Short Communication

A TAQMAN REAL-TIME RT-PCR ASSAY FOR DETECTION OF *GROUNDNUT RINGSPOT VIRUS*

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SUMMARY

Groundnut ringspot virus (GRSV) is a tospovirus species transmitted in a circulative propagative manner by several thrips species. Traditionally, GRSV is detected by enzyme-linked immunosorbent assay (ELISA) and reverse transcription polymerase chain reaction (RT-PCR), so this paper describes a rapid diagnostic system for the reliable detection of GRSV in plants and individual thrips by real-time RT-PCR. Watermelon GRSV infected leaves and fruits, and individual thrips (*Frankliniella schultzei*) collected from flowers on infected watermelons were used in the test. We designed a GRSV TaqMan assay to efficiently detect GRSV in all samples. The method had high specificity and could distinguish GRSV from the other tospovirus species, like *Tomato spotted wilt virus* (TSWV), *Tomato chlorotic spot virus* (TCSV) and *Zucchini lethal chlorosis virus* (ZLCV). The test will help us to collect epidemiological data for GRSV in plants and thrips worldwide.

Keywords: *Tospovirus,* GRSV*, Frankliniella schultzei*, real-time RT-PCR.

Groundnut ringspot virus (GRSV), a member of the genus *Tospovirus*, causes spotted wilt disease, one of the most serious diseases for *Solanaceae* in Brazil, Central America and South Africa (Pappu *et al.,* 2009). Tomato (*Solanum lycopersicum* L.) and sweet pepper (*Capsicum annuum* L.) are the most common crops affected by GRSV worldwide. In general, the symptoms are bronzing, mosaic, mosaic with ringspots on leaves, yellowing and stem necrosis. GRSV is an important threat to horticulture since, in addition to an overall reduction in crop yield, the marketing quality of the harvested product is seriously affected by pronounced symptoms on fruits (Webster *et al*., 2010).

Recently, GRSV was detected on watermelon [*Citrullus lanatus* (Thunb.) Matsum & Nakai] and cucumber (*Cucumis sativus* L.) in Brazil (Leão *et al*., 2014; Spadotti *et al*., 2014), becoming an emerging problem in cucurbits crops. GRSV is transmitted in a circulative-propagative manner by several thrips species, including *Frankliniella occidentalis* (Pergande), *F. schultzei* (Trybom), *F. intonsa* (Trybom) and *F. gemina* (Bagnall) (Wijkamp *et al*., 1995; de Borbón *et al*., 1999; Nagata *et al*., 2004; de Borbón *et al*., 2006). The virus multiplies in the insect, but it is not transmitted to the eggs (Wijkamp *et al*., 1995).

Traditionally, tospovirus species are detected by enzyme-linked immunosorbent assays (ELISA) (Resende *et al*., 1991; Webster *et al*., 2010), DOT-BLOT hybridization (Eiras *et al*., 2001) and conventional reverse transcription (RT) polymerase chain reaction (PCR) with generic (Eiras *et al*., 2001) or specific primers (de Breuil *et al*., 2007). Other methods, such as host range and electron microscopy, have also been utilized (Boari *et al*., 2002; Camelo-García *et al*., 2014).

Real-time RT-PCR detection methods have already proved to be an efficient tool for the detection of other plant tospoviruses (Roberts *et al*., 2000; Boonham *et al*., 2002; Chen *et al*., 2013; Boben *et al*., 2007). In this study, we describe a routine diagnostic system for the reliable detection of GRSV in plants and inside individual thrips by real-time RT-PCR (TaqMan). The method is very sensitive and suitable for large scale testing, and could be used as a tool for examining tospovirus interaction within thrips.

Watermelon leaves and fruits showing tospovirus-like symptoms were collected in two regions of São Paulo state (Marília and Presidente Prudente). The virus species infecting these plants was identified as GRSV by plate-trapped antigen (PTA)-ELISA and RT-PCR sequencing using generic primers (BR60 and BR65), which are located in the tospovirus nucleoprotein gene (Eiras *et al*., 2001). The specimens of *F. schultzei* were collected from flowers of GRSV-infected watermelon plants and stored in 70%

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ethanol until analysis. A previously characterized GRSV isolate transmitted mechanically to *Datura stramonium* L. from South Africa (SA-05 from Dr. G. Adam DSMZ PV0205) was used as a positive control.

The primers and probe for real-time RT-PCR assay were designed within the conserved regions of the nucleoprotein gene of the GRSV isolate deposited in GenBank (accession No. AF251271). The design of primers and probes were carried out using Primer Express™ software (PE-Biosystems). The GRSV-forward (5'- GAGACTTGCTATAATGCTTGGAGGTA-3') and GRSV-reverse (5'- TGGCAAGAACAACACTGATCATC-3') primer pair and probe (5'-ATCCCTCTCATTGCTTCTGTTGACAGCTTC- 3') were used.

Total RNA from fifteen watermelon leaves and fruits samples was extracted using Norgen's total RNA purification kit (Norgen Biotek Corporation, Canada), according to the manufacturer's instructions. Ten viruliferous thrips samples were used for RNA extraction using Trizol reagent, following the manufacturer's protocol, with some modifications. Briefly, individual thrips were placed in Eppendorf tubes (1.5 ml) and homogenized with a micropestle in 250 µl of extraction buffer (Trizol reagent), then incubated at room temperature for 5 min. Next, 50 µl of chloroform isoamyl alcohol (24:1) were added after 3 min at room temperature. After centrifugation at 12,000 *g* for 10 min at 4°C, the supernatant was moved to another Eppendorf tube containing 1 µl of glycogen (10 ng/ml) and mixed with 125 µl of cold isopropanol, then incubated for 10 min at room temperature. After a final centrifugation at 12,000 *g* for 10 min at 4°C, the pellets were washed with 100 µl of 70% ethanol, dried in a vacuum chamber and re-suspended in 8 µl of water Milli-Q following incubation for 15 min at 60°C.

First-strand cDNA was synthesized using 1 µl of total RNA from plants and 8 µl for thrips with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems), according to the manufacturer's instructions. Real-time RT-PCR reactions were performed in a 96-well plate (Bio-Rad, California, USA) that contained 1 µl cDNA, 0.3 µl of each primer (final primer concentration of 10 mM each), 0.2 µl of TaqMan probe (10 mM) and 5 µl of iTaq™ Universal Probes Supermix (Bio-Rad), in a final volume of 10 µl. The real-time RT-PCR amplifications were undertaken in a CFX Connect™ Real-Time PCR detection system (Bio-Rad) with CFX Manage Software (Bio-Rad). The amplification reactions were run at least in triplicate. The real-time RT-PCR reactions were performed under the following conditions: initial at 95°C for 3 min followed by 40 cycles consisting of denaturation at 95°C for 10 s, and annealing/synthesis at 60°C for 30 s.

The assay was subjected initially to an optimization in a 5-fold serial cDNA dilution of a *D. stramonium* positive sample to determine the end-point limit of detection and the linearity of the assay. The slope (–3.554) and correlation coefficient (91.1%) of the dilution curve showed that this assay could be used to detect target RNA even in low concentrations as found in the thrips vectors. The final concentration of primers and probe (10 mM) was suitable for detection of the virus in both plants and thrips.

An internal control was used in plant samples. All plant samples were tested with a primer-probe combination designed in a highly conserved region of the plant mtCOX1 gene (accession No. X83206) (Weller *et al*., 2000). We attempted unsuccessfully to use primers for actin gene amplification for *F. occidentalis* (Boonham *et al*., 2002); we also designed primers and probes on ribosomal RNA sequences (5.8S) we obtained from RT-PCR from RNA extracted from *F. schultzei* but, also this primer set did not work in the RNA sample extracted from single thrips stored in alcohol (data not shown).

The GRSV TaqMan assay successfully detected GRSV isolates from Brazilian watermelon leaves and fruits (Fig. 1A). Cycle threshold (Ct) value observed for infected watermelon samples was less than 20 cycles, showing the specificity of the primers and the sensibility of the probe. We included in our assay at least two phylogenetically distinct GRSV isolates, the ones present in our samples from São Paulo state, and the one from South Africa, that we used as positive control to set up the method. The internal control used was capable to amplify the mtCOX1 gene from the plant. The internal controls are naturally present in the biological sample. They are used as indicators of efficacy of nucleic acid extraction, quality of samples and quality of PCR.

In addition, the assay could detect GRSV in individual viruliferous thrips (Fig. 1B). While the RNA extracted from individual viruliferous thrips provided the lowest Ct-value (30.0), non-viruliferous thrips resulted in no detection of RNA in the assay conditions. Boonham *et al.* (2002) developed reliable RNA extraction and real-time RT-PCR methods for detecting TSWV from single thrips, and also observed low Ct-value (28.0). There was no suitable amplification with the internal controls used in the insect. A possible explanation could be that thrips stored in alcohol preserved the encapsidated virus RNA from degradation but this storage method was not enough to preserve the unstable actin mRNA present inside the thrips.

To avoid possible non specific amplification signal, we also performed the same assay including positive controls for other tospovirus species more common in Brazil, such as *Tomato spotted wilt virus* (TSWV), *Tomato chlorotic spot virus* (TCSV) and *Zucchini lethal chlorosis virus* (ZLCV). The results obtained showed that the primers and probes used in our study were able to detect only GRSV (data not shown).

Molecular-based methods may be more expensive and less time-consuming than the other techniques described earlier, especially for large-scale testing. Although traditional PCR techniques can also detect a low virus titer, they are not quantitative in nature, and more prone to false positive detection. Real-time PCR allows rapid detection

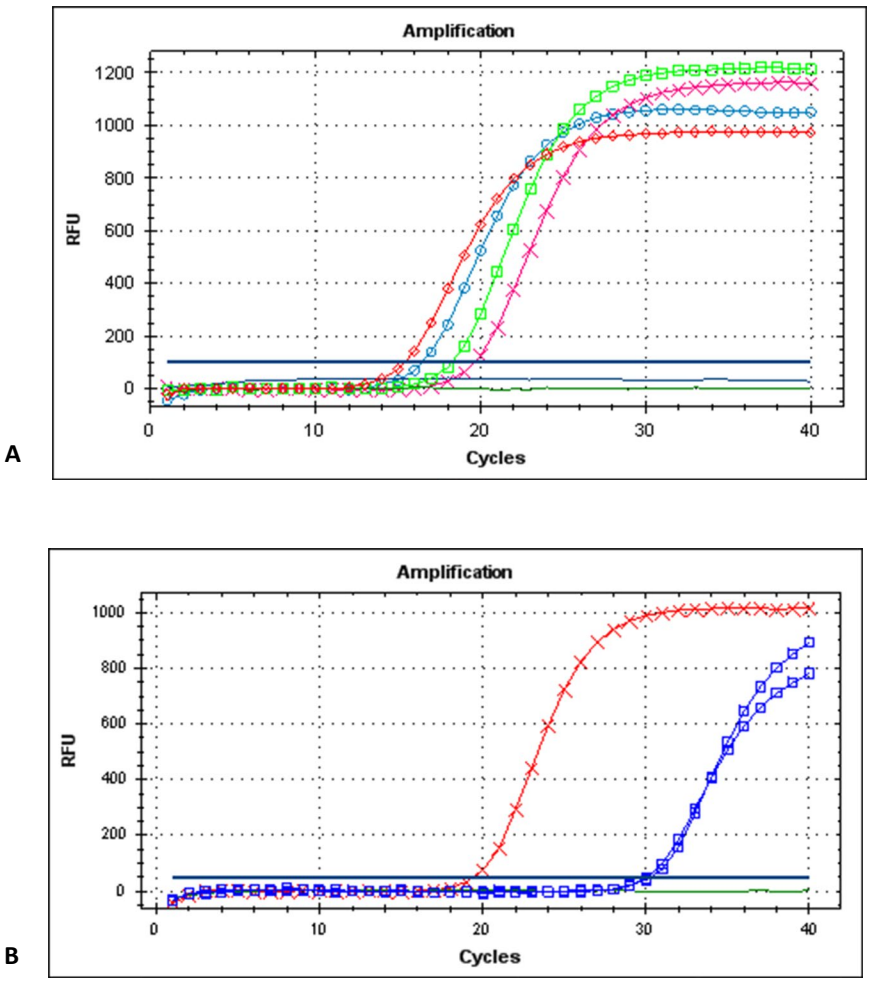

Fig. 1. Amplification plots showing the detection of GRSV in plants (A) and in individual thrips (B). (A) Plant positive sample infected by GRSV (◇), the watermelon leaves samples infected by GRSV (○; □), the watermelon fruit sample infected by GRSV (×), healthy and no-template controls (–). (B) The plant positive sample infected by GRSV (×), the positive thrips sample infected by GRSV (□), no infected thrips (–).

of target-specific amplicons and accurate quantification when used with a standard curve (Saponari *et al*., 2008). Chen *et al.* (2013) found that the real-time RT-PCR method measured more accurately low concentrations of *Impatiens necrotic spot virus* (INSV) than conventional RT-PCR. This high sensitivity was also reported in other plant viruses as described by Olmos *et al.* (2005), who compared real-time PCR with serological assays, and by Fabre *et al.* (2003), who compared it with RT-PCR and ELISA.

The successful use of real-time RT-PCR has been reported for the detection of TSWV and INSV in the plants and thrips vector (Roberts *et al*., 2000; Boonham *et al*., 2002; Chen *et al*., 2013). Detection of *Chrysanthemum stem necrosis virus*, CSNV (Boben *et al*., 2007) and *Iris yellow spot virus*, IYSV (Tiberini *et al*., 2012) in plants was also described. However, GRSV identification by this method has not been reported.

The real-time RT-PCR assay to detect GRSV described in this report was sensitive and specific: the method could detect a very low level of GRSV RNA in infected field tissue. High sensitivity allowed its use even when the virus titer was low as in the viruliferous thrips. Since absolute or relative quantitation of virus concentration was not the primary goal of our work, future research will be devoted to specific quantitative aspects of our detection methods in different hosts and thrips. Detection of GRSV in thrips has only been reported using ELISA (Wijkamp *et al*., 1995); but, according to the authors, false positive results were frequent due to the small size of thrips, low concentration of virus in thrips and interference of insect tissue with the immune reaction.

Thrips monitoring in greenhouses is usually done with the utilization of sticky traps as an important component of an integrated pest management system for tospovirus control. This procedure, however, does not provide any indication of the proportion of viruliferous insects at any time. Testing the trapped thrips using this method may be useful as an early warning of the presence of viruliferous thrips. A change in the thrips management system may then be able to prevent the spread of viruses in the very early stages of the infection process. Boonham *et al.* (2002) showed that it is possible to detect TSWV via real-time

RT-PCR in thrips trapped on sticky traps for 24 h and seven days after their collection.

Therefore, a real-time RT-PCR assay was used successfully to detect GRSV in plants and thrips. Furthermore, although additional efforts are needed, the optimization of primers for GRSV detection in real-time RT-PCR assays might serve as a useful tool in the identification studies, epidemiology and interaction of GRSV with different hosts.

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