Viroids are unencapsidated, small, single-stranded, circular RNAs that replicate autonomously when inoculated into their plant hosts, where they may elicit diseases. Citrus are natural hosts of several viroids belonging to the family Pospiviroidae, characterized by the presence of a central conserved region (CCR) and the absence of RNA self-cleavage mediated by hammerhead ribozymes (Flores et al., 2005). Citrus exocortis viroid (CEVd), hop stunt viroid, citrus bent leaf viroid, citrus dwarfing viroid, citrus bark cracking viroid and citrus viroid V have been included as distinct species in the Virus Taxonomy Scheme (www.ictvonline.org/virusTaxonomy.asp) (Di Serio et al., 2014). These viroids become systemic upon inoculation to their citrus hosts, which may express disease symptoms (sensitive hosts) or not (tolerant hosts). Most citrus species are tolerant, thus acting as reservoirs of viroids, which when transmitted either mechanically to sensitive species (Barbosa et al., 2005) or by grafting infected tolerant scions onto sensitive rootstocks, incite symptoms. Among the sensitive hosts, the selection 861-S1 of Etrog citron (Citrus medica) has been widely used in bioassays because it expresses viroid-specific symptoms and accumulates easily detectable viroid titres.

Experimental evidence indicating how viroids infect their hosts in a systemic manner was first provided by Palukaitis (1987), who identified the phloem as the pathway for long-distance movement of potato spindle tuber viroid (PSTVd), a result later confirmed by in situ hybridization (Zhu et al., 2001). Studies conducted with PSTVd indicate that cell-to-cell movement occurs through plasmodesmata and that it is mediated by specific viroid structural motifs (Ding et al., 1999). The infection process followed by viroids is assumed to involve: (i) intracellular movement to access their sub-cellular replication site, with the viroid progeny then moving through the cytoplasm; (ii) cell-to-cell movement via plasmodesmata and entry into the phloem; (iii) long-distance movement within the phloem to reach distal plant parts; and (iv) exit from the phloem to invade non-vascular cells (reviewed by Flores et al., 2005; Ding & Wang, 2009). The movement of viroids within their hosts has been addressed in herbaceous hosts (Qi et al., 2004; Zhong et al., 2007; Takeda et al., 2011) and implicitly assumed to operate in a similar manner in woody species.

Studies comparing the response of different citrus to viroid infection have shown that replication/accumulation depends on the viroid and the host (Vidalakis et al., 2004; Serra et al., 2008; Bani-Hashemian et al., 2010), with Citrus karna being identified as a host wherein
citrus viroids accumulate at unusually high concentrations in bark (Barbosa et al., 2002).

In an attempt to use *C. karna* seedlings for viroid bioindexing, an assay comparing viroid titre in *C. karna* and Etrog citron was performed. Briefly, blocks of three seedlings with a height of 20 cm were graft inoculated with citrus exocortis viroid (CEVd; E-117) (Gandía et al., 2005), hop stunt viroid 1 (HSVd-1; non-cachexia-inducing strain CVd-Ia-117) and HSVd-2 (cachexia-inducing strain X-707) (Palacio-Bielsa et al., 2004), citrus bent leaf viroid (CBLVd; CVd-Ia-117) (Foissac & Duran-Vila, 2000), citrus dwarfing viroid (CDVd; CVd-IIId) (Foissac & Duran-Vila, 2000), citrus viroid V (CVd-V) (Serra et al., 2008) or citrus bark cracking viroid (CBCVd; CVd-IV-Ca) (Francis et al., 1995) (three plants each), and maintained at 25–28 °C. Inoculated plants were assessed at monthly intervals over a 6-month period by Northern blot hybridization of leaf nucleic acid preparations (Semancik et al., 1975) using DIG-labelled viroid-specific DNA probes (Palacio-Bielsa et al., 1999; Murcia et al., 2009). Unexpectedly, detection of the inoculated viroids was erratic (data not shown). Therefore, the study was extended to compare viroid distribution in different tissues of inoculated plants. Northern blot hybridization of nucleic acid preparations from bark of young shoots generated strong signals in both hosts, regardless of the inoculated viroid (Fig. 1, lanes 1 and 3). However, preparations from fully expanded leaves devoid of petioles and midribs provided strong signals in Etrog citron (Fig. 1, lane 2) but, excepting CEVd, not in *C. karna* (Fig. 1, lane 4), wherein viroids remained undetectable in the two HSVd strains (HSVd-1 and HSVd-2) and CDVd. Thus, *C. karna* and Etrog citron behave differentially in terms of viroid accumulation in leaf tissues.

The above observations were further reinforced by analysis of protoplasts isolated from young expanded leaves sampled from the different viroids in infected Etrog citron and *C. karna*. Protoplasts were obtained essentially as described by Grosser & Gmitter (1990) using fully expanded young leaves cut into thin strips and incubated in 60 mm plates in a mixture of 2.5 ml of 0.6 M BH3 protoplast culture medium and 1.5 ml enzyme solution containing 0.7 mM mannitol, 12.0 mM CaCl₂, 6.0 mM MES, 1.4 mM NaH₂PO₄, 2 % (w/v) Onozuka RS cellulase and 2 % (w/v) Macerozyme R-10. In all instances, consistent protoplast yields were obtained after purification in a sucrose/mannitol gradient that was evaluated using a Fuchs-Rosenthal haematocytometer chamber. Northern blot hybridization of equalized preparations (2 × 10⁶ protoplasts ml⁻¹) revealed that viroids were only detected in those from Etrog citron, but not in those from *C. karna* (Fig. 1, lanes 5 and 6), suggesting that while viroids are efficiently downloaded from the phloem to adjacent cells in the former, they remain phloem restricted in the latter. The weaker hybridization signals observed in leaf blade samples of *C. karna* (Fig. 1, lane 4) are probably due to viroid accumulation in secondary veins.

To assess whether the finding that citrus viroids are phloem restricted in *C. karna* is an unusual characteristic of this genotype or a common behaviour of other citrus hosts, an additional study was performed using trifoliate orange (sensitive species) (Vernière et al., 2004) and sour orange (tolerant species). Seedlings of both species were graft inoculated with the same viroid sources of CEVd, HSVd, CBLVd, CDVd, CVd-V and CBCVd. Six months after inoculation, samples of bark, midribs and leaves (devoid of petioles and midribs) were tested separately by Northern blot hybridization with DIG-labelled viroid-specific DNA probes. Bark and midrib samples tested positive (Fig. 2,
lanes 1, 2, 4 and 5), whereas leaf samples tested negative or generated weak hybridization signals (Fig. 2, lanes 3 and 6). Further analysis of equalized preparations (2 x 10^6 protoplasts) of both species failed to detect viroid accumulation in all the viroid/host combinations examined (Fig. 2, lanes 7, 8, 9 and 10), therefore showing that citrus viroids are also phloem restricted in hosts other than C. karna.

These results indicate that viroid movement/accumulation in Etrog citron follows the characteristic pattern established for experimental herbaceous hosts: cell-to-cell movement, entry into the phloem, long-distance movement and trafficking through the bundle sheath into mesophyll (reviewed by Flores et al., 2005; Ding & Wang, 2009).

Indeed, all the viroids inoculated were detected in the bark, which contains the vascular bundles, as well as in mesophyll protoplasts. However, the other three citrus species tested, C. karna, sour orange and trifoliate orange, do not follow this model: the inoculated viroids were easily detected in tissues containing vascular bundles (bark, leaf midveins and even leaf blades devoid of midribs) but, in contrast to Etrog citron, not in mesophyll protoplasts. It should be noted that viroid titres in the leaf blades of C. karna, sour orange and trifoliate orange were rather low, suggesting that viroids were only present in the secondary veins – from where they were unable to exit and invade other cell types, including mesophyll cells – thus explaining why they could not be detected in protoplasts. Phloem-restricted infection is not exceptional in citrus and has been reported for viruses, like citrus tristeza virus (Fagoaga et al., 2011). The mature phloem contains the sieve tube, composed of a vertical column of elongated cells devoid of nuclei that are responsible for the long-distance movement of viruses and viroids, and companion cells with nuclei competent for virus and viroid replication. The high viroid titres found in the bark of C. karna indicate that viroid replication proceeds in an efficient manner, probably only in the phloem, with the companion cells and maybe the surrounding phloem parenchyma cells probably playing a key role.

To investigate the properties of the barrier that probably prevents viroid trafficking from the bundle sheath to adjacent tissues, an approach was undertaken using CEVd-infected C. karna, in which this viroid accumulates at titres as high as in Etrog citron (Fig. 1, lanes 1 and 3). Since Etrog citron is competent for viroid trafficking, we wanted to elucidate whether it could mediate viroid movement across the C. karna phloem/mesophyll barrier. One of the branches of each of two CEVd-infected C. karna seedlings was decapitated and side-grafted with an Etrog citron bud-stick (3 cm long and containing three to four buds) (Fig. 3a, 1) until leaves from the dormant buds of the bud-stick emerged (Fig. 3a, 2). These side-grafted plants, and two additional CEVd-infected Etrog citron and C. karna seedlings used as controls, were maintained for 3 months at 25–28 °C. After this period, the Etrog citron branch of the side-grafted plants expressed the characteristic CEVd symptoms. Northern blot hybridization of equalized preparations (2 x 10^6 protoplasts) isolated from young expanded leaves of the side-grafted plants (C. karna stock and Etrog citron branch), as well as from the C. karna seedling controls, revealed the presence of CEVd in mesophyll protoplasts of the C. karna stock (Fig. 3b, lanes 1 and 2) and the Etrog citron branch (Fig. 3b, lanes 5 and 6), but not in the corresponding C. karna seedling controls (Fig. 3b, lanes 3 and 4). These results showed that, in the experimental design tested, Etrog citron is able to transfer its viroid-trafficking ability to C. karna, and possibly facilitates CEVd replication in its mesophyll cells.
Because PSTVd trafficking between the phloem and non-vascular tissues of *Nicotiana tabacum* and *Nicotiana benthamiana* involves viroid sequences and structural motifs (Qi et al., 2004; Zhong et al., 2008), we examined the possibility that side-grafted Etrog citron could generate a CEVd progeny capable of trafficking from phloem to *C. karna* mesophyll. Nucleic acid preparations from bark and protoplasts of the two Etrog citrons, *C. karna* seedlings and side-grafted plants were subjected to RT-PCR in reaction mixtures (50 μl) containing 1.5 mM MgCl2, 0.12 mM dNTPs, 0.5 μM primers and 1 U *Pfu* DNA polymerase (Stratagene) as described by Bernad & Duran-Vila (2006). DNA products were cloned in pBluescript II KS (+) (Stratagene), digested with *EcoRV* and 10 clones from each plant and source were sequenced with an ABI PRISM 377 apparatus (Perkin Elmer). Sequence analysis of nucleic acid preparations from bark and protoplasts from two Etrog citrons revealed minor changes consistent with viroids being propagated in their hosts as quasispecies (reviewed by Flores et al., 2014). In contrast with the CEVd population from bark, in protoplasts, one substitution (129U→A) in the loop of the PR motif within the V domain, and a second identical substitution (185U→A) in the terminal loop of the TR domain, were found in all or most clones, respectively (Fig. 3c). Interestingly, the change 129U→A has been found previously in protoplasts from *Citrus amblycarpa*, which upon inoculation with a single CEVd sequence variant displayed a high genomic variability (Hajeri et al., 2011). We also observed minor changes in the bark population from the two *C. karna* plants. However, five clones presented the nucleotide substitution 129U→A with one of these also presenting 185U→A, both being characteristic of the CEVd population found in Etrog citron protoplasts.

![Image](https://www.microbiologyresearch.org/)

**Fig. 3.** (a) Budstick of Etrog citron grafted on a decapitated branch of *C. karna* infected with CEVd (1); 3 months later, Etrog citron branch expressed the characteristic CEVd symptoms (2). (b) Northern blot hybridization analysis of equalized preparations (2×10⁶ protoplasts) recovered from CEVd-inoculated plants: *C. karna* stock side-grafted with Etrog citron (1, 2); *C. karna* seedlings (3, 4); Etrog citron branch from the side-grafted *C. karna* (5, 6) and non-inoculated Etrog citron (7). Extract of bark tissue from CEVd-infected Etrog citron included as positive hybridization control (8). (c) Major nucleotide changes (U→A) mapping at the loop of the PR motif (V domain) and the TR domain identified in the CEVd populations infecting: (i) Etrog citron bark (parental isolate); (ii) Etrog citron mesophyll protoplasts; (iii) *C. karna* bark; (iv) bark of side-grafted *C. karna*; (v) mesophyll protoplasts of Etrog citron side-grafted on *C. karna*; (vi) mesophyll protoplasts of *C. karna* side-grafted with Etrog citron. Frequencies were calculated with the results of two independent cloning and sequencing analyses of 10 clones from each of the two plants tested.
We also examined the CEVd populations from bark and protoplasts of C. karna plants side-grafted with Etrg citron. The nucleotide substitution 129U→A was detected in one single clone out of the 20 analysed from the bark. This substitution was consistently found in protoplasts of grafted Etrg citron but not in those of C. karna, wherein a high variability at nucleotide positions 129 and 130 was observed. In all types of tissues, position 185 was highly polymorphic.

Therefore, the changes 129U→A and 185U→A identified in Etrg citron and in Etrg citron side-grafted on C. karna do not seem to be responsible for the trafficking ability, because they have also been found in the phloem-restricted viroid population of C. karna seedlings (Fig. 3c). These results rule out the hypothesis that the side-grafted Etrg citron provides CEVd variants with the ability to invade the C. karna mesophyll, and rather suggest that a translocatable factor from Etrg citron mediates viroid invasion of the leaf blade.

In summary, the present study shows that viroid movement in citrus hosts does not appear to follow the model proposed from studies in herbaceous hosts. The unexpected lack of viroid trafficking into the mesophyll observed in C. karna, sour orange and trifoliate orange reveals that viroids are phloem restricted. Reinforcing the notion that viroid phloem restriction is quite common in citrus, earlier studies indicated that viroids were readily detectable in bark but not in leaf blade tissues of 10 different citrus species (Barbosa et al., 2002). The results presented here reinforce the notion that in addition to viroid structural motifs, like those reported for PSTVd trafficking between the phloem and non-vascular tissues in N. tabacum and N. benthamiana (Qi et al., 2004; Zhong et al., 2008), host factors also play a crucial role in viroid trafficking. Indeed, host proteins mediating mobility of pathogenic RNAs have been reported previously: tomato VirP1 in association with the TR domain of PSTVd in intracellular transport (Maniataki et al., 2003; Kalantidis et al., 2007), cucumber CaPP2 protein (Gómez & Pallás, 2001; Owens et al., 2001) and tobacco Nt-4/1 protein in vascular transport (Solovyev et al., 2013). Presumably, an Etrg citron protein might operate in similar manner.

Acknowledgements

The work was supported by the Ministerio de Ciencia y Tecnología (Spain), grants AGL2005-01469, AGL2008-00596 and AGL2012-32429. S. M. B.-H. and G. P.-B. were recipients of fellowships from the Iran Citrus Research Institute and from the Conselleria de Agricultura – IVIA, respectively. The authors would like to thank Ricardo Flores for critical and constructive reading of the manuscript and Rosario Carbo for technical assistance.

References


