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Pomelo, a resistance variety to Citrus tristeza virus in peninsular Malaysia

Kavous Ayazpour, Kamaruzaman Sijam, Ganesan Vadamalai, Hawa Jaafar
Plant Protection Department
University Putra Malaysia
Serdang, Malaysia
Email: kayazpour@yahoo.com

***Abstract*- Citrus tristeza virus (CTV) is the most important viral disease of Citrus and is distributed worldwide. Results of ELISA and PCR methods showed that all *Citrus* varieties including *Fortunella* sp., *Citrofortunella microcarpa* and Citromelo in major citrus growing areas of Malaysia were infected with CTV in a high rate. In most areas, pomelo however was free of infection, but in Cameron Highlands, we found some strains of CTV that were severe to Citromelo and pomelo. Phylogeny studies revealed that these strains were familiar to CTV isolates from China and Japan and were very differ from CTV isolates of USA and New Zealand.**

Keywords- Citrus tristeza virus; Phylogeny analysis; Strain; CP gene; Malaysia

I. INTRODUCTION

Citrus tristeza virus (CTV) is a member of the genus *Closterovirus* (family Closteroviridae), which is distributed worldwide and causes one of the most economically important diseases of Citrus [1-6]. CTV particles are flexuous, threadlike, and 2000×10-12 nm in size [7-9]. They contain a positive-sense, single-stranded genomic RNA about 20 kb [8-11] with a molecular weight of 6.3-6.9×10^6 [12]. The RNA contains 12 open reading frames [1, 5, 11, 13] and encodes at least 19 proteins [1, 6, 8, 11]. Two of these are capsid proteins of 25 and 27 kDa, which coat respectively about 95% and 5% of the virus length [4, 7, 9, 14]. Members of Closteroviridae are unusual in their size, genomic composition and have a complex replication strategy [11]. CTV is phloem-limited and is transmitted in a semi-persistent manner by aphids such as *Aphis gossypii* [4, 15-17], *Toxoptera citricida* [4, 14-16, 18], *Aphis spiraecola* [15] and *Toxoptera aurantii* [15, 16]. *Toxoptera citricida* and *Aphis gossypii* are the most efficient vectors of CTV [4, 14-16, 19].

The virus is genetically and biologically diverse. Virus isolate, Citrus cultivar, rootstocks, time of infection, and environmental conditions can affect the symptoms [1, 8]. A complex range of symptoms is produced under field conditions. There are three economically devastating field symptoms caused by CTV. The first is decline and death of trees grafted onto sour orange rootstock. The second is stem pitting of scions, regardless of rootstock [1, 17, 18, 20, 21]. Trees affected with CTV stem pitting strains, have reduced fruit production and quality. A third type of symptoms can cause losses in tree nurseries and is called 'seedling yellows'. Symptoms of seedling yellows are leaf chlorosis and stunting of sour orange, grapefruit and lemon seedlings[9]. There were some reports on occurrences of CTV in Peninsular Malaysia that needs attention, as information on CTV in Malaysia is limited; Hence a research was carried out to determine distribution and host range of CTV in peninsular Malaysia.

II. MATERIALS AND METHODS

A. Plant materials

Field samples were obtained randomly from 340 Citrus trees, including *Citrus aurantifolia*, *C. sinensis*, *C. maxima*, *C. reticulata*, *C. hystrix, Citrofortunella microcarpa, Fortunella* sp., seven *Poncirus* sp. and 10 weeds including *Pasiflora* foetida*, Melothria pendula* and *Mikania micrantha* growing in Selangor, Pahang, Johor, Terengganu, Perak and Kedah states in Malaysia. Mature shoots and leaves of Citrus plants were gathered from all sides of the trees pointing east, west, south and northward and then samples mixed for the test. Petioles, midrib of leaves and bark of shoots were used to prepare extractions for ELISA test and extraction of total RNA for RT-PCR.

B. ELISA determination

To diagnose Citrus trees infected by CTV, direct double antibody sandwich (DAS) ELISA was performed [22]. In this study one polyclonal antiserum (Bioreba) was used. Extractions were prepared from 0.5 g of shoot barks, midribs and petioles in 5 ml 0f 1× PBST buffer (0.15 M NaCl; 0.015 M NaH_2PO_4; 0.05% Tween 20, pH 7.0). Positive reactions were defined as an OD_{405nm} two times higher than negative control.

C. Nucleic acid extraction from Citrus tissues

Total RNA (tRNA) was extracted from 0.2g of shoot barks, midribs and petioles. First tissues were pulverized with liquid nitrogen by pestle and mortar and then collected in 1.5 ml sterile eppendorf tube. Each sample was suspended in 400 µl TES buffer (100 mM Tris-HCl pH 8.0; 2mM EDTA; 2% w/v SDS) and 400 µl phenol/chloroform/isopropanol (25/24/1) and shook vigorously for 10 min. After centrifugation (14000 rpm) for 10 min, the supernatant (400 µl) was treated with 200 µl ethanol (99.8%) and used in RNeasy mini kit and tRNA extracted according to the manufacturer's instructions and was used as template for the amplification of the coat protein (CP) gene of CTV.

For amplification of the complete CP cistron (672bp) of CTV two primers were used based on Jiang *et al.* (2008) report [7]. The sense primer was CP1: 5′-ATG-GAC-GAC-

GAA-ACA-AAG-AA-3′ and the anti sense primer was CP2: 5′-TCA-ACG-TGT-GTT-GAA-TTT-CC-3′.

D. cDNA synthesis and polymerase chain reaction amplification

Using tRNAs extracted from Citrus tissues as templates and CP2 as primer cDNA was synthesized. The total reaction volume was 40 µl, which contained 50 mM Tris-HCl (pH 8.3), 50 mM KCl, 4 mM MgCl2, 10 mM DTT, 0.2 mM each of the four dNTPs, 1 µm CP2, 20 U reverse transcriptase, and 18.75 µl extracted RNA. First tRNA and primer were mixed gently, heated for 10 min at 65ºC, and then immediately cooled on ice. After this, other materials were added and the contents were mixed gently and incubated at 25 °C for 10 min, 42°C for 60 min and 72°C for 10 min., respectively. The PCR amplification was performed in 25 µl of reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.05 mM each of the four dNTPs, 2 mM MgCl2, 0.3 µM of each primer (CP1, CP2), 1.25 U Taq DNA polymerase (iNtRON Biotechnology) and 1-4 µl of RT mixture. The PCR cycling profile consisted of one cycle at 94 °C for 5 min., followed by 35 cycles of 94 °C for 30 sec, 56 °C for 1 min, and 72 °C for 1 min, with a final extension step at 72 °C for 10 min. PCR amplified fragments were separated in 1.2% agarose gels in Tris-borate (TBE) buffer (89 mM Tris; 89 mM boric acid; two mM EDTA, pH 8.3). After electrophoresis, the gels were stained in 0.5 µg/ml ethidium bromide and analyzed using BIO imaging system (Syngene). A 100 bp DNA Ladder (Fermentas) was used as a nucleic acid marker.

III. RESULTS

PCR results showed that these primers (CP1 and CP2) could amplify a part of the genome of all isolates of CTV and produced a band of about 672 bp in agarose gel electrophoresis (Figure **1**). The results of CTV infection survey by using ELISA and PCR methods showed that the virus was present in all samples from different varieties of *Citrus* collected from Peninsular Malaysia (Table 1). In Keluang area, virus infection was observed in three years old *C. hystrix*, *C. aurantifolia* and *C. microcarpa* trees. This suggests that these trees were propagated from CTV infected materials.

This survey showed that in almost areas, all pomelo trees were free of virus infection, except for Cameron Highland areas where CTV was detected in both pomelo and citromelo trees. The sequences of the coat protein gene of these isolates were compared with some other isolates in the world. Phylogeny tree of these isolates in comparison with 10 isolates from other countries showed that AMC2 and AMC7 much close to CT-W1, isolated from *Poncirus* in China and also close to NUagA isolate from Japan and ML12 isolate from China. They are grouped in one cluster. AMC6 and Bangalore isolate from India are placed in another cluster. Resistant break isolates (NZRB-M17, NZRB-G90 and NZRB-TH28) from New Zealand produce a completely separate cluster. These strains had been isolated from *Poncirus*. T36, T30 and T385 are grouped in another cluster and had the most distance from the others (Figure 2).

TABLE 1. TOTAL NUMBER OF SAMPLES, NUMBER OF CTV INFECTED PLANTS AND PERCENTAGE OF CTV INFECTION RESULTED FROM THE CTV INFECTION SURVEY FOR VARIOUS VARIETIES, PERFORMANCE BY ELISA AND PCR TESTS.

Variety	Total samples	CTV infected	%infection
C. sinensis	21	17	80.95
Lemons	7	4	57.14
Citrumelo	3	2	66.67
C. limonia	2	2	100
C. hystrix	22	21	95.45
C. aurantifolia	62	59	95.16
C. reticulata	66	44	66.67
Fortunella sp.	15	15	100
C. microcarpa	50	50	100
Poncirus	7	0	0
Mikania micrantha	3	0	0
Melothria pendula	3	0	0
Pasiflora foetida	4	0	O
C. maxima	92	2	2.02

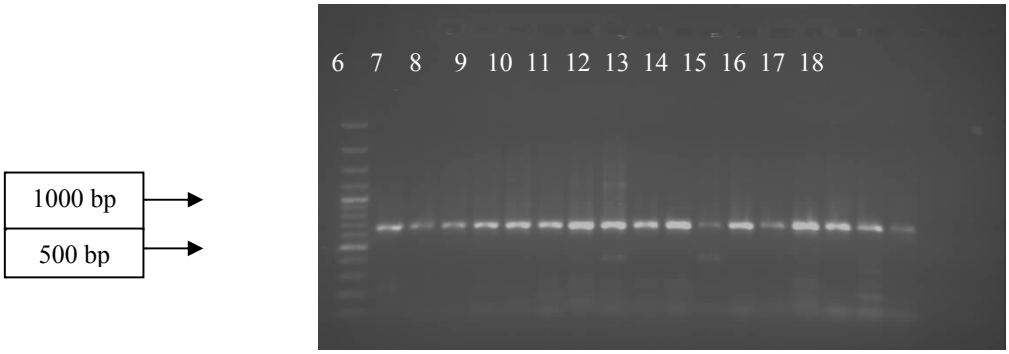

Figure 1. PCR product profile of some Malaysian citrus tristeza virus isolates in 1.2% agarose gel. M, molecular marker; line 1-17, infected plants; line 18, healthy plant.

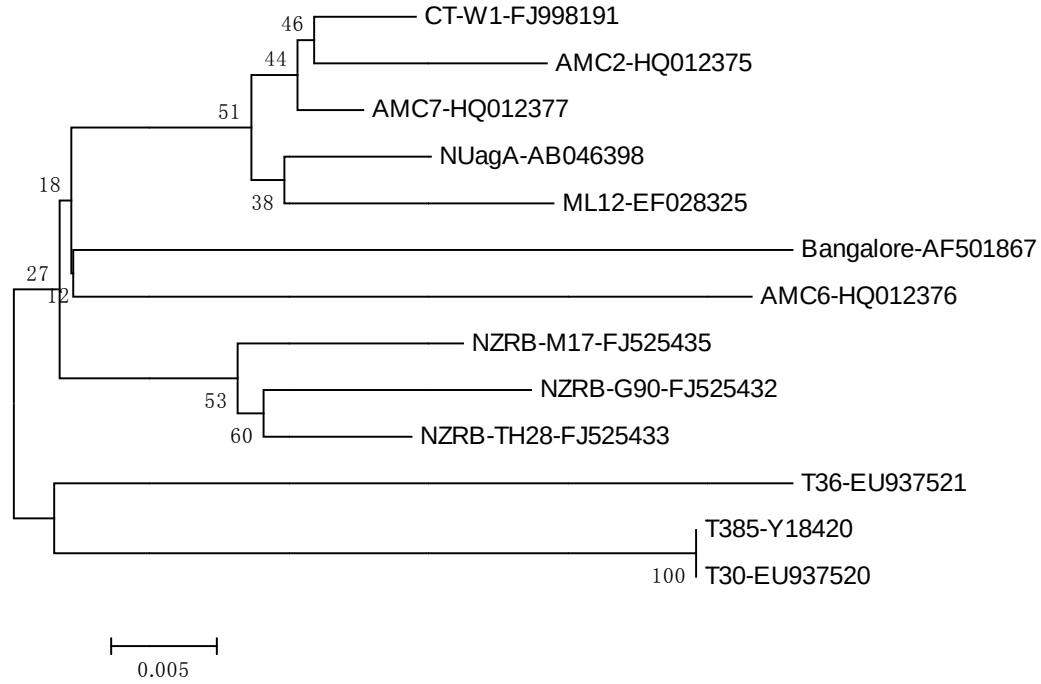

Figure 2. The bootstrap phylogenetic tree of pomelo and *Poncirus* isolates of citrus tristeza virus from Malaysia in comparison to 10 isolates from around the word, were conducted using MEGA software version 4 [23].

IV. Discussion

Although the CTV infection rate is very high in citrus growing areas of Malaysia but, the symptoms are hidden. According to our observations and discussion with *Citrus* growers in Malaysia, the *Citrus* trees are propagated by air layering method. Decline and death of trees could be accomplished when the trees are grafted onto sour orange rootstocks [1, 17, 18, 20, 21], so we cannot observe death or decline of trees in Malaysia, because they are propagated by air layering method. Stem pitting occurs on scions when grafted on rootstocks [1, 17, 18, 20, 21], however we cannot find this symptom in *Citrus* orchards of Malaysia.

Some species, such as Mexican lime (*C. aurantifolia*), are very sensitive to CTV infection and show disease symptoms with most CTV strains, whereas others, such as grapefruit and sweet orange (*C. sinensis*), are affected only by severe strains. Although sour orange is sensitive as a rootstock, seedlings accumulate virus at low titer with most CTV isolates. General resistance to CTV has been observed in trifoliate orange (*Poncirus trifoliata* (L.) Raf.), and resistance to some isolates occurrs in pomelo (*C. grandis)* and kumquat (*Fortunella crassifolia)* [13].

According to these results, it is supposed that in Malaysia pomelo in the most areas is resistant to CTV, but infected pomelos can be found in Cameron Highlands. We suppose that some aggressive strains have been present in Cameron highlands or imported from other countries, since these samples were collected from the citrus collection of Malaysian Agricultural Research and Development Institute (MARDI) of Cameron Highlands, or they were local strains, propagated in and limited to this area. So, it is recommended

to eradicate all citrus trees that are present in MARDI at Cameron highlands.

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