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Quantification and ecological study of '*Candidatus* Liberibacter asiaticus' in citrus hosts, rootstocks and the Asian citrus psyllid

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The use of proper management strategies for citrus huanglongbing (HLB), caused by '*Candidatus* Liberibacter asiaticus' (Las) and transmitted by Asian citrus psyllid (ACP) (*Diaphorina citri*), is a priority issue. HLB control is based on healthy seedlings, tolerant rootstock cultivars and reduction of ACP populations. Here, dynamic populations of Las in different citrus hosts and each instar of ACP were studied, together with the seasonal growth and distribution of Las in different tissues, using conventional and TaqMan real-time PCR. Different levels of susceptibility/tolerance to HLB were seen, resulting in different degrees of symptom severity and growth effects on hosts or rootstocks. Troyer citrange, Swingle citrumelo and wood apple were highly tolerant among 11 rootstock cultivars. Regarding distribution and seasonal analysis of Las, mature and old leaves contained high concentrations in cool temperatures in autumn and spring. Las was detected earlier through psyllid transmission than through graft inoculation, and the amounts of Las (AOL) varied in different hosts. Thus, different AOL (10^4–10^7 copy numbers μL^{-1}) and Las-carrying percentages (LCP; 40–53.3%) were observed in each citrus cultivar and on psyllids, respectively. Furthermore, both AOL and LCP were lower in nymphs than in adult psyllids, whereas the LCP of psyllids were not affected by increasing the acquisition-access time. The present study has significant implications for disease ecology. The combination of early detection, use of suitable rootstocks and constraint of psyllid populations could achieve better management of HLB disease.

Keywords: '*Candidatus* Liberibacter asiaticus', acquisition-access time, Asian citrus psyllid, dynamic population, huanglongbing, susceptibility/tolerance

Introduction

Citrus huanglongbing (HLB), also known as greening, is currently the most destructive disease in most citrus planting areas worldwide. Currently, three nonculturable phloem-limited Gram-negative α-proteobacteria are reported associated with HLB disease: '*Candidatus* Liberibacter asiaticus' (Las) from Asia, '*Candidatus* Liberibacter africanus' (Laf) from Africa and '*Candidatus* Liberibacter americanus' (Lam) from South America (Garnier *et al.*, 2000; Bové, 2006). The Asian citrus psyllid (ACP), *Diaphorina citri* (Hemiptera: Liviidae), is the main insect vector of '*Candidatus* Liberibacter' spp. in Asia and America, whereas the African psyllid, *Trioza erytreae* (Triozidae), is the main vector in Africa (Aubert, 1987; Halbert & Manjunath, 2004). The causal agent can infect all commercially important citrus cultivars, resulting in major reductions in fruit quality and yield and the appearance of foliar symptoms, such as irregular mottling and severe chlorosis with nutritional deficiency-like syndrome (McClean & Schwarz, 1970), followed by incomplete colouring of mature fruit (da Graça, 1991) and shortening of the lifespan of infected trees (Miyakawa, 1980). HLB is spread by vegetative propagation and insect vectors, and thus far, there is no cure for this severe disease. HLB has been the most important factor limiting citrus production worldwide.

Because there are no cures for HLB disease, prevention and vector control are critical for HLB integrated management. It is proposed that two important strategies for prevention are sensitive detection methods and a healthy seedling system. In recent years, quantitative detection methods have been developed and improved for more sensitive and specific purposes (Li *et al.*, 2006, 2009; Morgan *et al.*, 2012; Ananthakrishnan *et al.*, 2013; Hu *et al.*, 2013; Bertolini *et al.*, 2014; Kogenaru *et al.*, 2014; Feng *et al.*, 2015). Quantitative methods have been used to analyse the distribution of Las, showing uneven patterns in different infected

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tissues of various citrus cultivars (Tatineni *et al.*, 2008; Li *et al.*, 2009). Detection of the rate of Las multiplication has also been investigated, with a 100% infection rate seen at 120 days post-grafting inoculation, and a direct relationship between pathogen concentration and symptom expression (Coletta-Filho *et al.*, 2010). Real-time PCR combined with propidium monoazide (PMA) could detect live pathogens and benefit disease epidemiology and management studies (Hu *et al.*, 2013). Because numerous commercial citrus and rootstock cultivars are widely used and planted in fields and Las infection has commonly existed for decades in Taiwan, questions concerning the amounts of Las in these commercially important cultivars, and seasonal population variations of Las in different host tissues, should be answered.

In addition, several studies have shown the transmission patterns and characteristics of Las through ACP. ACP was shown to require hours of feeding to acquire Las, and Las only replicated in a small percentage of the whole population (Halbert & Manjunath, 2004; Pelz-Stelinski *et al.*, 2010). A period of days may be required before ACP adults acquire the capacity to transmit Las after feeding on infected citrus, but adults from nymphs could transmit Las into host plants as soon as they acquired Las on the last nymphal instar (Pelz-Stelinski *et al.*, 2010; Grafton-Cardwell *et al.*, 2013). The poor transmission of Las was observed when ACP acquired Las at the adult stage, unlike acquisition of Las at the nymphal instar (Inoue *et al.*, 2009; Pelz-Stelinski *et al.*, 2010). A recent study also indicated that Las replicated in both ACP nymphs and adults, but higher levels of Las were obtained when acquired by nymphs rather than adults, and the nymphal stage had a higher probability of Las inoculation into citrus plants (Ammar *et al.*, 2016). Furthermore, studies have also reported that ACP had less successful percentages of Las inoculation into hosts than inoculation via scion grafting, as every individual showed Las-positive signals based on PCR detection (Hung *et al.*, 2004; Ukuda-Hosokawa *et al.*, 2015). However, the different replication rates of Las in several citrus cultivars between graft inoculation and psyllid transmission and the replication characteristics of Las in psyllids at different instars or timing remain poorly understood.

The present study used conventional PCR and quantitative TaqMan real-time PCR methods to determine the levels of susceptibility/tolerance of six commercial citrus and 11 rootstock cultivars after 1 year of graft inoculation of Las and characterized its distribution in different tissues and seasonal populations in three citrus cultivars. In addition, the dynamic proliferation levels of Las were traced using conventional and quantitative methods in infected citrus plants under graft and psyllid inoculations. Furthermore, the amounts of Las-acquisition at different timing and instar stages of psyllids were determined. The results provided the absolute amounts and replication characteristics of Las in host plants, rootstocks or psyllids, which may illustrate the disease ecology of HLB and provide more information for better management using new tolerant rootstock cultivars and limiting the psyllid population in a more precise manner.

Materials and methods

Plant preparation

All the experimental citrus commercial cultivars were obtained by shoot-tip grafting from greenhouse-kept mother seedlings, and rootstock cultivars were obtained by using seedlings germinated from healthy seeds. All cultivars were confirmed as free from *Citrus tristeza virus* (CTV) and Citrus tatter leaf virus (CTLV). Experimental plants were kept in insect-free greenhouses with a 16 h light (28 °C)/8 h dark (24 °C) photoperiod and were regularly watered with commercial plant nutrients. For HLB inoculation, scions of six commercial citrus cultivars were tested: Ponkan mandarin (*Citrus reticulata*), Tankan mandarin (*C. reticulata*), Valencia sweet orange (*Citrus sinensis*), Wentan pomelo (*Citrus grandis*), Eureka lemon (*Citrus limon*) and kumquat (*Citrus japonica*). Healthy scions of each cultivar were propagated on virus-free rootstock seedlings of Sunki mandarin (*C. reticulata*) or Rangpur lime (*Citrus* × *limonia*). Furthermore, nine citrus rootstock cultivars: Sunki mandarin, Rangpur lime, calamondin (× *Citrofortunella microcarpa*), Volkamer lemon (*Citrus volkameriana*), Troyer citrange (*C. sinensis* × *Poncirus trifoliata*), Swingle citrumelo (*Citrus paradisi* × *P. trifoliata*), alemow (*Citrus macrophylla*), rough lemon (*Citrus jambhiri*), and bitter orange (*Citrus* × *aurantium*), together with two potential non-citrus rootstock cultivars, Chinese box orange (*Atalantia buxifolia*) and wood apple (*Limonia acidissima*), were used to test susceptibility in the study. For susceptibility/tolerance testing, infected buds used for grafting were obtained from greenhouse-kept Eureka lemons with severe symptoms that had been infected by Las strain II for at least 2 years, to minimize the effect of uneven distribution of Las in host.

For distribution and seasonal concentration of HLB, three Las-infected citrus species, 3-year-old Hong-Jian sweet orange (*C. sinensis*), 3-year-old Murcott tangor (*C. reticulata* × *C. sinensis*) and 10-year-old Peiyu pomelo (*C. grandis*) were selected and infection confirmed by PCR detection. The same weight of material from each host plant was collected seasonally from nine plant parts, including new flush, young leaf, midrib of mature leaf, mesophyll of mature leaf, midrib of old leaf, fruit, bark of young twig, bark of trunk and root.

Pathogen bacteria sources

Based on a previous study (Tsai *et al.*, 2008), Las strain II was used for graft and psyllid inoculation tests. This is the major strain infecting citrus in Taiwan and has a wide host range. The Las strain II was isolated and purified from different hosts.

Citrus psyllid preparation

Healthy citrus psyllids (*D. citri*) were collected from jasmine orange (*Murraya paniculata* var. *paniculata*) in a field in Chiayi county, Taiwan. A previous study confirmed that jasmine orange is a proper host to the citrus psyllid but is immune to Las bacteria (Hung *et al.*, 2000). No citrus plants were planted around the jasmine orange field and the psyllids were confirmed as Las-free by PCR and real-time PCR detection.

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Genomic DNA isolation

Plant DNA extraction

DNA extraction followed the method of Hung *et al.* (1999) with modifications. Leaf midrib (500 mg) was powdered in liquid nitrogen, and each sample was suspended in 1.5 mL DNA extraction buffer [1 M Tris-HCl (pH 8.0), 0.5 M EDTA, 5 M NaCl, 1% N-lauroylsarcosine] and transferred to a 1.5 mL Eppendorf tube. After incubation at 55 °C for 1 h, the sample was centrifuged at 4000 ***g*** for 5 min. The supernatant (800 μL) was collected, and 100 μL 5 M NaCl and 100 μL 10% CTAB (hexadecyl trimethylammonium bromide) in 0.7 M NaCl were added. The mixture was incubated at 65 °C for 10 min. The sample was subjected to one cycle of chloroform/isoamyl alcohol (24:1) extraction, and the aqueous supernatant was then extracted by an additional cycle of phenol/chloroform/isoamyl alcohol (25:24:1). The nucleic acids were precipitated by mixing 600 μL of the supernatant with 360 μL isopropanol followed by centrifugation at 12 000 ***g*** for 10 min. The pellets were washed with 70% ethanol, dried, and resuspended in 150 μL TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) as template solution.

Psyllid DNA extraction

DNA was extracted as described by Hung *et al.* (2004). Each psyllid was rinsed twice in 50 μL DNA extraction buffer [1 M Tris-HCl (pH 8.0), 0.5 M EDTA, 5 M NaCl, 1% N-lauroylsarcosine] for 5 min. The psyllid was put in a 1.5 mL Eppendorf tube containing 300 μL DNA extraction buffer, homogenized with a plastic rod, and incubated at 55 °C for 1 h. After phenol/chloroform/isoamyl alcohol extraction, the DNA was precipitated by mixing 200 μL of the supernatant with 500 μL 100% ethanol followed by centrifugation at 12 000 ***g*** at 4 °C for 10 min. The pellet was dried and resuspended in 10–20 μL TE buffer depending on extraction of adult psyllid or egg/nymph.

Conventional PCR detection of Las in citrus tissues and psyllid bodies

PCR-based detection of Las amplified a Las-specific fragment (226 bp) by using a previously designed primer pair (5′-CACC GAAGATATGGACAACA-3′; 5′-GAGGTTCTTGTGGTTTTT CTG-3′). PCR was performed using 25 μL reaction mixture containing 20 mM Tris-HCl pH 8.4, 50 mM KCl, 4 mM $MgCl_2$, 0.2 mM each dATP, dTTP, dCTP and dGTP, 50 ng of each primer, 0.75 U *Taq* DNA polymerase (Invitrogen) and 250 ng template DNA. Thermal cycling conditions were: one cycle at 94 °C for 3 min; 30 cycles at 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 2 min; and then 72 °C extension for 10 min. Reactions were carried out in a DNA thermal cycler 2720 (Applied Biosystems).

PCR products were analysed by gel electrophoresis using 1.4% agarose in 0.5 × TAE buffer (40 mM Tris-acetate, 1 mM EDTA pH 8.0). The products were then stained with ethidium bromide, visualized and analysed using AlphaEase FC image analysis software. The density of the PCR products was determined and represented by a pixel value with a range from 0 to 255.

Real-time PCR detection of Las

Las detection by real-time PCR assay was carried out as described by Feng *et al.* (2015). TaqMan primers/probe for Las detection were designed based on the Las *trmU-tufB-secE-nusG-rplKAJL-rpoB* gene cluster region of Las-infected Ponkan mandarin (TW2 isolate) by using custom TaqMan gene expression assays (Applied Biosystems). The primer pair (primer-F: 5′-AGGTTGGCTGTGTTAAATTTTTTTAAGCAA-3′ and primer-R: 5′-ACAATAACCGAAACCAAAACCTCACT-3′) was designed based on the *secE* gene region. The TaqMan probe (5′-ACGGCCAGAATATCTT-3′) was labelled at the 5′ end with 6-carboxyfluorescein (FAM) reporter dye and at the 3′ end with non-fluorescent quencher (NFQ) plus minor groove binder (MGB). To construct a standard curve, a partial sequence of the Las *secE* gene was amplified with TaqMan primers and purified using High Pure PCR Product Purification kit (Roche Applied Science). The PCR product was ligated into pCR2.1 vector and transformed into ECOS 9-5 competent cells (Invitrogen) according to the manufacturer's instructions. Tenfold dilutions of the Las plasmid DNA containing the *secE* gene partial sequence were used as standard samples, with one healthy citrus sample and ddH_2O as negative controls in each run for quantitative analysis of Las.

The TaqMan real-time PCR was performed by using the StepOne Real-Time PCR System (ABI) in 20 μL reaction mixture containing 2 × TaqMan Universal Master mix II with UNG (Applied Biosystems), 250 nM TaqMan MGB probe, 900 nM Las forward and reverse primer pair and 200 ng DNA template. The amplification cycles were 50 °C for 2 min, 95 °C for 10 min; then 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The average cycle threshold (C_t) value for Las detection was determined in triplicate for each sample. Data were analysed using StepOne v. 2.0 software. C_t values > 36.5 were considered negative.

Detection of Las growth curve in different citrus species by graft or psyllid inoculation

For graft inoculation, four citrus cultivars (Ponkan mandarin, Valencia sweet orange, Wentan pomelo and Eureka lemon) were grafted by using Las-infected scions of sweet orange. The study was performed in triplicate. Three weeks after graft inoculation, 0.15 g leaf midrib of each cultivar was analysed by conventional PCR and real-time PCR weekly, with healthy citrus samples as control. For psyllid inoculation, adult citrus psyllids were collected from a Las-infected sweet orange field in Chiayi, Taiwan and then transferred to an insect-proof cage for 1 day at 25 °C for rearing. Two groups each containing 15 psyllids were fed separately for 24 h on a Ponkan or Valencia plant in insect-proof cages. The study was performed in triplicate. Las detection in the two groups of psyllids was performed by conventional PCR and real-time PCR after 24 h feeding. Furthermore, after three weeks' psyllid inoculation, weekly detection of Las in each infected plant was performed to record its growth curve. Each PCR included healthy citrus samples as control.

Las acquisition test in psyllids

Adult citrus psyllids were collected in the field, confirmed as healthy by real-time PCR and transferred to an insect-proof cage containing a Valencia sweet orange plant severely infected with Las. Fifteen psyllids were randomly collected every 2 days and were separated into 11 Eppendorf tubes (10 tubes for one psyllid and one tube for five psyllids) for DNA extraction. Conventional PCR and real-time PCR were used to detect the amplification and carrying percentage of Las in psyllids.

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Quantitative determination of Las for different instars of nymphs of citrus psyllids

Citrus psyllids were collected from Las-infected citrus fields in Chiayi, Taiwan. Different instars of nymphs were separated by using a dissecting microscope, DNA was extracted and then Las detected by conventional PCR and real-time PCR. Due to the small amounts of DNA obtained from the instar of egg and first nymph, five to 10 samples were used as a unit for detection.

Results

Susceptibility/tolerance to HLB

Proliferation of Las and symptom development on six Las-infected citrus and 11 rootstock cultivars are summarized in Table 1 and Figures 1 and 2. Ponkan and Tankan mandarins were positive for Las using PCR at only 3 months after inoculation and initially showed mild chlorosis. The typical mottling symptoms were observed after 6 months, and strong PCR signals were also detected. No symptoms were noted on Valencia sweet oranges at 3 months after inoculation, but severe chlorosis symptoms and strong PCR signals were observed at 6 months after inoculation. Wentan pomelo and Eureka lemon were tolerant to HLB disease. PCR signals were first detected at 4 months after inoculation. Strong PCR signals and chlorosis symptoms appeared at 10 to 12 months after inoculation. Kumquat was a much more tolerant host among the six citrus cultivars examined. PCR signals were detected at 6 months after inoculation, and low concentrations of Las slowly increased for 1 year following inoculation. Only mild yellowing symptoms similar to those resulting from a lack of magnesium were observed on kumquat leaves.

Based on the symptom index and effect on growth, the 11 rootstock cultivars were separated into four types: (i) sensitive: slight PCR signals were detected for Sunki mandarin, calamondin and bitter orange at 4 months after inoculation, and strong PCR signals with severe mottling symptoms appeared at 10 months after inoculation. (ii) Intermediate tolerant: Volkamer lemon, alemow, rough lemon and Rangpur lime showed mild chlorosis and yellowing symptoms on the leaves at 6 months after inoculation. The index of symptoms remained at low levels, whereas the PCR signals were detected after 6 months and slowly increased until 10 months after inoculation. Furthermore, Chinese box orange, a potential rootstock cultivar, showed severe mottling at 6 months after inoculation, and the symptoms decreased to mild chlorosis on infected leaves for a long period of time. Chinese box orange was also considered as having intermediate tolerance to HLB disease. (iii) Highly tolerant. Troyer citrange and Swingle citrumelo only showed small leaves without any yellowing symptoms at 1 year after inoculation. PCR signals also

Table 1 Determination of symptom severity and pathogen proliferation trend on '*Candidatus* Liberibacter asiaticus' (Las)-infected citrus and rootstock cultivars

	Symptom index/PCR index after grafting (average)[a]						
	2M	3M	4M	6M	8M	10M	12M
Citrus cultivar							
Ponkan mandarin	0[b]/–[c]	1/+	2/++	3/+++	3/+++	3/+++	3/+++
Tankan mandarin	0/–	1/+	2/++	3/+++	3/+++	3/+++	3/+++
Valencia sweet orange	0/–	0/+	1/+	2/++	2/++	2/++	2/++
Wentan pomelo	0/–	0/–	0/+	1/++	2/++	2/+++	2/+++
Eureka lemon	0/–	0/–	0/+	1/+	2/++	2/+++	2/+++
Kumquat	0/–	0/–	0/–	0/+	1/++	2/++	2/++
Rootstock cultivar							
Sunki mandarin	0/–	nt	0/+	1/++	2/+++	3/+++	3/+++
Calamondin	0/–	nt	0/+	1/++	2/+++	3/+++	3/+++
Bitter orange	0/–	nt	0/+	1/+	2/++	3/+++	3/+++
Volkamer lemon	0/–	nt	0/–	0/+	1/++	2/++	2/++
Rough lemon	0/–	nt	0/–	0/+	1/+	1/+	2/++
Rangpur lime	0/–	nt	0/–	0/+	0/+	1/++	2/++
Alemow	0/–	nt	0/–	0/+	0/+	1/++	2/++
Chinese box orange	0/–	nt	1/+	2/++	3/++	2/++	2/++
Troyer citrange	0/–	nt	0/–	0/+	0/+	0/++	1/++
Swingle citrumelo	0/–	nt	0/–	0/+	0/+	1/++	1/+++
Wood apple	0/–	nt	0/–	0/–	0/+	0/+	0/+

M, months.

[a]All index values were determined as the average of four individual plants.

[b]Huanglongbing (HLB) symptom index decided at the 12th month: 0, healthy looking without symptoms; 1, mild chlorotic symptoms; 2, intermediate symptoms including chlorosis with intermediate dwarfing; 3, typical greening symptoms including leaf yellowing, leaf curling, vein yellowing and vein corking with distinct dwarfing.

[c]Pixel value (density count) index of the Las specific band on agarose gel measured by a densitometer: –, pixel value <50; +, 50–109; ++, 110–169; +++, 170–230.

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Figure 1 Symptom development and growth effects of six commercially important citrus cultivars infected with '*Candidatus* Liberibacter asiaticus'. Pon, Ponkan mandarin; Tan, Tankan mandarin; Val, Valencia sweet orange; WT, Wentan pomelo; EL, Eureka lemon and Kq, kumquat. The left plant of each pair is the healthy control. Photographs were taken 1 year after infection. [Colour figure can be viewed at wileyonlinelibrary.com]

illustrated low concentrations of Las in the two hosts. (iv) Resistant. Wood apples seldom showed mild yellowing and slight symptoms on the leaves, and the unstable PCR signal could only be detected at 8 months after grafting.

Pathogen quantity and effect on growth

At 1 year after inoculation, the mature leaves of each citrus and rootstock cultivar were examined to detect the absolute amounts of Las using real-time PCR (Table 2). In the commercial citrus cultivars, Ponkan and Tankan mandarins were the most susceptible to HLB disease, with Las populations at more than 8 million copies in Ponkan plants. The amounts of Las obviously decreased in tolerant types, such as Wentan pomelo. The Las population remained low in kumquat among the six citrus cultivars examined. The effect on the growth of each citrus and rootstock cultivar infected with Las are listed in Table 3 and Figure 3. Infected Ponkan and Tankan mandarins showed severe growth effects, with a 70% decrease in growth rate 1 year after inoculation compared to the healthy control. The growth rates decreased by 60% and 65% for sensitive types, such as Valencia sweet oranges, and tolerant types, such as Wentan pomelo, respectively. Although Eureka lemon and kumquat showed intermediate tolerance and tolerance to HLB, their growth rates still decreased by 30% and 42.2%, respectively, compared to those of healthy plants.

In the rootstock cultivars, sensitive types, such as Sunki mandarin, calamondin and bitter orange maintained higher amounts of Las compared to those with intermediate tolerance, which had 12–33% of the amounts of sensitive types and 1–12% of the amounts of highly tolerant types (Table 2). Las-infected rootstock cultivars showed severe wilting and typical leaf symptoms. The growth rates of Sunki mandarin, calamondin and bitter orange decreased 70–80% compared to those of healthy plants. Intermediate tolerant cultivars (Volkamer lemon, alemow, rough lemon, Rangpur lime and Chinese box

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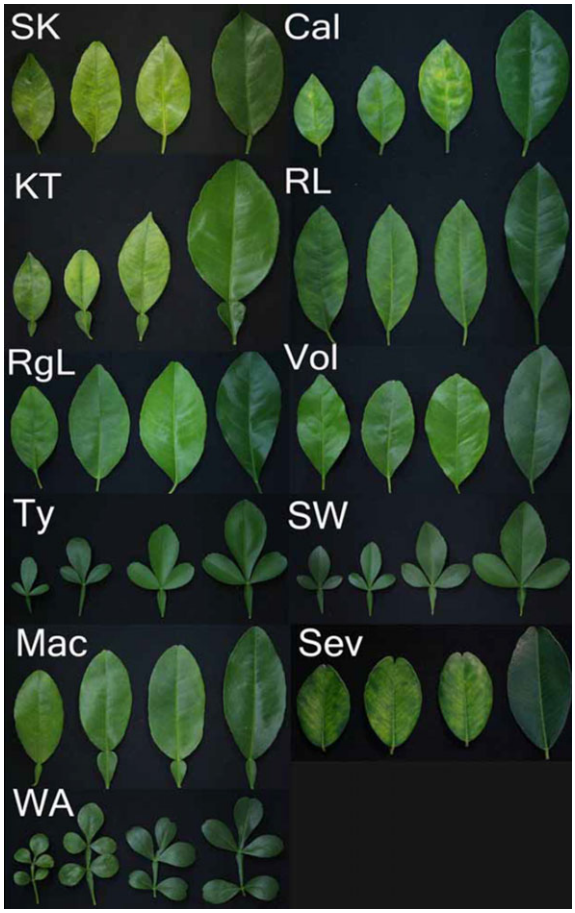

Figure 2 Leaf symptoms of 11 rootstock cultivars after '*Candidatus* Liberibacter asiaticus' inoculation. SK, Sunki mandarin; Cal, calamondin; KT, bitter orange; RL, Rangpur lime; RgL, rough lemon; Vol, Volkamer lemon; Ty, Troyer citrange; SW, Swingle citrumelo; Mac, alemow; Sev, Chinese box orange and WA, wood apple. The three leaves on the left of each set of four were all collected from the same plant; the leaf on the right is the healthy control. Leaves were photographed 1 year after infection. [Colour figure can be viewed at wileyonlinelibrary.com]

Table 2 Evaluation of the '*Candidatus* Liberibacter asiaticus' bacteria concentration in 1-year-old inoculated mature leaves of commercial citrus and citrus rootstock cultivars by TaqMan real-time PCR

	C_t mean	Absolute amount (copies)
Citrus cultivar		
Ponkan mandarin	20.96	8.31×10^6
Tonkan mandarin	22.82	2.40×10^6
Valencia sweet orange	25.16	5.01×10^5
Wentan pomelo	26.38	2.23×10^5
Eureka lemon	28.91	4.12×10^4
Kumquat	30.01	1.98×10^4
Ponkan mandarin healthy control	nd[a]	0
Tonkan mandarin healthy control	nd	0
Valencia sweet orange healthy control	nd	0
Wentan pomelo healthy control	nd	0
Eureka lemon healthy control	nd	0
Kumquat healthy control	nd	0
Rootstock cultivar		
Sunki mandarin	25.99	2.90×10^5
Calamondin	26.58	1.95×10^5
Bitter orange	26.61	1.91×10^5
Volkamer lemon	27.97	7.71×10^4
Rough lemon	28.86	4.26×10^4
Rangpur lime	29.09	3.66×10^4
Alemow	28.62	5.00×10^4
Chinese box orange	28.92	4.07×10^4
Troyer citrange	32.7	3.28×10^3
Swingle citrumelo	29.03	3.80×10^4
Wood apple	nd	0
Sunki mandarin healthy control	nd	0
Calamondin healthy control	nd	0
Bitter orange healthy control	nd	0
Volkamer lemon healthy control	nd	0
Rough lemon healthy control	nd	0
Rangpur lime healthy control	nd	0
Alemow healthy control	nd	0
Chinese box orange healthy control	nd	0
Troyer citrange healthy control	nd	0
Swingle citrumelo healthy control	nd	0
Wood apple healthy control	nd	0

[a]nd, not detected.

orange) showed a 40–50% decrease in growth rate in response to Las infection. Highly tolerant types, such as Troyer citrange and Swingle citrumelo, barely showed symptoms, and their growth rates had decreased 40–45% at 1 year after inoculation. Wood apple, as a resistant type, showed no symptoms and only an 18.75% decrease in the growth rate was observed under Las infection.

Tissue distribution and seasonal dynamics of Las

Comparisons of Las concentrations in different leaf and tissue parts of three Las-infected citrus cultivars are listed in Table 4. Similar results were obtained in each cultivar. High average Las concentrations were detected in mature and old leaves, whereas young leaves and new flush contained low Las concentrations. According to the PCR analysis, the Las concentrations, from high to low, were fruit, mature leaf, old leaf (completely yellowing), bark of young twig, root, new flush and bark of trunk. For the seasonal dynamics analysis, higher Las concentrations were detected in the cooler temperatures of autumn and spring, whilst lower concentrations of Las were detected in the high and low temperatures of summer and winter.

Periodic detection of graft inoculation and psyllid transmission of Las

Four Las graft-inoculated citrus cultivars (Ponkan mandarin, Valencia sweet orange, Wentan pomelo and Eureka lemon) were subjected to periodic detection of Las replication amounts on leaf midribs using real-time PCR. Las was detected after 21 days in Ponkan, 35 days in

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Table 3 Effect of huanglongbing (HLB) on the growth of commercial citrus and citrus rootstock cultivars in Taiwan

	Tree[a]	Original height (cm)	Average height in 1 year (cm)	Growth rate in 1 year (cm)	Disease index[b]
Citrus cultivar					
Ponkan mandarin	D	25	43.75	18.75	S
	H		85	60	H
Tonkan mandarin	D		39.5	14.5	S
	H		73	48	H
Valencia sweet orange	D		55.25	30.25	S
	H		90	75	H
Wentan pomelo	D		47	22	I
	H		87	62	H
Eureka lemon	D		88.75	63.75	I
	H		116	91	H
Kumquat	D		43.5	18.5	I
	H		57	32	H
Rootstock cultivar					
Sunki mandarin	D	50	68	18	S
	H		118	68	H
Calamondin	D		68.25	18.25	S
	H		122	72	H
Bitter orange	D		62.5	12.5	S
	H		110	60	H
Volkamer lemon	D		94	44	I
	H		125	75	H
Rough lemon	D		86	36	I
	H		120	70	H
Rangpur lime	D		95.75	45.75	I
	H		128	78	H
Alemow	D		98.5	48.5	I
	H		128	78	H
Chinese box orange	D		78	28	I
	H		103	53	H
Troyer citrange	D		102.25	52.25	M
	H		137	87	H
Swingle citrumelo	D		87.25	37.25	M
	H		118	68	H
Wood apple	D	40	52.5	32.5	H
	H		80	40	H

[a]H, healthy control; D, diseased.
[b]Disease index: S, severe; I, intermediate; M, mild; H, healthy.

Valencia sweet orange, 49 days in Eureka and 56 days in Wentan (Fig. 4a). The replication rates of Las in each cultivar showed similar trends. Las replicated more easily and faster in Ponkan and Valencia sweet orange than in Eureka and Wentan. In the first 2 months after graft inoculation, the replication rate of Las was faster in Valencia sweet orange than in Ponkan, but Las was detected earlier in Ponkan. The amounts of Las were nearly 10^4 copy numbers in Valencia sweet orange (2.34×10^4) and Ponkan (9.48×10^3) at 63 and 77 days after inoculation, respectively. The amounts of Las reached the highest level (1.9×10^7) at 154 days after inoculation. In contrast, the amounts of Las remained low (~10^3) in Eureka and Wentan during the first 7 weeks and increased up to 4.86×10^3 copy numbers at 5 months after graft inoculation. Symptoms were observed in Ponkan and Valencia sweet orange at 4–5 months after inoculation. The upper leaves of Ponkan showed vein yellowing, and small, hard and yellowing symptoms were observed on the young leaves at 6 months after inoculation. Similar symptoms were observed on the upper leaves of Valencia sweet orange and obvious vein yellowing was easily observed on new upper young leaves. In contrast, no obvious symptoms were observed in Eureka and Wentan at 6 months after inoculation (Fig. 5).

Las was detected earlier through psyllid transmission compared to graft inoculation at 21 days after infection (Fig. 4b). However, Las replicated slowly in Ponkan and Valencia sweet orange through psyllid transmission. The amounts of Las in Ponkan reached 1.33×10^3 copy numbers at 8 weeks after infection, whereas hundreds to thousands of copies of Las remained in Valencia sweet orange. At 4 months after infection on Ponkan, only the new young leaves on the ends of shoots showed small, hard leaves with vein yellowing symptoms. No obvious symptoms were observed on infected Valencia sweet orange (Fig. 5).

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Figure 3 Growth effects of 11 rootstock cultivars infected with '*Candidatus* Liberibacter asiaticus'. SK, Sunki mandarin; Cal, calamondin; KT, bitter orange; Vol, Volkamer lemon; RgL, rough lemon; RL, Rangpur lime; TC, Troyer citrange; SW, Swingle citrumelo; Mac, alemow; Sev, Chinese box orange; and WA, wood apple. The left plant of each pair is the healthy control. Photographs were taken 1 year after infection. [Colour figure can be viewed at wileyonlinelibrary.com]

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Table 4 Distribution and seasonal population dynamics of '*Candidatus* Liberibacter asiaticus' (Las) bacteria in three citrus species, Hong-Jian sweet orange, Murcott tangor and Peiyu pomelo

Season	Position	PCR index[a]		
		Hong-Jian	Murcott	Peiyu
Spring	New flush	±	±	−
	Young leaf	+	++	±
	Midrib of mature leaf	+++	+++	+++
	Mesophyll of mature leaf	++	++	++
	Midrib of old leaf	+++	nt	nt
	Fruit	+++	++ (flower)	+ (flower)
	Bark of young twig	+++	+++	++
	Bark of trunk	−	−	−
	Root	++	+	+
Summer	New flush	−	−	−
	Young leaf	±	±	±
	Midrib of mature leaf	+++	++	+++
	Mesophyll of mature leaf	++	++	++
	Midrib of old leaf	+++	nt	nt
	Fruit	+	+++	+++
	Bark of young twig	++	++	++
	Bark of trunk	−	−	−
	Root	+	+	+
Autumn	New flush	±	±	±
	Young leaf	++	++	+
	Midrib of mature leaf	+++	+++	+++
	Mesophyll of mature leaf	++	++	++
	Midrib of old leaf	+++	nt	nt
	Fruit	+++	+++	+++
	Bark of young twig	+++	+++	+++
	Bark of trunk	−	−	−
	Root	+	+	++
Winter	New flush	nt	nt	nt
	Young leaf	±	+	±
	Midrib of mature leaf	+++	+++	++
	Mesophyll of mature leaf	++	++	++
	Midrib of old leaf	+++	nt	nt
	Fruit	+++	+++	+++
	Bark of young twig	+++	+++	+++
	Bark of trunk	−	−	−
	Root	+	++	++

nt, not tested.

[a]PCR index: density count of the Las-specific band on agarose gel measured by a densitometer. Pixel value −, ≤14; ±, 15–49, +, 50–109; ++, 110–169; +++, 170–230. All values were determined as the average of four individual plants.

Quantitative detection of Las in citrus cultivars and Las-carrying percentages on psyllid populations in the field

The highest amounts of Las were detected in Murcott tangor (2.15×10^7), followed by Ponkan mandarin (2.42×10^6), Valencia sweet orange (1.06×10^6), calamondin (9.33×10^5), and Wentan pomelo (4.18×10^4; Table 5). These data indicated that each citrus cultivar had different responses to Las infection, resulting in varying levels of symptoms and proliferation. The average percentages of Las-carrying psyllids from the three infected fields ranged from 40% to 53.3% (Table 5).

Quantitative detection of Las and Las-carrying percentages on different instars of nymphs of psyllid

No Las was detected in the instars of egg and 1st nymph stages (Table 6). Tens and hundreds of copies of Las were detected in individual nymphs from the 2nd to 5th instars. The average percentage of Las-carrying instars of nymphs was 33.9%. These results indicated that the amounts of Las and Las-carrying individuals were both lower in nymphs than in adult psyllids.

Test of acquisition-access of Las in psyllids

The results of Las acquisition tests are summarized in Table 7. The amounts of Las (hundreds to thousands of copy numbers) had no obvious fluctuations within psyllids during the acquisition-access time. The percentages of Las-carrying psyllids were 40–60%, indicating that the increasing acquisition-access time did not affect the percentages.

Discussion

HLB, the main devastating and systemic disease on citrus worldwide, is caused by Las in most citrus planting areas. Thus far, strategies for the control of HLB rely on prevention methods, such as using tolerant rootstock cultivars, implementing a healthy seedling system, eliminating infected plants and intermediate hosts in the field and preventing insect vectors. However, the lack of knowledge regarding disease ecology and pathogen transmission might leave blind spots when establishing control methods. In the present study, the susceptibility/tolerance to HLB of commercially important citrus and rootstock cultivars was determined and the dynamic population of Las in citrus hosts or psyllids was demonstrated using conventional PCR (Hung *et al.*, 1999) and TaqMan real-time PCR (Feng *et al.*, 2015). The TaqMan real-time PCR developed here showed high specificity and sensitivity to the amounts of Las in citrus plants or even in one or a few psyllids. The present study provided more information on HLB based on the application of a quantitative detection method for tracking the dynamic proliferation of Las in citrus and psyllids.

Susceptibility/tolerance testing was carried out on the main commercial citrus and common rootstock cultivars in Taiwan. The determinations were based on the replication rates of Las, symptom severity and the growth rate on infected plants. Measures were taken to ensure that all experimental plants avoided any viral interference. Similar results between Las-infected plants in the

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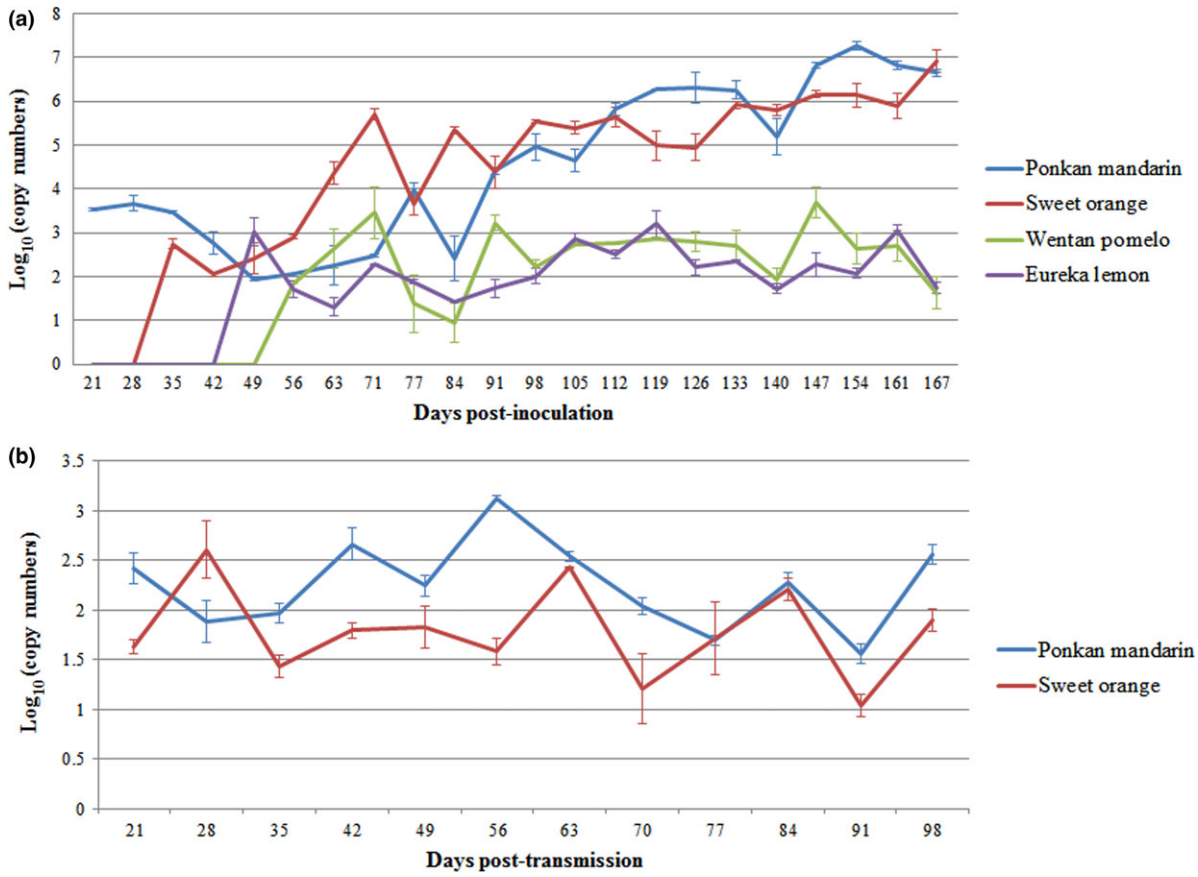

Figure 4 Quantitative detection using real-time PCR of the dynamic replication of '*Candidatus* Liberibacter asiaticus' (Las) in (a) four Las-inoculated citrus cultivars (Ponkan mandarin, Valencia sweet orange, Wentan pomelo and Eureka lemon) after graft inoculation, and (b) two citrus cultivars (Ponkan mandarin and Valencia sweet orange) after psyllid transmission. [Colour figure can be viewed at wileyonlinelibrary.com]

laboratory or field showed that Ponkan and Tankan mandarins are susceptible to Las and are unable to grow after infection. Valencia sweet orange is less susceptible, whereas Wentan pomelo and Eureka lemon are tolerant to Las and are able to grow slowly. Kumquat is more tolerant but is still infected through inoculation. The present study confirmed that Las strain II, isolated from the field in a previous study, showed high virulence and multiplied quickly in all commercially important citrus cultivars in Taiwan (Tsai *et al.*, 2008). The present study also illustrated a positive relationship between HLB symptom severity on citrus leaves and the proliferation rate of Las using PCR detection (Coletta-Filho *et al.*, 2010). In addition, more susceptible cultivars had much higher concentrations of Las. Based on the present pathological study, more molecular evidence is needed to elucidate the factors involved in different levels of susceptibility/tolerance of various cultivars while facing Las infection. It is assumed that different citrus cultivars might show differing gene expressions or responses against Las infection. Previous studies have also shown that many metabolites were detected at higher concentrations in the tolerant cultivars and might play roles in conferring tolerance to HLB (Albrecht *et al.*, 2016).

The analysis of the amounts of Las in different tissues and the seasonal population dynamics in host plants showed that Las was unevenly distributed in different tissues (Tatineni *et al.*, 2008; Li *et al.*, 2009). The effect of live or dead populations of Las was not considered in this study. According to Hu *et al.* (2014), the population trends between live and dead Las were similar (although with different titres) and so it was assumed that it would show little effect on the results. The highest amounts of Las were detected in the transportation tissues of fruit in every citrus cultivar. These results might explain the typical symptoms of Las-infected fruits, such as greening and thick skin, resulting in poor quality fruits. In contrast, no Las was detected in the bark of the stem base of citrus, suggesting that Las might not accumulate in the main transportation region of the stem base. It is assumed that new flush retained good growth potential and that Las moved too slowly to invade newly growing leaves. The uneven distribution of Las in different tissues might be because nutrition-rich propagated or metabolic tissues such as fruit, young and mature leaves are beneficial to Las proliferation and accumulation. In addition, previous studies analysing the dynamic population of Las revealed that the Las population decreased in the spring (Wang

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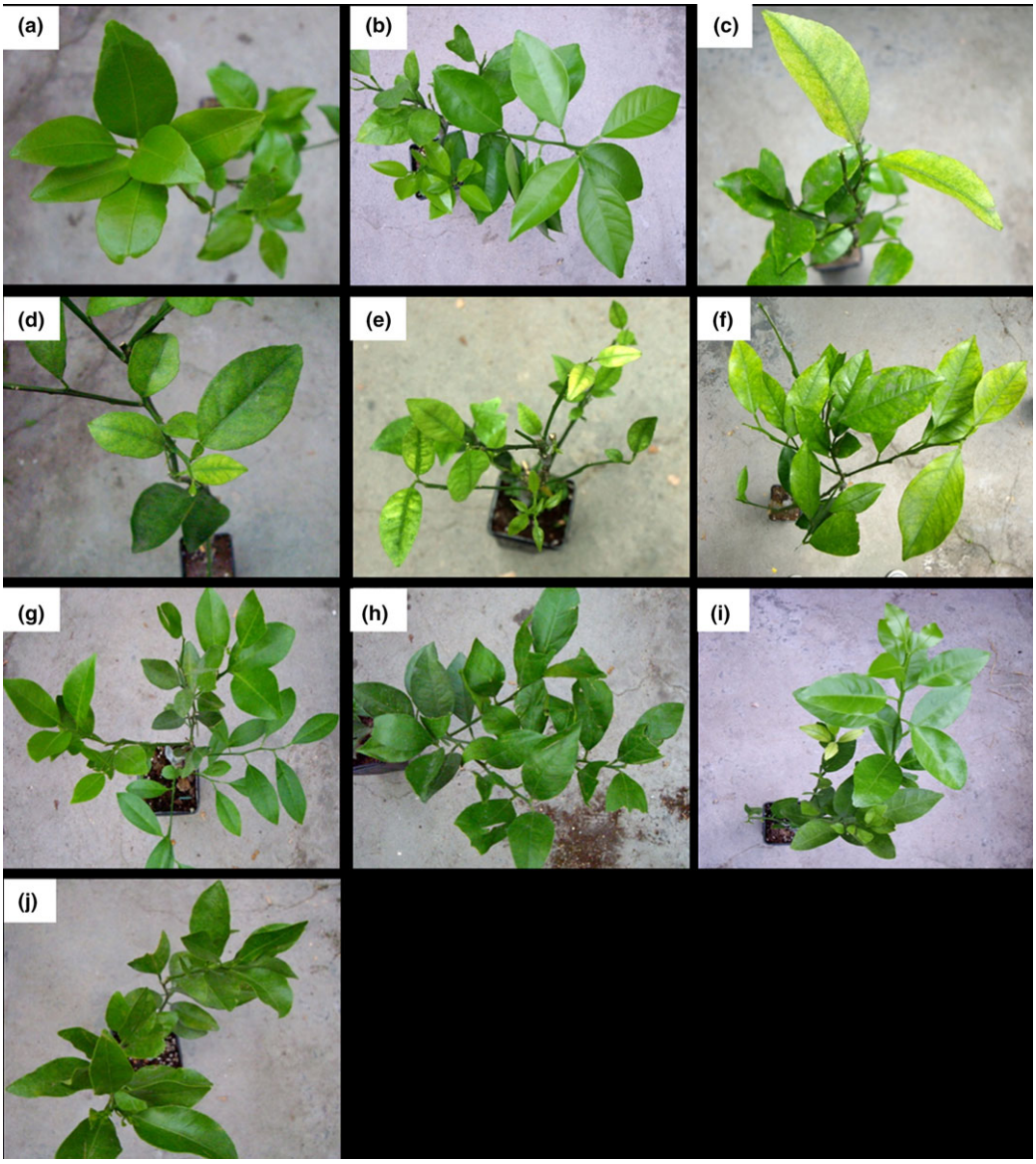

Figure 5 Symptom development on Ponkan mandarin (PM) and Valencia sweet orange (SO) infected with '*Candidatus* Liberibacter asiaticus' (Las) after graft inoculation and psyllid transmission. (a) PM before graft inoculation; (b) PM 3 months after graft inoculation; (c) PM 6 months after graft inoculation; (d) SO before graft inoculation; (e) SO 3 months after graft inoculation; (f) SO 6 months after graft inoculation; (g), PM 2 months after psyllid transmission; (h) PM 4 months after psyllid transmission; (i) SO 2 months after psyllid transmission; and (j) SO 4 months after psyllid transmission. [Colour figure can be viewed at wileyonlinelibrary.com]

Table 5 Quantitative detection of '*Candidatus* Liberibacter asiaticus' (Las) in different Las-infected citrus cultivars and determination of Las-carrying percentage on psyllid populations in fields by real-time PCR

Citrus cultivar	Source	Average of Las quantities in citrus (copies)	Average of Las quantities in psyllid (copies)[a]	Las-infected psyllids (%)
Ponkan mandarin	Chuchi village	2.42×10^6	3.41×10^5	42.9
Murcott tangor	Chiayi city	2.15×10^7	4.25×10^2	40.0
Valencia sweet orange	Chuchi village	1.06×10^6	1.98×10^4	53.3
Wentan pomelo	Chuchi village	4.18×10^4	–	–
Calamondin	Taipei city	9.33×10^5	–	–

[a]7, 5 and 38 psyllids were collected from Ponkan, Murcott and Valencia cultivars, respectively. No psyllids were found on Wentan or calamondin in the field survey. Mean copy numbers were obtained with triplicate assays for each sample extract by real-time PCR.

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Table 6 Quantitative determination of '*Candidatus* Liberibacter asiaticus' (Las) and Las-carrying percentage on different instars of psyllid nymphs collected from Las-infected sweet oranges in fields by real-time PCR

Instar	Numbers of psyllid tested	Average of Las quantities in psyllid (copies)	Las-infected psyllid (%)
Egg	10	0	0.0
1st	15	0	0.0
2nd	10	6.5	10.0
3rd	9	42.8	22.2
4th	6	331.1	66.7
5th	19	33.3	36.8

et al., 2006) or winter (Hu *et al.*, 2014), which differed from the data obtained in the present study. This finding suggests that different sampling methods and environments might affect investigations of the Las population. Generally, Las was easily detected in new flush in the spring and autumn rather than in the summer. This result agrees with Hung (2006), who illustrated the wide spread of HLB disease during the spring was because the highest populations of psyllid were observed in the sprouting period of Murcott tangor in the spring. Psyllids migrated and transferred Las much more frequently during this period time.

Previous studies have indicated that different citrus cultivars have different susceptibility or tolerance levels to HLB disease (Garnier *et al.*, 1984; Bové & Garnier, 2002). Similar results showed that Ponkan and Valencia sweet orange were more susceptible to HLB and Las using PCR at 3 and 5 weeks after grafting, respectively. In contrast, Las was detected at 7 and 8 weeks after grafting in Eureka and Wentan, respectively. Real-time PCR showed greater sensitivity for detecting Las earlier in these citrus hosts than conventional PCR, and Las was also amplified at different rates in each host. Although the uneven distribution of Las in different tissues while sampling (Huang, 1987) might lead to unreliable detection, the long-term detection still showed an obvious trend in Las amplification. Compared with the Las-grafting method, psyllid transmission resulted in faster Las amplification in citrus hosts during early infection, but the amounts of Las were lower after 3 months. The difference between grafting and psyllid inoculations might reflect different pathways of Las infection. Las could directly enter sieve tube cells and subsequently replicate via the stylet of the psyllid, whereas graft-inoculated Las could be slower to move in to the host after the graft heals. The amounts of Las in infected scions were 100 times greater than those in psyllids, indicating the different replication rates between these two vectors.

The results of the Las acquisition test in psyllids, which were similar to a previous study (Hung, 2006), indicated that continuing to acquire Las day after day did not obviously enhance the amounts or the Las-carrying percentages in psyllids. Even if healthy psyllids were forced to acquire Las on infected plants for an extended

Table 7 Quantitative detection of '*Candidatus* Liberibacter asiaticus' (Las) in psyllids individually with different acquisition-access time by real-time PCR

	Acquisition time (days)													
	2	4	6	8	10	12	14	16	18	20	22	24	26	28
Psyllid	Amount of Las [log(copy numbers)]													
1	0	0	2.32	0	2.27	2.84	0	2.21	0	0	0	2.00	1.65	3.14
2	2.40	0	2.58	2.13	1.87	2.16	0	0	0	0	0	2.09	0	0
3	0	2.33	0	0	0	0	0	2.17	0	0	0	1.83	0	2.58
4	2.22	0	3.42	0	0	0	0	0	2.46	2.58	1.90	0	0	0
5	2.35	3.01	1.55	0	0	0	2.16	0	2.52	2.76	1.89	0	2.61	0
6	0	3.21	1.88	0	0	2.26	0	2.16	0	2.23	3.13	0	0	2.07
7	2.16	2.75	0	2.27	1.78	0	2.01	0	0	2.60	1.84	1.82	1.63	2.42
8	0	0	0	2.04	0	1.90	2.27	2.56	2.11	2.87	0	1.89	1.44	0
9	0	0	0	0	2.10	0	2.33	0	3.54	0	1.97	2.15	1.63	2.48
10	0	0	0	2.36	2.16	0	2.41	1.58	0	0	0	0	0	0
Mean	2.29	2.93	2.82	2.22	2.07	2.34	2.26	2.24	3.02	2.66	2.52	1.98	2.05	2.69
Psyllids with Las (%)	40	40	50	40	50	40	50	50	40	50	50	60	50	50

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period, only a portion of the population would successfully acquire Las (Manjunath *et al.*, 2008). In contrast, the amounts of Las inside psyllids collected from infected fields were hundreds and thousands of times greater than in the psyllids from the acquisition test. However, the Las-carrying percentage of psyllids from the field also remained near 40–53.3%, showing results similar to those of psyllids from the acquisition test. According to real-time PCR detection, only 5.71% of these Las-carrying psyllids had Las copy numbers in the thousands. This phenomenon might be related to the feeding habits of psyllids. Similar evidence was also reported by Pelz-Stelinski *et al.* (2010). Ammar *et al.* (2017) demonstrated that the salivary glands and midgut of psyllids may act as transmission barriers that can impede translocation of Las within the vector, and this evidence could explain the low Las infection rate in vector psyllid. In addition, the results of detecting psyllid nymphs on infected citrus plants in the field using PCR revealed that nymphs (2nd to 5th instars) could carry Las, consistent with a previous study (Hung *et al.*, 2004). Furthermore, it was observed that the amounts of Las were lower in nymphs than in adult psyllids, indicating that Las could continually replicate during psyllid growth. These results were similar to those of a previous study showing high replication rates of Las in the nymph stages of psyllids (Ammar *et al.*, 2016b). It is concluded that most psyllids carried Las at the instar nymph stage and that Las replicates to high concentrations in adult instars, showing the capacity to transmit Las in the fields.

The present study evaluated the levels of susceptibility of different citrus and rootstock cultivars to HLB, which might help the establishment of healthy seedling systems to select proper tolerant cultivars as rootstocks. Hung (2006) showed that only one Las-carrying psyllid could transmit Las to infect citrus plants. However, only 12.9% of psyllids could successfully infect plants, and the amounts of Las in each psyllid were significantly different, consistent with the results of a previous study (Ukuda-Hosokawa *et al.*, 2015). The data also showed that a low proportion of nymph or adult psyllids in the population carried high amounts of Las, suggesting that the successful Las transmission rate was associated with the amounts of Las in each psyllid and the numbers of psyllid populations. Monitoring and decreasing the psyllid population numbers could effectively reduce the chance of Las-carrying individuals infecting citrus plants. This study, based on quantitative methods, is relevant not only to research on Las pathogenicity in citrus hosts or in psyllid vectors but also to efforts to enhance the current understanding of HLB disease ecology.

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A Note from the Senior Editor

Matt Dickinson

We would like to take this opportunity to acknowledge the hard work of all our Editorial Board members, technical readers and production team, and also the efforts of all the anonymous reviewers that we have used during the past year. We thank them all for their invaluable contributions, and for helping to maintain the standards and continued success of the Journal. In 2017, Richard Cooper, Monica Höfte and Tobin Peever stepped down from our Editorial Board and we particularly thank them for their work over many years.

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