Cochin China atalantia (*Atalantia citroides*) as a new alternative host of the bacterium causing citrus Huanglongbing

Y. C. Feng · C. H. Tsai · S. Vung · T. H. Hung · H. J. Su

Abstract Through graft inoculation and psyllid transmission experiments, the Cochin China atalantia (*Atalantia citroides*) was found to be a new alternative host of the fastidious bacterium (*Candidatus* Liberibacter asiaticus=Las) causing citrus Huanglongbing (HLB). The Las pathogen is commonly transmitted by Asian citrus psyllid (*Diaphorina citri*) in persistent manner. The Cochin China atalantia plants locally called wild lime (WL) including elliptic leaf (WL-1) and elongated leaf (WL-2) types are growing wildly in Cambodia and Cochin China of Southern Vietnam. The Las pathogen infected Cochin China atalantia plants by graft inoculation and psyllid transmission, and survived persistently. The Las was transmitted from Las-infected Cochin China atalantia plants of two types to healthy citrus plants (Ponkan mandarin) and atalantia plants through psyllid transmission. Therefore, the Cochin China atalantia plants of two types may serve as a new alternative host of Las. Higher titer of Las was detected in the Las-infected WL-1 atalantia plant which developed typical yellow mottling symptom, while lower titer of Las was detected in WL-2 atalantia plant developing mild chlorosis symptom.

Keywords Cochin China atalantia · *Atalantia citroides* · Alternative host · Huanglongbing · *Candidatus* Liberibacter asiaticus · *Diaphorina citri*

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Introduction

Citrus Huanglongbing (HLB, greening) is one of the most destructive diseases of citrus crop worldwide (Bové 2006). The disease infected all commercial citrus species and caused great damage to citrus industry by shortening tree lifespan and poor fruit yield and quality (Su 2008). The causal agent, *Candidatus* Liberibacter asiaticus (Las) belonging to Asian form, is a Gram-negative bacterium, phloem limited, non-culturable and has submicroscopic, pleomorphic and walled bodies inhabiting in the plant sieve tubes (Huang 1987). HLB disease has been spread commonly through infected budwood or seedlings via vegetative propagation, and transmitted by Asian citrus psyllid (*Diaphorina citri*) in the Asia (Capoor et al. 1967) and America (Halbert and Manjunath 2004), and African citrus psyllid (*Trioza erytreae*) in Africa (McClean and Oberholzer 1965) in persistent manner without transovarial passage (Su 2008).

The epidemiology of HLB is quite important for formulating disease management. The host plants of the Las bacterium and vector psyllid serving as reservoirs of Las, usually play an important role in the disease epidemiology and management. Las can grow well and survive in the citrus plants of commercial cultivars which serve as inoculum donor plants for psyllid transmission. Hung et al. (2000 and 2001) reported the two alternative hosts of Las bacterium which were the suitable hosts for citrus psyllids. The results demonstrated that Las could survive in Chinese box orange (CBO, *Severinia buxifolia*) persistently and in wood apple (WA, *Feronia limonia*) temporarily. However, Las was not detected in jasmine orange (JO, *Murraya paniculata*) and curry leaf (CL, *Murraya koenigii*) which were the good hosts of citrus psyllid. The above mentioned four hosts of psyllid were defined that CBO belonged to "persistent host", WA belonged to "transient host", and JO and CL belonged to "non-host" of Las. However, Damsteegt et al. (2010) reported *M. paniculata* and related species as potential hosts and reservoirs of Las causing HLB disease. Their research demonstrated that 34/36 plants of *M. paniculata* and 22/23 plants of *M. exotica* were infected by Las via psyllid transmission at a high rate of

Las-infection of the *Murraya* plants. Lopes et al. (2010) reported that *Ca*. L. americanus (Lam) was detected in 61/786 (7.8 %) plants, and *Ca*. L. asiaticus (Las) in 30/786 (3.8 %) plants of *M. exotica* in two surveys in Brazil. However, Walter et al. (2012) reported that low infection rates of Las detection were found in less than 1% of psyllids and 1.8 % of *M. paniculata*. They suggested that *M. paniculata* might serve as a minor source of Las inoculum for psyllid transmission.

Two types of Cochin China atalantia (*Atalantia citroides*) (Reuther et al. 1967) locally called wild lime (WL) including elliptic leaf (WL-1) and elongated leaf (WL-2) types (Fig. 5c, d) were found possibly to be an alternative host of Las in Cambodia by Su in 2005 (Su, unpublished data). Wild lime plants wildly grown in Cambodia were called "Krouch prey" in Khmar. The plants which belong to shrubs (WL-1) or trees (WL-2) are widely distributed in tropical Indo-China including Southern Vietnam (Cochin China) and Cambodia, and were introduced to Honduras, USA-Miami and Taiwan. The Chinese box orange (*S. buxifolia*), an alternative host of Las in Taiwan belongs to primitive citrus group of Subtribe Citrinae in Tribe Citreae. Bayer et al. (2009) reported the phylogenetic relationships among the members of the citrus subfamily Aurantioideae of Family Rutaceae, and found good graft-compatibility of citrus plants with *Severinia* and *Atalantia* plants. The consensus graft compatibility indicated that the phylogenetic relationship of *Severinia buxifolia* vs. *Atalantia citroides* was very close. The present investigation aimed at clarifying whether Cochin China atalantia close to CBO in phylogeny, is a new alternative host of Las and vector psyllid. The Cochin China atalantia seedlings were grown from seeds introduced from Cambodia through international collaboration. This study on the two types of Cochin China atalantia was made by graft and psyllid vector transmission of Las between citrus and Cochin China atalantia in alternative ways, i. e. Las transmission from citrus to Cochin China atalantia or Cochin China atalantia to citrus by graft- and psylla- inoculation.

Materials and methods

Plant preparation and graft-compatibility evaluation

The test plants including Chinese box orange (CBO, *S. buxifolia*), elliptic leaf (WL-1) and elongated leaf (WL-2) types of Cochin China atalantia (WL, *A. citroides*) and Ponkan mandarin (PM, *Citrus reticulata*) were used in experiments of graft- and psylla-inoculation. The test seedlings were grown mainly from seeds. The seeds of Cochin China atalantia were introduced from Royal University of Agriculture, Cambodia.

The compatibility among citrus plants and WL plants were evaluated by grafting WL plants with citrus scions. On the other hand, scions of two types of WL and CBO were grafted onto citrus rootstocks including carrizo citrange (*Poncirus trifoliata* x *C. sinensis*) and cleopatra mandarin (*C. reshni*), for the evaluation of graft compatibility. The grafted plants of WL on citrus rootstock or citrus on Cochin China atalantia rootstock were subjected to inoculation tests with Las pathogen in scions or vector psyllids.

Las transmission by graft-inoculation

When shoots of Cochin China atalantia (WL) grew up 20 cm, the Las-infected citrus scions (Las severe strain II) as inoculum were graft-inoculated onto WL plants of the two types, and PM and CBO served as control plants. The multiplication of Las in test plants was monitored by Q-PCR assay monthly after inoculation. The symptom expression of inoculated plans was also observed. The monitoring and observation started 2 months after inoculation and then periodically within one year.

For fulfilling Koch's postulates, back graft-inoculation with Las-infected WL scions were made onto citrus (PM) seedlings. Las infection in test plants was monitored by Q-PCR assay, and symptom expression on test plants was observed periodically.

Las transmission by vector psyllid

Duplicate healthy WL plants of two types were grown in insect cage for psyllid transmission. The Las-harboring psyllids (Asian citrus psyllid, *Diaphorina citri*) were collected from HLB-diseased Liucheng sweet orange trees in orchard. Over 100 of viruliferous psyllids were released onto the test plants for 30 days of inoculation feeding period in insect cage. The viruliferous percentage of 10 psyllids collected from insect cage, were examined by PCR assay. Efficiency of the vector transmission was affected by viruliferous percentage of psyllids. About 100 Las-harboring psyllids were released onto the healthy WL plants in the cages twice. All the WL plants were assayed by Q-PCR assay for monitoring Las infection periodically during 2 to 12 months after feeding inoculation.

Non-viruliferous psyllids obtained from propagation of citrus psyllids on health jasmine orange (*M. paniculata*) seedlings, were used for vector transmission of Las from Las-infected WL plant into healthy citrus plants (PM). The WL plants of two types graft-inoculated with severe Las-strain II were used as donor plants. The acquisition access periods of non-viruliferous psyllids were 2–4 weeks feeding on Las-infected WL plants of two types. The inoculation feeding period of viruliferous psyllids on receptor PM plants were more than 30 days. Las detections in all receptor PM plants by PCR assay were made periodically during 3 to 9 months after feeding inoculation.

Las detection by PCR and quantitative real-time PCR (Q-PCR) assays

The methods of Las detection in host plant and vector psyllid by PCR assay were described by Hung et al. (1999 and 2001).

DNA extraction from plant tissue: Leaf midrib (0.5 g) was powdered in liquid nitrogen, suspended with 2.7 ml DNA extraction buffer [100 mM Tris-HCH (pH 8.0), 100 mM EDTA, 250 mM NaCl] and mixed well with 0.3 ml 10% Sarkosyl (N-Lauroylsarcosine). Then, the sap was transferred to 1.5 ml eppendorf tube. After incubation at 55°C for 1 h, the sample was centrifuged at 6000 rpm for 5 min. The 800 μl supernatant was transferred into new eppendorf tube, before adding 100 μl 5 M NaCl and 100 μl 10 % CTAB (hexadecyl-trimethyl-ammonium-bromide) in 0.7 M NaCl. The mixture was incubated at 65°C for 10 min. Equivalent volume of chloroform/isoamyl alcohol (24: 1) was added into each sample. After vortexing well, the mixture was centrifuged at 11,000 rpm for 10 min. The aqueous supernatant was re-extracted by equivalent volume of phenol/chloroform/isoamyl alcohol (25: 24: 1). The nucleic acids were precipitated by mixing 600 μl of supernatants with 360 μl of isopropanol. After gently mixing, the mixture was centrifuged at 11,000 rpm for 10 min at 4°C. The pellet was washed with 70% ethanol, dried, and suspended in 100 μl of TE buffer which served as template for PCR amplification. DNA extraction from psyllid: Place one psyllid in an eppendorf tube containing 70 μl DNA extraction buffer (as mentioned above). One psyllid in eppendorf tube was homogenized with a plastic pestle on ice. After adding 200 μl DNA extraction buffer and 30 μl 10 % Sarkosyl (final 1 %), the mixture in tube was incubated at 55°C for 1 h. After adding 200 μl phenol/chloroform/isoamyl alcohol (25:24:1) in an eppendorf tube of each sample, the mixture in tube was mixed violently and centrifuged at 12,000 rpm for 10 min. The upper phase of 200 μl was transferred into a new tube, and 500 μl of 95~100 % EtOH was added. After gently mixing, the mixture was centrifuged at 12,000 rpm for 15 min at 4°C. The DNA pellet was dissolved in 20–50 μl of TE buffer after vacuum-drying.

PCR assay

The 2 μl of DNA extract as the template was added into 20 μl of PCR reaction mixture containing 10X PCR buffer、25 mM MgCl$_2$、10 mM dNTPs、10 μM HLB primer pair and 1 units of Taq DNA polymerase (Super Therm DNA Polymerase, BERTEC). The amplification of PCR was performed with specific HLB primer pair (HLB-226 F: 5′-CACCGAAGAT ATGGACAACA-3′; HLB-226 R: 5′-GAGGTTCTT GTGG TTTTTCTG-3′). The PCR thermal cycle consisted of initial denaturing of 94°C for 3 min; 30 cycles of denaturing at 94°C for 1 min, annealing at 60°C for 1 min and extension at 72°C for 1 min; the final extension at 72°C for 10 min. PCR reactions were carried out in PCR Thermal Cycler (GeneAmp 2720, Applied Biosystem Corp., Norwalk, CT, USA). Amplified PCR products were subjected to electrophoresis analysis in 1.5% agarose gel, for obtaining specific DNA amplicon size of 226 bp. Gel was stained with ethidium bromide, and visualized under an UV densitometer supplied by AlphaEase®FC Image Analysis Software.

Q-PCR assay

Specific primer pair and TaqMan probe oligonucleotides of Las detection for Q-PCR were designed based on the 'Ca. L. asiaticus' trmU-tufB-secE-nusG-rplKAJL-rpoB gene cluster region of HLB-Las infecting Ponkan mandarin (TW2 isolate) in Custom TaqMan® Gene Expression Assays by Applied Biosystems International, USA. The HLB Q-PCR primer pair (primer-F: 5′-AGGTTGGCTGTGTTAAATTTTTTTAAGC AA-3′ and primer-R: 5′-ACAATAACCGAAACCAAAAC CTCACT-3′) was designed base on the secE gene region. The HLB-TaqMan probe, 5′-ACGGCCAGAATATCTT-3′, was labelled at 5′-end terminal nucleotide with 6-carboxy-fluorescein (FAM) reporter dye and labelled at 3′ -end terminal nucleotide with non-fluorescent quencher (NFQ) plus minor groove binder (MGB). The HLB-Las plasmid constructed with partial sequence of secE gene was used for preparing a standard curve of Q-PCR by series dilution. The PCR product amplified with HLB Q-PCR primers was clarified by using a High Pure PCR Product purification kit (Roche Applied Science, Mannheim, Germany). Cloning was performed by ligation into the pCR®2.1 vector and transformed into the ECOS™ 9–5 Escherichia coli competent cells by heat shock according to the manual of manufacturer (Invitrogen, Carlsbad, CA, USA). DNA sequencing was operated in a DNA sequencer (ABI PRISM 3730).

The TaqMan Q-PCR amplifications were performed by using StepOne Real-Time PCR System (ABI) in 20 μl reaction-volume consisting reagents: 2X TaqMan® Universal Master mix II with UNG (Applied Biosystems International, USA), 250 nM HLB-TaqMan® MGB probe, 900 nM HLB Q-PCR forward and reverse primer pair and 200 ng DNA template. The amplification cycles were 50°Cfor 2 min, 95°Cfor 10 min, 40 cycles of 95°Cfor 15 s, 60°Cfor 1 min. Tenfold dilutions of the HLB-Las plasmid DNA (inserted partial sequences of secE gene) were used as standard samples, with one healthy sample and ddH$_2$O as negative controls in each run for quantitative analysis of HLB-Las. The average cycle threshold (Ct) value was determined with triplicate for each sample by Q-PCR for Las detection. Data were analyzed using StepOne Software v2.0. Results of Q-PCR with 'Ca. L. asiaticus' levels at Ct value >36.5 were considered "negative". Samples with amplification results indicated as "Und" mean undetermined.

Sensitivity test of PCR assay with HLB specific 226-primer pair was compared with Q-PCR assay targeting the secE gene of HLB-Las. Tenfold serial dilution obtaining 200 ng (10°) to 200 fg (10^{-6}) of total DNA extracts from HLB-Las infected Ponkan mandarin was used for evaluating the sensitivity of PCR and Q-PCR assays.

Results

Specificity and sensitivity of Q-PCR

Development of standard curve of Q-PCR

The HLB-Las plasmid constructed with partial sequence of *sec*E gene was used for developing a standard curve of Q-PCR. HLB-Las primers and probe were used for targeting the *sec*E region of Las. The logarithmic standard curve developed by using eight dilutions (10^8-10^1) of plasmid DNA, revealed cycle threshold (Ct values) ranging from 12.9 to 35.6, showing the high accuracy ($R^2=0.999$) over a wide range of concentrations in Las detection by Q-PCR assay (Fig. 1a).

Specificity test of Q-PCR assay for Las detection

For the specificity test of primers and TaqMan probe in Q-PCR assay, the total DNA extracts from Las-infected Ponkan mandarine (PM), Liucheng sweet orange (LSO), Wentan pummelo (WP), Eureka lemon (EL) revealed the positive Las-detection, while those of the healthy sample and ddH$_2$O as control showed negative detection. In Q-PCR assay, the four Las-infected citrus samples with Ct values ranging from 19.3 to 28.8, while the healthy sample showed over level of Ct value 36.5, and Ct value of ddH$_2$O was undetermined ($R^2=0.999$). The level of Ct value more than 36.5 in Q-PCR assay was considered "negative" detection of Las (Fig. 1b).
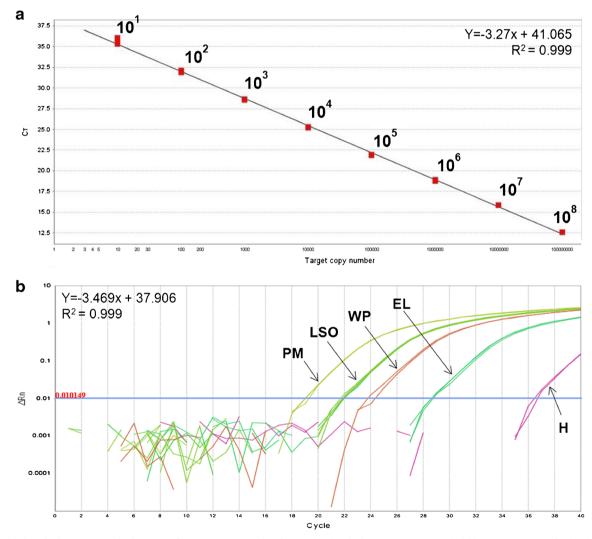

Fig. 1 (a) Standard curves to calculate *sec*E fragment copy number in tenfold serial dilutions (10^8–10^1) by Q-PCR assay with TaqMan symstem. (b) The specificity test of TaqMan primer-probe by Q-PCR assay using total DNA extracts from Las infected Ponkan mandarine (PM), Liucheng sweet orange (LSO), Wentan pummelo (WP), Eureka lemon (EL), served as the positive templates, and the healthy sample and ddH$_2$O served as negative controls

Sensitivity test of Q-PCR assay for Las detection

Sensitivity of conventional PCR assay with HLB specific 226-primer pair was compared with that Q-PCR assay targeting the *sec*E gene region for Las detection. DNA templates were diluted in tenfold serial dilutions obtaining 200 ng ($10°$) to 200 fg (10^{-6}) from Las-infected Ponkan mandarin. The Q-PCR assay could detected Las-DNA in lowest concentration of template DNA at 10^{-5} dilution (2 pg) showing the linear relationship (R^2=0.999) (Fig. 2a), while PCR assay showed detectable lowest concentration of template DNA at 10^{-4} dilution of Las-DNA (20 pg) (Fig. 2b).

Las transmission from citrus plants to Cochin China atalantia plants of two types by graft-inoculation

The graft compatibility between citrus cultivars and Cochin China atalantia plants (WL) may affect the transmission of Las by graft inoculation. The two types of WL and CBO grafted onto different citrus rootstocks (cleopatra mandarin and carrizo citrange) showed good compatibility by normal growth. Yoshida (1996) reported that species of Tribe Citreae (Aurantioideae, Rutaceae) including *A. citroides* (WL), *S. buxifolia* (CBO) and *F. limonia* (WA), showed good graft compatibility with citrus scions. The same was true in the present study. In graft-inoculation, the citrus (Eureka lemon) scions as the inoculum grafted onto WL plants of two types showed high compatibility. The citrus scions of Eureka lemon grew on WL plants of two types as well as CBO plants. The scions of two-type WL grafted on citrus rootstocks grew normally too (Fig. 3).

Accordingly, the transmission of Las within the combinations of citrus and Cochin China atalantia plants was effective. The Las-infected citrus scions (Eureka lemon) as inoculum were grafted onto the seedlings of two-type of WL, CBO and PM. The Las detections in the four test plants were performed by Q-PCR assay 2 to 12 months after graft inoculation (Table 1, a). The Las was exactly detected in the four test plants by Q-PCR showing Ct values ranging from 22 to 27.5, 3 months after inoculation with Las-infected citrus scion in the primary inoculation. The Las was able to grow and survive persistently until 12th month by showing Ct values ranging from 21.2 to 27.1. The Las multiplied up to the highest titer at Ct value 20.6 and induced typical symptoms in PM citrus plant within 4 months after graft inoculation. While, lower titers of Las were detected in the other non-citrus plants with mild chlorotic symptom, such as CBO (Ct value 22.7), WL-1 (Ct value 23.9) and WL-2 (Ct value 27.7). In view of Las detection by Q-PCR in the 12th month, the titers of Las were PM (Ct value 21.2)>CBO (Ct value 22.1)>WL-1 (Ct value 23.4)>WL-2 (Ct value 27.1). HLB-Las infected citrus PM plant and alternative host CBO plant by inducing HLB symptom. It also caused the new alternative host Cochin China atalantia plants of two types including WL-1 plant to show yellow mottling symptom and WL-2 plant mild chlorosis at the 6th month after graft inoculation (Fig. 4a). The Las could survive persistently in the non-citrus plants including CBO and Cochin China atalantia of the two types.

Fig. 2 (a) Quantitative real-time PCR (Q-PCR) assay targeting the *sec*E gene product for Las detection, templates in tenfold serial dilution ($10°$–10^{-6}) of total DNA extract from HLB-Las infected Ponkan mandarin. Copy numbers of the *sec*E gene fragment of Las by Q-PCR with HLB TaqMan primer/probe. The detectable lowest dilution was 10^{-5} (2 pg) by Q-PCR assay. (b) Comparative sensitivity test of conventional PCR assay with HLB specific 226-primer pair. The detectable lowest dilution was 10^{-4} (20 pg) by PCR assay. The three-dimensional view of gel on lower picture was showing clear vision at 10^{-4} dilution. M, 100 bp DNA ladders (GeneDireX). The PCR amplicon size of Las is indicated on the right side
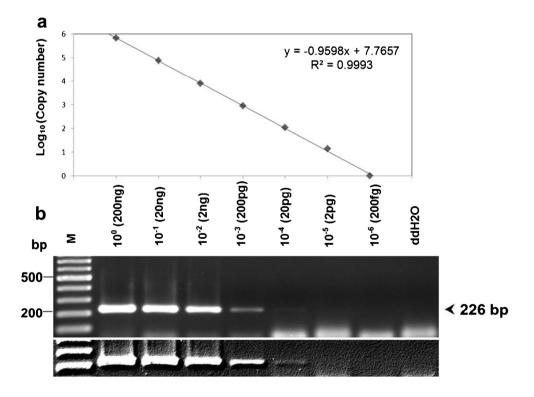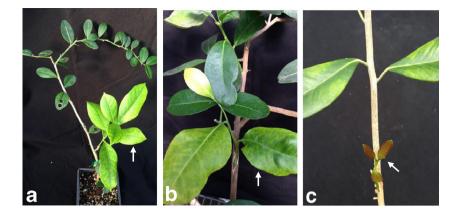

$$y = -0.9598x + 7.7657$$
$$R^2 = 0.9993$$

◄ 226 bp

Fig. 3 Las-infected citrus scions (↑) of Eureka lemon graft-inoculated onto Chinese box orange (**a**), WL-1 (**b**) and WL-2 (**c**) of Cochin China atalantia plants showing good compatibility by normal growth (↑)

Las transmission from two types of Cochin China atalantia back to citrus by graft inoculation

The Las-infected scions of Cochin China atalantia plants (WL-1 and WL-2) were grafted back to healthy WL-1 and WL-2 plants and PM citrus plants as controls. The multiplication of Las in test plants was monitored by Q-PCR assay. The all test plants showed the first detectable titer of Las by Q-PCR with Ct values of 26.4 (PM), 27.4 (WL-1) and 30.4 (WL-2) 3 months after inoculation (Table 1, b). It implied that Las in Cochin China atalantia scion was completely transmitted back to another Cochin China atalantia plants and citrus plants. The concentration of Las bacterium in PM citrus seedling was higher than that in the WL seedlings of the two

types. The WL-1 donor scion showed higher titer of Las in PM receptor plant with Ct value 26.4 than PM plant with Ct value 29.6 inoculated with WL-2 donor scion in 3 months after inoculation. This implied that Las concentration was higher in WL-1 scion than in WL-2 scion. Presumably, Las multiply better in WL-1 atalantia plant than in WL-2 atalantia plant. Typical HLB symptom developed on WL-1 plant (Ct value 22.2) in 12th month, while WL-2 plant (Ct value 24.9) showed mild mottling (Table 1, b). Based on the results mentioned above, the Cochin China atalantia plants were demonstrated as potential alternative hosts of Las, serving as donor plant. The Cochin China atalantia plants could serve as reservoirs of Las which was maintained positive detection up to 5 years (Fig. 4b to d).

Table 1 Detection of HLB-Las (*Ca. L.* asiaticus) by Q-PCR and symptom expression in Cochin China atalantia plants of elliptic leaf and elongated leaf types, Chinese box orange and mandarin plants graft-inoculated with Las strain II

Donor plant[a]	Recipient plant[b]	Las detection by Q-PCR (Ct) / Symptom index[c]									
		2 M		3 M		4 M		6 M		12 M[d]	
a. Primary graft-inoculation											
Eureka lemon	PM	36.8	/ 1	22.0	/ 2	20.6	/ 3	22.4	/ 3	21.2	/ 3
	CBO	37.3	/ 0	23.6	/ 1	22.7	/ 1	23.4	/ 2	22.1	/ 3
	WL-1	Und	/ 0	22.1	/ 1	23.9	/ 1	25.4	/ 2	23.4	/ 3
	WL-2	Und	/ 0	27.5	/ 1	27.7	/ 1	26.8	/ 1	27.1	/ 2
b. Back graft-inoculation											
WL-1	PM	Und	/ 0	26.4	/ 1	24.7	/ 1	23.4	/ 2	21.4	/ 3
	WL-1	37.2	/ 0	27.4	/ 1	24.8	/ 2	23.0	/ 2	22.2	/ 3
WL-2	PM	Und	/ 0	29.6	/ 1	27.1	/ 1	25.6	/ 2	22.4	/ 2
	WL-2	37.3	/ 0	30.4	/ 0	26.2	/ 0	26.4	/ 1	24.9	/ 2

[a] Eureka lemon plants inoculated with Las strain II were used as donor plants in primary graft-inoculation (a), and Las-infected Cochin China atalantia (*A. citroides*) plants of two types including WL-1 (elliptic leaf) and WL-2 (elongated leaf) were used as donor plants in back graft-inoculation (b)

[b] Receptor plants: Ponkan mandarin (PM, *C. reticulata*), Chinese box orange (CBO, *S. buxifolia*) and Cochin China atalantia plants of two types (WL-1 and WL-2)

[c] Las detection/Symptom index: The average cycle threshold (Ct) value was determined with triplicate for each sample by Q-PCR assay for Las detection; Ct value > 36.5 was considered to be "negative". "Und" (undetermined) of Ct value indicated no Las-template amplification. Symptom index: 0, healthy looking; 1, mild chlorotic symptom; 2, mild mottling or chlorosis with leaf atrophy; 3, HLB symptoms including yellow mottling with leaf curling and leaf atrophy

[d] M: months after graft inoculation. Las detection of test plants by Q-PCR assay from the 2nd to 12th month after graft inoculation
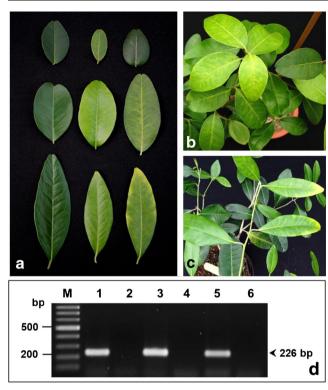
Fig. 4 (**a**) Leaf symptoms developed on leaves of Las-inoculated plants including Chinese box orange (CBO, upper), Cochin China atalantia plants of elliptic type (WL-1, middle) and elongated type (WL-2, bottom) 6 months after graft inoculation. The Las-infected leaves (right two) of CBO and WL-1 showing yellow mottling and atrophy, and those of WL-2 showing chlorotic symptom. Healthy leaves without symptoms are on left. (**b**) Las-inoculated WL-1 plant showing yellow mottling symptom on upper leaves persistently during 5 years after inoculation. (**c**) Las-inoculated WL-2 plant showing mild mottling and atrophy symptoms on upper leaves persistently during 5 years after inoculation. (**d**) Las-detection by PCR assay in the graft-inoculated alternative host plants including CBO (Lane 1), WL-1 (Lane 3) and WL-2 (Lane 5) of Cochin China atalantia, showing positive detection with amplicons of 226 bp. Healthy leaves of CBO (Lane 2), WL-1 (Lane 4) and WL-2 (Lane 6) of Cochin China atalantia, showing negative detection. Lane M, 100 bp DNA ladders (GeneDireX)

Las transmission to Cochin China atalantia of two types by citrus psyllid

Healthy Cochin China atalantia plants of the two types (WL-1 and WL-2) were prepared in insect cage for psyllid transmission. Viruliferous psyllids harboring Las were collected from the HLB-diseased Liucheng sweet orange trees in the field. Over 100 of viruliferous psyllids were transferred into insect cage and fed on test plants over 30 days of inoculation feeding period two times. The viruliferous psyllids colonized well on leaves of WL-1 (Fig. 5a) and WL-2 plants (Fig. 5b). Ten psyllids collected from insect cage by random were assayed by the PCR for examining the percentage of Las-harboring. The percentages of psyllids harboring Las detected by PCR assay were 20% in the first round and 60% in the second round.
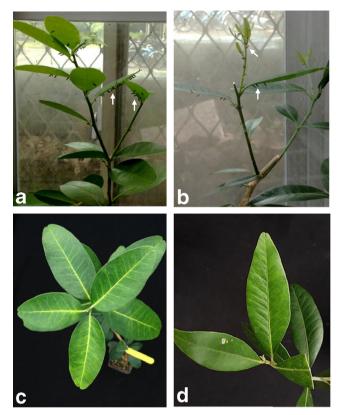

Fig. 5 Las transmission by viruliferous citrus psyllid (*D. citri*) from the field into Cochin China atalantia plants of the two type: Las-harboring psyllids (↑) feed on healthy WL-1 plant (**a**) and WL-2 plant (**b**) of Cochin China atalantia in insect cage. Yellow mottling symptom developed on leaves of WL-1 (**c**), and mild chlorosis developed on leaves of WL-2 (**d**) 6 months after psyllid transmission

Table 2 Transmission of HLB-Las into the two types of Cochin China atalantia (*A. citroides*) by viruliferous citrus psyllids from the field and Q-PCR assay monitoring Las in Cochin China atalantia plants

Receptor plant[a]	Las detection after viruliferous psyllid inoculation by Q-PCR (Ct) / Symptom index[b]					
	2 M	3 M	4 M	6 M	8 M	12 M
WL-1 (1)	37.4	38.3	35.1	27.6/ 2	26.6	26.4
WL-1 (2)	37.3	38.9	35.7	30.6/ 1	29.2	29.4
WL-2 (1)	36.8	29.5	20.9	23.2/ 1	22.3	21.8
WL-2 (2)	36.5	26.9	28.9	27.2/ 1	25.2	22.6

[a] Duplicate receptor plants of Cochin China atalantia (WL-1 and WL-2) were subjected to vector transmission of viruliferous citrus psyllids (*D. citri*). Viruliferous citrus psyllids from infected Liucheng sweet orange trees in the field were collected two times. The percentage of Las-harboring in psyllids were 20% in first round and 60% in second round. Sets of 100 viruliferous citrus psyllid adults were transferred onto Cochin China atalantia plants for inoculation feeding 4 weeks each round, in two times

[b] Las detection/Symptom index: Detection of Las in test plants by Q-PCR assay from the second month to the twelfth month (M) after inoculation feeding. Cycle threshold (Ct) values of Q-PCR and symptom index were ditto in Table 1

Q-PCR assay for detection of Las in the Cochin China atalantia plants of the two types inoculated by vector transmission with viruliferous psyllids was performed monthly within one year. No Las was detected in the 2nd month after inoculation as all Ct values were >36.5. Positive detection of Las was found first in two plants of WL-2 in low titer with Ct values of 29.5 and 26.9 in the 3rd month after inoculation feeding. Considerable titers of Las were detected in WL-1 plants with mild chlorosis or yellow mottling symptom with Ct values of 27.6 and 30.6, and in WL-2 plants with mild mottling with Ct values of 23.2 and 27.2, in 6 months after inoculation feeding (Fig. 5c, d). Positive detection of Las in high titers was found in all Cochin China atalantia plants particularly in WL-2 with Ct values of 21.8 and 22.6 persistently over 12 months in this experiment (Table 2). It was assumed that WL-2 Cochin China atalantia was more tolerant to Las by producing milder symptom in comparison with WL-1 Cochin China atalantia.

Las transmission via citrus psyllid was made by using Las-infected Cochin China atalantia as donor plants. Non-viruliferous psyllids were obtained from the offspring psyllids fed on jasmine orange (*M. paniculata*) seedlings immune to Las in insect cages. Some psyllids collected from the cage by random were confirmed to be free of Las by PCR assay. The non-viruliferous psyllids were fed on Las-infected WL-1 and WL-2 donor plants for acquisition feeding 2–4 weeks in insect cages. Considerable numbers of psyllids acquiring Las were fed on healthy PM seedlings for inoculation feeding of 2 weeks, one to three times. After vector inoculation, the receptor PM plants were grown outside insect cages in a greenhouse. PCR detection of Las in the all citrus receptor test plants were made monthly after inoculation feeding, and symptom expression was also observed. Positive detections of Las by PCR were found in the all PM receptor plants 9 months after inoculation feeding with the psyllids acquiring Las from WL-1 and WL-2 donor plants (Table 3). However, first positive detection of Las was found in PM receptor plant inoculated with over 100 psyllids of acquisition feeding on WL-1 donor plants for 4 weeks, and symptom express of yellow mottling was observed 5 months after inoculation feeding. As a whole, the Cochin China atalantia plants of two types could serve as donor plants of Las source for psyllid transmission to citrus plants, and also served as receptor plants susceptible to Las-transmission by citrus psyllids. It can be concluded that the Cochin China atalantia plants of two types are the alternative host plants of Las and the Las-infected Cochin China atalantia plants serve as donor plants for psyllid transmission of Las causing citrus HLB disease.

Discussion

Citrus HLB disease was commonly transmitted via vegetative propagation with Las-infected budwood, and spread by psyllid vector in the field. The epidemiology of HLB is primarily important for formulating disease management. The potential hosts of the Las pathogen including citrus and alternative host plants and the psyllid vector serve as reservoirs of Las, and usually play an important role in the disease epidemiology. Some citrus relative plants in the Family Rutaceae may be the alternative hosts of psyllids

Table 3 Transmission of HLB-Las by non-viruliferous citrus psyllids from Las-infected Cochin China atalantia plants of two types into Ponkan mandarin plants

Donner plant[a]	AAP[b] (week)	No. of psyllids[c] on/off	Recipient plant[d]	Las detected by PCR[e]			Symptom expression
				3 M	5 M	9 M	
WL-1							
	2 W	65/34	PM-1	−	−	+	chlorosis
	2 W	20/9	PM-2	−	−	+	chlorosis
	4 W	>100	PM-3	−	+	+	yellow mottling
WL-2							
	2 W	91/66	PM-1	−	−	+	chlorosis
	2 W	>100	PM-2	−	−	+	chlorosis
	2 W	>100	PM-3	−	−	+	chlorosis

[a] Donor plants: Cochin China atalantia plants of WL-1 (elliptic leaf) and WL-2 (elongated leaf) types of inoculated by graft inoculation with Las strain II

[b] AAP: acquisition access period, weeks

[c] Total number of citrus psyllids (*D. citri*) transferred onto PM seedlings for inoculation feeding for 2 weeks, No. of psyllids feeding <u>on</u>/ No. of psyllid removing <u>off</u> at the beginning and end of inoculation-feeding, 2–4 weeks

[d] Las-susceptible receptor plants were Ponkan mandarin (PM) seedlings

[e] Las detection in PM receptor plants by PCR assay periodically (M, months) after inoculation feeding, Las-specific amplicon of PCR at 226 bp, +, positive detection; −, negative detection

vector as well as Las. Therefore, searching the new alternative hosts of Las and psyllid is quite important for the management of HLB disease. Hung et al. (2000 and 2001) reported that Chinese box orange (*S. buxifolia*) was an alternative host of HLB bacteria in Taiwan by graft and psyllid transmission experiments. The citrus psyllids were commonly seen on CBO which served as reservoirs and inoculum sources of Las in citrus areas in Taiwan (Lin et al. 1973).

The Cochin China atalantia plants of two types, locally called "Krouch prey" in Khmar and wild lime (WL) in English, are growing wildly or as ornamental plant in Cambodia and Cochin China area of Southern Vietnam. The present investigation through binational collaboration reveals that Cochin China atalantia is the new host plant of HLB pathogen and psyllid vector by experiments of graft and psyllid transmission. The Cochin China atalantia plants of two types are shrub (elliptic leaf type, WL-1) or tree (elongated leaf type, WL-2) which widely distributed in the tropical Indochina including Cochin China of South Vietnam and Cambodia. Cochin China atalantia plants (*A. citroides*) belong to Subtribe Citrinae, Tribe Citreae and Family Rutaceae.

Using CPDNA (chloroplast DNA) sequences, a molecular phylogeny of orange Subfamily Aurantioideae reveals close phylogenic relationship among *S. buxifolia*, *A. citroides* and *Citrus* spp. which belong to Tribe Citreae (Bayer et al. 2009). Yoshida (1996) reported good graft compatibility of citrus plants grafted onto *S. buxifolia* and *A. citroides* which were susceptible to *Citrus tristeza virus* (CTV). Subtribe Citrinae includes genus *Severinia* belonging to primitive citrus group, genus *Atalantia* belonging to near citrus group, and true citrus group includes genera of *Citrus*, *Fortunella* and *Poncirus*. In view of the close phylogeny among the three citrus groups, HLB pathogen (Las) might have citrus hosts and the alternative host plants including CBO and WL which are the suitable host plants of citrus psyllid vector. The Las multiply fast in citrus (PM), *Severinia* (CBO) and elliptic leaf type of Cochin China atalantia (WL-1) of which high detectable titer of Las was detected 3 months after graft inoculation, while Las multiplication was slow in elongated leaf type of Cochin China atalantia (WL-2) containing high detectable titer of Las 6 months after graft inoculations. The Las-infected WL-1 plants produced typical HLB symptoms including yellow mottling and atrophy leaf, but WL-2 plants developed mild chlorosis symptom and showed more tolerant to Las infection than WL-1 plant. Las survived persistently in Cochin China atalantia plants of the two types as alternative hosts of Las. Accordingly, the Cochin China atalantia plants serve as potential donor plants of psyllid transmission. The Las-infected Cochin China atalantia plants of the two types (WL-1 and WL-2) could serve as donor plants for psyllid transmission of Las to citrus plants (PM) in the present experiment. The citrus psyllids showed good colonization on the two types of Cochin China atalantia plants. Therefore, the Cochin China atalantia plants might serve as potential donor plants for psyllid transmission of Las in the field.

Lopes et al. (2010) reported that jasmine orange (*M. paniculata*) was associated with Liberibacter in Brazil. Low incidence of Las in *M. paniculata* and the associated psyllids (*D. citri*) were reported in Florida, U. S. A. by Walter et al. (2012). However, no positive detection of Las was found in the wild jasmine orange plants, good hosts of citrus psylla by PCR assay in Taiwan. So far, no positive infection of Las was detected in jasmine orange plants exposed to Las-harboring psyllids for long period in insect cages.

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