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Preliminary identification of Citrus tristeza virus (CTV) vectors in Peninsular Malaysia

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Citrus tristeza virus (CTV) is a member of genus *Closterovirus* with long flexuous virions, approximately 2000 X 11 nm and monopartite genome and is vectored by aphids. CTV spreads into new regions via movement of infected propagating materials and within a citrus growing area by aphid dispersion. Efficiency of CTV transmissibility is affected by the species of aphids, the source plant at acquisition feeding and the CTV isolate. The BrCA is the most efficient aphid vector of CTV. In areas where BrCA is not present, *Aphis gossypii* is the most efficient vector implicated. In major citrus growing areas of Peninsular Malaysia, all Citrus varieties and its hybrides including *Fortunella* sp., *Citrofortunella microcarpa* and Citromelo were infected with CTV in a high rate. Survey of CTV vector (s) in Peninsular Malaysia revealed that at least there is one efficient vector (*A. gossypii*) of CTV in citrus growing areas.

Key words: Citrus Tristeza virus, Aphids, transmission, Peninsular Malaysia.

INTRODUCTION

Viruses are obligate intracellular parasites which use host cell's metabolic machinery and multiply inside living host cells. Viruses cannot penetrate the intact plant cuticle and the cellulose cell wall, so they must have ways to invade hosts and be transmitted among hosts in order to survive (Hull, 2009). Members of family Closteroviridae have very large positive-sense single stranded RNA (ssRNA) genomes which are transmitted by insect vectors as well as through grafting. Based on vector type and genome partite, this family is divided to three viral genera, namely *Closterovirus*, type species Beet Yellow Virus (BYV), has aphid vectors (Homoptera: Aphididae) and mono-partite genome; *Crinivirus*, type species Lettuce Infectious Yellow Virus (LIYV), is vectored by whiteflies (Homoptera: Aleyrodidae) and *Ampelovirus*, type species Grapevine Leaf Roll-associated Virus 3 (GLRaV-3), is transmitted by mealy bugs (Hemiptera: Pseudococcidae) (Martelli et al., 2000; Mayo, 2002).

Citrus tristeza virus (CTV) is a member of genus *Closterovirus* which has long flexuous virions, approximately 2000 X 11 nm and monopartite genome and is vectored by aphids. CTV is an important phloem limited virus in citrus growing regions and cause damage to citrus production worldwide (Bar-Joseph et al., 1989, 1983, 1981).

According to persistence level of noncircular viruses in vectors, they are subdivided into two groups, namely non-persistent and semi-persistent. Non-persistent viruses are called stylet-borne by some authors as they are carried at the tips of the stylets and semi-persistent virus

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Table 1. Discriminating properties of nonpersistent and semipersistent types of transmission.

Property	Non persistent	Semi persistent
Preacquisition starvation effect	Present	Absent
Acquisition and inoculation thresholds	Seconds	Minutes
Optimal acquisition access period	Short (Seconds)	Long (Hours)
Retention of inoculativity by feeding aphids	Minutes	Hours to days
Acquisition and inoculation tissues	Epidermis	Phloem

Source: Fereres and Collar (2001).

as foregut-borne because they are carried on the cuticular lining of the anterior alimentary canal (Fereres and Collar, 2001). The differences between non-persistent and semi-persistent viruses are described in Table 1.

CTV spreads into new regions (long spread) via movement of infected propagating materials such as seedlings and bud woods. Aphid dispersion of virus is important within a citrus growing area (short spread). The principal vectors of CTV are *Toxoptera citricida* (Kirkaldy), the brown citrus aphid (BrCA), *Aphis gossypii* (Glover), the melon cotton aphid, and *A. spiraecola* (Patch), the spirea or green citrus aphid (Herron et al., 2006; Roistacher and Bar-Joseph, 1987). The other aphid species with limited transmission reported are: *Toxoptera aurantii* (Fonscolombe), black citrus aphid, in Florida (Norman and Grant, 1956), *Myzus persicae* (Sulzer), green peach aphid, *Aphis craccivora* (Koch), groundnut aphid, and *Dactynotus jacae* (Linnaeus) from India (Varma et al., 1965, 1960). Efficiency of CTV transmissibility is affected by the species of aphid, by the source plant at acquisition feeding and the CTV isolate. The BrCA is the most efficient aphid vector of CTV. In areas where BrCA is not present, *A. gossypii* is the most efficient vector implicated (Bar-Joseph and Loebenstein, 1973; Bar-Joseph et al., 1989; Bar-Joseph et al., 1983).

In major citrus growing areas of Peninsular Malaysia all Citrus varieties and its hybrides including *Fortunella* sp., *Citrofortunella microcarpa* and Citromelo in major citrus growing areas of Malaysia were infected with CTV in a high rate (Ayazpour et al., 2011). This experiment was done to detect vector (vectors) of citrus tristeza virus at the preliminary stage in Peninsular Malaysia.

MATERIALS AND METHODS

Aphid sources

During visits to the citrus groves in Peninsular Malaysia, a survey was done to find aphids which had colonized citrus trees, citrus orchard weeds and crop plants near citrus orchards. Five different types of aphids were collected from citrus, rice, eggplant, maize and weeds. Aphids were collected from shoots of colonized plants, placed in cool ice plastic bags and transferred to microbiology laboratory of University Putra Malaysia for further examination.

Feeding Aphids with citrus plants

In the laboratory, the back of the aphids were touched by a gentle brush to excite them to bring out the stylet from plants tissues. Two hundred of these aphids were fed on harvested CTV infested leaves (Figure 1A) or on CTV infested *Citrus aurantifolia* (Figure 1B) for more than 24 h. Then they were divided into two groups. The first group was tested immediately for CTV infection, while the second group after 3 h of fasting and 5 min of feeding on CTV free leaves of citrus plants. In each group, 50 aphids were used for ELISA tests and 50 aphids for RT-PCR test. The 50 groups of aphids were divided into five subgroups (each subgroup=10 aphids) and then used for extraction of RNA for RT- PCR or sap extraction for ELISA test.

Sap extraction for ELISA

Sap extraction was done as described by Fabre et al. (2003). 10 aphids were placed in a 1.5 µl micro centrifuge tube, some liquid nitrogen added and ground with a small glass pestle. Then 150 µl of extracted buffer (PBS, 0.05% (v/v) Tween 20 and 2% (w/v) polyvinylpyrolidone) was added. 100 µl of extracted sap was used for ELISA test.

Extraction of total RNA from Aphids

RNA extraction was done as per the procedure by Fabre et al. (2003) with a little modification. Ten aphids were transferred inside a 1.5 µl micro centrifuge tube and some liquid nitrogen was added. Aphids were crushed properly by a small glass pestle and then mixed with 50 µl of phenol/chloroform/isoamyl alcohol (25:24:1), vortexed gently for 1 min and centrifuged at 14,000 rpm for 20 min at 4°C. Nucleic acids present in the aqueous phase were precipitated in the presence of 10 mg of glycogen by adding 2 volumes of cold absolute ethanol and 0.1 volume of 3 M sodium acetate, pH 5.5. Mixture was kept at -/20°C for 4 h and then centrifuged at 14,000 rpm for 20 min. The supernatant was discarded and nucleic acids were washed with 70% ethanol, dried, suspended again in 20 µl of RNase-free water and stored at -/20°C until used (Fabre et al., 2003). Total RNAs were used for cDNA making and PCR method.

ELISA and RT-PCR

Direct double antibody sandwich (DAS) ELISA was performed using a CTV polyclonal antiserum (Bioreba) to diagnose aphids infected by CTV. Positive reactions were defined as an OD405 nm 2 times higher than the healthy control (Huang et al., 2004).

cDNA was synthesized by using tRNA extracted from aphids as

Figure 1. Feeding aphids with harvested leaves (A) and citrus plant (B).

templates and CP_2 as the primer. The total reaction volume was 40 µl, which contained 50 mM Tris-HCl (pH 8.3), 50 mM KCl, 4 mM $MgCl_2$, 10 mM DTT, 0.2 mM each of the four dNTPs, 1 µm CP2, 20U Reverse Transcriptase, 18.75 µl extracted RNA. First tRNA and primer were mixed gently and heated for 10 min at 65°C and then put on ice immediately. 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 $MgCl_2$, 0.3 µM of each primer (CP1, CP2), 1.25U Taq DNA polymerase (iNtRON Biotechnology) and 1 to 4 µl of cDNA. The PCR cycling profile was one cycle at 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 56°C for 1 min, and 72°C for 1 min, with a final extension step at 72°C for 10 min. Aliquots of PCR amplified fragments were separated in 1.2% agarose gel in Tris-borate (TBE) buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.3). After electrophoresis, the gels were stained in 0.5 µg/ml ethidium bromide and then analyzed using BIO imaging system (Syngene). A 100 bp DNA Ladder (Fermentas) was used as a nucleic acid marker.

RESULTS

Species of Aphids

After preparing of proporsions, aphids were identified using the book of aphids on the world's herbaceous plants and shrubs (Blackman and Eastop, 2006). *A. gossypii* was verified by Associated Professor Hafidzi bin Mohd Noor at the plant protection department of University Putra Malaysia. Only one aphid which colonized citrus was found and it was identified as *Toxoptera aurantii* (Figure 2). There were differences between pterostigma, wing veins and antenna of *T. citricida* and *T. aurantii*. Figure 3 shows wing veins and antenna of *T. aurantii* collected from Cameron Highlands. The other identified aphids were *Aphis craccivora* (Koch) on rice, *A. gossypii* (Glover) on eggplant, *Rhopalosiphum maidis* (Fitch) on maize and *Macrosiphum euphorbiae* Thomas) on weeds.

Detection of CTV Infection in Aphids by ELISA and RT-PCR methods

The ELISA method could not find CTV infection in aphids in all the cases in this study. At the initial stage of monitoring, immediately after feedings aphids with CTV infected tree materials, using CP1 and CP2 primers, all the samples showed infection. However after fasting and short feeding on healthy citrus materials, only *A. gossypii* samples produced PCR products with approximately 670 bp on agarose gel.

DISCUSSION

Results of RT-PCR showed that all aphids were infected by citrus tristeza virus immediately after 24 h of feeding on CTV infected plant materials, but after 3 h of fasting and a short time feeding on healthy plants, only *A. gossypii* remained infected. These results are in line with the definition of semi persistent transmission which explains that retention time for non-persistent transmission

Figure 2. *Toxoptera aurantii* on a citrus tree in Cameron highlands.

is very short and infection requires only brief and shorter than one minute stylet penetration because virus is stylet borne. Meanwhile infection and retention time for semi-persistent transmission is longer and sometimes aphid remains contaminated for more than two days after infection (Katis et al., 2007).

There were differences between identification of aphids CTV infection by ELISA and RT-PCR methods. RT-PCR could show the infection whereas ELISA could not. It means that RT-PCR detects tristeza virus scrupulously and is stricter than ELISA.

Based on the results of this experiment, we could consider *A. gossypii* as the vector of CTV in Peninsular Malaysia. As Table 1 shows CTV infection rate is very

Figure 3. Wing veins (above) and antenna (below) of *T. aurantii.*

high in citrus orchards in Peninsular Malaysia. As infected propagated materials are very important in Peninsular Malaysia, perhaps the vectors do not play a principal role in the transmission and distribution of CTV in citrus groves in Malaysia.

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