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Short communication

Citrus leaf blotch virus invades meristematic regions in *Nicotiana benthamiana* and citrus

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SUMMARY

To invade systemically host plants, viruses need to replicate in the infected cells, spread to neighbouring cells through plasmodesmata and move to distal parts of the plant via sieve tubes to start new infection foci. To monitor the infection of *Nicotiana benthamiana* plants by *Citrus leaf blotch virus* (CLBV), leaves were agroinoculated with an infectious cDNA clone of the CLBV genomic RNA expressing green fluorescent protein (GFP) under the transcriptional control of a duplicate promoter of the coat protein subgenomic RNA. Fluorescent spots first appeared in agroinfiltrated leaves 11–12 days after infiltration, indicating CLBV replication. Then, after entering the phloem vascular system, CLBV was unloaded in the upper parts of the plant and invaded all tissues, including flower organs and meristems. GFP fluorescence was not visible in citrus plants infected with CLBV-GFP. Therefore, to detect CLBV in meristematic regions, Mexican lime (*Citrus aurantifolia*) plants were graft inoculated with CLBV, with *Citrus tristeza virus* (CTV), a virus readily eliminated by shoot-tip grafting *in vitro*, or with both simultaneously. Although CLBV was detected by hybridization and real-time reverse transcription-polymerase chain reaction (RT-PCR) in 0.2-mm shoot tips in all CLBV-inoculated plants, CTV was not detected. These results explain the difficulty in eliminating CLBV by shoot-tip grafting *in vitro*.

Systemic infection of plants by viruses requires the replication of the viral genome in infected cells, cell-to-cell movement in the inoculated leaf and long-distance transport to start new infection sites. For cell-to-cell movement, viruses cross through plasmodesmatal intercellular connections. Long-distance movement requires the entry of the virus into phloem sieve tubes from adjacent companion cells, fast motion through the connected sieve elements and subsequent unloading into new adjacent cells to start again cell-to-cell movement and to invade neighbouring cells of distal plant tissues (Harries and Ding, 2011, Scholthof, 2005). Although most viruses use phloem transport to move systemically, some have been shown to traffic through the xylem (Moreno *et al*., 2004; Opalka *et al*., 1998; Verchot *et al*., 2001). Plant viruses usually encode movement proteins (MPs) which, in cooperation with some host proteins, facilitate their translocation through plasmodesmata and the vascular system (Lucas, 2006; Taliansky *et al*., 2008).

Citrus leaf blotch virus (CLBV), family *Betaflexiviridae* (Adams *et al*., 2012; Martelli *et al*., 2007) , has a single-stranded, positive-sense genomic RNA (gRNA) of 8747 nucleotides with three open reading frames (ORFs) and untranslated regions (UTRs) at the 5′ and 3′ ends of the gRNA, as described previously (Galipienso *et al*., 2001; Renovell *et al*., 2010; Vives *et al*., 2001, 2002). Although preliminary data on CLBV accumulation in different plant organs have been reported (Ruiz-Ruiz *et al*., 2009), invasion of growing shoots by CLBV has not yet been examined.

Methods for virus localization in infected cells include electron microscopy or *in situ* techniques for the detection of either viral RNA or virus-encoded proteins (Amari *et al*., 2009; Appiano and Pennazio, 1972; Leisner *et al*., 1992). An alternative approach has been to introduce the reporter gene *β-glucuronidase* into the viral genome, thus allowing histochemical localization of virus-infected cells (Chapman *et al*., 1992; Dolja *et al*., 1992). However, all of these techniques are invasive and do not allow the real-time observation of virus movement. The introduction of the jellyfish *green fluorescent protein* (*GFP*) gene into viral genomes as an *in vivo* reporter has increased the ability to observe inter- and intracellular events accompanying virus infection by fluorescence observation or confocal microscopy (Baulcombe *et al*., 1995; Cheng *et al*., 2000; Folimonova *et al*., 2008; Haupt *et al*., 2001; Santa Cruz *et al*., 1996; Silva *et al*., 2002).

Several reports have documented that most viruses and viroids are unable to invade the apical meristems of infected shoots (Al-Kaff and Covey, 1996; Di Serio *et al*., 2010; Faccioli *et al*., 1988; Gosalvez-Bernal *et al*., 2006; Hull, 2002; Walkley and Webb, 1968). Generally, a variable region of 100–1000 μm remains virus free, a feature that has been exploited to recover virus-free plants from infected varieties by growing excised shoot tips in appropriate tissue culture media (Matthews, 1991). Similarly, shoot-tip

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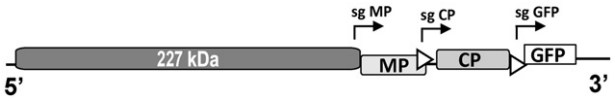


Fig. 1 Outline of the infectious *Citrus leaf blotch virus* (CLBV) clone *clbv3′pr*-GFP expressing the green fluorescent protein (GFP). Grey boxes represent the CLBV open reading frames (ORFs) (227-kDa polyprotein containing the replicase domains; MP, movement protein; CP, coat protein) and the white box the *gfp* gene. Arrows indicate transcription of subgenomic RNAs (sg). White triangle represents the promoter of the CP subgenomic RNA, duplicated to express GFP.

grafting *in vitro* has been used to recover virus-free plants from infected citrus cultivars (Navarro *et al*., 1975); however, CLBV was difficult to eliminate by this procedure (Navarro and Juárez, 2007), suggesting that it might be able to replicate very close to the meristem.

In this work, we examined CLBV spread during systemic infection of *Nicotiana benthamiana* plants in nearly real-time conditions using an infectious cDNA clone engineered to express GFP (*clbv3′pr*-GFP clone) (Fig. 1; Agüero *et al*., 2012; Vives *et al*., 2008). This clone, maintaining all genes and controller regions of the wild virus, was agroinoculated in *N. benthamiana* plants as described in Agüero *et al*. (2012). The viral progeny produced by this construct is referred to as CLBV-GFP. The infection pathway was initially monitored, observing GFP expression in inoculated plants illuminated with a long-wavelength UV lamp (Black RayR model B100AP, UV Products, Upland, CA, USA) and capturing images with a CANON EOS 300D digital camera using a yellow filter (Jos. Schneider Optische Werke, B + W Filter, Bad Kreuznach, Germany). Infection was first detected in agroinoculated leaves at approximately 11 or 12 days post-inoculation (dpi) by the appearance of bright green fluorescent spots (Fig. 2a) that increased in number and size in the following days (Fig. 2b). Discontinuous fluorescent foci were later observed in veins of different size, including the midrib (Fig. 2c), suggesting that the virus was loaded into the sieve tubes and that minor and major veins may be entry points for long-distance infection of *N. benthamiana* plants, as reported for other plant viruses (Cheng *et al*., 2000; Silva *et al*., 2002). Detailed observation with a stereomicroscope (Leica Microsystems, Heerbrugg, Switzerland) using a high-energy light source and a GFP filter revealed clusters of infected cells beside the vein foci (Fig. 2d), suggesting that CLBV-GFP arrived to veins via cell-to-cell movement. Within cells, confocal laser scanning microscopy (Leica TCS-SL, Mannheim, Germany) showed that fluorescence was located in the cytoplasm and the nucleus, but not in the vacuoles of infected epidermal cells, with the most intense fluorescence being observed in the nucleus (Fig. 2e). This GFP accumulation does not mean that the virus replicates in the nucleus.

Systemic viral infection was detected at 18 dpi by the presence of new fluorescent areas in the stem, petiole and veins of the upper leaves (Fig. 2f), and then in other leaf tissues (Fig. 2g), indicating that, once the virus enters the vascular system, it can move long distances in a short time. For other plant viruses, cell-to-cell movement has been reported to occur at a rate of approximately 25 μm/h (Derrick *et al*., 1992; Mise and Ahlquist, 1995; Szécsi *et al*., 1999), whereas movement through the vascular system occurs at a rate of centimetres per hour (Nelson and van Bel, 1998). Generally, fluorescence was not uniform throughout the stem, petioles or leaf veins, with scattered fluorescent spots of variable size and intensity being observed (Fig. 2f). This distribution is consistent with CLBV-GFP delivery into surrounding cells after long-distance transport via the phloem vessels. Observation of stem cross-sections from infected plants with a stereomicroscope showed GFP fluorescence in the phloem and pith, indicating the presence of the virus in these tissues (Fig. 2h). Stem cross-sections from healthy plants showed red fluorescence caused by chlorophyll, or a faint yellow fluorescence in xylem and parenchyma cells, probably caused by phenolic compounds (Fig. 2i). In the petiole of infected leaves, GFP fluorescence was observed in the phloem and, to a lesser extent, in parenchyma (Fig. 2j), whereas no fluorescence was observed in identical sections from healthy plants (Fig. 2l). At the most intense fluorescence spots, in either the stem (data not shown) or petioles, additional GFP fluorescence was detected in the parenchyma and epidermal cells (Fig. 2k), probably as a result of cell-to-cell movement.

After vascular transport of the virus through the stem, it leaves the phloem to establish new infection foci in sink tissues. In young developing leaves, GFP fluorescence was first seen mainly in the midrib (class I vein) and class II veins. The first indication of virus exit to the mesophyll of these leaves was the appearance of disperse fluorescent flecks in the lamina, suggesting that virus unload did not occur uniformly (Fig. 2m). Fluorescent foci associated with virus unload were mainly observed in class III and, occasionally, class II veins at later stages of infection, but rarely in smaller veins. Similar results were reported by Roberts *et al*. (1997) comparing phloem unload of GFP-tagged *Potato virus X* (PVX) and the fluorescent solute carboxyfluorescein in *N. benthamiana* plants. Both virus and carboxyfluorescein were predominantly unloaded from class III veins, with minor veins (classes IV and V) playing no role in the process. From the initial site of CLBV unload, the infection spread by cell-to-cell movement, as indicated by the progressive appearance of GFP fluorescence in the interveinal mesophyll tissue (Fig. 2g). The invasion pattern depends on the developmental stage of the leaf at the time of infection. The three leaves immediately above those agroinoculated did not show any fluorescence, indicating that these leaves were already developed and not at the sink stage when CLBV reached the vascular system. In the next two or three following upper leaves, the virus invaded only their basal region, but never reached the leaf apex (Fig. 2n), suggesting that the virus arrived to these leaves when they were at the sink–source transition, as

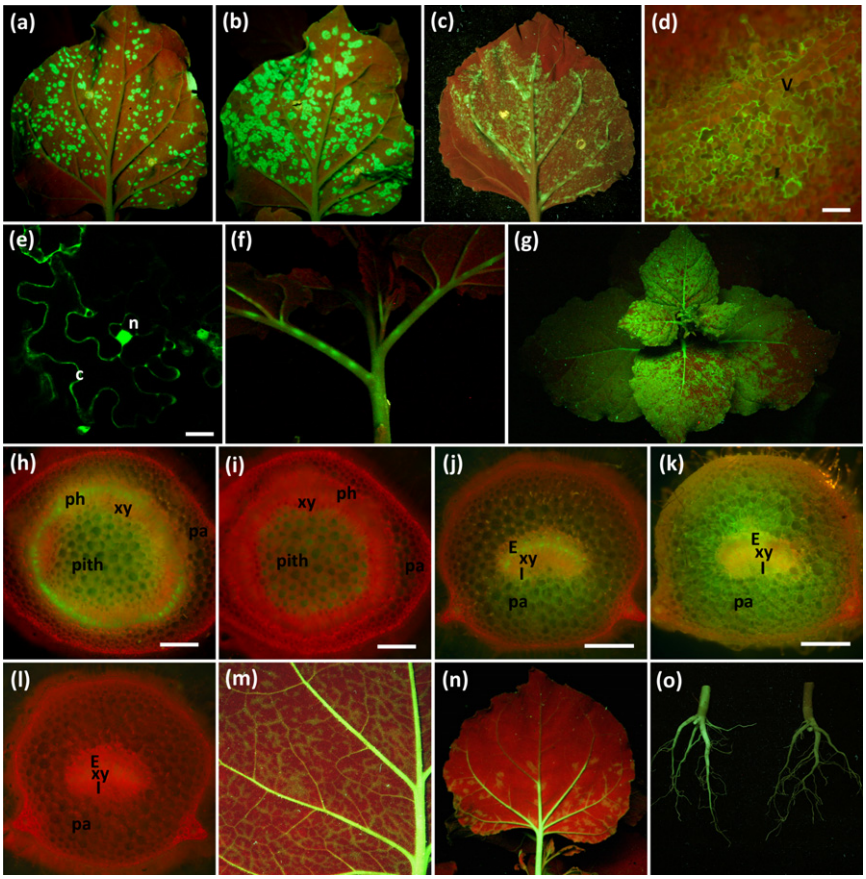


Fig. 2 Expression of the green fluorescent protein (GFP) in *Nicotiana benthamiana* plants agroinoculated with the infectious *Citrus leaf blotch virus* (CLBV) clone *clbv3′pr*-GFP. (a, b) Infection foci in agroinoculated leaves at 13 and 16 days post-inoculation (dpi), respectively. (c) Virus loading into the phloem vessels at different sites of an agroinoculated leaf. (d) Fluorescence focus beside a vein observed in a fluorescence stereomicroscope. Bar, 80 μm (e) Epidermal cells observed by confocal microscopy. Bar, 10 μm. n, nucleus; c, cytoplasm. (f) GFP accumulation in stem, petiole and veins of upper leaves at 18 dpi. (g) Plant systemically infected at 25 dpi. (h, j) Cross-sections of stem and petiole from an infected plant. (k) Similar petiole cross-section from an intense fluorescence spot. (i, l) Similar stem and petiole cross-sections from a healthy plant. Bar, 500 μm. E, external phloem; I, internal phloem; pa, parenchyma; ph, phloem; xy, xylem. (m) Upper leaf of infected plants at 20 dpi. (n) Leaf undergoing the sink–source transition at 20 dpi. (o) Roots of infected (left) and healthy (right) plants. Photographs a–c, f, g and m–o were taken with a digital camera using UV light and a yellow filter, d and h–l with a fluorescence stereomicroscope and e with a confocal laser scanning microscope.

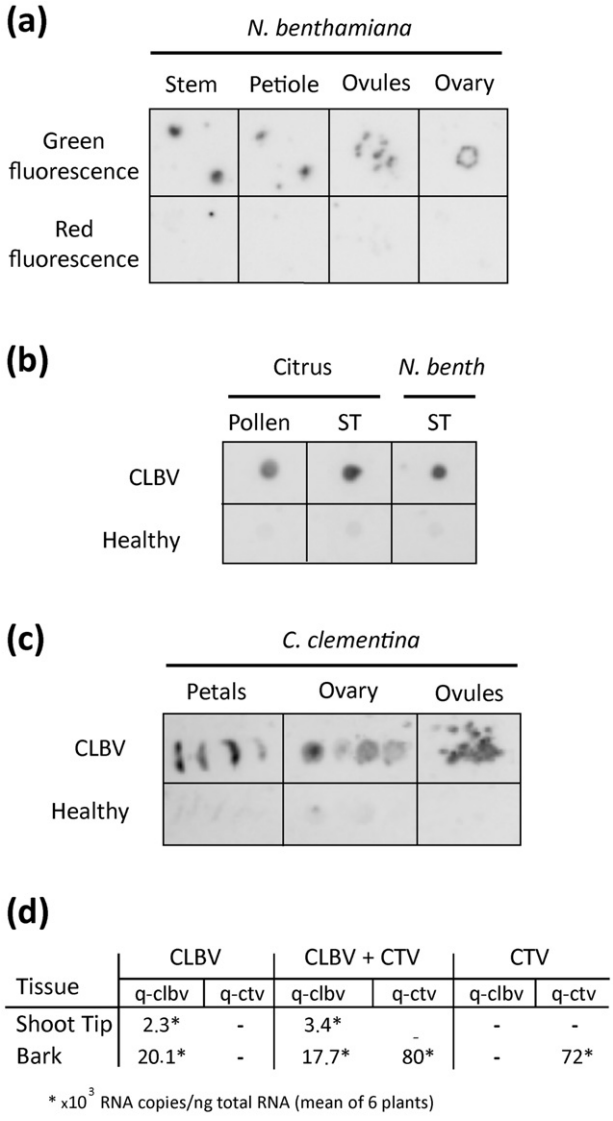
described previously by Roberts *et al*. (1997) for PVX. The virus would be unable to advance through the veins, but it would still move from cell to cell from some basal class III veins, invading the mesophyll around the unloading point (Fig. 2n). CLBV also invaded and accumulated in *N. benthamiana* roots, as detected by real-time reverse transcription polymerase chain reaction (rtRT-PCR) with a TaqMan probe targeted to the CLBV ORF1 (Ruiz-Ruiz *et al*., 2009) (data not shown), and by strong green GFP fluorescence observed in infected plants in comparison with the weak yellowish fluorescence shown by healthy plant roots (Fig. 2o).

Cell-to-cell and long-distance movement of free GFP has been observed in plants after biolistic bombardment of sink leaves with a plasmid encoding the *gfp* gene (Itaya *et al*., 1998; Oparka *et al*., 1999), and in plants expressing the *gfp* gene under the control of the companion cell-specific *AtSUC2* promoter (Imlau *et al*., 1999). In order to confirm that GFP fluorescence observed in plants agroinoculated with the *clbv3′pr*-GFP clone is caused by the presence of CLBV, tissue print hybridization was performed with fresh sections of different plant organs and a digoxigenin (DIG)-labelled RNA probe specific for the 3′ UTR of the gRNA (Agüero *et al*., 2012). CLBV was detected in all tissues showing GFP fluorescence, whereas no hybridization signal was obtained in nonfluorescent tissues (Fig. 3a). These results confirmed that the GFP fluorescence observed was caused by virus spread rather than by independent translocation of the GFP protein. Moreover, GFP fluorescence faded in a couple of weeks, suggesting that the bright fluorescence observed in the initial stages of infection was a result of GFP expression during CLBV replication.

To explore the possibility that CLBV could invade meristems, shoot tips of infected *N. benthamiana* plants were examined by confocal laser scanning microscopy. Two kinds of meristem were observed.

(i) Shoot apical meristems (SAMs) located at the tip of growing stems and surrounded by the leaf primordia (Fig. 4a). A strong fluorescent signal was observed in both SAM and leaf primordia with GFP fluorescence being located mainly in the cytoplasm (Fig. 4b,c), whereas no GFP fluorescence was observed in shoot tips from noninfected plants (data not shown). In these meristems, cells divide actively and different stages of the cell cycle were observed (Fig. 4c).

(ii) Latent axillary meristems located in leaf axils (Fig. 4d). Cells in these meristems are quiescent and GFP fluorescence appeared mainly in the nuclei (Fig. 4e,f). Because GFP fluorescence expressed by CLBV-GFP was detected in the corpus cells of both meristems, where no vascular tissue is present, the virus probably



(d)

Tissue	CLBV		CLBV + CTV		CTV	
	q-clbv	q-ctv	q-clbv	q-ctv	q-clbv	q-ctv
Shoot Tip	2.3*	-	3.4*	-	-	-
Bark	20.1*	-	17.7*	80*	-	72*

* $\times 10^3$ RNA copies/ng total RNA (mean of 6 plants)

Fig. 3 *Citrus leaf blotch virus* (CLBV) detection by molecular hybridization in infected tissues of *Nicotiana benthamiana* and citrus plants. (a) Imprints of different tissues from *N. benthamiana* plants agroinoculated with the CLBV infectious clone *clbv3′pr*-GFP showing green (green fluorescent protein, GFP) or red (chlorophyll) fluorescence. (b) Dot-blot hybridization using total RNA extracted from healthy or CLBV-infected *Citrus clementina* (pollen), Mexican lime (shoot tips, ST) or *N. benthamiana* (ST) plants. (c) Tissue-print hybridization of petals, ovary or ovules from healthy or CLBV-infected *C. clementina* plants. The membranes were hybridized with a digoxigenin (DIG)-labelled RNA probe specific for the CLBV 3′ untranslated region (UTR). (d) Detection and absolute quantification of genomic RNA copies of CLBV (q-clbv) or *Citrus tristeza virus* (CTV) (q-ctv) in 0.2-mm shoot tips or bark of Mexican lime plants infected with CLBV, CTV or co-inoculated with both viruses. –, not detected.

moves from cell to cell from the infected protophloem to meristematic cells.

As Imlau *et al*. (1999) showed that GFP can spread by passive diffusion through expanding tissues, we attempted to confirm the presence of CLBV in SAM cells by analysing total RNA extracts from infected and healthy meristematic tissue (about 0.2-mm shoot tips) by dot-blot hybridization and rtRT-PCR. CLBV was readily detected by both techniques in the meristems of infected plants (Fig. 3b and data not shown), confirming that the GFP fluorescence observed was caused by virus spread rather than by passive translocation of the GFP protein from neighbouring regions.

In CLBV-GFP-infected citrus plants, GFP fluorescence was not observed, probably as a result of the instability of the construct (Agüero *et al*., 2012). Therefore, to examine CLBV infection in citrus meristematic regions, six Mexican lime [*Citrus aurantifolia* (Chrism.) Swing.] plants were graft inoculated with CLBV, with the T318A isolate of *Citrus tristeza virus* (CTV), a phloem-restricted virus that is easily eliminated by shoot-tip grafting *in vitro*, or co-inoculated simultaneously with both viruses. CLBV was detected by dot-blot hybridization (Fig. 3b) and rtRT-PCR (Ruiz-Ruiz *et al*., 2009) (Fig. 3d) in 0.2-mm shoot tips from citrus plants inoculated with CLBV or CLBV plus CTV, whereas CTV was not detected by rtRT-PCR (Ruiz-Ruiz *et al*., 2007) in similar shoot tips from plants inoculated with CTV or CTV plus CLBV. These results explain, in part, the difficulty in eliminating CLBV by shoot-tip grafting *in vitro*. Most virus and viroids are eliminated by this technique in more than 90% of micrografted plants. However, depending on the citrus genotype, CLBV is usually eliminated in only 10%–50% of micrografted plants and, in some genotypes, no CLBV-free plants were recovered.

In most plant–virus combinations, viruses are not detected in SAM (Hull, 2002), but it is unknown whether this absence of virus is a result of the inability of viruses to replicate in meristematic tissues or whether the presence of some active or passive barrier impairs their entry to SAM. An attractive hypothesis to explain the ability of meristematic cells to avoid viral infection is that a strong defence mechanism would allow newly differentiated tissues to develop virus free. It has been reported that an RNA-mediated surveillance system protects the shoot tip from viral infection (Foster *et al*., 2002). The RNA-dependent RNA polymerase 6, which is an integral part of the RNA silencing machinery, is involved in the exclusion of viruses (Qu *et al*., 2005; Schwach *et al*., 2005) and viroids (Di Serio *et al*., 2010) from *N. benthamiana* meristems. To counteract RNA silencing, most plant viruses encode proteins that act as suppressors of the host antiviral defence. *Tobacco rattle virus* (TRV) can invade SAM cells using a 16K suppressor protein encoded by its genome. This protein showed a weak suppressor activity in agroinoculated *N. benthamiana* 16c plants in comparison with the p19 protein of *Tomato bushy stunt virus* (Martín-Hernández and Baulcombe, 2008). The

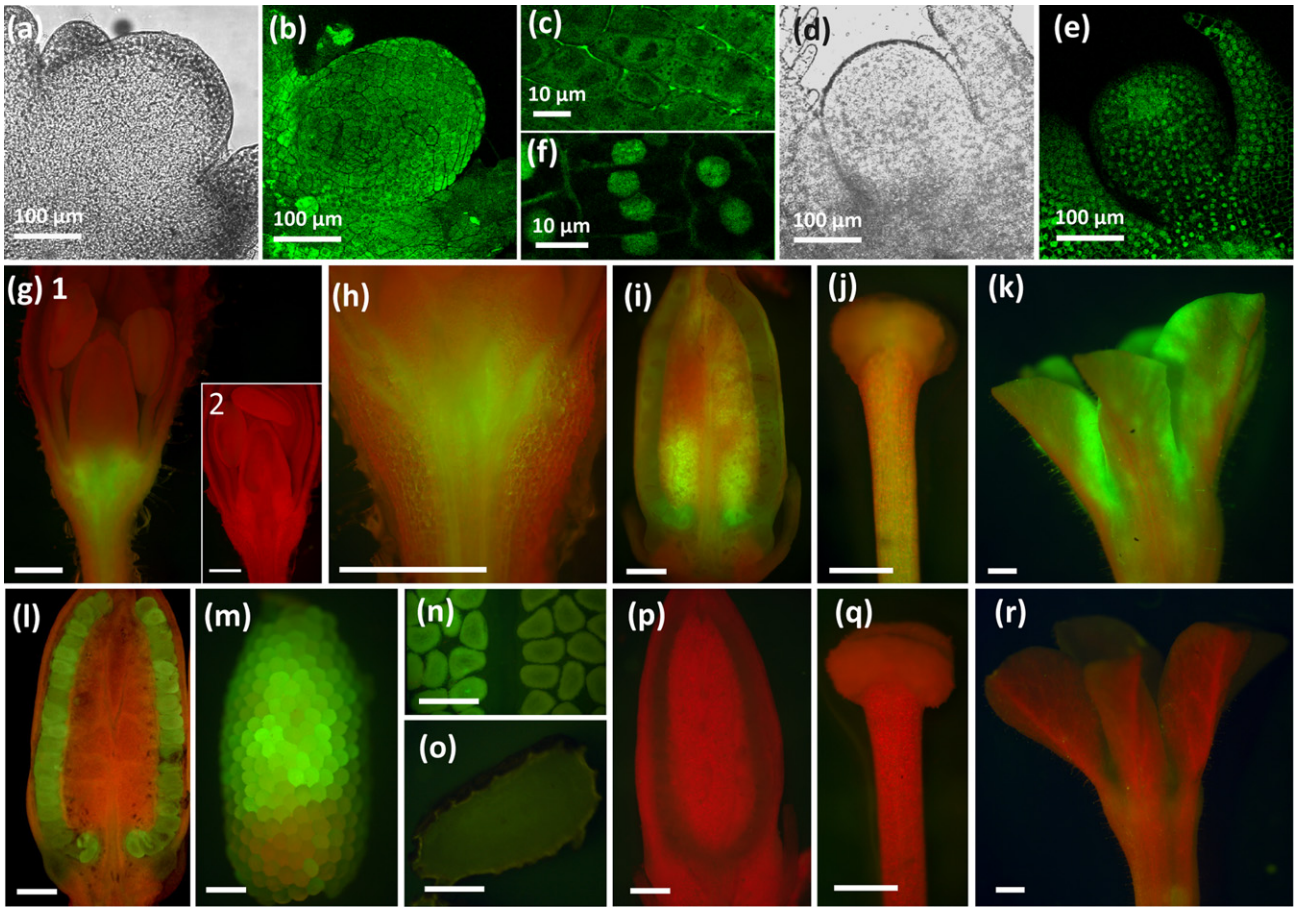


Fig. 4 Green fluorescent protein (GFP) detection in meristems and floral organs of *Nicotiana benthamiana* plants agroinoculated with the infectious *Citrus leaf blotch virus* (CLBV) clone *clbv3'pr*-GFP. (a–c) Shoot apical meristem (SAM). (d–f) Axillary meristem. (g, h) Longitudinal sections of flowers from an infected plant at early developmental stages (g1 and h) and a similar section from a healthy plant (g2). (i–k) Developing ovary, style, stigma and corolla from an infected plant, and similar organs from a healthy plant (p–r). (l, m) Longitudinal section of a mature ovary from an infected plant (l) and a similar ovary after eliminating the carpel (m). (n) Young seeds from infected (left) and healthy (right) plants. (o) Mature seed from a healthy plant. Images (b, c, e, f) were captured using a confocal laser scanning microscope, (a, d) with a light microscope and (g–r) with a fluorescence stereomicroscope. Bars in the latter images, 500 µm.

authors suggested that the weak suppressor activity of 16K might be a crucial evolutionary factor, as a strong suppressor activity would allow high virus accumulation in meristematic cells, probably causing severe damage to infected plants. Similarly, the CLBV MP is a weak silencing suppressor (Renovell *et al*., 2012), and could be the factor responsible for the viral invasion of meristematic cells without causing important symptoms in most citrus hosts (Galipienso *et al*., 2000). However, the 16K protein of TRV is able to act *in trans*, whereas the MP of CLBV is not. Thus, although plants co-infected with TRV and PVX accumulated PVX in 53% of the meristems, this virus was excluded from the meristems in plants co-infected with PVX and a *16k* mutant of TRV (Martín-Hernández and Baulcombe, 2008). However, in plants co-inoculated with CLBV and CTV, the latter virus was never detected in meristematic tissues.

Extensive examination of longitudinal sections of *N. benthamiana* flowers with a fluorescence stereomicroscope revealed that CLBV-GFP reaches the flower through phloem channels (Fig. 4g1, h), and then invades the ovary (Fig 4i), style and stigma (Fig. 4j), sepals and petals (Fig. 4k). In mature ovaries, strong GFP fluorescence was observed in ovules (Fig. 4l,m), indicating preferential accumulation of the virus in these organs. The ovary, style, stigma and petals of healthy plants showed red fluorescence (Fig. 4g2, p–r). The anthers of healthy plants showed a strong green fluorescence, probably as a result of the accumulation of phenolic compounds in the pollen exine, which hampered the detection of GFP fluorescence in infected plants (data not shown). The presence of CLBV in ovary and ovules was confirmed by tissue-print hybridization (Fig. 3a) and rtRT-PCR (data not shown).

CLBV infection in flower organs of citrus was examined by dot-blot hybridization with total RNA extracted from pollen (Fig. 3b), and by tissue-print hybridization with petals, ovary and ovules (Fig. 3c) from CLBV-infected *C. clementina*. The virus was detected in all of these tissues, indicating that CLBV invades floral organs, including pollen, at least in citrus.

Finally, GFP fluorescence was also observed in young seeds of CLBV-infected *N. benthamiana* plants (Fig. 4n, left), in comparison with the weak yellowish fluorescence caused by the presence of lignin in healthy seeds (Fig. 4n, right), indicating that CLBV-GFP is able to invade the seed coat. However, cross-sections of dry seeds from infected or healthy plants showed similar yellowish fluorescence (Fig. 4o and data not shown). This finding indicates that, although CLBV is able to infect maternal seed tissues, it would be excluded from the embryo, suggesting that embryos are not symplastically connected with the maternal tissue. In addition, we did not observe seed transmission in more than 100 seedlings obtained from CLBV-GFP-infected *N. benthamiana* plants after fluorescence observation and RT-PCR analyses (Agüero *et al*., 2012), whereas a low rate of seed transmission (about 2.5%) was observed previously in citrus plants (Guerri *et al*., 2004).

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REFERENCES

Adams, M.J., Candresse, T., Hammond, J., Kreuze, J.F., Martelli, G.P., Namba, S., Pearson, M.N., Ryu, K.H., Saldarelli, P. and Yoshikawa, N. (2012) Family *Betaflexiviridae*. In: *Virus Taxonomy: IXth Report of the International Committee on Taxonomy of Viruses* (King, A.M.Q., Adams, M.J., Carstens, E.B. and Lefkowitz, E.J., eds), pp. 920–941. London: Elsevier Academic Press.

Agüero, J., Ruiz-Ruiz, S., Vives, M.C., Velázquez, K., Navarro, L., Peña, L., Moreno, P. and Guerri, J. (2012) Development of viral vectors based on *Citrus leaf blotch virus* to express foreign proteins or analyze gene function in citrus plants. *Mol. Plant–Microbe. Interact.* **25**, 1326–1337.

Al-Kaff, N.S. and Covey, S.N. (1996) Unusual accumulations of *Cauliflower mosaic virus* in local lesions, dark green leaf tissue, and roots of infected plants. *Mol. Plant–Microbe. Interact.* **5**, 357–363.

Amari, K., Burgos, L., Pallás, V. and Sánchez-Pina, M.A. (2009) Vertical transmission of *Prunus necrotic ringspot virus*: hitch-hiking from gametes to seedling. *J. Gen. Virol.* **85**, 761–768.

Appiano, A. and Pennazio, S. (1972) Electron microscopy of potato meristem tips infected with *Potato virus X*. *J. Gen. Virol.* **14**, 273–276.

Baulcombe, D.C., Chapman, S.N. and Santa Cruz, S. (1995) Jellyfish green fluorescent protein as a reporter for virus infections. *Plant J.* **7**, 1045–1053.

Chapman, S., Kavanagh, T. and Baulcombe, D. (1992) Potato virus X as a vector for gene expression in plants. *Plant J.* **2**, 549–557.

Cheng, N.H., Su, C.L., Carter, S.A. and Nelson, R.S. (2000) Vascular invasion routes and systemic accumulation patterns of tobacco mosaic virus in *Nicotiana benthamiana*. *Plant J.* **23**, 349–362.

Derrick, P.M., Barker, H. and Oparka, K.J. (1992) Increase in plasmodesmatal permeability during cell-to-cell spread of tobacco rattle virus from individually inoculated cells. *Plant Cell*, **4**, 1405–1412.

Di Serio, F., Martínez de Alba, A.E., Navarro, B., Gisel, A. and Flores, R. (2010) RNA-dependent RNA polymerase 6 delays accumulation and precludes meristem invasion of a viroid that replicates in the nucleus. *J. Virol.* **84**, 2477–2489.

Dolja, V.V., McBride, H.J. and Carrington, J.C. (1992) Tagging of plant potyvirus replication and movement by insertion of β-glucuronidase into the viral polyprotein. *Proc. Natl. Acad. Sci. USA*, **89**, 10 208–10 212.

Faccioli, G., Rubies-Autonell, C. and Resca, R. (1988) Potato leafroll virus distribution in potato meristem tips and production of virus-free plants. *Potato Res.* **31**, 511–520.

Folimonova, S.Y., Folimonov, A.S., Satyanarayana, T. and Dawson, W.O. (2008) *Citrus tristeza virus*: survival at the edge of the movement continuum. *J. Virol.* **82**, 6546–6556.

Foster, T.M., Lough, T.J., Emerson, S.J., Lee, R.H., Bowman, J.L., Forster, R.L.S. and Lucas, W.J. (2002) A surveillance system regulates selective entry of RNA into the shoot apex. *Plant Cell*, **14**, 1497–1508.

Galipienso, L., Navarro, L., Ballester-Olmos, J.F., Pina, J.A., Moreno, P. and Guerri, J. (2000) Host range and symptomatology of a graft-transmissible pathogen causing bud union crease of citrus on trifoliate rootstocks. *Plant Pathol.* **49**, 308–314.

Galipienso, L., Vives, M.C., Moreno, P., Milne, R.G., Navarro, L. and Guerri, J. (2001) Partial characterisation of citrus leaf blotch virus, a new virus from Nagami kumquat. *Arch. Virol.* **146**, 357–368.

Gosalvez-Bernal, B., García-Castillo, S., Pallás, V. and Sánchez-Pina, M.A. (2006) Distribution of carnation viruses in the shoot tip: exclusion from the shoot apical meristem. *Physiol. Mol. Plant Pathol.* **69**, 43–51.

Guerri, J., Pina, J.A., Vives, M.C., Navarro, L. and Moreno, P. (2004) Seed transmission of *Citrus leaf blotch virus*: implications in quarantine and certification programs. *Plant Dis.* **88**, 906.

Harries, P. and Ding, B. (2011) Cellular factors in plant virus movement: at the leading edge of macromolecular trafficking in plants. *Virology*, **411**, 237–243.

Haupt, S., Duncan, G.H., Holzberg, S. and Oparka, K.J. (2001) Evidence for symplastic phloem unloading in sink leaves of barley. *Plant Physiol.* **125**, 209–218.

Hull, R. (2002) *Matthews' Plant Virology*, 4th edn. New York: Academic Press.

Imlau, A., Truernit, E. and Sauer, N. (1999) Cell-to-cell and long distance trafficking of the green fluorescent protein in the phloem and symplastic unloading of the protein into sink tissues. *Plant Cell*, **11**, 309–322.

Itaya, A., Woo, Y.M., Masuta, C., Bao, Y., Nelson, R.S. and Ding, B. (1998) Developmental regulation of intercellular protein trafficking through plasmodesmata in tobacco leaf epidermis. *Plant Physiol.* **118**, 373–385.

Leisner, S.M., Turgeon, R. and Howell, S.H. (1992) Long distance movement of cauliflower mosaic virus in infected turnip plants. *Mol. Plant–Microbe. Interact.* **5**, 41–47.

Lucas, W.J. (2006) Plant viral movement proteins: agents for cell-to-cell trafficking of viral genomes. *Virology*, **344**, 169–184.

Martelli, G.P., Adams, M.J., Kreuze, J.F. and Dolja, V.V. (2007) Family *Flexiviridae*: a case study in virion and genome plasticity. *Annu. Rev. Phytopathol.* **45**, 73–100.

Martín-Hernández, A.M. and Baulcombe, D.C. (2008) Tobacco rattle virus 16-kilodalton protein encodes a suppressor of RNA silencing that allows transient viral entry in meristems. *J. Virol.* **82**, 4064–4071.

Matthews, R.E.F. (1991) Virus-free seed. In: *Plant Virology* (Matthews, R.E.F., ed.), pp. 599–630. New York: Academic Press.

Mise, K. and Ahlquist, P. (1995) Host-specificity restriction by bromovirus cell-to-cell movement protein occurs after initial cell-to-cell spread of infection in nonhost plants. *Virology*, **206**, 276–286.

Moreno, I.M., Thompson, J.R. and García-Arenal, F. (2004) Analysis of the systemic colonization of cucumber plants by *Cucumber green mottle mosaic virus*. *J. Gen. Virol.* **85**, 749–759.

Navarro, L. and Juárez, J. (2007) Shoot-tip grafting *in vitro*. In: *Citrus Genetics, Breeding and Biotechnology* (Khan, I., ed.), pp. 45–140. Wallingford, Oxfordshire: CABI Publishing.

Navarro, L., Roistacher, C.N. and Murashige, T. (1975) Improvement of shoot-tip grafting *in vitro* for virus-free citrus. *J. Am. Soc. Hortic. Sci.* **100**, 471–479.

Nelson, R.S. and van Bel, A.J.E. (1998) The mystery of virus trafficking into, through and out of vascular tissue. *Prog. Bot.* **59**, 476–533.

Opalka, N., Brugidou, C., Bonneau, C., Nicole, M., Beachy, R.N., Yeager, M. and Fauquet, C. (1998) Movement of rice yellow mottle virus between xylem cells through pit membranes. *Proc. Natl. Acad. Sci. USA*, **95**, 3323–3328.

Oparka, K.J., **Roberts, A.G.**, **Boevink, P.**, **Santa Cruz, S.**, **Roberts, I.M.**, **Pradel, K.S.**, **Imlau, A.**, **Kotlizky, G.**, **Sauer, N. and Epel, B.** (1999) Simple, but not branched, plasmodesmata allow the nonspecific trafficking of proteins in developing tobacco leaves. *Cell*, **97**, 743–754.

Qu, F., **Ye, X.**, **Hou, G.**, **Sato, S.**, **Clemente, T.E. and Morris, T.J.** (2005) RDR6 has a broad-spectrum but temperature-dependent antiviral defense role in *Nicotiana benthamiana*. *J. Virol*. **79**, 15 209–15 217.

Renovell, A., **Gago, S.**, **Ruiz-Ruiz, S.**, **Velázquez, K.**, **Navarro, L.**, **Moreno, P.**, **Vives, M.C. and Guerri, J.** (2010) Mapping the subgenomic RNA promoter of the *Citrus leaf blotch virus* coat protein gene by *Agrobacterium*-mediated inoculation. *Virology*, **406**, 360–369.

Renovell, A., **Vives, M.C.**, **Ruiz-Ruiz, S.**, **Navarro, L.**, **Moreno, P. and Guerri, J.** (2012) The *Citrus leaf blotch virus* movement protein acts as silencing suppressor. *Virus Genes*, **44**, 131–140.

Roberts, A.G., **Santa Cruz, S.**, **Roberts, I.M.**, **Prior, D.A.M.**, **Turgeon, R. and Oparka, K.J.** (1997) Phloem unloading in sink leaves of *Nicotiana benthamiana*: comparison of a fluorescent solute with a fluorescent virus. *Plant Cell*, **9**, 1381–1396.

Ruiz-Ruiz, S., **Moreno, P.**, **Guerri, J. and Ambrós, S.** (2007) A real-time RT-PCR assay for detection and absolute quantitation of *Citrus tristeza virus* in different plant tissues. *J. Virol. Methods*, **145**, 96–105.

Ruiz-Ruiz, S., **Ambrós, S.**, **Vives, M.C.**, **Navarro, L.**, **Moreno, P. and Guerri, J.** (2009) Detection and quantitation of *Citrus leaf blotch virus* by TaqMan real-time RT-PCR. *J. Virol. Methods*, **160**, 57–62.

Santa Cruz, S., **Chapman, S.**, **Roberts, A.G.**, **Roberts, I.M.**, **Prior, D.A.M. and Oparka, K.J.** (1996) Assembly and movement of a plant virus carrying a green fluorescent protein overcoat. *Proc. Natl. Acad. Sci. USA*, **93**, 6286–6290.

Scholthof, H.B. (2005) Plant virus transport: motions of functional equivalence. *Trends Plant Sci.* **10**, 376–382.

Schwach, F., **Vaistij, F.E.**, **Jones, L. and Baulcombe, D.C.** (2005) An RNA-dependent RNA polymerase prevents meristem invasion by potato virus X and is required for the activity but not the production of a systemic silencing signal. *Plant Physiol*. **138**, 1842–1852.

Silva, M.S., **Wellink, J.**, **Goldbach, R.W. and van Lent, J.W.M.** (2002) Phloem loading and unloading of *Cowpea mosaic virus* in *Vigna unguiculata*. *J. Gen. Virol*. **83**, 1493–1504.

Szécsi, J., **Ding, X.S.**, **Lim, C.O.**, **Bendahmane, M.**, **Cho, M.J.**, **Nelson, R.S. and Beachy, R.N.** (1999) Development of tobacco mosaic virus infection sites in *Nicotiana benthamiana*. *Mol. Plant–Microbe. Interact*. **12**, 143–152.

Taliansky, M., **Torrance, L. and Kalinina, N.O.** (2008) Role of plant virus movement proteins. *Methods Mol. Biol*. **451**, 33–54.

Verchot, J., **Driskel, B.A.**, **Zhu, Y.**, **Hunger, R.M. and Littlefield, L.J.** (2001) Evidence that soilborne wheat mosaic virus moves long distance through the xylem in wheat. *Protoplasma*, **218**, 57–66.

Vives, M.C., **Galipienso, L.**, **Navarro, L.**, **Moreno, P. and Guerri, J.** (2001) The nucleotide sequence and genomic organization of *Citrus leaf blotch virus*: candidate type species for a new virus genus. *Virology*, **287**, 225–233.

Vives, M.C., **Galipienso, L.**, **Navarro, L.**, **Moreno, P. and Guerri, J.** (2002) Characterization of two kinds of subgenomic RNAs produced by *Citrus leaf blotch virus*. *Virology*, **295**, 328–336.

Vives, M.C., **Martín, S.**, **Ambrós, S.**, **Renovell, A.**, **Navarro, L.**, **Pina, J.A.**, **Moreno, P. and Guerri, J.** (2008) Development of a full-genome cDNA clone of *Citrus leaf blotch virus* and infection of citrus plants. *Mol. Plant Pathol*. **9**, 787–797.

Walkley, D.G.A. and Webb, M.J.W. (1968) Virus in plant apical meristems. *J. Gen. Virol*. **3**, 311–313.

