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Short communication

A simple, rapid and inexpensive method for localization of *Tomato yellow leaf curl virus* and *Potato leafroll virus* in plant and insect vectors

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A B S T R A C T

A simple, rapid, inexpensive method for the localization of virus transcripts in plant and insect vector tissues is reported here. The method based on fluorescent in situ hybridization using short DNA oligonucleotides complementary to an RNA segment representing a virus transcript in the infected plant or insect vector. The DNA probe harbors a fluorescent molecule at its 5′ or 3′ ends. The protocol: simple fixation, hybridization, minimal washing and confocal microscopy, provides a highly specific signal. The reliability of the protocol was tested by localizing two phloem-limited plant virus transcripts in infected plants and insect tissues: *Tomato yellow leaf curl virus* (TYLCV) (*Begomovirus: Geminiviridae*), exclusively transmitted by the whitefly *Bemisia tabaci* (Gennadius) in a circulative non-propagative manner, and *Potato leafroll virus* (*Polerovirus: Luteoviridae*), similarly transmitted by the aphid *Myzus persicae* (Sulzer). Transcripts for both viruses were localized specifically to the phloem sieve elements of infected plants, while negative controls showed no signal. TYLCV transcripts were also localized to the digestive tract of *B. tabaci*, confirming TYLCV route of transmission. Compared to previous methods for localizing virus transcripts in plant and insect tissues that include complex steps for in-vitro probe preparation or antibody raising, tissue fixation, block preparation, sectioning and hybridization, the method described below provides very reliable, convincing, background-free results with much less time, effort and cost.

Localization of plant viruses or their transcripts in plant and insect tissues is an essential research objective for understanding pathways of viral pathogenicity in the plant, the virus's association with the tissues of its host, and paths of virus acquisition, retention and transmission in the insect vector. Plant viruses differ in their ability to infect plant tissues, depending strongly on their mode of transmission, the plant tissues they infect and in which they replicate, and the mode by which they spread in the plant. In insect vectors, virus location depends upon its transmission mode (non-persistent, semi-persistent, persistent, circulative non-propagative or circulative propagative), and how close the association is between the virus and its vector. An essential step in understanding the mode by which plant viruses are transmitted by vectors is localization of these viruses or their transcripts in the vectors' tissues.

Tomato yellow leaf curl virus (TYLCV-IL) belongs to the genus *Begomovirus*, family *Geminiviridae* and has a genome encoded by one circular ssDNA molecule of ~2700 nucleotides encapsidated in geminate particles (~20 nm × 28 nm). TYLCV is the most devastating viral disease complex of cultivated tomato (*Solanum lycopersicum*) in tropical and warm temperate regions of the world, where losses of up to 100% are incurred. TYLCV has been recorded in Europe, Mediterranean countries, parts of sub-Saharan Africa, Asia, Australia, and the Caribbean (Czosnek and Laterrot, 1997). It has also been reported locally in the USA (Ling et al., 2006; Polston et al., 1999, 2002). Begomoviruses infect only dicotyledonous plants, exhibit tissue tropism in the phloem and are transmitted exclusively by the whitefly *Bemicia tabaci* (Frohlich et al., 2002; Ghanim et al., 2001a).

Luteoviruses are a group of simple non-enveloped, icosahedral particles ~25 nm in diameter encapsidating one positive ssRNA molecule of ~6000 nucleotides. *Potato leaf roll virus* (PLRV), which belongs to the genus *Polerovirus,* family *Luteoviridae*, damages potato production and is a phloem-limited virus which causes losses of two types: yield reduction and poor-quality tubers due to net necrosis.

A model describing the translocation of luteoviruses in aphids has been proposed (Gray and Gildow, 2003). In many of its steps, this model resembles begomovirus translocation in whiteflies (Ghanim et al., 2001a, 2001b; Hunter et al., 1998; Rosell et al., 1999). Two specific barriers need to be crossed during circulative transmission of viruses in whiteflies and aphids: the gut/hemolymph and the hemolymph/salivary gland. Crossing these barriers is an active process and is probably mediated by (unknown) receptors which recognize the virus capsid and translocate the virions in coated vesicles. While TYLCV is thought to cross *B. tabaci* midgut (Czosnek et

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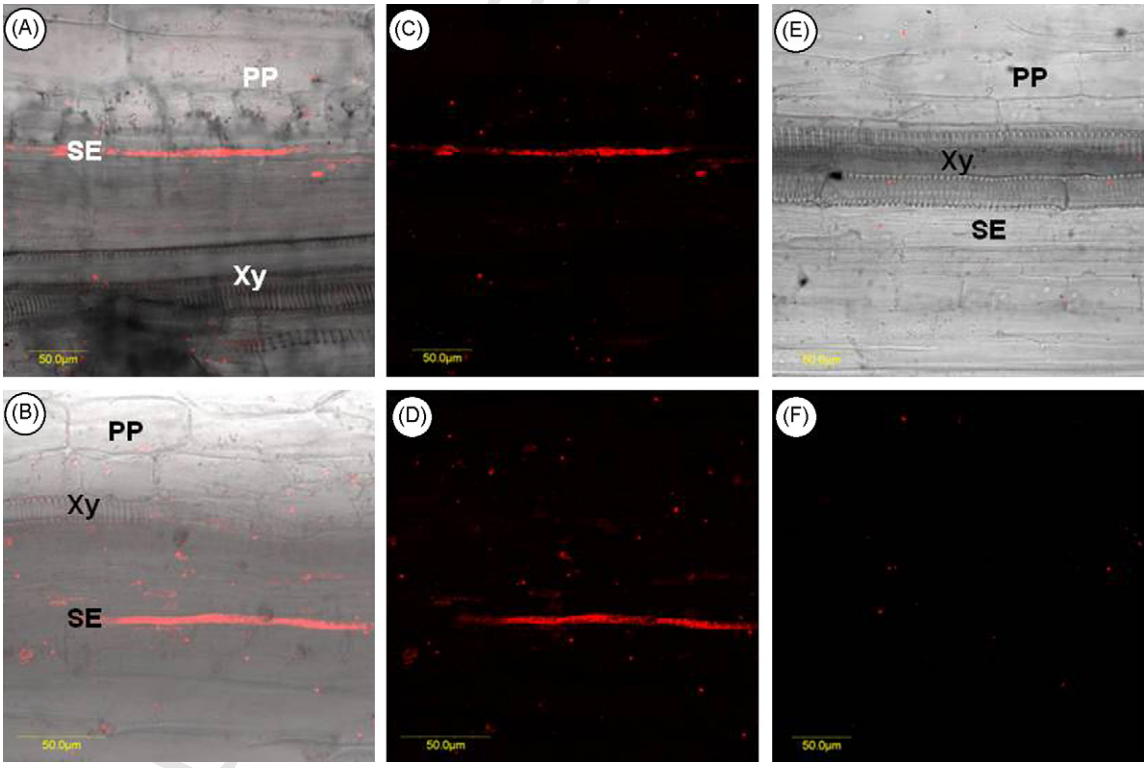
al., 2002; Ghanim et al., 2001a) and be transmitted through the primary salivary gland (Ghanim et al., 2001a), PLRV is thought to cross the posterior part of *M. persicae*'s midgut and hindgut and transmitted through the accessory salivary glands (Garret et al., 1993; Gildow, 1982, 1993; Gildow and Gray, 1993; Gray and Gildow, 2003).

Several reports have described the localization of TYLCV and PLRV, as well as related viruses, in plants and insect vectors. However, those studies made use of time-consuming and costly protocols. Some of the methods, which in many cases involved transmission electron microscopy (TEM), were also used for subcellular localization of the viruses, for example localization of TYLCV in *B. tabaci* using specific primary antibodies against the virus coat protein and secondary antibodies conjugated to gold particles for viewing under TEM (Czosnek et al., 2002; Ghanim and Medina, 2007). Other methods used in situ RNA synthesis and hybridization on thick microscopic sections to localize viral transcripts in *B. tabaci* primary salivary glands (Brown and Czosnek, 2002). TYLCV was further localized in infected thick plant sections using a TYLCV-specific biotinylated probe and visualized under a light microscope with silver-enhanced streptavidin-gold particle conjugates (Michelson et al., 1997). PLRV localization in *M. persicae* tissues has been conducted using specific antibodies against viral proteins and the signal was visualized using TEM and secondary antibodies conjugated to gold particles. In this case, visualization was conducted in insect sections that had been prepared and sectioned for TEM analysis (Garret et al., 1993). This virus was also localized in plant tissues using specific antibodies against viral proteins and immunohistochemistry with secondary antibodies, followed by visualization under TEM (Schmitz et al., 1997) or under light microscope with silver enhancement (van den Heuvel et al., 1995). Since all of these methods for localizing PLRV, TYLCV and other plant viruses are laborious, costly and time-consuming, a method for plant virus transcript localization in plants and insects was developed, knowing that localization protocols for such virus transcripts are often needed and are a limiting factor in studying virus-plant and virus-vector interactions.

The short oligonucleotide probe Cy3-5′-GGAACATCAGGG-CTTCGATA-3′ which is complementary to 20 base pairs in the coat protein sequence of TYLCV was synthesized. The probe was conjugated with the fluorescent dye cyanine 3 (Cy3) at its 5′ end. The second oligonucleotide probe Cy3-5′-TTTCCATTTCCCTTCCACAG-3′ was also synthesized based on the PLRV sequence, and was also conjugated with Cy3 at it 5′ end. These probes are very short and their ability to penetrate plant and insect tissues after fixation is expected to be high. Hand-cut longitudinal sections of tomato (*Lycopersicon esculentum* cv. Beefsteak) stems and leaves, three weeks after inoculation with TYLCV and showing disease symptoms were prepared. TYLCV-infected plants were prepared using *B. tabaci*-mediated inoculation of healthy plants with 10 viruliferous whiteflies for each plant, and for a 48 h inoculation access period. For PLRV, hand-cut sections from PLRV-infected *Physallis floridana* plants, three weeks after *M. persicae*-mediated inoculation were prepared, using the same protocol for *B. tabaci*-mediated inoculation of tomato plants. Since *P. floridana* does not show disease symptoms, its infectivity was verified using RT-PCR with PLRV-specific primers: PLRV_b 5′-CGCGCTAACAGAGTTCAGCC-3′ and PLRV_ab 5′-GCAATGGGG GTCCAACTCAT-3′. For TYLCV localization in dissected *B. tabaci* midguts, the midguts were dissected from *B. tabaci* that had acquired the virus from infected plants which show disease symptoms during a 48-h acquisition access period as described previously (Ghanim et al., 2001b), and the hybridization procedure was performed on microscopic slides with the same solutions used for the plant sections. Plant sections were fixed in Carnoy's fixative (chloroform:ethanol:glacial acetic acid, 6:3:1, v/v) for 2 h at



Q1 **Fig. 1.** Hand-cut leaf sections from tomato plants infected with TYLCV. The phloem sieve elements (SE) are infected and accumulation of the virus transcripts is observed (red signal). (A) and (C) Localization of TYLCV transcripts in SE shown in bright-field and confocal sections, respectively. (B) and (D) Another view of TYLCV transcript localization in infected plant in bright-field and confocal sections, respectively. (E) Control bright-field section from non-infected plant and (F) control confocal section from TYLCV-infected and RNase-digested plant. Xy, xylem; PP, parynchema. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

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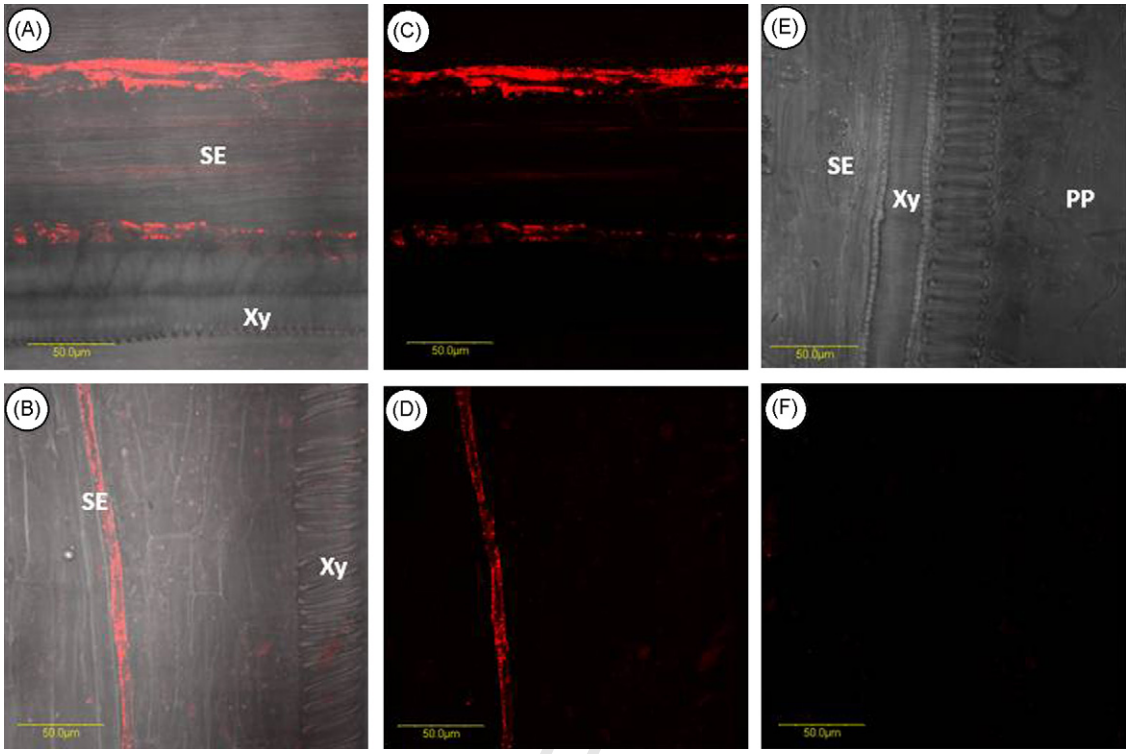

Fig. 2. Hand-cut leaf sections from *P. floridana* plants infected with PLRV. Phloem sieve elements (SE) are infected and accumulation of the virus transcripts is observed (red signal). (A) and (C) Localization of PLRV transcripts in SE shown in bright-field and confocal sections, respectively. (B) and (D) Another view of PLRV transcripts localization in infected plant in bright-field and confocal sections, respectively. (E) Control bright-field section from non-infected plant and (F) control confocal section from PLRV-infected and RNase-digested plant. Xy, xylem; PP, parynchema. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

room temperature, while midguts were fixed for 5 min. Plant sections and midguts were then washed three times, for 1 min each, in hybridization buffer (HB) [20 mM Tris–HCl pH 8.0, 0.9 M NaCl, 0.01% (w/v) sodium dodecyl sulfate (SDS), 30% (v/v) formamide], hybridized overnight with 10 pmol fluorescent probe/ml in HB, washed three times, 1 min each, in HB, mounted whole and viewed under an IX81Olympus FluoView500 confocal microscope. Specificity of detection was confirmed using the following controls: no probe, RNase-digested, and healthy plants, or *B. tabaci* that had not acquired the virus. Fig. 1 shows the specific localization of TYLCV transcripts in sieve elements of infected tomato seedlings while other vascular and surrounding plant tissues were free of virus transcripts, confirming that TYLCV is limited to the phloem. The same results were obtained with sections prepared from PLRV-infected *P. floridana* plants (Fig. 2), supporting the results of TYLCV localization in tomato. A time course experiment was performed to

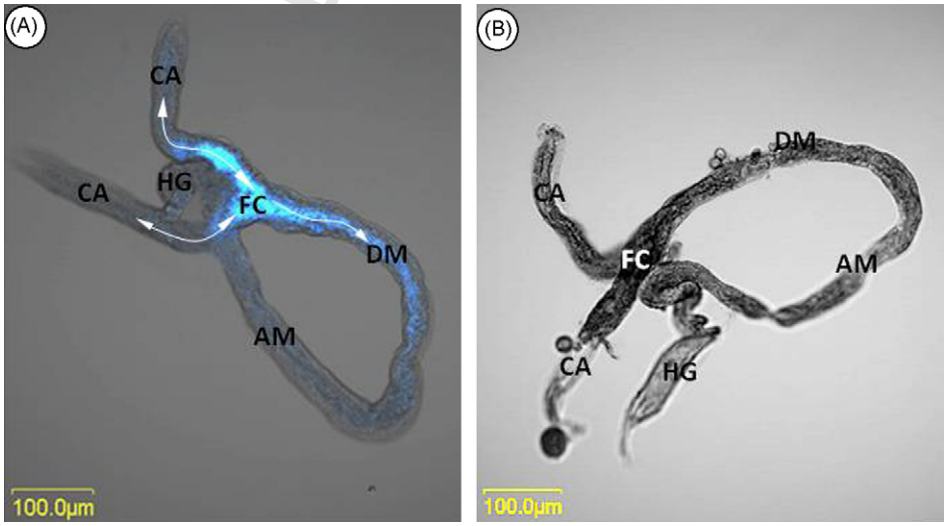

Fig. 3. Dissected and fluorescent in situ hybridization (FISH)-labeled midgut loops with TYLCV-specific probe from viruliferous (A) and non-viruliferous (B) *B. tabaci* adult females showing specific localization of the virus transcripts in the filter chamber (FC) and the caeca (CA) (blue signal). Curved arrows show the route of TYLCV movement when it reaches the FC. HG, hindgut; DM, descending midgut; AM, ascending midgut. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

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test the minimal time in which TYLCV and PLRV transcripts can be detected using the method described above. 8 days after TYLCV inoculation and 10 days after PLRV inoculation, sufficient signals for both viruses could be detected using this method. Fig. 3 shows specific localization of TYLCV transcripts in the filter chamber area of *B. tabaci* midgut. This result confirmed a previous hypothesis that the route of TYLCV translocation in *B. tabaci* is from the esophagus to the midgut, reaching the filter chamber first where most of the virus is absorbed to the hemolymph, while the remaining viruses or virus transcripts move on to the caeca and are pushed into the descending midgut and finally the ascending midgut, where the lowest concentration of virus is observed (Fig. 3) (Ghanim et al., 2001b).

In summary, a simple method for the localization of plant virus transcripts in plant and insect vector tissues was described. This method requires a net processing time of about 30 min, and it does not suffer from the drawbacks of other methods such as high background signal, long processing time for specimens and the use of numerous and costly materials for the analysis. Although this method is described for localization of TYLCV and PLRV, it might be adapted for localization of other viruses and their transcripts. It is sufficient for general viewing of virus transcript location in the plant and the insect tissues, but may be developed into further suitable localization methods using our probe format: for example, these probes can be conjugated to molecules such as biotin, which in turn can be targeted using gold particles conjugated to streptavidin, resulting in highly specific localization with minimal background. This latter modification can be suitable for subcellular localization of viral transcripts, and not only for general viewing. Although the concentration of viral transcripts needed for sufficient detection using this method was not examined, the fact that detection is only possible after 8 days with TYLCV, and after 10 days with PLRV suggests that high concentration of viral transcripts are needed for sufficient viewing of the signal.

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