strips, which are usable in the field. Altogether, we believe that our test is closer to field deployment for the following reasons: (i) It can be coupled with rapid nucleic acid extraction, (ii) it is based on isothermal amplification, and (iii) its results can be read by the naked eye using LFA. To our knowledge, this is the first report of a CRISPR-based test to detect ToBRFV developed to be as close as possible to field deployment. Although its applicability under field conditions is still debatable, this work paves the way for further improvements, which could soon lead to simple and effective tests for the detection of ToBRFV in the field.

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