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Citrus Bent Leaf Viroid

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Abstract – Citrus bent leaf viroid (CBLVd) from genus Apscaviroid, is one of the widely distributed viroids among the seven citrus viroids. It is comprised of three variants: Citrus viroid-Ia (CVd-Ia) (327 - 329 nucleotides), Citrus viroid-Ib (CVd-Ib) (315 - 319 nucleotides) and Citrus viroid-I-low sequence similarity (CVd-I-LSS) (325 - 330 nucleotides). Virulence of CBLVd totally expressed on citrus plants. Etrog citron (*Citrus medica* (L.)) coinfected with CBLVd, Citrus exocortis viroid (CEVd), Citrus viroid III (CVd-III) and Citrus viroid V (CVd-V) showed epinasty, leaf rolling, and stunting. CBLVd has been reported to reduce the canopy proportion and fruit production of citrus trees inserted on trifoliate orange rootstock. Moreover, citrus tree infected with singly CBLVd or in combinations with CEVd, Hop stunt viroid (CVd-II) and CVd III induced dwarfing have been associated with poor development of the root system. Reverse-transcriptase polymerase chain reaction (RT-PCR) amplification and multiplex reverse-transcriptase polymerase chain reaction (multiplex RT-PCR) amplification have been widely used to detect citrus viroids including CBLVd. As citrus viroids are emerging threats in citrus groves, therefore, this review covers the evolution, geographical distribution and epidemiology, economic impact and symptomatology, host range and transmission, detection, and management will be helpful in formulating the integrated management strategies for CBLVd.

Keywords: CBLVd, citrus, multiplex RT-PCR, RT-PCR, viroids

Introduction

Citrus bent leaf viroid (CBLVd) has been reported in most citrus producing countries. It has three variants included Citrus viroid-Ia (CVd-Ia) (327 - 329 nucleotides), Citrus viroid-Ib (CVd-Ib) (315 - 319 nucleotides) and Citrus viroid-I-low sequence similarity (CVd-I-LSS) (325 - 330 nucleotides). Schlemmer, Roistacher and Semancik (1985) published a paper in which they described CVd-Ib (formerly reported as CVaV) was isolation from Eureka lemon (*Citrus limon* (L.) Burm.) grafted on sweet orange (*Citrus sinensis* (L.) Osb.) in California during the mid-1950s. They revealed the band of this isolate migrated more rapid than the respective circular and linear molecules of the Citrus

exocortis viroid in 5% PAGE under denaturing conditions. In addition, Schlemmer et al. (1985) also reported this isolate was able to mechanically transmit on Etrog citron (*Citrus medica* (L.)) and caused uneven development of the leaf curling (reason to be named as Citron variable viroid (CVaV)). CVd-Ia was revealed to have the same electrophoretic mobility with RNA-I during the analysis of exocortis isolates from Spain by Duran-Vila, Flores and Semancik in 1986. Two years later, Duran-Vila, Roistacher, River-Bustamante and Semancik (1988) designated CVaV as CV-Ib. They proved CV-Ia isolate migrated slightly slower than CV-Ib when subjected to sPAGE. Besides, CV-Ia and CV-Ib both had the sequence with high similarities, the similar rates of elution from CF-11 cellulose and the similar host range - Etrog citron (Duran et al., 1988). In 2000, Ito, Ieki, and Ozaki examined that CVd-I-like RNA failed to reactive with CVd-I-specific primer pairs, but detected by sPAGE led them to further purify and back inoculate the RNA into Etrog citron using the stem-slash inoculation method (Garnsey and Muller, 1988). Effect of CBLVd on citrus plants had been studied since 1977 (Nauer, Roisteacher, Calavan & Carson, 1988). The treated Etrog citron exhibited modest leaf bending resulted from a point mid-vein necrosis which was similar to the symptom appeared on Etrog citron infected with CVd-I. However, in comparison, treated Etrog citrons have more acute epinasty. Ito et al (2000) designated this viroid as CVd-I-LSS attributing to its lower sequence similarity among other CVd-I variants.

Taxonomic position and nucleotide sequence

Family: *Pospiviroidae*
Genus: *Apscaviroid*
Species: Citrus bent leaf viroid
Variant: Citrus viroid-Ia, Citrus viroid-Ib, Citrus viroid-I-low sequence similarity

The nucleotide sequence of CVd-Ia is 327 - 329 nucleotides, CVd-Ib is 315 - 319 nucleotides (Figure 1), and CVd-I-LSS is 325 - 330 nucleotides (Figure 2) (Ashulin, Lachman, Hadas, and Bar-Jospeh, 1991; Ben-Shaul et al., 1995; Hataya, Nakahara, Ohara, Ieki, and Kano, 1998; Ito et al., 2000; Semancik, Rakowski, Bash, and Grumpf, 1997). A part of central conserved region (CCR) of Apple scar skin viroid (ASSVd) and the pathogenicity (P) and terminal left (T1) domain of Citrus exocotis viroid (CEVd) make up a chimeric viroid, CVd-Ib (Ashulin et al., 1991). Previous research finding into the P domain of CVd-Ib have a high degree of sequence homology with the P domain of CEVd have been consistent with the outcome from Ben Shaul et al. (1995). Hadas, Bar-Joseph, and Semancik (1989) concluded that P domain in CVd-Ib could play a vital role in pathogenicity on Etrog citron. In addition, Sano, Candresse, Hammond, Diener, and Owens (1992) reported the T1 loop and the T2 loop also make a significant contribution to viroid pathogenicity. Although both CVd-Ia and CVd-Ib are different in size and recognisable nucleotide changes, but they have similar biological properties (Ashulin et al., 1991). Hataya et al. (1998) proposed CVd-Ia could be an imitation of CVd-Ib by incomplete sequence duplications occurred in the T2 domain.

Sequence of CVd-I-LSS was 82-85% similar to that of CVd-Ia and CVd-Ib, which was lower than 90% nucleotide sequence similarity fixed as the definite border separating viroid species from variants (Ito, 2003), placing this viroid as a new variant of CBLVd (Ito et al., 2000). The nucleotide changes between these three CBLVd variants were occurred within the pathogenicity (P), variable (V), terminal right (T2) domains and around the central conserved region (CCR) of the upper and lower strands of the predicted rod-like secondary structure (Wu et al., 2014).

Geographical distribution and epidemiology

CBLVd was initially isolated from Eureka lemon in Ventura County, California, United States of America (USA) (Schlemmer et al., 1985). CBLVd is widely distributed in different citrus producing countries as shown in Table 1.

Viroid/ Isolate	Nucleotides	Country	Reference
CVd-Ib	318 bp	China	Hataya et al. (1998)
CBLVd-Iran	230 bp	Kohgiluyeh-Boyerahmad, Iran	Mazhar, Bagherian, Salahi Ardakani, and Izadpanah (2014)
CVd-I-LSS isolate AK-4	327 bp	Zhejiang, China	Wu et al. (2014)
CBLVd-225A	318 bp	Israel	Ashulin et al. (1991)
CVd-Ia isolate Jp	328 bp	Japan	Hataya et al. (1998)
CVd-I-LSS	327 bp	Japan	Ito et al. (2000)
CBLVd variant E83AK	328 bp	Japan	Ito et al. (2002)
CVd-Ia isolate P2	329 bp	Philippines	Hataya et al. (1998)
CVd-Ib	320 bp	Nicoya, Costa Rica	Villalobos, Rivera, and Hammond (1997)
CBLVd clone A16-1-1	328 bp	Punjab, Pakistan	Cao et al. (2009)
CVd-l-LSS isolate SL-4	329 bp	Punjab, Pakistan	Wu et al. (2014)
CBLVd (E117)	328 bp	Spain	Hashemian et al. (2009)

Table 1: CBLVd isolates reported in different countries

Economic impact and symptomatology

Impact of CBLVd on citrus plants had been studied since 1977 (Nauer et al., 1988). Duran-Vila, Flores, and Semancik (1986) described Etrog citron showed modest epinasty and point necrosis of the mid-vein of Etrog citron when infected with CBLVd. Roistacher, Bash and Semancik (1993) paid an attention on the co-infection of complex viroid on citrus plants and found out synergism among CVd-Ib, CVd II and CVd III significantly responsible for the reduction of the canopy volume and both the trunk section of the trifoliate orange (*Poncirus trifoliate* (L.) Raf.) scion and rootstock. This study came to the agreement with studies by Vernière, Botella, Dubois, Chabier, and Duran-Vila

(2002) and Vernière et al. (2004, 2006). In addition, Ito et al. (2002) revealed the exocortis-like symptoms occurred on Etrog citrons were co-infected with CBLVd, CVd-II, CVd-III, and Citrus viroid IV (CVd-IV). Vernière et al. (2006) reported the synergism of CBLVd, CVd-III, and CVd-IV caused an exocortis-like symptoms. The discovery of CVd-I-LSS led to the experimental investigations of its effect on Etrog citron, highlighting it caused the indistinguishable symptoms on Etrog citron as CBLVd variants did, but with the exception of a more acute epinasty (Ito et al., 2000). Vernière et al. (2004) proposed those citrus viroids from the genus Apscaviroid resulted in stunting and fruit yield reduction without induced any specific diseases. Serra, Barbosa, Daros, Flores, and Duran-Vila (2008) proved the synergism among CBLVd and Citrus viroid V (CVd-V) enhanced leaf symptoms and very noticeable stunting in the Etrog citron.

Host range and transmission

Citrus plants are the known to be the natural hosts of CBLVd. The viroid can be experimentally graft-transmitted to Etrog citron, a biological indicator (Calavan, Frolich, Carpenter, Roistacher, and Christiansen, 1964; Malfitano, Barone, Duran-Vila, and Alioto, 2005; Vernière et al., 2002). Some citrus varieties which are susceptible to CBLVd are shown in Table 2.

Viroid/ variants	Host	Symptom expression	Reference
CBLVd	'Biondo Commune' sweet orange, 'Tarocco' sweet orange, 'Ovale di Sorrento' lemon, 'Zagara' lemon	Asymptomatic	Malfitano et al. (2005)
CBLVd	'Foster pink' grapefruit (*Citrus paradise*)	Kassala disease	Mohamed, Bani Hashemian, Dafalla, Bové, and Duran-Vila, (2009)
CBLVd	'Sinnari' sweet orange	Gummy bark	Mohamed et al., (2009)
CBLVd	Willow leaf mandarin orange (*Citrus. reticulata*)	Cachexia	Mohamed et al. (2009)
CBLVd	'Washington navel' sweet orange	Asymptomatic	Murcia, Serra, Olmos, and Duran-Vila (2009)
CBLVd	'Valencia' sweet orange, 'Rubi' sweet orange, Tahiti lime (*Citrus latifolia*), Mexican lime (*Citrus aurantifolia*)	Asymptomatic	Murcia et al. (2009)
CVd-Ia variant	'Red blood' sweet orange, 'Kinnow' mandarin orange	Asymptomatic	Wu et al. (2014)
CVd-Ib variant	Chinese mandarin hybrids (Nishirkaori)	Asymptomatic	Wu et al. (2014)
CVd-Ib variant	Lemon (*Citrus limon* (L.) Burm. f.)	Asymptomatic	Villalobos, Rivera, and Hammond (1997)

CVd-I-LSS variant	'Shiranui' mandarin orange	Asymptomatic	Ito, Namba, and Ito (2003)
CVd-I-LSS variant	'Succri' sweet orange, 'Red blood' sweet orange, 'Kinnow' mandarin orange, Sweet lime (*Citrus limettioides*), Chinese mandarin hybrids (Akemi), Chinese mandarin hybrids (Nishirkaori)	Asymptomatic	Wu et al. (2014)

Table 2: Reported host range of CBLVd

All citrus viroids included CBLVd are transmitted to citrus plants by sap (Ferguson and Grafton-Cardwell, 2014), budwood and grafting (Ferguson and Grafton-Cardwell, 2014; Targon et al., 2005). Pruning and harvesting operation were also responsible for transmission of CBLVd (Barbaso et al., 2005, Hataya et al., 1998). CBLVd is not seed transmitted in citrus (Hammond and Owens, 2006).

Detection

CBLVd can be detected through biological indexing on Etrog citron (Calavan et al., 1964; Malfitano et al., 2005; Vernière et al., 2002) and Parson's special mandarin (Roistacher, Blue, and Calavan, 1973). It is time consuming (Cohen et al., 2006) as several months are needed to incubate the inoculated indicator species at 28-30^0 C (Bernard and Duran-Vila, 2006). The simplicity and sensitivity of RT-PCR offered an alternative way for routine viroid detection (Cohen et al., 2006). Multiplex reverse transcription polymerase chain reaction (multiplex RT-PCR) was lately developed and employed to detect the presence of viroid species simultaneously (Al-Harthi, Al-Sadi, and Al-Saady, 2013; Al-Shariqi et al., 2013; Ito, Ieki, and Ozaki, 2002). However, the sensitivity and reliability of this method was attributed to the titre and distribution of viroids in the host plants (Palacio, Foissac, and Duran-Vila, 2000). In addition, PCR diagnosis associated with the difficulty to differentiate real infections and false positives (Bernard and Duran-Vila, 2006). Palacio et al. (2000) then suggested the coupling use of the biological indexing with molecular analysis for viroid diagnosis because of their specificity and sensitivity. Moreover, presence of viroid circularity and linear form was revealed by sPAGE (Duran Vila et al., 1988; Malfitano et al., 2005; Pagliano et al., 2000). This method was used together with Northern dot or slot-blot hybridization that performed with digoxigenin (DIG)-labeled viroid specific probes generated by PCR (Mohamed et al., 2009; Murcia et al., 2009) to know the composition of the viroid species present in one tree (Pagliano et al., 2000). Consequently, DIG labeled CVd-multiprobe was developed to detect the presence of any citrus viroids regardless of their species (Cohen et al., 2006). Cohen et al. (2006) explained the budwood source trees that were identified with the infection of any of the citrus viroid practically will be discarded, thus the viroid species was not a matter to be concerned. Molecular hybridization of imprinted membranes was also employed to detect viroids because it required minimal sample manipulation (Targon et al., 2005) and ease the preparation of large quantity of samples in short time (Pagliano et al., 2000).

Management

Bud-wood source trees infected with any of the known citrus viroids should be discarded according to the common practice in the most of the citrus certification programs (Cohen et al., 2006). Other control measures are control the importation of infected citrus varieties (Ferguson and Grafton-Cardwell, 2014), certified bud-wood production and nurseries practices to prevent infections (Targon et al., 2005). Barbosa et al. (2005) suggested the disinfection of tools used in pruning and harvesting operation should be compulsory in certification programs. Harvesting and pruning operations are running during the late autumn and winter in the temperate country as citrus viroids are in low titers in the dormant tree, probably unfavourably mechanically transmitted (Barbosa et al., 2005).

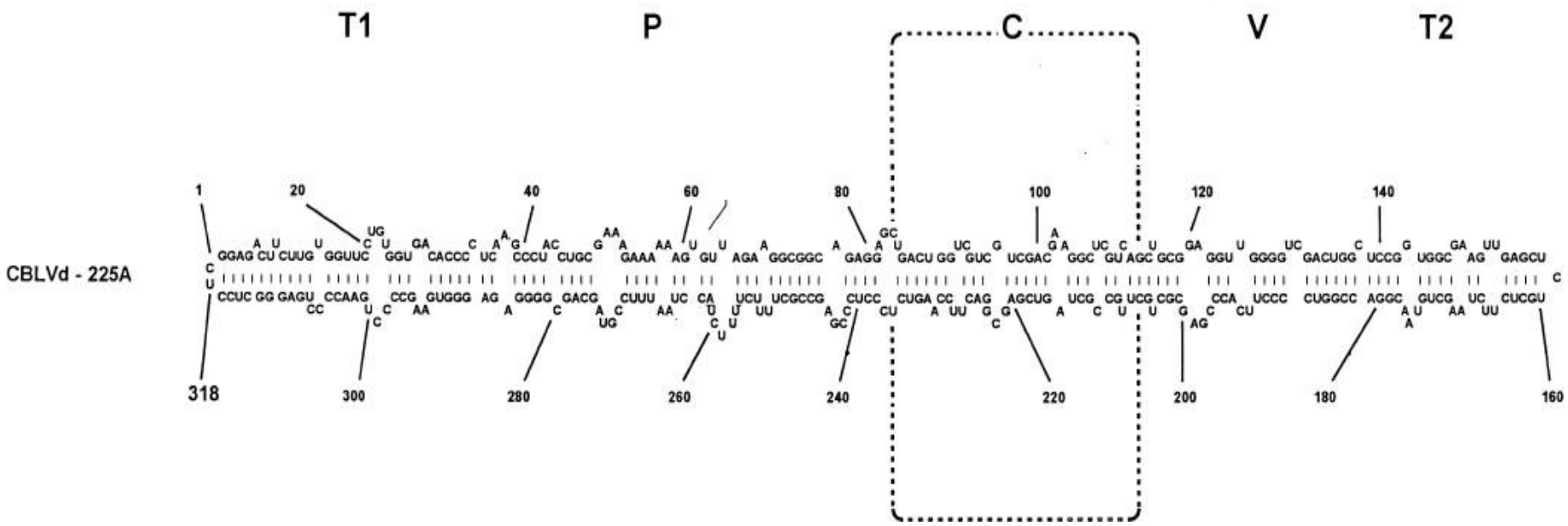

Figure 1: Nucleotide sequence and secondary structure of CVd-Ib variant (CBLVd - 225A isolate) of CBLVd (Ashulin et al., 1991). The five domains, C (central), P (pathogenic), V (variable), T1 (terminal left), and T2 (terminal right) are arranged according to Keese and Symons. (1985).

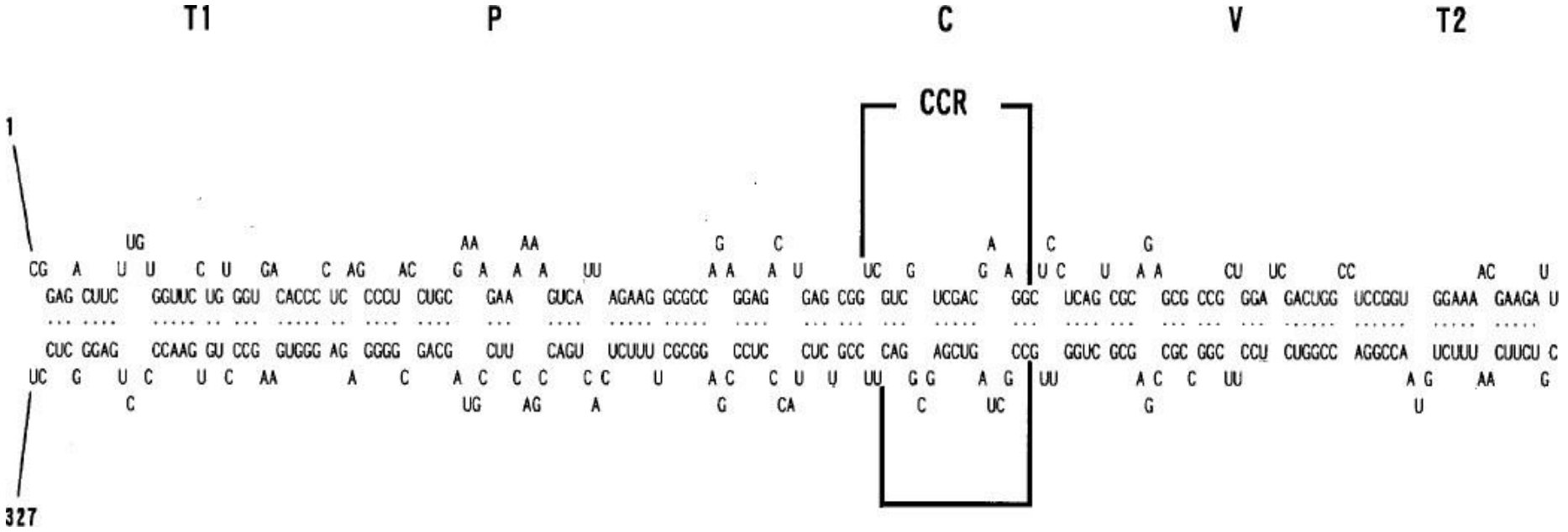

Figure 2: Nucleotide sequence and secondary structure of CVd-I-LSS variant of CBLVd (Ito et al., 2000). The five domains, C (central), P (pathogenic), V (variable), T1 (terminal left), and T2 (terminal right) are arranged according to Hataya et al. (1998).

Conclusion

CBLVd has three variants which are CVd-Ia (327 - 329 nucleotides), CVd-Ib (315 - 319 nucleotides) and CVd-I-LSS (325 - 330 nucleotides). They are distributed within the main citrus producing countries such as China, Costa Rica, Iran, Japan, Pakistan, Spain and USA. Viroid pathogenicity is significantly attributed to the T1 loop and the T2 loop of CBLVd. Moreover, CBLVd fostered leaf epinasty on Etrog citron, and caused stunting and reduced fruit yield on citrus plants. Citrus plants are the only host for CBLVd. In addition, CBLVd is transmitted via sap and mechanical tools in citrus. RT-PCR and multiplex RT-PCR are predominantly employed in detection of CBLVd. Regulatory and cultural practices are imposed to control and eradicate CBLVd.

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