**UNIVERSITI PUTRA MALAYSIA**

***DETECTION, CHARACTERIZATION AND PATHOGENICITY OF CITRUS VIROIDS IN PENINSULAR MALAYSIA***

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# DETECTION, CHARACTERIZATION AND PATHOGENICITY OF CITRUS VIROIDS IN PENINSULAR MALAYSIA

By

**KHOO YING WEI**

**Thesis submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirement for the Degree of Master of Science**

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

**DETECTION, CHARACTERIZATION AND PATHOGENICITY OF CITRUS VIROIDS IN PENINSULAR MALAYSIA**

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**November 2017**

**Chairman: Ganesan Vadamalai, PhD**
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Citrus from *Rutaceae* family is a nutritious fruit that traded globally. Citrus viroids are known to pose a threat to citrus production. These viroids are distributed worldwide, but yet to be reported in Malaysia. Lack of information on citrus viroids in Malaysia is of concern for the local citrus production. In view of this, the objectives of this study were (a) to detect and characterize citrus viroids in Peninsular Malaysia using Multiplex RT-PCR, RT-PCR, cloning and sequencing, (b) to test the pathogenicity and to determine host range of the citrus viroids on seedlings of lemon, chili pepper, cucumber, tobacco and tomato. Leaves from 133 citrus plants showing viroid-like symptom such as stunting, leaf bending, leaf epinasty, leaf necrosis, leaf yellowing and petiole necrosis in Johor (Muar), Malacca (Cheng), Pahang (Jaya Gading, Sri Damai and Semambu), Perak (Bagan Serai and Kamunting) and Selangor (Kuala Selangor, Sabak Bernam, Serdang and Kajang) were collected. Complementary DNA of samples was synthesized using random hexamer by Avian Myeloblastosis Virus Reverse Transcriptase (AMV-RT). Multiplex RT-PCR was performed to detect *Citrus exocortis viroid* (CEVd), *Citrus bent leaf viroid* (CBLVd), *Hop stunt viroid* (HSVd) and *Citrus dwarfing viroid* (CDVd) using specific primer sets of these viroids. Results of molecular detection by Multiplex RT-PCR showed that 23 samples were positive for CBLVd but not CEVd, HSVd and CDVd on *Citrus aurantifolia*, *C. hystrix*, *C. jambhiri*, *C. maxima*, *C. microcarpa* and *C. sinensis* from Johor, Malacca and Selangor respectively. CBLVd was detected in 17.3% out of the total sample. RT-PCR was carried out to amplify the full length of CBLVd using CBLVd specific primer sets. The amplicons were cloned and sequenced. Sequence analysis of 12 clones showed that the CBLVd isolates from this study were 328 nt in size with 99-100% of sequence homology to CBLVd isolate Jp (AB006734). Of total 12 clones, substitution mainly occurred in the Pathogenicity and Variable domains of the secondary structure of seven isolates. Results from phylogenetic analysis of the Malaysian CBLVd isolates with isolates from China, Japan, Pakistan and Spain showed that the Malaysian isolates formed same clade with Japan isolates (AB006734). Pathogenicity study showed that lemon seedlings inoculated with plasmid containing a CBLVd insert (MyMuar01/14) expressed symptoms such as leaf epinasty, leaf bending, leaf yellowing and midvein

necrosis over a 12 month observation period. No stunting expressed in CBLVd inoculated lemon seed-grown seedlings over 12 months. CBLVd were detected in 1, 2 and 3 month inoculated lemon seedlings between 3 to 6 months post-inoculation by RT-PCR assay. Cloning and sequencing of the amplicons showed the presence of CBLVd with more than 99% sequence similarity with CBLVd variant (MyMuar01/14) that was used for the inoculation. Host range study showed that chili pepper, cucumber, tobacco and tomato were not suitable alternate host for CBLVd as there were no symptoms expressed during 3 month post-inoculation observation. RT-PCR assay also failed to detect the CBLVd RNA in the host tested. In summary, CBLVd was detected in citrus grown in Peninsular Malaysia. Twenty-three citrus plants that included *C. microcarpa*, *C. maxima*, *C. aurantifolia*. *C. hystrix* and *C. sinensis* and *C. jambhiri* were positve for CBLVd. In this study, the CBLVd variants were in size of 328 nt and had 99-100% similarity with CBLVd isolate Jp. Substitution of nucleotide of CBLVd variants in this study occurred in mainly P and V domain. CBLVd isolate Malaysia formed same clade with Japan isolates. CBLVd replicated in lemon seedlings with expressed symptoms, but no sign of stunting over 12 months. CBLVd did not replicate in chili pepper, cucumber, tobacco and tomato during 2 month post-inoculation. This is the first report of CBLVd variants in Malaysia.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

**PENGESANAN, PENCIRIAN DAN KEPATOGENAN VIROID LIMAU DI SEMENANJUNG MALAYSIA**

Oleh

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Limau daripada famili *Rutaceae* merupakan buah berkhasiat yang didagang di seluruh dunia. Viroids limau dikenali memberi ancaman kepada penghasilan sitrus. Walaupun viroids tersebar di seluruh dunia, tetapi masih tidak dilaporkan di Malaysia. Kekurangan maklumat tentang viroid sitrus menjadi kebimbangan kepada penghasilan sitrus di Malaysia. Oleh yang demikian, objektif utama kajian ini adalah (a) mengesan dan mencirikan viroid sitrus di Semenanjung Malaysia menggunakan RT-PCR multipleks, RT-PCR, pengklonan dan penjujukan, (b) menguji kepatogenan dan julat perumah sitrus viroid pada lemon, cili, timun, tembakau dan tomato. Sampel daun diperoleh daripada 133 tanaman sitrus yang mempunyai ciri-ciri simptom viroid seperti bantut, bengkok daun, epinasti daun, nekrosis daun, daun kuning dan nekrosis petiol di Johor (Muar), Melaka (Cheng), Pahang (Jaya Gading, Sri Damai dan Semambu), Perak (Bagan Serai dan Kamunting) dan Selangor (Kuala Selangor, Sabak Bernam, Serdang dan Kajang). Komplementari DNA sampel disintesis dengan menggunakan hexamer rawak bersamaan dengan Avian Myeloblastosis Virus Reverse Transcriptase (AMV-RT). RT-PCR multipleks dipraktikkan untuk mengesan *Citrus exocortis viroid* (CEVd), *Citrus bent leaf viroid* (CBLVd), *Hop stunt viroid* (HSVd) dan *Citrus dwarfing viroid* (CDVd) melalui penggunaan set primer spesifik viroid- viroid tersebut. Keputusan pengesanan secara molekul daripada RT-PCR multipleks menunjukkan bahawa 23 sampel adalah positif CBLVd, tetapi negatif CEVd, HSVd dan CDVd pada tanaman limau seperti *C. aurantifolia*, *C. hystrix*, *C. jambhiri*, *C. maxima*, *C. microcarpa* dan *C. sinensis* daripada Johor, Malacca dan Selangor. Peratusan kelaziman CBLVd adalah 17.3% pada keseluruhan sampel. RT-PCR digunakan untuk mengamplifikasikan jujukan sepenuh CBLVd melalui set primer spesifik CBLVd. Amplikon diklon dan dibuat jujukan. Analisis jujukan 12 klon menunjukkan bahawa isolat CBLVd dalam kajian ini bersaiz 328 nt dengan 99-100% persamaan jujukan dengan CBLVd jenis Jp (AB006734). Daripada 12 klon, penggantian hanya wujud pada domain 'Pathogenicity' dan 'Variable' di struktur sekunder tujuh isolat. Hasil kajian daripada pohon filogenik, isolat CBLVd Malaysia dibandingkan dengan isolat dari China, Japan, Pakistan dan Sepanyol menunjukkan bahawa isolat Malaysia membentuk klad bersama dengan isolate Japan (AB006734). Kajian kepatogenan menunjukkan anak pokok diinokulasi

plasmid yang mengandungi selitan CBLVd (MyMuar01/14) mengekspreskan simptom epinasti daun, daun bengkok, daun kuning dan nekrosis midvein selama 12 bulan pemerhatian. Simptom bantut tidak diekpresikan pada anak pokok lemon yang diinokulasi dengan CBLVd selama 12 bulan. CBLVd dikesan dalam anak pokok lemon yang diinokulasi dengan CBLVd yang berumur 1,2 dan 3 bulan di antara 3 hingga 6 bulan per-inokulasi menggunakan RT-PCR. Pengklonan dan penjujukan amplikon menunjukkan kehadiran CBLVd dengan lebih 99% persamaan jujukan dengan varian CBLVd (MyMuar01/14) yang digunakan sebagai inokulum. Kajian julat perumah menunjukkan bahawa cili, timun, tembakau dan tomato adalah tidak sesuai sebagai perumah alternatif CBLVd kerana tiada simptom diekspres selama pemerhatian 3 bulan pre-inokulasi. Pengesahan menggunakan RT-PCR juga gagal mengesan RNA CBLVd dalam kajian perumah. Secara ringkas, CBLVd dikesan di Semenanjung Malaysia dengan saiz 328 nt dan persamaan jujukan 99-100% dengan isolat Jp CBLVd. Nukleotida penggantian varian CBLVd dalam kajian ini berlaku di domain 'Pathogenicity' dan 'Variable'. Isolat CBLVd Malaysia membentuk klad bersama dengan isolate Japan. CBLVd berreplikasi dalam lemon dengan menunjukkan simptom tetapi tiada simptom bantut selama 12 bulan per-inokulasi. CBLVd tidak berreplikasi dalam tanaman cili, timun, tembakau dan tomato dalam 12 bulan per-inokulasi. Laporan ini adalah laporan pertama varian CBLVd di Malaysia.

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I certify that a Thesis Examination Committee has met on 5 November 2017 to conduct the final examination of Khoo Ying Wei on his thesis entitled "Detection, Characterization and Pathogenicity of Citrus Viroids in Peninsular Malaysia" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

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| | |
|---|---|
| ANOVA | Analysis of variance |
| AMV-RT | Avian myeloblastosis virus reverse transcriptase |
| ASSVd | Apple scar skin viroid |
| bp | Base pair |
| BLAST | Basic local alignment search tool |
| cm | centimeter |
| CA | Chloroform: isoamyl alcohol |
| CBCVd | *Citrus bark cracking viroid* |
| CBLVd | *Citrus bent leaf viroid* |
| CBVd-I | *Coleus blumei*-I viroid |
| CCCVd | *Coconut cadang-cadang viroid* |
| CCR | Conserved central region |
| cDNA | Complementary deoxyribonucleic acid |
| CDVd | *Citrus dwarfing viroid* |
| CEVd | *Citrus exocortis viroid* |
| CLVd | *Columnea latent viroid* |
| CMV | *Cucumber mosaic virus* |
| CRD | Completely randomized design |
| CSVd | *Chrysanthemum stunt viroid* |
| CTV | *Citrus tristeza virus* |
| CVd-OS | *Citrus viroid VI* |
| CVd-V | *Citrus viroid V* |
| CVd-VI | *Citrus viroid VI* |
| CVMV | *Chili veinal mottle virus* |
| dATP | Deoxyadenosine triphosphate |
| dCTP | Deoxycytidine triphosphate |
| dGTP | Deoxyguanosine triphosphate |
| dTTP | Deoxythymidine triphosphate |
| DNA | Deoxyribonucleic acid |
| EDTA | Ethylenediaminetetraacetic acid |
| EtBr | Ethidium bromide |
| EtOH | Ethanol |
| g | Gram |
| *g* | Relative centrifugal force |
| GYSVd | *Grapevine yellow speckle viroid* |
| ha | Hectare |
| HSVd | Hop stunt viroid |
| Jp | Japan |
| LiCl | Lithium chloride |
| M | Moles/ liter (Molarity) |
| mg | Milligram |
| $MgCl_2$ | Magnesium chloride |
| min | Minute |

| | |
|---|---|
| mL | Milliliter |
| mM | Millimolar |
| mt | Metric ton |
| Multiplex RT-PCR | Multiplex reverse transcriptase polymerase chain reaction |
| NaCl | Sodium chloride |
| ng | Nanogram |
| P | Pathogenicity |
| pmol | Picomole |
| PCA | Phenol: chloroform: isoamyl alcohol |
| PCFVd | *Pepper chat fruit viroid* |
| PLMVd | *Peach latent mosaic viroid* |
| PLRV | *Potato leafroll luteovirus* |
| PSTVd | *Potato spindle tuber viroid* |
| PVP | Polyvinylpyrrolidone |
| PVY | *Potato virus Y* |
| RNA | Ribonucleic acid |
| RNAsin | RNase inhibitor |
| rpm | Revolutions per minute |
| RT-PCR | Reverse transcriptase polymerase chain reaction |
| RM | Ringgit Malaysia |
| sec | second |
| SDS | Sodium dodecyl sulfate |
| TASVd | *Tomato apical stunt viroid* |
| TBE | Tris-borate EDTA |
| TCDVd | *Tomato chlorotic dwarf viroid* |
| TESLP | Tris-HCl, EDTA, SDS, LiCl, PVP |
| TL | Terminal left |
| TPMVd | *Tomato planta macho viroid* |
| TR | Terminal right |
| Tris-HCl | *Tris*(hydroxymethyl) aminomethane *hydrochloride* |
| TVMV | *Tobacco veinal mosaic virus* |
| µL | Microliter |
| µM | Micromolar |
| UV | Ultraviolet |
| V | Variable |
| V | Voltage |
| X-gal | 5-bromo-4-chloro-3-Indolyl –D-galactopyranoside |

# CHAPTER 1

## INTRODUCTION

Citrus from Rutaceae family is a fruit crop that trades globally in worldwide commercial market with its known nutritive value (Ladaniya, 2010). The citrus industries achieved a worldwide total production of 121,273.2 thousand mt in 2014 (FAOSTAT, 2015). Citrus is also an important economic fruit crop in Malaysia. It is among eight major fruits emphasized under the Ninth Malaysia Development Plan for domestic and export markets (Mohd. Salleh and Mohd. Yusof, 2006). Citrus species such as *Citrus aurantifolia* (key lime), *C. hystrix* (kaffir lime), *C. limon* (lemon), *C. maxima* (pomelo), *C. microcarpa* (calamondin), *C. reticulata* (Mandarin orange) and *C. sinensis* (sweet orange) are planted in Malaysia (Chooi, 1994; Md Othman *et al*., 2016).

Citrus production is affected byvariouspest and diseases including viroid diseases. Citrus viroids cause devastating impact in citrus industries by reducing yield and plant health. To date, citrus naturally played host to viroids, namely *Citrus exocortis viroid* (CEVd) and *Hop stunt viroid* (HSVd) from genus Pospiviroid, *Citrus bark cracking* (CBCVd) from genera Cocaviroid, *Citrus bent leaf viroid* (CBLVd), *Citrus dwarfing viroid* (CDVd), *Citrus viroid V* (CVd-V) and *Citrus viroid VI* (CVd-VI or CVd-OS) from genus Apscaviroid (Ito *et al*., 2002b). Citrus viroids induced symptoms including stunting, leaf yellowing, leaf epinasty, necrosis of midvein, petiole and leaf tip, gumming and browning of phloem tissues, wood pitting and bark cracking on citrus plants (Hutton *et al.,* 2000; Palacio-Bielsa *et al.,* 2004; Malfitano *et al.,* 2005; Ramachandran *et al*., 2005). Moreover, some crops such as apricot, cucumber, grapevine, peach, pear, plum and tomato were also reported to harbor these viroids (Kofalvi *et al*., 1997; Verhoeven *et al*., 2004).

Previous studies to diagnose citrus viroid infection have been practiced by indexing on biological indicator, Arizona 861-S1 Etrog citron (*C. medica* L.) and sequential polyacrylamide gel electrophoresis (sPAGE) (Ito *et al*., 2002b; Wang *et al*., 2009). Lately, molecular tools such as reverse-transcriptase polymerase chain reaction (RT-PCR) and Multiplex RT-PCR (Yang *et al*., 1992; Ito *et al*., 2002b) have been employed in the detection and characterization of citrus viroids. For instance, RT-PCR was adopted in citrus viroid indexing program in Florida and citrus budwood certification programin Texas (Sieburth *et al*., 2002; Kunta *et al*., 2007). Besides, Ito *et al*. (2002b) has employed a Multiplex RT-PCR to detect six citrus viroids and *Apple stem grooving virus* (ASGV).

Citrus industry in Malaysia had been infected by *Citrus tristeza virus* (CTV). Thus, it is possible that both citrus viruses and viroids are present synergistically in the citrus plants as there has been a report of both plant viruses and viroids detected simultaneously in the same plants (Hao *et al*., 2016). Citrus viroids have been reported

worldwide and hosted in citrus and other agricultural crops. However, there was no report about citrus viroids in Malaysia, so the present research was undertaken to study the present of citrus viroids in Malaysia. In view of this, the objectives of this study are:

1. To detect and characterize citrus viroids in Peninsular Malaysia using Multiplex RT-PCR, RT-PCR, cloning and sequencing.

2. To test the pathogenicity and host range of the citrus viroids on seedlings of lemon, chili pepper, cucumber, tobacco and tomato.

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