Commentary

Multiplex Detection of Citrus Viroids: Advances and Future Application

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Abstract | Two citrus viroids, *Citrus exocortis viroid* (CEVd) and *Hop stunt viroid* (HSVd), have been reported decades ago without further pathological and genetic researches in Taiwan. Here, bioassay, multiplex RT-PCR and multiplex real-time RT-PCR were used to detect the current status of the two viroids showing HSVd was more prevalent than CEVd and simultaneous infections were common in field. Uneven distributions of both viroids also occurred in different tissues of citrus. Two multiplex molecular detection methods provide the understanding of the genetic diversities among viroid isolates and quantify viroids in citrus host. Our field survey can help clarify citrus-viroid relationships and develop proper prevention strategies in the near future.

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Introduction

Citrus species are natural hosts of at least seven viroids in the family Pospiviroidae: *Citrus exocortis viroid* (CEVd, genus *Pospiviroid*), *Citrus bent leaf viroid* (CBLVd, CVd-I-b, genus *Apscaviroid*), *Hop stunt viroid* (HSVd, CVd-II, genus *Hostuviroid*), *Citrus dwarfing viroid* (CDVd, CVd-III, genus *Apscaviroid*), *Citrus bark cracking viroid* (CBCVd, CVd-IV, genus *Cocadviroid*), *Citrus viroid V* (CVd-V, genus *Apscaviroid*), and *Citrus viroid VI* (CVd-VI, genus *Apscaviroid*; initially named *Citrus viroid original sample*, CVd-OS) (Duran-Vila et al., 1988; Foissac and Duran-Vila, 2000; Ito et al., 2001; Serra et al., 2008). Viroids are small, circular, single-stranded noncoding RNAs that only infect plants. With tiny genome sizes (246–401 nt) and simple structures, viroids are the smallest known agents that infect hosts and cause disease. Because they lack gene-encoded proteins to provide specific functions, viroids depend on host-encoded factors and enzymes for replication (Diener et al., 2001; Flores et al., 2005; Ding et al., 2005). Viroid replication occurs in specific subcellular compartments and trafficking throughout the plant, leading to complete systemic infection (Ding and Itaya, 2007a; Ding and Itaya, 2007b; Ding, 2009).

CEVd induces initial bark shelling and produces subsequent sloughing symptoms on trifoliate orange (*Poncirus trifoliata* [L.] Raf.), Troyer citrange, and Rangpur lime (*Citrus* × *limonia* Osb.), all widely used as rootstocks in commercial orchards (Duran-Vila et al., 1986; Fawcett and Klotz, 1948). HSVd variants with corresponding disease being known as cachexia induces discoloration, gumming, browning of phloem tissue, wood pitting, bark cracking, and stunting symptoms in mandarin (*C. reticulata* Blanco), clementine (*C. clementina* Hort. ex Tan.), satsuma (*C. un-*

shiu [Macf.] Marc.), alemow (*C. macrophylla* Webster), Rangpur lime, kumquat (*Fortunella* spp.), and mandarin hybrids such as tangelo (*C. paradisi* Macf. × *C. tangerina* Hosrt. ex Tan.) (Serra et al., 2008).

Currently, one-step RT-PCR and real-time RT-PCR are used for multiplex detection of citrus viroids infection (Papayiannis, 2014; Bernad and Duran-Vila, 2006; Wang et al., 2009). However, different viroids infections in variance of citrus cultivars and further distribution of viroids in citrus remain to be determined.

Recently, the bioassay method first confirmed on indicator plants such as Etrog citron Arizona 861-S and *Gynura aurantiaca* for CEVd infection and *Cucumis sativus* for HSVd infection showing typical symptoms of epinasty and stunting. However, the disadvantage of time-consuming highlights the development of molecular multiplex method that could simultaneously detect the two viroids was therefore necessary. We simultaneously detected total 689 of CEVd and HSVd isolates by one-step multiplex RT-PCR for the first time from seven citrus cultivars. The results found CEVd and HSVd infection rates were 30.4% and 32.2%, respectively. In addition, different rootstocks appeared to affect infection rates. Finally, viroid disease symptoms were frequently (up to 53.7%) due to co-infection with CEVd and HSVd, with HSVd more common than CEVd. Furthermore, quantification of the data revealed that the two viroids were similar in abundance and that both were unevenly distributed across the different citrus tissues. In all five citrus cultivars tested, root tissues had the highest concentrations of viroids, while leaf tissues displayed no viroid detection signals. We determined that twig bark is the best material for sampling viroids for assays using multiplex real-time RT-PCR (Lin et al., 2015).

In conclusion, two viroids had similarly uneven distributions in different citrus tissues and the underground parts contain higher concentrations of two viroids. The fact that nearly 35% of citrus plants in Taiwan are infected by a combination of CEVd and HSVd suggests that viroid diseases will become an increasingly important and urgent global issue. The results of this study should provide a solid basis for exploration of the two citrus viroids and for understanding the relationship between viroids and citrus plants. Among two molecular detection methods, the biggest challenge is the rapid mutation rates of viroid's sequence which increases the difficulty of designing new detection primers and decreases the specificity of the present primers (Gago et al., 2009). In future applications, both of the multiplex conventional and quantitative RT-PCR should be expanded the detection abilities to all seven citrus viroids. The advanced technology as next generation sequencing (NGS) might be also applied on the citrus diseases caused by viroids. Combining with its mass quantities of sequences data and recent release of citrus genome, the interaction between viroid and host or the influence of multiple viroids infection could be estimated. Further studies must be performed on the dynamical titers of viroids while infecting different cultivars of citrus and should also focus on the ecology and epidemiology of viroids using multiplex real-time RT-PCR method.

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