Molecular Plant Pathology

MOLECULAR PLANT PATHOLOGY (2011) **12**(2), 203–208 DOI: 10.1111/J.1364-3703.2010.00662.X

Short communication

Two nucleotide positions in the *Citrus exocortis viroid* RNA associated with symptom expression in Etrog citron but not in experimental herbaceous hosts

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SUMMARY

Citrus exocortis viroid (CEVd) is the causal agent of exocortis disease of citrus. CEVd has a wide host range that includes woody and herbaceous species. A new CEVd strain ($CEVd^{COL}$), phylogenetically clustering with CEVd variants of Class A inducing severe symptoms in tomato, was identified in Colombia and shown to induce only extremely mild symptoms in Etrog citron indicator plants. Using site-directed mutagenesis, two nucleotide substitutions (314A → G and 315U → A) in the lower strand of the P domain of the predicted $CEVd^{COL}$ secondary structure resulted in a severe artificial $CEVd^{MCOL}$ variant. Conversely, two nucleotide exchanges (314G → A and 315A → U) in the same region of the severe variant $CEVd^{E-117}$ resulted in a symptomless artificial $CEVd^{ME-117}$ variant. Infectivity assays conducted with the natural and mutated variants showed that all induced severe symptoms in *Gynura aurantiaca*, tomato and chrysanthemum. This is the first report of the identification of pathogenic determinants of CEVd in citrus, and shows that these pathogenicity determinants are host dependent.

Citrus exocortis viroid (CEVd) is the causal agent of exocortis disease of citrus, a bark scaling disorder that affects, among others, trifoliate orange [*Poncirus trifoliata* (L.) Raf.] and its hybrids (Troyer and Carrizo citranges) and Rangpur lime (*Citrus limonia* Osb.), which are all widely used as rootstocks in commercial orchards. CEVd belongs to the genus *Pospiviroid*, family *Pospiviroidae*, and is a covalently closed, circular RNA with about 370 nucleotides and a highly base-paired, rod-like secondary structure (revised by Duran-Vila and Semancik, 2003). Like the other members of this genus, the predicted secondary structure of CEVd conforms to the model of the five structural domains (Terminal left, T_L; Pathogenicity, P; Central, C; Variable, V; and Terminal right, T_R) defined by Keese and Symons (1985), with a central conserved region (CCR) and a terminal conserved region (TCR) within the C and T_L domains, respectively (Fig. 1) (Flores *et al*., 2005). CEVd has a wide experimental host range, including woody and herbaceous species, with sensitive hosts displaying symptoms of stunting, leaf epinasty and distortion. Etrog citron (*Citrus medica* L.) has been widely used for biological indexing purposes and, following infection, displays a severe syndrome characterized by pronounced stunting, strong leaf epinasty, and midvein, petiole and stem necrosis. Based on the symptoms induced in tomato (*Solanum lycopersicum* L.) as an experimental host, CEVd variants have been classified into severe 'Class A' and mild 'Class B' (Visvader and Symons, 1985). Such variants have been found to differ in a minimum of 26 nucleotides, mainly affecting two regions (P_L and P_R) located, respectively, in the P and V domains of the viroid secondary structure (Fig. 1). Infectivity assays conducted with chimeric cDNA clones have shown that the changes in the P_L region are responsible for symptom modulation (Visvader and Symons, 1986). This result was further confirmed using *Gynura aurantiaca* as an experimental host, in which a set of five nucleotides located in the P domain discriminated between variants inducing severe symptoms and those inducing mild symptoms (Chaffai *et al*., 2007; Skoric *et al*., 2001). The limited information available regarding the modulation of symptom expression of CEVd in citrus indicates that 'Class A' and Class B' variants induce similar symptoms in trifoliate orange (Vernière *et al*., 2004), suggesting that the pathogenicity determinants identified using tomato and *G. aurantiaca* do not necessarily affect symptom expression in citrus hosts.

Recently, an unusual CEVd isolate ($CEVd^{COL}$) has been identified in a symptomless Etrog citron tree from Colombia. Unexpectedly, the consensus sequence of $CEVd^{COL}$ (EU512994), constructed with the sequences of full-length clones showing some variability, was highly similar (96.5%) to the reference sequence (M30868) of 'Class A' variants (Murcia *et al*., 2007). As viroids infect their hosts as populations of closely related variants, the dominant CEVd variant present in $CEVd^{COL}$ was identified as follows. Briefly, nucleic acid preparations from the

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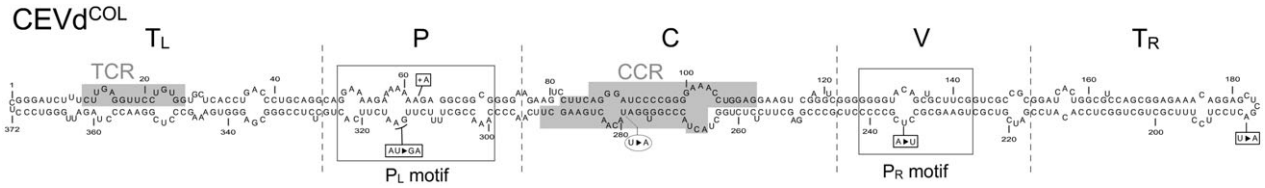

Fig. 1 Proposed secondary structure of minimum free energy of CEVd^{COL}. Discontinuous lines divide the secondary structure into the five structural domains (Terminal left, T_L; Pathogenicity, P; Central, C; Variable, V; and Terminal right, T_R). Conserved regions [central conserved region (CCR) and terminal conserved region (TCR)] are shaded in the T_L and C domains. P_L and P_R are boxed in the P and V domains. Boxed nucleotides (square boxes) show nucleotide changes found in CEVd^{COL} when compared with the severe variant CEVd$^{E\text{-}117}$. Boxed nucleotide (round box) shows the nucleotide change found in the CEVd variants recovered from inoculated tomatoes.

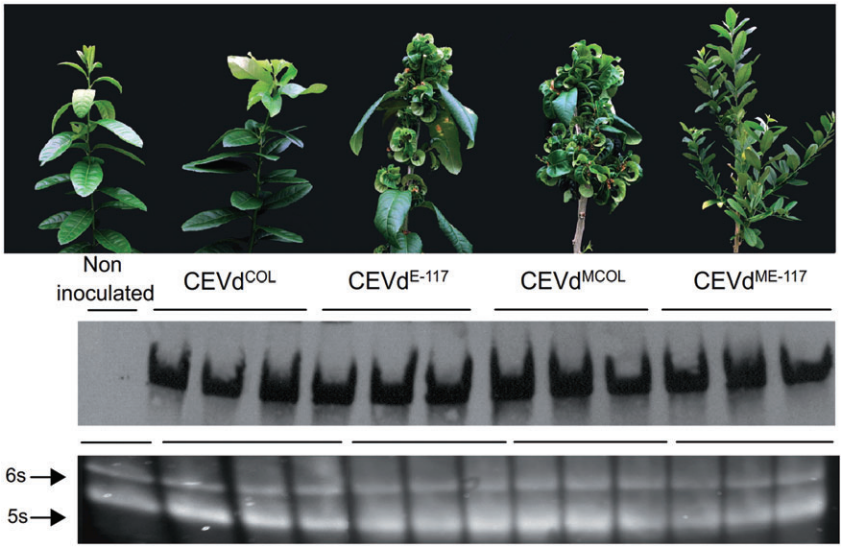

Fig. 2 Top: symptom expression in Etrog citron plants infected with natural variants (CEVd^{COL}, CEVd$^{E\text{-}117}$) and the corresponding mutated variants (CEVd^{MCOL}, CEVd$^{ME\text{-}117}$) obtained by site-directed mutagenesis. Bottom: viroid titres in plants infected with CEVd^{COL}, CEVd$^{E\text{-}117}$, CEVd^{MCOL} and CEVd$^{ME\text{-}117}$, as determined by Northern blot hybridization with CEVd-specific DNA probes. Ethidium bromide staining of a nondenaturing polyacrylamide gel shows that RNA levels (6S and 5S RNAs) in all preparations are comparable.

symptomless Etrog citron infected with CEVd^{COL} were subjected to reverse transcriptase-polymerase chain reaction (RT-PCR) using primers CEVdF1 and CEVdR1, as described by Bernad and Duran-Vila (2006), and the DNA amplicons were cloned in Bluescript II KS+ plasmid (Promega®, Madison, WI, USA). The dominant CEVd variant, identified by sequencing six clones, was found to be identical to the consensus sequence reported by Murcia *et al*. (2007).

To compare the biological properties of this new variant (CEVd^{COL}) with that of CEVd$^{E\text{-}117}$, a variant which induces severe symptoms in Etrog citron as well as in other citrus hosts, and is characterized as 'class A' with a nucleotide identity of 98.2% to the reference sequence (M30868) (nucleotide changes are boxed in Fig. 1) (Duran-Vila and Semancik, 1990; Gandía *et al*., 2005; Vernière *et al*., 2004), an assay was performed to compare symptom expression on Etrog citron. The inocula consisted of *in vitro*-synthesized dimeric transcripts of each variant. Briefly, monomeric DNAs of each clone were recovered as blunt-end PCR products using the phosphorylated primers CEVd-R1 and CEVd-F1 and *Pfu* DNA polymerase. The amplification products were ligated with T4 DNA ligase and the dimeric molecules were cloned into pBluescript II KS (+) digested with *Eco*RV. The recombinant plasmids from transformed cells were sequenced to verify the head-to-tail organization of the dimeric inserts and, according to their orientation, these plasmids were linearized with *Eco*RI and used as a template in transcription reactions with 1 mM deoxynucleoside triphosphates (NTPs), 1 mM dithiothreitol (DTT) and 50 U of RNA polymerase T7 to produce dimeric transcripts. Three Etrog citrons were slash-inoculated using 50 ng of each transcript per plant, and kept for 6 months in the glasshouse at 28–32°C; three additional noninoculated plants were maintained under the same conditions as controls. Infection was confirmed by Northern blot hybridization, as described by Murcia *et al*. (2009b) (data not shown), and the stability of the progeny was assessed by sequencing RT-PCR amplicons from nucleic acid extracts of each plant. In order to monitor symptom expression, all the plants were cut at the level of the second internode and the symptoms in the second flush of growth were evaluated. Three months later, plants infected with CEVd$^{E\text{-}117}$ disclosed the characteristic CEVd syndrome (Fig. 2). In contrast, plants infected by CEVd^{COL} disclosed an almost imperceptible leaf distortion (Fig. 2). Northern blot hybridization analysis (Murcia *et al*.,

2009b) of these plants using a CEVd-specific probe showed that infected plants accumulated similar viroid titres, and that the drastic difference between the severe symptom expression in CEVd$^{E\text{-}117}$-infected plants and the virtually symptomless condition in CEVd^{COL}-infected plants was unrelated to viroid titres (Fig. 2). Therefore, subtle differences in the nucleotide composition of CEVd$^{E\text{-}117}$ and CEVd^{COL} must be responsible for their distinct biological properties.

In order to verify which nucleotide changes were responsible for the differences in biological properties observed, an approach using site-directed mutagenesis was used. As the P_L region located in the P domain has been demonstrated to be responsible for symptom modulation in herbaceous hosts (Visvader and Symons, 1986), the changes identified in positions 314 and 315 of the lower strand of this region were chosen to synthesize two mutants. Mutant CEVd^{MCOL} was designed by introducing, into the sequence of CEVd^{COL}, the substitutions 314G → A and 315A → U, which are characteristic of CEVd$^{E\text{-}117}$. Similarly, the mutant CEVd$^{ME\text{-}117}$ was designed by introducing, into the sequence of CEVd$^{E\text{-}117}$, the substitutions 314A → G and 315U → A, which are characteristic of CEVd^{COL}. These mutants were generated following a PCR-based protocol (Byrappa *et al*., 1995) with minor modifications (Gago *et al*., 2005). Briefly, 5 ng of plasmid pBluescript II KS (+) containing the full-length sequences of either CEVd^{COL} or CEVd$^{E\text{-}117}$ were amplified using each pair of adjacent phosphorylated primers in which the appropriate changes (shown in bold) had been introduced in the forward primers F-MCO (5′**AT**ATCTTCACTGCTCTCCGGGCG3′) and F-ME117 (5′**GA**ATCTTCACTGCTCTCCGGGCG3′). In both instances, the reverse primer was CEVd-R (5′AAGAAAAGCGGTTTGGGGTTGAAGC3′). The PCR cycling profile to amplify the complete plasmid with *Pfu* Turbo DNA polymerase consisted of 30 cycles of 30 s at 94°C, 30 s at 60°C and 3.5 min at 72°C, with an initial denaturation at 94°C for 2 min and a final extension at 72°C for 10 min. After electrophoresis in 1% agarose gels, PCR-amplified products of plasmid length were purified with the QIAquick kit (Qiagen®, Valencia, CA, USA), circularized with T4 ligase and used for transformation. Sequencing confirmed that the plasmids contained inserts with only the desired mutations. Dimeric transcripts of each mutant were generated with the strategy described above, using, as template, the plasmid containing the insert of the mutants (CEVd^{MCOL} and CEVd$^{ME\text{-}117}$). Three Etrog citron plants were slash-inoculated with 50 ng of each transcript per plant and kept for 6 months in the glasshouse at 28–32°C; three plants inoculated with CEVd^{COL}, three plants inoculated with CEVd$^{E\text{-}117}$ and three noninoculated plants were maintained under the same conditions as positive and negative controls. Infection was confirmed by Northern blot hybridization (data not shown), and the stability of the progeny was assessed by sequencing RT-PCR amplicons obtained with nucleic acid preparations from each plant. In order to monitor viroid-induced symptoms, all the plants were cut at the level of the second internode, and the second flush of tissue was evaluated for symptom expression. Three months later, positive control plants displayed the characteristic symptoms of CEVd$^{E\text{-}117}$ and CEVd^{COL}, as described above. Plants infected with CEVd^{MCOL} disclosed the characteristic syndrome induced by CEVd$^{E\text{-}117}$ (Fig. 2), whereas plants infected with CEVd$^{ME\text{-}117}$ were symptomless (Fig. 2) and indistinguishable from the noninoculated controls. Northern blot hybridization showed that the differences observed in symptom expression were unrelated to viroid titres (Fig. 2) and were therefore a result of differences in nucleotides 314 and 315, acting as pathogenicity determinants. However, it should be mentioned that, although plants infected with CEVd$^{ME\text{-}117}$ were indistinguishable from negative controls, plants infected with CEVd^{COL} displayed an extremely subtle leaf distortion. This observation suggests that other positions in the viroid molecule may also play a role in the modulation of symptom expression.

Although the molecular bases involved in symptom expression are still unknown, it is generally accepted that viroid-induced symptoms are caused by specific interference with host gene expression. This hypothesis is supported by the results obtained from macroarray-based and differential display approaches, which showed that the regulation of specific host genes was altered in viroid-infected plants (Itaya *et al*., 2002; Tessitori *et al*., 2007). Studies conducted with different strains of *Potato spindle tuber* (PSTVd), the type member of the genus *Pospiviroid*, allowed the identification of the virulence-modulating (VM) region located in the P domain, in which as few as one or two nucleotides appear to be responsible for symptom modulation (Góra *et al*., 1996; Góra-Sochacka *et al*., 1997; Lakshman and Tavantzis, 1993; Owens *et al*., 1991). This supports the hypothesis that specific viroid sequences/structures (Owens *et al*., 1996) probably interact with yet-to-be-identified host factors. The results reported here on the modulation of symptom expression in CEVd-infected Etrog citron plants show the same trend as those described for PSTVd. It was also observed that CEVd^{COL}-infected plants displayed extremely subtle leaf distortion, suggesting that other positions of the viroid molecule may also play a role in the modulation of symptom expression, as already reported for the T_L, T_R and C domains of PSTVd (Qi and Ding, 2003; Sano *et al*., 1992) and for the T_L domain of *Citrus viroid V* (CVd-V) and *Citrus dwarfing viroid* (CDVd), two members of the genus *Apscaviroid* (Murcia *et al*., 2009a; Serra *et al*., 2009).

As most of the information available regarding the identification of pathogenicity determinants of viroids has been obtained using experimental herbaceous hosts, additional assays were performed to determine the effect of inoculation of CEVd^{COL}, CEVd$^{E\text{-}117}$, CEVd^{MCOL} or CEVd$^{ME\text{-}117}$ into *G. aurantiaca*, chrysanthemum (*Chrysanthemum morifolium*) and tomato plants. Nucleic acid preparations from infected Etrog citron plants were used to inoculate these three herbaceous hosts. The inoculated plants

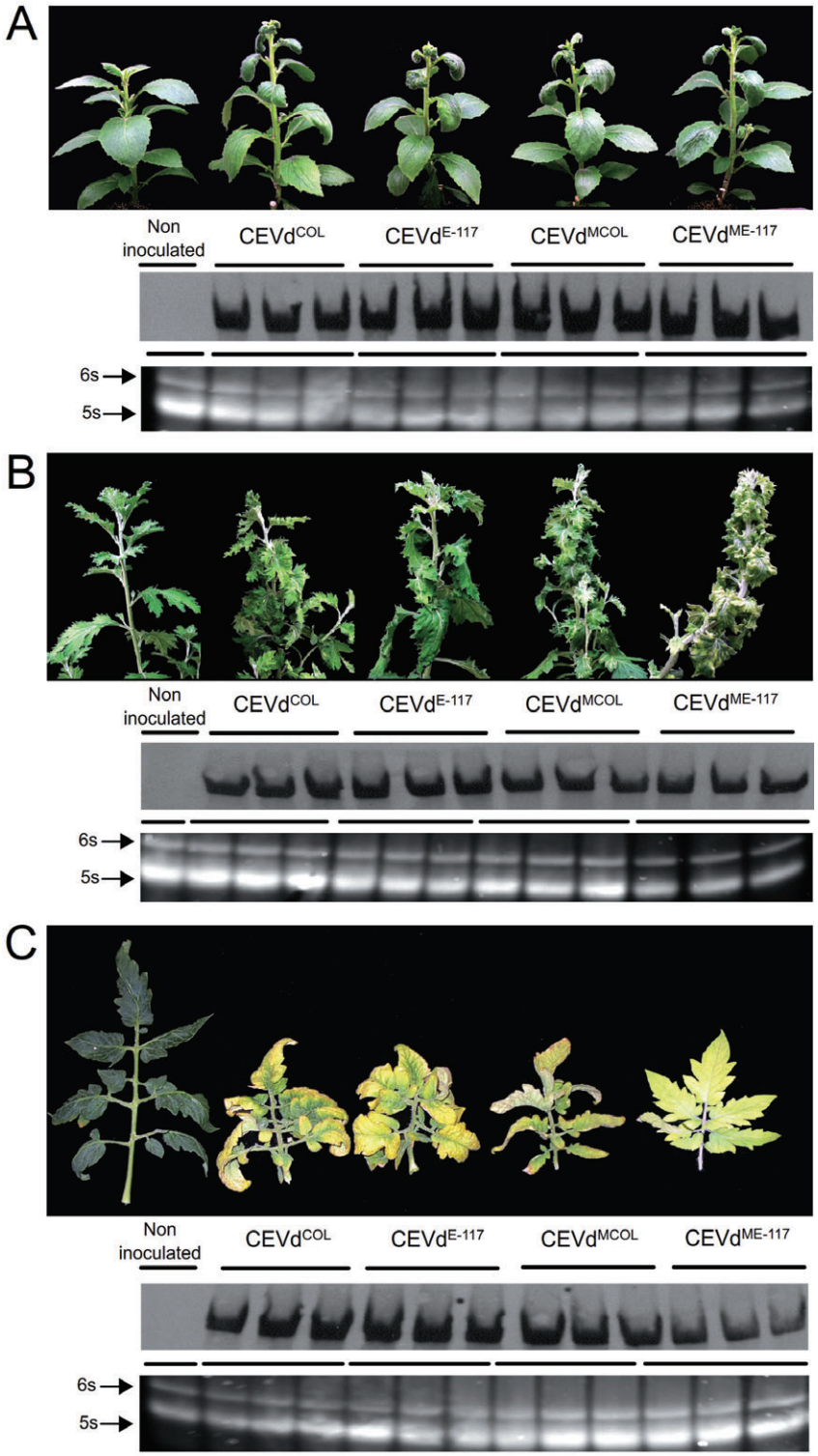

Fig. 3 Top: symptom expression in *Gynura aurantiaca* (A), *Chrysanthemum morifolium* (B) and *Solanum lycopersicum* (C) infected with natural variants ($CEVd^{COL}$, $CEVd^{E-117}$) and the corresponding mutated variants ($CEVd^{MCOL}$, $CEVd^{ME-117}$) obtained by site-directed mutagenesis. Bottom: viroid titres in plants infected with $CEVd^{COL}$, $CEVd^{E-117}$, $CEVd^{MCOL}$ and $CEVd^{ME-117}$, as determined by Northern blot hybridization with a CEVd-specific DNA probe. Ethidium bromide staining of a nondenaturing polyacrylamide gel shows that RNA levels (6S and 5S RNAs) in all preparations are comparable.

(five plants per inoculum source and host species) and five noninoculated controls were maintained for 3 months in the glasshouse at 28–32°C. Three months later, infection was assessed by Northern blot hybridization, which showed that most infected plants accumulated similar viroid titres (Fig. 3). However, unlike that observed with Etrog citron, all CEVd-infected plants disclosed symptoms regardless of the inoculum source (Fig. 3). Within each host, the symptoms induced by natural variants and by artificial mutants were indistinguishable from each other. It should be noted that, although tomato plants infected with $CEVd^{ME\text{-}117}$ presented slightly lower viroid titres, the symptoms were indistinguishable from those induced by the other CEVd variants ($CEVd^{COL}$, $CEVd^{E\text{-}117}$ and $CEVd^{MCOL}$). The stability of the inoculated variants in each host was assessed by RT-PCR and amplicon sequencing, showing that the symptoms observed were not associated with reversion events. It should be noted, however, that all the CEVd variants recovered from tomato contained a 279U → A transition in the lower strand of the C domain (boxed in Fig. 1) that did not affect symptom expression. As already reported by Semancik *et al*. (1993), this change may be the result of differences in host selection pressures.

The overall results reported here, in addition to identifying the first pathogenic determinants of CEVd in citrus, illustrate that the modulation of symptom expression is host dependent. Although the natural variants, $CEVd^{COL}$ and $CEVd^{E\text{-}117}$, and their respective mutants, $CEVd^{MCOL}$ and $CEVd^{ME\text{-}117}$, induced different responses in Etrog citron, their effect on the experimental herbaceous hosts tested was always severe. Recently, a new CEVd variant recovered from citrus (Bernad *et al*., 2005) has been shown to act as a very mild strain in herbaceous hosts, whereas it induced severe symptoms in Etrog citron (L. Bernad *et al*., in preparation). The lack of correlation between symptom expression in Etrog citron and other experimental hosts has also been found in a field assay in which, over a 12-year period, the response of clementine trees on trifoliate orange inoculated with a severe (Class A) CEVd variant was compared with that of trees inoculated with a mild (Class B) CEVd variant (Vernière *et al*., 2004). The two CEVd variants induced comparable reductions in tree size and harvest, and no differences in agronomic parameters were observed.

The mechanisms underlying viroid pathogenesis are still unclear, and different hypotheses have been advanced on how viroid infection elicits the cascade of events leading to symptom expression in sensitive hosts. As viroid RNAs must interact with host proteins to produce a systemic infection, it is plausible that interactions of this kind could be the primary signal of pathogenesis. X-Ray crystal and nuclear magnetic resonance studies have shown that most RNA loops and bulges are highly structured motifs stabilized by non-Watson–Crick base pairing, base stacking and other noncanonical interactions; these motifs most probably serve as the major sites for RNA–RNA, RNA–protein and RNA–small ligand interactions (Leontis *et al*., 2006). Although the nucleotide changes associated with pathogenesis in Etrog citron do not alter the predicted secondary structure of CEVd, they modify the primary structure of a specific loop and may affect its internal structure. A second mechanism in viroid pathogenesis involves RNA silencing and proposes that small viroid-derived RNAs (vd-sRNAs) guide the RNA-induced silencing complex to inactivate certain messenger RNAs of the host (Wang *et al*., 2004). Our results are also consistent with this sequence-specific mechanism, because it is possible that vd-sRNAs from $CEVd^{E\text{-}117}$ and $CEVd^{ME\text{-}117}$ might target different host RNAs.

From a practical point of view, one should be cautious when using biological indexing for viroid detection. As shown in this work, certain viroid variants may infect and replicate latently in Etrog citron, the most widely used indicator species for viroid detection in citrus certification programmes. We recommend the concomitant use of bioassays and additional molecular methods.

ACKNOWLEDGEMENTS

This research was supported by grant AGL2008-01491 from the Ministerio de Educación y Ciencia. P. Serra and L. Bernad are recipients of fellowships from the Consellería de Agricultura—IVIA. The authors would like to acknowledge J.M. Bové for critical reading of the manuscript and R. Carbó for technical assistance.

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