Research Article

In situ localization of citrus exocortis viroid RNA using an optimized RNAscope™ assay

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Citation: Stanton, D., Harper, S. J, Cowell, S. J, & Brlansky, R. (2021). In situ localization of citrus exocortis viroid RNA using an optimized RNAscope™ assay. Journal of Citrus Pathology, 8(1). http://dx.doi.org/10.5070/C48153251 Retrieved from https://escholarship.org/uc/item/93h2n20h

Abstract

Due to their small size, locating pathogenic RNAs, such as viroids, in plant tissue and cell organelles has been difficult. Viroids are small circular single-stranded RNA plant pathogens that reduce plant growth, vigor, and yield in economically important crops such as potato, tomato, hops and citrus. Viroid infections in plants are largely diagnosed by dot blot hybridization, polyacrylamide gel electrophoresis (PAGE) or gels, or real-time quantitative polymerase chain reaction (qPCR). Because traditional plant *in situ* hybridization studies for viroids are often limited by the lack of signal amplification and binding specificity due to the small target sequence, we examined the use of RNAscope™ (Advanced Cell Diagnostics Inc., Newark, CA). This *in situ* hybridization method increases the detection by amplifying the signal of a single target, to detect the cellular distribution of citrus exocortis viroid (CEVd) with higher sensitivity and specificity. We found that after optimization, CEVd was localized in nuclei of infected cells as clearly distinguishable punctate red dots visible with light microscopy at low magnification, suggesting that the RNAscope™ can be used to study viroids *in situ*.

Keywords: CEVd, Plant Pathogen

Introduction

Viroids are unique plant pathogens, with a small circular single-stranded RNA genome of between ~250-433 nucleotides in length that does not encode for any proteins. Despite this, they can induce severe and economically damaging symptoms on their plant hosts through interference with host gene regulatory pathways (Tsagris et al. 2008; Navarro et al. 2012; Najar et al. 2018).

Their small size and lack of a coat protein makes cellular localization difficult, hindering studies on their biology and pathogen-host interactions. Historically, localization of viroids has relied on the hybridization of complementary cDNA or RNA probes, labeled with biotin or digoxigenin, and detected through a labeled secondary antibody by light or electron microscopy (Harders et al. 1989; Bonfiglioli et al. 1996). This approach has significant limitations in sensitivity and specificity, requiring careful optimization of the protocol for accurate results.

More recently, a new *in situ* hybridization (ISH) methodology, RNAscope™ ISH, was developed (Wang et al. 2012). This approach uses a double Z-probe design strategy in which two short independent probes hybridize in tandem to the target RNA so that signal amplification can occur, increasing specificity and signal strength by preventing amplification of non-specific signals. This approach has been used for the detection of various RNA targets in clinical histological studies (Brostoff et al. 2014; Carter et al. 2015) but has only seen limited use in the localization of plant pathogens (Bergua et al. 2016; Munganyinka et al. 2018).

Therefore, here we examined the use of the RNAscope™ for the localization of citrus exocortis viroid (CEVd) in citrus, one of its natural hosts. This pathogen causes the scaling and splitting of bark on trifoliate orange (*Poncirus trifoliata* L.) and hybrid rootstocks, reducing tree vigor and growth. Previous attempts at CEVd localization have either focused on experimental host species (Bonfiglioli et al. 1996) or used synthesized digoxigenin-labeled riboprobes that may have limited sensitivity or specificity (Lin et al. 2015). In this study, we report the successful utilization of RNAscope™ ISH to detect and locate CEVd in infected citrus, and the visualization of the positive reaction at low magnifications in the nuclei of the infected plants.

Materials and Methods

Six-month old sweet orange (*Citrus sinensis* L.) seedlings were graft-inoculated with CEVd and grown under greenhouse conditions. Successful infection was confirmed three months post inoculation using a CEVd-specific real-time quantitative polymerase chain reaction (qPCR) assay as previously described (Monger et al. 2010). Parallel inoculation of citrus tristeza virus (CTV) T36-like

isolate CTV (Satyanarayana et al. 2001) was performed as a positive control, and infection confirmed using a T36-genotype specific qPCR assay as described (Harper et al. 2014). Healthy sweet orange was used as a negative control for the *in situ* assays.

To measure CEVd titer in different tissues, leaf lamina and petiole, young green stem/branch, hardened stem/branch, main stem, taproot, and secondary root tissues were sampled from three CEVd infected citrus plants. Total RNA was extracted as previously described (Harper et al. 2014), and viroid concentration was quantitated using the Monger et al. (2010) assay against two reference genes in technical replicates of three (Harper et al. 2014). Data was log-transformed and averaged across the three biological replicates for each tissue type.

Tissue samples of petioles, stems and roots of CEVd-inoculated plants, as well as uninfected negative controls, and CTV-infected positive controls were fixed in 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield PA, USA) in 1 x Dulbeccos's phosphate buffer (1xDPBS) (Thermo Fisher Scientific, Waltham, MA, USA) for 24-72 hours at 4ºC. Sections were then washed three times in 1xPBS for 30 minutes, and then dehydrated in an ethanol (EtOH) series of 30%, 50%, 70%, 85%, 95%, twice in 100%, 100% for 30 min per stage, then finally overnight in 100% EtOH. Sections were briefly rinsed in 100% EtOH and transitioned from 100% ethanol to 100% *tert*-butanol through increasing the concentrations of 3:1,1:1, and 1:3 of ethanol to *tert*-butanol at room temperature for 8-16 hours per stage. The sections were cleared in 100% *tert*-butanol for an hour before paraffin infiltration. Finally, sections were infiltrated with increasing concentrations (3:1, 1:1, 1:3) of *tert*-butanol to Paraplast Plus paraffin (Thermo Fisher Scientific, Waltham, MA, USA) for 24 hours per stage, and then incubated for 24 hours in three rounds of 100% paraffin. Sections were then embedded in 7 mm molds with paraffin and allowed to harden at room temperature for 24 hours.

The blocks were trimmed into trapezoids, and 10 µm sections were cut using a Leica 2155 microtome and placed on a drop of water on Biobond 360 slides (Electron Microscopy Sciences, Hatfield, PA, USA). The sections were fixed onto the slide by incubating the slides for two hours on a 60 ºC hotplate and then incubated overnight on a slide warmer set at 37 ºC. Slides were de-waxed in three changes of 100% Histoclear II (National Diagnostics, Atlanta GA, USA) for one hour each. The slides were rinsed in 100% EtOH for 5 minutes and allowed to air dry.

The sections on the slides were pre-treated according to the RNAscope™ 2.5 Assay protocol (Advance Cell Diagnostic Inc (ACD Inc.), Hayward, CA, USA). The protease treatment was omitted. Mounted CEVd, CTV positive, or uninfected tissue sections, were covered with 3-4 drops of the CEVd probe (V-CEVd), designed upon request by ACDBio. This probe contained double Z pairs that bound to the target within nucleotide 2 to 368 of the CEVd reference sequence J02053 (Gross et al. 1982), or a CTV probe available in the ACD catalog (V-CTV-T36) respectively (Bergua et al. 2016). Each proprietary probe design results in 6-20 different Z pairs per target sequence. In our case, ACD designed seven probes against the CEVd target sequence. Because the probes are proprietary, the exact target and the length for each individual Z pair was not shared by ACD. The probe design is explained by Wang and others (2012) to include the design of each individual Z probe, how they bind complementary to the target sequence, and how the two Z probes create a 28-base hybridization site for binding the preamplifier. The preamplifier has 20 binding sites that allows for sequential hybridization of up to 20 binding sites for the labeled probe. Slides were incubated at 40 °C for one hour rather than the recommended two hours as this did not affect target detection.

After probe incubation and preamplifier hybridization, a signal scaffold was built using the amplification reagents Amp1-6. Incubation times for reagents Amp 1, 3, and 5 were shortened to 20 minutes, while incubation with Amp 2 and 4 were shortened to 10 minutes. Amp 6 hybridization and Fast Red incubation times were performed as per the manufacturer's instructions. probes were further hybridized using the RNAscope™ 2.5 HD red detection kit (ACD Bio). This amplification scaffold allows for the enzyme and substrate to bind to multiple sites targeting a single probe in order to enhance the signal-to-noise-ratio through amplifying the signal without amplifying the background. This allows up to 8000 labels for each target sequence (Wang et al. 2012).

Following colorimetric detection, sections were rinsed in 100% xylene for one minute, followed by a one-minute wash in dH_2O. Sections were mounted with EcoMount (Biocare Medical, Pacheco CA, USA) and a coverslip was added. The sections were observed under an Olympus BX61 microscope (Olympus, Center Valley PA, USA) using 10x, 20x, and 40x objectives and images were captured using an OMAX A35140U camera (OMAX, Irvine, CA, USA) .

Results

In this study, we wanted to determine whether the RNAscope™ could be used to localize viroids as a tool for further research. We confirmed that CEVd was present in inoculated plants by Real-time quantitative PCR (qPCR) at three months post-inoculation. All tissues sampled, including leaf lamina and petiole, young shoots, older hardened or lignified stems, and the root system were infected with CEVd (Figure 1).

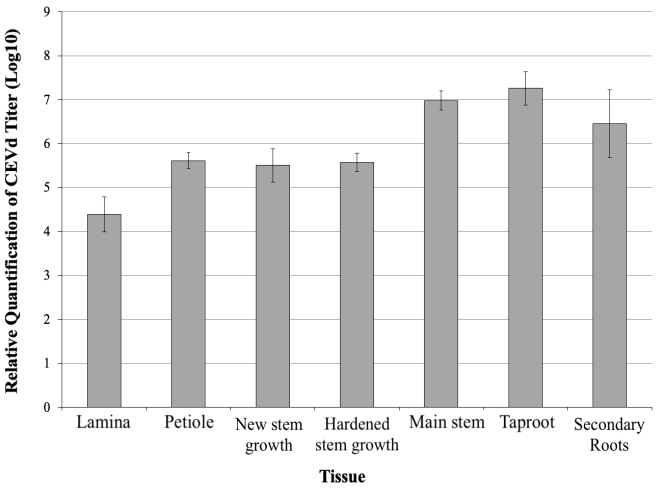

Figure 1. CEVd titer in different tissues of *Citrus sinensis* at three months post-inoculation, as determined by qPCR.

We selected petiole, mature hardened shoot, and taproot cross sections to perform CEVd localization. We found that detection with RNAscope™ was effective at producing clear, visible punctate red dots in infected tissues that corresponded to expected CEVd presence (Figure 2). CEVd probes were found to be specific, with punctate red dots were only observed in CEVd infected tissue, and not in viroid-free controls in petiole, stem and root tissue and were comparable with the CTV positive controls (Figure 3 A and B). CEVd stem tissue treated with the V-CTV-T36 probe and CTV stem tissue treated with the V-CEVd probe were used as negative controls. we did not observe any red punctate dots in the negative controls (Figure 3 C and D).

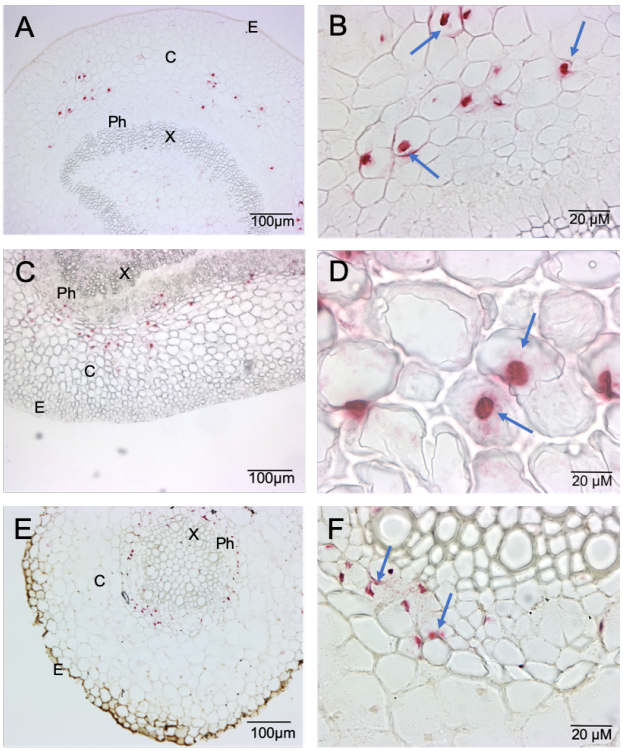

Figure 2. Citrus exocortis viroid labeled in citrus tissues using RNAscope™ Fast RED: Petioles (A&B), Stem (C&D), and Root (E&F) CEVd infected cells labeled with the V-CEVd probes in stem cortex tissue, blue arrow indicating infected nuclei. E - epidermis, C - Cortex, Ph - phloem, and X – xylem.

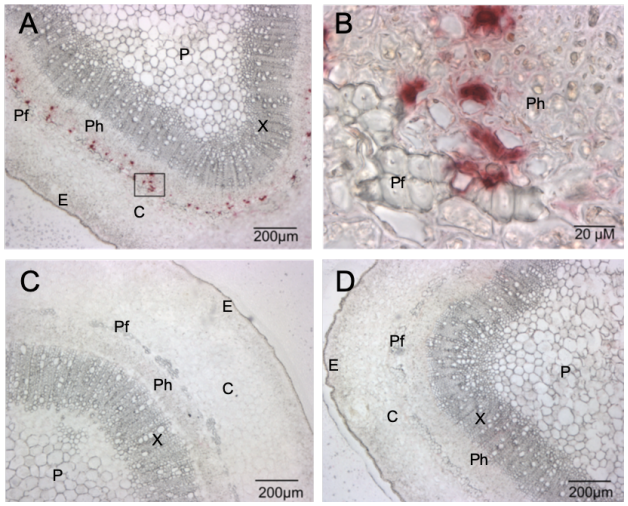

Figure 3. Stem tissue infected with Citrus tristeza virus was detected using the V-CTV-T36 probe as a positive control (A & B). Negative controls are as follows: CEVd stem tissue treated with the V-CTV-T36 probe (C) and CTV stem tissue treated with the V-CEVd probe (D). E - epidermis, C - Cortex, Pf - phloem fibers, Ph - phloem, X - xylem, P= Pith.

Discussion

Our qPCR data confirmed the presence of CEVd in all tissues sampled. We observed a general gradient of increasing titer, over a range of two orders of magnitude, from the leaf tissue to the roots (Figure 1). Based on the qPCR results, root, stem, and petiole tissues were selected to observe localization of CEVd in these tissues.

RNAscope™ provided superior signal amplification and after development with the Fast Red substrate, punctate red dots were visible in infected tissues. Labeling was concentrated in the nuclei of infected cells, which is to be expected as this is the site of replication for pospiviroids (Tsagris et al. 2008). Faint punctate red dots also were observed in the cytoplasm and plasmodesmata. CEVd was primarily found in collenchyma tissue adjacent to the phloem, to an extent of 5-6 cell layers (Figure 2). Interestingly, despite having only 7 oligo double Z pairs in the V-CTV-T36 probe, signal amplification was found to be as effective as the CTV probe with 20 oligo double Z pairs (Figures 2 & 3), suggesting that shorter sequences can successfully be targeted using this method.

We did find, that optimization and reduction of the reaction steps and methodically performing the rinse steps did reduce the nonspecific binding of probes in stem and petiole tissue, such as was observed in the original RNAscope™ CTV study (Bergua et al. 2016). Here, reducing hybridization times as described above limited labeling of CTV to the phloem, and as clear and distinct punctiform dots (Figure 3). Furthermore, we observed non-specific binding of the Fast Red dye along the cell walls in root tissue using unoptimized amplification conditions, which similarly, did not occur when the amplification step

reaction times were optimized. Additionally, no labeling was observed in the negative controls because this kit prevents amplification of non-specific targets. Therefore, RNAscope™ provides a higher sensitivity and specificity over traditional hybridization techniques.

Localizing viroid RNA in plant tissue is an essential tool in understanding the biology of these pathogens, and here we present an optimized approach for the use RNAscope™ that is less cumbersome than traditional RNA hybridization. Our study underlines the need for optimization of hybridization methods in given tissue types, as a poor optimization can lead to off target effects, but properly optimized, extension of this approach could also allow the detection of many smaller RNA targets in plant tissue.

Acknowledgments

Financial support for this work was provided by the Mid Florida Citrus Foundation.

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