Detection by PCR of *Candidatus* Liberibacter asiaticus, the bacterium causing citrus huanglongbing in vector psyllids: application to the study of vector–pathogen relationships

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Citrus huanglongbing (HLB), previously called greening, is a serious citrus disease in Asia, eastern and southern Africa. It is caused by *Candidatus* Liberibacter asiaticus (Las), a phloem-limited, nonculturable bacterium transmitted by the Asian citrus psyllid (*Diaphorina citri*) in Asia. A PCR-based assay was developed for monitoring Las in vector psyllids using a rapid DNA extraction from psyllid bodies and PCR amplification. The entire procedure for Las detection in psyllids can be completed within 5 h. Using this method, Las can be accurately detected in psyllid adults as well as nymphs in different instar stages. The assay is sensitive enough for Las detection in single-psyllid extract from adult, fifth, fourth and third instars. In a transovarial transmission experiment, Las was not detected in eggs or in offspring produced by Las-carrying psyllid females. In a retention test, the Las-carrying psyllids remained Las-positive for 12 weeks after they were moved to common jasmine orange, a Las-immune plant. From these experimental results it was concluded that Las persists in the Asian citrus psyllid vector, but is not transovarially transmitted by the vector. These data help in understanding epidemiological characteristics of Las and psyllids in citrus HLB.

Keywords: Asian citrus psyllid, *Candidatus* Liberibacter asiaticus, citrus huanglongbing, mycoplasma, phytoplasma

Introduction

Citrus huanglongbing (HLB), also known as citrus greening, is a severe citrus disease, especially in Asian countries. It is caused by nonculturable bacteria of the *Candidatus* genus Liberibacter (Garnier *et al.*, 2000) that inhabit citrus phloem, retarding growth of the plant and causing the incomplete colouring of mature citrus fruits (da Graca, 1991). In Asia the pathogen of HLB was categorized as the *Candidatus* species Liberibacter asiaticus (Las) (Jagoueix *et al.*, 1997; Garnier *et al.*, 2000). Las infects most citrus cultivars and causes substantial economic losses by shortening the lifespan of infected trees (Miyakawa, 1980). It is spread by vegetative propagation and insect vectors, two reasons why HLB has become such a difficult disease to control. HLB can be categorized into two types, Asian and African, based on the influence of temperature on host symptoms. The Asian type, in which HLB symptoms

can occur at temperatures above 30°C, is heat-tolerant; and the African type, in which no symptoms appear above 30°C, is heat-sensitive (Bové *et al.*, 1974). Although the fastidious bacterium causing HLB has not yet been successfully cultured *in vitro*, it has been characterized by molecular methods and tentatively assigned to the genus *Candidatus* Liberobacter (Jagoueix *et al.*, 1994; Murray & Schleifer, 1994). By comparison of the 16S/23S rDNA sequences and ribosomal protein genes, strains composing *Ca.* Liberobacter were further categorized as two different species, *Ca.* Liberobacter asiaticum for the Asian pathogen and *Ca.* Liberobacter africanum for the African pathogen (Jagoueix *et al.*, 1997). Following the rules of the International Code of Nomenclature of Bacteria, the two bacterial species have now been renamed *Ca.* Liberibacter asiaticus and *Ca.* Liberibacter africanus (Garnier *et al.*, 2000). The Asian citrus psyllid, *Diaphorina citri*, is the vector of *Ca.* Liberibacter asiaticus (Capoor *et al.*, 1967; Chen *et al.*, 1973) and the African citrus psyllid, *Trioza erytreae*, for *Ca.* Liberibacter africanus (McClean & Oberholzer, 1965). HLB has been the most important factor limiting citrus production in Asia.

Detection of Las is difficult because of its low concentration and uneven distribution in its citrus hosts

(McClean, 1970; Huang, 1979). A rapid and sensitive assay based on the polymerase chain reaction (PCR) has been developed for Las detection in citrus plants (Jagoueix *et al*., 1996; Hocquellet *et al*., 1999; Hung *et al*., 1999b). However, detailed research associated with monitoring Las in the Asian citrus psyllid is rare (Bové *et al*., 1993). In this study, a simple method sufficient for Las detection in psyllids was developed by combining rapid preparation of DNA extracts from psyllid bodies and a PCR assay. This method is sensitive enough to detect Las in a single adult psyllid. Use of this sensitive Las-detection method in psyllids enabled research to be conducted on vector–pathogen relationships. Detection of Las in psyllids reported in this paper is applicable for monitoring Las in adult and nymphal psyllids, and was also a useful tool for studying transovarial transmission and Las retention in adults. The results reported here complement earlier psyllid–Las relationships that were carried out by traditional bioassays and electron microscopy (Xu *et al*., 1988). Understanding the vector–pathogen relationship of psyllids should facilitate control of HLB disease.

Materials and methods

Sources of psyllids and plant materials

The original source of psyllids (*D. citri*) in this study was from a common jasmine orange (CJO, *Murraya paniculata* var. *paniculata*). According to previous studies (Miyakawa, 1980; Garnier & Bové, 1993; Hung *et al*., 2000), CJO is a host for psyllids but is immune to Las. Collected psyllids were transferred to another CJO in an insect-proof cage at 25°C for rearing. The CJO plant was trimmed monthly to stimulate new growth, based on a previous report (Lin *et al*., 1973) that the sprouting of CJO induces the oviposition of psyllids to produce many offspring.

A Las-infected Luchen sweet orange (LSO, *Citrus sinensis*, 50 cm tall, 1 year old) was used as the source of Las. A healthy LSO (50 cm tall, 1 year old) obtained by the shoot-tip grafting technique (Murashige *et al*., 1972) was used as negative control in this study.

Acquisition of Las by adult psyllids

A total of 50 Las-free adult psyllids were transferred to the Las-infected LSO and placed in an insect-proof cage at 25°C for acquisition feeding. Because adult psyllids tend to feed on the very young parts of hosts where the level of Las is low (McClean, 1970; Huang, 1979), young shoots of the Las-infected LSO were removed, forcing the insects to feed on mature leaves and enhance acquisition. An aspirator was used to collect and transfer psyllids. After 2 weeks the psyllids were collected for DNA extraction and Las detection. As a negative control, a further 50 adult psyllids were transferred from a CJO to a healthy LSO and subjected to the same treatments and tests.

Collection of nymphal psyllids for Las detection

One hundred adult psyllids reared on the CJO plant were transferred to a Las-infected LSO plant with abundant young shoots to accelerate egg production. The adult psyllids were removed after 4 weeks. Nymphs that hatched from these eggs on the diseased LSO plant were collected for Las detection. The five nymphal instars of Asian citrus psyllid can be differentiated by their distinct morphological characteristics (Lin *et al*., 1973; Yang, 1984). Nymphs were collected with a fine-tip brush and placed in an Eppendorf tube for DNA extraction. As a negative control, another 100 adult psyllids from the healthy CJO plant were transferred to a healthy LSO plant. Nymphs from eggs on the healthy LSO plant were collected and tested as the corresponding negative controls.

Transovarial passage tests

Two experiments were devised for the transovarial passage tests. The objective of the first experiment was to determine if Las could be detected in eggs. After a 2 week acquisition feeding period on the Las-infected LSO plant, 50 adult females were moved to a CJO plant with many young shoots to facilitate oviposition. Twenty adult females were collected for Las detection from this egg-laying population. Their eggs were then collected from shoots of the CJO plant and used for PCR assay. Eggs were combined and used for DNA extractions to counteract the low efficiency of DNA extraction from single eggs. Total DNA was extracted from one egg or from batches of five, 10, 25, 50 and 100 eggs, and the amount of DNA for each sample was determined using a spectrophotometer (GeneQuant II RNA/DNA Calculator, Pharmacia Biotech, Cambridge, UK). The DNA extracted from single and multiple egg samples was analysed by PCR. The experiment was replicated four times.

The objective of the second experiment was to monitor the presence of Las in psyllid offspring. The offspring were produced by the Las-carrying psyllids as described above. After oviposition, all the Las-carrying adult females were removed from the CJO plant. The eggs were kept on CJO shoots for hatching to produce psyllid offspring. After developing into adults, they were subjected to the Las test. The experiment was replicated four times.

Retention tests of psyllids for Las carrying

After a 2-week acquisition-feeding period, 220 Las-carrying adult psyllids on the diseased LSO plant were collected for the Las-retention tests. Psyllids were transferred to a CJO plant in an insect-proof growth chamber at 25°C. Immediately before they were transferred, 20 adults were tested for Las to determine the percentage of the population carrying Las. All CJO shoots were removed to avoid offspring production. To determine whether citrus psyllids retain Las in a persistent, semipersistent or nonpersistent manner, 20 adults were taken from the population and tested for Las at 2, 4, 6, 8, 10 and 12 weeks after transfer. Four experimental replicates were carried out.

DNA extraction from psyllid bodies and citrus tissues

For the detection of Las in psyllids, a simple protocol was designed to extract DNA from psyllids for the PCR test. Each psyllid was put in a 1·5 mL Eppendorf tube containing 300 μL DNA extraction buffer (0·1 M Tris-HCl pH 8·0, 0·05 M EDTA, 0·5 M NaCl, 1% *N*-lauroylsarcosine), 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 and 500 μL 100% ethanol followed by centrifugation at 12 000 *g* at 4°C for 10 min. The pellet was dried and resuspended in 15–50 μL volume of distilled water to serve as template for PCR amplification. The amount of extracted DNA was measured using a spectrophotometer for quantification of the DNA template. DNA extracts from citrus tissues were prepared using the method described by Hung *et al.* (1999b).

Primers and thermal cycles

A primer pair (5′-CAC CGA AGA TAT GGA CAA CA-3′; 5′-GAG GTT CTT GTG GTT TTT CTG-3′) for PCR-based detection of Las was derived from the sequence of a cloned Las-specific DNA fragment (Hung *et al.*, 1999a) and designed to amplify a Las-specific DNA fragment (226 bp) by PCR. The assay has proven to be Las-specific and sensitive for Las detection in citrus tissues (Hung *et al.*, 1999b). 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 units *Taq* DNA polymerase (Invitrogen, Carlsbad, CA, USA) 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; followed by a 72°C extension for 10 min. Reactions were carried out in a DNA Thermal Cycler 2400 (Perkin Elmer, Norwalk, CT, USA).

Analysis of PCR products by electrophoresis

PCR products were analysed by gel electrophoresis using 1·4% agarose in TAE buffer (40 mM Tris-acetate, 1 mM EDTA pH 8·0). After electrophoresis (100 V for 30 min), products in the gel were stained with ethidium bromide (0·5 μg mL^{-1}), visualized and analysed using the ALPHAIMAGER 2000 Documentation & Analysis System (Alpha Innotech Co., San Leandro, CA, USA). A 100 bp DNA ladder set (Promega, Madison, WI, USA) was included to determine fragment size.

Results

Las detection in single and multiple adult psyllids

Forty-four adult psyllids on the Las-infected LSO plant were collected for DNA extraction as well as Las detection after a 2-week acquisition-feeding period. Las-carrying psyllids were detected by PCR assay using the Las-specific

Figure 1 Detection of Las in single and multiple adult psyllids by PCR using a Las-specific primer pair. The template was 250 ng DNA extracted from 1, 3, 5, 10 and 25 adults. PCR products were analysed by electrophoresis in a 1·4% agarose gel, and positive results were recognized by the appearance of the Las-specific 226 bp band. DNA samples extracted from one, three, five, 10 and 25 adults inhabiting the Las-infected citrus plant were Las-positive (lanes 1, 3, 5, 7 and 9, respectively), whereas those from one, three, five, 10 and 25 adults inhabiting the healthy citrus plant were Las-negative (lanes 2, 4, 6, 8 and 10). Tissues from Las-infected and healthy citrus leaves were included in this test (lanes 11 and 12) for comparison. Lane M, 100 bp DNA ladder.

primer pair with our DNA extraction procedure. DNA extracts were prepared from single or multiple (three, five, 10, 25) adults. The results of Las detection in single and multiple psyllid adults are shown in Fig. 1. A PCR product of correct size was amplified from all psyllids from Las-infected plants, whereas no fragment was obtained from psyllids exposed to healthy plants. Las detection in an individual adult showed an equal signal to that in multiple adults when the DNA template of PCR is standardized (Fig. 1). Signal strength from PCR-amplified fragments on the gel did not appear to differ among Las detections in one, three, five, 10 and 25 adults.

Las detection in nymphal psyllids

The different nymphal instars collected from a Las-infected citrus plant were separated and tested for the presence of Las. Before PCR was conducted, the quantity of DNA extract from different instars was estimated by a spectrophotometer (Table 1). Detectable amounts of DNA were obtained from individual nymphs of the third, fourth or fifth instars. According to the average results of 10 single-nymph extractions, approximately 250 ng DNA could be obtained from a third instar; 360 ng from a fourth instar; and 1040 ng from a fifth instar. However, DNA from individuals of the first and second instars was undetectable. To gather more DNA from the first two instars, DNA was extracted from multiple nymphs. A minimum of either five second instars or 10 first instars was necessary to obtain enough DNA ($\approx$200 ng) for spectrophotometer monitoring.

Las was detected in individual third, fourth and fifth instars (Fig. 2). Ten nymphs of each different instar were collected for the individual tests. Seven, six and four individuals in tests of fifth, fourth and third instars, respectively, were Las-positive. Las was not detected in individuals of the second or first instars. The total DNA extracted from individual first and second instars may not be enough to serve as PCR templates. The results in Fig. 3

Table 1 Amount of DNA obtained from individual or multiple eggs, nymphs and adults of Asian citrus psyllid (*Diaphorina citri* Kuwayama) using a rapid DNA extraction method

| Number of psyllids tested | Amount (μg) of DNA extract[a] | | | | | | |
| | Egg | Instar | | | | | Adult |
		1st	2nd	3rd	4th	5th	
1	–[b]	–	–	0·25	0·36	1·04	9·18
				(0·02)	(0·04)	(0·09)	(0·83)
5	–	–	0·68	1·52	2·17	5·21	42·24
			(0·06)	(0·13)	(0·18)	(0·42)	(2·42)
10	–	0·23	1·50	2·83	3·25	10·11	88·91
		(0·02)	(0·12)	(0·22)	(0·25)	(0·61)	(4·56)
25	0·18	1·00	3·73	6·85	7·35	24·35	228·52
	(0·02)	(0·08)	(0·30)	(0·48)	(0·50)	(1·23)	(16·07)
50	0·45	2·41	6·66	13·51	15·89	47·13	NT[c]
	(0·03)	(0·15)	(0·41)	(0·81)	(0·83)	(2·83)	
100	1·20	4·38	12·34	25·72	30·13	91·04	NT
	(0·08)	(0·24)	(0·59)	(1·52)	(1·62)	(6·27)	

[a]Means (SD) of 10 replicates of eggs, nymphs or adults. The amount of DNA extract was calculated from the OD_{260}.
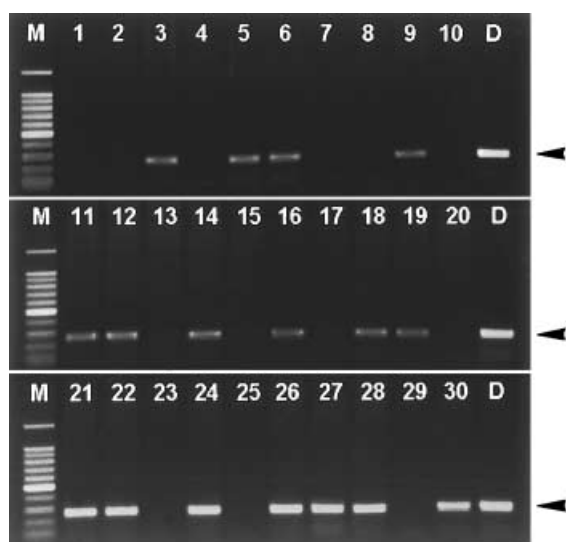[b]–, not detectable.
[c]NT, no test.

Figure 2 PCR detection of Las in individual psyllids of the third, fourth and fifth instars. Approximately 250 ng of DNA extract was used for the PCR template of each sample. PCR products were analysed by electrophoresis in a 1·4% agarose gel, and positive results were recognized by the appearance of the Las-specific 226 bp (arrowed) band. Ten nymphs of each different instar inhabiting the Las-infected citrus plant were collected for the individual tests. Lane M, 100 bp DNA ladder; lanes 1–10, 10 randomly sampled nymphs of the third instar; lanes 11–20, 10 randomly sampled nymphs of the fourth instar; lanes 21–30, 10 randomly sampled nymphs of the fifth instar; lane D, a Las-infected citrus sample as positive control.
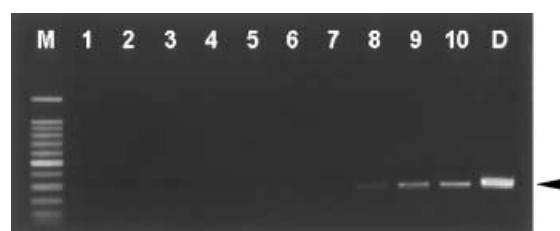

Figure 3 PCR detection of Las in single and multiple first and second instar psyllids. The template DNA for PCR was extracted from one, five, 10, 50 and 100 nymphs colonizing the Las-infected citrus plant. All the DNA was used for PCR templates in the samples of one, five and 10 first instars as well as single second instars. Approximately 250 ng DNA was used for PCR templates in the other samples. PCR products were analysed by electrophoresis in a 1·4% agarose gel, and positive results were recognized by the appearance of the Las-specific 226 bp (arrow) band. In the first instar the samples of one, five, 10, 50 and 100 nymphs were Las-negative (lanes 1–5, respectively). In the second instar, Las was not detected in the samples of one and five nymphs (lanes 6 and 7), whereas Las was detected in samples of 10, 50 and 100 nymphs (lanes 8–10). Lane D, a Las-infected citrus sample. Lane M, 100 bp DNA ladder for size markers.

show that Las could be detected in the second instar when 10 or more nymphs were combined for DNA extraction. However, samples of first instars were always negative for Las, even when 100 nymphs were extracted for PCR. Standardization of PCR template prepared from 25 nymphs resulted in Las detection in different instars (Fig. 4). Las was detected from the second to fifth instars, but the signal strength of the PCR band observed in the gel varied among the four different instars. The signal was faint for the second instar, moderate for the third, robust for the fourth, and the strongest for the fifth instar. Compared to detection in the adult sample, the fifth instar had an equally strong signal.

Tests for transovarial passage of Las

To determine whether transovarial passage of Las occurs in *D. citri*, the eggs oviposited by Las-carrying females

| Week(s)[a] | Percentage of Las-carrying psyllids[b] in populations A–E[c] | | | | | Table 2 Retention of Las by adult psyllids over time |
	A	B	C	D	E	
0[d]	75	70	65	55	75	
2	75	75	60	60	70	
4	65	65	60	55	65	
6	70	70	55	60	65	
8	65	70	65	50	60	
10	75	60	55	50	70	
12	70	70	60	50	75	

[a]Tests were conducted every 2 weeks after the psyllid population was transferred to a Las-immune common jasmine orange (CJO) plant.
[b]Twenty adults were sampled and tested for Las from a population of adult psyllids each time.
[c]Five populations (A–E) of adult psyllids were collected from different Las-infected citrus plants for Las.
[d]In week 0 the test was conducted immediately before the psyllid population was transferred to a CJO plant.

Figure 4 Comparative sensitivity of PCR detection of Las in different instars and adults of the psyllids. DNA was prepared from 25 psyllids, and 250 ng of DNA was used for the PCR template. The Las-specific 226 bp (arrow) band was amplified from samples of the second (lane 3), third (lane 5), fourth (lane 7) and fifth (lane 9) instars, as well as from adults (lane 11) reared on the Las-infected citrus plant. Las could not be detected in the sample of the first instar from the diseased citrus host (lane 1). The samples of nymphal and adult psyllids collected from the healthy citrus plant were the corresponding negative controls (lanes 2, 4, 6, 8, 10, 12 for first, second, third, fourth, fifth instars and adults, respectively). Lane M, 100 bp DNA ladder for size markers.

were tested by the PCR method described above. Twenty adult females were sampled and tested for Las before the egg test. Fifteen (75%) females tested positive for Las, but Las was not detected in their eggs. Las detection in an individual or five, 10, 25, 50 and 100 egg batches was negative. Spectrophotometric measurement indicated that the DNA extracts from 25 or more eggs were enough to obtain the detectable value of OD_{260} (Table 1). Approximately 180 ng of DNA extracts was obtained from 25 eggs; 450 ng from 50 eggs; and 1200 ng from 100 eggs. However, the PCR fragment of Las could not be amplified from DNA derived from multiple eggs.

Another experiment for transovarial passage was the offspring test. The eggs produced by Las-carrying females on a CJO plant were kept in an insect-proof chamber to produce offspring after removing all the parental psyllids. Six weeks later, a total of 45 adults emerged. Las was not detected by PCR in any of the offspring (data not shown).

Las-carrying retention test of adult psyllids

Results of the retention test are shown in Table 2. Twenty adult psyllids collected from the Las-carrying population (220 adults) were tested for Las immediately before the retention test, and 15 of 20 (75%) adults were Las-positive (population A). The percentage of Las-carrying adults did not differ significantly over the 12 weeks. The proportion of Las-carrying adults in population A, for example, was 65–75% during the 12-week experimental period. The experiments were repeated four times (psyllid populations B–E), with similar results. Adult psyllids had Las in their bodies for more than 12 weeks even though they were moved to a Las-immune plant (CJO).

Discussion

The newly devised method described here is efficient for Las detection in psyllids. It consists of three major steps: extraction of total nucleic acids from psyllid bodies; PCR amplification; and analysis of PCR products by electrophoresis. The entire procedure can be completed within 5 h: 1·5 h for extraction of nucleic acids; 3 h for PCR; 0·5 h for electrophoresis. The process is more efficient than the protocol designed for Las detection in citrus tissues (Hung *et al.*, 1999b). The entire procedure takes 6–7 h for Las detection in citrus tissues. This method is simple, efficient, and can be used on a large number of psyllid samples in a short period of time.

The highly sensitive PCR-based assay can detect Las in a single adult psyllid. The signal strength of the PCR band on gel obtained from the single-adult test is similar to that obtained from multiple-adult tests. Thus this assay should be helpful in determining the proportion of Las-carrying individuals in a field population of psyllids. The method is currently being used to study the seasonal dynamics of Las in psyllid populations on citrus orchards in Taiwan.

Las could be detected in nymphal psyllids inhabiting diseased citrus plants. This shows that both adult and nymphal psyllids can carry Las in their bodies, agreeing

with the previous bioassay results (Xu *et al*., 1988). Previous research demonstrated that Las-carrying adult psyllids were effective vectors that transmitted Las to host plants. Plants inoculated by Las-carrying adults tested Las-positive by the PCR assays 2 months after inoculation, and showed evident HLB symptoms 8 months after inoculation (Hung *et al*., 2001). Although the nymphs hardly move, they soon become Las-carrying adults with the ability to fly and transmit Las to other citrus plants. Thus the control period of vector psyllid should include the nymphal stages.

The single-psyllid PCR-based test is also suitable for screening third, fourth and fifth instars (Fig. 2). However, Las was not detected using individual first or second instars. The amount of DNA extracted from individuals of the first and second instars was too low to monitor using a spectrophotometer. The multiple-nymph test was therefore adopted for Las detection in the first two instars. Las could be detected when 10 or more second-instar nymphs were combined in one sample for DNA extraction (Fig. 3). This indicates that psyllids acquired few Las through acquisition feeding between the first and second instars. Las was always detected in samples of 10 nymphs of the second instar which were kept on Las-infected citrus shoots, whereas Las was not detected in any of the multiple-nymph first instar samples. Based on these data, it is concluded that psyllids can carry Las in either adult or nymphal stages, except in the first instar.

Las detections among five instars showed different signal strength in the PCR-based assays (Fig. 4). The signal strength of the PCR band on the gel increased with each instar. Detection in the second instar had the weakest signal, whereas the fifth instar gave a signal which was as strong as that obtained with adults. Thus the amount of Las DNA appears to increase through vector metamorphosis. This may be indirect evidence to suggest the propagative nature of Las in vector psyllids.

In the Las-retention test, psyllids maintained Las-carrying ability for 12 weeks, even though acquisition feeding of Las had been terminated. According to a previous study (Lin *et al*., 1973), the Asian citrus psyllid has an adult lifespan of ≈90 days. The retention test was carried out for 84 days, which covered most of the lifespan. Thus it can be inferred that Las persists in the Asian citrus psyllid vector. In general, vectors carrying persistent pathogens need more time than vectors carrying nonpersistent pathogens to acquire the pathogens through acquisition feeding. Vectors carrying persistent pathogens also need a longer period to transmit the pathogens to host plants successfully (Nault & Ammar, 1989). Vector control with insecticides is usually considered feasible for controlling the diseases caused by insect-borne pathogens that are transmitted in a persistent manner.

Although Asian citrus psyllids can carry Las throughout their adult lives, they cannot directly transmit Las to their offspring. A similar phenomenon appears in pear decline, another disease caused by a psyllid-borne phytoplasma (Hibino *et al*., 1971; Davies *et al*., 1995). If an insect vector has the property of transovarial passage, disease control is more difficult. Fortunately, the results reported here showed that Asian citrus psyllids cannot transovarially transmit Las to their offspring although the pathogen is persistent in the adult vectors.

PCR-based assays have been proven effective for studying other insect-borne phytoplasma-caused diseases such as aster yellows and sugarcane white leaf. The aster yellows phytoplasma was detected in nymphs and adults of the leafhopper vector *Scaphoideus titanus* by PCR, using phytoplasma 16Srl group-specific primers (Alma *et al*., 1997). Likewise, the phytoplasma causing sugarcane white leaf disease was detected in nymphs and adults of the leafhopper vector *Matsumuratettix hiroglyphicus* (Hanboonsong *et al*., 2002). However, unlike the psyllid vectors of Las, both these leafhoppers tested positive for transovarial transmission by PCR.

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