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Reverse transcriptase recombinase polymerase amplification for detection of tomato spotted wilt orthotospovirus from crude plant extracts

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Virus detection in early stages of infection could prove useful for identification and isolation of foci of inoculum before its spread to the rest of susceptible individuals via vectoring insects. However, the low number of viruses present at the beginning of infection renders their detection and identification difficult and requires the use of highly sensitive laboratory techniques that are often incompatible with a field application. To obviate this challenge, utilized Recombinase Polymerase Amplification, an isothermal amplification technique that makes millions of copies of a predefined region in the genome, to detect tomato spotted wilt orthotospovirus in real time and at the end point. The reaction occurs isothermically and can be used directly from crude plant extracts without nucleic acid extraction. Notably, a positive result can be seen with the naked eye as a flocculus made of newly synthesized DNA and metallic beads. The objective of the procedure is to create a portable and affordable system that can isolate and identify viruses in the field, from infected plants and suspected insect vectors, and can be used by scientists and extension managers for making informed decisions for viral management. Results can be obtained in situ without the need of sending the samples to a specialized lab.

Tomato spotted wilt orthotospovirus (TSWV) causes extensive losses worldwide to various crops and ornamentals[1] that can account to as much as 1 billion dollar[2]. These losses are in part due to the generalist nature of the virus that can infect up to 1000 species of plants, including monocots and dicots[3]. This plant pathogen has one of the largest host ranges on record for plant viruses[4]. by more than a dozen thrips species[5], such as *Franklinella occidentalis*, *F. fusca*, *F. gemina*, *Thrips setosus* and *Thrips tabaci*[6]. The virus has an ambisense ssRNA genome, comprised of 3 segments named by size small (S), medium (M) and large (L), surrounded by coat proteins and enclosed by a lipidic envelope[3,7]. Like for other plant viruses, virus and vector exclusion and eradication are the most effective control measures. The use of ultraviolet-reflective mulches, for instance, has been proven to be useful in avoiding primary spread[8].

Early detection of TSWV and other plant pathogens is critical for adequate selection and deployment of counter measures[2]. Common techniques for detection of TSWV include molecular methods such as RT-PCR[9], and Next Generation Sequencing[10]; nevertheless, they necessitate expensive lab equipment, can be time consuming and require trained personnel[11]. On the other hand, immunology-based methods are most commonly used, some examples are immunostrips[12] and ELISA[13]. Unlike molecular methods, immunology methods do not offer a high degree of sensitivity, leading to the possibility of false negative and missed virus detection. Losses caused by a lack of detection can be significant, especially for an economically important virus, such as TSWV. Providing a field-based, inexpensive, sensitive, and easy-to-use detection assay would allow to test infected material, even before the onset of symptoms, and would result in better disease management.

Recombinase polymerase amplification (RPA)[14] is a nucleic acid sequence based amplification (NASBA)[15] that allows amplification, at low constant temperature[16]. Unlike Loop-mediated Isothermal amplification (LAMP), another isothermal amplification technique which requires a temperature of 65 °C, RPA can perform well at temperatures close to 37 °C, and even ambient temperature, although with lower efficiency[17]. Because of this,

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RPA forgoes the need of a thermal cycler and bulky equipment and can be used in the field[18,19]. Even more conveniently, all reaction components can be lyophilized, allowing room temperature storage and transport under field conditions, and the assay can be performed by untrained personnel[16]. Mechanistically, RPA exploits a recombination and repair system found in phages[20]; one of its reagents consist of a nucleoprotein complex (recombinase + primer) that stabilizes ssDNA between the primers, followed by extension by DNA polymerase. The end-point product is usually tagged with fluorescein on one side and a biotin that is attached to the reverse primer, and therefore, can be detected using a commercially available Amplification detection chambers, here referred to as lateral flow devices[21] which contain proprietary mixes of antibodies that detect marked amplification products[22].

In cases in which there is the need to follow RPA in real time, the design of the probe can be modified to move the FAM molecule closer to the recombination site and quenched in the intact probe. In this way, as the reaction happens, the FAM is cleaved and its concentration can be detected with a fluorometer, as directly proportional to the amount of amplicon being produced[23]. Advantages of reverse transcriptase recombinase polymerase amplification RT-RPA are its readiness and affordability, specificity, sensitivity, speed and easiness of use[24] as well as the possibility of multiplexing[25]. Notably, the sensitivity and specificity of this reaction are comparable and sometimes even superior to that of PCR[18], in some cases up to tenfold[26]. Because of these reasons, RT-RPA is becoming a favorite technique for performing lab-on-a-chip microfluidics analyses that require minimum incubation and can give real-time measurements[16].

Binding-flocculation detection of amplicons is a cheaper and equally sensitive approach for detection of RPA products. This techniques exploits the property of aggregation of newly-synthesized nucleic acid and magnetic beads, which form stable floccules under acidic conditions, indicating a positive result[27]. Interestingly, this same principle can be used for a variety of applications, including the detection of methylation[28].

In this work, we have developed a RT-RPA assay for specific detection of TSWV. The assay results can be interpreted through quantitative real time RPA or end point assays like lateral flow strip assay and flocculation assay. We envision that such a test will become a useful addition to the toolkit already in use for TSWV diagnosis.

Results

End-point RPA can be visualized in amplification detection chambers. Crude extract from a leaf infected with TSWV was used for RT-RPA reaction and the results of the amplification were visualized in a lateral flow device (Agdia, Elkhart, IN). As seen in Fig. 1, both the control (top) and the test line (bottom line) were visible in the chamber where the extract from the infected leaf was loaded (left chamber). This result, indicates a positive amplification, confirming the presence of the dual labelled RT-RPA products and thus of TSWV in the mixture. A negative control, made with the crude extract of a non-infected plant, and one where water was used instead of leaf extract were also subjected to the same procedure. Results in Fig. 1 (center and right chambers respectively) show that for both controls only the control line (top line) was visible, indicating a valid test but negative result.

End-point RT-RPA can be visualized by flocculation assay. After performing the RT-RPA amplification, the tubes were removed from the thermal block, SPRI (Solid Phase Reversible Immobilization) magnetic beads were added to the amplified fraction, washed with ethanol, followed by the addition of the acetate-based crowding agent. The tube that contained the crude extract of the TSWV infected *N. benthamiana* showed a stable flocculus on the bottom of the tube that did not break, despite flicking the tube repeatedly (Fig. 2, left tube). Since the flocculus collects all the beads in the mixture in one small mass, the solution in the tube becomes clear. On the other hand, the vessel containing the non-target controls, did not form a stable flocculus and, due to the even distribution of beads in the mixture, the mixture remained opaque (Fig. 2, center and left tube).

TSWV RT-RPA assay can detect multiple TSWV isolates in multiple plant species. To ensure that our RT-RPA reaction would detect different TSWV isolates and in different plant species, we repeated the

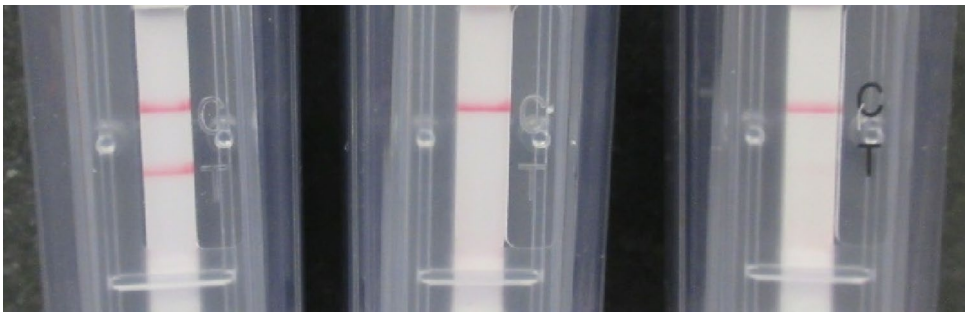

Figure 1. Result of end-point RT-RPA) in Lateral flow devices (Agdia, Elkhart, IN). Left: positive result for crude extract of a TSWV infected *N. benthamiana* leaf, where both the control (top) and the test line (bottom) are visible. Center and Right: negative results for crude extract of an uninfected leaf and water respectively, where only the control line (top) is visible. NTC1: healthy *N. benthamiana*, NTC2: water.

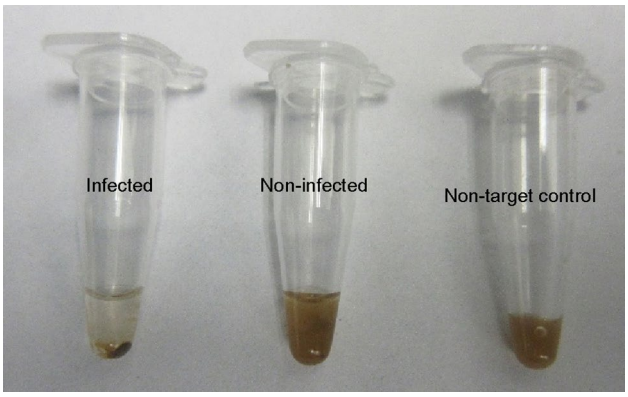

Figure 2. Result of end-point RT-RPA with SPRI beads. Left: positive result, after rehydrating the pellet and the amplification reaction, added SPRI beads react with the amplicon and form a stable flocculus in presence of an acetate-based crowding agent. Center and left: Negative results NTC1: healthy *N. benthamiana*, NTC2: water, the beads are spread throughout the solution making it opaque.

RT-RPA reaction for two more TSWV isolates: TSWV-Chrys5, isolated from Chrysanthemum and maintained in *Nicotiana benthamiana*, TSWV-Tom2, isolated from *Solanum lycopersicum* and maintained in *N. benthamiana*, and TSWV-PA01, isolated from *Capsicum anuum* and maintained in *Emilia sonchifolia*. All isolates were originally detected via immunostrips (Agdia, Elkhart, IN) and isolated to single lesions to ensure the presence of a single viral species. Our RT-RPA assay was able to detect all the three isolates in all 3 plant species, with different sensitivity. Nevertheless, the reaction for TSWV-PA01 was rather weak, when visualized in the amplification detection chambers (Fig. 3), but equally positive, when using the flocculation assay (Fig. 4).

TSWV specific RT-RPA can be visualized by fluorescence in real time. The RT-RPA amplification was followed in real time using the SYBR/FAM channel of a Bio-Rad CFX 96 thermal cycler (Bio-Rad, Hercules, CA). As expected, a positive RT-RPA reaction showed an amplification curve similar to curves typically generated by qRT-PCR (quantitative reverse-transcriptase real time PCR) for the crude extract of a TSWV *N. benthamiana* leaf (Fig. 5). The two negative controls, containing both the crude extract of an uninfected leaf and water (non-target controls) did not show an amplification curve.

Standard curve. For standard curve analyses, a qRT-PCR product was purified from a completed PCR reaction; then its concentration was measured and used to make serial dilutions. The number of copies of template was calculated using the Eq. (1). The standard curve, calculated by making a linear regression of the obtained Cq in the y-axis vs. the $\log_{10}$ of the calculated number of copies in the x-axis, is shown in Fig. 6.

Quantification of starting material. Fifty microliters of the TSWV infected *N. benthamiana* extract were subjected to RNA extraction, followed by a one-step real time PCR (Fig. 7). The calculated Cq for TSWV in 50 µl of lysate was close to 1.61, which interpolated in the standard curve regression Eq. (1), shown in Fig. 6, gave an approximate of 6.02333E + 12 copies of the amplified portion of the TSWV L genomic segment). Since only 2 µl of this solution were used for the crude extracts and all its dilutions, this gives an estimated initial concentration of 2.40933E + 11 genomic segments.

Sensitivity assay. Serial dilutions of the crude extracts used for the above experiments were subjected to RT-RPA and visualized in a detection chamber (Agdia, Elkhart, IN). After 20 min of incubation, all the dilutions of the crude extract from the TSWV infected plant, from undiluted to 10^{-12} (~ 1 copy of TSWV genomic segment) showed two bands, indicating a positive result and establishing the theoretical sensitivity of the assay at 1 viral copy. The negative controls showed only the control line (Fig. 8).

Replicas of the 1:10 dilutions were interpreted with the flocculation assay, showing similar results, all the dilutions, up to 10^{-12} showed a dark precipitate that remained stable even after flicking the tubes repeatedly (Fig. 9).

Finally, the results for real time RT-RPA for some of the above dilutions from undiluted to 10^{-20} in 10^{-4} increments showed differential amplification curves (Fig. 10).

Discussion and conclusions

Plant viruses are a threat to food security, because of the huge amount of losses (sometimes as high as 100%, for the case of Tomato spotted wilt virus (TSWV) they cause on crops. As viruses are cellular parasites, no curative measures are available for treating plants, other than prophylactic interventions aimed at controlling their vector and avoid transmission to new hosts[29]. RT-RPA has been already used for detection of other plant viruses, such as Rose rosette virus[11], Plum pox virus[30], Little cherry virus-2[31], Yam mosaic virus[32] as well as detection at species/subspecies level of Potato virus Y and Wheat dwarf virus[33]. This study provides an RT-RPA assay specific for rapid detection of TSWV both in real time and at the end point.

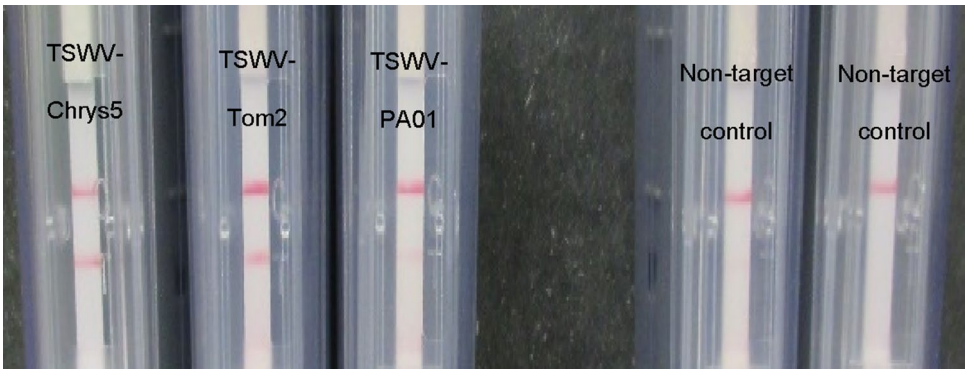

Figure 3. Right: RT-RPA positive result for three isolates of TSWV revealed with an amplification detection chambers. TSWV-Chrys5, TSWV-Tom2, TSWV-PA01 (faint test band), NTC1: healthy *N. benthamiana*, NTC2: water.

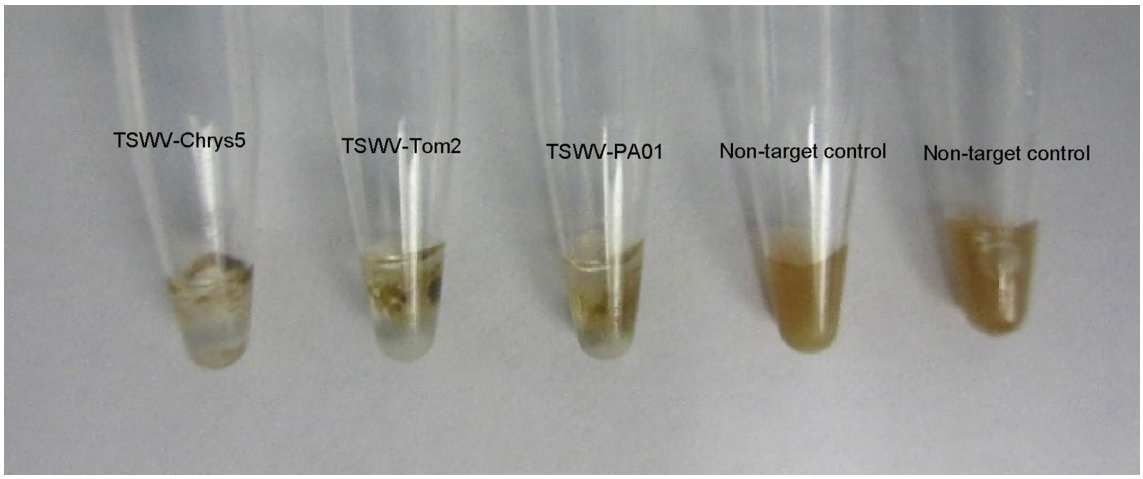

Figure 4. RT-RPA results for three isolates of TSWV revealed with a flocculation assay. TSWV-Chrys5, TSWV-Tom2, TSWV-PA01, NTC1: healthy *N. benthamiana*, NTC2: water.

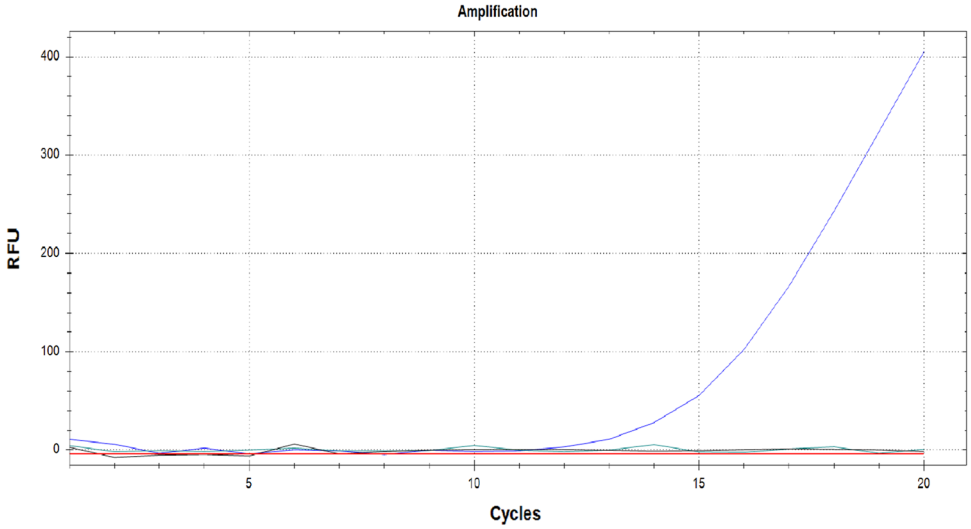

Figure 5. Result of real-time RT-RPA visualized in the SYBR/FAM channel of a Bio-Rad CFX96 real time PCR thermal cycler kept isothermally at 39 °C. Like qRT-PCR, an amplification curve is visible (blue). The non-target controls (black and green) did not produce an amplification curve in the 20 min the reaction took place. Black lines NTC1: healthy *N. benthamiana*, NTC2: water.

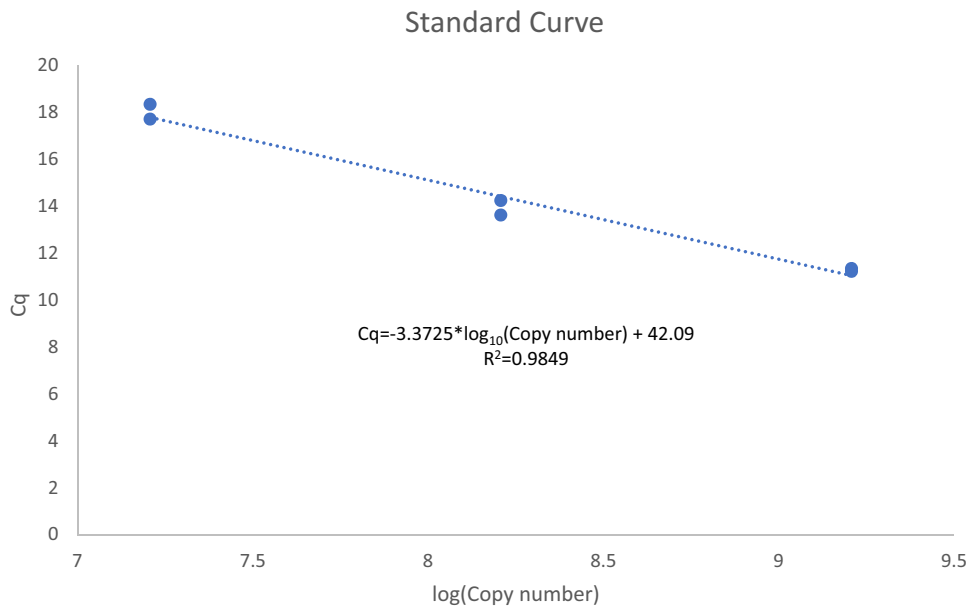

Figure 6. Standard curve computed for absolute quantification. The curve was built by purifying the qRT-PCR amplicon and making tenfold serial dilutions of it. The obtained Cq for the dilutions was used for a linear regression with the estimated concentrations (measured by nanodrop).

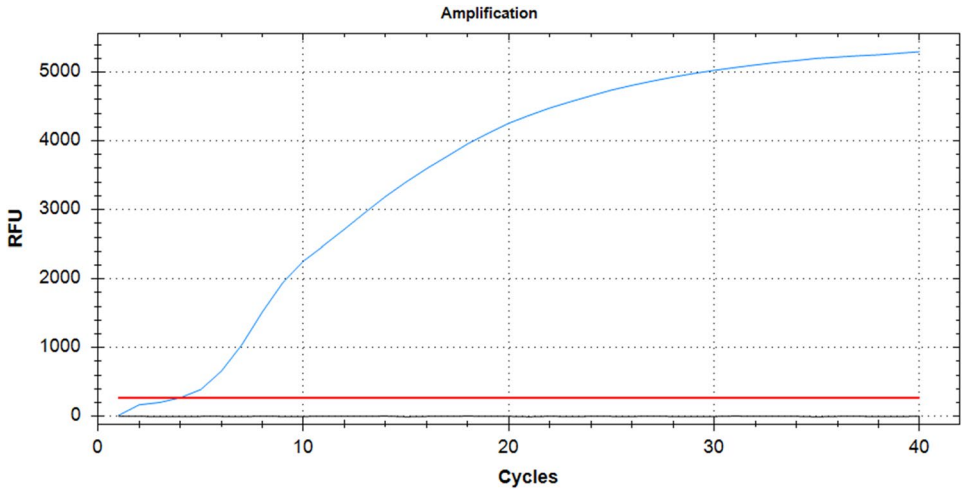

Figure 7. qRT-PCR Amplification curve. RNA was extracted from 50 µl of plant lysate and amplified via one-step qRT-PCR for 40 cycles. The amplification curve is visible with a Cq of 1.61 cycles.

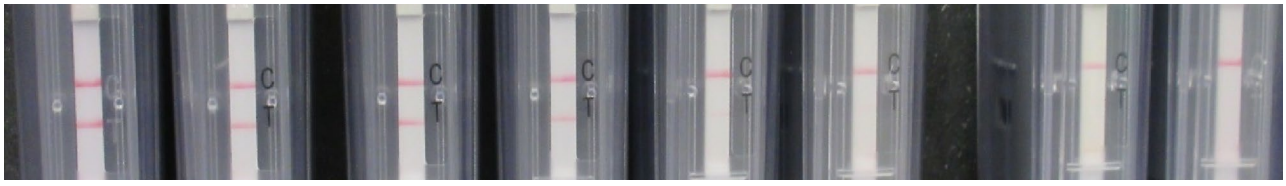

Figure 8. Serial dilutions of a crude extract visualized in lateral flow devices. From left to right, undiluted sample, 10^{-4} (2^7 copies), 10^{-8} (2^4 copies), 10^{-12} (~ 1 copy) 10^{-16} (out of range), 10^{-20} (out of range) all showing both control and test lines indicating a positive result. To the rightmost side, the two non-target controls NTC1: healthy *N. benthamiana*, NTC2: water were tested, which only showed the control line.

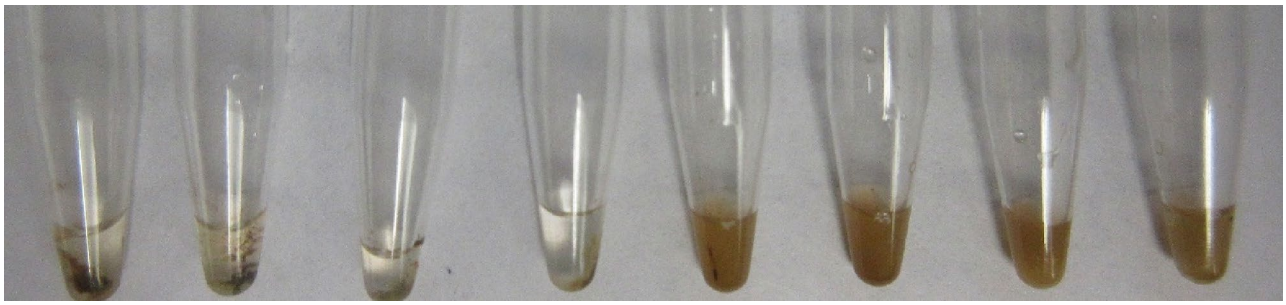

Figure 9. Serial dilutions of a crude extract visualized with SPRI beads. From left to right, undiluted sample, 10^{-4} (2^7 copies), 10^{-8} (2^4 copies), 10^{-12} (~ 1 copy) 10^{-16} (out of range), 10^{-20} (out of range), all showing a flocculus indicating a positive result due to the presence of the amplicon; 10^{-16} and 10^{-20} show no amplification. Last two tubes: NTC1: healthy *N. benthamiana*, NTC2: water.

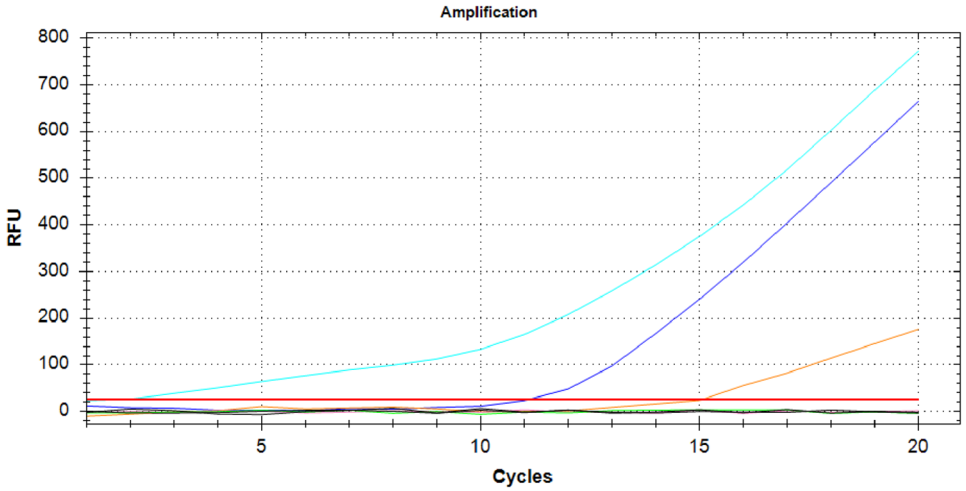

Figure 10. Serial dilutions of a crude extract visualized in the SYBR/FAM channel of a Bio-Rad CFX96 real time PCR thermal cycler kept isothermally at 39 °C From left to right, teal: Crude extract, blue: 10^{-4} (2^7 copies), orange: 10^{-8} (2^4 copies), pink: 10^{-12} (~ 1 copy), green:10^{-16} (out of range), yellow: 10^{-20} (out of range), Black: NTC1: healthy *N. benthamiana*, NTC2: water.

The results of the RT-RPA molecular detection technique developed in this study prove that crude extracts of TSWV infected plants can be used directly for sensitive and reliable detection of the virus with a portable but robust technique. We applied three different ways of checking the outcome of RT-RPA, this is a novelty since the standard way involves exclusively the use of lateral flow devices, however, with the application of the flocculation assay and proving any real-time capable thermal cycler can read detect in real time the presence of the amplicon, we expect this can move the field forward. Our results were confirmed by qRT-PCR amplification. In the case of qRT-PCR, it is noteworthy that the appearance of amplification curves obtained was atypical and not linear, suggesting that this assay is not the ideal one to quantify an RT-RPA product, but it should be used more as a qualitative tool for assessing the presence or absence of target. This result is expected since the design of this assay is suitable for use with a fluorometer and was not intended to be used in real time PCR thermal cyclers. The produced RT-RPA amplicons could also be visualized and characterized using agarose electrophoresis, nonetheless, as other researchers report, the assay can have a carryover of protein that can impose a limitation in the proper migration of the amplified nucleic acid during the electrophoresis if a purification is not performed[11].

The approximate cost of each single use lateral flow device at the time of preparation of this manuscript was around $5. On the other hand, 5 ml of SPRI beads solution costed around $120, nonetheless, each reaction only demands around 1.8 µl of beads, meaning that the solution should last for at least 10,000 reactions, and the cost becomes around 12 cents per reaction. The real time RT-RPA assay cost calculated here does not include the cost of a fluorescence reader .The Real time version is not cheap, the production of 100 mmoles of the real-time probe costed around $500[27]. As seen by Zhang et al.[30] the level of sensitivity of RT-RPA is very high, and allowed us to reach the theoretical detection of one copy of TSWV RNA segment. This unsurpassed sensitivity is intrinsic to RT-RPA, that combines nucleic acid amplification with serological detection.

In conclusion, we were able to design a molecular technique for the rapid and sensitive detection of Tomato spotted wilt orthotospovirus that is portable and can be interpreted using three different tools: lateral flow devices, SPRI beads + crowding agent and fluorometry, using a modified probe. All these techniques can be

used interchangeably for a qualitative detection of TSWV and can be scaled up for lab-on-a-chip applications or point-of-care detection of virus, to make quicker informed decisions in resource-limited environments, and without the need of trained personnel.

Materials and methods

***N. benthamiana* rearing and infection by TSWV-Tom2 and TSWV Chrys5.** *N. benthamiana* seeds were germinated in Sunshine #4 aggregate plus soil mix (Sungro, Agawam, MA) for 1 week and the resulting seedlings were transplanted in 4″ square pots and reared for 2 weeks. After this, 18 plants were inoculated using around 1 g of TSWV-Chrys 5 infected *N. benthamiana* tissue homogenized with mortar and pestle into chilled Paul's buffer (5 mM diethyldithiocarbamate –DIECA-; 1 mM Disodic Ethylenediaminetetraacetate –EDTA-; 5 mM sodium thioglycolate in 0.05 M phosphate buffer pH 7)[34,35] amended with a dash of carborundum and activated charcoal. Other 18 *N. benthamiana* plants were inoculated using TSWV-Tom2. All plants were kept in a growth chamber at 60% relative humidity and a photoperiod of 16/8 h day/night for at least 7 days.

Similarly, seeds of *E. sonchifolia* were germinated in petri dishes for 1 week and transferred to pots of Sunshine #4 aggregate plus soil mix (Sungro, Agawam, MA) and reared for 2 weeks in a 16/8 h day/night photoperiod, relative humidity was not controlled for this case. With the described process, TSWV-PA01 was inoculated and harvested 7 days after.

***E. sonchifolia* rearing and infection by TSWV-PA1.** Similarly, seeds of *E. sonchifolia* were germinated in petri dishes for 1 week and transferred to pots of Sunshine #4 aggregate plus soil mix (Sungro, Agawam, MA) and reared for 2 weeks in a 16/8 h day/night photoperiod, relative humidity was not controlled for this case. Following the described process, TSWV-PA01 was inoculated, and infected leaves were harvested 7 days after.

Sample homogenization. Leaf tissue of *N. benthamiana* or *E. sonchifolia* was collected 7 days post infection, transferred to an extraction bag (Bioreba, Switzerland) and mixed with 5 ml of phosphate buffered saline (VWR, West Chester, PA), hand homogenized using a hand-held tissue homogenizer (Agdia, Elkhart, IN) and kept at room temperature for the remainder of the experiment. Serial dilutions of this extract were made, when necessary, by mixing 20 µl of extract with 180 µl of nuclease-free water and repeating this process in series for up to 20 times.

Amplification. Endpoint: AmplifyRP Acceler8 (Agdia, Inc., Elkhart, IN) reaction pellets were rehydrated accordingly to the manufacturer's instructions. Additionally, iScript Reverse Transcriptase (Bio-Rad, Hercules, CA), was provided externally for converting RNA into cDNA, alongside modified primers and a the Acceler8 probe, both provided by Integrated DNA Technologies (IDT, Coralville, IA).

The probe and primers were designed according to the Assay Design Help Book from Agdia (https://d163axztg8am2h.cloudfront.net/static/doc/b0/4f/4e8d445a51f202f9f57f24a74c8d.pdf). The special considerations to design the probe are here summarized: Less than 50 nt long (42), idSp is the abasic spacer (also known as THF) site that replaced a T, there are 30 spaces between the Fluorophore (FAM) and the abasic site and at least 15 from this site and the 3' spacer (Fig. 11).

The primers were designed using Primer Blast[36] automatic parameters namely, the target was set to TSWV with accession NC_002050.1with the option of searching for other isolates from the Refseq Representative Genome Database by limiting the organism field to *Tomato spotted wilt orthotospovirus* (taxid: 1933298), the maximum, optimum and minimum primer sizes were set to 30, 33 and 36 nt respectively, the size of the amplicon was limited from 100 to 230 nt. The best primers and probe candidates, based on the criteria above, were selected. Finally, a biotin was added to the 5' end of the reverse primer (Table 1).

For performing the reaction, according to the manufacturer's procedure, dry reaction pellets in 0.2 ml tubes from the AmplifyRP® Discovery Kits (Agdia, Elkhart, IN) were hydrated with: 5.75 µl of rehydration buffer; 0.5 µl of each, primers at 10 µM; 0.25 µl of 10 µM probe, 0.5 µl of iScript Reverse Transcriptase (Bio-Rad, Hercules, CA), were mixed with a 2 µl of crude plant extract and with a reaction pellet. Following manufacturer's suggestions, the last 0.5 µl of 280 mM Magnesium acetate (MgOAc) were added to the lid of the microfuge tube and spun down to complete the 10 µl reaction.

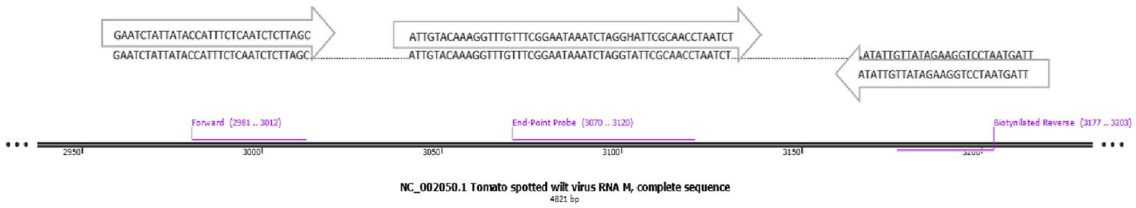

Figure 11. Schematic of the locations of primers and probes. Upper: Forward primer, probe and 5' biotinylated reverse primer respectively. Lower: Map of the M genomic segment of TSWV zoomed between bases 2950 and 3200, indicating primers and probe locations in purple. An abasic site on the middle of a probe is needed for a nuclease to cleave and release the blocking group, allowing the polymerase contained as well in the mixture to extend the probe[24]. The reverse primer contains a 5' biotin label and it forms with the probe a dual labeled FAM-biotin amplicon that can be detected with antibodies[37]

Assay	Name	Sequence 5'-3'
Lateral flow and flocculation	Forward	GAATCTATTATACCATTTCTCAATCTCTTAGC
	Reverse	Biotin-ATATTGTTATAGAAGGTCCTAATGATT
	End-point probe	FAM-ATTGTACAAAGGTTTGTTTCGGAATAAATCTAGG/idSp/ATTCGCAACCTA ATCT/C3-Spacer
Real-time	Real-time probe	ATTGTACAAAGGTTTGTTTCGGAATAAATC[FAM-dT]AGG[THF]AT[BHQ-dT] CGCAACCTAATCT/C3-Spacer

Table 1. Details for primers and probe used in the present study.

Upon closing the vessel, the reaction was maintained at 39 °C for 20 min in a portable thermal block following the manufacturer recommendations (Agdia, Elkhart, IN), and the results interpreted afterward. For non-target controls, water was used instead of the plant extract, as well as the extract from a healthy plant.

Revealing of results: amplification detection chambers. The 0.2 ml tubes were collected from the portable thermal block and inserted in the designated slot in the Amplification detection chambers (Agdia, Elkhart, IN). After this, the tube was smashed into the amplification detection chamber, freeing both the RT-RPA product and the proprietary solution and this device was left undisturbed while the liquids moved upwards through the lateral flow immunostrip for 20 min.

Revealing of results: flocculation assay. For cheaper and in-field visualization methods, we also tried the method of Ref.[38] for which, two volumes of SPRI (Solid Phase Reversible Immobilization) magnetic beads (Applied Biological Materials, Richmond, BC, Canada) were added to the amplified RT-RPA products, incubated for 5 min and then vortexed thoroughly for 10 s.

The tubes containing the mixture were placed on a magnetic rack and the supernatant was discarded and replaced by 50 µl of 80% ethanol, the mixture was then incubated for 5 min and vortexed 10 s again. Finally, the tubes were placed into the magnetic rack again and the ethanol was discarded, the tubes were open to air dry for 5 min and 50 µl of crowding solution comprised of 3 M sodium acetate and 20% Tween.

Real time. Like in the End point assay, pellets of AmplifyRP Acceler8 (Agdia, Inc., Elkhart, IN) were rehydrated using rehydration buffer, MgOAc, primers, XRT probe and reverse transcriptase. The XRT probe was designed according to the instructions from the Assay design book from Agdia, and synthesized by Integrated DNA Technologies (IDT, Coralville, IA).; the same primers as the end-point assay were used.

The XRT probe has the same sequence as the designed Acceler8 probe but contains a few modifications for being used in real-time, according to the Assay design book. Namely: the FAM was moved from the 5' end to one side of the abasic site and attached to a T, a black hole quencher was added to the other side of the abasic site and attached to a T nucleotide as well. See table.

For this case, to every dried amplification pellet, 5.75 µl of rehydration buffer were added; 0.5 µl of each, primers at 10 µM; 0.25 µl of 10 µl XRT probe, 0.5 µl of iScript Reverse Transcriptase (Bio-Rad, Hercules, CA), were mixed with a 2 µl of crude plant extract and with a reaction pellet. Following manufacturer's suggestions, the last 0.5 µl of 280 mM Magnesium acetate (MgOAc) were added to the lid of the microfuge tube and spun down to complete the 10 µl reaction.

For measuring the production of FAM, the vessels were inserted in a Bio-Rad CFX-96 system for real time PCR with a custom-made program that consisted of 20 cycles at 39 °C, with a plate read step every minute. The channel for SYBR/FAM that measures emission at 520 nm was used for collecting fluorescence.

Standard curve. For standard curve analyses, a qRT-PCR product was purified from a complete PCR reaction using the Zymo DNA Clean and Concentrator™-5 kit (Zymo Research, Irvine, CA) and eluted in 10 µl of nuclease-free water; then its concentration was measured by a Nanodrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE). Serial dilutions were prepared by adding 10 µl of the original amplicon to 90 µl of nuclease free water for each case. Then, the number of copies of template was calculated using the following formula:

$$\text{Number of copies} = \frac{(\text{amount in ng} * 6.022 * 10^{23})}{(\text{size in bp} * 1 * 10^{9} * 650)} \quad (1)$$

Where $6.022*10^{23}$ corresponds to the Avogadro's number for number of molecules per mol, 10^9 nanograms of exist in one gram and 650 is the average molecular weight in Daltons or g/mol of a pair of bases of DNA (double stranded). The standard curve was made by making a linear regression of the obtained Cq in the y-axis vs. the $\log_{10}$ of the calculated number of copies in the x-axis.

Quantification of starting material. Fifty microliters of *N. benthamiana* lysate were transferred to a 1.5 ml microfuge tube and amended with three volumes of TRIzol LS (Life technologies) and incubated for 3 min. Then, a volume of 100% ethanol was added and vortexed for 10 s. Finally, Directzol (Zymo Research) kit was used to extract the total RNA, following the manufacturer instructions.

The total RNA was checked in a Nanodrop (ThermoFisher Scientific) spectrophotometer and diluted to 250 ng/µl to be used in real time PCR. The qRT-PCR reaction was performed using a the iTaq™ Universal SYBR® Green One-Step Kit , containing 10 µl of 2X (Bio Rad, Hercules, CA), 0.25 µl of iTaq™ Universal SYBR® Green (Bio-Rad, Hercules, CA), 1X iScript Reverse Transcriptase (Bio-Rad, Hercules, CA), 10 mM of the primers L_TSTW_Fw_Short 5'-GCTCTCCTRTCCACCATCTAC-3'; L_TSTW_Rv_Short 5'-TTGCTGTCAAGGCAC ACATTTT-3 designed to amplify a 136 bp amplicon in the TSWV L genomic fragment. This set of primers has been validated and is routinely used in our laboratory.

The cycling conditions for the qRT-PCR included a reverse transcription at 50 °C for 10 min, an initial denaturation at 95 °C for 1 min, followed by 40 cycles of denaturation at 95 °C for 10 s, and combined annealing/extending at 60 °C for 30 s, according to the manufacturer's instructions.

Sensitivity assay. Serial dilutions of crude extract were made by mixing 10 µl of lysate with 90 µl of nuclease-free water, and then 10 µl of this solution were mixed with 90 µl more and so on (1:10). From each dilution, 2 µl were taken directly and mixed with the master mix for each RT-RPA reaction.

Sources of experimental plant material. All plants and seeds used in the present study belong to the Rosa Lab at the Pennsylvania State University. This plant material is available from the Corresponding Author upon request.

Statement of ethical use of experimental organisms. The authors confirm that the experiments that used plants ant their parts in the present study comply with International, National and Institutional Guidelines.

Data availability

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

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Author contributions

J.F.I.M. Performed research and experimental design. C.R. Oversaw the research and provided advise. Both authors contributed equally for the preparation of the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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